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Isolation and functional characterization of *MxCS1*: a gene encoding a citrate synthase in *Malus xiaojinensis*

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Abstract

Iron is one of the essential micronutrients required by all living organisms. In this study, we isolated a gene encoding putative citrate synthase (CS) from *Malus xiaojinensis*, designated as *MxCS1*. The *MxCS1* gene encodes a protein of 473 amino acid residues with a predicted molecular mass of 52.5 kDa and a theoretical isoelectric point of 8.67. The expression of *MxCS1* was enriched in the leaf rather than in phloem and root, however, its expression was hardly detected in the xylem. The gene expression was strongly induced by Fe stress treatment in the *M. xiaojinensis* seedlings. Over-expression of *MxCS1* improved Fe deficiency tolerance in transgenic *Arabidopsis*. We argued that *MxCS1* is a new member of the CS genes, and it may function as a regulator in response to iron stress in plants.

Additional key words: gene expression, iron deficiency, transgenic *Arabidopsis.*

Introduction

Iron is an essential element for all plants. Iron has poor solubility in most soil (Guerinot and Yi 1994), particularly in calcareous soil where the concentration of free Fe is far below concentration optimal for plant growth (Han *et al*. 1994a,b). Fe-deficiency induces chlorosis, which is a worldwide problem for crop production (Chouliaras *et al*. 2004). Iron deficiency, especially in North China, can largely limit the growth, yield and quality of apples. To avoid Fe deficiency, plants have developed adaptable mechanisms for the efficient absorption and use of Fe (Benderliev *et al*. 2003), *e.g.*, plants produce more ferric reductase-oxidase (FRO) under Fe-deficiency, to reduce Fe^{III} to Fe^{II} (Li *et al*. 2006, Zhang *et al*. 2009). Nicotianamine (NA) can chelate Fe^{II} for the Fe transport through phloem (Stephan and Scholz 1993, Stephan *et al.* 1994). Additionally, citric acid can chelate Fe^{III} and transport it through xylem (Cataldo *et al*. 1988) where the pH is about 5.5 - 6.0 (Hell and Stephan 2003). The *Arabidopsis* mutant, *frd3*, has provided molecular evidence of the role of citric acid in long-distance iron transport (Rogers and Guerinot 2002, Green and Rogers 2004, Durrett *et al*. 2007). However, the role of citrate synthase (CS) has not been determined yet.

To identify Fe-efficient genotypes, we have collected more than 40 samples from various species and ecotypes of genus *Malus* and indicated that *M. xiaojinensis* is a Fe-efficient apple genotype (Han *et al*. 1998). However, the role of CS in *M. xiaojinensis*, has not yet been confirmed. The *CS* gene plays a key role in synthesizing CS, but the relation between the *CS* and iron transport remains unclear. In this study, we isolated a *CS* gene from *M. xiaojinensis*, designated as *MxCS1*. We detected the expression of *MxCS1* in different organs, and found relationship between the expression of *MxCS1* and applied Fe concentration. The most importantly, we found that over-expression of the *MxCS1* can improve tolerance to Fe deficiency in transgenic *Arabidopsis*.

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Abbreviations: BA - 6-benzylaminopurine; CaMV - the cauliflower mosaic virus; CS - citrate synthase; IBA - indolebutyric acid; MS - Murashige and Skoog; ORF - open reading frame; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcriptase-polymerase chain reaction; WT - wild type.
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Materials and methods

Malus xiaojinensis (a wild apple rootstock, Han *et al*. 1994a) seedlings were rapidly propagated on Murashige and Skoog (MS) medium with 0.5 mg dm^{-3} 6-benzyl-aminopurine (BA) and 0.5 mg dm^{-3} indolebutyric acid (IBA) for one month, and then returned to MS + 1.0 mg dm^{-3} IBA for another and a half months for rooting. Finally the seedlings were grown in Hoagland nutrient solution for one month. Plants were exposed to different Fe concentration in Hoagland nutrient solutions (4, 40 and 160 μM) when the plants have 8 - 9 mature leaves (fully expanded).

