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Systems analysis of the responses to long-term magnesium deficiency and restoration in *Arabidopsis thaliana*

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Summary

- Unravelling mechanisms that control plant growth as a function of nutrient availability presents a major challenge in plant biology. This study reports the first transcriptome response to long-term (1 wk) magnesium (Mg) depletion and restoration in *Arabidopsis thaliana*.
- Before the outbreak of visual symptoms, genes responding to Mg starvation and restoration were monitored in the roots and young mature leaves and compared with the Mg fully supplied as control.
- After 1 wk Mg starvation in roots and leaves, 114 and 2991 genes were identified to be differentially regulated, respectively, which confirmed the later observation that the shoot development was more affected than the root in Arabidopsis. After 24 h of Mg resupply, restoration was effective for the expression of half of the genes altered. We emphasized differences in the expression amplitude of genes associated with the circadian clock predominantly in leaves, a higher expression of genes in the ethylene biosynthetic pathway, in the reactive oxygen species detoxification and in the photoprotection of the photosynthetic apparatus. Some of these observations at the molecular level were verified by metabolite analysis.
- The results obtained here will help us to better understand how changes in Mg availability are translated into adaptive responses in the plant.

Introduction

Plants require magnesium (Mg) to harvest solar energy and to drive photochemistry. This is probably one the most important physiological functions of this metal as the central atom of chlorophyll (Wilkinson *et al.*, 1990; Hörtensteiner, 2009). Signs of Mg deficiency in most plants usually manifest belatedly as a chlorophyll breakdown between the veins and make their appearance first in mature leaves, systematically progressing from these towards the youngest ones (Bennett, 1997; Hermans & Verbruggen, 2008). The knowledge about Mg^{2+} uptake by roots, transport to shoots and recycling between organs is relatively limited (Gardner, 2003; Karley & White, 2009). The few physiological reports essentially describe an early impairment in sugar partitioning (in Arabidopsis, Hermans & Verbruggen, 2005; bean plants, Fisher & Bremer, 1993; Cakmak *et al.*, 1994a,b; rice, Ding *et al.*, 2006; spinach, Fisher *et al.*, 1998; spruce, Mehne-Jakobs, 1995 and sugar beet, Hermans *et al.*, 2004, 2005). One dramatic effect of Mg starvation is sugar accumulation in source leaves, before any noticeable effect on photosynthetic activity. Sugar accumulation in source leaf tissues, rather than a reduction in the amount of Mg available for chlorophyll biosynthesis, could be at the root of the decrease in chlorophyll content (Hermans *et al.*, 2004; Hermans & Verbruggen, 2005). A later effect of Mg deficiency is a reduction of plant growth and a modification of

the root (R) to shoot (S) biomass allocation. However, observations of the effect of Mg shortage on R : S vary according to the plant model studied and the age of the plant. Early studies report a severe decrease in the root biomass of bean plants (Cakmak *et al.*, 1994a,b) and spinach (Fisher & Bremer, 1993). More recent studies report the absence of an effect on the root system of sugar beet (Hermans *et al.*, 2004, 2005), Arabidopsis (Hermans & Verbruggen, 2005) and rice (Ding *et al.*, 2006) grown hydroponically. Carbon allocation to the youngest leaves is more affected than that to the roots in *Arabidopsis thaliana*, which could explain why Mg deficiency reduces the growth of young leaves more than the growth of roots (Hermans & Verbruggen, 2005; Hermans *et al.*, 2006). In the present study, we further investigated the transcriptomic response to Mg deficiency in roots and leaves of Arabidopsis, before the outbreak of the visual symptoms described.

Materials and Methods

Hydroponics culture

Five-week-old *Arabidopis thaliana* Heynh Columbia (Col-0) mature plants grown hydroponically were subjected to Mg starvation. The growth conditions and the composition of the nutrient solution are described in Hermans *et al.* (2010).

Xylem sap collection

Xylem sap was obtained by using root pressure exudation after decapitation of the shoot. Briefly, the rosette was cut with a razor blade and the root placed in a pressure chamber, with the hypocotyl of the plant sticking out. Pneumatic pressure (10–15 bar) was applied. The first emerging droplets were discarded to prevent contamination of the xylem sap with contents from damaged cells.

Mineral content analysis

Samples were harvested and dried at 60°C, digested with nitric acid and assayed by atomic absorption (AAS) or inductively coupled plasma mass spectrometry (ICP-MS).

Ethylene measurement

Plants were placed on to round Petri dishes filled with 50 ml of nutrient solution and capped into 0.5 l vials. The vials were tided together with two metal pieces. Ethylene was measured using the ETD300 photoacoustic ethylene detector (Sensor-Sense, Nijmegen, the Netherlands). A valve control box allowed automated sampling of gas production under a continuous flow rate of 1 l h^{-1}. The ethylene production of six vials was then measured in rotation over a 10 min period during 24 h.

Glutathione and ascorbate measurement

A fine powder of 50 mg of leaves was mixed with 1 ml of ice-cold 6% metaphosphoric acid. The suspension was then centrifuged at 14×10^3 ***g*** for 15 min at 4°C. The supernatant was collected and further analysed. The concentrations and redox states of glutathione and ascorbate were determined with high-pressure liquid chromatography (HPLC) analysis essentially as described by Semane *et al.* (2007).

Genome-wide expression

Whole-genome Agilent Arabidopsis 3 60-mer oligo 44K microarrays were used to assess transcript expression levels in the root and young mature leaf samples (Agilent Technologies Inc., Palo Alto, CA, USA). The procedure is described in Hermans *et al.* (2010). The array design can be accessed via the VIB Microarray Facility (VIB MAF) website (http://www.microarrays.be). All hybridizations were performed at the VIB MAF in Leuven, Belgium, according to standard protocols available at the VIB MAF website.

Extraction of total RNA and reverse transcription and semiquantitative PCR

For confirmation of microarray data, the material from the two experiments used for microarray hybdridization and from one additional independent experiment were used. Tissue samples were ground in liquid nitrogen and total RNA was subsequently extracted with TRIzol reagent from 100 mg of tissue powder according to the manufacturer's instructions (RNeasy, Qiagen). Owing to the high content of proteoglycans and polysaccharides, a second purification was performed (Minielute, Qiagen). Reverse transcription was done starting from 1 μg RNA using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany). Quantitative PCR (qPCR) reactions were performed with the LightCycler 480 (Roche) as described in Hermans *et al.* (2010). The primers used to amplify the different sequences are listed in Supporting Information, Table S9.

