BIOLOGIA PLANTARUM 59 (4): 654-660, 2015 DOI: 10.1007/s10535-015-0543-9

Characterization of transgenic *Poncirus trifoliata* overexpressing the ferric chelate reductase gene *CjFRO2* from *Citrus junos*

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Abstract

Iron deficiency chlorosis occurs frequently in calcareous soils. The transformation of plants with ferric chelate reductase genes (*FROs*) provides a potential strategy to alleviate plant chlorosis under iron deficiency. A *CjFRO2* gene isolated from *Citrus junos* Sieb. ex Tanaka was introduced into *Poncirus trifoliata* (L.) Raf *via Agrobacterium*-mediated transformation. The transgene integration and expression were confirmed by PCR, Southern blot, and real-time PCR analyses. Hydroponic- and soil-grown transgenic plants were tested for their tolerance to iron deficiency. Compared with nontransgenic (NT) *P. trifoliata* plants, a rhizosphere acidification capacity in the transgenic lines increased, and a ferric chelate reductase activity in roots was up to 3.39- and 2.93-fold higher in a hydroponic solution and soil, respectively. A transgenic line TO-8, which reacted similarly in hydroponics and soil, appeared tolerant to the iron deficiency. Its leaf chlorophyll and ferrous ion content was significantly higher than in NT. These results indicate that tolerance to the iron deficiency in *P. trifoliata* could be improved through the genetic engineering.

Additional key words: chlorosis, iron deficiency, real-time PCR, rhizosphere acidification, Southern blot.

Introduction

Iron is fairly abundant in soils, usually exceeding plant requirements. However, iron in calcareous soils is present mainly in the form of insoluble ferric ion, which results in a low iron availability for plants. Reduction of ferric ion to ferrous ion at root surface is an obligatory process for iron acquisition from soils (Chaney *et al*. 1972). Plants, including citrus, respond to iron deficiency with a series of physiological processes involving many iron-responsive genes. Ferric chelate reductase genes (*FROs*) encode plasma membrane ferric chelate reductase (FCR) which is responsible for reduction of the insoluble ferric ions to the soluble ferrous ions. Thus, it may be possible to increase plantability to absorb iron through overexpression of *FROs*. Many *FROs* have been isolated from iron-efficient species (Dancis *et al*. 1990, 1992, Robinson *et al*. 1999, Waters *et al*. 2002, Li *et al*. 2004) and some of them have been transformed into different plant species to alter their iron metabolism. The constitutive expression of *FRE1* and *FRE2* encoding ferric reductases from yeast in transgenic tobacco increased both FCR activity in roots and leaf iron content (Samuelsen *et al*. 1998). The introduction of *FRO2* from *Arabidopsis thaliana* (*AtFRO2*) into soybean (Vasconcelos *et al*. 2006), apple (Yang *et al*. 2009), and *Arabidopsis* (Connolly *et al*. 2003) significantly enhanced ferric reduction in roots and leaves of transgenic plants and reduced iron deficiency chlorosis (IDC). These reports demonstrate that the genetic manipulation of *FROs* may yield lines with an improved tolerance to iron defficiency.

Citrus tolerance to iron deficiency is determined by the rootstock. The performance of different rootstocks under iron deficiency conditions is highly variable. *Poncirus trifoliata* is a widely used commercial rootstock, but it is very susceptible to iron deficiency. *Citrus junos* is an iron-efficient plant that is able to use iron from soils with low iron availability. Four weeks after iron stress, FCR activity can be stimulated to

Submitted 5 September 2014, *last revision* 6 March 2015, *accepted* 16 March 2015.

Abbreviations: Chl - chlorophyll; GFP - green fluorescence protein; IDC - iron deficiency chlorosis; FCR - ferric chelate reductase; *FRO* - ferric chelate reductase gene; NT - nontransgenic *Poncirus trifoliate.*

Acknowledgements: We thank Prof. Xiaochun Zhao from the Citrus Research Institute of the Southwest University for reading critically the manuscript. This research was supported by the National High Technology Research and Development Program of China (Grant No. 2011AA100205)

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~20 times the normal activity in *C. junos*, but only ~3 times in *P. trifoliata* (Li *et al*. 2002) These results indicate that an obvious increase in FCR activity is an important factor for tolerance of *C. junos* to iron deficiency.