Nested PCR was performed to obtain a partial sequence of *MxCS1* by using the first strand cDNA of *M. xiaojinensis* as a template. Two degenerate primers corresponding to the amino acid sequences ELIPEQQE [5'-GAAT(A)TGATTCCAGAACAACAGGAA(C/G)C-3'] and C-terminal WPNVDAHS [5'-CACTGTGA(G)GCA TCT(A)ACATTG(T)GGCCA-3'] were used for the first PCR, and then the WPNVDAHS primer and another primer corresponding to the WETSLLDP sequence [5'-TGGGAAACCTC(A/G)CTTA(G)CTTGACCCA(G/T) GA-3'] were used for the second PCR.

For 3' rapid amplification of cDNA ends (RACE), two gene-specific primers were designed: GSP1, 5'-AGAATG CCAGAAAGTATTACCTGCT-3', and GSP2, 5'-TTG CACAAGTGCCAGTAGTAGCTGC-3'. For the 5'RACE, the primers GSP3, 5'-GCAGGTAATACTTTCTGG CATTCTG-3', and GSP4 5'-CATAAGCTGGAA CTACAGCACGAGT-3' were used. The RACE reactions were performed with RACE cDNA amplification kit (*Invitrogen*, Beijing, China) according to the manu-facturer′s instruction. A single, full-length, cDNA sequence was obtained by combining the 5'-RACE fragment, cDNA fragment and 3'-RACE fragment together. A pair of primers (F1, 5'-TTCACA TTTAAAACGTAATTGAGTT-3' and F2 5'-AGAAGT GCTTGGACTTTATTTTAAC-3') were designed to amplify the putative 5' and 3' untranslated region (UTR) of the full-length cDNA sequence. The resulting DNA fragments and RACE products were gel purified and cloned into the pMD18-T vector (*Takara Biotechnology*, Dalian, Japan) and sequenced (*Invitrogen*).

Total RNA was extracted separately from roots and leaves using the CTAB method (Zhang *et al*. 2005). First strand cDNA was synthesised with 1 μg total RNA and 0.001 cm^3 superscript II enzyme (*Invitrogen*) according to the protocol from manufacturer. As a control, the actin rRNA gene was amplified from *M. xiaojinensis* tissues using the following primers: ApAts1, 5'-CTACAA AGTCATCGTCCAGACAT-3' and ApAtR1, 5'-TGG GATGACATGGAGAAGATT-3'. The primers used for detecting *MxCS1* gene expression were as follows: forward, 5'- GTATTCTTCAGGAGCGTCACC -3' and reverse, 5'-GCTCGGTCCCATATCAACTG-3'. All excised samples were frozen in liquid nitrogen and immediately stored at -80 °C for RNA extraction. Samples including root, xylem, phloem and leaves (partly expanded new leaves) of normal Fe treatment (40 μM); roots and leaves taken from treated plants under low (4 μM) and high (160 μM) Fe treatments for 0, 12, 24 and 48 h respectively. The PCR was performed as follows: pre-denaturation at 94 °C for 3 min, followed by 30 cycles of 40 s at 94 °C, 40 s at 50 °C, 90 s at 72 °C for *MxCS1* (25 cycles for actin) and a final extension of 7 min at 72 °C. The amplified products were detected using a 1.1 % agarose gel electrophoresis. All RT-PCR experiments were repeated at least three times.

To construct an expression vector for transformation of *Arabidopsis*, restriction enzyme cut sites of *Xba*I and *Sac*I were added into *MxCS1* cDNA at both 5' and 3' end by PCR. To construct the pBI121-*MxCS1* vector, the products of PCR and pBI121 were digested by *Xba*I and *Sac*I, and ligated together through the replacing of *GUS* gene. The *MxCS1* gene driven under the cauliflower mosaic virus (CaMV) 35S promoter was introduced into *Arabidopsis thaliana* ecotype Columbia (Col-0) plants by *Agrobacterium*-mediated GV3101 transformation (An *et al*. 1988) using the vacuum infiltration method. Transformants were selected on MS medium containing 50 mg dm^{-3} kanamycin.

The T_3 generation plants were used in the subsequent experiments. For the growth assay, T_3 transgenic plants of lines, control plant only transformed only PBI121 vector line and wild-type seeds were placed on MS agar plates for germination. After 4 d, 30 germinated seedlings from each line were carefully transferred to new MS agar plates supplemented with 4 (low), 100 (normal) or 400 μM (high) Fe concentration. After 10 d, the fresh mass of seedlings was measured. Three replicates were conducted and the standard errors were calculated.

