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ClpC1, an ATP-dependent Clp protease in plastids, is involved in iron homeostasis in *Arabidopsis* leaves

Huilan Wu[1], Yanyan Ji[1], Juan Du[1,2], Danyu Kong[1,2], Hui Liang[1] and Hong-Qing Ling[1,*]

[1]*The State Key Laboratory of Plant Cell and Chromosome Engineering, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, China and* [2]*Graduate School of the Chinese Academy of Sciences, Yuquan Road, Shijingshan District, Beijing 100049, China*
** For correspondence. E-mail hqling@genetics.ac.cn*

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• *Background and Aims* Iron (Fe) is necessary for plant growth and development. Although it is well known that Fe deficiency causes chlorosis in plants, it remains unclear how the Fe homeostasis is regulated in mesophyll cells. The aim of this work was to identify a gene related to Fe homeostasis in leaves.
• *Methods* A spontaneous mutant *irm1*, which revealed typical Fe-deficiency chlorosis, was found from *Arabidopsis thaliana*. Using map-based cloning, the gene responsible for the altered phenotype of *irm1* was cloned. The expression of genes was analysed using northern blot hybridization and multiplex RT-PCR analysis. Further, GUS staining with transgenic promoter-GUS lines and transient expression of the fusion protein with GFP were used for detecting the expression pattern of the gene in different tissues and at different developmental stages, and for the subcelluar localization of the gene product.
• *Key Results* A point mutation from G to A at nucleotide 2317 of *ClpC1* on chromosome V of *Arabidopsis* is responsible for the *irm1* phenotype. The leaf chlorosis of the mutant *irm1* and *clpc1* (a T-DNA-inserted null mutant of *ClpC1*) could be converted to green by watering the soil with Fe solution. The expression intensity of ferric reductase *FRO8* in *irm1* and *clpc1* was disordered (significantly higher than that of wild type).
• *Conclusions* The glycine residue at amino acid 773 of ClpC1 is essential for its functions. In addition to its known functions reported previously, ClpC1 is involved in leaf Fe homeostasis, presumably via chloroplast translocation of some nuclear-encoded proteins which function in Fe transport.

Key words: *Arabidopsis*, ClpC1, iron homeostasis, chloroplasts, Clp protease.

INTRODUCTION

Iron (Fe), an essential mineral nutrient for plant growth and development, functions as a component of many important enzymes and proteins involved in fundamentally biochemical processes, such as respiration and photosynthesis (Briat *et al.*, 1995). Deficiency of Fe leads to chlorosis in young leaves and retarded growth, consequently resulting in reduced crop productivity and food quality. Although Fe is one of the six most abundant elements in the earth's crust, Fe deficiency is a worldwide problem for crop production on calcareous soils because it exists primarily in an oxidized ferric form [Fe(III)], which is not available to plants due to its low solubility. Therefore, plants have to activate specific mechanisms to mobilize and take up Fe from the soil. Dicotyledonous plants such as *Arabidopsis* use a reduction mechanism known as the strategy I mechanism (Römheld and Marschner, 1986) to acquire Fe from soils under stress of Fe limitation effectively. The reduction of ferric to ferrous Fe on root surface and subsequent transfer of ferrous ion into root cells are mainly accomplished by plasmalemma ferric chelate reductase FRO2 (FERRIC REDUCTASE/OXIDASE 2) (Robinson *et al.*, 1999) and metal transporter IRT1 (IRON-REGULATED TRANSPORTER 1) (Eide *et al.*, 1996; Henriques *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002). The expression of the two major Fe uptake genes in *Arabidopsis* is regulated by transcription factors FIT, bHLH38 and bHLH39 (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005, 2008). FIT interacts with bHLH38 or bHLH39, forming heterodimer FIT-bHLH38 or FIT-bHLH39, which directly functions in the activation of *IRT1* and *FRO2* transcription (Yuan *et al.*, 2008).

In the cell, Fe is compartmentalized into different organelles, such as chloroplasts, mitochondria and vacuoles, for utilization or storage. In rapidly growing leaves, about 80 % of the Fe is localized in the chloroplasts, where Fe is involved in electron transport, photosynthesis, haem biosynthesis and Fe–S clusters (Kim and Guerinot, 2007). Although the necessity for Fe in the chloroplast has been clearly established, the processes involved in Fe homeostasis in this organelle are still relatively unknown. Recently, it was shown that *AtPIC1* is involved in chloroplast Fe transport and that *AtFRO7* is responsible for Fe reduction in chloroplasts (Duy *et al.*, 2007; Jeong *et al.*, 2008). The mitochondrion is also an organelle with a high demand for Fe (Briat *et al.*, 2007) but the Fe transporters and reductase(s) involved have not been identified. Generally, it is unclear how Fe is regulated across the envelopes of chloroplasts and mitochondria.

ClpC [the subfamily C of Clp (caseinolytic protease) proteins] is a component of ATP-dependent proteases. As a molecular chaperone it plays an important role in protein quality control (Adam and Clarke, 2002; Hengge and Bukau, 2003; Constan *et al.*, 2004). In higher plants, there are two chloroplast-localized ClpC proteins (ClpC1 and ClpC2).

These two proteins possess similar functions because overexpression of *ClpC2* was able to complement the *clpc1* mutant to wild type (Kovacheva *et al.*, 2007). Knocking out *ClpC2* by T-DNA insertion, the mutant did not show distinct phenotypic changes (Park and Rodermel, 2004; Kovacheva *et al.*, 2007), whereas loss of function of *ClpC1* in the *clpc1* mutant showed as clearly stunted growth with pale green leaves and a substantial reduction in accumulation in PSI and PSII (Constan *et al.*, 2004; Sjogren *et al.*, 2004). These suggest that *ClpC1* is more important than *ClpC2* in their biological functions.

Here, a new *ClpC1* mutant was characterized and the glycine at the position of amino acid 773 of ClpC1 was identified as essential because substitution of the glycine to arginine via a point mutation exhibited the same phenotypes as the loss-of-function mutant generated by T-DNA insertion. It was also found that the leaves in the two mutants could be changed from pale green to a normal shade of green by watering the soil with Fe, indicating that ClpC1 is involved in Fe homeostasis in leaves.

MATERIALS AND METHODS

Plant growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Columbia), *irm1* mutant, transgenic and T-DNA insertion lines *clpc1* (SALK_014058, obtained from the Nottingham Arabidopsis Stock Center) were surface-sterilized with 3 % commercial bleach for 10 min and then washed three times with distilled water. After vernalization at 4 °C in the dark for 2 d, the seeds were sown on plates with MS basal salt medium (Murashige and Skoog, 1962) supplemented with 3 % sucrose and 0·6 % phytogel at pH 5·8 and germinated at 23 °C with a 16-h light period for 7 d. The seedlings were then used for further analysis.

For seed harvest and hybridization, *Arabidopsis* plants were grown in potting soil mixture and kept in growth chambers at 22 °C with illumination at 120 mmol m^{-2} s^{-1} for a 16-h daily light period. The relative humidity was approx. 70 % ($\pm$ 5 %). For nutrition experiments, seedlings were grown in potting soil watered with 100 μM FeEDTA, 100 μM $MnSO_4$ or 30 μM $ZnSO_4$ every 3 d.

Map-based cloning of IRM1

The *irm1* (Columbia background) mutant was crossed with Landsberg *erecta* to create mapping populations. The plants with mutation phenotypes were selected and used for genetic mapping of *IRM1* with simple sequence length polymorphism (SSLP) markers, which were designed based on information from the Monsanto Arabidopsis Polymorphism Database (Jander *et al.*, 2002).

