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ORIGINAL ARTICLE

Bicarbonate affects the expression of iron acquisition and translocation genes involved in chlorosis in *Medicago lupulina*

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Abstract

Iron (Fe) deficiency chlorosis (FDC) in plant is associated with high bicarbonate concentration in calcareous soil and irrigation water, which leads to new leaf yellowing and lessens crop yield and quality. However, little is known about whether the chlorosis under bicarbonate stress resulted from blocking root–shoot Fe translocation or root Fe absorption. Moreover, the molecular response of chlorotic leaf under bicarbonate stress has been rarely reported on. The purpose of this study was to investigate the effect of bicarbonate on Fe acquisition, Fe translocation as well as Fe accumulation in roots, normal leaf (NL) and chlorotic leaf (CL) of *Medicago lupulina*. Seeds were grown with and without Fe and $NaHCO_3$ (Fe and Bic) in the nutrient solution for 10 d. Fe content, gene expression and enzymatic activity in different tissues were determined. A factorial statistical design with two factors (Fe and Bic) and two levels of each factor was adopted: + Fe, − Fe, + Fe + Bic and − Fe + Bic. Results indicated that bicarbonate stress increased the expression of genes *MlHA1*, *MlFRO1* and *MlIRT1* related to Fe acquisition and promoted the Fe absorption from solution. Furthermore, the presence of bicarbonate stress inhibited the expression of *MlMATE66* in roots, prevented the Fe translocation from roots to developing leaf, brought about Fe accumulation in roots and reduced the Fe content in new leaf. Generally, according to our results, bicarbonate could prevent Fe translocation from roots into developing leaf, decrease Fe bioavailability and induce chlorosis in *M. lupulina*.

Keywords Fe uptake · Ferric reductase · Iron transporter · *MATE66* · Fe storage

Introduction

Iron (Fe) is the fourth abundant element in the Earth's crust accounting for 5% (Souri et al. 2018; Mengel et al. 2001), while serving as an essential mineral microelement in plant

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growth and development. The alkaline and calcareous soil representing about one-third of the Earth's soil contains bicarbonate ion (HCO^{3-}) (Chen and Barak 1982), which is the most important inducing factor of Fe-deficient chlorosis in plant. It can inhibit Fe uptake by roots and Fe translocation into shoots and leaves (Shahsavandi et al. 2020) and then cause losses in the production of crops (Tato et al. 2013).

Fe plays a fundamental role in plant metabolism, for instance, electron transfer for photosynthesis or respiration (El-Gioushy et al. 2021; Roschzttardtz et al. 2013). Fe is also a key element in photosynthesis and carbon dioxide assimilation to ensure electron flow through the PS II-b6f-PSI complexes (Briat et al. 2015). Moreover, numerous enzymes in most organisms (including plants) cannot achieve their biological functions without Fe (Connorton et al. 2017).

When plants are under bicarbonate stress, many morphological and physiological responses are induced in roots to increase iron transport and uptake (García et al. 2014; Wang et al. 2020). Bicarbonate is widely reported to affect the ferric reductase oxidase (*FRO*) and H^+-ATPase (*HA*) activity, including inhibition or promotion. Lucena et al. (Lucena et al. 2007) reported that bicarbonate inhibited the expression of *FRO* and *HA* in some species such as *Cucumis sativus*, *Pisum sativum* and *Lycopersicon esculentum*. In contrast, the above genes were induced by bicarbonate and enhanced *FRO* and *HA* in *Citrus limon* roots (Martínez-Cuenca et al. 2013). Robinson et al. (Robinson et al. 1999) reported that the *AtFRO2* is expressed mainly in roots in *Arabidopsis thaliana*. In contrast to *A. thaliana*, *LeFRO1* in *L. esculentum* and *PsFRO1* in *P. sativum* are expressed in both roots and leaves (Waters et al. 2002). In roots epidermal cells, Fe^{3+} is reduced to Fe^{2+} by *FRO,* which is transported into roots by iron-regulated transporter 1 (*IRT1*) (Mora-Córdova et al. 2022). The expression of *IRT1* in roots had been reported on the previous study under bicarbonate stress (García et al. 2014; Lucena et al. 2007; Martínez-Cuenca et al. 2013; Hsieh and Waters 2016). The zinc/iron-regulated transporter-like protein (*ZIP*) family is involved in metal transport (Curie et al. 2000; López-Millán et al. 2004), which can transport divalent cations such as Fe^{2+}, Zn^{2+} and Mn^{2+}. A couple of *ZIP* family transcription factor genes, such as *HD-ZIP* (Ariel et al. 2007) and *bZIP* (Liu et al. 2014), were identified as alkali stress-responsive genes (Cao et al. 2016). Although a number of previous studies have been reported about the roles of related genes in plant bicarbonate stress, little is known regarding their roles in bicarbonate stress. Therefore, it is urgent to understand the basic mechanism of plant responses to bicarbonate stress.