Chlorophyll content was measured according to the method of Aono *et al.* (1993). Fe concentrations in leaves were measured according to Kojima and Iida (1986) and Takahashi *et al.* (2003). The CS activities in leaves were measured according to Bryan *et al.* (2001). Assays for the content of citric acid were performed by *o*-phthal-dialdehyde (OPA) derivatisation, reverse-phase high performance liquid chromatography (HPLC) separation and fluorescence detection of the OPA derivatives. In all experiments pure citric acid was used (*Sigma*, St. Louis, USA) as an external standard (Zhang *et al*. 2009).

Results

By designing primers to the homologous regions of CS, partial cDNA of *MxCS1* gene was isolated from *M. xiaojinensis* using PCR. The full-length cDNA was obtained by employing rapid amplification of 3'-cDNA

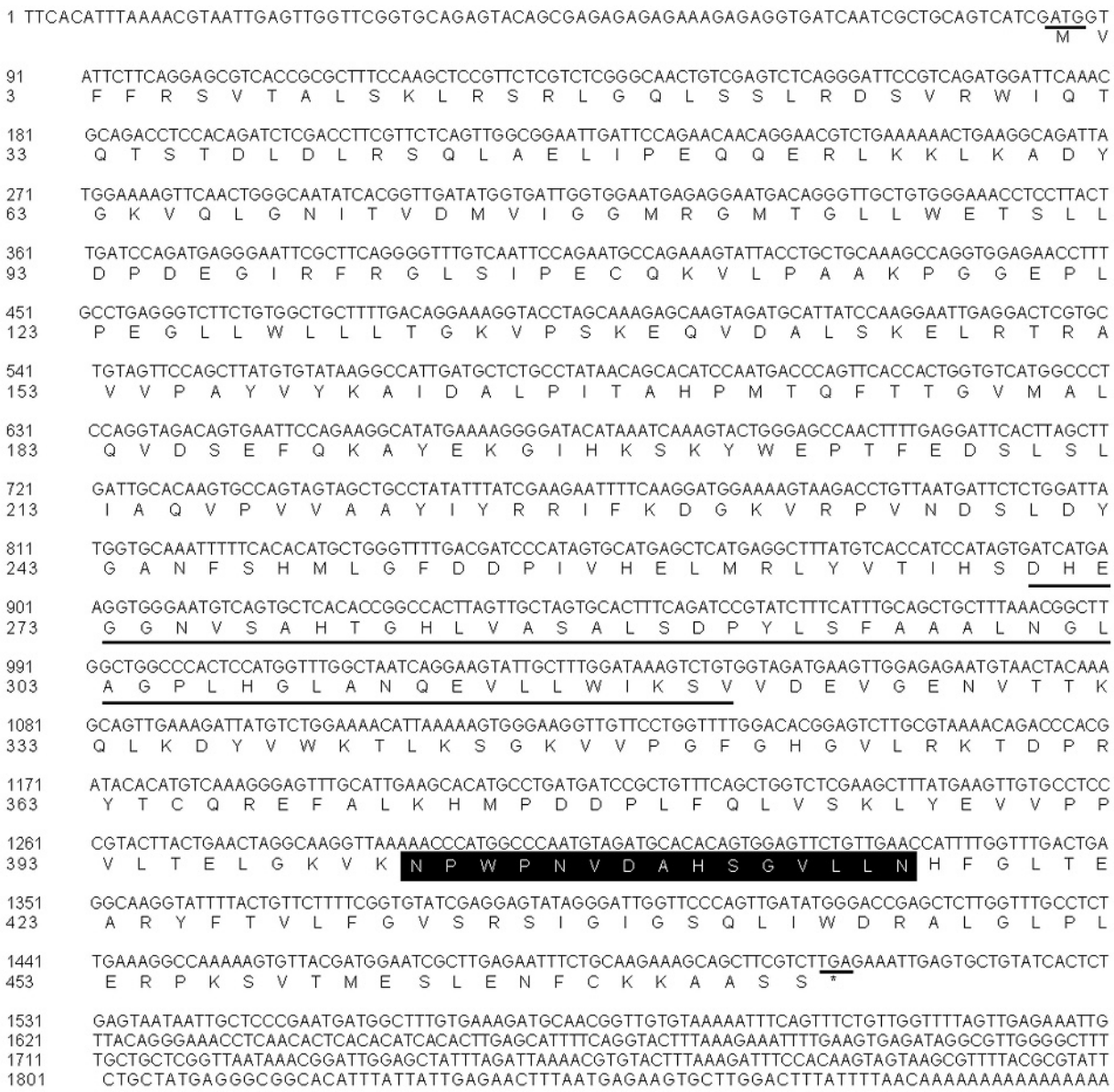

Fig. 1. Nucleotide and deduced amino acid sequences of *MxCS1* (*GenBank* accession No. HM459855). The citrate synthase domain is *underlined*, The PWPN-motif is highlighted with *white letters in the black background*.

end (3'-RACE) and 5'-cDNA end (5'-RACE). Sequence analysis showed that the *MxCS1* cDNA was 1882 bp, including a complete open reading frame of 1422 bp flanked by a 85 bp 5'-UTR and a 375 bp 3'-UTR (Fig. 1; Genbank accession No. HM459855). The predicted protein of MxCS1 comprises 473 amino acids with a predicted molecular mass of 52.5 kDa, and a theoretical isoelectric point of 8.67.

To investigate the evolutionary relationship among plant CS proteins, 8 genes of CS proteins from different species were analysed by *DNAman* analyse software (Fig. 2). The deduced amino acid sequence of MxCS1 included one conserved CS domain in the C-terminal region (Fig. 2*A*) and a PWPN-box in the N-terminus (Fig. 2*B*) contained the plant-specific WPNVDAHS sequence which serves as a DNA-binding motif of CS (Christelle *et al*. 2002, Takita *et al*. 1999). The PWPN-box in the N-terminus functions as a transcriptional repression domain.

