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RESEARCH PAPER

Establishment of an *in planta* magnesium monitoring system using *CAX3* promoter-luciferase in *Arabidopsis*

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Abstract

The direct determination of elemental concentrations in plants is laborious. To overcome this, a novel monitoring system for magnesium (Mg) in plants was established. Mg deficiency-induced genes were identified by microarray analysis and transgenic lines that expressed luciferase (LUC) under the control of the Mg deficiency-inducible *CAX3* promoter were established. The transgenic lines showed a clear response under low Mg conditions, and the degree of luminescence reflected the accumulation of endogenous *CAX3* mRNA. The *CAX3* expression pattern was also examined in a previously characterized low Mg-sensitive mutant, *mrs2-7*. In *mrs2-7* mutant plants, *CAX3* expression was more than three times higher than in the wild-type. In addition, *CAX3* expression was negatively correlated with the shoot Mg concentration. Together, these results indicate that *CAX3* transcription is a quantitative marker of the Mg status in *Arabidopsis*.

Key words: CAX3, deficiency, luciferase, magnesium.

Introduction

The direct determination of essential nutrient concentrations is an effective way to examine the nutritional status of plants. For example, inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectrometry, and X-ray fluorescence analysis can be used to determine the actual amount of various elements (Salt *et al.*, 2008). However, preparation of the sample in most of these approaches is laborious and time-consuming; moreover, imaging under viable conditions and determining the tissue concentration is difficult.

Measuring the level of transcription of nutrient-responsive genes is another effective way to determine the nutritional status of plants. To make it easier to observe the transcript level, reporter genes such as β-glucuronidase (GUS), green fluorescent protein (GFP), and luciferase (LUC) have been used. Reporter genes are fused with the promoter of a nutritional status-responsive gene, enabling quicker and easier observation of nutritional status compared with direct measurements. To date, reporter gene activity has been used to assess the phosphate, sulphate, and nitrate status in plants, and it has been used for mutant screening. González *et al.* (2005) used the phosphate starvation-responsive gene *IPS1* fused with GUS to observe the phosphate status in *Arabidopsis*, and they screened for mutants displaying constitutive *IPS1* expression. Similarly, the promoter of *SULTR1;2*, a sulphate deficiency-induced gene, was fused with GFP, and SLIM1 (Maruyama-Nakashita *et al.*, 2006), a transcription factor involved in sulphate assimilation, was identified through screening based on GFP fluorescence. Among the reporter genes, LUC is widely used as a reporter in mutant screening because the background is quite low, non-invasive observations can be made, and the dynamic range is wide (de Ruijter *et al.*, 2003). The nitrate-responsive gene *NRT1.2* was fused with LUC and mutants with an

altered nitrate response were isolated (Girin *et al.*, 2010). While reporter genes are a powerful tool for determining nutritional status, they have been applied in a limited number of cases.

Magnesium (Mg) is an essential macronutrient that is required for many enzymatic reactions, including those involving ATPases, kinases, the protein synthetic machinery, and polymerases. Mg, positioned in the centre of chlorophyll, is also a component of the photosynthetic machinery in plants. A characteristic visual symptom of Mg deficiency is intervenial chlorosis in the leaves due to a decrease in chlorophyll, which appears 15 d after the removal of Mg in *Arabidopsis* (Hermans and Verbruggen, 2005). A decrease in Mg in the shoot was observed 1 d after the removal of Mg, following an increase in the sugar content (day 12) and decrease in chlorophyll (Hermans *et al.*, 2010*a*; Hermans and Verbruggen, 2005). A change in the level of transcription has been observed in as little as 24 h. Hermans *et al.* (2010*a*, *b*) performed a microarray analysis of hydroponically-grown *Arabidopsis* plants subjected to short- (4–24 h) and long-term (1 week) Mg deficiency, and they identified Mg deficiency-responsive genes. Genes involved in ethylene biosynthesis, reactive oxygen species (ROS) detoxification, photoprotection of the photosynthetic apparatus, the ABA response, and the circadian rhythm showed an altered expression pattern in response to low Mg. These data indicate that transcriptomic changes are the earliest phenotype of Mg deficiency and can be used as a marker, although their responsiveness is not specific to low Mg (Hermans *et al.*, 2010*b*).

In the present work, low Mg-induced genes were identified by microarray analysis and transgenic lines were established carrying LUC as a reporter, driven by the promoter of the low Mg-responsive gene *calcium/proton exchanger 3* (*CAX3*). The transgenic lines emitted luminescence in response to low Mg. It was also found that *CAX3* expression was correlated with the shoot Mg concentration. Using these transgenic lines, one can readily evaluate the Mg status of plants and isolate mutants with an altered response to low Mg.

