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ORIGINAL ARTICLE

Overexpression of *Citrus junos* mitochondrial citrate synthase gene in *Nicotiana benthamiana* confers aluminum tolerance

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Abstract Aluminum (Al) toxicity is one of the major factors that limit plant growth in acid soils. Al-induced release of organic acids into rhizosphere from the root apex has been identified as a major Al-tolerance mechanism in many plant species. In this study, Al tolerance of Yuzu (*Citrus Junos* Sieb. ex Tanaka) was tested on the basis of root elongation and the results demonstrated that Yuzu was Al tolerant compared with other plant species. Exposure to Al triggered the exudation of citrate from the Yuzu root. Thus, the mechanism of Al tolerance in Yuzu involved an Al-inducible increase in citrate release. Aluminum also elicited an increase of citrate content and increased the expression level of mitochondrial citrate synthase (CjCS) gene and enzyme activity in Yuzu. The *CjCS* gene was cloned from Yuzu and overexpressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*-mediated methods. Increased expression level of the *CjCS* gene and enhanced enzyme activity were observed in transgenic plants compared with the wild-type plants. Root growth experiments showed that transgenic plants have enhanced levels of Al tolerance. The transgenic *Nicotiana* plants showed increased levels of citrate in roots compared to wild-type plants. The exudation of citrate from roots of the transgenic plants significantly increased when exposed to Al. The results with transgenic plants suggest that overexpression of mitochondrial CS can be a useful tool to achieve Al tolerance.

Keywords Aluminum tolerance · Citrate · Citrate synthase · Overexpression · Yuzu

Abbreviations

35S	Cauliflower mosaic virus 35S promoter
CS	Citrate synthase
DEPC	Diethyl pyrocarbonate
GUS	β-Glucuronidase
NPTII	Neomycin phosphotransferase
ORF	Open reading frame
RACE	Rapid amplification of cDNA ends
YADE	Y-shaped adaptor-dependent extension

Introduction

Aluminum (Al) toxicity is one of the major factors limiting productivity of many crops grown in acid soils which cover about 40% of the world's arable land (von Uexkull and Mutert 1995). In acid soils (pH < 5.0), Al becomes soluble and available to plants in the Al^{3+} and $Al(OH)^{2+}$ forms (Kinraide 1991). Micromolar concentrations of Al^{3+} in soil solution can result in the inhibition of root growth, disturbance of cell division and disruption of the cytoskeleton, and hence reduce crop yield. Al toxicity is also associated with decreased uptake and content of some cations, and leads to cation-deficiency symptoms (Foy 1984; Rengel and Robinson 1989; Rengel 1990; Robinson and Rengel 1991; Mariano and Keltjens 2005).

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Although most plants are sensitive to Al, several plant species show tolerance to Al toxicity in acid soils (Kochian 1995; Matsumoto 2000; Barcelo and Poschenrieder 2002; Kochian et al. 2005). One mechanism of Al tolerance is the chelation of Al by organic anions within root cells or in the rhizosphere (Taylor 1991). Exudation of a variety of organic anions such as citrate, malate, or oxalate has been reported in several plant species upon exposure to Al (Miyasaka et al. 1991; Basu et al. 1994; Pellet et al. 1995; Larsen et al. 1998). Although a rapid release of organic anions (malate) was observed in near-isogenic, Al-tolerant wheat (*Triticum aestivum*) lines (Delhaize et al. 1993), several lines of evidence suggested that a lag phase might also exist between exposure of roots to Al and excretion of organic anions. Citrate exudation from roots of rye was observed only after 10 h of exposure (Li et al. 2000). Delayed exudation of organic anions could possibly be due to alteration of cellular metabolism leading to biosynthesis and release of anions. Al-activated malate transporter 1 (*ALMT1*), which is responsible for malate release, has been identified in wheat by a subtraction approach (Sasaki et al. 2004). An *Arabidopsis* homolog of the wheat *ALMT1* gene, designated *AtALMT1*, played a specific role in malate release from roots in *Arabidopsis* Al tolerance by encoding an Al-activated root malate efflux transporter (Hoekenga et al. 2006).

Citrate is reported to be involved in ameliorating the Al toxicity. Accumulation and/or efflux of citrate can be enhanced by increasing the activities of enzymes involved in citrate synthesis, such as citrate synthase (CS), malate dehydrogenase. CS is a key enzyme involved in condensation of oxaloacetate (OAA) and acetyl CoA to produce citrate. Tobacco and papaya plants overexpressing a bacterial CS gene exhibited citrate overproduction and enhanced tolerance to Al (de la Fuente et al. 1997). Delhaize et al. (2001) could not confirm these findings on tobacco lines expressing bacterial CS at higher levels. The authors also reported that *CS*-expressing alfalfa did not show an improved Al tolerance. When a mitochondrial CS of *Arabidopsis thaliana* was introduced into carrot cells, the transgenic cell lines had increased citrate efflux in cultured carrot cells and high growth rates in Al-phosphate medium (Koyama et al. 1999). Anoop et al. (2003) reported that overexpression of *Arabidopsis* mitochondrial *CS* genes in canola also resulted in an enhanced citrate efflux and increased resistance to Al.

Yuzu (*Citrus junos* Sieb. ex Tanaka) is a medium-sized spiny tree and worthy of trial as an acid fruit for the home garden in subtropical or warm-temperate climates that are too cold to permit the growth of other acid citrus fruits. Yuzu was employed as a rootstock for Satsuma orange in Japan. Tree on this stock are vigorous, resistant to drought. In this paper, Al tolerance of Yuzu was tested on the basis of root elongation assay. The results indicated that Yuzu showed Al tolerance compared with other plant species and Al-induced alteration in efflux involved in the Al tolerance. The *CjCS* gene was isolated and introduced into *Nicotiana benthamiana* plants controlled by CaMV 35S promoter. The transgenic plants overexpressing *CjCS* gene showed enhanced Al tolerance, enhanced levels of citrate and citrate exudation in roots.