To obtain transgenic *P. trifoliata* having an increased tolerance to iron deficiency, *CjFRO2* was introduced into *P. trifoliata* through *Agrobacterium*-mediated transformation. Then, the characteristics of transgenic plants, such as rhizosphere acidification ability, FCR activity, and content of chlorophyll (Chl) and ferrous ions were investigated.

Materials and methods

Production of transgenic plants: Seeds of *Poncirus trifoliata* (L.) Raf were collected from the National Citrus Germplasm Repository, Chongqing, China. Germination of seeds, preparation of *Agrobacterium*, and transformation of explants were performed according to Dutt and Grosser (2009). A transformation vector was pBICjFRO2-EGFP (Fig. 1). When the stems of adventitious shoots reached 1.0 cm, they were excised from the explants for rooting. A rooting medium was a half-strength Murashige and Skoog (1962) medium containing 0.5 mg dm^{-3} indole-3-butyric acid, 0.2 mg dm^{-3} α-naphthylacetic acid and 8 g dm^{-3} agar. After hardening for 7 d, plantlets with 2-cm-long roots were transferred to a 28 °C greenhouse. Nontransgenic *P. trifoliata* (NT) was used as control.

Detection of green fluorescence protein (GFP) in transformed shoots was performed using a *Leica M165FC* fluorescence stereomicroscope equipped with a GFP filter, a 480/40 nm excitation filter, and a 510 nm barrier filter (*Leica Microsystems*, Wetzlar, Germany).

Fig. 1. The T-DNA region of pBICjFRO2-EGFP plasmid. The T-DNA harbors NOSP-*NPTII*, CAMV35S-*EGFP*, and CAMV35S-*CjFRO2* genes. A 786-bp 35S-CjFRO2 cassette (marked with a *black line*) will hybridize to a probe. RB - right T-DNA border, LB - left T-DNA border, NOS-P - nopaline synthase promoter, NOS-T - nopaline synthase terminator, CAMV 35S - *Cauliflower mosaic virus* 35S promoter, *NPTII* - neomycin phosphotransferase II gene, *EGFP* - enhanced green fluorescence protein gene, *CjFRO2* - ferric chelate reductase gene from *Citrus junos*, *Vsp*I, *Cal*I, and *Kpn*I - restriction enzyme sites for *Vsp*I, *Cal*I, and *Kpn*I, respectively.

PCR was used to detect the integration of a foreign gene into the citrus genome. Genomic DNA was extracted from plants showing GFP fluorescence and from NT using a plant genomic DNA extraction kit (*Aidlab*, Beijing, China). A primer pair P1/P2 (5′-GGAAGGTGGCTCCTACAAATGC-3′ and 5′-ACT TGGCTTGCCATACTTTCTCTCC-3′) was used to amplify 35S-CjFRO2 cassette and the expected fragment was 786 bp.

To investigate the T-DNA copy number in the transgenic plants, Southern blot analysis was performed according to the instructions of *DIG High Prime* DNA labeling and detection *Starter Kit I* (*Roche*, Basel, Switzerland). Genomic DNA (50 μg) from the transgenic plants and NT were digested with *Kpn*I restriction enzyme (Fig. 1), separated on 0.8 % (m/v) agarose gels, UV cross-linked to *Hybond-N*$^+$ nylon membranes, and hybridized with a *DIG*-labeled probe. The DNA fragment of the probe was amplified from the pBICjFRO2-EGFP plasmid using the primer pair P1/P2.

For real-time PCR analysis, total RNA was prepared from roots and leaves of the transgenic plants and NT. Experiments were performed as described by Meng *et al.* (2014). An *actin* gene was used as internal control. Primer pairs for the *CjFRO2* and *actin* genes were C1/C2 (5′-CTGCGGATAGAGAGCTCG-3′ and 5′-GATTAT CAGCCAACCCAG-3′) and A1/A2 (5′-CATCCCTCA GCACCTTCCAGC-3′ and 5′-CCAACCTTAGCACTT CTCCATGTC-3′), respectively.