Results

Physiological characterization of plants starved of Mg for 1 wk

Magnesium deficiency in *A. thaliana* plants usually results in symptoms of sugar accumulation first, and eventually chloroses in young mature leaves and a higher R : S ratio within a 2 wk time-frame in the experimental setting

described by Hermans & Verbruggen (2005). Here, the focus was on the transcriptomic response to long-term Mg deprivation but preceding the outbreak of visual symptoms, and the transcriptomic response to Mg restoration (resupply of Mg to Mg-deficient plants). Earlier transcriptomic responses (within hours) to Mg deprivation have been studied elsewhere (Hermans *et al.*, 2010).

Five-week-old *A. thaliana* (Col-0) plants grown hydroponically were subjected to Mg starvation (complete omission of Mg from the nutrient solution) during a 1 wk period. At day 7, half of the Mg-starved plant population was resupplied with Mg for 8 and 24 h (Fig. 1a). After 8 d treatment, the individual biomass and the chlorophyll content in young mature leaves were unaltered (Table 1). Marked chlorosis was only visible after 2 wk of treatment (Fig. 1b). The Mg concentration in roots and the entire rosette decreased by 69 and 59%, respectively, compared with controls fully supplied with Mg at day 8 (Fig. 2a,b). We selected that stage of deficiency for further transcriptomic studies because, while no visual symptoms in leaves (Fig. 1b) were perceptible, Mg content was already considerably decreased in both organs (Fig. 2a,b). The concentrations of other minerals were measured in roots and young mature leaves. Ca, Fe and Cu concentrations increased and K decreased in both organs, while Zn increased in roots and decreased in young mature leaves (Fig. 2d). In this experiment, the restoration of Mg deficiency was also studied. Twenty-four hours after Mg resupply, the Mg concentration increased by 125% in roots, 75% in the rosette and 127% in the xylem sap, compared with Mg-deficient samples (Fig. 2a–c).

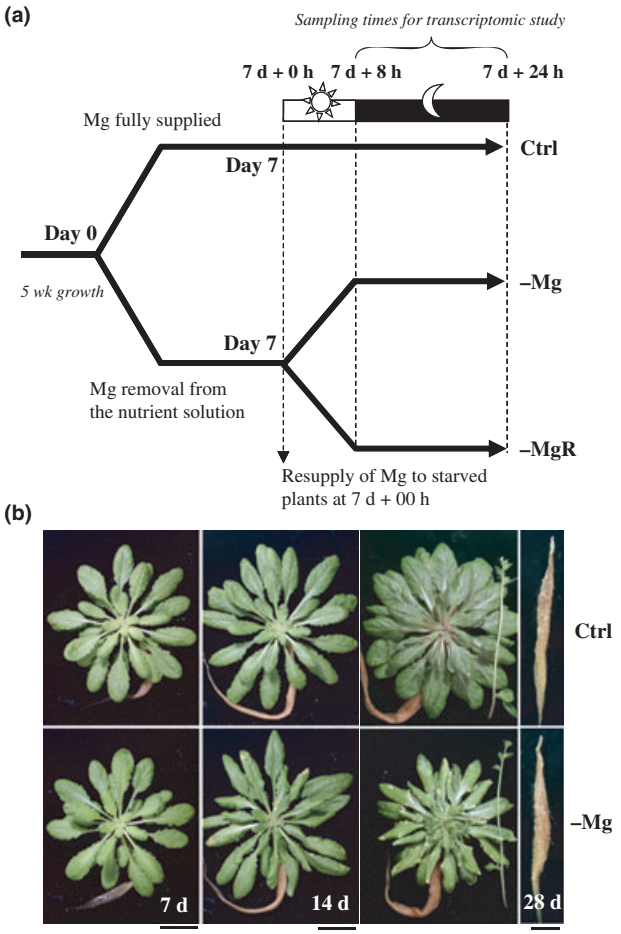

Fig. 1 Experimental design for studying long-term magnesium (Mg) deficiency and restoration in *Arabidopsis thaliana*. (a) Plants were grown hydroponically for 5 wk. At day 0, the plant population was divided into two groups: one fully supplied (Ctrl) and one Mg-starved (–Mg). At the start of the light period of day 7 (7 d + 00 h), a subgroup of Mg-starved plants was resupplied with Mg in the nutrient solution (–MgR). Sampling points for the array experiments were 7 d + 8 h (end of the light period) and 7 d + 24 h (end of the dark period). (b) Colour pictures of Arabidopsis plants supplied with (Ctrl) or starved of (–Mg) Mg after 7, 14 and 28 d of treatment. Bars, 2 cm.

Transcriptome changes associated with long-term Mg deficiency

The tissues analysed were the roots and the young mature leaves that were previously identified as target organs undergoing the most drastic decreases in Mg concentration (Hermans & Verbruggen, 2005; Hermans *et al.*, 2010). The expression of 33 939 features in roots and 32 993 in young mature leaves was monitored using 14 Agilent Arabidopsis 3 60-mer oligo 44K chips, according to a loop design (Fig. S1). In this way, genes responding to Mg starvation (−Mg) in roots and young mature leaves at the time points 7 d + 8 h (end of the 8 h light period) and 7 d + 24 h = 8 d (end of the 16 h dark period) were monitored and compared with the fully supplied as control (Ctrl) (Fig. 1). A summary of the complete transcriptomic analysis is presented in Table S1. Changes in expression of 1155 and 11 930 genes across the two sampling time points in roots and leaves, respectively, differed significantly ($P <$ 0.001; FDR (false discovery rate) = 0.0209 and 0.0011) depending on the Mg treatment (Table S1). Another 1782 and 4738 genes showed significant changes ($P < 0.001$; FDR = 0.0009 and 0.0010, respectively) in expression in roots and young mature leaves, respectively, depending on the Mg treatment but irrespective of the sampling time (Table S1). Duplicated hybridization values of genes probed more than once were eliminated. From the combined sets of genes, 222 and 7723 annotated genes showed ≥ twofold change in expression upon Mg starvation ($|\Delta S| = |\log_2 S_{-Mg} - \log_2 S_{Ctrl}| > 1$, where S is the hybridization signal) for at least one time point in roots and young mature leaves, respectively, and were kept for further analysis (Table S1). Interestingly, they were proportionally more up-regulated than down-regulated genes in the roots, contrary to the response observed in leaves (Fig. 3a).