Materials and methods

Root elongation experiments

Aluminum tolerance of Yuzu (*Citrus junos* Sieb. ex Tanaka; obtained from College of Horticulture and Landscape Architecture, Southwest University, Chongqing, China) was quantified by the root growth elongation assay described by measuring elongation of Wenzl et al. (2001). Scarified seeds of Yuzu were surface-sterilized in 70% (v/v) ethanol for 1 min and 2% (w/v) NaOCl, 0.1% (v/v) Triton X-100 for 15 min. After 6 days of germination in 200 μM $CaCl^2$ (pH 4.2), uniform seedlings were transferred to constantly aerated control and Al solutions. The seedlings were left to grow in a growth chamber at 24°C and a 12-h diurnal cycle with a photon-flux density of 120 μmol m^{-2} s^{-1}. Root elongation was measured after 2 days, during which the pH of the control and Al treatments remained constant. Data from three independent experiments were pooled to compute relative root elongation according to the formula described by Wenzl et al. (2001). Comparison of Al resistance of Yuzu with that of other species was performed by using the method described by Wenzl et al. (2001).

Isolation of total RNA and genomic DNA from Yuzu

Total RNA was extracted from young leaves of Yuzu using RNA extraction kit (Promega, Madison, WI, USA). The DNase-treated RNA was stored in DEPC-treated sterile water at −70°C until used. Genomic DNA was extracted from young leaves of Yuzu using DNA extraction kit (Promega). The quality and concentration of genomic DNA were determined by agarose gel electrophoresis and spectrophotometer analysis.

Generation of cDNA clone of *CjCS*

cDNA synthesis was performed according to the manufacture's guidelines of the 3′RACE kit (TaKaRa, Dalian, China) in which the oligo dT-adaptor primer was provided as the forward primer. According to the conserved sequence of the *AtCS* (GenBank accession number: AY085647) and *CmCS*

(GenBank accession number: U19481) genes, a degenerate primer P1 [5′-GACATGGTAAT(T/C)GG(T/C)GG(A/G)AT G-3′] was designed for the 3′RACE. Polymerase chain reaction (PCR) was carried out in a reaction volume of 50 µl under the following condition: cDNA was denatured at 94°C for 30 s, followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 2 min), and by a final extension at 72°C for 7 min. The amplified product was purified, cloned into vector pMD18-T (TaKaRa), and sequenced. Based on the sequence of the 3′RACE product, the full-length gene was subsequently cloned by PCR walking by using the Y-shaped adaptor-dependent extension (YADE) method as previously described by Deng et al. (2008). Restriction enzymes *Eco*RV, *Sca*I, and *Hin*dIII were used to digest citrus genomic DNA and linked to appropriate Y-shaped adaptors with compatible terminals. The adaptor-linked citrus genomic DNA was used as template to perform sequential linear and exponential amplification. P2 (5′-AGCC AGTCATTCCTCTCTTCCCG-3′), P3 (5′-CAGGAATAG ACAGACCCCGAAAGC-3′), and Y-shaped adaptor primer (5′-CGGTAGGATCCCCAGAAC-3′) were designed to amplify the 5′-upstream sequence of the *CjCS* gene. For the YADE method, linear amplification was performed at 95°C for 5 min, followed by 40 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 3 min of polymerization at 72°C and then 72°C for 3 min. Exponential amplification was performed at 95°C for 5 min, followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 2 min of polymerization at 72°C and then 72°C for 10 min.

By comparing and aligning the sequence of YADE and 3′RACE products, the ORF region of *CjCS* was obtained by reverse transcription-PCR (RT-PCR) using a pair of primers P4 (5′-ATGGCGTTCTTCAGGAGCGT-3′) and P5 (5′-TTACTCAATCCAATCTAAAG-3′). The PCR program was: 95°C for 5 min followed by 30 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 60°C, and 2 min of polymerization at 72°C and then 72°C for 10 min. The PCR product was purified, ligated into pMD18-T vector and sequenced. The DNA sequences were analyzed with DNAStar software. A BLAST search was performed at http://www.ncbi.nlm.nih.gov/blast/. Sequences were aligned using the by Clustal method, using the MegAlign program in the DNAStar software.

Quantitative real-time PCR

Total RNA was isolated from 2-cm root apex of seedlings of Yuzu according to the manufacture's guidelines of RNA extraction kit (Promega). DNase treatment was used to eliminate DNA contamination from RNA samples and then 2 µg of total RNA were used to synthesize cDNA with ImProm-II TM Reverse Transcription System (Promega).

Quantitation of *CjCS* mRNA expression was carried out using iCycler™ Real Time System (Bio-Rad Laboratories, Richmond, CA, USA). The citrus ubiquitin gene (Gao et al. 2006), used as reference gene, was amplified in parallel with the target gene allowing gene expression normalization and provide quantification. Primer sequences were as follows: ubiquitin-F (5′-TCTTCGCAGGAAAGCAAC-3′) and ubiquitin-R (5′-CCTCAGACGCAAAACCAG-3′). CjCS-F (5′-C CCAGATTATGTGTACAAGGCC-3′) and CjCS-R (5′-AC GTACGCAGCTACTACCGGCA-3′). Real-time PCR was done using the SYBR Premix EX Tag Master mixture kit (TaKaRa) following the manufacture's recommendations. Approximately 25 µl of reaction volume was used for the real-time PCR assay that consisted of 2× SYBR Premix Ex Tag Master mixture, 100 nM of primers, and 1 µl of template (the equivalent of 50-ng total RNA). The thermal conditions consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, 55°C for 30 s, and 72°C for 20 s. All reactions were carried out in triplicate to ensure the reproducibility of the results. The amplicon was verified by melting curve analysis following the PCR reaction. For verification of the correct amplification product, PCR reactions were analyzed on an ethidium bromide-stained 2% agarose gel. Analysis of data was performed using iCycler software version 3.0. PCR efficiencies of target and reference genes were assayed by generating standard curves based on serial dilutions of plasmids. Amplification efficiency was automatically calculated using iCycler software version 3.0. Data normalization was accomplished using the endogenous control (ubiquitin gene) and the normalized values were subjected to a 2-ΔΔCt formula to calculate the fold change between the control and experiment groups (Livak and Schmittgen 2001).

