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Mg deficiency interacts with the circadian clock and phytochromes pathways in Arabidopsis

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Abstract

Little is known about the relationship between nutrition and the circadian clock in plants. The first global transcriptomic study in plants of the response to magnesium deficiency (–Mg) revealed that the circadian clock was affected in the *Arabidopsis thaliana* model species. Interactions between the circadian clock and Mg status were here investigated in the light of recent knowledge. We highlight the wide disturbances caused by –Mg within the central oscillator and, reciprocally, the probable pervasive influence of the circadian clock on the response to –Mg. We provide evidence that light signalling pathways are likely to be involved in the input of Mg status to the circadian oscillator and that they interact with the circadian clock to coregulate an important part of the transcriptomic response. We further studied *PIF3 LIKE 1* (*PIL1*) because it strongly and early responded, before the core genes of the circadian oscillator, and was a representative regulator of light signalling that interacts with the circadian oscillator. Furthermore, the far-red light-responsive genes, which are related to *PIL1*, were more enriched among the –Mg-deregulated genes than those responding to red, blue and intense lights. Finally, *pil1* mutants had an altered response to –Mg notably by losing the upregulation of *PSEUDO-RESPONSE REGULATOR 9*, a core circadian oscillator. In short, we have further characterised the interactions between the Mg status and the circadian clock and identified the involvement of light signalling pathways in the response to Mg status. In particular, we have illustrated the role of a light-signalling component in the regulation of the circadian oscillator and physiological processes during Mg starvation.

KEYWORDS

Arabidopsis, circadian clock, light input, Mg deficiency, microarray expression data

1 | INTRODUCTION

The study of magnesium (Mg) nutrition in plants is a pressing topic. Magnesium deficiency (–Mg) is a frequently occurring limiting factor in cropping systems (Wang et al., 2020). Furthermore, decreases of Mg contents in food crop put at risk a great number of people in modern societies for hypomagnesemia (DiNicolantonio, O'Keefe, & Wilson, 2018). Even so, our understanding of the mechanisms regulating Mg concentration in plants, which are our main food source, is still limited today (Guo, Nazim, Liang, & Yang, 2016). Low Mg availability impacts the plants in many physiological processes such as starch metabolism, carbon partitioning, photosynthesis, growth or even senescence (Hermans & Verbruggen, 2005; Tanoi & Kobayashi, 2015). This nutrient plays a key role, particularly in the production and use of energy: it is the chelating agent for most of the ATP (thus promoting its bioactivity), the central element of

chlorophyll, and the cofactor and allosteric modulator of enzymes involved in glycolysis (Hermans, Conn, Chen, Xiao, & Verbruggen, 2013).

In 2010, we studied the transcriptomic response to magnesium deficiency in the model species *Arabidopsis thaliana* (Hermans, Vuylsteke, Coppens, Craciun, et al., 2010; Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010). This analysis suggested a role of the circadian clock in the response to −Mg based on three observations. First, the expression of components of the circadian oscillator was altered, early in roots and at long-term but more widely in leaves. Second, the effect of the time of the day had a significant impact on the transcriptomic response to Mg deficiency, with the differentially expressed genes (DEGs) at dawn and dusk being different in number and showing relatively little overlap. Finally, most of the processes related to −Mg symptoms, such as the accumulation at dawn of soluble sugars and starch in the leaves, overproduction of ethylene, oxidative stress and chlorosis are also regulated by the circadian clock. Recently, Feeney et al. (2016) showed that intracellular concentrations of Mg influenced the properties of the central oscillator in organisms phylogenetically as distant as the humans and the pico-algae *Ostreococcus tauri*. In addition, we recently showed that magnesium deficiency lengthened the autonomous period of the circadian oscillator in *Arabidopsis thaliana* seedlings (de Melo et al., 2020).

Unravelling how the circadian clock can perceive nutritional stresses and thereby allow the plant to keep physiologically in phase with the ever-changing environment is a challenging question (Haydon, Román, & Arshad, 2015; Seo & Mas, 2015; Webb, Seki, Satake, & Caldana, 2019). Circadian clocks are complex systems that have evolved independently in the major kingdoms of life in response to day and night cycles. In plants, the core circadian oscillator contains a set of transcription factors and cofactors that interact in complex feedback loops through successive expression through the day. The transcription factors that form the circadian oscillator can drive free running oscillations of up to one third of the transcriptome under constant conditions (Covington, Maloof, Straume, Kay, & Harmer, 2008).

We have re-examined the response to Mg deficiency in the light of new knowledge about the circadian oscillator (Sanchez & Kay, 2016; Webb et al., 2019). In the last decade, new components of the circadian clock have been characterised, adding (co)activators and histone modifiers to the well-known (co)-repressors composing the central oscillator (Nohales & Kay, 2016); also, transcriptional targetsof the main components of the central oscillator have been identified (Ezer et al., 2017; Graf et al., 2017; Liu, Newton, Liu, Shiu, & Farré, 2016; Nagel et al., 2015). This allowed us to further study the possible interactions between the circadian clock and Mg deficiency, with four main questions to address: (a) How widely is the core circadian oscillator impacted by −Mg?; (b) Which signalling pathways that interact with the circadian oscillator are targeted by −Mg?; (c) Is the circadian oscillator likely to regulate the transcriptomic response to −Mg?; and (d) Which biological processes may be affected by −Mg through the action of the circadian clock? By addressing these questions, we found in particular that light-responsive pathways might not only be involved in the mediation of the Mg status to the circadian oscillator but also in the interaction with the circadian clock to regulate the transcriptomic response to Mg starvation. We therefore further studied *PIF3 LIKE 1* (*PIL1*), a regulator of the far-red light (FRL) signalling, which appeared especially disturbed during −Mg.

2 | MATERIALS AND METHODS

2.1 | Update of microarray expression data and DEGs analysis

The dataset published by Hermans, Vuylsteke, Coppens, Cristescu, et al. (2010) was used. Probe sets that were left unannotated were updated based on the description of the "A-DORD-1-Agilent Arabidopsis 3 Oligo Microarray 4x44K" microarray available on the *ArrayExpress* database (Athar et al., 2019). Probe sets that had a hybridisation signal close to the background in more than 2/3 of the measurements were excluded. Genes were considered as DEGs if they were significantly misregulated, according to the statistical tests described below, with an absolute expression $\log_2$ fold-change of ≥1 under long-term magnesium deficiency compared to the control in at least one of the two time points (after 7 days +8 hr, at dusk, and/or 7 days +24 hr, at dawn). The significance of the misregulation was assessed by two different Wald tests that studied the impact of the treatment on the gene expression (a) independent from and (b) dependent on the time. Different to Hermans, Vuylsteke, Coppens, Cristescu, et al. (2010), we corrected the *p*-values for multiple testing in order to decrease the risk of incorrectly identifying a DEG. The threshold of the Bonferroni-corrected *p*-value in either one of the two Wald tests was set to .05. The genes that were overlooked in 2010 because of a lack of annotations and the updated microarray expression data are presented in the Tables S1 and S2, Supporting Information, respectively. Methodological details are provided in Data S1 and Figure S1.