The previous study focused on the Fe uptake from soil and physiological responses to bicarbonate stress in roots. However, the effect of carbonate on Fe transport from roots to leaves and the effect of bicarbonate on Fe homeostasis in cells are still poorly understood. Fe is translocated into leaves from roots by carrier complexes on the xylem (Hell and Stephan 2003), which is usually a citrate and iron transport protein (*ITP*) (Kruger et al. 2002; Yokosho et al. 2009). It is proved that the multidrug and toxin extrusion (*MATE*) transporter involved in the transport of citrate, which translocates Fe from roots to shoot in *Oryza sativa* and *A. thaliana* (Yokosho et al. 2009; Durrett et al. 2007). In legume species, the citrate transporter gene (*MtMATE66* and *LjMATE1*) is identified in *Medicago truncatula* and *Lotus japonicus* (Wang et al. 2017; Takanashi et al. 2013). Function analysis showed that *MtMATE66* is primarily expressed in root epidermal cells and involved in chlorotic symptom under Fe deficiency (Wang et al. 2017). The iron transporter protein in plants is the natural resistance-associated macrophage protein (*NRAMP*) family (Curie et al. 2000) and Yellow Stripe-Like (*YSL*) protein family (Curie et al. 2001). In *A. thaliana*, *AtYSL* genes (*AtYSL1*, *AtYSL2*, and *AtYSL3*) have been identified, and *YSL1* and *YSL3* protein transported iron between vascular tissues (Waters et al. 2006). *NRAMP3* transported Fe from cotyledons to roots, and its expression was inhibited by bicarbonate in *Citrus* (Martínez-Cuenca et al. 2013). However, whether iron deficiency chlorosis results from blocking iron translocation from roots under bicarbonate stress is still unclear. Furthermore, ferritin is ubiquitous iron storage proteins in the plastids and plays an important role in iron homeostasis (Harrison and Arosio 1996; Petit et al. 2001). The previous report showed that the transcriptional level of soybean ferritin gene is regulated in response to iron overload (Lescure et al. 1991). Santos et al. (Santos et al. 2015) found that ferritin was highly expressed in "Fe-inefficient" plant roots, which can resist Fe deficiency chlorosis. However, the relationship between the expression of ferritin and bicarbonate stress remains unknown.

Although the above cited literature had reported the expression of genes related to iron uptake (*FRO1* and *IRT1*) under Fe deficiency or bicarbonate stress, the expression profiles of gene related to iron translocation and accumulation are unclear under bicarbonate stress. At the same time, does bicarbonate stress lead to the inability of plants to absorb iron from the external environment, or does the iron absorbed by plant roots fail to transfer from plant roots to leaves? These issues are also unclear. Thus, this paper aimed to test whether bicarbonate impairs Fe mobilization from roots to leaves or affects the expression of storage-related genes in *M. lupulina* roots or chlorotic leaf. To this end, the effect of bicarbonate on the expression *ZIP* family, *MATE66* and ferritin was studied in roots, normal leaf and chlorotic leaf. In addition, the influence of Fe uptake-related genes (*FRO*, *HA* and *IRT*) and enzyme activity (*FRO*) was also determined in the present study.

Materials and methods

Plant growth, iron deficiency and bicarbonate treatment

The seeds of *Medicago lupulina* were collected from Wanfenglin Scenic Spot, Xingyi City, Guizhou Province, China (104° 56′ 2.868″ E, 25° 0.3672′ 0″ N). The average altitude is 1200 m. *M. lupulina* was cultured in the Key Laboratory of National Forestry and Grassland Administration on Biodiversity Conservation in Karst Mountainous Areas of Southwestern China (106° 38′ 34.62″ E, 26° 23′ 11.148″ N). The *M. lupulina* seeds were soaked in concentrated sulfuric acid for 7 min and then washed three times with distilled water. The seeds were planted in a plastic pot containing perlite with 1/4 MS solution in hydroponics. They were cultured in a chamber with 16/8 h light/dark at 22 °C/20 °C, irradiance of 350 μmol m^{-2} s^{-1} and air humidity of 60–70%. After seeding growth for 30 d, the solution with 10 Mμ Fe (+Fe), without Fe (–Fe), +Fe with 10 mM $NaHCO_3$ or $KHCO_3$ (+Fe+Bic), and –Fe with 10 mM $NaHCO_3$ or $KHCO_3$ (–Fe+Bic) were replaced. All treatments were carried out in three replications. Root, normal leaf and chlorotic leaf (Fig. 2A) were collected at 10 d for gene expression analysis by RT-PCR. Root and shoot were collected at 10 d for determination of biomass and Fe content.

The measurement of biomass and SPAD

The shoot and root were dried at 65 °C to a constant weight and weighed for biomass. SPAD values in all treatments were measured by a Minolta SPAD chlorophyll meter.

Determination of enzyme activity

FRO activity was measured using whole roots of individual *M. lupulina*; after treatment for 10 days, roots were rinsed in deionized water and submerged in 20 mL assay solution (1 mM MES buffer, pH 5.5, 150 μm Fe (III)–EDTA and 200 μM ferrozine). Ferrozine-Fe (II) was measured by absorbance at 562 nm (subtracting blanks of assay solution with no plants) (Hsieh and Waters 2016).

Identification of genes-related Fe uptake and phylogenetic analysis

These genes were obtained from *M. truncatula* (http://www.medicagogenome.org, Mt4.0v2) and NCBI database to identify the genes related to iron uptake. The identified sequences of *M. truncatula* were used as query sequences to identify corresponding genes from *M. lupulina* transcriptome database by a local BLAST program. These sequences are listed in Supplementary Table 1. In addition, *A. thaliana* isoforms were also obtained from the Arabidopsis Information Resource (http://www.arabidopsis.org/). Among the identified genes, the ZIP transporter is a multi-gene family. Thus, they were subjected to phylogenetic analysis. Trees were constructed using the neighbor-joining method as implemented in the ClustalW program. The following parameters were set during the construction of the phylogenetic tree, for example, substitution, Poisson model, complete deletion, replication and bootstrap analysis with 1000 replications.