Enzyme assay of CS

One gram of Yuzu roots was ground in liquid nitrogen and homogenized with 2 ml of ice-cold extraction buffer [50 mM Hepes, 0.5% (w/v) Triton-X, 1-mM EDTA, 1-mM iodoacetamine, and 10% (w/v) glycerol]. The extract was centrifuged for 10 min at 4°C, and the supernatant was collected and desalted by passing through PD-10 columns (Bio-Rad Laboratories) equilibrated with 10 mM Hepes and eluted with 2 ml of 10 mM Hepes. The reaction mix for CS enzyme assay consisted of 1 mM 5,5′-dithio-bis(2-nitrobenzoic acid) in Tris–Cl (pH 8.1), 10 mM acetyl CoA, and the enzyme. The reaction was started by the addition of 10 mM OAA, and increase in absorbance due to deacetylation of acetyl CoA was measured at 412 nm (Srere et al. 1963).

Plasmid construction and plant transformation

The coding region of the Yuzu *CjCS* gene was amplified from cDNA with the primers CjCS-F1 (5′-CGGATCCATGGCG

TTCTTCAGGAGCGT-3′) and CjCS-R1 (5′-AGAGCTCTTACTCAATCCAATCTAAAG-3′). *Bam*HI and *Sac*I restriction sites are underlined. PCR was performed for 35 cycles (denaturing at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s). PCR products were applied to a DNA sequencer to confirm their sequences. The *Bam*HI/*Sac*I amplified fragment was cloned downstream of the CaMV 35S promoter of binary vector pBI121 (Clontech, San Francisco, CA, USA), followed by a nopaline synthase terminator. Subsequently, the 35S::*CjCS* fragment was excised by *Hin*dIII/*Eco*RI and ligated to binary vector pCAMBIA1305.1 (Cambia, Canberra, Australia) which was digested with *Hin*dIII and *Eco*RI. The resulting plasmid was named pCJCS (Fig. 5a). The pCJCS plasmid was transformed into *N. benthamiana* plants by *Agrobacterium tumefaciens*-mediated method described by Deng et al. (2006). The plants were grown in a greenhouse under constant conditions: photoperiod of light:dark 16:8 h. Natural light was supplemental with white fluorescent lights to provide light intensity of 200–300 μmol s^{-1} m^{-2}.

Reverse transcription-polymerase chain reaction and CS enzyme assay

Total RNA was extracted from roots of wild-type and transgenic plants using RNA extraction kit (Promega). For RT-PCR reactions, cDNA synthesis was performed with the ImProm-II TM Reverse Transcription System (Promega). The primers, CjCS-F2 (5′-TGGGCAACAGTCTAATCTCAG-3′) and CjCS-R2 (5′-GACCCCACTAGCAAATTGAGT-3′), were designed specifically to amplify the *CjCS* cDNA. The primers, TAC9-F (5′-ATGCCCTCCCACATGCTATTC-3′) and TAC9-R (5′-AACATGGTAGAGCCACCACTA-3′) were used to amplify the *TAC9* cDNA used for a control (Deng et al. 2006). RT-PCR for *CjCS* cDNA was conducted for 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s, and finally 7 min at 72°C. PCR for *TAC9* expression was conducted under the above conditions but for only 25 cycles of amplification. CS enzyme activities in roots of wild-type and transgenic plants were determined according to the procedure described above.

Root elongation assay

Nicotiana benthamiana plants were tested for their sensitivity to Al using a procedure described by Deng et al. (2006). A plate assembly consisted of a 3-mm-thick glass plate, three squares of 1-mm-thick chromatography paper (3 MM CHR; Whatman, Maidstone, UK). The chromatography sheets were saturated with 1/6 MSB5 medium and then set on the glass plate. Sterilized seeds were germinated and plated on the chromatography sheets. The plates were inclined at an angle of >80º in a sterilized plant growth rack containing 20 ml of 1/6 MSB5 medium (pH 4.3). Roots of the seedlings grown over the plate elongated in a straight line, enabling easy handling of the seedlings and accurate measurements of root lengths. After 5 days of growth, the young seedlings were transferred to a second plate, placed in a marked line on three new chromatography sheets. This new plate was inclined in the growth rack containing 1/6 MSB5 solution medium (pH 4.3) with or without Al. After exposure to the treatment solution for 2 days, the final root lengths (between the root apex and marked line) of ten plants, selected on the basis of seed availability, were measured. Root growth in each treatment was calculated relative to the control.

Citrate analysis

Root tissues (approximately 0.2 g) of citrus and *N. benthamiana* plants were ground with liquid nitrogen, homogenized with 1 ml of 80% (v/v) ethanol, and vortexed thoroughly. The samples were centrifuged for 2 min at 16,000*g* and the supernatant was vortexed and boiled at 80°C for 15 min. Samples were centrifuged at 16,000*g* for 5 min, the supernatant was collected and passed through 0.45-μm filters, and 100 μl was used in citrate assay as described by Delhaize et al. (1993). Citrate efflux from whole roots was determined by immersing the roots of 10–15 seedlings in 50 ml of continuously aerated solution and the resulting solution was measured for citrate assay as described by Delhaize et al. (1993).