Vector constructions and Arabidopsis *transformation*

The P_{ClpC1}::*GUS* construction was generated by fusing the *ClpC1* promoter (1·699 kb), which was amplified with primers 5′-ccatcatcttggGTCATCCTCTGTTTCTCAAG-3′ and 5′-cccgggGACTTCCTAAATAAATT-3′ from genomic DNA, in front of the *β-glucuronidase* (*GUS*) coding sequence in pCAMBIA1381 vector. To generate complementation lines of the *irm1* mutant, a 7·3-kb fragment including the native promoter and the *ClpC1* gene was cut out from BAC (bacterial artificial chromosome) clone K3K7 with restriction endonucleases *Sal*I and *Bgl*II and cloned into pCAMBIA1391 vector. *Arabidopsis* transformation via *Agrobacterium tumefaciens* strain (GV3101) was carried out by the floral dip method (Clough and Bent, 1998).

Subcellular localization of ClpC1

To investigate the subcellular location of ClpC1, the coding sequence of *ClpC1* was amplified from *A. thaliana* cDNA by PCR using the 5′-aagcttATGGCTATGGCCACAAGGGT-3′ and 5′-GGATCCAATGTAATTGTGACCAAG-3′ primers. The PCR product was cloned into the pGEM T-easy vector (Promega, USA). After verification by sequencing, the sequence of *ClpC1* was excised by *Hin*dIII and *Bam*HI and cloned into the pJIT163–*hGFP* vector to generate the ClpC1–GFP fusion protein (GFP at the C-terminus of ClpC1) expression plasmid pJIT163–*ClpC1–hGFP*. For transient expression in onion epidermis cells, 5 μg of the pJIT163–*ClpC1–hGFP* plasmid was used to bombard the onion epidermis cells according to a described procedure (Weigel and Glazebrook, 2002). For transient expression in cowpea protoplasts, 10–15 mg of the purified pJIT163–*ClpC1–hGFP* plasmid was used to transform cowpea mesophyll protoplasts by PEG transformation according to a described procedure (Shah *et al.*, 2002). The GFP fluorescence in onion epidermis cells and in cowpea protoplasts were detected using a confocal laser scanning microscope (Olympus, FV500, Japan). The GFP fluorescence was observed at an excitation wavelength of 488 nm and an emission wavelength of 506–538 nm, and the autofluorescence of chloroplasts was observed at an excitation wavelength of 488 nm and an emission wavelength of 664–696 nm.

GUS activity assay

Five independent transgenic lines were selected for the GUS staining analysis. Seedlings were grown on MS plates without antibiotics until they reached three-to-four-true-leaf stage. One true leaf from a single plant was excised and examined by histochemical GUS staining. Seedlings with positive GUS signals were transferred to soil and grown further for GUS assays on the developing seeds. The progenies of these plants were plated on MS medium and sampled during germination and early seedling stages. For the metal-deficiency experiments, 4-d-old seedlings were transferred onto MS medium lacking Fe, zinc (Zn) or manganese (Mn). After growing for 4 d in the growth chamber with a 16-h light period, plants were harvested. For the histochemical GUS assay, plant tissues were stained by submersion in staining solution (Jefferson *et al.*, 1987).

Northern blot analysis

Total RNA was extracted using the Trizol reagent from roots and shoots of plants cultivated in MS medium with and

TABLE 1. *Primers used in the Multiple RT-PCR experiment in this paper*

Gene name	Forward primer sequence	Reverse primer sequence
FRO6	5′-AGGTGACACTATAGAATACACATTTGTCTCCTCCAAGA-3′	5′-GTACGACTCACTATAGGGAATAGCCTAAACCTCGGGA-3′
FRO7	5′-AGGTGACACTATAGAATATCGGTGATCAAATCCTCACA-3′	5′-GTACGACTCACTATAGGGACAGCTGCAGAAACAACTCCA-3′
FRO8	5′-AGGTGACACTATAGAATAATGGCTTGCCACATTAGTCC-3′	5′-GTACGACTCACTATAGGGAATAGTTGCAGCGAAACCACC-3′
PIC1	5′-AGGTGACACTATAGAATAACTGCTAGTGGGATTGCTGC-3′	5′-GTACGACTCACTATAGGGACCGAGTAGTGCTGATCCCAT-3′
ClpC1	5′-AGGTGACACTATAGAATAGTGCGACAGGCAATGAATG-3′	5′-GTACGACTCACTATAGGGACCCTCACCAATAAGACCA-3′
ClpC2	5′-AGGTGACACTATAGAATAGCTCTTCTTACACCTCCTTT-3′	5′-GTACGACTCACTATAGGGAATCATCTTCACACGCCCT-3′
Nramp3	5′-AGGTGACACTATAGAATATTGCGTTTTTAGATCCAGGG-3′	5′-GTACGACTCACTATAGGGAAACCAAAAGACCCATTGCTG-3′
Nramp4	5′-AGGTGACACTATAGAATAAGCAAATCATGGGCAGTTTC-3′	5′-GTACGACTCACTATAGGGAGAAGCCACTAGTTGCCAAGG-3′
ACTIN2	5′-AGGTGACACTATAGAATACTGGATTCGCTGGAGATGAT-3′	5′-GTACGACTCACTATAGGGACTTCAGGAGCAATACGGAGC-3′

without Fe supply following the instructions of the manufacturer (Invitrogen, USA). For northern blot analysis, 10 μg of total RNA was denatured and electrophoresed on a 1·2 % 3-(*N*-morpholino)-propane-sulfonic acid/formaldehyde/agarose gel, and then blotted onto a nylon Hybond-N^+ membrane according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). The probes, which were amplified by PCR using primers (5′-GTTCCCAATGATGGTCGTAGC-3′ and 5′-TTGATGCTTGACTCCGAGGTT-3′ for *AtFRO2*, 5′-TCTCTCCAGCAACTTCAACT-3′ and 5′-AATGACTCGG TATCGCAAGA-3′ for *AtIRT1*, and 5′-CCGGAGATAGATA GAGAGAG-3′ and 5′-CGATTCCAGTACCCTCACCA-3′ for *ClpC1)* from cDNA, were labelled with [^{32}P]dCTP. The hybridization, probe labelling and membrane washing were performed as described by Church and Gilbert (1984). Subsequently, the membranes were exposed to Fujifilm (BAS-SR2025, Tokyo, Japan) in an X-ray imaging plate under −70 °C for 1–3 d.

Multiplex RT-PCR analysis

For expression analysis of *PIC1*, *FRO6*, *FRO7* and *FRO8*, total RNA was extracted from the leaf of 2-week-old seedlings grown on soil. For expression analysis of *ClpC1*, *ClpC2*, *NRAMP3* and *NRAMP4*, RNA was extracted from 3-d-old seedlings grown on an MS plate. The expression pattern of these genes was detected using the GenomeLab GeXP analysis system multiplex RT-PCR assay (Beckman, Fullerton, CA, USA) according to the protocol described by Chen *et al.* (2007). The housekeeping gene *ACTIN2* (*At1g49240*) was used as the control. The gene expression data obtained from multiplex RT-PCR were normalized via dividing the peak area value of each gene by the peak area of *ACTIN2*. The primers used in the multiplex RT-PCR reactions are listed in Table 1.