Materials and methods

Plant growth conditions and media

Arabidopsis thaliana accession Col-0 obtained from laboratory stock was used for the physiological experiments. T-DNA insertion mutants of *MRS2-7* were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. For the *MRS2-7* mutants, homozygous lines of *mrs2-7(1)* (SALK_064741) and *mrs2-7(2)* (SALK_090559) were used (Gebert *et al.*, 2009). For hydroponic culture, urethane foam, which was covered with polyethylene mesh to prevent the seeds from dropping into the foam, was used for the seed beds. The urethane was set into a transparent plant box with a lid. The seeds were sown on the urethane surface and grown in MGRL solution (without sucrose) (Fujiwara *et al.*, 1992) under a 16/8 h light/dark photoperiod. For agar culture, the seeds were surface-sterilized and grown on MGRL medium containing 1% sucrose (catalogue no. 84097-250G; Sigma, St Louis, MO, USA) solidified with 1.2% purified agar (catalogue no. 01056-15; Nacalai Tesque, Kyoto, Japan) under a 16/8 h light/dark photoperiod. For the hydroponic and agar growth experiments, ultra-pure water was used.

Mg concentration determination by ICP-MS

Plants were washed with distilled water three times, dried at 65 °C, and digested with concentrated nitric acid. After digestion, the sample was dissolved in 0.08 N HNO_3 containing 1 ppb indium as an internal standard and subjected to Mg determination by ICP-MS (SPQ9700; SII, Chiba, Japan).

Microarray analysis and real-time PCR

Total RNA was prepared from the plants using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) and was treated with RNase-free DNase (Qiagen). Microarray analysis was performed using Affymetrix (Santa Clara, CA, USA) ATH1 GeneChips following the manufacturer's protocol. Total RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent kit, diluted 10-fold, and used for real-time PCR analysis with the Dice system (Takara, Ohtsu, Japan) and SYBR Premix Ex Taq II (Takara). The sequences of the primers used in this study are provided in Supplementary Table S1 at *JXB* online. The primers used for *Elongation factor 1α* (*EF1α*, internal standard) were described previously by Takano *et al.* (2006).

Establishment of plants carrying the CAX3-promoter LUC construct and luminescence imaging

Genomic DNA was amplified by PCR using the primers 5′-CACCTGTACAGTTGTACGAATGGTC-3′ and 5′-CTCCAC-GATACTTCCCATGT-3′. The resulting fragment was cloned into pENTR-D/TOPO (Invitrogen, Tokyo, Japan) followed by the destination vector pGWB535 (Nakagawa *et al.*, 2007) to generate the fusion construct with LUC. Col-0 plants were transformed by the floral dip method using *Agrobacterium tumefaciens* strain GV3101:pM90. T_3 or T_4 homozygous lines were selected and used in our experiments.

For luminescence imaging, the plants were sprayed with 1 mM D-luciferin (Wako, Osaka, Japan) containing 0.01% Triton X-100. After 5 min in the dark, the luminescence was observed using LAS3000 (Fujifilm, Tokyo, Japan) and LAS4000 (GE Healthcare, Tokyo, Japan).

Results and discussion

Identification of low Mg-responsive marker genes

To identify low Mg-induced genes, *Arabidopsis* was grown in hydroponic culture. In hydroponic culture, plant growth was strongly inhibited without added Mg (0 μM in Fig. 1A), and Mg concentration-dependent phenotypes were observed (Fig. 1A, B). The Mg concentrations in plants grown at 15 μM Mg were one-fifth of those in plants grown at a normal Mg concentration (1500 μM). Both our growth and Mg concentration data show that the plants were Mg deficient.

Microarray analysis was performed using plants grown in the presence of 15 and 1500 μM (normal) Mg (Fig. 1D; see Supplementary Table S2 at *JXB* online). While the expression of a small number of genes was suppressed by low Mg, a lot of genes were induced under low Mg conditions (Fig. 1D). For a Mg deficiency marker, the fold induction and signal intensity must be high under low Mg conditions. Based on that criterion and/or gene function, five genes were selected for further analysis (a detailed description of