Comparing the amino acid sequences of MxCS1 with the CS proteins from other species, we found that MxCS1 had a high identity to the CS protein family. Additionally, a phylogenetic tree (Neighbour-Joining) was constructed with the full-length amino acid residues (Fig. 2*C*). The results showed that MxCS1 from *M. xiaojinensis* and CS1 from peach (Christelle *et al*. 2002; AF367444.1) clustered together, followed by clustering of grapefruit (U19481.1) and orange (GQ372880.1). However, *Arabidopsis* (NM_129998.3), carrot (Takita *et al*. 1999; AB017159.1), rice (AF302906.1) and tobacco (X84226)

were grouped into another cluster.

The expression profile of the *MxCS1* in various *M. xiaojinensis* tissues was investigated using the semi-quantitative RT-PCR. Expression of *MxCS1* was enriched in leaf; *MxCS1* expression was also expressed in root and the phloem of stem, but was nearly undetectable in the xylem (Fig. 3*A*). The results showed that *MxCS1* mRNA increased in roots and leaves under a low iron concentration (4 μM) (Fig. 3*B*) at the beginning, but decreased little at 48 h; on the contrast, the expression decreased in the roots and leaves under a high iron concentration (160 μM; Fig. 3*C*).

In order to investigate the role of *MxCS1* in response to Fe stress, we generated transgenic *Arabidopsis* plants over-expression of *MxCS1* under the control of the CaMV 35S promoter. Among 10 transformant lines, six of them (OE-1, OE-2, OE-5, OE-7, OE-8 and OE-9) were confirmed by using RT-PCR analysis, the line of WT (wild-type) and line of vector (transformed only pBI121 vector) were acted as contrapositions (Fig. 4*A*).

No significant difference in appearance between WT, vector lines and *MxCS1*-OE *Arabidopsis* lines were observed. The T_3 transgenic lines OE-1 and OE-5, WT and vector seedlings were placed on MS agar plates supplemented with 4, 100 and 400 μM Fe. Up to 100 μM Fe, all types of *Arabidopsis* grow well. WT and vector lines have obvious chlorosis appearance, but *MxCS1*-OE lines have no obvious chlorosis appearance under Fe-deficiency (4 μM). *MxCS1*-OE lines have better appearance than others on high Fe concentration (400 μM). No difference between WT and vector in response to Fe stress was observed.