Iron stress tests: After being grown in the greenhouse for 2 months, the transgenic and NT plants were transferred to a hydroponic solution and calcareous soil to evaluate their tolerance to iron deficiency. For hydroponics, the plants were cultured in a Hoagland nutrient solution with 100 μM ferrous ethylene diamine tetraacetic acid (EDTA) until new roots and shoots sprouted. Iron-deficient and iron-sufficient treatments were then carried out according to the method of Chaney *et al*. (1992) with minor modifications using 10 μM ferric diethylene triamine pentacetate acid (DTPA)/10 mM $NaHCO_3$ and 100 μM ferric-DTPA/10 mM $NaHCO_3$ supplements, respectively. The same transgenic lines were grown in a hydroponic box. The solutions were replaced every 15 d. For soil culturing, soil (pH 8.1) was collected from the alkaline purple soil orchard of Citrus Research Institute, Chinese Academy of Agricultural Sciences, using the method described by Cao *et al*. (2011). The transgenic plants and NT were grown in individual plastic feeding blocks (10 × 10 × 30 cm) and

irrigated only with water for 15 d. Then, the plants were irrigated every 15 d with an iron-free Hoagland nutrient solution adjusted to pH 8.1 with 1 mM NaOH.

Root proton exudation and FCR activity of the transgenic and NT plants were measured according to Egilla *et al*. (1994) and Li *et al*. (2002), respectively, 7 and 14 d after the iron stress treatments. When the new leaves of NT became yellow except the veins which were still green, representing the third stage of IDC (Cao *et al*. 2011), the youngest fully expanded leaves were excised to analyse Chl and active ferrous ions content. The Chl content was measured as described by Hiscox and Israelstam (1979) method with a UV/Vis spectrophotometer (*TU-1901*, *PGeneral*, Beijing, China). The ferrous ions content was determined using an atomic absorption spectrophotometer (*AA-800*, *Perkin Elmer*, Waltham, MA, USA) as described by Ren *et al*. (2005).

Statistical analysis: In this study, there were five replications per experiment, and three independent experiments were performed per transgenic line. The statistical analysis was performed using *SPSS v. 13.0* statistical software. Significance of differences was analysed using the *t*-test, and $P < 0.05$ was considered significant.

Results

The 35S-CjFRO2 cassette was introduced into *P. trifoliata* by *Agrobacterium*-mediated transformation. Using *GFP* as reporter gene, callus masses with green fluorescence at the cut end of the explants were visible 9 d after co-cultivation, and adventitious buds were induced in the shoot regeneration culture. Most of them grew normally and developed well. Finally, 20 *GFP*-positive transgenic lines, named TO-1 to TO-20, were obtained. A 786-bp 35S-CjFRO2 specific product was detected by PCR in all *GFP*-positive lines, whereas no corresponding product was amplified from NT (Fig. 2*A*). The Southern blot analysis shows that transgenic lines contained one to three copies of the transgene (Fig. 2*B*). These results indicate that *CjFRO2* was successfully integrated into the genome of *P. trifoliata*. The expression of *CjFRO2* in roots and leaves of 20 transgenic lines was evaluated by real-time PCR. All the transgenic lines showed the expression of *CjFRO2* in both roots and leaves (Fig. 2*C*).