Elemental analysis

Wild-type and *irm1* were grown on soil under a 16-h light period. Plant tissues were pooled from three plants and assayed as a group. Each experimental set was composed of five groups. Leaves were collected when plants started to bolt, and dried overnight in an oven at 65 °C after washing with ultra-pure water. Seeds were harvested when they were fully dry. The concentrations of various metals were determined using inductively coupled plasma spectroscopy (Perkin Elmer, USA) according to methods published previously (Yuan *et al.*, 2008). The means and standard deviations were calculated from three biological replications.

Electron microscopy

For observation of chloroplast structure, new true leaves of the seedlings grown on soil were harvested. Leaves were cut into approx. 1-cm-long segments and then washed in 0·5 mM $CaSO_4$. The segments were fixed overnight in 0·1 mM potassium phosphate buffer, pH 7·4, containing 0·2 % (w/v) glutaraldehyde and 1·5 % (w/v) paraformaldehyde. After being rinsed three times in 0·1 mM potassium phosphate buffer, pH 7·4, the tissue was dehydrated through a graded ethanol series of 20 % and 40 %, and post-fixed in 0·25 % (w/v) osmium tetroxide for 2 h in 40 % ethanol at 4 °C. Leaf segments were washed again in 40 % ethanol three times (each 10 min) and treated in a solution with 0·3 % (w/v) uranyl acetate in 40 % ethanol for 2 h at 4 °C. The material was washed again twice in 40 % ethanol (10 min each) and once in 50 % ethanol for 10 min. The samples were then dehydrated in an ethanol series of 75 % and 90 %, twice at 100 %, for 30 min each, infiltrated with LR White resin, and polymerized at 50 °C for 24 h in vacuum. Ultra-thin sections were cut with a microtome (Ultracut E; Reichert, Vienna) and stained with uranyl acetate and lead citrate. Semi-thin sections were stained with toluidine blue. Sections used for electron microscopy were examined in an electron microscope (EM 902; Zeiss, Jena, Germany).

Chlorophyll isolation and quantification

Whole plants of wild type, *irm1* and *clpc1* grown under normal and Fe-supplemented (100 μM FeEDTA) conditions were harvested 4 weeks after germination and weighed. They were then ground with sand in 80 % acetone. The ground tissue was spun at approx. 2000 *g* for 5 min to remove the sand and other debris from the extracted chlorophyll. The supernatant was then measured spectrophotometrically at 645 nm and 663 nm, respectively. The amount of chlorophyll (mg g^{-1}) in each sample was determined using the equation given in Arnon (1949).

RESULTS

Isolation and characterization of irm1 *mutant*

A plant with chlorotic leaves was found from the segregation population of a heterozygote T-DNA insertion line of

AtFRO4 in *A. thaliana* ecotype Columbia (Col-0) grown in a growth chamber. PCR analysis showed that no T-DNA fragment was inserted in *AtFRO4* of this plant (data not shown). To purify the genetic background, the mutant was backcrossed with Col-0 five times. Then, it was used for further analysis. The mutant displayed yellow cotyledons at an earlier stage of germination (3 d) on MS medium than wild type (Fig. 1A). With extension to germination, the cotyledons of the mutant turned increasingly green (Fig. 1B–D). By the 10th day, the mutant plant revealed green cotyledons and true leaves like the wild type on MS medium (Fig. 1E). After transplantation to soil, the mutant showed pale green leaves and retarded growth (Fig. 1F). To test whether the pale green of the mutant phenotype on soil is due to deficiency of some mineral nutrients, additional Fe, Zn and Mn were supplied to the soil by watering. Interestingly, the pale leaves of the mutant turned to green 3 d after watering with Fe, but not by watering with Zn or Mn (Fig. 1G). Therefore, this mutant was called *i*ron-*r*escued *m*utant 1 *(irm1)*. Subsequently, *irm1* was crossed with wild-type Col-0. The F_1 plants displayed normal growth like the wild type and segregation of wild type and mutant phenotype was observed in F_2 at a ratio of 3 : 1 (data not shown), demonstrating that the altered phenotype in *irm1* is caused by a recessive mutation in a single nuclear-encoded gene. To determine whether the mutation of *IRM1* affects the expression of Fe-uptake genes, the expression of *FRO2* and *IRT1* was examined by northern-blot analysis. As shown in Fig. 2A, the expression pattern and intensity of *FRO2* and *IRT1* in *irm1* was the same as the wild type, indicating that the mutation of *IRM1* did not affect the expression of the two marker genes for Fe uptake. To demonstrate further whether *IRM1* is involved in Fe homeostasis, the Fe content of mutant seeds and leaves was determined. The Fe content in leaves of *irm1* was about 20 % higher than that of wild type (Fig. 2B) whereas no difference was observed in seeds between the mutant and wild type (Fig. 2C). The content of Zn and Mn in leaves was also measured. It was higher in leaves of *irm1* than that of wild type, but the difference was not significant (Fig. 2B).

Considering the pale green phenotype of *irm1* leaves of soil-grown plants, their chlorophyll content was determined. Consistent with the phenotype, the chlorophyll content of *irm1* ($132{\cdot}3 \pm 8{\cdot}03$ μg Chl g^{-1} f. wt) was approx. 43 % lower than that of wild type ($232{\cdot}3 \pm 5{\cdot}83$ μg Chl g^{-1} f. wt). Further, the chloroplast ultrastructure in young leaves of *irm1* plants grown on soil was investigated using transmission electron microscopy. Chloroplasts of *irm1* revealed a shape similar to those seen in wild type, such as characteristic lens-like shape and well-developed thylakoid membrane system with numerous grana stacks (Fig. 3A, B). However, the leaves of the *irm1* mutant revealed fewer chloroplasts per cell ($4{\cdot}2 \pm 0{\cdot}3$) than those of the wild type ($9 \pm 0{\cdot}8$; Fig. 3C, D). When watered with Fe, the chloroplast number in leaf cells of *irm1* was restored to the level of the wild type (Fig. 3E, F).

A point mutation in ClpC1 *is responsible for the* irm1 *phenotype*

To identify the gene responsible for the *irm1* phenotype, *irm1* was crossed with Landsberg *erecta* and mapped using