Determination of Fe contents

The shoot and root were collected in all treated plant. The samples were dried at 65 °C. The dried samples were digested for 2–5 h in 50% perchloric acid and concentrated sulfuric acid. Then, the digested solution was filtered through filter paper and adjusted to 50 mL volumes with addition of deionized water. An inductively coupled plasma-optical emission spectrometer was applied to measure the Fe contents (ICP-OES, Thermo Elemental-IRIS Advantage, USA).

Gene expression analysis using qRT-PCR

The total RNA of the samples was extracted using an Omni-Plant RNA Kit (Kangwei, Beijing, China) and was reverse-transcribed using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR Kit (Transgen, Beijing, China) following the manufacturer's protocol. Actin was used as the reference for gene expression normalization. The gene-specific primers are listed in Supplementary Table 1. RT-PCR was carried out using a Rotor-Gene Q real-time PCR system (Qiagen, Germany) and 10 μL 2×TransStart Top/Tip Green qPCR SuperMix (Transgen, Beijing, China) according to the manufacturer's instructions. The relative expression levels of the genes normalized to the expression level of actin were calculated from cycle threshold values using the $2-\Delta\Delta Ct$ method (Livak and Schmittgen 2001). All expression levels in each treatment were log2-transformed. (transcript level).

Statistical analysis

One-way analysis of variance (ANOVA), performed using SPSS (version 18.0) was used to determine differences among groups. Data were presented as means ± standard deviation (SD), with values followed by $P<0.05$ considered statistically significant. The results were visualized using sigmaplot version 12.5.

Results

Physiological responses to bicarbonate stress in *M. lupulina*

The growth of *M. lupulina* was not significantly affected in Fe-deficient plant for 10 d compared with Fe-supplied plant. For example, shoot biomass (Fig. 1C), root biomass (Fig. 1D) and chlorophyll content (Fig. 1E) were not significantly changed. In the presence of bicarbonate, shoot biomass was decreased. Still, it did not show a significant change (Fig. 1C). In contrast, root biomass was significantly inhibited (Fig. 1A, D). Furthermore, chlorophyll content was significantly decreased in bicarbonate-supplied plant (Fig. 1E). Chlorosis symptom was observed in new leaf of *M. lupulina* (Fig. 1B). To investigate the effect of cation on the growth of *M. lupulina*, sodium bicarbonate ($NaHCO_3$) and potassium bicarbonate ($KHCO_3$) were compared in the present study. Statistical analysis showed no significant difference in biomass and chlorophyll content (Fig. 1).

Identification of genes related to iron uptake in *M. lupulina*

One ferric reductase gene (*MtIRO*), one iron transporter gene (*MtIRT1*), one H^+-ATPase gene (*MtHA1*), six ZIP family genes (*MtZIP*), three ferritin genes (*MtFer1-3*) and one citrate transporter gene (*MtMATE66*) were screened from *M. truncatula* (Table 1). The identified sequences of *M. truncatula* were used as query sequences to identify iron uptake genes from *M. lupulina* transcriptome database by a local BLAST program. The results showed that 11 genes related to iron uptake and translocation were screened. However, the homologous genes of *MtZIP4* and *MtZIP6* in *M. lupulina* were not found. The identification ratio of these genes to the corresponding homologous genes in *M. truncatula* was higher than 75% (Table 1). The phylogenetic tree analysis of ZIP family in *A. thaliana*, *M. truncatula* and *M. lupulina* showed that the members of ZIP gene family in *M. lupulina* were orthologous genes with *M. truncatula* (Sup Fig. 1).