Results

Aluminum tolerance of Yuzu

Al tolerance of Yuzu was tested on the basis of root elongation. Exposure of the seedlings to solutions containing Al^{3+} activities ranging from 0 to 200 μM only caused a modest inhibition of root growth (Fig. 1), generally causing about 60% root growth inhibition at an Al^{3+} activity of 50 μM, and about 80% inhibition in solutions with an Al^{3+} activity of 100 μM. These results established that the Yuzu was Al-tolerant compared with other crop species reported in the literature (for comparison, see Wenzl et al. 2001). Citrate content of root was investigated in response to Al treatment. As shown in Fig. 2a, the citrate content in root was low at 35 and 50 μM Al, but significantly increased at 100 μM Al. A time course analysis of citrate content was performed in response to Al^{3+} stress (100 μM). The citrate content in root was low during the first 12 h after the start of exposure to Al, but significantly increased at 24 h (Fig. 2b). Al also triggered the secretion of citrate from

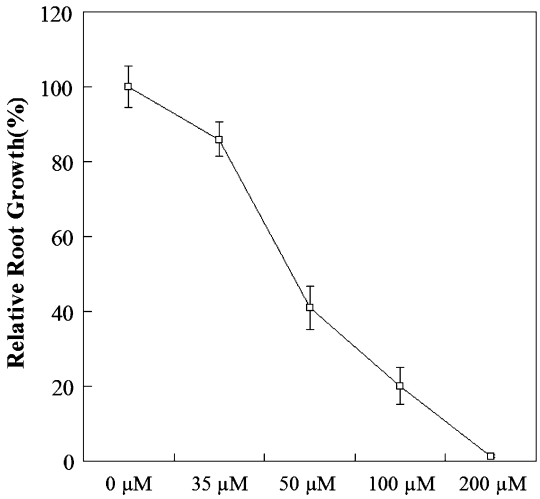

Fig. 1 Effect of Al on the root elongation in Yuzu. Seedlings were cultivated in 200 μM $CaCl_2$ solution (pH 4.2) without Al^{3+} for 5 days, and then transferred to $CaCl_2$ solution (pH 4.2) containing varying activities of Al^{3+}, ranging with 0–200 μM, for an additional 2 days. Relative root growth was expressed as a percentage of root growth in the absence of Al. *Vertical bars* represent SE of three independent experiments in which ten plants were used for each determination

roots of Yuzu. Exposure to increasing levels of Al elicited an increase in secretion of citrate from the root. The amount of citrate secreted from roots increased by 65% at 35 μM Al and was 2.7-fold higher at 100 μM Al (Fig. 2c). The time course analysis showed the amount of secretion of citrate was low during the first 8 h after the start of exposure to Al, but significantly increased at 12 h (Fig. 2d). This pattern of citrate efflux by Al stress is a typical pattern II response. These results indicated that an Al-inducible secretion of citrate from roots is involved in Al tolerance in Yuzu.

Cloning and characterization of CjCS from Yuzu

An alignment of the AtCS and CmCS sequences was used to design degenerate PCR primer in highly conserved regions. The degenerated primer P1 was used for the amplification of 3′ end of *CjCS* cDNA from Yuzu. A single fragment was obtained and DNA sequencing showed that the fragment of 1,486 bp contained a 1,179-bp ORF and a 3′-untranslated region of 307-bp downstream from the stop codon, but lacked the start codon as well as 5′-untranslated region. BLAST search at NCBI website confirmed that the fragment produced by 3′RACE had high levels of similarity with other mitochondria CS genes. According to the 3′RACE fragment, two specific primers (primer 2 and primer 3) were designed to clone the 5′-flanking region of *CjCS* using the YADE method (Deng et al. 2008). A distinct fragment was obtained when the genomic DNA digested by *Sca*I was used as a template for the PCR reaction. Nucleotide sequence analysis showed that the amplified fragment was of 349 bp and contained a 103-bp overlap with the 3′RACE fragment. The ORF of *CjCS* was