2.2 | Definition of circadian clock-regulated and light-regulated genes

We defined potential targets for each of the considered transcriptional regulators of the core circadian oscillator as genes (a) whose oscillating expression profile is maintained in continuous light (Covington et al., 2008), (b) whose expression is disturbed in circadian oscillator mutants (Carré, Adams, & Veflingstad, 2014; Gendron et al., 2012; Graf et al., 2017; Hsu, Devisetty, & Harmer, 2013; Mockler et al., 2007; Nakamichi et al., 2009, 2012; Strasser, Alvarez, Califano, & Cerdán, 2009) and (c) which have been predicted as direct targets of the transcriptional regulators of the core circadian oscillator according to data of ChIP sequencing (chromatin immunoprecipitation followed by massive sequencing; Adams et al., 2018; Ezer et al., 2017;

Huang et al., 2012; Kamioka et al., 2016; Liu et al., 2016; Nagel et al., 2015; Nakamichi et al., 2009; Nohales et al., 2019).

In addition, we considered DEGs under red light (RL; Hu, Su, & Lagarias, 2009; Paik et al., 2019; Tepperman, Hwang, & Quail, 2006) and blue light (BL; Wang et al., 2016) versus darkness, and under white light + far-red light (FRL; Lim et al., 2018) as well as high white light (HWL; fluence rate of 1,000 μE emitted by metal halide light bulbs) versus white light for normal growth (WL; fluence rate of 100 μE; Kleine, Kindgren, Benedict, Hendrickson, & Strand, 2007), as well as potential targets of regulators of the phytochromes signalling (Burko et al., 2020; Chen et al., 2014; Hu et al., 2009; Jung et al., 2016; L. Li et al., 2012; Lim et al., 2018; Major et al., 2017; Nassrallah et al., 2018; Pfeiffer, Shi, Tepperman, Zhang, & Quail, 2014; Tepperman et al., 2006) (Table S4).

Further methodological details are provided in Tables S3 and S4.

2.3 | Gene sets enrichment analysis

Unilateral Fisher's tests were performed to assess the enrichment of different gene sets among the four clusters of DEGs upon long-term magnesium deficiency in leaves: genes downregulated or upregulated at dusk or dawn (i.e., after 7 days +8 hr or 7 days +24 hr of treatment). The considered gene sets consisted either of the expressed genes that are potential targets of the core circadian oscillator, light-responsive genes or genes involved in light signalling pathways. The reference list corresponded to the whole set of expressed genes that were identified as described in section 2.1.

2.4 | Gene sets network analysis

The significantly up/downregulated genes at dusk and/or dawn, after long-term Mg starvation, in Arabidopsis young mature leaves, were analysed using the PlantGSEA toolkit (Yi, Du, & Su, 2013) to identify enriched gene sets. These gene sets consisted of published lists of genes responsive to specific treatments or modulated by regulators of interest. The results were visualised in Cytoscape (Shannon, 2003) with the EnrichmentMap app (Merico, Isserlin, Stueker, Emili, & Bader, 2010), which allows to connect between overlapping gene sets. To ease the functional interpretation of the results, the network was also built based on enriched gene ontology (GO) terms with BiNGO Cytoscape app (Maere, Heymans, & Kuiper, 2005), which can be used together with EnrichmentMap. All the above-mentioned tools were run with default parameters.

2.5 | GO analysis

GO term enrichments were assessed by Fisher's tests using Panther (Mi et al., 2017). We analysed the set of circadian regulated genes that are down(up)regulated when taking the whole set of −Mg down(up) regulated genes as reference. Results were visualised using Revigo (Supek, Bošnjak, Škunca, & Šmuc, 2011).

2.6 | Analyses of the *pil1* mutants

2.6.1 | Plant material

The SALK_043937c (*pil1*-4) (Li et al., 2014) line in Arabidopsis Col-0 background was obtained from the Nottingham Arabidopsis Seed Stock Centre (Nottingham, UK). Garlic line 438c01 (*pil1*-1) (Salter, Franklin, & Whitelam, 2003) in Arabidopsis Col-0 background was obtained from Prof. K.A. Franklin (University of Bristol, UK). The two mutant lines were genotyped with the primer sequences obtained from the Salk Institute Genomic Analysis Laboratory website: http://signal.salk.edu/tdnaprimers.2.html (Figure S2).

2.6.2 | Plant culture

Plants were grown as described in Hermans, Vuylsteke, Coppens, Cristescu, et al. (2010).

2.6.3 | Quantitative RT-PCR analysis

Total RNA was extracted from young mature leaves of 5-week-old plants grown hydroponically using the Aurum Total RNA Mini Kit (Bio-Rad). The first-strand cDNA was synthesised using GoScript Reverse Transcription System (Promega). Quantitative PCR analyses were carried out with SYBR Green reagent (Affymetrix). *UBIQUITIN 10* (*UBQ10*) and *CYCLIN-DEPENDENT KINASE A* (*CDKA*) were used as reference genes. Sequences of primers for *UBQ10*, *CDKA* and *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) are described in Hermans, Vuylsteke, Coppens, Cristescu, et al. (2010).

2.6.4 | Starch iodine staining

Starch was visualised by iodine staining according to the procedure described in Hermans and Verbruggen (2005).

2.6.5 | Sugars and chlorophyll determination

Young mature leaves were harvested, flash-frozen and ground in liquid nitrogen. Chlorophylls were determined according to the procedure developed in Misyura, Colasanti, and Rothstein (2013). Soluble sugars (fructose, glucose and sucrose) and starch were analysed as described in Hermans and Verbruggen (2005).

2.6.6 | Mineral analysis

Leaf samples were dried prior to ICP-MS (Elan DRCe; PerkinElmer) analysis at the Centre for Excellence in Molecular Plant Sciences (CEMPS), Chinese Academy of Sciences (Shanghai, China).

3 | RESULTS AND DISCUSSION

3.1 | Disturbance of the circadian oscillator genes during magnesium deficiency

In young mature leaves, we identified 6,584 DEGs (Tables S1 and S2) corresponding to 36.57% of the expressed genes. Among these, 6,213 were common with the 7,587 DEGs identified in 2010. We found 16 DEGs that were unannotated in 2010 but none of them have a known biological function. As in 2010, we could highlight that the effect on the transcriptome was stronger at dusk than at dawn: 3,027 and 2,556 genes were downregulated or upregulated at dusk while 1,715 and 2,043 genes were downregulated or upregulated at dawn (Table S2) (Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010).