Table 1 Physiological parameters measured in young mature leaves and roots of *Arabidopsis thaliana* after 8 d of magnesium (Mg) starvation

	Young mature leaves		Roots	
	Ctrl	−Mg	Ctrl	−Mg
Individual dry biomass (mg)	218 ± 18	216 ± 14	44 ± 3	41 ± 1
Chlorophyll (nmol g^{-1} FW)	357.7 ± 34.0	339.3 ± 22.6	–	–
Total ascorbate (μmol g^{-1} FW)	1.100 ± 0.104	1.128 ± 0.081	0.087 ± 0.013	0.120 ± 0.006
DHA : ASC (oxidized : reduced)	0.31 ± 0.05	1.08 ± 0.40*	2.48 ± 0.55	1.98 ± 0.42*
Total glutathione (μmol g^{-1} FW)	0.091 ± 0.011	0.092 ± 0.006	0.010 ± 0.003	0.014 ± 0.001
GSSG : GSH (oxidized : reduced)	0.46 ± 0.10	0.79 ± 0.25*	0.62 ± 0.21	0.56 ± 0.03
	Whole plant			
Ethylene production (ng C_2H_4 h^{-1} g^{-1} FW)	0.29 ± 0.06	0.69 ± 0.11*		

DHA : ASC, dehydroxyascorbate : ascorbate ratio; GSSG : GSH, oxidized : reduced glutathione ratio; Ctrl, plants fully supplied with Mg; −Mg, Mg-deficient plants. *Statistically significant ($P < 0.05$) differences between treatments. $n \geq 3$ ± SE.

Because the effect of Mg starvation was different depending on the time of day, only genes differentially regulated by Mg depletion simultaneously at both time points were analysed in more detail. The differential expression of 124 (12 down-regulated and 102 up-regulated) and 2991 (1474 down-regulated and 1517 up-regulated) genes resulting from Mg depletion in roots and leaves, respectively, did not rely on the time of sampling (Fig. 3a). A survey of the functional distribution of these genes was carried with the MIPS Functional Catalogue (http://mips.gsf.de/proj/funcatDB). The distribution of the down- and up-regulated genes between the different MIPS categories is presented in Fig. S2. In roots and leaves globally, the most important and significant differences compared with the whole genome representation were in the 'metabolism', the 'cell rescue, defence and virulence', the 'protein with binding function or cofactor requirement' and the 'subcellular localization'.

Transcriptome changes associated with Mg restoration

After 7 d of starvation, Mg was resupplied to deficient plants (−MgR) and the restoration response was monitored after 8 and 24 h of Mg addition (Figs 1a, S1). Transcripts for which restoration was effective showed a <1.5-fold difference in expression between Mg-resupplied and control samples ($|\Delta S| = |\log_2 S_{-MgR} - \log_2 S_{Ctrl}| < 0.58$) (Fig. S3). The expression of 18 genes in roots was restored after 8 h, whereas the number increased to 59 genes after 24 h of resupply, representing 14 and 47%, respectively, of the genes altered by Mg deficiency (Tables 2, S4). In young mature leaves, 207 genes were effectively restored after 8 h, and 1373 genes after 24 h, which represent about 7 and 46%, respectively, of the genes altered by Mg deficiency (Tables 2, S5).

Confirmation of microarray expression patterns

The hybridization signals of selected Mg-responsive genes with different degrees of induction or repression (Tables S2, S3) were further confirmed by reverse transcription-qPCR (RT-qPCR) in three biological replicates. A good correlation was found between the quantified signals for five genes in roots and 12 genes in leaves (Table S9, Fig. S4).

Remarkable responses to Mg deficiency

Survey of the circadian rhythm Because the experimental array design covered the end of the light period (8 h) and the end of the dark period (24 h), the pace of the circadian clock could be assessed. In leaves fully supplied with Mg, the behaviour of the clock central oscillator genes *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL 1* (*LHY1*) and *TIMING OF CAB 1/ PSEUDO-RESPONSE REGULATOR 1* (*TOC1/PRR1)* was in agreement with previous findings (Dodd *et al.*, 2005; Allen *et al.*, 2006; James *et al.*, 2008; Knight *et al.*, 2008). *CCA1* and *LHY1* expressions were low at dusk (end of the light period) and high at dawn (end of the dark period), whereas *TOC1* phased reversely in leaves (Fig. 4a). In long-standing Mg-deficient organs, the expression levels of *CCA1* were higher at dusk and lower at dawn than in fully supplied organs, with more important amplitude variations observed in leaves than in roots. Remarkably, the expression levels of *PHYTOCHROME INTERACTING FACTOR 3-LIKE 1* (*PIL1*) were also lower but restored after Mg resupply in leaves (Table 2). To increase the temporal resolution, the expression of *CCA1*, *LHY1*, *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) and *PIL1* was monitored in an independent experiment every 4 h over 2 d starting from day 6 of treatment (Fig. 4b). Upon Mg deficiency, *CCA1* and *LHY1* expression in leaves had a slower