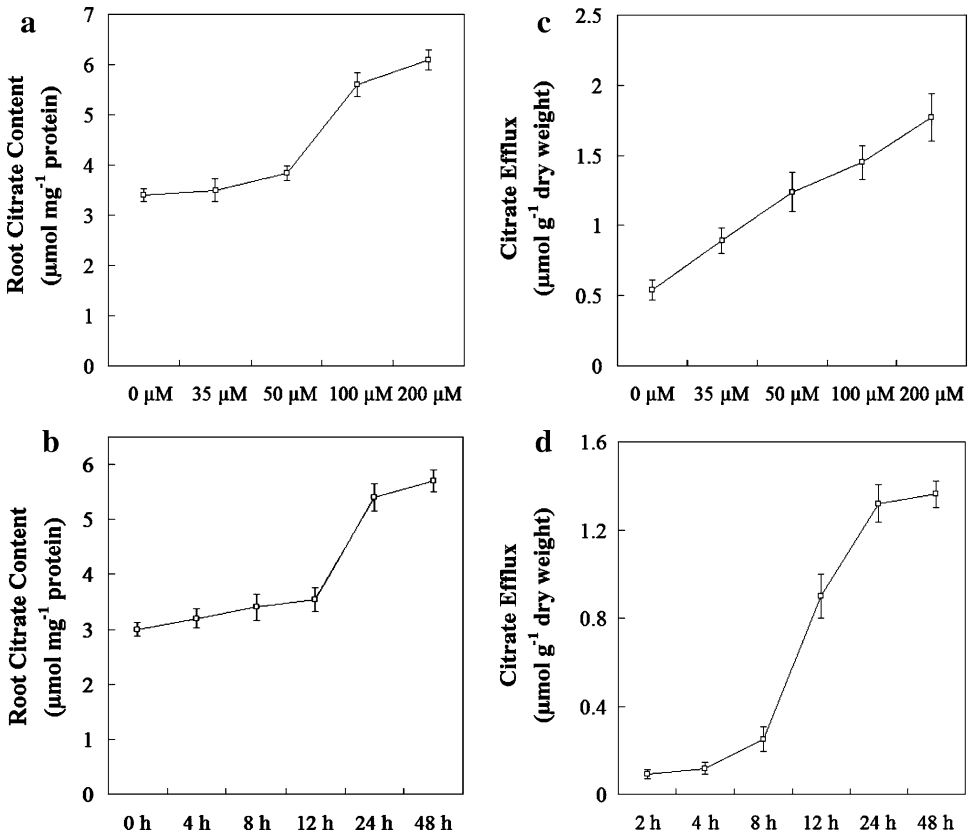

Fig. 2 Effect of Al on citrate content (**a, b**) and citrate efflux (**c, d**) from Yuzu. **a** Citrate content in root tissues of Yuzu. Root tissues from seedlings of Yuzu were collected after 24-h treatment in response to different concentrations of Al^{3+} and used in citrate estimation. **b** Time course of effect of Al on citrate content of Yuzu. The roots were exposed to 100 μM Al^{3+} for 0, 4, 8, 12, 24, and 48 h, respectively, then the tissues were collected. **c** Citrate exudation from root of Yuzu. Root exudates from seedlings of Yuzu were analyzed for levels of citrate released after 24-h treatment in response to different concentrations of Al^{3+}. **d** Time course of Al-stimulated efflux of citrate in Yuzu. The roots were exposed to 100 μM Al^{3+} for 2, 4, 8, 12, 24, and 48 h, respectively, before collecting. *Vertical bars* represent SE ($n = 3$)

obtained by RT-PCR using primer 4 and primer 5. The ORF of *CjCS* consisted of 1,396-bp nucleotides encoding a protein of 465 amino acids with a calculated molecular mass of 51.7 kDa and an isoelectric point (pI) of 6.66. An alignment of the amino acid sequences of CjCS with the cloned mitochondria CS from many organisms was conducted using DNAStar software shown in Fig. 3. The result revealed that the CjCS showed high degree of similarity with other mitochondrial CS.

Al treatment increases the CjCS gene expression and enzyme activity

To determine whether *CjCS* expression was increased by Al treatment, the citrus plants were grown in nutrient media supplemented with a range of Al concentration for 24 h. *CjCS* mRNA levels were determined by real-time PCR using RNA extracted from root. As shown in Fig. 4a, the expression levels of *CjCS* was low at 0, 35, and 50 μM Al, but significantly increased at 100 μM Al. Moreover, a time course analysis of *CjCS* expression was performed in response to Al^{3+} stress (100 μM). *CjCS* mRNA levels increased at 8 h time point and reached a maximum at 24 h (Fig. 4b). These results indicated that *CjCS* expression was induced by high concentration of Al. The time course of effect of Al on the activity of CjCS was investigated in root of Yuzu. The activity of CjCS increased obviously at 12–24 h.

Construction of transgenic plants overexpressing CjCS

To investigate effects of constitutive expression of *CjCS* on Al tolerance in whole plants, the CaMV 35S promoter was used to drive expression of *CjCS*. The gene cassette 35S::*CjCS* was inserted into a binary expression vector pCAMBIA, and the resulting construct was obtained and named pCJCS (Fig. 5a). *Agrobacterium*-mediated transformation of *N. benthamiana* with the 35S::*CjCS* gene cassette resulted in 26 independent kanamycin-resistant lines. The primary transformants were screened by histochemical assay of GUS activity (data not shown). Ten GUS-positive lines were tested for their segregation ratios in T1 generation. Among these independent transgenic plants, five lines showed segregation ratios of 3:1 (GUS^+:GUS^-) indicating a single independent integration loci. The single locus homozygous lines were selected for a detailed analysis.