Root proton exudation from the transgenic lines was

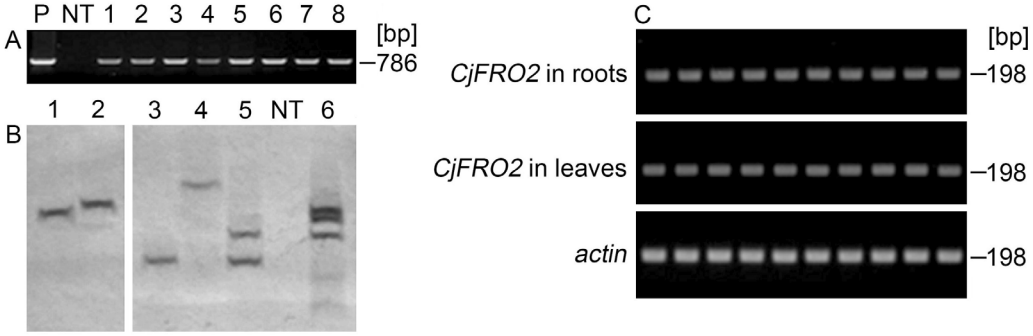

Fig. 2. Production of transgenic *Poncirus trifoliata* using *CjFRO2* gene from *Citrus junos*. *A* - The PCR amplification of 35S-CjFRO2 cassette in plasmid pBICjFRO2-EGFP (P), non-transgenic (NT), and transgenic lines (1 - 8). *B* - The Southern blot of transgenic *Poncirus trifoliata* plants; 1 to 6 - transgenic lines TO-1, TO-2, TO-4, TO-8, TO-11, and TO-13, respectively. *C* - The real-time PCR analysis of *CjFRO2* in roots and leaves of transgenic plants. An *actin* gene was amplified as template control.

investigated using bromocresol purple as pH indicator after the plants endured the iron stress for 7 d. The initial pH (6.2) of the medium led to a purple colour. When pH declines to 5.2, the medium becomes yellow. The medium around the excised roots of the transgenic lines and NT turned yellow 3 h after incubation. Compared with NT, the colour was more intense and extensive in transgenic lines TO-2, TO-4, and TO-8 cultured in the iron-deficient hydroponics (Fig. 3*A*) and TO-2, TO-8, and TO-16 cultured in the soil (Fig. 3*B*). These results indicate that transgenic *P. trifoliata* overexpressing *CjFRO2* acquired an enhanced rhizosphere acidification capacity.

FCR activity was examined in the transgenic lines after 14 d of growth under the iron-deficiency. For the iron-deficient hydroponics, the FCR activity varied from 0.32 ± 0.03 to 1.20 ± 0.05 nmol g^{-1}(f.m.) min^{-1}. Transgenic lines TO-2, TO-4, and TO-8 displayed 3.39-, 2.09-, and 3.0-fold higher FCR activities than NT, respectively (Fig. 4*A*). Increased FCR activities were also observed in the soil culture. In particular, the FCR activity in transgenic line TO-8 was 2.93-fold higher than that in NT (Fig. 4*A*). These results indicate that FCR activity could be increased using expression of *CjFRO2* in the transgenic plants.

To screen for transgenic lines with an enhanced

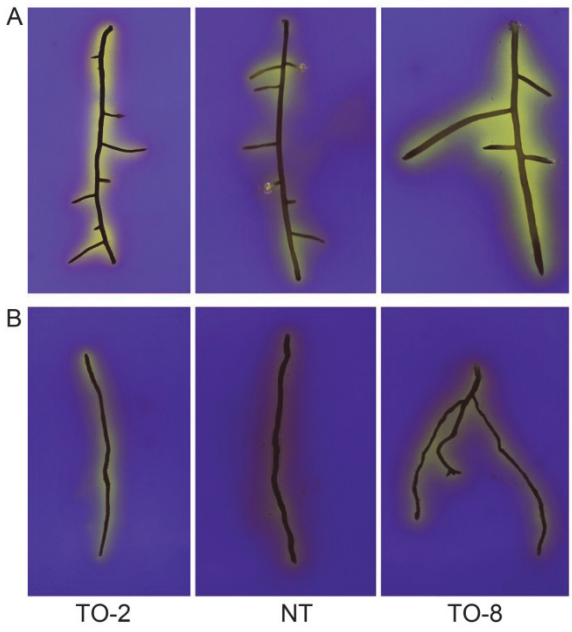

Fig. 3. The visualization of rhizosphere acidification in excised roots of transgenic *Poncirus trifoliata* lines TO-2 and TO-8, and NT 7 d after being transferred into an iron-deficient solution (*A*) and soil (*B*). Colour changes of the medium around roots represent the pH change. *Yellow regions* indicate acidification.