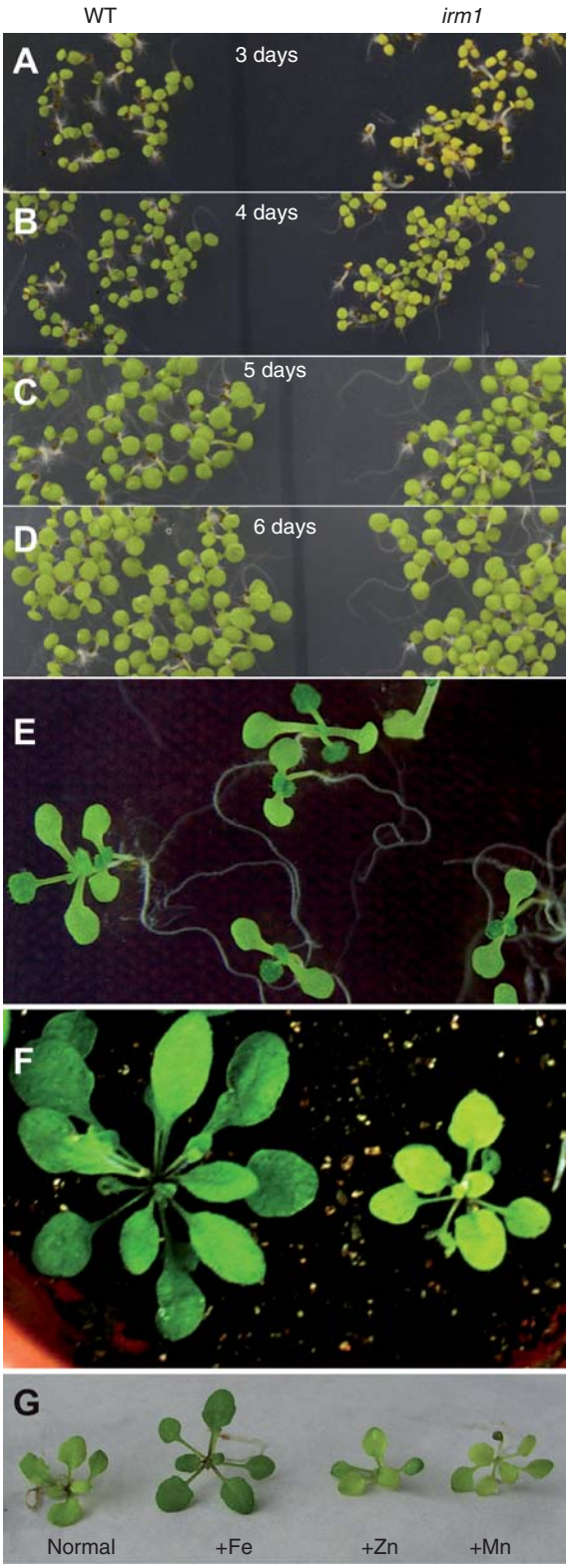

FIG. 1. Phenotypic characterization of *irm1* mutant: (A–D) phenotype comparison between *irm1* and wild type at an early stage of germination (3, 4, 5 and 6 d) on an MS agar plate; (E) *irm1* seedlings grown on an MS agar plate for 10 d; (F) wild type and *irm1* grown on soil for 2 weeks; (G) phenotype of *irm1* on soil (normal) and when watered with 100 μM FeEDTA (+Fe), 30 μM $ZnSO_4$ (+Zn) or 100 μM $MnSO_4$ (+Mn). The picture was taken on the 3rd day after treatment.

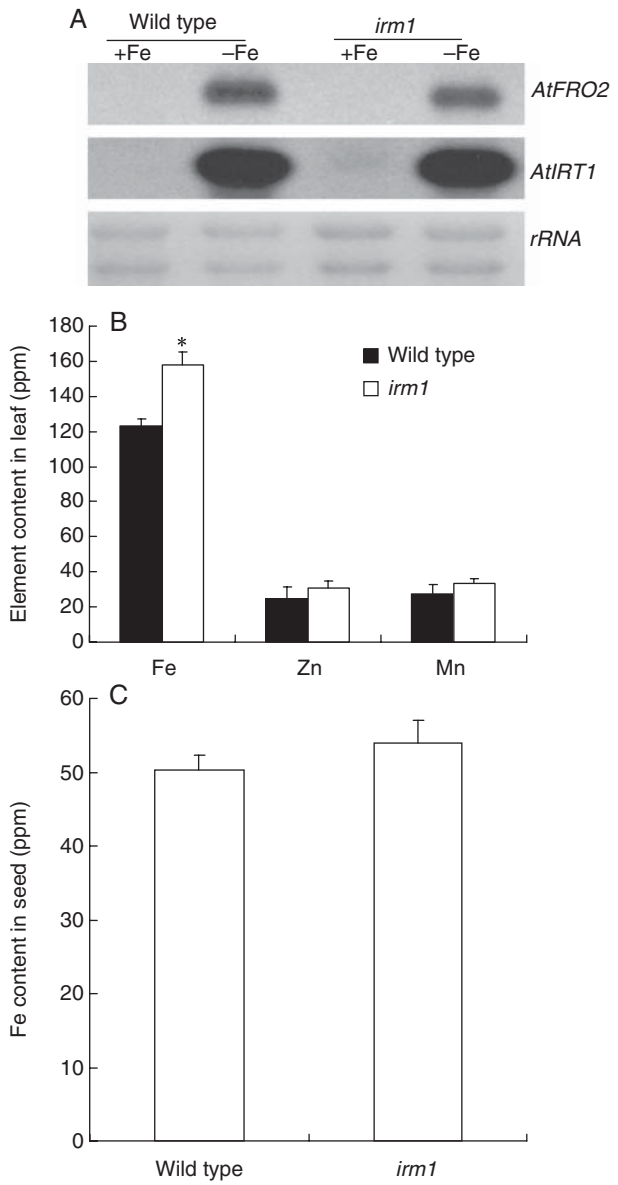

FIG. 2. Fe-deficiency responses and Fe content in wild type and *irm1*: (A) northern blot analysis of Fe uptake genes *FRO2* and *IRT1* in roots of seedlings exposed to Fe deficiency (−) and sufficiency (+); (B) contents of Fe, Zn and Mn in leaves of seedlings growing on soil for 2 weeks; (C) Fe content in seeds. Data are shown as means ± standard error of the mean ($n = 4$).

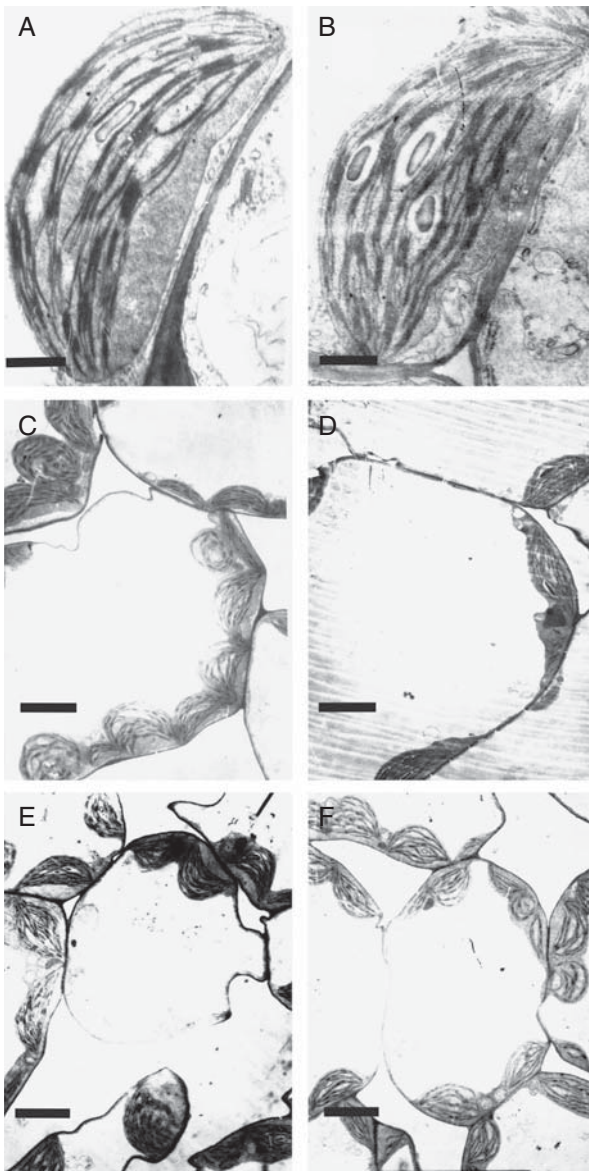

FIG. 3. Transmission electron micrographs of chloroplast structure (A, B) and mesophyll cells (C–F) of *irm1* and wild type: (A and B) chloroplast structure of young leaves of wild type (A) and *irm1* (B); (C and D) an overview of a mesophyll cell of young leaves of wild type (C) and *irm1* (D); (E, F) transmission electron micrographs of a mesophyll cell of wild type (E) and *irm1* (F) grown on soil watered with Fe solution. Scale bar = 1 μm.