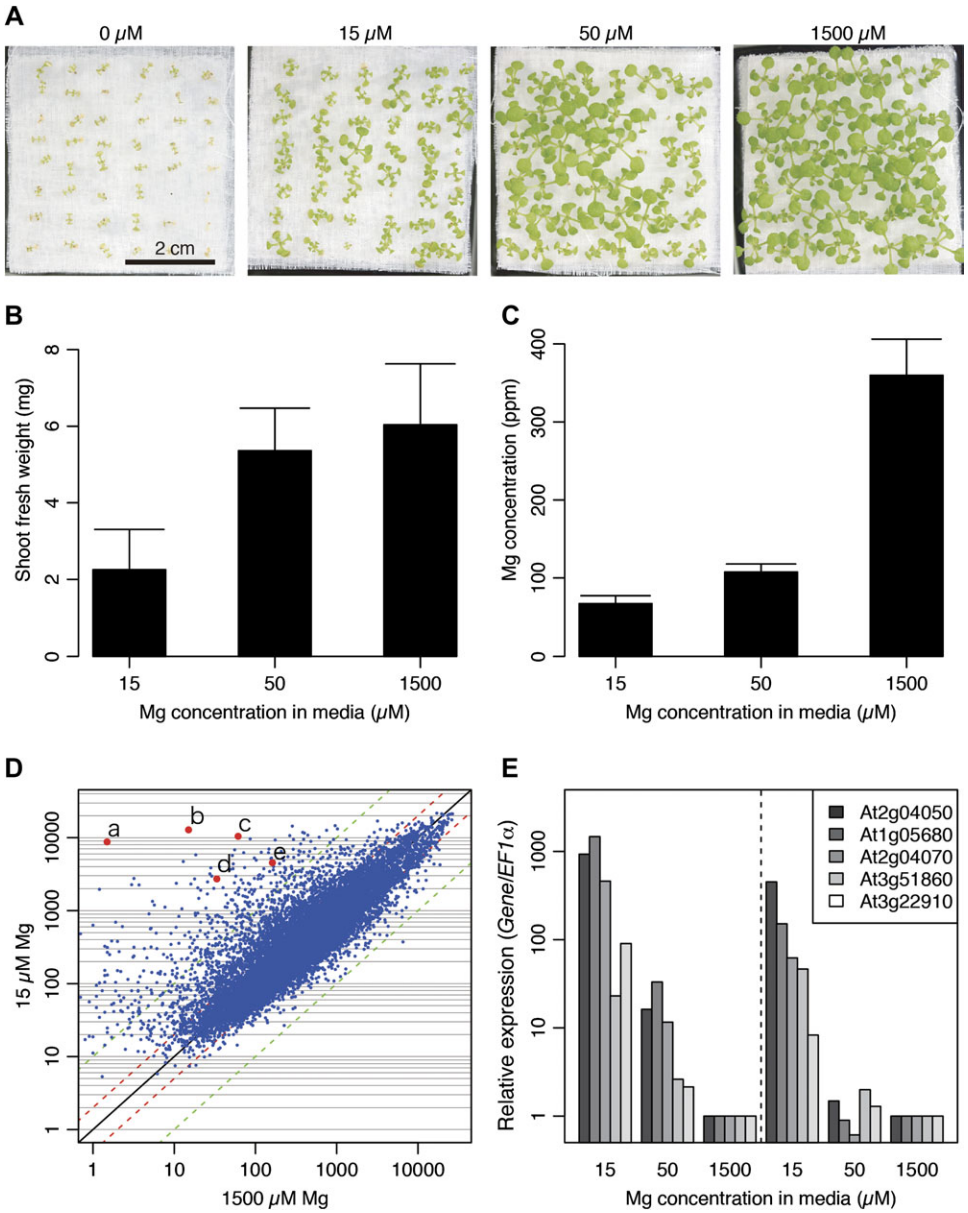

Fig. 1. Screening for marker genes of Mg deficiency. (A) Plants were grown by hydroponic culture in the presence of several concentrations of Mg for 2 weeks. (B) The shoot fresh weight of the plants shown in (A) (n=10). (C) Shoot Mg concentrations of the plants shown in (A) (n=5). The values in (B) and (C) represent means ±SD (n=10 and 5). (D) Scatter plot of the signal intensity values. Total RNA was prepared from shoots grown at 15 and 1500 μM Mg by hydroponic culture and subjected to microarray analysis. Red points show the genes used in our subsequent experiments [a, *AtDTX3* (At2g04050); b, *UGT72E* (At1g05680); c, *AtDTX1* (At2g04070); d, *CAX3* (At3g51860); and e, *ACA13* (At3g22910)]. Red and green dashed lines represent differences of 2-fold and 10-fold from the centre line, respectively. (E) Confirmation of expression by real-time PCR. Total RNA was prepared from two independent experiments and the gene expression assessed. The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 1500 μM Mg.

the genes is given in Supplementary Table S1 at *JXB* online). All of the genes selected were induced by short- or long-term Mg deficiency (Hermans *et al.*, 2010*a*, *b*). Three of them are co-expressed according to the ATTED-II database (Obayashi *et al.*, 2007): two MATE-type transporters [AtDTX3 (At2g04050) and AtDTX1 (At2g04070)] and a UDP-glucronosyl/glucosyl family member [UGT72E4 (At1g05680)], which showed low expression in the presence of a normal Mg concentration and strong expression under low Mg conditions. The other two were CAX3 (At3g51860), a vacuolar calcium (Ca)/proton exchanger (Cheng *et al.*, 2005), and ACA13 (At3g22910), a putative Ca-transporting P2-type ATPase. These genes were selected based on the relationship between Ca and Mg; the Ca content in plants is positively correlated with the Mg content (Broadley *et al.*, 2004; Baxter, 2009). This relationship was observed at the genetic levels. First, the growth of double mutants for *CAX3* and *CAX1*, a homologue of *CAX3*, was partially