tolerance to iron deficiency, they were under conditions simulating a calcareous soil. After 3 months of the Fe-deficiency, transgenic lines TO-2 and TO-8 showed alleviated IDC with young leaves that were still green compared with young leaves of NT which became yellow (Fig. 5*A*). However, the other transgenic lines displayed serious chlorotic symptoms. The young leaves of transgenic lines TO-2 and TO-8 were used to investigate Chl content. The Chl content in transgenic lines TO-2 and TO-8 was 2.62 ± 0.05 and 2.19 ± 0.09 mg g^{-1}(f.m.), respectively (Fig. 4*B*), which was significantly different from that in the NT plants. Chlorotic symptoms appeared slowly in plants in the iron-sufficient hydroponic solution, with the visual chlorosis of NT reaching the third stage of IDC after five months. At this point, the young leaves of transgenic lines TO-2 and TO-8 were still green (Fig. 5*B*), and their Chl content was 2.95 ± 0.06 and 2.66 ± 0.04 mg g^{-1}(f.m.), respectively (Fig. 4*B*). However, unlike with the hydroponics, four months after being grown in the soil with pH of 8.1, only transgenic line TO-8, with a Chl content of 3.04 ± 0.16 mg g^{-1}(f.m.) (Fig. 4*B*), appeared tolerant to the iron deficiency (Fig. 5*C*). Transgenic line TO-2 displayed the same chlorotic symptoms as NT (Fig. 5*C*), and its Chl content was not significantly different compared with NT (Fig. 4*B*).