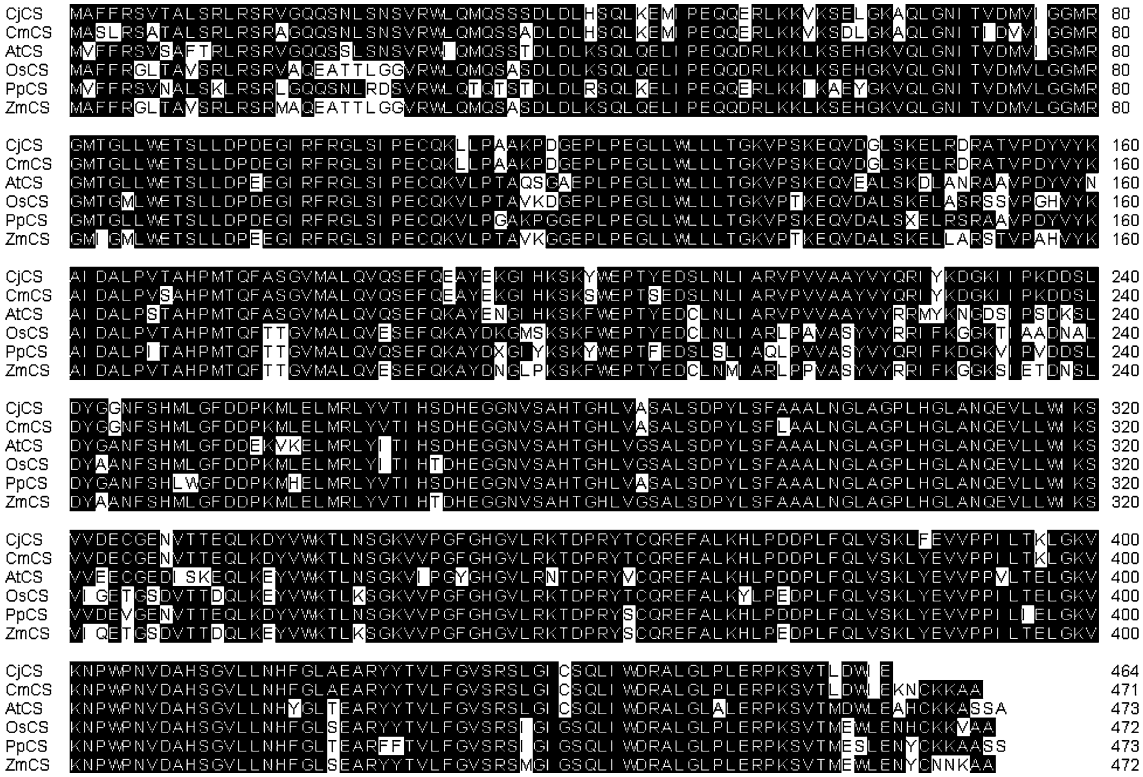

Fig. 3 Multiple alignment of deduced amino acid sequences of Yuzu CjCS and other mitochondrial CS. The identical amino acids were indicated with *white foreground and black background*. The different amino acids were indicated with *black foreground and white background*. The plant mitochondrial CSs used for alignment were: CjCS (GenBank accession no. AAR88248); CmCS (GenBank accession no. P49298); AtCS (AAK62463); OsCS (AAG28777); PpCS (AAL11504); ZmCS (NP001132846). *At Arabidopsis thaliana*, *Cj Citrus Junos*, *Cm Citrus maxima*, *Pp Prunus persica*, *Os Oryza sativa*, *Zm Zea mays*

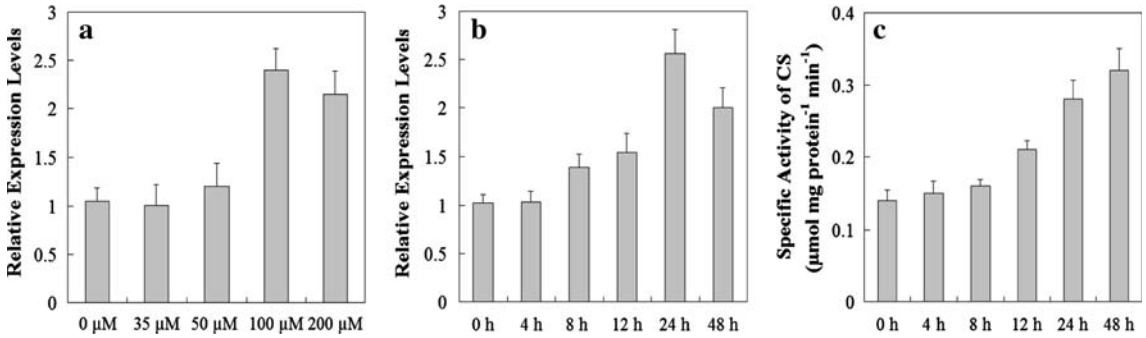

Fig. 4 Analysis of *CjCS* gene expression and enzyme activity in Yuzu. **a** Real-time PCR analysis of *CjCS* expression in Yuzu roots in response to different concentrations of Al^{3+} after 24 h treatment. **b** Time course of *CjCS* expression in Yuzu roots in response to 100 μM Al^{3+} treatment. Total RNA was prepared from the 2-cm root apex region of seedlings. The citrus ubiquitin gene was used as internal standard. **c** CS enzyme activity was measured in root of Yuzu. Data are mean ± SE for three independent experiments

CjCS steady-state mRNA levels were examined in three single locus homozygous lines by using semi-quantitative RT-PCR method. Three transgenic lines showed an accumulation of *CjCS* transcripts (Fig. 5b). By contrast, the CjCS amplicon was not detected in non-transgenic plants. CS enzymes activity was measured in the wild-type and transgenic lines (Fig. 5c). Transgenic lines C3, C6, and C10 showed a significant increase in CS enzyme activity compared with the wild-type plants.

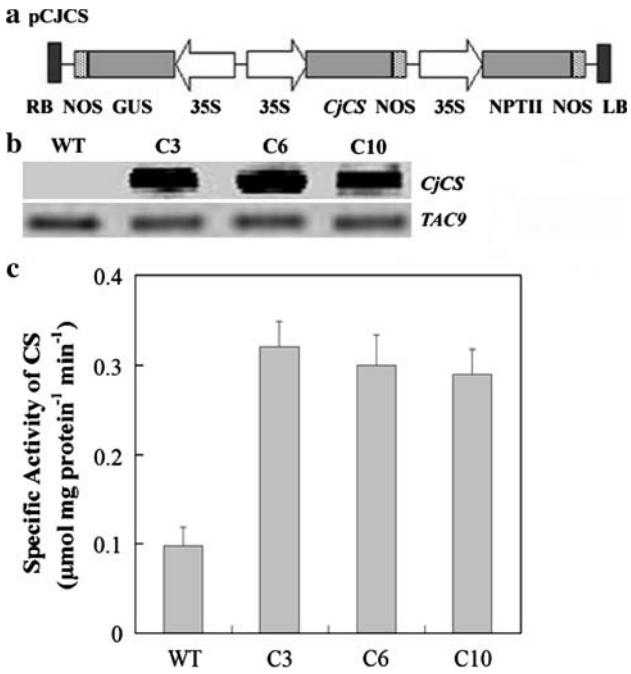

Fig. 5 Construction of transgenic plants overexpressing CjCS. **a** Schematic representation of T-DNA region of binary vector pCJCS for *N. benthamiana* plant transformation. Expression of the *CjCS* gene is driven by the CaMV 35S promoter. NOS is the terminator of the nopaline synthase gene. *RB* and *LB* represent *right* and *left border* sequences of a T-DNA, respectively. **b** Semi-quantitative RT-PCR analysis of wild-type and transgenic plants overexpressing *CjCS* gene. Amplification of *TAC9* cDNA was used as an internal standard. **c** CS enzyme activity in roots of wild-type and transgenic plants. *Vertical bars* represent SE ($n = 4$)