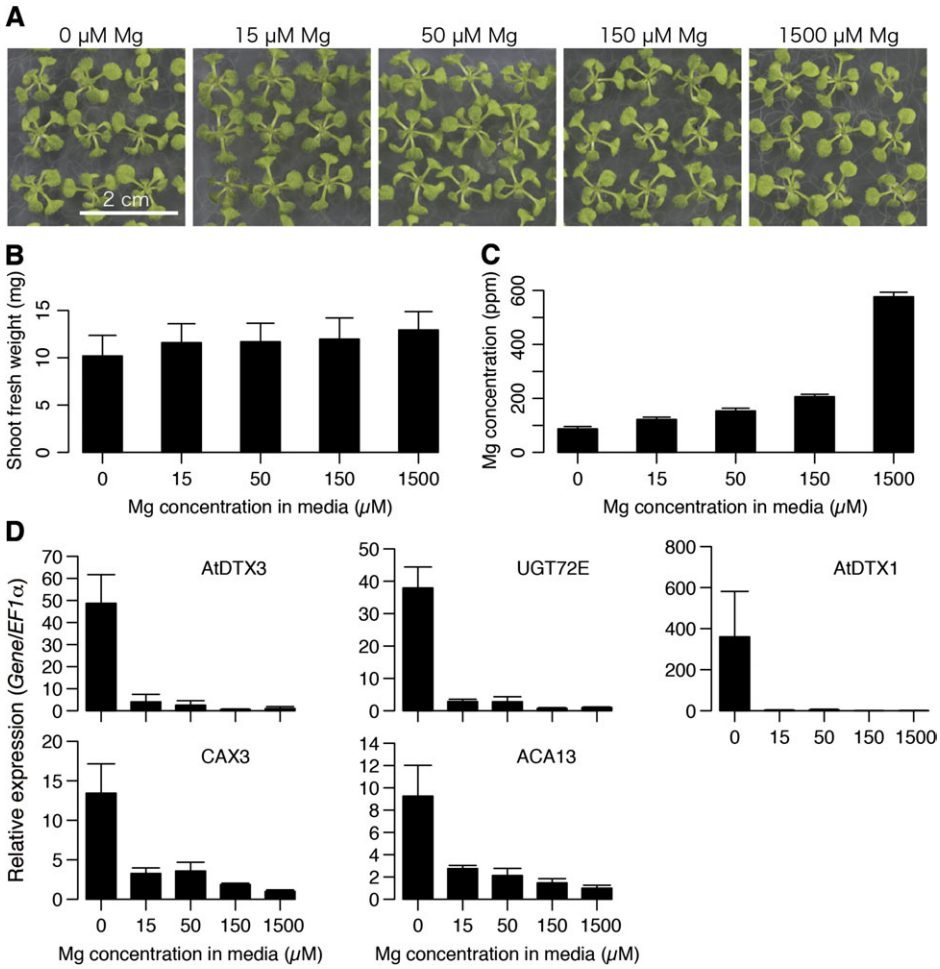

Fig. 2. Growth phenotype of wild-type plants by agar culture. (A) Plants were grown on agar medium containing various concentrations of Mg for 2 weeks. The shoot fresh weight (B) and Mg concentrations (C) of the plants (A) were then determined (n=10 and 5, respectively). (D) Expression levels of selected marker genes in the plants (A) (n=4). The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 1500 μM Mg. The data represent means ±SD.

recovered by excess Mg (Cheng *et al.*, 2005). Second, disruption of *CAX1* conferred serpentine (low Ca:Mg ratio) tolerance to plants (Bradshaw, 2005). Third, the expression levels of an Mg transporter family (MRS) were high in *cax1*/*cax3* double mutant plants (Conn *et al.*, 2011). These data suggest a strong relationship between Ca and Mg.

To confirm the microarray analysis results, real-time PCR was performed using samples prepared from two independent experiments. All of the genes were induced by low Mg (Fig. 1E), as observed in our microarray analysis (Fig. 1D), indicating that they may be suitable as markers of the Mg status in *Arabidopsis*.

The functions of these genes are unclear in terms of the Mg deficiency response. AtDTX1, AtDTX3, and UGT72E4 are induced by salt and UV-B according to the Bio-Array Resource database (http://esc4037-shemp.csb.utoronto.ca/welcome.htm), and by excess boron (Kasajima and Fujiwara, 2007). It has been shown that UGT72E4 expression is also induced by hydrogen peroxide, and that it is involved in auxin homeostasis, through ROS (Tognetti *et al.*, 2010). Because Mg deficiency as well as salt and UV-B have been suggested to induce ROS (Cakmak and Kirkby, 2008), these three genes are likely induced by ROS. *CAX3* and *ACA13* are induced by excess Ca (Chan *et al.*, 2008). Considering that there is no report of Mg transport activity in either transporter and that the Mg concentration is positively correlated with Ca, these transporters may maintain Ca homeostasis disrupted by Mg deficiency.