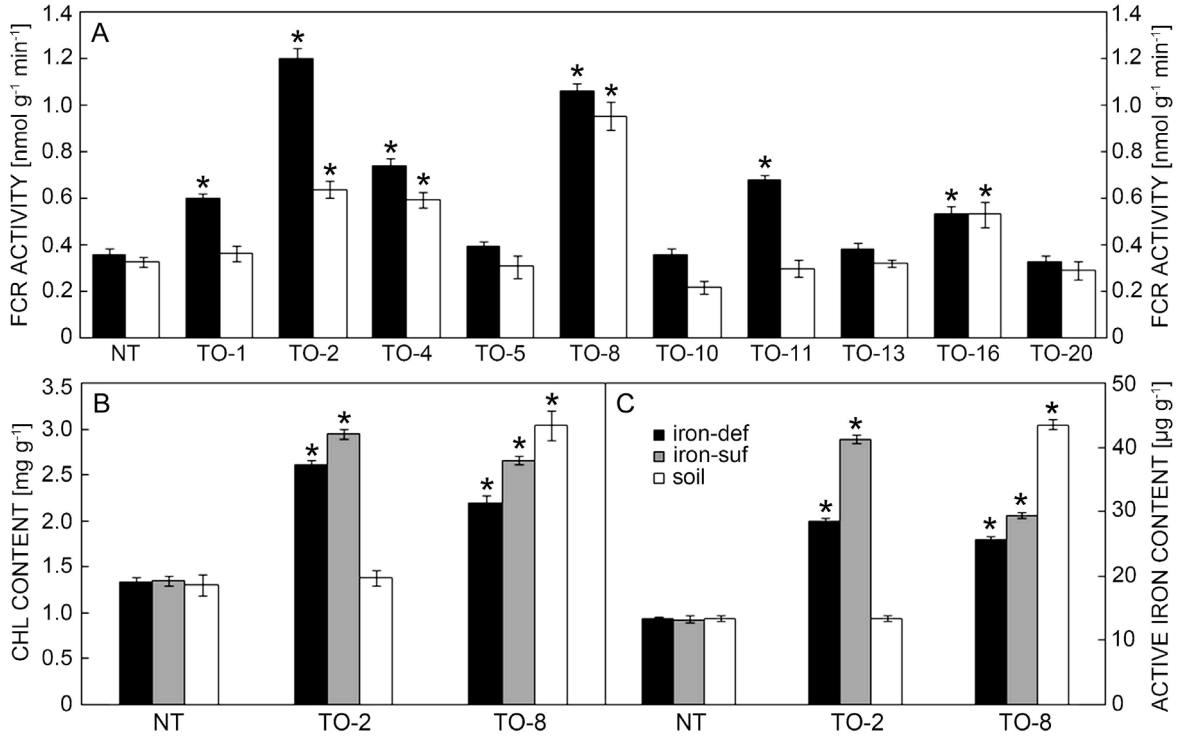

Fig. 4. FCR activity and Chl and ferrous ions content in transgenic *Poncirus trifoliata* lines. *A* - FCR activity in transgenic lines TO-1, TO-2, TO-4, TO-5, TO-8, TO-11, TO-13, TO-16, and TO-20, and NT after being grown in an iron-deficient solution and soil with pH of 8.1 for 14 d. *B* and *C* - Chl and active iron (ferrous ions) content in transgenic lines TO-2 and TO-8, and NT grown in both iron-deficient and iron-sufficient solutions and in a soil until the visual chlorosis rate of NT reached the third stage. Means ± SD, $n = 5$. * - indicates a significant difference at $P < 0.05$ between transgenic plants and NT.

The leaf active ferrous ions content in TO-2 and TO-8 transgenic lines and NT was measured simultaneously with the Chl content. In the iron-deficient hydroponic solution, the ferrous ions content in transgenic lines TO-2 and TO-8 was 28.48 ± 0.43 and 25.60 ± 0.45 μg g^{-1}(f.m.), respectively, whereas it was only 13.35 ± 0.26 μg g^{1}(f.m.) in the NT plants (Fig. 4*C*). There was a significantly higher accumulation of ferrous ions in transgenic lines TO-2 and TO-8 compared with NT. Similar results were observed under the iron-sufficient hydroponic solution, with accumulations of 41.21 ± 0.61 and 29.40 ± 0.45 μg g^{-1}(f.m.) ferrous ions in transgenic lines TO-2 and TO-8, respectively (Fig. 4*C*). Under the soil-based conditions, the leaf content of ferrous ions in transgenic line TO-8 was 43.51 ± 0.76 μg g^{-1}(f.m.), which was significantly higher than that in NT (Fig. 4*C*). These results show that the overexpression of *CjFRO2* increased the ability of roots to use iron in transgenic *P. trifoliata*.

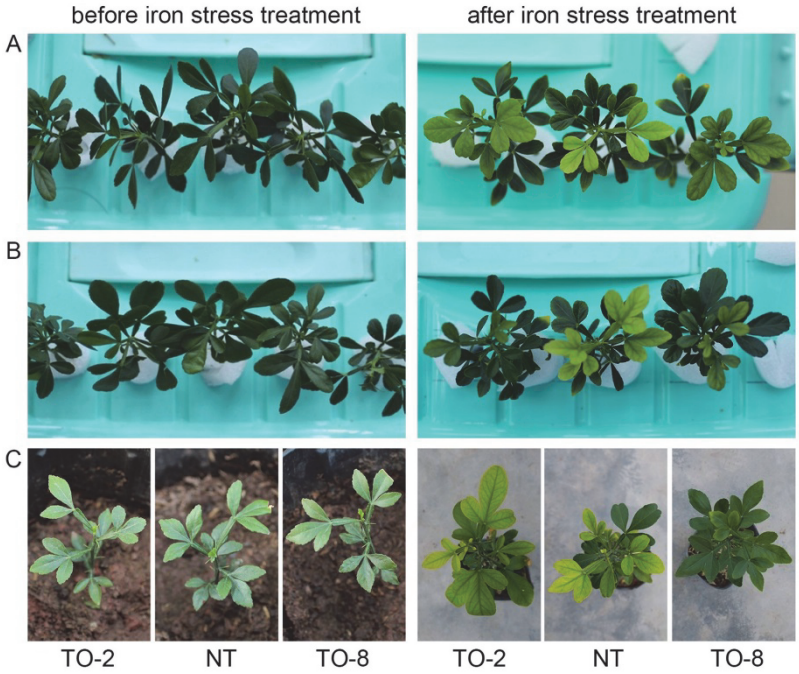

Fig. 5. The phenotypes of transgenic *Poncirus trifoliate* lines TO-2 and TO-8, and NT before (l*eft*) and after (*right*) being grown in iron-deficient (*A*) and iron-sufficient (*B*) solutions and in a soil (*C*).