SSLP (single sequence length polymorphic) markers. Approximately 3200 chlorotic F_2 mutant progenies were genotyped with SSLP markers, and *irm1* was mapped to a 150-kb interval between the SSLP markers K16E14-1 and MWD22-1 at the bottom of chromosome V, which includes three BACs (K16E14, K3K7 and MWD22). To detect the candidate gene of *irm1*, the 42 predicted open reading frames in the interval were amplified and sequenced. Comparison with the known genomic sequence of Col-0 revealed that *ClpC1* in *irm1* showed a substitution of G to A at nucleotide 2317, resulting in conversation of glycine to arginine at amino acid 773 of the protein (Fig. 4A), whereas no sequence difference was observed among the other 41 open reading frames between *irm1* and wild type, implying that *ClpC1* is the candidate for *irm1*. The sequences of ClpC1 homologues from different phylogenetic kingdoms were obtained from the database and compared at amino acid level. It revealed that the glycine at position of amino acid 773 is highly conserved among the proteins of ClpC (Fig. 4B). The results indicate that the glycine at amino acid 773 may be essential for the function of ClpC1, strongly suggesting that the point mutation resulting in conversion of glycine to arginine at amino acid 773 in ClpC1 is the reason for the altered phenotypes in the mutant *irm1*.

To confirm the hypothesis that *ClpC1* is the gene of *irm1*, a 7·3-kb fragment including the native promoter and the coding sequence of *ClpC1* was cut out from the BAC clone K3K7 and

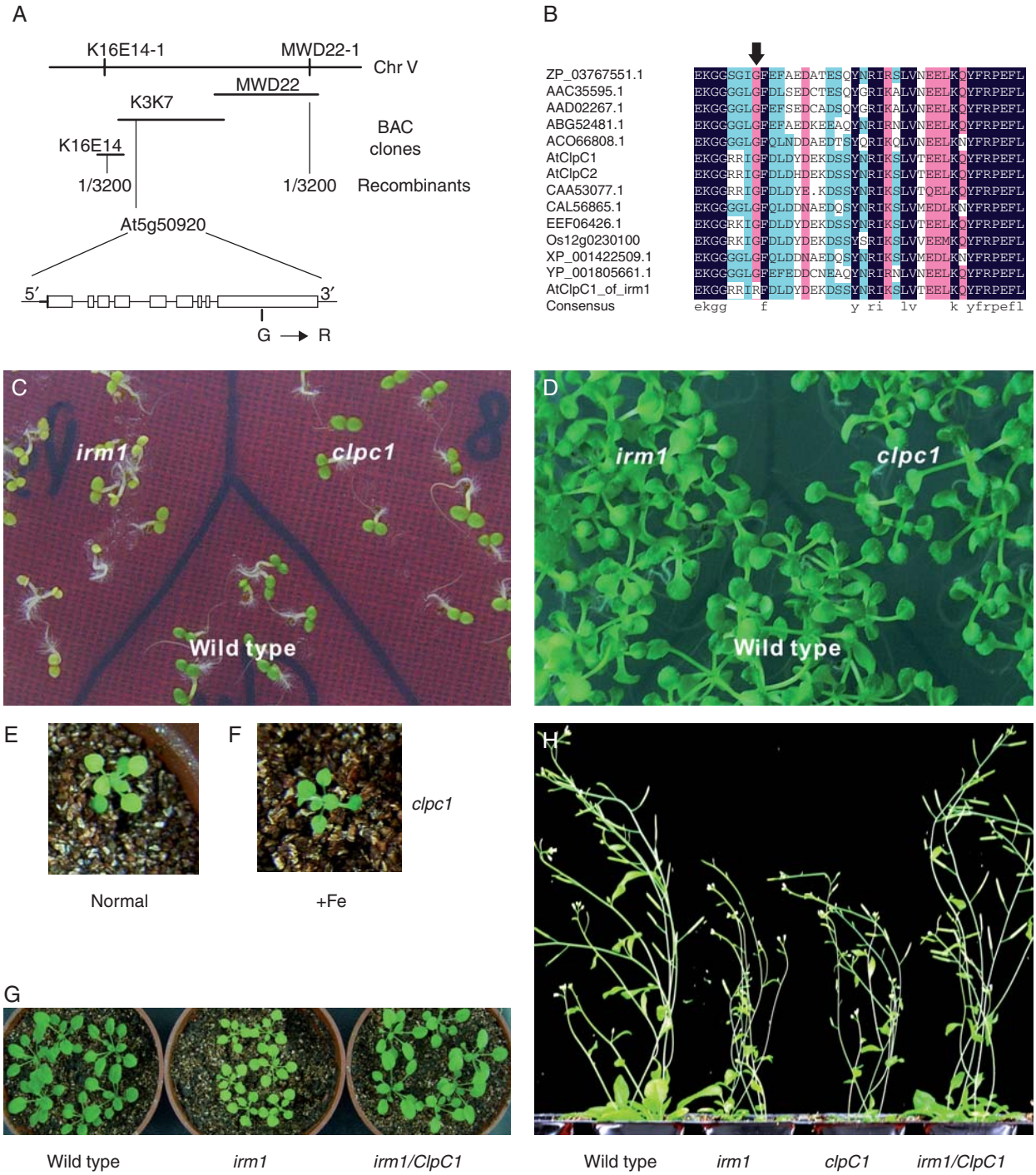

FIG. 4. (A) The schematic outline of genetic and physical maps of *IRM1* and the structure of the candidate gene At5g50920 (*ClpC1*) localized on BAC clone K3K7 (the open boxes represent exons and the lines among the open boxes represent introns). The site of mutation G → R is shown. (B) Sequence alignment of ClpC proteins from *Arabidopsis* (AtClpC1 and AtClpC2), *Nostoc azollae* (ZP_03767551·1), *Trichodesmium erythraeum* (ABG52481·1), *Guillardia theta* (AAC35595·1), *Rhodomonas salina* (AAD02267·1), *Micromonas* sp. *RCC299* (AC066808·1), *Populus trichocarpa* (EEF06426·1), *Brassica napus* (CAA53077·1), *Ostreococcus tauri* (CAL56865·1), *Oryza sativa* (Os12g0230100), *Ostreococcus lucimarinus CCE9901* (XP_001422509·1) and *Cyanothece* sp. *ATCC 51142* (YP_001805661·1). The amino acid indicated by arrow is the mutation site found in the *irm1* mutant. (C and D) Phenotypic comparison of *irm1*, *clpc1* (a T-DNA insertion mutant of ClpC1) and wild type grown on an MS agar plate for 4 d (C) and for 10 d (D). (E and F) Phenotypic recovery of leaf chlorosis of *clpc1* in soil, 3 d after being watered with Fe solution (100 μM FeEDTA) (+Fe). (G and H) Phenotypic complementation by expression of *ClpC1* in an *irm1* mutant at early (G) and a late stage (H).

introduced into the *irm1* genome by *Agrobacterium tumefaciens*-mediated transformation. The transgenic plants exhibited normal growth like the wild-type (Fig. 4G, H), indicating that the defect functions in *irm1* was complemented by expression of *ClpC1*. The chlorophyll content of the transgenic plants also recovered to the same level as wild type (data

not shown). These results demonstrate that a single base change (G to A) at nucleotide 2317 of *ClpC1* in *irm1* is responsible for the altered phenotypes (chlorosis and retarded growth).

