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Isolation and characterization of *PbCS2* gene regulated by iron deficiency and auxin-based systemic signals in *Pyrus betulifolia*

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ABSTRACT

Iron (Fe) is an essential micronutrient for plants and citric acid acts as a chelate substance for the long-distance transmission of Fe. Citrate synthase (CS) protein is a key enzyme in the synthesis of citric acid. Using PCR amplification, a gene encoding a putative citrate synthase was isolated from *Pyrus betulifolia*, a widely-used rootstock native to China, and designated it as *PbCS2*. This gene was 1422 bp in length with an open reading frame encoding a protein of 473 amino acids. The deduced *PbCS2* protein contains a conserved citrate synthase domain and conservative WPNVDAHS sequence existing in the PWPN-box. Phylogenetic analyses clearly demonstrated that *PbCS2* had the highest homology with rosaceous CS proteins. The expression of *PbCS2* was enriched in roots, leaves and phloem, but relatively weak in xylem. Fe deficiency induced substantial up-regulation of the *PbCS2* expression in the roots. The split-root experiments showed that Fe deprivation in one part of the root system caused dramatic up-regulation of the *PbCS2* expression in the Fe-sufficient portion, indicating that the *PbCS2* expression was triggered by systemic signals. Furthermore, supplying auxin to the de-topped shoot could recover the Fe deficiency-caused up-regulation of the *PbCS2* expression in the Fe-supplied part of the root system. By contrast, NPA application (an auxin transport inhibitor) to the shoot apex blocked up-regulation of the *PbCS2* expression in the untreated portion. These results strongly indicated that Fe deficiency-caused alterations of the *PbCS2* expression were mediated by IAA.

1. Introduction

Iron is an essential nutrient element for plants and participates in many important physiological and biochemical processes (Briat et al., 1995). Fe deprivation-induced chlorosis is a very common disorder in crops growing on alkaline and calcareous soils (Tagliavini and Rombolà, 2001). This limitation particularly affects productivity of fruit tree species, stunting tree growth and development, decreasing fruit yield and quality as well as limiting life span of orchards. In response to low Fe stress, plants have developed two types of adaptive mechanisms to absorb Fe: Strategy I and Strategy II (Marschner et al., 1986; Römheld, 1987; Schmidt, 2003). Dicotyledonous and non-grass monocotyledonous species appear to adopt Strategy I for Fe mobilization and acquisition (Kim and Guerinot, 2007; Schmidt, 1999). Strategy I plants activate a three-step process that contains plasmalemma H^+-ATPase-mediated proton extrusion mobilizing the insoluble ferric hydroxide complexes, ferric chelate reductase (FCR) converting ferric chelates to ferrous chelates, and metal transporters transporting ferrous chelates (Brüggemann et al., 1990; Buckhout et al., 1989; Dell'Orto et al., 2000; Robinson et al., 1999). However, Strategy II is only used by graminaceous species that can directly acquire Fe^{3+} using phytosiderophore (PS) that belongs to the mugineic acid family, and chelate Fe^{3+} is then used to form Fe^{3+}-PS complexes (Kobayashi et al., 2006; Roberts et al., 2004).

One widely applied solution to enhancing resistance to Fe deficiency chlorosis is to choose the rootstock genotypes that are efficient in Fe acquisition. So far, several studies have focused on several Fe deficiency-tolerant rootstocks in fruit crops (Cinelli and Loreti, 2002; Han et al., 1998; Jiménez et al., 2011; Martínez-Cuenca et al., 2013a, 2016). In the Mediterranean area, the almond × peach hybrid GF 677 is widely used as a rootstock for peaches and nectarines. Unlike other rootstocks sensitive to Fe shortage, GF 677 rootstock is able to carry out the adaptive mechanisms like enhanc-

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ing the root proton extrusion and inducing the FCR activity to avoid low Fe stress in soil (Gogorcena et al., 2005; Jiménez et al., 2008; Molassiotis et al., 2006). *Malus xiaojinensis* is a Fe-efficient genotype found recently and could be used as an apple rootstock in Northern China. It has been proved that under Fe-deficient conditions, the expressions of Fe uptake- and transport-related genes are significantly upregulated in *M. xiaojinensis* (Xu et al., 2011; Yang et al., 2015; Zha et al., 2014a; Zhang et al., 2013). A latest study has evaluated the new citrus hybrids' tolerance to Fe deficiency, suggesting that enhancing root proton extrusion and FCR activity could contribute some tolerance under Fe-deprived conditions (Martínez-Cuenca et al., 2016). These results suggest that the use of Fe-efficient rootstock for enhancing Fe uptake and transmission would offer an effective and practical way to cope with Fe deficiency chlorosis.

Acetyl CoA and oxaloacetate are synthesized into citric acid (CA) by means of catalysis of citrate synthase (EC2.3.3.1). CA can chelate Fe for carrying out Fe transport through the xylem (Cataldo et al., 1988) where the pH value ranges from 5.5 to 6 (Hell and Stephan, 2003). The previous studies once reported that CA contents in fruit trees (such as kiwifruits, citrus and apples) increased significantly under Fe-deficient conditions (Han et al., 2014; Martínez-Cuenca et al., 2013b; Rombolà et al., 2002). The recent studies have showed that Fe deficiency also induces CS activity and the expression of CS gene in *M. xiaojinensis* (Zha et al., 2014b). Fe deficiency-induced changes of CS activity and expression also exist in Strategy II plant species (López-Millán et al., 2012). Additionally, it is proved that increased expression of *MxCS1* could contribute to Fe deficiency tolerance in transgenic tobacco and *Arabidopsis* plants. Interestingly, overexpression of *MxCS1* also resulted in early-flowering, morphologically abnormal flowers, and increased contents of Fe, Zn, Mn, and Cu in young flowers and leaves of transgenic tobaccos (Han et al., 2013).