Among the 28 genes of the core circadian oscillator (Table S5) (Lee, Hong, & Seo, 2019; Nohales & Kay, 2016), 15 were differentially expressed (Figure 1), which represents a higher proportion of DEGs in comparison with the whole transcriptome (15/28 = 53.6% vs. 37.2%). *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *PSEUDO-RESPONSE REGULATOR 3*, *5*, *9* (*PRR3*, *5*, *9*), *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *EARLY FLOWERING 3*, *4* (*ELF3*, *4*) were previously identified by Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010. We found seven additional DEGs in the circadian oscillator: *REVEILLE 8* (*RVE8*), *RVE4*, *NIGHT-LIGHT INDUCIBLE AND CLOCK-REGULATED 2* (*LNK2*), *BROTHER OF LUX ARRHYTHMO* (*BOA*), *JUMONJI DOMAIN CONTAINING 5* (*JMJD5*), *TEOSINTE BRANCHED 1* (*TCP20*) and *SAP30 FUNCTION RELATED 1* (*AFR1*). JMJD5 and AFR1 are histone modifiers while the others are (co)-activators for the expression of other genes of the central oscillator. In particular, *RVE8*, *RVE4* and *LNK2*, which were also differentially expressed, can exhibit antagonistic roles with *CCA1* and *LHY* (Jones, Morohashi, Grotewold, & Harmer, 2019; Lee et al., 2019; Nohales & Kay, 2016).

As previously observed, the newly identified genes in the circadian oscillator tended either to be downregulated under –Mg at the time point of their highest expression in control conditions or upregulated under –Mg at the time point of their lowest expression in control conditions (Figure 1). *JMJD5* is the only exception, being repressed at both time points. *RVE8* and *BOA*, together with *CCA1*, *PRR9*, *PRR5* and *EARLY-FLOWERING 4* (*ELF4*), showed the highest fold-changes (Figure 1).

We did not re-analyse the roots in this article. When we updated the data following the same procedure as applied to young mature leaves, we found that the circadian clock was not responding to –Mg in roots. This confirmed the results obtained previously by Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010.

3.2 | Genes of multiple input pathways to the circadian oscillator are impacted upon –Mg

A challenging question remains the understanding of how the circadian oscillator responds to the Mg status. It can be expected that Mg feeds the circadian oscillator through pathways related to the biological processes disturbed during –Mg. Haydon et al. (2015) suggested that the circadian clock was actually responding to the accumulation of sucrose in the leaves during Mg starvation. This might play a role but it is unlikely that only sugars inform the circadian clock. Indeed, *BASIC LEUCINE ZIPPER 63* (*bZIP63*), which mediates the input of sucrose to the circadian oscillator (Frank et al., 2018), is downregulated at dawn (Figure 2) but its target, *PRR7*, is only weakly disturbed at dawn during –Mg (log_2 fold-change was of –0.44 at that time point). We looked for input signals to the circadian clock other than sugars. In total, we identified 14 other genes that might regulate components of the core oscillator (Figure 2). These have been described as input signals associated with temperature (*HEAT SHOCK TRANSCRIPTION FACTOR B2B*, *HEAT SHOCK PROTEIN 70*, *SUPERSENSITIVE TO ABA AND DROUGHT 1*, *COLD REGULATED GENE 27*, *28*), light (*CRYPTOCHROME 2*, *PHYTOCHROME A*, *ELONGATED HYPOCOTYL 5*, *PHYTOCHROME INTERACTING TRANSCRIPTION FACTORS 1*, *3*, *5*, *PIF3 LIKE 1*), abscisic acid (*ABA RELATED*) and redox status of the chloroplast (*SUPPRESSORS OF PIN1 OVEREXPRESSION 1*). In particular, the downregulation at dawn of *COLD REGULATED GENE 27*

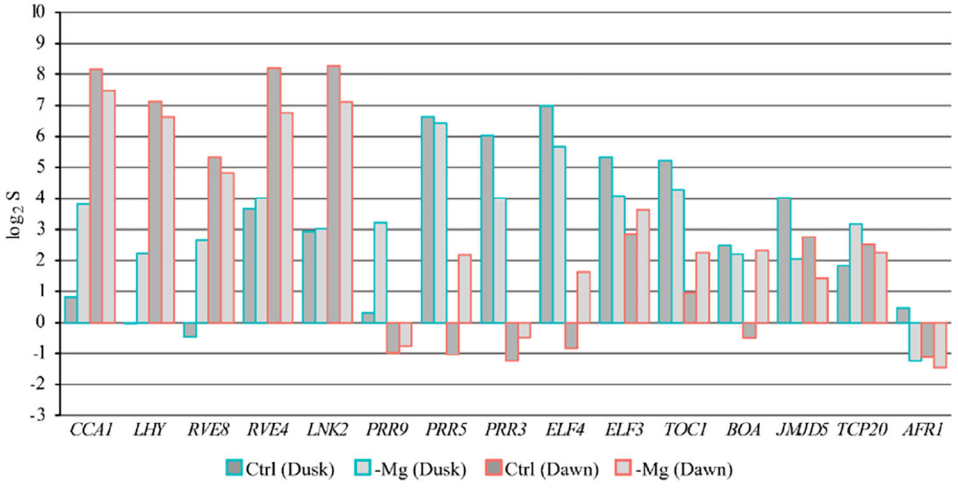

FIGURE 1 Response of the circadian oscillator components to –Mg. The log_2 hybridisation signal (Log_2 S) is presented for each gene at dusk (7 days +8 hr of treatment) and dawn (7 days +24 hr) in control (Ctrl) and magnesium deficiency (–Mg) conditions. n = 2 biological × 3 technical repeats. All genes presented are DEGs (p-value ≤.05; absolute log_2 fold-change of the expression in Ctrl with respect to that in –Mg ≥1 at least one time point)

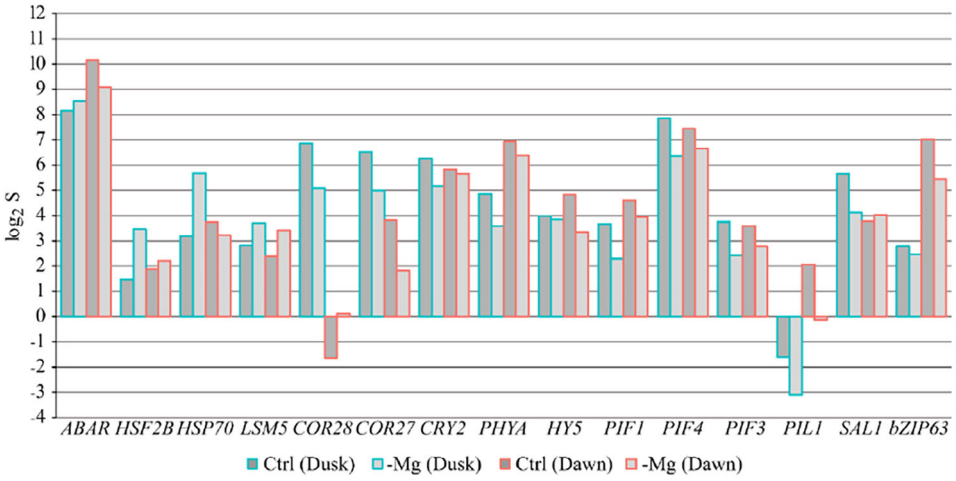

FIGURE 2 Analysis of the input pathways to the clock during –Mg. The $\log_2$ hybridisation signal (Log_2 S) is presented for each gene at dusk (7 days +8 hr of treatment) and dawn (7 days +24 hr) in control (Ctrl) and magnesium deficiency (–Mg) conditions. n = 2 biological × 3 technical repeats. All genes presented are DEGs (p-value ≤.05; absolute $\log_2$ fold-change of the expression in Ctrl with respect to that in –Mg ≥1 at least one time point)