Phenotypes on agar medium

To determine whether agar could be used to test for Mg deficiency, *Arabidopsis* plants were grown on agar medium. Because it has been reported that agar is contaminated with elements, including Mg (85.3–295.4 μM in 1.2% agar; Jain *et al.*, 2009), purified agar was used in our experiments. The plants were grown on medium containing several concentrations of Mg (Fig. 2A). There was a slight reduction in shoot fresh weight depending on the Mg concentration in medium (Fig. 2B), although there were few visual symptoms under low Mg conditions (Fig. 2A). The shoot Mg concentration in the absence of Mg (0 μM) was five times

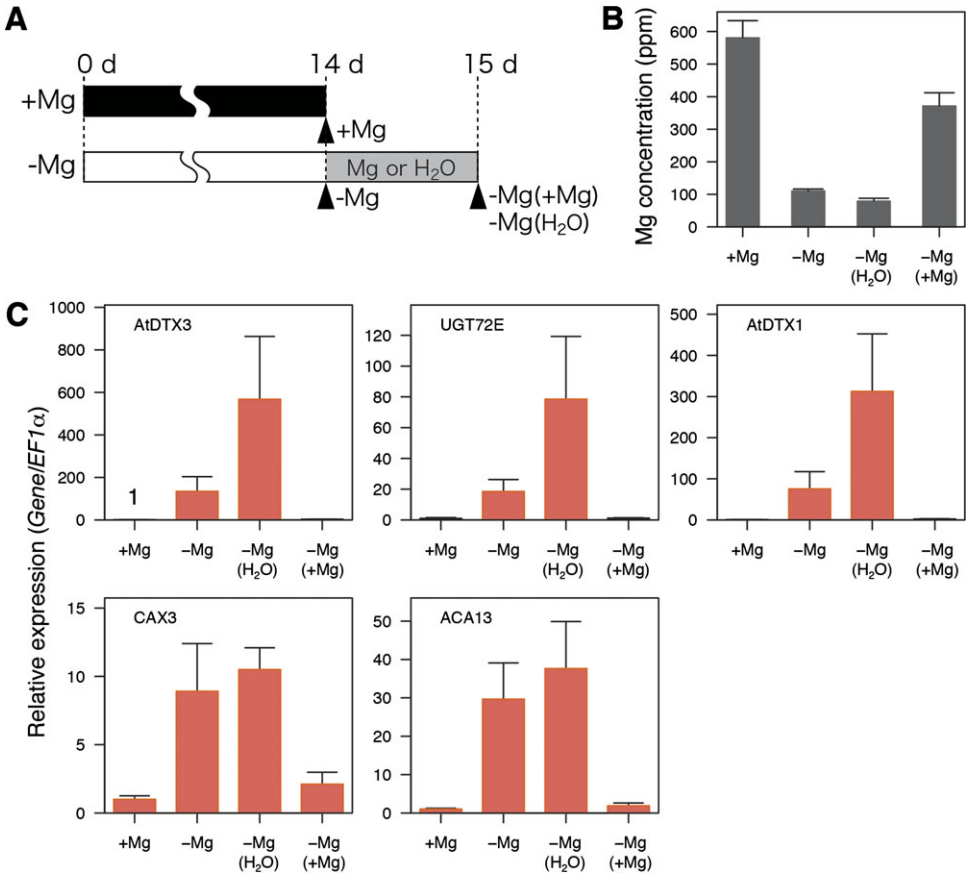

Fig. 3. Rapid response to Mg. (A) Schematic diagram of the experiments. Seeds were sown on agar medium with (+Mg) or without Mg (–Mg) and grown for 2 weeks. Next, Mg was added to the media at a final concentration of 1500 μM [–Mg(+Mg)]. As a control, an equal amount of dH_2O was added [–Mg(H_2O)] to the media. After 24 h, the plants were harvested and the Mg concentration was determined (B) (n=5). (C) Analysis of expression by real-time PCR (n=4). The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 1500 μM Mg (+Mg). The data represent means ±SD.

lower than that with 1500 μM Mg (Fig. 2C), and the shoot Mg content was correlated with the Mg concentration in the medium, indicating that agar medium is sufficient to lower the Mg concentration in plants.

The expression levels of marker genes induced by low Mg were determined by real-time PCR (Fig. 2D) under agar growth conditions. All of the low Mg-induced genes were negatively correlated with the shoot Mg content (Fig. 2C), although the level of induction was smaller than that in plants grown by hydroponic culture. These data indicate that agar medium can be used to assess the Mg status of plants in studies focused on gene expression rather than on visual phenotypes.

The rapid change in plant gene expression in response to Mg was also examined. After growth without Mg for 2 weeks, Mg was added to the medium at a final concentration of 1500 μM. After 24 h, the Mg content and gene expression levels were determined (Fig. 3). The shoot Mg concentration was increased, and reached levels similar to that in plants grown at a normal Mg concentration for 2 weeks (Fig. 3B). Consistent with the shoot Mg concentration, the transcript levels of marker genes were suppressed by supplementation with Mg. These data indicate a rapid response by the genes to the shoot Mg concentration.