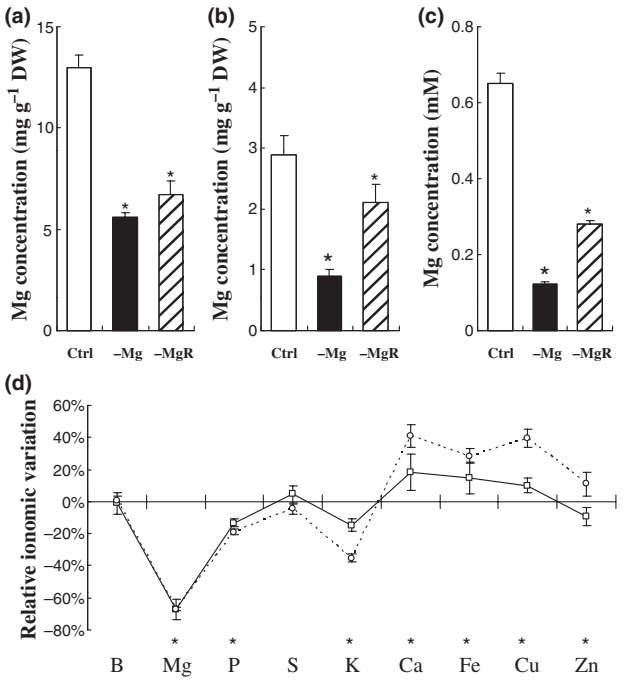

Fig. 2 Mineral profile in *Arabidopsis thaliana* plant organs upon magnesium (Mg) starvation and restoration. Mg concentration in rosette (a), roots (b) and xylem sap (c). Open bars, Mg fully supplied control (+Mg); closed bars, Mg-deficient (−Mg); hatched bars, Mg-starved and resupplied (−MgR). Mg was removed from the nutrient solution at day 0 and resupplied at day 7 for 24 h. $n = 5 \pm$ SE. (d) Ionomic variation in roots (squares, solid line) and young mature leaves (circles, dashed line) at 7 d + 24 h of Mg-deficiency treatment. Values are expressed relative to control plants fully supplied with Mg. $n = 8 \pm$ SE. Asterisks indicate statistically significant differences ($P < 0.05$) between treatments. (d) Significant differences are indicated for both organs.

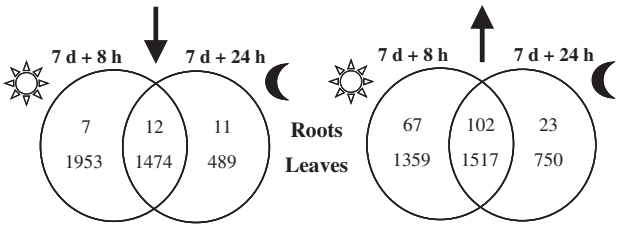

Fig. 3 Long-term impact of magnesium (Mg) starvation on the transcriptome of *Arabidopsis thaliana*. Venn diagram of negatively (↓) and positively (↑) regulated genes after 7 d + 8 h and 7 d + 24 h, corresponding, respectively, to the end of the light and dark periods. Note that 77 genes in leaves show reverse regulation between the two time points (Supporting Information, Table S3) and are not taken into account. Mg-deficiency treatment was induced at day 0 by removing Mg from the nutrient solution.

decay in the morning, similar to the effect observed in roots after short-term Mg depletion (Hermans *et al.*, 2010). *PRR9* and *PIL1* expressions were higher and lower, respectively, with an unchanged phase in Mg-deprived leaves.

Upon Mg starvation, we observed twice the number of genes differently regulated in leaves after 8 h (end of the light period) compared with 24 h (end of the dark period) (Fig. 3), underlining the importance of the light period in the response to Mg depletion. The evening element (EE: *AAAATATCT*) and CCA1-binding site (CBS: *AAAAAATCT*) are important, respectively, for the evening- and morning-specific transcription of target genes (Harmer *et al.*, 2000; Michael & McClung, 2002). The 1500 bp of upstream sequence of the differentially regulated genes at the two time points by Mg starvation was analysed with the Regulatory Sequence Analysis Tools' DNA pattern matching function (van Helden, 2003). A lower frequency of the EE but no change in the frequency of the CBS motif was found in the promoter region of the Mg-regulated genes compared with the whole genome (Table S6). Nevertheless, several Mg-regulated genes were identified with a high number of these motifs, such as *LHCA5* encoding a protein belonging to the light-harvesting antenna of photosystem I (Storf *et al.*, 2005) (Table S6).

Ethylene production upon Mg deficiency Ethylene plays an important role in the regulation of many growth and developmental processes of higher plants (Pierik *et al.*, 2006; Yoo *et al.*, 2009). In this study, four genes encoding isoforms of the 1-aminocyclopropane-1-carboxylic acid synthase (ACS) family were strongly induced by Mg starvation at both time points: *ACS11* was up-regulated in both roots and leaves, while *ACS2*, *ACS7* and *ACS8* were up-regulated in leaves (Tables S2, S3, Fig. S4). To investigate the correlation between *ACS* gene induction and endogenous ethylene production, we measured the gas emission by plants. After 7 d treatment, ethylene production was doubled in Mg-starved plants compared with controls (Table 1).

Response to oxidative stress upon Mg deficiency Around 50 genes in roots and leaves from the MIPS functional category 'oxygen and radical detoxification' were differentially regulated by Mg-deficiency treatment (Table S7). Several genes involved in the redox control of the cell were strongly up-regulated: *THIOREDOXIN* (*TRX*), *GLUTAREDOXIN* (*GRX*) (Gelhaye *et al.*, 2005) and *GLUTATHIONE S-TRANSFERASE TAU* (*GSTU*) (Dixon *et al.*, 2009). In view of this indication of antioxidative mechanism enhancement, we measured the key antioxidant molecules ascorbate and glutathione. After 1 wk of Mg-deficiency treatment, the total ascorbate and glutathione pools were not different in leaves compared with controls, but their oxidation state was significantly ($P < 0.05$) increased, as shown by the dehydroxyascorbate : ascorbate (DHA : ASC) and oxidized : reduced gluthatione (GSSG : GSH) ratios (Table 1). In Mg-deficient roots, the total ascorbate pool was slightly increased, and a significantly lower ratio of DHA : ASC was found, while the observed decrease in GSSG : GSH was not significant.