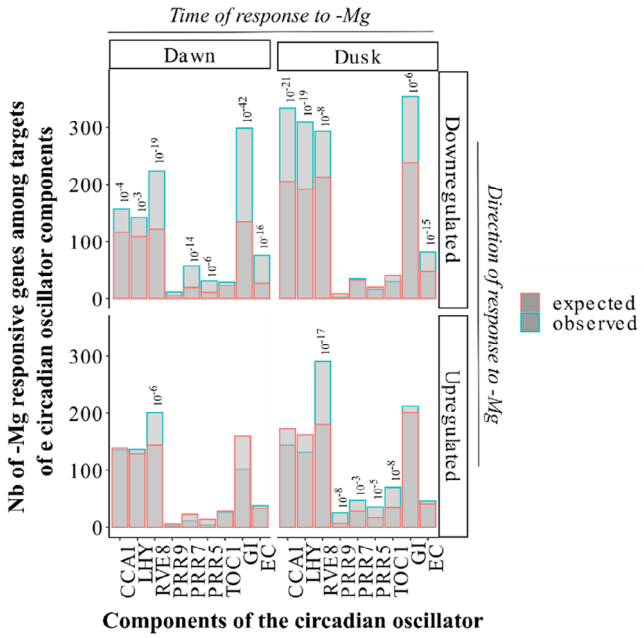

FIGURE 3 Enrichment of potential targets of components of the circadian oscillator among the differentially expressed genes upon –Mg. The number (Nb) of the potential targets of the different core clock transcriptional regulators that are observed among the –Mg down/upregulated DEGs is compared to the expected number at each time point, that is, the proportion of the transcriptome that is down/upregulated at the considered time point multiplied by the total number of potential targets. p-values of significant Fisher's test for enrichment (≤10^{-3}) are indicated on the top of the bars

and *28*, which are transcriptional repressors, might partly explain the upregulation at dawn of *PRR5* and *TOC1* (Nohales & Kay, 2016).

Interestingly, these results allow to highlight that –Mg is likely to interact with multiple signalling pathways, including pathways whose associated signals (as light or temperature) are not disturbed during Mg starvation. It can be postulated that these pathways are related to –Mg by the physiological processes they commonly target. In fact, a gene set enrichment analysis (GSEA) of the –Mg upregulated and downregulated genes (at dusk and/or at dawn; Table S6) allowed us to identify overlaps between response to long-term Mg starvation and responses to biotic, cold, drought and oxidative stresses as well as to potassium (K), phosphorus (P) and nitrogen (N) deficiencies. The latter are all known to impact, similarly to –Mg (Hermans & Verbruggen, 2005), photosynthesis, sugar metabolism and/or resource allocation (Ballaré, 2014; Dumont & Rivoal, 2019; Foyer & Shigeoka, 2011; Hafsi, Debez, & Abdelly, 2014; Hammond & White, 2007; Kurepin et al., 2013; McAllister, Beatty, & Good, 2012; Zargar et al., 2017).

Regarding the circadian clock, it will be interesting to determine whether the response of the central oscillator to –Mg is regulated predominantly by one particular pathway or whether it requires the interactions of different input signals. We can foresee that the light and/or temperature pathways play an important role because they are the strongest signals for entraining the circadian oscillator (De Caluwé et al., 2017). In strong support, the published gene set that overlapped the most with the potential targets of the circadian clock regulated by –Mg matched the *PHYTOCHROME INTERACTING FACTORs* (*PIFs*)-regulated genes, known to be induced during seedlings de-etiolation (Figure S3).

3.3 | Genes regulated by components of the circadian oscillator are enriched among the –Mg responsive genes

To assess a potential role of the circadian clock during –Mg, Hermans, Vuylsteke, Coppens, Cristescu, et al. (2010) performed an analysis of the promoters of the –Mg responsive genes. No enrichment of CCA1 and LHY binding sites was found. Other components of the circadian oscillator could not be evaluated at that time because their binding sites were not known. Recent data allowed us to update and extend this analysis. To do so, we changed the approach to identify transcriptional targets of the circadian clock more confidently. Instead of predicting targets by promoter analysis, which has many drawbacks (Aerts, 2012), we integrated three different kinds of data:

(a) expression data in continuous light, (b) expression data in circadian oscillator mutants and (c) genome-wide maps of the binding sites of components of the circadian oscillator. Confident sets of target genes were obtained for nine core components or complexes of the circadian oscillator: CCA1, LHY, RVE8, PRR9, PRR7, PRR5, TOC1, the evening complex (EC; formed by ELF3, ELF4 and LUX) and GIGANTEA (GI). As outcome a total of 3,391 genes were likely regulated by the circadian oscillator. We found that 48.9% (1,680/3,434) of these potential circadian clock-regulated genes were also regulated by Mg deficiency (Table S7). This represents an enrichment compared to the 37.2% observed at the whole transcriptome level. Furthermore, the 1,680 predicted circadian clock-regulated genes correspond to 27% of the total number of −Mg DEGs. We observed an enrichment of potential targets for all considered core circadian oscillator components (Figure 3). Even though this does not prove any cause–consequence relationship, it supports the hypothesis according to which the circadian clock is involved in the regulation of the transcriptomic response to −Mg. In particular, we found out correspondence between changes in expression of circadian core components and cognate targets. Indeed, among the −Mg downregulated genes, we found an enrichment of the targets of the following repressors, whose corresponding genes were upregulated: (a) CCA1 and LHY (cf. at dusk), (b) the EC (cf. at dawn) and (c) PRR5 (cf. at dawn). Among the upregulated genes, the targets of the TOC1 repressor, whose corresponding gene was downregulated at dusk, were also enriched.

Other components of the circadian oscillator showed, however, contradictions between the enrichment results and their transcriptional response to −Mg. For instance, while *GI* transcripts did not respond to long-term −Mg treatment, the predicted targets of GI were the most significantly enriched (at dawn, among the downregulated genes: p-value of 10^{-42}). This might be interpreted as an indication of post-transcriptional regulation. In support of the latter hypothesis GI is modulated by protein–protein interactions (Nohales et al., 2019).

Similarly, the potential targets of PRR7 were enriched among the −Mg responsive genes, although *PRR7* was not in the list of DEGs. However, for this gene, the adjusted p-value was 3×10^{-4} and the $\log_2$ fold-change of expression was −0.89 at dusk, close to our DEG definition.