In plants, much evidence has indicated the existence of systemic signals that could trigger Fe-deficient responses and plant hormones are involved in response to Fe limitation. In split-root experiments, an early study with sunflower plants showed that one part of the root system growing in a culture solution containing Fe could develop Fe efficiency responses when the other portion was not supplied with Fe (Romera et al., 1992). Similarly, a recent study with *M. xiaojinensis* also indicated that systemic signaling may be involved in regulation of adaptive responses to Fe deficiency (Wu et al., 2012). In sunflower and bean plants, it was found that root proton extrusion induced by Fe deficiency can be prevented by removing the shoot apex (Landsberg, 1981a, 1984). In the same way, this fact has also been reported recently that the removal of the shoot apex inhibits up-regulation of both root FCR activity and proton extrusion caused by Fe deficiency in *M. xiaojinensis*, suggesting that the shoot apex has played an important role in the regulation of the Fe-deprived responses. Furthermore, exogenous application of NAA to the de-topped shoots could recover Fe-deficient responses, suggesting that auxin (IAA) may be involved in the regulation of original signals that activate the Fe-starved responses (Wu et al., 2012). A previous study in bean plants has also reported that removal of the shoot tip or application of 2-chloro-9-hydroxyfluorenecarboxylic acid-(9)-methylester onto the shoot apex could block the increase of FCR activity in the Fe-limited roots (Li et al., 2000). In contrast, the presence of the shoot tip in cucumber and sunflower plants was not essential to the development of the Fe-deficient responses (Romera et al., 1992).

Pyrus betulifolia is a widely used pear rootstock in North and Northwest China, accounting for over 80% of pear production. The chlorosis is a physiological disorder cased by Fe deficiency and happens commonly on this rootstock. So far most of the researches have focused on the prevention and correction of the disorder by growing practices, such as applying Fe fertilizer in the soil, trunk injection, foliar Fe application, but the effectiveness is unsatisfactory because those districts possess lime soil and high pH. The development for advanced technology depends upon in-depth study on the mechanism. Therefore, it is essential for us to reveal the molecular mechanism of Fe absorption and utilization by *P. betulifolia*. Unfortunately, there is few research on it so far, especially for *PbCS2* gene. In the present study, we have isolated and characterized the *PbCS2* gene from *P. betulifolia* and investigated the expression level of *PbCS2* in different organs. The relationship was found between the expression of *PbCS2* and systemic signals activated by Fe deprivation. In addition, it was proved that the expression of *PbCS2* was significantly induced by IAA under Fe deficiency. This study will provide a valuable clue to in-depth investigation disclosing the mechanisms of adaptation to low Fe stress in *P. betulifolia*.

2. Materials and methods

2.1. Plant materials and growth conditions

P. betulifolia seedlings with 3–5 leaves were transferred to an aerated Hoagland nutrient solution for 1 week. Thereafter, the solution was switched to a standard Hoagland nutrient solution with pH 6.3 (Fe Source: Fe^{3+}-EDTA), which was replaced once a week. These plantlets were hydroponically cultured in a greenhouse at a regime of 23–25 °C/day and 19–21 °C/night with a photoperiod of 14-h cool-white fluorescent light at 2000 Lx and 10-h dark at a relative humidity between 50 and 70%. The Fe treatments were started when the plants grew 8–10 leaves. The samples were frozen in liquid nitrogen immediately after samples were collected and then stored at −80 °C for RNA isolation.

For low Fe stress treatment, all intact plant roots were grown in Hoagland solution containing 4 μM Fe with 40 μM Fe serving as a control. Each treatment was carried out with three replicates. The root samples were collected at 0, 1, 3, 6, 9 days after treatment.

2.2. Split-root experiments

In the split-root experiment, three treatments were performed: (a) Both portions of the root system were cultured with Fe-sufficient treatment (40 μM Fe); (b) one portion of the root was supplied with low-Fe solution (4 μM Fe) and the other portion remained as before; (c) the Fe-limited portion of the root system was removed after the Fe-deficient treatment was conducted for 1–3 days respectively, and the other portion was still kept in Fe-sufficient solution.

2.3. Shoot-apex treatment with IAA and NPA

Based on above treatment (b) of the split-root experiment, the shoot-apex removal experiment was performed and the following four treatments were designed: (i) the split-root treatment (b) was remained as a control; (ii) the shoot apex was removed completely; (iii) NAP was applied to the shoot apex; and (iv) IAA was applied to the de-topped shoot. The samples from Fe-sufficient portion of the root system were harvested at 0, 1, 3, 6, 9 days after treatment.

2.4. Isolation and real-time PCR expression analysis of PbCS2

RNA was extracted from young leaves (newly unfolded), mature leaves (fully expanded), lateral roots, phloem and xylem of the stem by using the CTAB method with some modifications (Zhang et al., 2004). Approximately 1 μg of DNase-treated RNA (TIANGEN, Beijing, China) was reverse transcribed by using an oligo-dT primer and reverse transcriptase (TaKaRa Dalian, China) in a reaction volume of 20 μL. The cDNA synthesis was used to clone