Table 2 List of genes in roots and young mature leaves of *Arabidopsis thaliana* differentially regulated by magnesium (Mg) starvation and whose expression is restored following the resupply of Mg. AGI code: Arabidopsis Genome Initiative locus code

AGI code		Description	Gene Ontology (GO) biological process	$\log_2 S_{Ctrl}$		$\log_2 S_{-Mg}$		$\log_2 S_{-MgR}$		Deficiency			Re-supply	
				8 h	24 h	8 h	24 h	8 h	24 h	$\Delta S_{8\ h}$	$\Delta S_{24\ h}$	$\Sigma\Delta$	$\Delta S_{8\ h}$	$\Delta S_{24\ h}$
Roots: down-regulated genes restored at 7 d + 24 h														
At2g43580		Chitinase, putative	Cell wall catabolic process	−0.4	−0.3	−1.5	−1.6	−1.6	−0.2	−1.1	−1.3	**−2.5**	−1.1	0.1
At1g11920		Pectate lyase family protein similar	–	1.4	1.6	0.3	0.5	−0.2	1.1	−1.2	−1.1	**−2.3**	−1.6	−0.5
Roots: up-regulated genes restored at 7 d + 8 h (top 10)														
At5g43650		Basic helix-loop-helix (bHLH) family protein	Regulation of transcription	−3.1	−2.4	−1.5	−0.2	−2.6	−2.3	1.6	2.1	**3.7**	0.5	0.1
At2g30670		Tropinone reductase, putative	Metabolic process	4.8	4.4	6.6	6.3	5.4	4.7	1.8	1.9	**3.7**	0.6	0.3
At5g60350		Unknown protein	–	0.2	0.1	1.7	1.9	0.4	−0.1	1.5	1.8	**3.3**	0.2	−0.2
At2g30660		3-hydroxyisobutyryl-coenzyme A hydrolase, putative	Metabolic process	2.6	1.8	4.2	3.4	2.8	1.6	1.6	1.6	**3.2**	0.2	−0.1
At5g52760		Heavy metal-associated domain-containing protein	Metal ion transport	0.1	−0.1	1.6	1.5	0.6	−0.2	1.5	1.7	**3.2**	0.4	0.0
At2g04050		MATE efflux family protein	Multidrug transport	−2.9	−2.9	−1.7	−1.2	−2.8	−3.1	1.2	1.7	**2.9**	0.2	−0.2
At5g22520		Unknown protein	–	−1.4	−0.8	0.2	0.5	−1.0	−1.1	1.5	1.3	**2.9**	0.3	−0.2
At2g30750	CYP71A12	Cytochrome P450 71A12	Response to bacterium	0.4	−0.2	1.6	1.2	0.4	0.4	1.2	1.4	**2.6**	0.1	0.6
At1g35230	AGP5	Arabinogalactan-protein 5	Response to cyclopentenone	−3.3	−3.2	−2.0	−1.8	−3.1	−2.9	1.2	1.3	**2.5**	0.2	0.2
At4g14370		Disease resistance protein (TIR-NBS-LRR class)	Defence response	−2.3	−2.6	−1.1	−1.2	−1.9	−2.4	1.1	1.4	**2.5**	0.3	0.1
Leaves: down-regulated genes restored at 7 d + 8 h (top 10)														
At2g46970	PIL1	Phytochrome interacting factor 3-like 1	Regulation of transcription	−1.6	2.1	−3.1	−0.1	−1.8	1.6	−1.5	−2.2	**−3.7**	−0.2	−0.5
At3g52370	FLA15	Fasciclin-like arabinogalactan protein 15 precursor	Cell adhesion	1.8	−0.2	0.3	−2.4	1.5	−0.6	−1.4	−2.2	**−3.7**	−0.2	−0.4
At5g03760	CSLA9	Cellulose synthase like A9	Response to bacterium	5.9	5.8	4.5	3.6	5.4	5.3	−1.4	−2.3	**−3.7**	−0.5	−0.5
At3g47360	HSD3	Hydroxysteroid dehydrogenase 3	Metabolic process	−0.4	2.1	−2.5	0.7	−0.8	2.2	−2.1	−1.4	**−3.5**	−0.4	0.1
At3g15115		Unknown protein	–	0.7	2.9	−0.7	0.8	0.5	2.3	−1.5	−2.0	**−3.5**	−0.3	−0.5
At2g32990	GH9B8	Glycosyl hydrolase 9B8	Carbohydrate metabolic process	4.4	3.3	3.0	1.3	4.0	3.2	−1.5	−2.0	**−3.4**	−0.4	−0.1
At2g05070	LHCB2.2	Light-harvesting chlorophyll *a*/*b*-binding 2	Photosynthesis	−0.1	1.5	−1.9	−0.2	−0.4	1.1	−1.8	−1.7	**−3.4**	−0.3	−0.5
At1g58170		Disease resistance-responsive protein-related	Defence response	0.8	0.6	−0.6	−1.5	0.7	0.2	−1.4	−2.1	**−3.4**	−0.1	−0.4
At1g45010		Unknown protein	–	3.6	2.3	2.0	0.5	3.0	1.9	−1.7	−1.8	**−3.4**	−0.6	−0.4
At2g35860	FLA16	Fasciclin-like arabinogalactan protein 16 precursor	Cell adhesion	5.5	5.2	4.2	3.1	5.1	4.8	−1.3	−2.1	**−3.4**	−0.4	−0.4
Leaves: up-regulated genes restored at 7 d + 8 h (top 10)														
At1g20520		Unknown protein	–	−3.4	−2.8	0.9	−1.4	−2.9	−2.7	4.3	1.4	**5.8**	0.5	0.1
At5g66650		Unknown protein	–	−0.4	−0.4	2.6	1.5	0.1	−0.4	3.0	1.9	**4.8**	0.5	0.0
At1g14480		Ankyrin repeat family protein	–	1.8	0.5	4.1	3.0	1.9	0.9	2.2	2.6	**4.8**	0.1	0.4
At2g38790		Unknown protein	–	3.7	1.8	6.7	3.2	4.0	2.1	3.0	1.3	**4.4**	0.3	0.2

Table 2 (Continued)