The *MxCS1*-OE lines had higher fresh mass and higher chlorophyll contents than the WT and vector lines

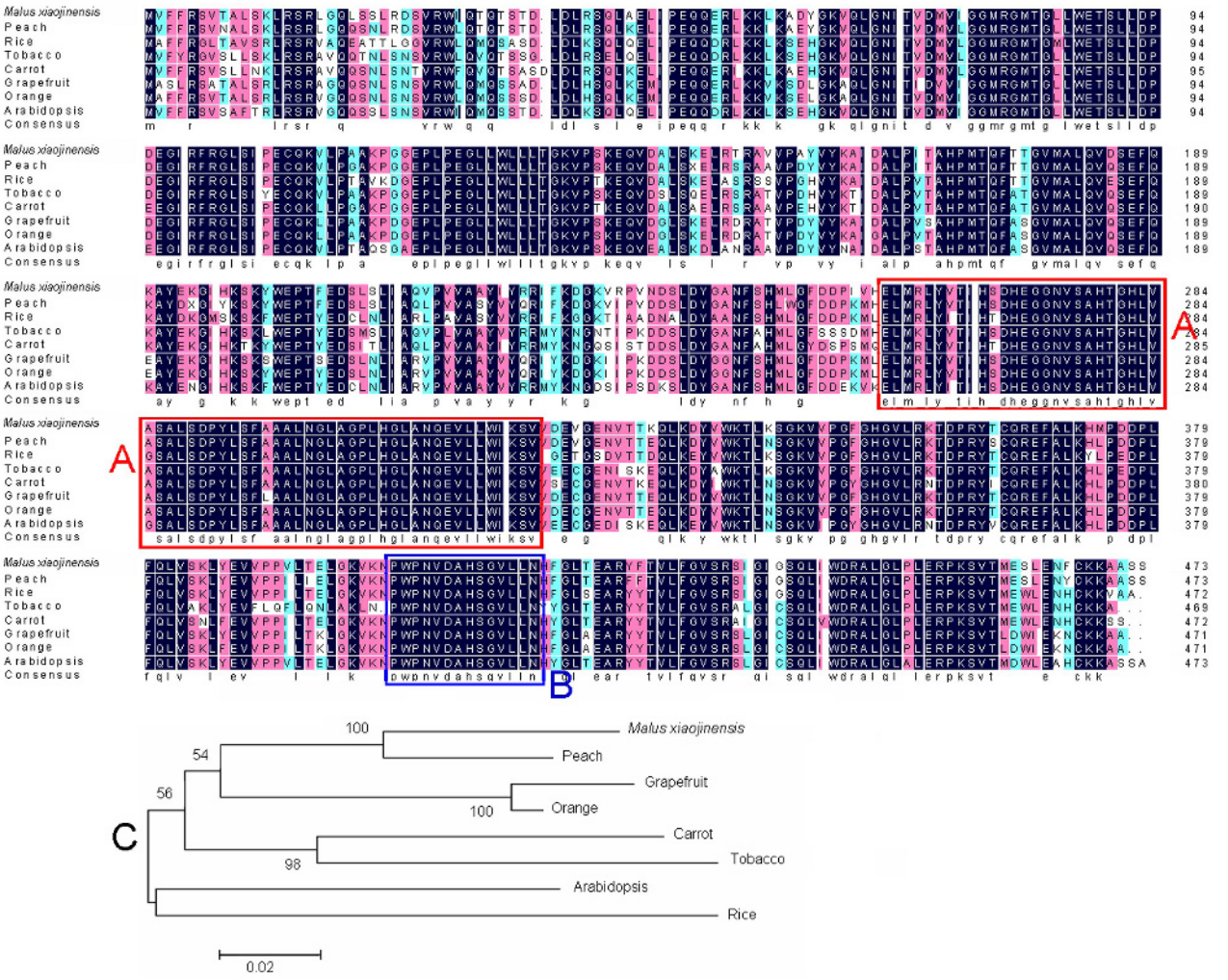

Fig. 2. Comparison and phylogenetic relationship of MxCS1 with reported citrate synthase proteins from other species. Alignment of MxCS1 with other plant citrate synthase proteins. Positions containing identical residues are *shaded in navy blue*, while conservative residues are shown in *green* (*top*). Panel *A* (in *red lines*) - partial alignment of MxCS1 (citrate synthase domain) with other plant citrate synthase proteins. Panel *B* (in *blue lines*) - PWPN-box of MxCS1. *C* - Phylogenetic tree of MxCS1 and other plant citrate synthase proteins. The tree was constructed by the Neighbour-Joining method with *MEGA* program (*v. 4.0*). Branch numbers are represented as a percentage of bootstrap values in 1000 sampling replicates and scale indicates branch lengths.

(Table 1), especially when they were exposed to low or high Fe concentrations. The *MxCS1*-OE lines also showed higher CS activities, and Fe and citric acid contents (Table 1).

These results suggested that *MxCS1* might be one of the upstream regulator genes of Fe stress, and the over-expression of *MxCS1* can enhance the Fe stress tolerance in *Arabidopsis*.

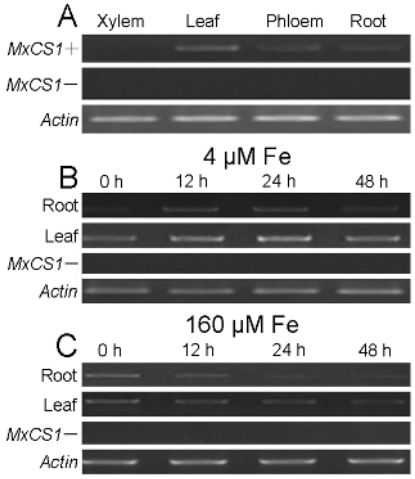