Further, T-DNA insertion *ClpC1*-mutant line *clpc1* (*hsp93-v-2*, SALK_014058; Kovachva *et al.*, 2005) was obtained from Nottingham Arabidopsis Stock Center and characterized. As shown in Fig. 4C–E, *clpc1* displayed a similar phenotype to *irm1* (yellow cotyledons at an early stage of germination, chlorotic leaves and retarded growth on soil), and its leaf chlorosis was also restored to normal by watering with Fe (Fig. 4E, F). However, *irm1* showed much stronger mutation phenotypes than *clpc1* (Fig. 4C, H). Considering the function redundancy of *ClpC1* and *ClpC2*, it is speculated that the phenotypic difference between *irm1* and *clpc1* may be caused by the different expression levels of *ClpC2* in the two mutant plants, so the *ClpC2* expression levels in wild type, *irm1* and *clpc1* were analysed. As shown in Fig. 5A, the expression level of *ClpC2* in plants of *clpc1* is significantly higher than that of *irm1* or wild type.

Further, the chlorophyll content was determined in wild type, *irm1* and *clpc1* plants under normal and Fe-supplemented (100 μM) conditions in the soil. The chlorophyll *a* and *b* content in *irm1* and *clpc1* was significantly lower than in wild type under normal growth conditions, but increased obviously when the soil was watered with Fe, especially in *clpc1*, where the chlorophyll *a* and *b* content even reached the same level as wild type (Fig. 5B, C). The ratio of chlorophyll *a* and *b* in wild type (1·82 ± 0·12), *irm1* (1·82 ± 0·05) and *clpc1* (1·85 ± 0·06) was not altered.

Expression profile of ClpC1 *and the localization of its protein*

In previous studies of *ClpC1*, the focus was only on its role in chloroplast development (Constan *et al.*, 2004; Sjogren *et al.*, 2004; Kovacheva *et al.*, 2005, 2007; Nakagawara *et al.*, 2007). Given the Fe-related phenotype in *irm1*, it was of great interest to determine whether *ClpC1* expression is related to Fe status. Therefore, RNA-blot analysis was performed with total RNA extracted from the roots and shoots of *Arabidopsis* seedlings exposed either to Fe-sufficient or -deficient conditions. *ClpC1* mRNA was detected in shoots and roots, and it was more abundant in shoots than that in roots (Fig. 6). No difference in *ClpC1* expression intensity was observed between the Fe-sufficient and -deficient conditions in either shoots or roots (Fig. 6).

To localize *ClpC1* expression in plant tissues, the putative promoter (1699 bp of genomic DNA sequence upstream of the transcription start site of *ClpC1*) was amplified and fused with the coding sequence of *GUS*. The *ClpC1* promotor::*GUS* construct (P_{ClpC1}::*GUS*) was introduced into *Arabidopsis* and GUS activity of stable transformants was analysed. Consistent with the results of northern-blot analysis, GUS activity was detected in both root and shoot, and its expression abundance was not affected by the status of Fe, Zn or Mn (Fig. 7A1–4). *GUS* expression was also strongly detected in germinating seeds, cotyledons (Fig. 7A5–8), seedlings, filaments and siliques (Fig. 7A9–11).

Previous biochemical work indicated that ClpC1 protein localized to the chloroplasts (see review, Adam *et al.*, 2006).

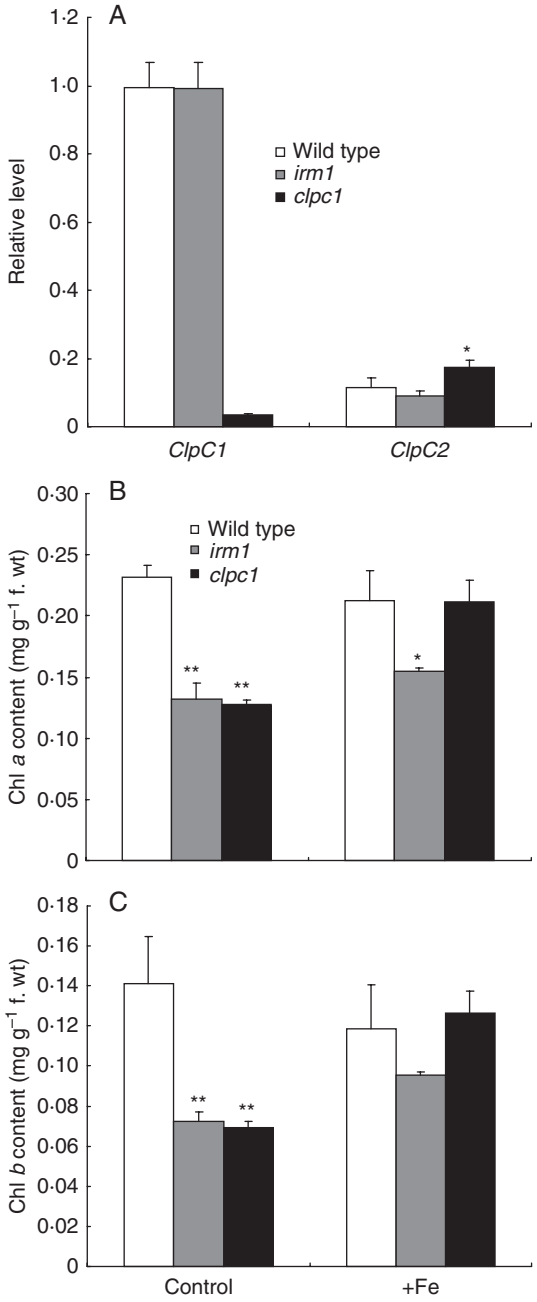

FIG. 5. Analysis of gene expression and chlorophyll content in wild type, *irm1* and *clpc1*: (A) *ClpC1* and *ClpC2* expression level in wild type and the *irm1* and *clpc1* mutants; (B) chlorophyll *a* content in wild type, *irm1* and *clpc1*; (C) chlorophyll *b* content in wild type, *irm1* and *clpc1*.

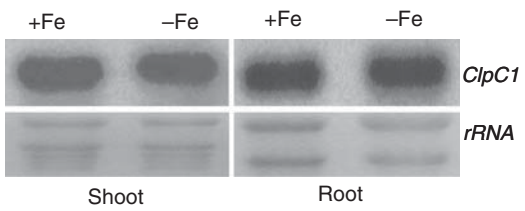

FIG. 6. Northern blot analysis of *ClpC1* expression in roots and shoots under Fe-sufficient (+Fe) and -deficient conditions (−Fe).