AGI code		Description	Gene Ontology (GO) biological process	$\log_2 S_{Ctrl}$		$\log_2 S_{-Mg}$		$\log_2 S_{-MgR}$		Deficiency			Re-supply	
				8 h	24 h	8 h	24 h	8 h	24 h	$\Delta S_{8\ h}$	$\Delta S_{24\ h}$	$\Sigma\Delta$	$\Delta S_{8\ h}$	$\Delta S_{24\ h}$
At1g15580	AUX2-27/IAA5	Auxin-induced protein 2-27/Indoleacetic acid-induced protein 5	Response to auxin stimulus	2.0	1.2	5.0	2.6	2.5	1.1	3.0	1.4	**4.4**	0.5	−0.1
At3g09032		Unknown protein	–	1.3	−1.0	3.8	0.8	1.4	−0.8	2.5	1.8	**4.3**	0.1	0.2
At5g51190		AP2 domain-containing transcription factor	Regulation of transcription	2.5	2.0	4.1	4.5	2.6	2.3	1.6	2.5	**4.1**	0.1	0.3
At1g27770	ACA1/PEA1	Auto inhibited Ca^{2+}-ATPase/Plastid envelope ATPase 1	Calcium ion transport	4.5	4.4	6.7	6.1	4.8	4.9	2.2	1.7	**3.9**	0.3	0.5
At5g52710		Heavy metal-associated domain-containing protein	Metal ion transport	−3.2	−3.0	−0.4	−1.9	−3.1	−3.0	2.8	1.1	**3.9**	0.1	0.0
At3g50800		Unknown protein	–	1.2	2.4	3.7	3.8	1.6	2.5	2.5	1.4	**3.9**	0.4	0.1

ΔS corresponds to difference in hybridization signal. For full list of genes, see Supporting Information Tables S4 and S5.

Discussion

The objective of this work is to understand how changes in Mg availability are translated into adaptive responses in plants. Some remarkable transcriptomic responses to Mg deprivation and restoration in roots and leaves were highlighted here, before the outbreak of the deficiency symptoms. Contrasting adaptations were found between these two organs, with the root transcriptome less severely affected.

Mg deficiency affects the root transcriptome moderately

We have consistently observed an impact of Mg starvation on aerial but not on root biomass production in *A. thaliana*, reflected by higher R : S ratio (Hermans & Verbruggen, 2005; Hermans *et al.*, 2006). The present transcriptomic analysis further supported the observation that Mg starvation affects the root development less than the shoot development in Arabidopsis (Fig. 1b). Indeed, the transcriptome of the roots was barely affected compared with the leaves (Fig. 3), notwithstanding the proportional decrease of Mg concentration in these two organs (Fig. 2a,b). These results were clearly distinct from reports on N, P and K deficiencies, which have an unambiguous impact on the root transcriptome at an early stage of induction, and eventually on root morphology (Armengaud *et al.*, 2004; Shin & Schachtman, 2004; Misson *et al.*, 2005; for review, see Hermans *et al.*, 2006; Iyer-Pascuzzi *et al.*, 2009). Here, very few Mg-regulated genes were found to be involved in root development (Tables 2, S4).

We noted that, by comparison, the expression of a large number of genes was restored within hours of resupply after starvation of other macroelements (e.g. N and P) and that the transcriptome is usually restored more quickly in roots than in shoots (Armengaud *et al.*, 2004; Bi *et al.*, 2007). Here, too, restoration in roots (14%) was proportionally more effective than in young mature leaves (7%) after 8 h, and almost as effective (*c.* 50%) in both organs after 24 h (Tables 2, S4, S5).

Ionomic adjustment to Mg depletion

Salt *et al.* (2008) emphasized the considerable interest in the functional connection between the genome and the ionome, which is defined as the mineral nutrient (both essential and nonessential) composition of an organism. After 1 wk of treatment, Mg starvation had a profound impact on the ionome, noticeably by decreasing Mg concentration but also by increasing the concentration of calcium, iron and copper in root and shoot tissues (Fig. 2d). Plants also lowered concentrations of a number of other elements (potassium, phosphorus in roots and leaves; zinc in