Another unexpected enrichment was obtained for PRR9. This repressor was upregulated during Mg deficiency at dusk, but its potential targets were overrepresented among the genes upregulated at dusk. This might be because of interactions with PRR7, whose corresponding gene was repressed, as almost all the genes regulated by PRR9 are also targeted by PRR7 (Liu et al., 2016).

Potential targets of the transcriptional activator RVE8 are enriched at all time-points in both directions of regulation (up and down). This might be related to its antagonistic action to CCA1 and LHY, which complexifies its regulatory network (Nohales & Kay, 2016) and the interpretation of the data.

3.4 | The −Mg responsive circadian targets are specifically associated to multiple biological processes

We have shown evidence to support a role for all the surveyed circadian oscillator components in −Mg response. Future research is needed to understand the disturbance of the circadian clock and its impact on the response to changing Mg status. A first evaluation can be obtained, however, by examining the biological functions associated to the −Mg DEGs that are potentially regulated by the circadian clock.

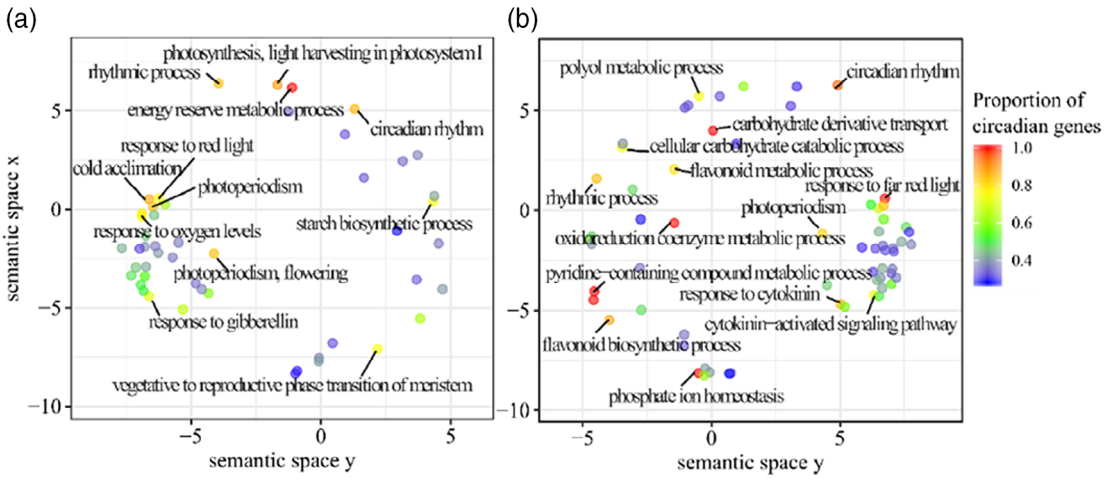

FIGURE 4 Enriched gene ontology (GO) annotations within the potential targets of transcriptional regulators of the core clock that are differentially expressed during −Mg. Only the differentially expressed target genes for transcriptional regulators associated to an enrichment among the whole (a) downregulated or (b) upregulated DEGs in young mature leaves at dawn and/or at dusk are included (Fisher's test p-value ≤.05). The enriched GO annotations are represented as circles that cluster together when they are associated to close biological processes. The colour of a circle is related to the proportion of potential circadian-clock targets among the −Mg downregulated (a) or upregulated (b) genes that belong to the enriched GO term that is considered. If this proportion is higher than 70%, then the circle is labelled with its corresponding biological process

The target genes of all core components of the circadian oscillator were pooled together according to the direction of their regulation. GO analyses were performed to assess to which specific biological processes potential targets of the circadian oscillator were associated during Mg deficiency (Table S8).

Genes that were downregulated belong to categories related to photosynthesis, starch biosynthesis, light sensing, development and response to gibberellin, cold and oxygen levels (Figure 4a). Indeed, known Mg deficiency symptoms are starch accumulation, chlorosis and degradation of the photosynthetic apparatus (Hermans et al., 2013). Therefore, the circadian clock might play a role in the appearance and/or evolution of those symptoms.

Upregulated targets of circadian oscillator components have designated functions in pyridine-containing compound biosynthesis, light sensing, oxidoreduction coenzyme metabolism, phosphate ion homeostasis, sugar derivative transport, flavonoid biosynthesis and cytokinin-activated signalling pathway (Figure 4b). Flavonoid biosynthesis, together with oxidation–reduction coenzyme metabolism, might be related to the oxidative stress (Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010). Phosphate homeostasis is to be linked to the decrease of P content which was observed in the plant during Mg deficiency (Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010).

Interestingly, the –Mg downregulated genes related to starch metabolism appeared as those showing the biggest overlap with the potential targets of the circadian clock responding to –Mg (Figure S3). Therefore, the circadian oscillator might modulate starch accumulation, after a first outbreak because of a limitation of the sucrose export from source leaves (Hermans & Verbruggen, 2005). We can expect an adaptation of the nocturnal degradation rate of the transient starch, which is known to be under the control of the circadian oscillator (Graf, Schlereth, Stitt, & Smith, 2010).

Photosynthesis appeared also highly connected to the circadian clock in the context of Mg starvation, likely through interactions between the circadian oscillator and phytochrome B and/or PIFs-regulated genes (Figure S3). At the transcriptomic level, the circadian clock might regulate photosynthesis during –Mg by modulating the expression of –Mg DEGs such as *SIGMA FACTOR 5* (*SIG5*) (Dodd, Kusakina, Hall, Gould, & Hanaoka, 2014)—encoding for a subunit of the chloroplastic RNA polymerase, *STATE TRANSITION 7* (*STN7*) (Dodd et al., 2014)—related to the cycle of repair of the light-damaged photosystems II, *CHLOROPLAST RNA BINDING* (*CRB*) (Dodd, Belbin, Frank, & Webb, 2015)—whose corresponding protein binds to the chloroplastic ribosomes and is necessary for proper functioning of the chloroplast, *thioredoxins f* and *m* (*ATF1*, *ATF2*, *ATHM1*, *ATHM2*) (Farré & Weise, 2012)—involved in light activation of photosynthesis enzymes, as well as components of the light harvesting complexes of the photosystems I and II, of the Calvin cycle and chlorophyll biosynthesis pathway (Farré & Weise, 2012). Regulation of genes related to sugar metabolism, senescence (Hermans et al., 2013) and transpiration (such as *ABAR*) might be also relevant.

Such results support a role of the circadian clock during Mg starvation. However, cross-talks with signalling pathways are likely to occur and some of the observed enrichments can rather come from the involvement of the circadian clock in the considered processes in general, independently from the treatment.

3.5 | Importance of the light pathways in the response to Mg deficiency

We identified light pathways as possible mediators of the Mg status to the circadian oscillator (Figure 2 and Figure S3). Furthermore, many –Mg DEG regulated by components of the circadian oscillator were also regulated by light (Figure 4). This fostered further analyses, because light is one of the strongest input signals for entraining the circadian oscillator (De Caluwé et al., 2017).

We have analysed among –Mg DEG those that were also regulated by blue (BL), red (RL), far-red (FRL) or high white (HWL) light.