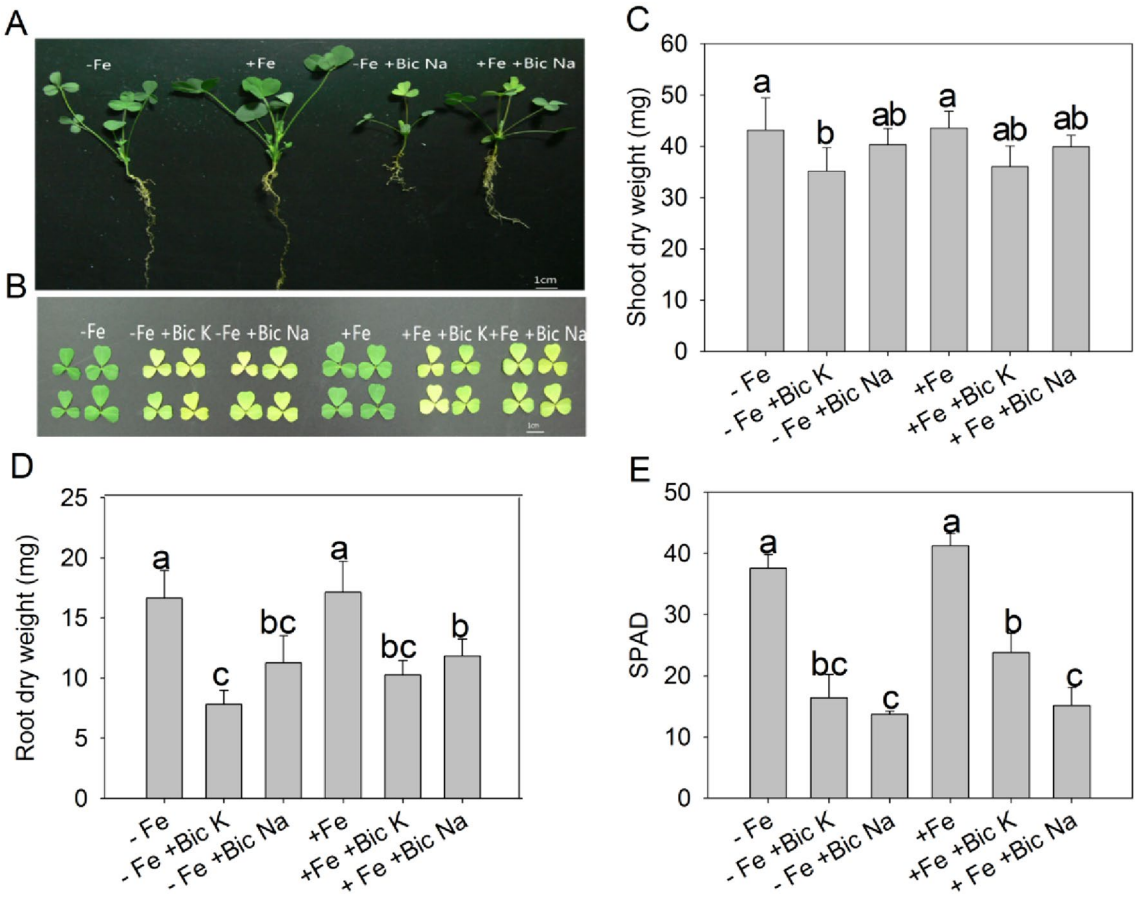

Fig. 1 Biomass **C** and **D** and SPAD **E** value of *M. lupulina* under iron deficiency and bicarbonate treatment. **A** and **B** indicate plant growth phenotype. Data are mean ± SD ($n = 3$). Lowercase represents the differences between different treatments ($p < 0.05$)

Table 1 List of iron response genes in *M. lupulina* and its similarity with its *M. truncatula* orthologs. *MlZIP4* and *MlZIP6* are not identified in *M. lupulina*

Name	Locus name	Putative name	Gene ID	% Identity	E-value
MtFRO1	AY439088	*MlFRO1*	c7926	92.0	0.0
MtIRT1	KX641478	*MlIRT1*	c55416	96.0	1E-122
MtHA1	AJ132892	*MlHA1*	c27873	75.0	0.0
MtZIP1	AY339054	*MlZIP1*	c21788	97.0	0.0
MtZIP3	AY339055	*MlZIP3*	c20978	99.2	0.0
MtZIP4	AY339056	–	–	–	–
MtZIP5	AY339057	*MlZIP5*	c18508	95.0	0.0
MtZIP6	AY339058	–	–	–	–
MtZIP7	AY339059	*MlZIP7*	c26957	94.0	0.0
MtFer1	Medtr4g014540	*MlFer1*	c26800	93.0	0.0
MtFer2	Medtr5g083170	*MlFer2*	c26726	95.0	0.0
MtFer3	Medtr7g069980	*MlFer3*	c27260	97.0	0.0
MtMATE66	Medtr2g097900	*MlMATE66*	c11995	95.0	0.0

FRO activity and gene expression profiles under bicarbonate stress

The activity of FRO exudate from roots of Fe-deficient plants was slightly higher than that of Fe-supplied plant. Meanwhile, application of bicarbonate ($KHCO_3$ and $NaHCO_3$) significantly increased the FRO activity from *M. lupulina* roots; for example, the FRO activity with $NaHCO_3$ and $KHCO_3$ increased 2.1 and 1.87 times than that without bicarbonate under Fe-deficiency, respectively (Fig. 2B). The same results were also shown in Fe-supplied plants (Fig. 2B). In Fe-deficient plants, the expression levels of *MlFRO1* in roots or leaves, but no significant changes were found between normal leaf (NL) and chlorotic leaf (CL) (Fig. 2C). Interestingly, bicarbonate significantly enhanced *MlFRO1* expression in CL (Fig. 2C). In contrast to *MlFRO1*, the expression of *MlHA1* showed no significant changes between CL and NL in bicarbonate-supplied plants, but its expression in roots was sharply increased under bicarbonate supply, especially in Fe-deficient and bicarbonate-treated plants (Fig. 2D).

Iron transporter responses to bicarbonate stress

Five genes related to iron transporter were identified based on the *M. lupulina* transcriptome database, including one iron transporter (*MlIRT1*) and four metal transporter family (*MlZIP*) (Table 1). The expression of *MlIRT1* in