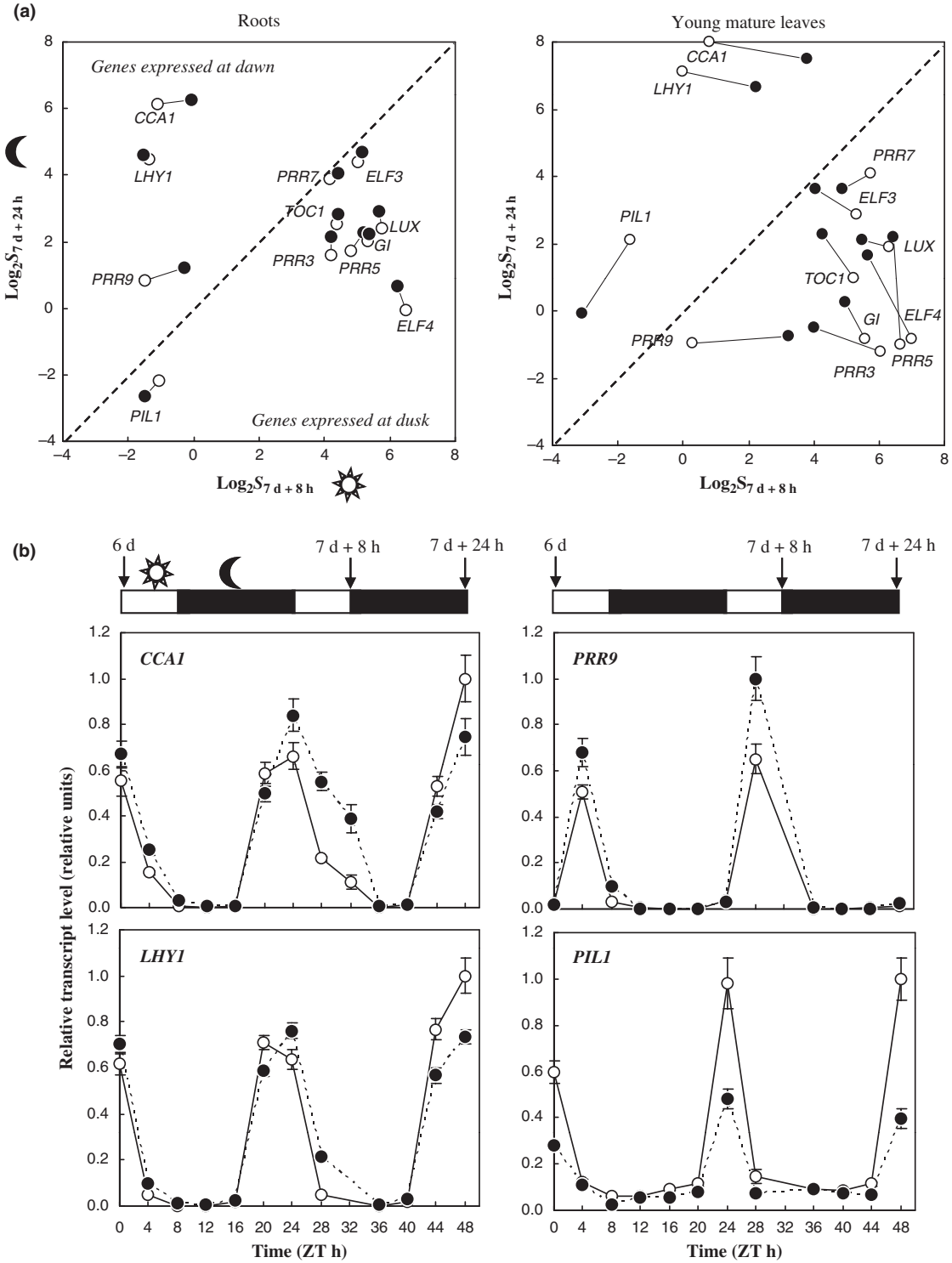

Fig. 4 Survey of the expression of circadian clock genes upon magnesium (Mg) starvation in *Arabidopsis thaliana*. (a) Phasing of circadian clock genes in roots and young mature leaves. The microarray hybridization signals (*S*) of genes at 7 d + 8 h (end of the light period = dusk) are plotted vs the signals at 7 d + 24 h (end of the dark period = dawn). The diagonal dashed lines indicate equal expression signals at dusk and dawn. In general, clock-associated genes tend to be closer to the diagonal upon Mg starvation, except for *PRR9* in leaves. (b) Monitoring of *CCA1*, *LHY1*, *PRR9* and *PIL1* expression in young mature leaves by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Leaves were harvested from plants fully supplied with Mg (Ctrl) and Mg-deficient (−Mg) plants from day 6 of treatment every 4 h during 2 d. There was an average of three pooled plants ± SE (each sample was assessed by three technical replicates). Plants were grown in short-day conditions (8 h light : 16 h darkness). Mg-deficiency treatment was induced at day 0 by removing Mg from the nutrient solution. Open circles, control; closed circles: Mg-starved samples. ZT, Zeitgeber time.

leaves). Many transporters involved in metal ion homeostasis have been identified in the Arabidopsis genome (Maser *et al.*, 2001; Krämer *et al.*, 2007). It is worth mentioning that Mg depletion did not induce the expression of genes encoding permeases potentially mediating Mg transport, such as the *MITOCHONDRIAL RNA SPLICING 2/MAGNESIUM TRANSPORTER* (*MRS2/MGT/CorA*) family (Li *et al.*, 2001, 2008; Gardner, 2003; Drummond *et al.*, 2006; Gebert *et al.*, 2009) and *MAGNESIUM/PROTON EXCHANGER 1* (*MHX1*) (Shaul *et al.*, 1999; David-Assael *et al.*, 2005) (Table S8). The only gene in leaves significantly down-regulated at the two time points was *MRS2-9* (Table S8). How the activity of Mg transporters is regulated is not understood, but this study shows little evidence of transcriptional regulation, as in microorganisms (Gardner, 2003 and references therein). The transcriptomic response to Mg deficiency is unique, because deficiencies of other major elements (N, P, K, S) trigger the expression of genes encoding these ion permeases in order to increase the root uptake capacity (Maruyama-Nakashita *et al.*, 2003; Misson *et al.*, 2005; Iyer-Pascuzzi *et al.*, 2009; Jung *et al.*, 2009).

One of the categories containing the highest number of Mg-regulated genes was the 'cellular transport, transport facilities and transport routes' (Fig. 3b). Genes encoding ion transporters were induced by Mg deficiency in young mature leaves, such as *CALCIUM EXCHANGER 3; 4; 7* (*CAX3*; *CAX4*; *CAX7/CCX1*), *PHOSPHATE TRANSPORTER 2* (*PT2/PHT1;4*), *SELENATE RESISTANT 1/ SULFATE TRANSPORTER 1;2* (*SEL1/SULTR1;2*), *CALCIUM-TRANSPORTING ATPASE* (*ACA1*; *ACA12*; *ACA13*) and *CATION/H^+ EXCHANGER 17; 18* (*CHX17*; *CHX18*) (Tables 2, S3). Those transporters may potentially play a role in the observed ionomic alteration upon Mg depletion (Fig. 2d).

The amplitude but not the phase of the circadian clock is altered in long-standing Mg-deficient plants

After 7 d of Mg starvation, the expression of several circadian clock-associated genes was dysregulated in the roots and in the leaves (Fig. 4a). The amplitude in the peak expression of clock-associated genes was altered but not their phase (Fig. 4b). Although the effect was less pronounced in roots, it appeared at an earlier time (8 h after the removal of Mg from the nutrient solution) than in leaves (Hermans *et al.*, 2010). Interestingly, *PIL1* encoding a putative bHLH transcription factor and identified as a *TOC1/PRR1*-interacting protein (Makino *et al.*, 2002) also showed an important decrease in the morning peak expression at an early stage (6 d of treatment) and was quickly restored after Mg resupply (Fig. 4b, Table 2).

Gating is the process of resetting the clock in response to environmental cues (Millar, 2004; Más & Yanovsky, 2009). While there is a large body of knowledge concerning the plant clock's response to light and temperature (Gould *et al.*, 2006), little is known about the response to nutrient status. Recently, an interplay was shown between the Arabidopsis circadian clock and the nitrogen assimilatory pathway (Gutiérrez *et al.*, 2008) and iron homeostasis (Duc *et al.*, 2009).

What, therefore, might be the consequences for Mg-deficient plants of a defect in the circadian clock? There are several lines of evidence of a possible link between the alteration of diurnal rhythm and symptoms of Mg deficiency. It has been shown that an incorrect match between endogenous rhythms and the environment reduces leaf chlorophyll content, CO_2 fixation and biomass production and that plants overexpressing *CCA1* contain less chlorophyll (Dodd *et al.*, 2005). Mg deficiency, in its turn, is reported to reduce the abundance of chlorophyll, increase starch accumulation in leaves and reduce shoot biomass production in Arabidopsis (Hermans & Verbruggen, 2005; Hermans *et al.*, 2006; Gaude *et al.*, 2007). Our previous works showed that the expression of *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2/LHCB1.1*) lost rhythmicity in 11 d Mg-deficient Arabidopsis plants, which did not yet display lower chlorophyll content (Hermans & Verbruggen, 2005). Oscillations of *CAB* promoter activity (Xu *et al.*, 2007) and also of other genes, such as *ELF3* (Carré, 2002), are actually dependent on the rhythmic expression of *CCA1*. Similarly, we found in this study the down-regulation at both time points of several *CHLOROPHYLL A/B BINDING PROTEINS* and *LIGHT-HARVESTING CHLOROPHYLL-PROTEIN COMPLEXES I & II* (Table S3).