Overall, Mg DEGs responding also to light represented 69% of the whole –Mg DEGs. In addition, we noticed specific enrichments in genes responding to light quality or intensity (Figure 5). The enrichments in genes differentially expressed under FRL (in comparison with white light) were especially high, with a *p*-value even reaching $10^{-\infty}$ when considering the overlap between the FRL and –Mg (at dusk) downregulated genes. This might indicate a repression of the

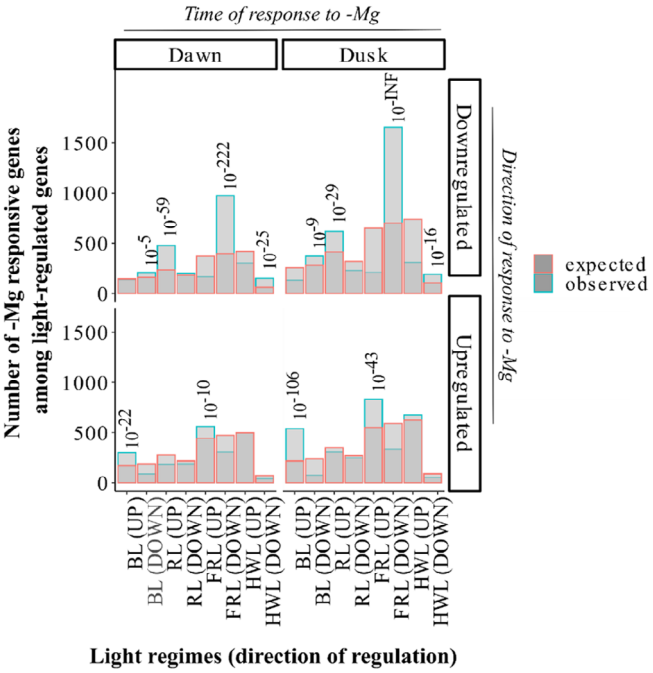

FIGURE 5 Enrichment of light-regulated genes among the differentially expressed genes (DEGs) upon –Mg. At each time point, the number (Nb) of the light-responsive genes (that are upregulated or downregulated by the considered light treatments) that are observed among the –Mg down/upregulated DEGs is compared to the expected number, that is, the proportion of the transcriptome that is down/upregulated at the considered time point times the total number of light-responsive genes. *p*-values of significant Fisher's test for enrichment ($\leq 10^{-3}$) are indicated on the top of the bars. BL, blue light; FRL, far-red light; HWL, high white light; RL, red light

phytochromes, supported by the reduced levels of transcripts of phytochrome A during −Mg, at dawn (Figure 2). Furthermore, consistent with that hypothesis, we found among −Mg downregulated genes enrichments of direct targets of phytochromes A and B as well as downstream regulators such as CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), ELONGATED HYPOCOTYL 5 (HY5) and FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) (Table S9). However, we obtained also enrichments suggesting a repression of the PHYTOCHROME INTERACTING FACTORs 1, 3, 5 and 7 (PIF1,3,5,7) (Table S9), which is in contradiction with a repression of the phytochromes, which are their negative regulators (Shin, Anwer, & Davis, 2013).

The light pathways are also likely to interact with the output pathways of the circadian clock for co-orchestrating the transcriptomic response to −Mg. The proportion of light-responsive genes among the potential targets of the circadian oscillator reached 77% when considering the whole set of potential targets and climbed to 88% when considering only the −Mg DEGs (Table S2). In particular, the PRRs and GI might regulate the transcriptomic response to −Mg in interaction with PIL1 and the PIFs (Li et al., 2014; Nohales et al., 2019; Shor, Paik, Kangisser, Green, & Huq, 2017) and CCA1/LHY, with DET1 (Lau & Deng, 2012).

We further studied *PIL1*, because its response to −Mg in young mature leaves of Arabidopsis was remarkable: it was disturbed before any gene of the circadian oscillator and showed the highest fold-change in expression amid the genes inputting the light to the circadian clock (Hermans, Vuylsteke, Coppens, Cristescu, et al., 2010). PIL1 is known to act as a negative regulator of shade avoidance, forming with