LUC luminescence in response to low Mg

Based on the expression pattern and gene annotation, five marker genes were divided into two groups; *AtDTX1*, *AtDTX3*, and *UGT72E4*, and *CAX3* and *ACA13*. From each of these two groups, one gene was selected showing the highest induction by low Mg. *AtDTX1* and *CAX3* were induced by low Mg by 400-fold and 13-fold, respectively. To monitor gene expression in response to low Mg, LUC was used as a reporter gene. For the construction of promoter-LUC plants, genomic regions from –2077 and –2553 to –1 from the first ATG in *AtDTX1* (At2g04070) and *CAX3* (At3g51860) were used as the promoter region, respectively. Although homozygous lines for both constructs were obtained, LUC luminescence in response to low Mg was observed only in the *CAX3* transgenic lines (Fig. 4; data not shown). Three independent lines (4-1, 6-5, and 9-3) were used in our subsequent experiments. After 2 weeks of growth with or without Mg, LUC luminescence was observed using a CCD camera. As shown in Fig. 4A, the level of luminescence was high under low Mg conditions in all lines compared with that in plants grown under normal Mg conditions. The transcription of *LUC* was induced by low Mg, and the level of induction was similar

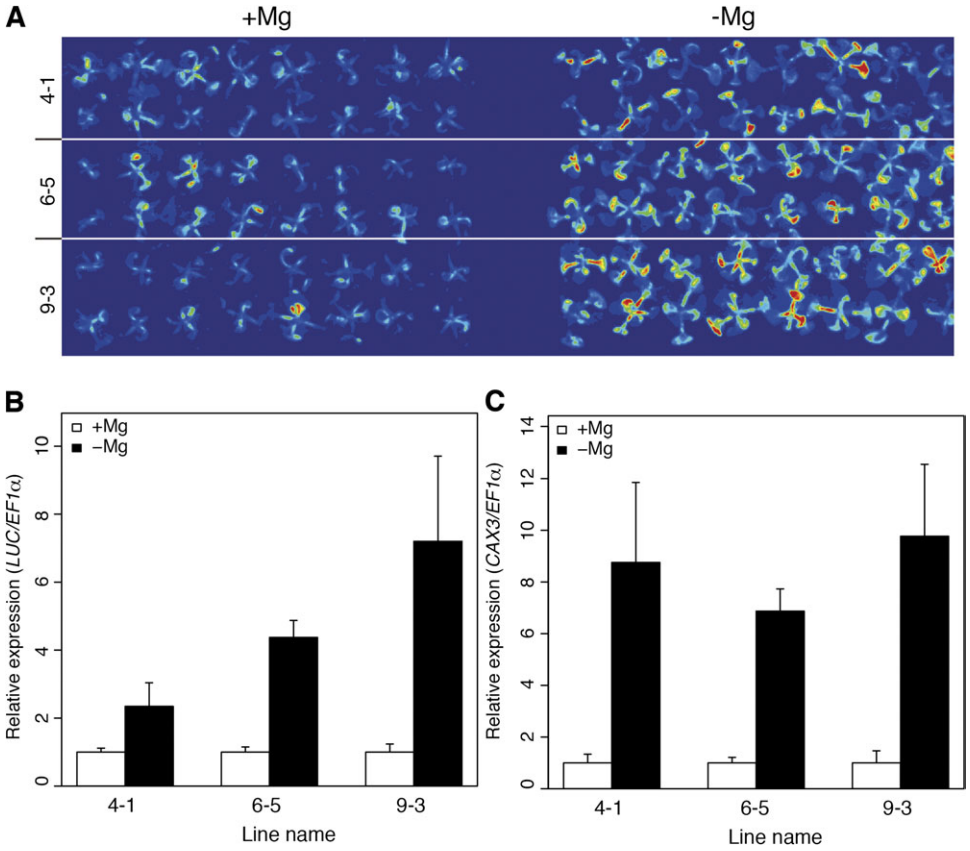

Fig. 4. LUC luminescence of transgenic plants carrying *CAX3* promoter-LUC. (A) Luminescence from plants grown with or without Mg. The seeds were sown on medium with or without Mg. After 2 weeks, a luciferin solution was sprayed onto the plants and the luminescence was observed using an LAS3000 imaging system. The expression levels of *LUC* (B) and *CAX3* (C) in the transgenic plants shown in (A). The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value at 1500 μM Mg (+Mg). The data represent means ±SD (n=3).

to that for *CAX3* (Fig. 4B, C), indicating that LUC luminescence reflected the level of transcription of endogenous *CAX3*. It should be noted that the luminescence does not completely reflect the endogenous *CAX3* expression in terms of expression site: The induction of LUC luminescence was not observed in roots (see Supplementary Fig. S1 at *JXB* online), while the *CAX3* transcript was highly accumulated in roots (Shigaki and Hirschi, 2000).