Overexpression of *CjCS* confers tolerance to Al

In order to study whether *CjCS* overexpression could improve Al tolerance in *N. benthamiana* plant, the effect of Al on root growth in 35S::*CjCS* plants was evaluated. Wild-type and transgenic plants C3, C6, and C10 were grown in nutrient media supplemented with a range of Al concentrations (0, 35, 50, and 100 μM) for 2 days. The results showed that the inhibition of Al-induced root elongation was significantly different between transgenic and wild-type plants (Fig. 6). Treatment of wild-type plants with 35 μM Al^{3+} resulted in a significant reduction in root growth rate by 80% compared with the untreated controls, whereas representative transgenic lines showed about 40% reduction in root growth rate under the same conditions. When exposed to 100 μM Al^{3+}, root growth in wild-type plants was inhibited completely, while roots of all the three transgenic lines showed some elongation (Fig. 6). The results demonstrated that overexpression of *CjCS* could confer tolerance to Al in *N. benthamiana* plants.

Overexpression of *CjCS* in *N. benthamiana* leads to altered citrate production

Citrate content and exudation of citrate from roots into the rhizosphere of transgenic lines C3 and C10 were compared with that of wild-type plants. There was no significant increase in citrate content with increasing external Al concentrations in roots of wild-type and transgenic plants (C3 and C10), whereas the citrate content in transgenic lines was significantly higher compared with wild-type plants (Fig. 7a). A time course analysis of citrate content was performed in response to Al^{3+} stress (50 μM). There was no significant increase in citrate content with the increase of exposure time in roots of wild-type and the transgenic plants C3 and C10, whereas the citrate content in C3 and C10 was significantly

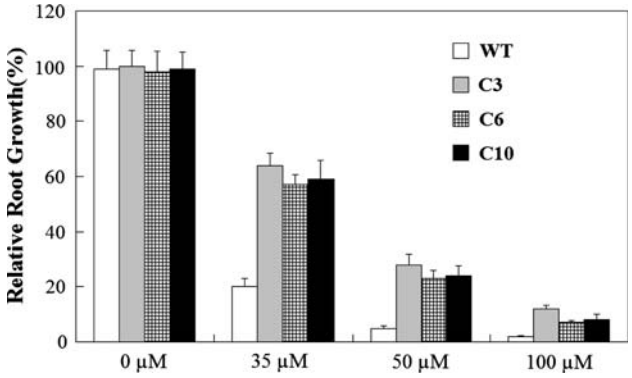

Fig. 6 Root growth of wild-type and transgenic plants overexpressing CjCS in nutrient solution containing Al^{3+}. Seedlings were grown on nutrient solution without Al^{3+} for 5 days, and then transferred to nutrient solution containing varying concentrations of Al^{3+}, ranging with 0–100 μM, for an additional 2 days. Relative root growth was expressed as a percentage of root growth in the absence of Al. Results are mean ± SE of three independent experiments in which ten plants were used for each determination

higher compared with wild-type plants (Fig. 7b). Moreover, we investigated whether overexpression of CjCS would increase the amounts of citrate secreted from roots. The secretion of citrate from roots of the transgenic lines C3 and C10 significantly increased with increasing external Al concentrations (Fig. 7c). The time course analysis also showed the secretion of citrate from roots of transgenic lines C3 and C10 significantly increased with the increase of exposure time (Fig. 7d). The results demonstrated that overexpression of CjCS could increase citrate content in root and improve the amounts of citrate exuded from roots in *N. benthamiana*.

Discussion

The present study showed that Yuzu was highly Al-tolerant compared with other crop species. Al treatment induced an increase of citrate content and enhanced citrate exudation