Ethylene production upon Mg deficiency

Interrelationships between hormonal stimuli and nutritional homeostasis are well depicted (Hermans *et al.*, 2006; Rubio *et al.*, 2009 and references therein). Our data support a key role of ethylene in the Mg starvation response, as the expression levels of several genes encoding enzymes (*ASC2*; *7*; *8*; *11*) in the C_2H_4 biosynthetic pathway were enhanced (Tables S2, S3, Fig. S3) and Mg-deficient plants produced twice as much gas as control plants (Table 1). Mineral deprivations of P (Borch *et al.*, 1999), K (Shin & Schachtman, 2004) and Fe (Romera *et al.*, 1999) are also reported to induce ethylene overproduction. Although ethylene is generally depicted as a leaf senescence inducer (Jing *et al.*, 2005), ethylene-regulated stress responses also seem essential for stress tolerance. Recently, ethylene-insensitive mutants were shown to be more prone to chlorophyll breakdown and shoot growth inhibition compared with the wild-type in response to K deficiency (Jung *et al.*, 2009). We can also emphasize a possible link between the circadian clock and ethylene upon Mg

deficiency. Undeniably, the expression of *ACS8* and ethylene production are controlled by the circadian clock (Thain *et al.*, 2004; Covington *et al.*, 2008). Interestingly, it is also shown that *CCA1*-overexpressing seedlings produced double the amount of ethylene with no hint of rhythmicity (Thain *et al.*, 2004).

Enhancement of the defence mechanisms against oxidative stress and photoprotection of the photosynthetic apparatus upon Mg deficiency

We identified several genes that potentially detoxify or alleviate the function of reactive oxygen species and higher oxidation state of the leaf ascorbate and glutathione pools (Tables 1, S7). Indeed it is documented that Mg-deficient plants have a markedly increased antioxidative capacity (Cakmak & Marschner, 1992; Tewari *et al.*, 2006). Cakmak & Kirkby (2008) further supported the idea that carbohydrate accumulation and impairment of CO_2 fixation in Mg-deficient leaves could cause an over-reduction in the electron transport chain that potentiates the generation of reactive oxygen species.

It is also possible that higher iron and copper concentrations in shoots upon Mg deficiency (Fig. 2d) induce an oxidative stress, as the accumulation of these metals within the cell can be toxic (Connolly & Guerinot, 2002; Draobkiewicz *et al.*, 2004). Interestingly, we observed the induction in the leaves of *FERRITIN 1* (*FER1*), which encodes a well-characterized chloroplastic iron-storage protein accumulating upon iron excess (Murgia *et al.*, 2007). FER1 is involved in the onset of senescence, and its iron-detoxification function during that stage is required when reactive oxygen species accumulate (Murgia *et al.*, 2007).

Transcriptomic observations indicate an early protection of the photosynthetic apparatus and the enhancement of mechanisms preventing the accumulation of free chlorophyll (Table S3). We monitored the up-regulation of *EARLY LIGHT-INDUCED PROTEIN 1* and *2* (*ELIP1*; *2*) and their restoration upon Mg resupply (Table S3). According to Hutin *et al.* (2003), ELIPs fulfil a photoprotective function that could involve either the binding of chlorophylls released during turnover of pigment-binding proteins or the stabilization of the proper assembly of those proteins. ELIPs could also work as sensors that modulate chlorophyll synthesis to prevent accumulation of free chlorophyll, and hence to prevent photooxidative stress (Rossini *et al.*, 2006; Tzvetkova-Chevolleau *et al.*, 2007). *MULTIDRUG RESISTANCE PROTEIN 3* (*MRP3*), which encodes a vacuolar ABC transporter that can transport cholorophyll catabolites (Tommasini *et al.*, 1998), but is also involved in detoxification of metals (Zientara *et al.*, 2009), was also strongly induced in leaves and repressed after 8 h restoration of Mg (Table S5).

Conclusion

The present global transcriptomic study identified Mg starvation targets, which involve the circadian clock, the redox control of the cell and the protection of the photosynthetic apparatus. Mg starvation signalling also seemed to depend on ethylene. The information obtained here gives new insights into the transcriptional response to Mg shortage and resupply and sheds light on how changes in Mg availability are translated into adaptive responses. The dysfunction of the circadian clock, which may in turn regulate part of the responses to Mg deficiency, opens new routes of research to understand how plants regulate growth as a function of nutrient availability. The molecular knowledge gained in Arabidopsis could help in the future in the development of strategies to improve the tolerance to Mg starvation in Brassica crops, which are close relatives to the model species.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Experimental loop arrays design comparing control, Mg-starved and Mg-resupplied samples.

Fig. S2 MIPS functional distribution of the Mg deficiency-regulated genes.

Fig. S3 Effect of Mg resupply on the expression of differentially regulated genes by Mg starvation.

Fig. S4 Microarray data reliability assessment.

Table S1 Summary of the transcriptomic analysis procedure and cut-off criteria applied.

Table S2 List of genes differentially regulated by Mg starvation in roots.

Table S3 List of genes differentially regulated by Mg starvation in young mature leaves.

Table S4 List of genes whose expression is restored after Mg resupply in roots.

Table S5 List of genes whose expression is restored after Mg resupply in young mature leaves.

Table S6 Promoter region analysis for evening element (EE) and CCA1-binding site (CBS) motifs in genes differentially regulated by Mg starvation.

Table S7 List of genes of the 'oxygen and radical detoxification' MIPS category.

Table S8 List of genes encoding permeases potentially implicated in Mg transport.

Table S9 List of primers used for the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay and microarray data of reconfirmed genes.

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