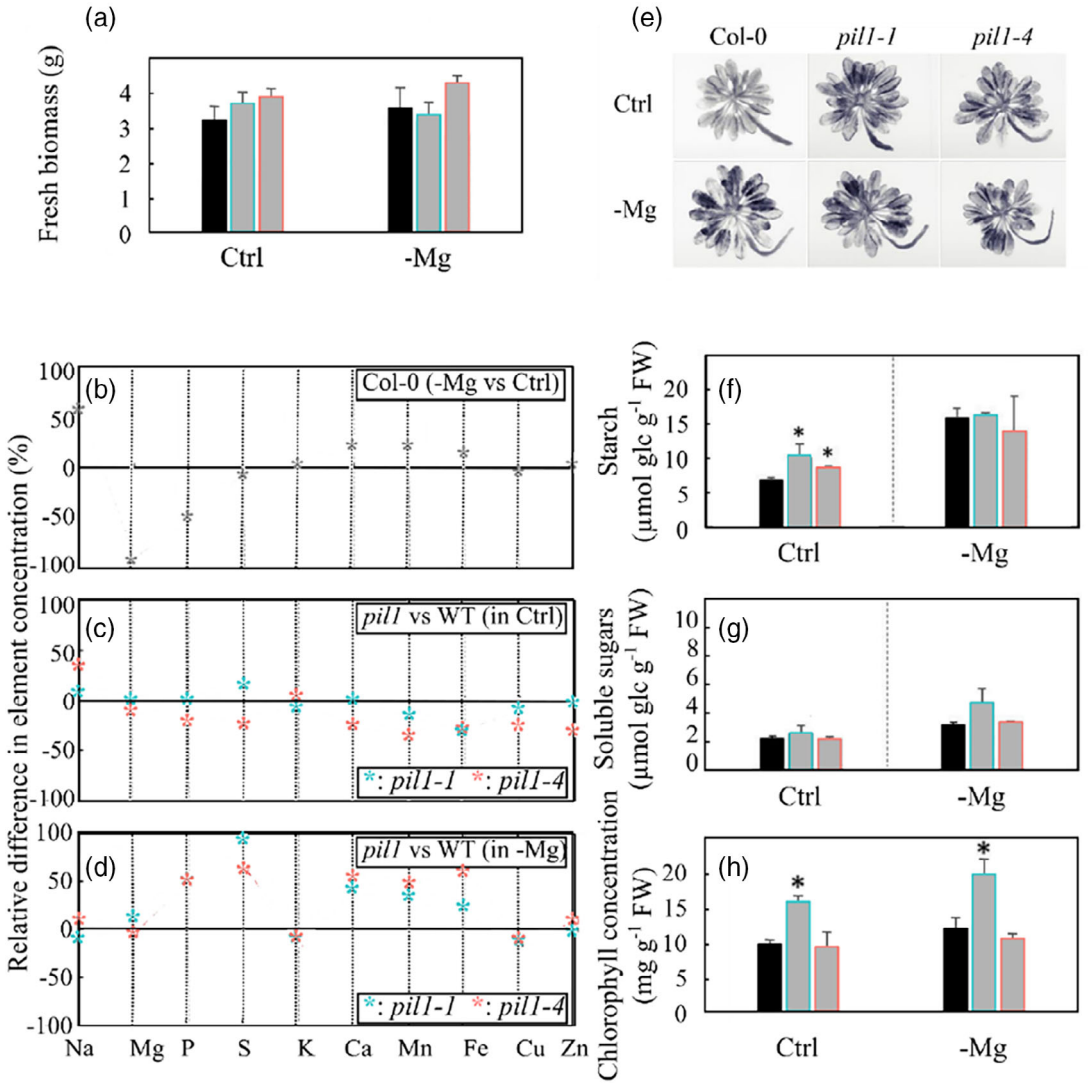

FIGURE 6 Physiological parameters measured in *pil1* mutants after 2 weeks of −Mg. (a) Individual fresh biomass, $n = 3$. Relative difference in mineral concentrations in young mature leaves of (b) Col-0 (wild-type) plants in −Mg compared to control condition (Ctrl); *pil1-1* and *pil1-4* mutants compared to Col-0 in (c) control condition and (d) −Mg. (e) Pictures of rosettes stained with a Lugol solution to visualise starch distribution. (f) Starch, (g) soluble sugars and (h) chlorophyll concentrations in young mature leaves. Blue, *pil1-1*; red, *pil1-4*. Concentrations are expressed per fresh biomass of young mature leaves, $n = 3$, $^*p < .001$ in comparison with the wild type

LONG HYPOCOTYL IN FAR-RED (HFR1) a heterodimer repressing the PIFs (Luo et al., 2014). Furthermore, PIL1 can directly interact with PIF5 (Li et al., 2014) and the circadian oscillator component TOC1, even though the function of these interactions remains unclear (Luo et al., 2014; Makino, Matsushika, Kojima, Yamashino, & Mizuno, 2002). The literature is however still scarce and the potential targets of PIL1 have not been described yet. This prevented us from evaluating the potential role of PIL1 on the −Mg transcriptomic response, as we did for other regulators of the RL/FRL signalling (Table S9).

Plants with PIL1 loss-of-function (pil1-1 and pil1-4) did not show any growth defect, in control or −Mg, in comparison with the wild type (Figure 6). However, these mutants showed an altered mineral profile, notably for the P content (Figure 6b–d), and a lower increase of starch content during −Mg than the wild type, which can be related to significantly higher control concentration compared to the wild type (Figure 6f). Starch metabolism and P homeostasis were predicted to be targeted by the circadian clock during −Mg (Figure 4). This suggests an interplay of PIL1 (and therefore of the light pathways) with the circadian clock at the level of biological processes related to symptoms of Mg deficiency. In addition, the interactions between PIL1 and the photosynthesis during −Mg remain to be explored in more details. The chlorophyll concentrations in young mature leaves of plants subjected to 2 weeks of −Mg were not modified by the treatment (Figure 6h), despite changes in the expression of genes related to photosynthesis appeared a week before (Figure 4 and Figure S3). Furthermore, the *pil1* mutants did not show lower concentrations of chlorophyll than the wild type, which was unexpected for mutants in which the action of PIFs is exacerbated (Toledo-Ortiz et al., 2014). The chlorophyll concentrations of *pil1-1* were even higher than that of WT in control and −Mg conditions. Therefore, the functions of PIL1 might be different in mature plants and seedlings, the latter showing a phenotype in accordance with disturbances of the phytochrome B signalling (Salter et al., 2003).

To investigate the possible role of PIL1 in mediating Mg status to the circadian clock level, we studied the response of *PRR9* in *pil1* mutants, as *PRR9* has been suggested to be regulated by PIL1 (Li et al., 2014). In *pil1* mutants *PRR9* was downregulated under −Mg conditions, whereas it was upregulated in the wild type (Figure 7). This was observed in night/day cycles as well as in continuous light during the 2 days of treatment, even though the levels of expression of *PIL1* were decreasing sharply in the first 24 hr of continuous light, not only in −Mg but also in control condition. These results are contradictory to the theory according to which the downregulation of *PIL1* by −Mg causes the upregulation of *PRR9*. It is worth mentioning that *PRR9* shows actually a complex regulation by RL/FRL pathways. It might be upregulated by the phytochromes A and B, by COP1 and DE-ETIOLATED1 (DET1) (Luo et al., 2014; Makino et al., 2002; Nassrallah et al., 2018; Paik et al., 2019), whereas it might be regulated in contradictory directions by the PIFs (Oh, Zhu, & Wang, 2012). *PRR9* might be therefore located at a regulatory node in the RL/FRL pathways, whose regulators might