The specificity of the CAX3 response was observed using promoter-LUC transgenic lines. It has been reported that *CAX3* was induced by excess Ca, Mg, Na, and phosphate deficiency, and *cax3* mutant plants showed sensitivity to low pH and NaCl stress (Shigaki and Hirschi, 2000; Zhao *et al.*, 2008; Liu *et al.*, 2011). The transgenic lines were grown on a variety of conditions: 50 mM and 100 mM NaCl, excess Ca (10 mM), phosphate deficiency, and low pH (pH 4.2). The induction of luminescence was observed by excess Ca and phosphate deficiency (see Supplementary Fig. S1 at *JXB* online). There are two apparent inconsistencies in our experiments with previous reports. One is that NaCl did not induce the luminescence. The other is that the induction of luminescence was observed in shoots under phosphate deficiency conditions, whereas the *CAX3* transcripts were accumulated only in roots by phosphate deficiency (Liu *et al.*, 2011). This might be due to the possibilities that the promoter region we used is not long enough to reflect the endogenous expression of *CAX3* fully or that the details of the growth conditions in the previous reports could be different from ours.

Although the *cax3* mutant plants showed phenotypes under the conditions in which the *CAX3* expression was affected, Mg deficiency did not affect the growth of *cax3* mutant plants in our experimental conditions (see Supplementary Fig. S2 and Supplementary Table S3 at *JXB* online).

Characterization of mrs2-7 *mutant plants*

The best way to demonstrate the availability of *CAX3* as a marker of the Mg status in plants is to assess the behaviour of *CAX3* in known mutants showing an altered phenotype in response to low Mg. *MRS2-7*, a Mg transporter localized to the endomembrane, is the only gene the disruption of which inhibited plant growth under low Mg conditions. Gebert *et al.* (2009) examined the phenotypes of plants grown by hydroponic culture and found few differences in the shoot element content under normal conditions. As the growth conditions of Gebert *et al.* (2009) were different from ours and they did not examine the Mg concentration under low Mg conditions, *mrs2-7* mutant plants (*mrs2-7[1]* and *mrs2-7[2]*) were first characterized under our experimental conditions. When grown on agar

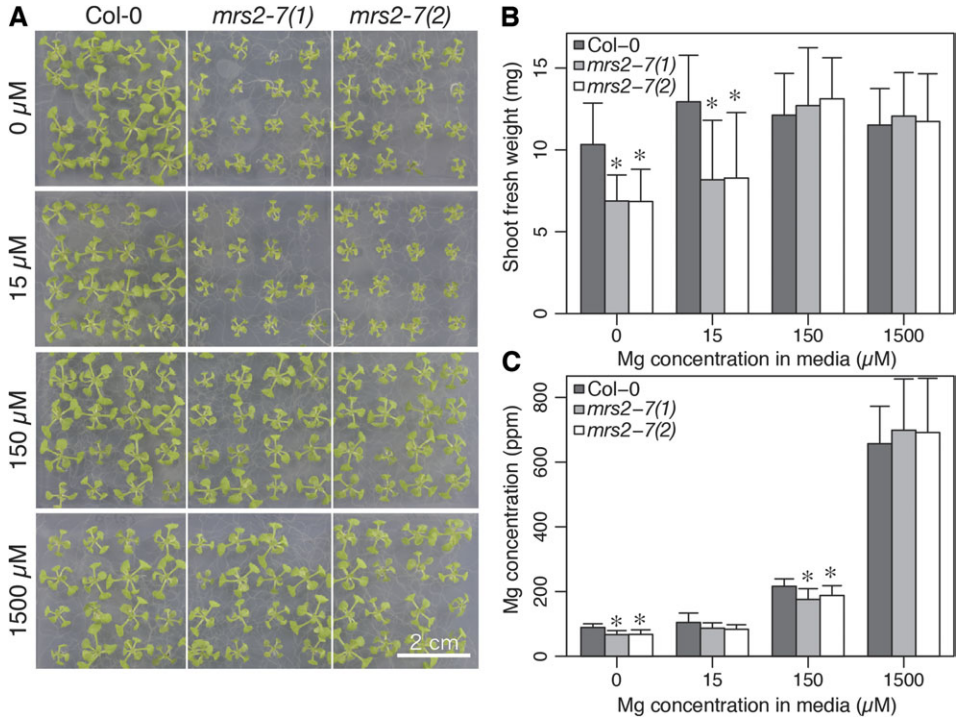

Fig. 5. Characterization of *mrs2-7* mutant plants on agar medium. (A) Growth phenotype of *mrs2-7* on agar. The seeds were sown on agar media containing the indicated concentrations of Mg. After 2 weeks, the shoot fresh weight (B) (n=20) and Mg concentrations (C) (n=10) were measured. The data represent means ±SD. Asterisks indicate a significant difference compared with Col-0 plants ($P < 0.05$, Student's t test).