Discussion

Plants often suffer from many environmental stresses, which negatively affect their growth and even survival. Many studies have shown that genetic transformation may offer an efficient way to produce new genotypes with enhanced stress resistance (Liu *et al*. 2013, Pal *et al*. 2013, Meng *et al*. 2014). In this study, we introduced gene *CjFRO2* from tolerant *C. junos* into iron-susceptible *P. trifoliata*. Then, we investigated the tolerance of the transgenic plants to iron deficiency using hydroponics and soil culture. Our data show that the transgenic plants expressing *CjFRO2* could acquire an increased tolerance to the iron deficiency.

Plants use different strategies, such as proton exudation and ferric ion reduction, under iron-limiting conditions. An increased proton exudation precedes the enhancement of FCR activity in the roots of *C. junos* under iron deficiency, and the region of proton exudation overlaps the area reduced by FCR (Li 2002). Iron-efficient plants can use iron from soils with a low iron availability. Under iron stress, proton exudation and FCR activities of iron-efficient plants are much greater than those of iron-susceptible plants (Li *et al*. 2001, 2003). Our data show that the rhizosphere acidification ability of TO-2 and TO-8 transgenic lines was greater than that of the NT plants under the iron stress (Fig. 3). In addition, the FCR activities in transgenic lines TO-2 and TO-8 increased in comparison to the NT plants (Fig. 4*A*). The increased proton exudation and FCR activities in these transgenic lines could result in the generation of more ferrous ions for root absorption. Lack of change in pH of the xylem sap and leaf apoplast (Nikolic and Römheld 1999, 2002) may facilitate iron trafficking. The expression of foreign *FRO* in leaves of transgenic plants can increase leaf iron reductase capacity (Vasconcelos *et al*. 2006) resulting in more ferrous ions absorbed into leaf mesophyll cells. These integrated processes can

ensure an effective utilization of iron in transgenic plants. As result, these transgenic lines exhibited an enhanced Chl and ferrous ions content (Fig. 4*B,C*) and alleviated IDC (Fig. 5). These results are consistent with a previous study where FCR activity and Chl and ferrous irons content are enhanced in transgenic lines expressing *AtFRO6* (Li *et al*. 2011). Our results indicate that the enhanced tolerance to the iron deficiency in the transgenic lines investigated was due to increased proton exudation and FCR activities which were induced by the *CjFRO2* transformation.

Under the soil culture, transgenic line TO-2, which appeared tolerant to the iron deficiency in the hydroponic solution (Fig. 5*A,B*), displayed chlorotic symptoms in new leaves (Fig. 5*C*). The content of Chl and ferrous ions in transgenic line TO-2 was not significantly different when compared with the NT plants (Fig. 4*B,C*). Brand *et al*. (2002) also noted that lines of rough-seeded lupins react differently in a solution than in soil. A possible explanation is that the increased proton exudation in the transgenic lines caused the direct acidification of the nutrient solution resulting in the pH decrease (M'Sehli *et al*. 2008). However, the high pH (8.1) of the soil in this study could decrease more slowly by *in vivo* root surface FCR activity in the intact transgenic lines (Susín *et al*. 1996). Therefore, the transgenic lines grown in the solution absorbed more iron than in the soil, resulting in more transgenic lines appearing tolerant to the iron deficiency. Although the solution- and soil-based culturing methods correlated, the latter is preferable because the soil culture was better representative of the high calcareous arable soils in the citrus-growing area of China.

In conclusion, our study shows that the over-expression of *CjFRO2* in *P. trifoliata* enhanced root proton exudation and FCR activity, which led to the increased content of Chl and ferrous ions, and reduced IDC. Ultimately, based on the solution- and soil-based culturing methods, transgenic line TO-8 possessed an enhanced tolerance to iron deficiency. Thus, we can produce transgenic citrus rootstocks with an improved tolerance to iron deficiency through the genetic manipulation of *FROs*.

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