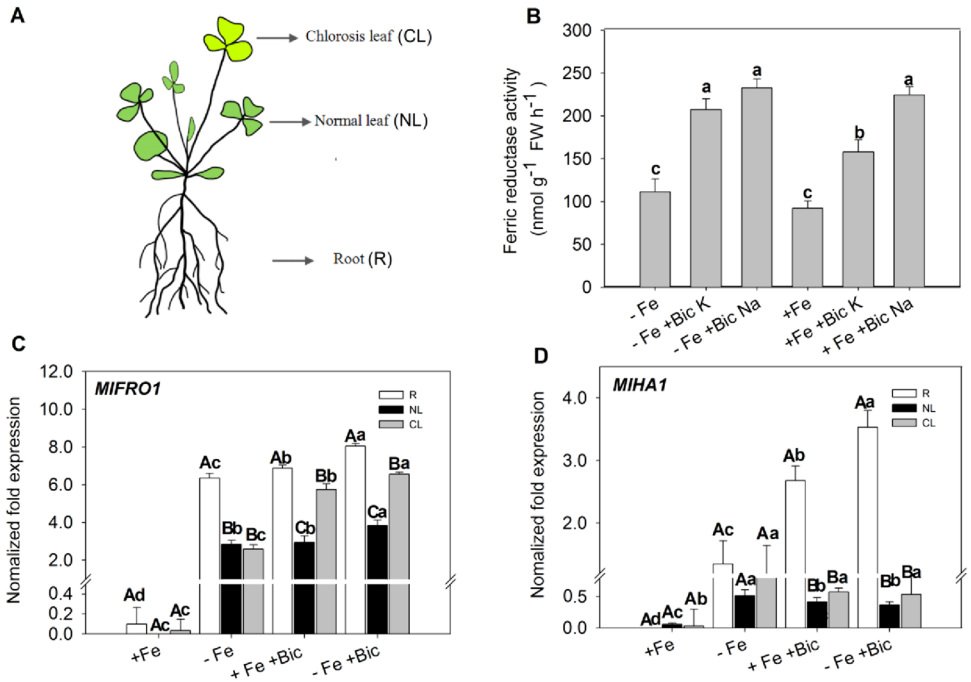

Fig. 2 FRO activity **B** and gene expression levels of *MlFRO1* **C** and *MlHA1* **D** of *M. lupulina* under iron deficiency and bicarbonate treatment. **A** indicates sampling from different tissues, root (R), normal leaf (NL) and chlorotic leaf (CL). Data are mean ± SD ($n = 3$). Uppercases on bar represent the differences between tissues under the same treatment ($p < 0.05$). Lowercases on bar represent the differences between different treatments in the same tissue ($p < 0.05$)

roots, NL and CL was significantly up-regulated under Fe-deficiency compared with Fe supply. In addition, its expression level was further enhanced by bicarbonate (Fig. 3A). In the *MlZIP* family, the expression profiles of *MlZIP3* (Fig. 3C) and *MlZIP7* (Fig. 3E) were similar to that of *MlIRT1* in roots and leaves under Fe-deficient and bicarbonate-treated plants. Furthermore, in contrast, the expression of *MlZIP1* (Fig. 3B) and *MlZIP5* (Fig. 3D) was suppressed in Fe-deficient or bicarbonate-supplied plants.

Effects of bicarbonate stress on Fe translocation

Fe usually combines a citrate in plants to form a complex, which is translocated from roots to leaves. The present study identified the gene encoding citrate transporter (*MtMATE66*) in *M. lupulina*. Its expression was significantly down-regulated in bicarbonate-supplied root, compared with Fe supply or Fe deficiency, indicating Fe translocation was inhibited by bicarbonate. Furthermore, *MlMATE66* in CL was significantly higher than in NL