Fig. 3. Expression patterns of *MxCS1*. *A* - Expression patterns of *MxCS1* in xylem, leaf, phloem and root under normal iron concentration (40 μM). *B* - Expression patterns of *MxCS1* in a low concentration of Fe (4 μM) in root and leaf at 0, 12, 24 24 and 48 h. *C* - Expression patterns of *MxCS1* in root and leaf with a high-concentration of Fe (160 μM) at the same time points. Ethidium bromide staining of PCR products using *MxCS1*-specific primers with (*top*) and without (*middle*) prior reverse transcription, and the RT-PCR products of the actin rRNA gene (ApAts1 and ApAtR1 primers) amplified from *M. xiaojinensis* tissues (*bottom*).

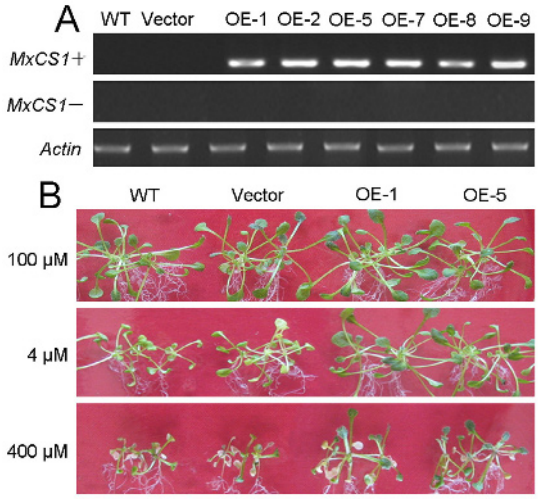

Fig. 4. Expression of *MxCS1* in transgenic *Arabidopsis* (*A*). The expression level of *MxCS1* in wild type (WT), line transformed only PBI121 vector and *MxCS1*-OE transgenic T_1 lines. The results of semi-quantitative RT-PCR. Ethidium bromide staining of PCR products using *MxCS1*-specific primers with (*top*) and without (*middle*) prior reverse transcription, and the PT-PCR products with actin rRNA gene (ApAts1 and ApAtR1) primers (*bottom*). Over-expression of *MxCS1* in *Arabidopsis* improved Fe tolerance (*B*). Seedlings of WT, vector and T_3 *MxCS1*-OE lines (OE-1 and OE-5) germinated and grown on MS medium supplied with 4, 100 or 400 μM Fe for 10 d. All treatments are repeated at least three times and representative results shown here.