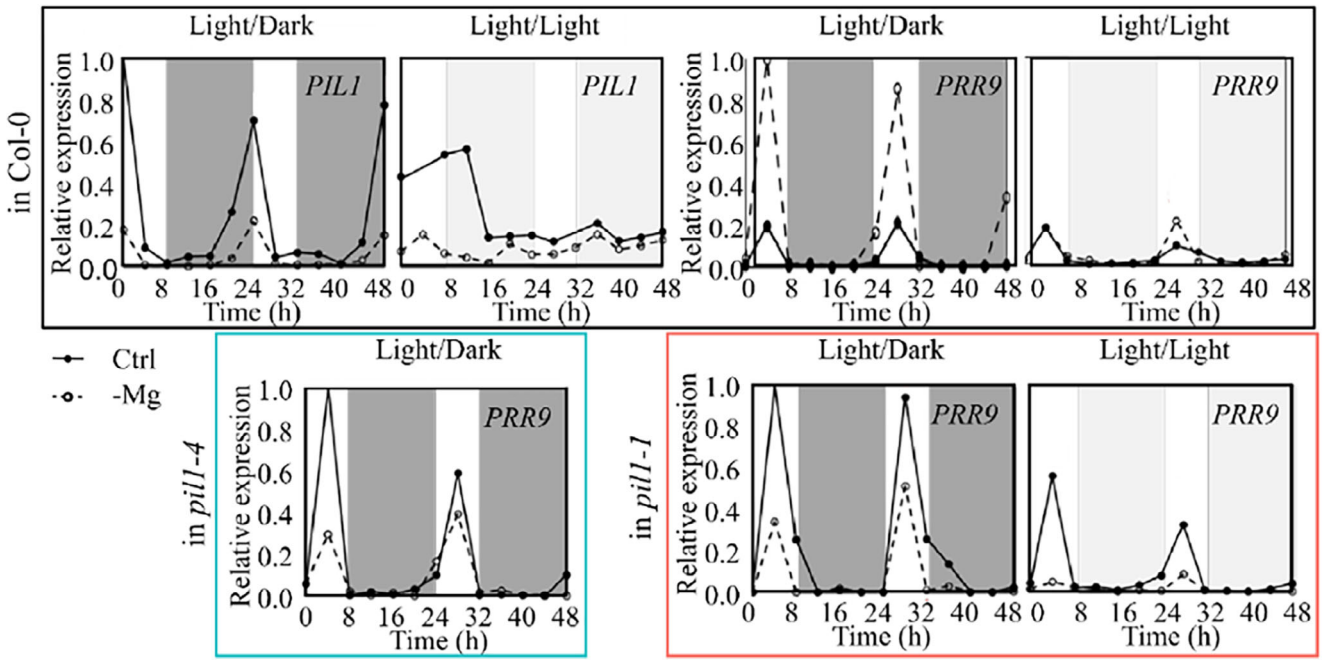

FIGURE 7 Disturbance of the response of the circadian clock to −Mg in the *pil1* mutants. Expression of *PIL1* and *PRR9* in young mature leaves after a week of Mg deficiency in Col-0 wild type and *pil1-1* mutant in short-days (8 hr light: 16 hr darkness) and continuous light, and in *pil1-4* in short days. The experiment has been duplicated with three pooled plants for each time point. Similar results have been obtained. Results from one experiment are shown. Solid line, fully supplied control; dashed line, Mg deficiency. *n* = 3 technical replicates ± *SE*. Dark grey background indicates night period; light grey indicates the subjective night; white background indicates the light period. The levels of expression of a gene in a given genotype are relative to the maximum level of expression that is observed in this genotype (in light/dark or light/light and in control or −Mg conditions)

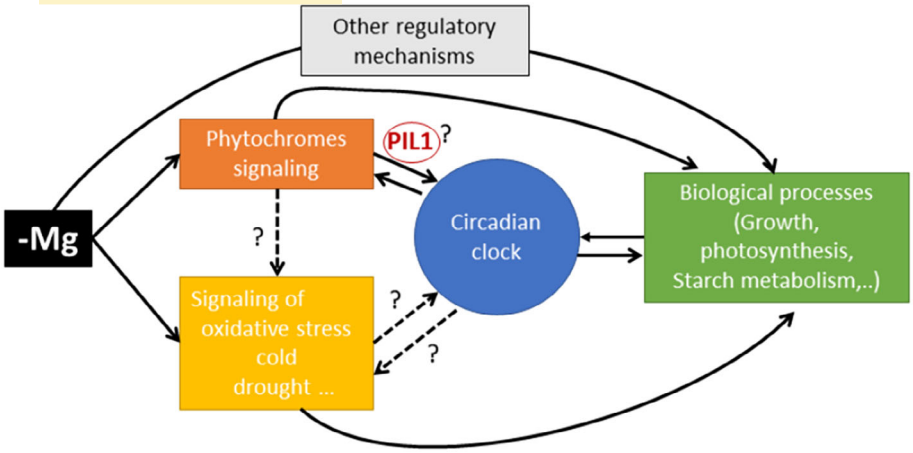

FIGURE 8 Model of probable interactions involved during Mg deficiency. We hypothesise that Mg deficiency can disturb many biological processes (light, abiotic stresses, etc.). An important overlap was identified between on one hand the circadian regulated genes and the other hand the phytochromes regulated genes among the −Mg differentially expressed genes (DEGs). The role of PIL1 in signalling −Mg stress to the clock is proposed. The exact interactions between −Mg, multiple stress signalling, the circadian clock and biological processes might involve multiple layers of regulation. Furthermore, Mg deficiency can have a direct impact on protein function as allosteric activator of enzymes and necessary chelator for ATP activity (see the "other regulatory mechanism" box in the diagram)

have opposing regulatory actions. The interactions between PIL1 and *PRR9* during −Mg might be partly mediated through COP1 and the PIFs, which are known to interact with PIL1 (Toledo-Ortiz et al., 2014) and for which we observed enrichments of potential targets within −Mg-regulated genes (Table S9). We can therefore suggest from our observations that altered response of the circadian clock to Mg starvation in *pil1* mutant are because of a deregulation of components of the RL/FRL. In a similar way, iron deficiency is signalled to the circadian oscillator by a pathway dependent on the FRL and RL phytochromes A and B photoreceptors (Salomé, Oliva, Weigel, & Krämer, 2012). It would be valuable to evaluate whether Fe and Mg share common components in their input pathways to the circadian oscillator.

In the future, it will be interesting to further study the role of PRR9 in the response to Mg starvation. This transcription factor is known to be a positive regulator of the senescence (Kim et al., 2018); therefore, its upregulation might enforce the cross-links between circadian clock, light pathways and −Mg. We did not observe an enrichment of PRR9 transcriptional targets among the −Mg responsive genes but the set of its potential targets should be considered cautiously. In fact, related ChIP-seq data predicted a number of targets of PRR9 notably lower compared to the other PRRs (Liu et al., 2016), which might be attributed to sensitivity limitations of the method rather than to a biological reality.

4 | CONCLUSIONS

Recent data published in the literature allowed us to develop new angles of analysis of a former pioneer transcriptomic study of the long-term Mg deficiency in Arabidopsis. We could further study the interactions with the circadian clock that were previously highlighted. Taking into consideration the components identified over the last past 10 years, we confirmed that in leaves, the circadian oscillator was widely impacted at the transcriptional level by Mg starvation, in a bigger extent than the whole transcriptome. The transcriptomic and physiological responses to magnesium deficiency appear diverse and, accordingly, we found that multiple signals induced by −Mg might feed into the circadian oscillator. We could however put the emphasis on the possible role of the light signalling pathways. Interactions between light and Mg deficiency were already evidenced several decades ago by the observation that the severity of the symptoms of −Mg was increasing with the light intensity (Marschner & Cakmak, 1989). In this article, the study of the possible cross-talks between the circadian clock and the transcriptomic response to Mg starvation in young mature leaves led us to highlight in particular the pathways of RL/FRL signalling. This was further supported by the altered response to −Mg of *PRR9* in loss-of-function mutants for PIL1, which is a protein involved in the RL/FRL signalling pathway mediated by the phytochromes. These observations strengthen the hypothesis that phytochromes are regulatory hubs not only involved in the perception of the light but integrating multiple signals (Carvalho, Campos, & Azevedo, 2011; Devireddy, Liscum, & Mittler, 2020; Qiu, Li, Kim, Moore, & Chen, 2019). The regulatory role of the circadian clock in the context of Mg starvation will need more in-depth research. Our analysis of the −Mg responsive potential direct targets of the circadian oscillator suggests a possible role of this biological oscillator in the regulation of processes impacted by Mg starvation, such as starch metabolism, ion homeostasis, redox status or photosynthesis. However, the regulation of biological processes by the circadian clock appears very complex, as the circadian clock may act at multiple levels, not only transcriptional, and multiple stress signalling pathways are likely to modulate its action. Our major findings are summarised and supplemented by open questions in Figure 8. Future research will provide us with advances in our understanding of the mechanisms by which the circadian clock helps the plants to cope with their ever-changing environment.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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