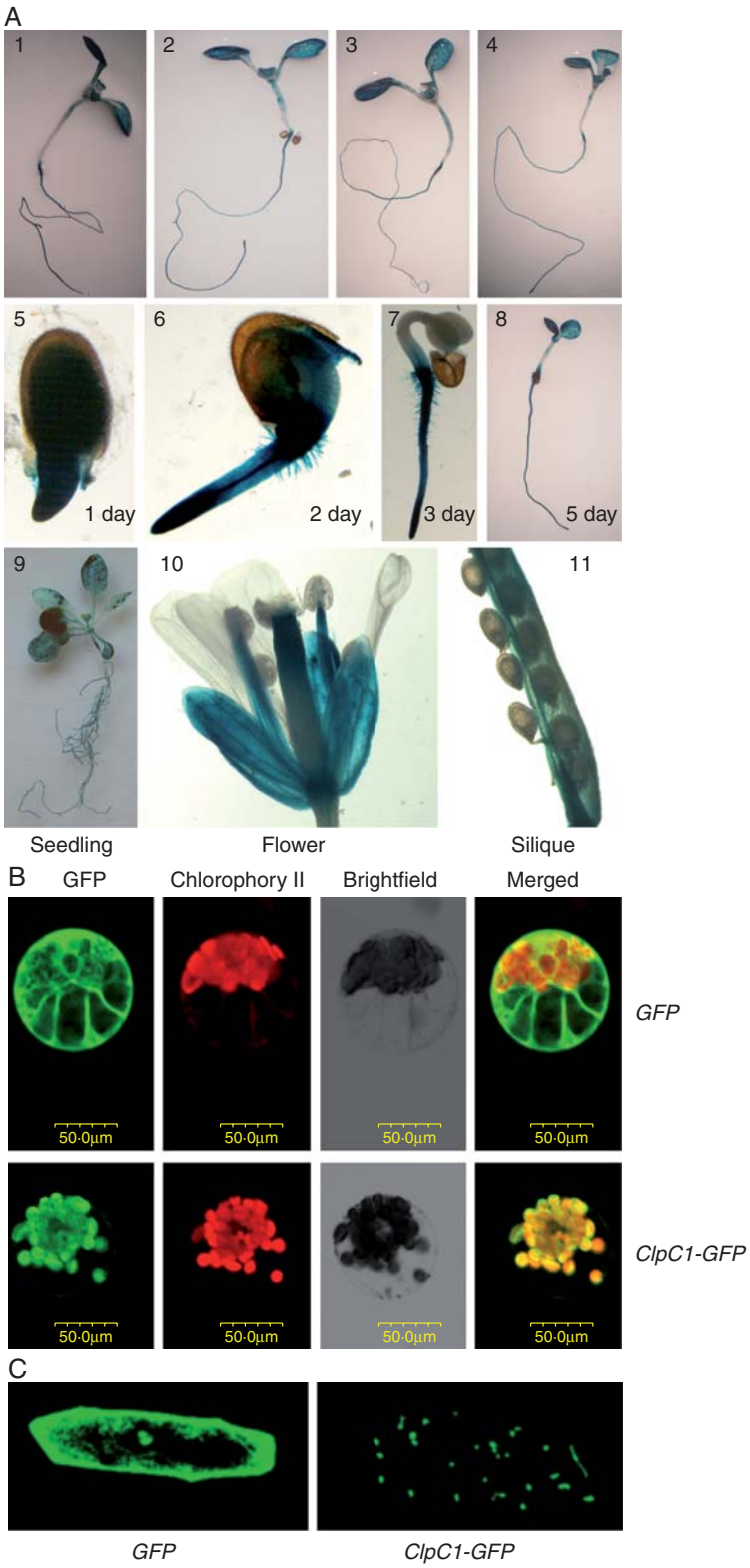

FIG. 7. Expression pattern analysis of *ClpC1* and subcellular localization of the ClpC1 protein. (A) Histochemical analysis of *ClpC1* expression pattern using *ClpC1* promoter-GUS transgenic lines: (1–4) GUS staining of the seedlings treated with Fe (2), Zn (3) or Mn (4) deficiency for 1 week; (5–8) GUS staining in the seedlings at 1, 2, 3 and 5 d after germination; (9–11) GUS staining of seedlings at the five-leaf stage and flowers and siliques from plants grown on soil. (B) Subcellular localization of ClpC1 by transient expression of *ClpC1–GFP* in cowpea protoplasts and in onion epidermis cells. The gene *GFP* (control) and *ClpC1–GFP* (a fusion gene of *ClpC1* and *GFP*) under control of the 35S promoter were separately introduced into cowpea protoplasts using PEG transformation and expressed. (C) *GFP* (control) and *ClpC1–GFP* were transiently expressed in onion epidermis cells introduced by bombardment. The GFP signal is localized in chloroplasts of cowpea protoplasts (B) and in plastids of onion epidermis cells (C) when transformed with 35S::*ClpC1–GFP*, whereas the GFP signal was spread through the whole cytoplasm in the cells that were transformed with 35S*::GFP*. Scale bar = 50 μm.

To confirm this, an expression plasmid containing a *ClpC1–GFP* fusion protein controlled by 35S promoter was created and transformed into cowpea protoplasts and onion epidermis cells for transient expression analysis. As shown in Fig. 7B, the ClpC1–GFP protein was clearly localized in chloroplasts of the transformed cowpea protoplasts (Fig. 7B) and in plastids of onion epidermis cells (Fig. 7C).

Expression of genes related to Fe homeostasis in the irm1 *mutant*

In *A. thaliana*, there exist eight ferric-chelate reductase genes. Of them, *FRO6*, *FRO7* and *FRO8* mainly expressed in leaves, are speculated to be involved in Fe reduction in the chloroplasts and mitochondria (Wu *et al.*, 2005; Mukherjee *et al.*, 2006; Jeong *et al.*, 2008). *PIC1* is localized to the inner envelope of the chloroplast and is critical for chloroplast development (Duy *et al.*, 2007). So, the expression of the four genes in the seedling leaves of *irm1* and wild type grown on soil were analysed by multiplex RT-PCR. As shown in Fig. 8A, the expression intensity of *FRO8* in *irm1* and *clpc1* was significantly higher than that of wild type, whereas *FRO6* displayed a lower expression in the two mutants than that of wild type, especially in *clpc1*. Same as *FRO8*, *FRO7* also showed a higher expression in *irm1* and *clpc1* than that in wild type, but the difference was not significant. For *PIC1*, no expression difference was observed between the mutants and wild type (Fig. 8A).

NRAMP3 and NRAMP4 are two Fe transporter proteins localized on the vacuole membrane and involved in mobilization of Fe stored in the vacuole during germination (Thomine *et al.*, 2003; Lanquar *et al.*, 2005). Considering the yellow phenotype of *irm1* at an early stage of germination, the expression of the two genes was checked in 3-d-old seedlings. The expression level of *NRAMP3* and *NRAMP4* in *irm1* and *clpc1* was clearly lower than that of wild type, although the difference was not significant (Fig. 8B).