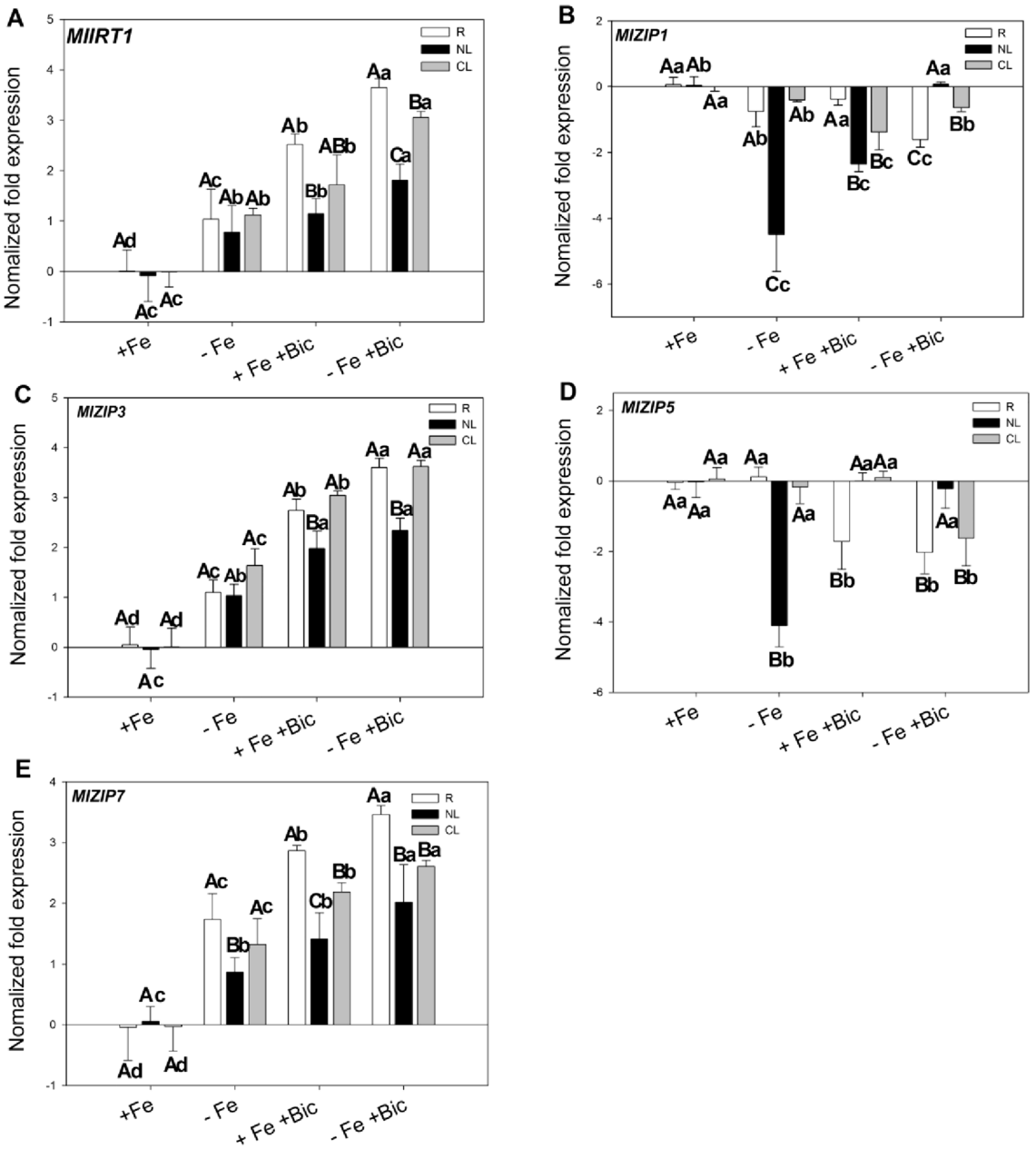

Fig. 3 The expression profiles of iron transporter gene of *MlIRT1* **A**, *MlZIP1* **B**, *MlZIP3* **C**, *MlZIP5* **D** and *MlZIP7* **E** in *M. lupulina* under iron deficiency and bicarbonate treatment. Data are mean ± SD ($n = 3$). Uppercases on bar represent the differences between tissues under the same treatment ($p < 0.05$). Lowercases on bar represent the differences between different treatments in the same tissue ($p < 0.05$)

(4A). To analyze whether bicarbonate hinders the combination of ferritin to Fe, three genes encoding ferritin (*MlFer1, MlFer2* and *MlFer3*) were down-regulated in roots, CL and NL under Fe deficiency and bicarbonate stress (Fig. 4B, C and D), indicating Fe in roots does not combine with ferritin. Fe content analysis showed that Fe was lower in both Fe-deficient and bicarbonate-supplied plants (Fig. 5A). Further analysis for proportion of Fe content in different tissues to total Fe content showed that Fe content in bicarbonate-supplied roots was higher than that in Fe-deficient roots and Fe supply (Fig. 5B), suggesting bicarbonate blocks Fe translocation from roots.

Discussion

It is well known that chlorosis occurs in calcareous soil, but its physiological and molecular basis has not been fully revealed. Therefore, the physiological and molecular responses to bicarbonate stress were studied in this work.

Bicarbonate-induced chlorosis in *M. lupulina*

After the seedlings were subjected to Fe deficiency, Fe in old leaf might translocate to new leaf by *MtMATE66*, which was up-regulated in chlorotic leaf (Fig. 4A). Therefore, chlorophyll content in Fe-deficient plant showed no changes when compared with Fe-supplied plants (Fig. 1). The inhibition of root growth in bicarbonate-supplied plants is consistent

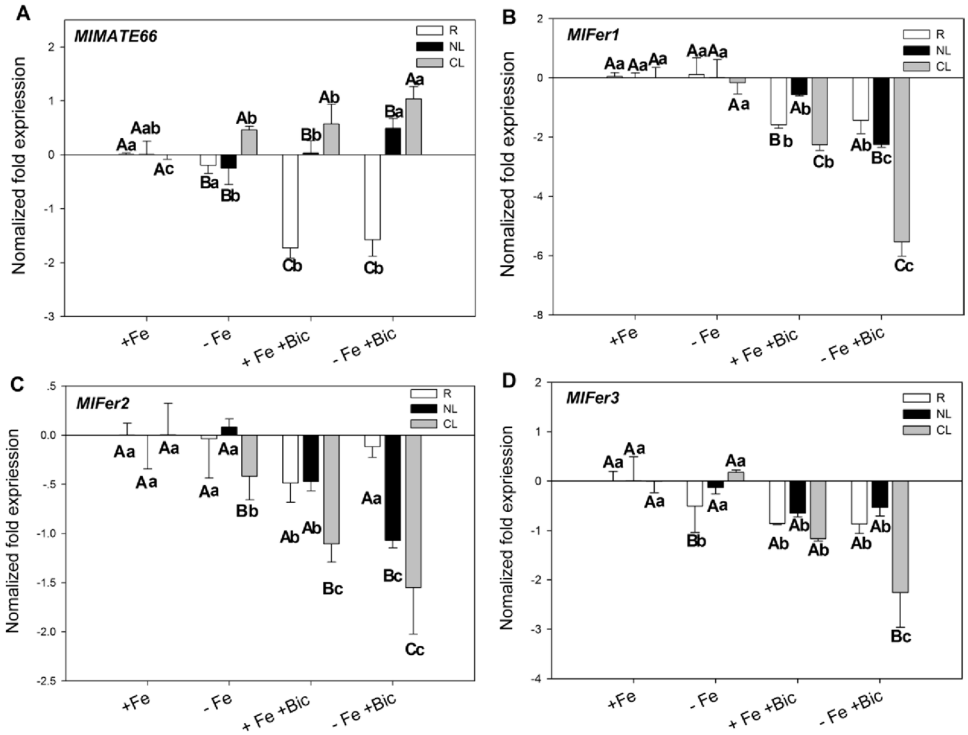

Fig. 4 The expression level of citrate transporter gene of *MlMATE66* **A**, *MlFer1* **B**, *MlFer2* **C** and *MlFer3* **D** in *M. lupulina* under iron deficiency and bicarbonate treatment. Data are mean ± SD ($n = 3$). Uppercases on bar represent the differences between tissues under the same treatment ($p < 0.05$). Lowercases on bar represent the differences between different treatments in the same tissue ($p < 0.05$)