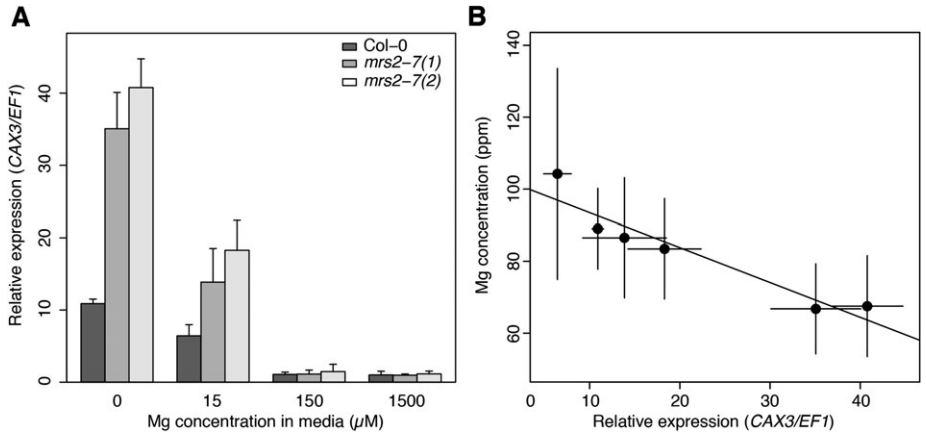

Fig. 6. Expression of *CAX3* in *mrs2-7* mutant plants. (A) The seeds were sown on media containing the indicated concentrations of Mg. After 2 weeks, the shoots were subjected to expression analysis by real-time PCR. The mRNA levels were normalized to the level of *EF1α* and expressed as a ratio relative to the value in Col-0 grown in the presence of 1500 μM Mg. The data represent means ±SD (n=4). (B) Negative correlation between *CAX3* expression and the Mg concentration in shoots grown at 0 and 15 μM Mg. Our CAX3 expression data are shown in Fig. 6A; our Mg concentration data are shown in Fig. 5C. The correlation factor was –0.94.

medium, the mutant plants displayed a clear visual phenotype after 2 weeks (Fig. 5A) and the shoot fresh weight was lower than in the wild type (Fig. 5B), indicating that our medium may be used to assess Mg deficiency in plants.

It was also found that the Mg contents were low in the mutant plants compared with the wild type only under low Mg conditions (Fig. 5C), supporting the function of *MRS2-7* as an Mg transporter at low Mg concentrations. These data indicate that *mrs2-7* may be used as a model of the altered response to low Mg.

Finally, *CAX3* expression was examined in *mrs2-7* mutant plants to determine the utility of using *CAX3* expression to monitor the Mg status in plants. After growing wild-type and *mrs2-7* mutant plants in the presence of several concentrations of Mg, *CAX3* expression was assessed by real-time PCR (Fig. 6A). At 0 μM Mg, *CAX3*

transcription was induced by 35 to 41-fold in the *mrs2-7* plants, while the transcript level in the wild type was 11-fold. The *CAX3* transcript level in *mrs2-7* is more than three times higher than that in the wild type. These data indicate that *CAX3*-promoter LUC transgenic lines can be used as a tool to isolate mutants with an altered response to conditions of Mg deficiency.

Under those conditions in which *CAX3* induction was observed (0 and 15 μM Mg), there was a negative correlation between *CAX3* expression and the shoot Mg concentration (Fig. 6B), indicating that the *CAX3* transcript level reflects the shoot Mg concentration. In addition, the change in *CAX3* expression (5-fold) was larger than the change in Mg concentration (1.7-fold). Thus, CAX3 expression amplifies the difference in Mg concentration. The negative correlation and clear difference in *CAX3* expression under low Mg conditions may make it possible to observe the Mg status in plants with ease and precision.

Conclusions

In this work, Mg deficiency-inducible genes, including *CAX3*, were identified and *CAX3* promoter-LUC transgenic lines were established. The transgenic lines showed a clear response to low Mg in terms of luminescence, and the expression pattern was similar to that of endogenous CAX3. In addition, CAX3 transcription was strong in the Mg deficiency-sensitive mutant *mrs2-7* compared with the wild type. These data indicate the efficacy of using *CAX3* expression to assess the Mg status of plants and transgenic lines for Mg mutant screening.

Although *CAX3* induction is not specific to low Mg (it is induced by excess Ca, Mg, and Na) (Shigaki and Hirschi, 2000), when combined with other experimental approaches, including the direct determination of the Mg concentration and phenotype under Mg-limited conditions by hydroponic culture, it may be possible to isolate genuine Mg-related mutants. Mutant plants have been prepared for screening by EMS mutagenesis to examine the mechanism of Mg homeostasis in plants.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Primer sequences for real-time PCR.

Supplementary Table S2. Microarray data.

Supplementary Table S3. Components of the media used in Fig. S1.

Supplementary Fig. S1. Specificity of *CAX3* expression.

Supplementary Fig. S2. Phenotype of *cax3* mutant plants.

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