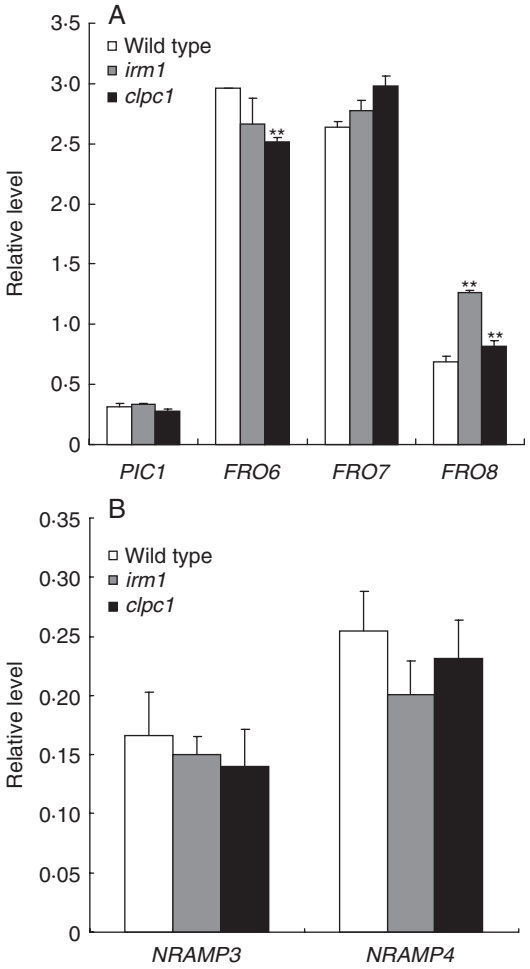

FIG. 8. Relative quantitative analysis of the expression intensities of *PIC1*, *FRO6/7* and *FRO8* in leaves of 2-week-old plants grown on soil (A), *NRAMP3* and *NRAMP4* in cotyledons of 3-d-old seedlings (B) by multiplex RT-PCR analysis.

DISCUSSION

The glycine residue at amino acid 773 of ClpC1 is essential for its functions

In this work, the spontaneous mutant *irm1* found in the Columbia ecotype of *A. thaliana*, which showed yellow cotyledons at an early stage of germination, chlorotic phenotype and retarded growth on soil, was characterized. Map-based cloning confirmed that a single nucleotide change (G to A) at nucleotide 2317 of *ClpC1*, resulting in conversation of glycine to arginine at amino acid 773 of the protein, is the reason for the altered phenotypes of *irm1*. Sequence comparison revealed that the glycine residue at amino acid 773 of ClpC1 is highly conserved among ClpC proteins from different phylogenetic kingdoms (Fig. 4B). Additionally, *irm1* exhibited similar phenotypes to *clpc1*, a null mutant of *ClpC1* generated by T-DNA insertion. These results imply that the glycine at amino acid 773 is an essential amino acid residue for the function of ClpC1, and substitution of the glycine to arginine due to the point mutation from G to A in *irm1* led to loss of function, although its expression abundance was not altered (Fig. 5A).

ClpC1 is involved in leaf Fe homeostasis of Arabidopsis

Previous studies demonstrated that ClpC1, as a stromal molecular chaperone, is required for importing protein to the chloroplast, and plays a vital role in chloroplast development and its functions. Knockout of *ClpC1* by T-DNA insertion in *Arabidopsis* resulted in a lower protein translocation rate in isolated chloroplasts, reduced plant growth and chloroplast development (Constan *et al.*, 2004; Sjögren *et al.*, 2004; Kovacheva *et al.*, 2007). Here, we strongly suggest that the ClpC1 protein is also involved in Fe homeostasis in *Arabidopsis* leaves for the following reasons: (*a*) the point mutation from G to A at nucleotide 2317 of *ClpC1* is responsible for the altered phenotypes in the *irm1* mutant because the expression of wild-type *ClpC1* in *irm1* could fully convert the mutant to wild type; (*b*) the mutant exhibited Fe-deficiency chlorosis, although the Fe content in its leaves was significantly higher than that of wild type; (*c*) watering with an Fe solution could turn the chlorotic leaves of the T-DNA knockout mutant *clcp1* and the mutant *irm1*, growing on soil, from yellow to green; (*d*) the expression of the genes, such as *FRO6*, *FRO7*, *FRO8*, *NRAMP3* and *NRAMP4*, related to Fe

homeostasis in leaves and to Fe mobilization during germination was disordered. Considering the higher Fe content in *irm1* leaves (Fig. 2B), and no changes in the expression pattern of the Fe uptake genes *FRO2* and *IRT1* in the *irm1* mutant (Fig. 2A), we speculate that ClpC1 may be involved in Fe homeostasis in leaf cells other than absorption and translocation from roots to shoots. ClpC1 is localized to chloroplasts and plastids (Fig. 7B and C), accumulates mainly in the stroma (Shanklin *et al.*, 1995), and is also associated with the chloroplast protein translocation machinery in the inner envelope (Nielsen *et al.*, 1997). It functions by controlling protein quality and translocation of precursor proteins encoded by nuclear genes. Loss of ClpC1 results in lower protein translocation rates in chloroplasts (Constan *et al.*, 2004; Kovacheva *et al.*, 2005). As chlorosis of the mutant *irm1* and *clpc1* leaves was removed by watering the plants with Fe solution, it is reasonable to assume that ClpC1, as a molecular chaperone, is involved in translocating some nuclear-encoding proteins that function in Fe transport to the chloroplasts in leaf cells. In the mutants, the nuclear-encoding genes *FRO6*, *FRO7* and *FRO8*, which are Fe^{3+}-chelate reductase and mainly express in leaves (Wu *et al.*, 2005; Mukherjee *et al.*, 2006), revealed a disordered expression (lower for *FRO6*, higher for *FRO7* and *FRO8*) in comparison with wild type (Fig. 8). Therefore, the disorder of Fe homeostasis in *irm1* leaves might, directly or indirectly, be the result of translocation of these or some other unknown proteins, from the cytoplasm into the chloroplasts, being affected, and, consequently, reducing their ability to transport Fe into the chloroplasts, resulting in the Fe-deficiency phenotype even though there is a high Fe content in the leaves. However, to understand how ClpC1 functions in the regulation of Fe homeostasis in *Arabidopsis* leaves needs to be studied in more detail.

Mobilizing Fe from vacuoles and transferring it into the cytoplasm is an essential process for seed germination (Lanquar *et al.*, 2005). NRAMP3 and NRAMP4, two metal transporters localized on the vacuole membrane, are involved in this process because knockout of the two genes leads to the arrest of germination under low Fe nutrition. In mutant *irm1* and *clpc1*, the expression intensity of *NRAMP3* and *NRAMP4* was lower than that of wild type on the third day of germination (Fig. 8B). According to this, we speculate that the yellow cotyledons of *irm1* and *clpc1* at an early stage of germination might be due to reduced Fe mobilization from the vacuole via the reduced expression of *NRAMP3* and *NRAMP4* in seedlings. As Fe acquisition is shifted from seed to culture medium at the late stage of germination, the *irm1* seedlings will restore their normal phenotype (Fig. 1A–E). As shown in Fig. 4C, *irm1* displayed a stronger phenotype than *clpc1*. This can be explained by the reduced expression of *NRAMP4* in *clpc1* being much less than that in *irm1* (Fig. 8B). It is possible to explain that the increased expression of *ClpC2* in *clpc1* (not in *irm1*) is the reason for its chlorotic cotyledon phenotype being alleviated (Figs 4C and 5A). ClpC2 is a homologue of ClpC1 and reveals functional redundancy with ClpC1 (Kovacheva *et al.*, 2007). However, more experiments are needed to confirm it.

In summary, the spontaneous mutant *irm1* is caused by a single nucleotide substitution of G to A at nucleotide 2317 of *ClpC1*, resulting in conversion of glycine to arginine at amino acid 773. This also demonstrated that the glycine at amino acid 773 is an essential amino acid residue for ClpC1 functions. Here, it has been discovered that ClpC1, in addition to its known functions reported previously, is involved in Fe homeostasis in leaf cells of *Arabidopsis*, based on the fact that chlorotic leaves of *clpc1* and *irm1* plants grown on soil could be turned green by watering them with an Fe solution. The observed results of this work should shed new light on understanding the functions of ClpC1.

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