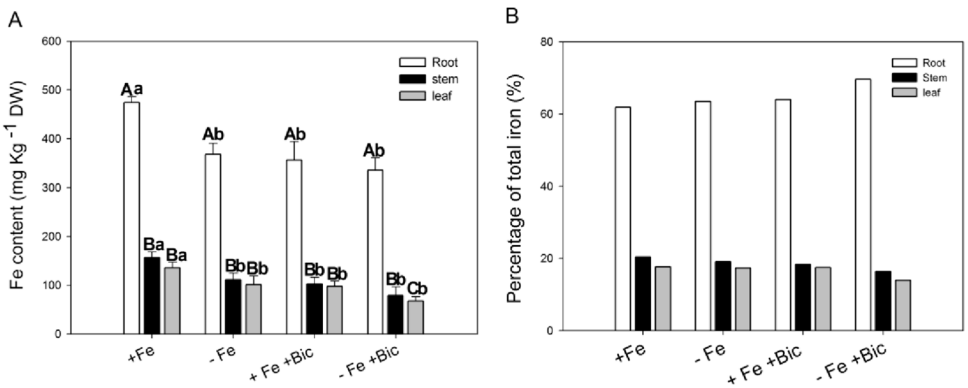

Fig. 5 Fe content **A** and percentage of total iron **B** in root, stem and leaf of *M. lupulina* under iron deficiency and bicarbonate treatment. Data are mean ± SD ($n = 3$). Uppercases on bar represent the differences between tissues under the same treatment ($p < 0.05$). Lowercases on bar represent the differences between different treatments in the same tissue ($p < 0.05$)

with previous reports on other crops (Alhendawi et al. 1997; Mengel et al. 1984). The inhibition was considered that bicarbonate affects cell elongation in roots (Lee and Woolhouse 1969) and was induced by accumulation of organic acid in roots, such as malate, succinate and citrate (Guo-hui 2012; Yang et al. 1994; Hajiboland et al. 2005). In addition, the new leaf exhibited chlorotic symptoms and lower chlorophyll content with bicarbonate in both Fe-deficient and Fe-supplied plants (Fig. 1A, B and E), indicating that bicarbonate induced chlorotic symptoms of new leaf.

Bicarbonate enhanced FRO activity and proton exudation

Bicarbonate stress increases pH in soil and changes the redox state of iron in soil (Mengel et al. 1984; Konrad Mengel 1994). Furthermore, it also directly affects the physiological and biochemical reaction in roots. Bicarbonate treatment significantly increased FRO activity in both Fe-supplied plants and Fe-deficient plants, indicating that bicarbonate enhanced FRO activity in *M. lupulina* roots for reduction of Fe^{3+} (Fig. 2B). The results conflict with the reports of Hsieh (Hsieh and Waters 2016). In his report, Fe-deficient plants were designed as those with 0.5 M Fe. However, in this study, 0 M Fe was designed as an iron deficiency condition. Together, these results suggested that bicarbonate increased or decreased FRO activity depending on the Fe concentration in the environment (García et al. 2014; Lucena et al. 2007; Romera et al. 1997; Waters et al. 2007). In Fe-deficient plants, the expression of *MlFRO1* in roots and leaves was up-regulated, but their expression in roots was higher than that in leaves. It is suggested that the expression of *MlFRO1* is consistent with its role in the reduction of rhizosphere Fe^{3+} (Waters et al. 2002). The higher expression of *MlFRO1* in chlorotic leaf with bicarbonate may increase the FRO activity in leaf apoplast, indicating a partial reduction of Fe^{3+} in leaf cells (Waters et al. 2002). Fe deficiency and bicarbonate supply induced significant *MlHA1* expression levels in roots, especially in bicarbonate-supplied plants (Fig. 2D), indicating H^+ was secreted from roots for acidification of rhizosphere soil with bicarbonate (Martínez-Cuenca et al. 2013). However, the expression of *MlHA1* in NL and CL showed no significant changes under bicarbonate treatment. It suggested that bicarbonate did not change apoplast pH in leaves (Nikolic and Römheld 2002).

Fe uptake response to bicarbonate stress

When plants are subjected to Fe deficiency and bicarbonate, the secretion of FRO and H^+ from roots increases Fe^{2+} availability, then Fe^{2+} is uptaken by root systems depending on iron transporter and metal transporter. Currently, the expression of *MlIRT1* in roots was higher than that in leaves with Fe deficiency or bicarbonate, showing that *MlIRT1* was mainly attributed to Fe transportation into roots from soil (Vert et al. 2002). In addition, higher *MlIRT1* expression levels in CL with bicarbonate indicated that the *MlIRT1* functions as Fe transporter in new leaf with bicarbonate supply. *ZIP* is a metal transporter family, which transports a variety of metal ions, such as Fe^{2+}, Zn^{2+} and Mn^{2+} (López-Millán et al. 2004). Phylogenetic tree analysis showed that *MlZIP1*, *MlZIP3*, *MlZIP5* and *MlZIP7* were homologous with *MtZIP1*, *MtZIP3*, *MtZIP5* and *MtZIP7*, respectively (Fig. S1). In the present study, RT-PCR analysis showed that the expression of *MlZIP1* and *MlZIP5* was down-regulated in Fe-deficient and bicarbonate-supplied roots and leaves (Fig. 3B and D). It is suggested that they are not involved in Fe transport. In fact, *AtZIP1* functions as a transporter of Zn^{2+} and responds to zinc deficiency in *A. thaliana* (Grotz et al. 1998), which is an ortholog *MtZIP1*. In addition, *MtZIP5* is down-regulated under Mn deficiency (López-Millán et al. 2004). López-Millán et al. (López-Millán et al. 2004) suggested that *MtZIP3* was induced in roots and leaves in response to Zn^{2+} deficiency. Although the expression of *MlZIP3* and *MlZIP7* was up-regulated in Fe-deficient and bicarbonate-supplied plants (Fig. 3C and E), *MtZIP3* was reported to be up-regulated under Zn^{2+} deficiency (López-Millán et al. 2004) and *MtZIP7* mainly contributed to the transportation of Mn^{2+} (López-Millán et al. 2004). Therefore, this study did not clearly indicate whether *MlZIP3* and *MlZIP7* are involved in Fe transport.