Table 1. Effects of different Fe concentrations (4, 100 and 400 μM) on fresh mass [mg plant^{-1}], contents of chlorophyll [mg g^{-1}(f.m.)], CS activities [U g^{-1}(f.m.)], Fe content [mg g^{-1}(g.m.)] and citric acid content [mg g^{-1}(f.m.)] of *Arabidopsis* transformants and WT. All parameters were measured 10 d after Fe application. Each value represents the mean of three experiments with 10 replicates in each. Means within a line followed by same letters are not significantly different at $P < 0.01$.

Parameter	4 μM				100 μM				400 μM			
	WT	vector	OE-1	OE-5	WT	vector	OE-1	OE-5	WT	vector	OE-1	OE-5
Fresh mass	241b	253b	454a	482a	521a	532a	545a	537a	127c	132c	281b	263b
Chlorophyll	0.63c	0.61c	1.08b	1.11b	0.94a	0.97a	2.06a	2.02a	0.97b	1.01b	1.86a	1.84a
CS activity	922b	961b	3291a	3422a	523c	561c	1013b	1124b	922b	934b	2934a	2713a
Fe content	42d	43d	75c	79c	84c	85c	85c	86c	136b	140b	189a	187a
Citric acid	62c	66c	198a	201a	52c	56c	101b	112b	39d	41d	123b	131b

Discussion

In this study, we isolated a CS protein gene from *M. xiaojinensis*, designated as *MxCS1*. The *MxCS1* gene encodes a protein of 473 amino acid residues with a predicted molecular mass of 52.5 kDa and theoretical isoelectric point of 8.67. Sequence homologous analysis displayed that there were 86.5 and 92.6 % of nucleotide and amino acid homology between *MxCS1* and the *CS1* peach gene respectively. The MxCS1 included one conserved CS domain in the C-terminal region and a PWPN-box in the N-terminus (Takita *et al.* 1999, Christelle *et al.* 2002). These results demonstrated that MxCS1 is a novel protein of CS family.

The expression of *MxCS1* was enriched more in the leaf than that in root and phloem, but can be hardly detected in the xylem. This expression pattern indicated that *MxCS1* may play an important role in active organs.

When treated with different Fe concentration, the expression of *MxCS1* in roots and leaves were affected markedly. It is possible that MxCS1 plays a key role in regulating Fe stress responses in *M. xiaojinensis*. Results showed that the content of mRNA of *MxCS1* increased in roots and leaves under low Fe treatment after 0, 12 and 24 h, while decreased a little after 48 h. When exposed to Fe-deficiency, *M. xiaojinensis* probably increases the expression of *MxCS1* to accelerate the synthesis of CS and citric acid, which consequently promotes the uptake of Fe from poor Fe environment (Tiffin 1970). A possible explanation for the lowered expression of *MxCS1* after 48-h treatment is that there has been enough citric acid accumulation for the Fe absorption at this point. Conversely, the expression of *MxCS1* in roots and leaves were down-regulated at high Fe concentration with the aim to reduce the synthesis of CS and citric acid so that the uptake of Fe from this environment is decreased.

Over-expression of *MxCS1* in transgenic *Arabidopsis* enhanced the tolerance to Fe stress (deficiency as well as surplus). It is possible that *MxCS1* plays a crucial role in helping plants to survive from Fe stress by regulating the synthesis of citric acid. Higher content of citric acid in *MxCS1*-OE *Arabidopsis* helped to extract Fe from poor Fe environment. Meanwhile, high concentration of citric acid is also helpful in chelating redundant Fe when plants were exposed to high Fe (Cataldo *et al*. 1988, Todorovsky *et al*. 2002). Therefore, this theory explained why transgenic tobacco showed higher tolerance to high Fe stress. Moreover, the higher Fe content induced by high concentration of citric acid leads to the higher content of chlorophyll in lines over-expressed *MxCS1*, since Fe is a necessary for its synthesis.

To our knowledge, this work is the first report on isolation and function characterization of the CS protein gene from *Malus*. Clarifying the role of the different domains of *MxCS1* in stress response will be helpful in breeding stress-resistant *Malus* by gene transfer. However, further experiments are required to identify other functions of *MxCS1* gene.

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