Bicarbonate blocked Fe translocation from root

Fe is usually combined with ligands or transporters to form a complex transporter, which is translocated into leaf tissue through the xylem. Currently, the *NRAMP* and *YSL* genes are not identified from the *M. lupulina* transcriptome data (Data are not shown). RT-PCR analysis showed that *MlMATE66* was mainly expressed in roots (Fig. 4A). The results were consistent with those reported by Yokosho (Yokosho et al. 2009). The expression of *MlMATE66* was significantly down-regulated in the roots of bicarbonate-treated plants and up-regulated in chlorotic leaf. It is suggested that bicarbonate blocks the Fe translocation from roots, which results in Fe accumulation in roots (Fig. 5B). Of course, higher expression of *MlMATE66* in chlorotic leaves with bicarbonate was involved in Fe translocation between leaf tissue. Three ferritin genes (*MlFer1-3*) were identified in *M. lupulina* and RT-PCR analysis showed that all of them were down-regulated in bicarbonate-supplied plants, especially in chlorotic leaf (Fig. 4B, C, D). It is shown that Fe did not combine with ferritin in the roots.

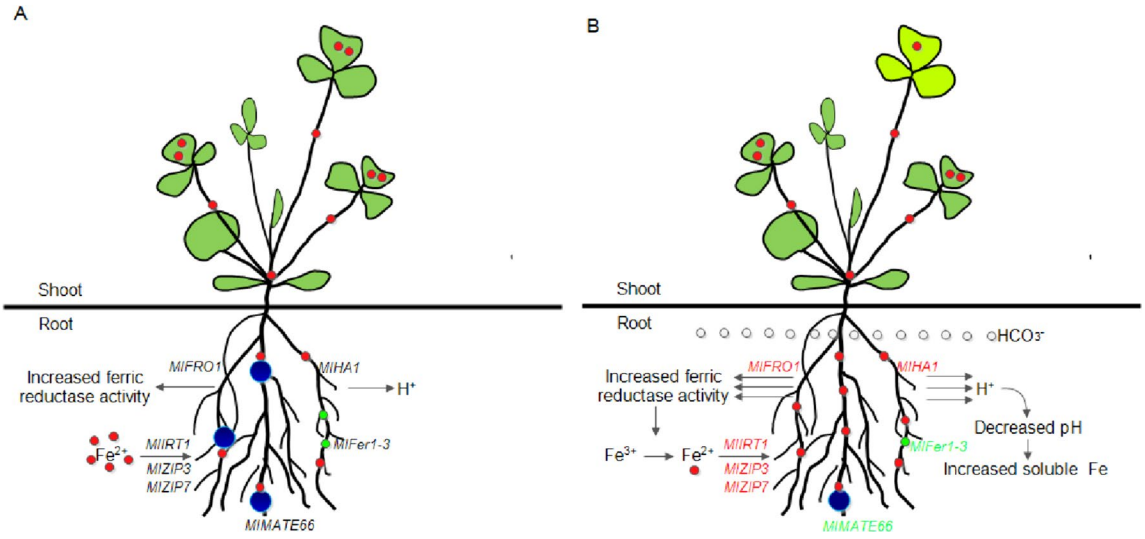

Fig. 6 Schematic diagram of iron uptake and translocation in *M. lupulina* under bicarbonate stress. **A** and **B** indicate the normal growth condition and bicarbonate stress, respectively. Hollow circle represents HCO^{3-}. Red circle represents $Fe^{2+.}$ Blue circle represents citrate transporter. Green circle represents ferritin. Genes with red represents up-regulation and genes with green represent down-regulation

Conclusions

In conclusion, bicarbonate stress reduces the iron (Fe) availability in the root environment, which enhances the expression of genes related to Fe acquisition, such as *MlHA1*, *MlFRO1* and *MlIRT1*, resulting in Fe uptake from soil. More importantly, bicarbonate stress inhibits the expression of *MlMATE66* in roots and prevents the Fe translocation from roots to developing leaf, resulting in Fe accumulation in roots and reducing Fe content in new leaf. Therefore, chlorotic symptoms of iron deficiency induced by bicarbonate are triggered in the root (Fig. 6). Additionally, the functions of *MlZIP3* and *MlZIP7* under bicarbonate stress remain unclear in the present study. Our studies broaden our knowledge level about iron acquisition-related genes and further reveal the mechanism of iron deficiency chlorosis grown in calcareous soil (enrichment bicarbonate).

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Author contribution statement Ximin ZHANG conceived and designed the experiments. Lunxian LIU performed the experiments on RT-PCR. Zhimeng SU performed the experiments on biomass and enzyme activity. Ming TANG designed the primers. Jing TANG identified the genes. Ximin ZHANG, Lunxian LIU, Meifeng CHEN and Xiaorong XU wrote the paper. Yin YI and Jiyi GONG revised the paper. All authors have read and approved the manuscript.

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Data availability The raw data used in the present study are available within the article and Supplementary File.

Declarations

Conflict of interest All authors declare that they have no conflicts of interest.

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