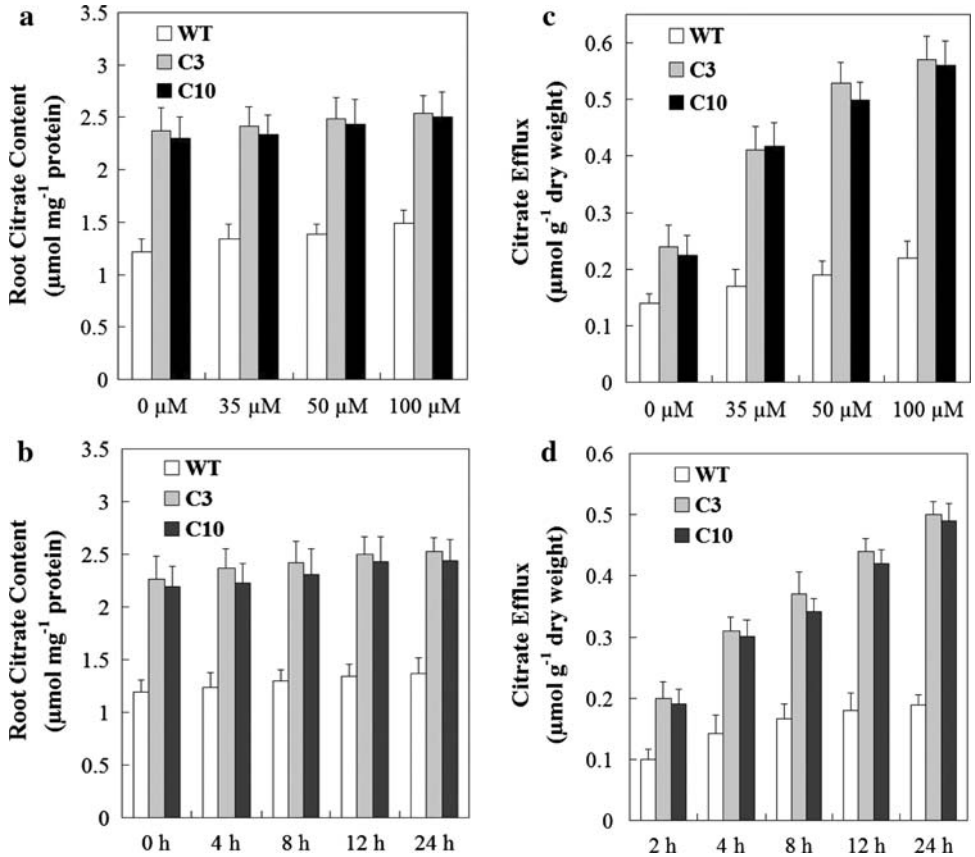

Fig. 7 Effect of Al on citrate content (**a, b**) and citrate efflux (**c, d**) from wild-type and transgenic plants overexpressing *CjCS* gene. **a** Citrate content in root tissues of wild-type plants and transgenic lines C3 and C10. Root tissues from seedlings of wild-type and transgenic plants were collected after 24-h treatment in response to different concentrations of Al^{3+} and used in citrate estimation ($n = 10$). **b** Time course of effect of Al on citrate content of wild-type and transgenic plants. The roots were exposed to 50 μM Al^{3+} for 0, 4, 8, 12, and 24, respectively, and the root tissues were collected. **c** Citrate exudation from root of wild-type and transgenic plants. Root exudates from seedlings of wild-type and transgenic plants were analyzed for levels of citrate released after 24-h treatment in response to different concentrations of Al^{3+}. **d** Time course of Al-stimulated efflux of citrate in wild-type and transgenic plants. The roots were exposed to 50 μM Al^{3+} for 2, 4, 8, 12, and 24, respectively, before the root tissues were collected. *Vertical bars* represent SE ($n = 3$)

from root of Yuzu. Al also increased the CjCS gene expression and enzyme activity in Yuzu. The results indicated Al-inducible increase in citrate release involved in Al tolerance in Yuzu. Root growth experiments showed that overexpression of *CjCS* gene in *N. benthamiana* resulted in increased tolerance to Al. The plants with overexpression of *CjCS* had enhanced concentration of citrate in roots, increased citrate exudation from roots.

Wenzl et al. (2001) compared the Al resistance of signalgrass, a highly Al-resistant grass, with other species, such as *Arabidopsis*, wheat and triticale. The Al^{3+} activity inhibiting root elongation by 50% ($\{Al^{3+}\}_{50}$), which calculated for each species from data in the literature, was used to quantitative comparison. The $\{Al^{3+}\}_{50}$ value of signalgrass (49.5) was several times greater than that of Al-resistant genotypes of other species (Wenzl et al. 2001). In this study, exposure of the Yuzu to solutions containing 50 μM Al^{3+} only caused about 60% inhibition of root growth. We calculated the $\{Al^{3+}\}_{50}$ value of Yuzu was about 40. This result demonstrated Yuzu had high Al tolerance compared with other crop species reported in the literature. The Al-activated efflux of organic acid anions from roots is now a well established mechanism that is proposed to be used by a range of Al-tolerant plants (Ma et al. 2001). The organic acids include malate in wheat (Delhaize et al. 1993), citrate in *Cassia tora*, snapbean, maize, and soybean (Ma et al. 1997a; Miyasaka et al. 1991; Pellet et al. 1995; Yang et al. 2000) and oxalate in buckwheat and taro (Ma et al. 1997b; Ma and Miyasaka 1998; Zheng et al. 1998). In this study, we showed that Yuzu strongly increased citrate release under moderate Al-toxic conditions (35 and 50 μM, Figs. 1, 2c). This indicates that Al-inducible citrate release is involved in Al tolerance of Yuzu.

Ma et al. (2001) have classified two patterns of Al-stimulated efflux of organic acids on the basis of the timing of efflux. In pattern I, there was no discernible delay between the addition of Al and the onset of organic acid efflux such as in tobacco (Delhaize et al. 2001), wheat (Ryan et al. 1995), buckwheat (Zheng et al. 1998), and barley (Zhao et al. 2003). Thus, Al may activate an already expressed organic acid transporter in such species. Sasaki et al. (2004) has identified an Al-activated malate transporter gene, *ALMT1* in wheat by a subtraction approach, which is responsible for malate release. A multidrug and toxic compound extrusion (MATE) family gene has been identified responsible for the Al-activated citrate secretion in barley (Furukawa et al. 2007; Wang et al. 2007). In pattern II, there is a marked lag phase between the addition of Al and excretion of organic anions. In plant species such as rye (Li et al. 2000), triticale (Ma et al. 2000), and *Cassia tora* (Yang et al. 2006), the efflux of organic acids was delayed by several hours, and Al might induce the expression of genes and synthesis of proteins involved in metabolism of organic acids, and/or transport of organic acids which were necessary for efflux of organic acids across the root cells. In the present study, the amount of secretion of citrate was low during the first 8 h after the start of exposure to Al, but significantly increased at 12 h in root of Yuzu. This pattern of the citrate secretion induced by Al stress is a typical pattern II response. In this study, *CjCS* gene expression was induced at 100 μM Al, the toxic level of which was strong (Figs. 1, 4a). When Yuzu was treated with 100 μM Al, the onset of induction of *CjCS* gene expression and CS activity was started at 8 and 12 h after the treatment, respectively (Fig. 4b, c). The citrate content increased in roots at 24 h after the treatment (Fig. 2b). It can be concluded that, under strong Al-toxic condition, the induction of *CjCS* expression leading to citrate synthesis alteration contributes to the citrate release in Yuzu. On the other hand, the citrate content and *CjCS* gene expression was slightly increased at 50 μM Al, but not at 35 μM (Figs. 2a, 4a). From this result, we cannot conclude that the mechanism of Al tolerance in Yuzu involves an Al-inducible alteration of the citrate synthesis. The time course analysis with 100 μM Al showed that citrate efflux increased at 12 h before root citrate content increases (24 h) (Fig. 2b, d). It is speculated that the transport of citrate across the membrane might be responsible for the efflux of citrate in Yuzu roots. Yang et al. (2006) provided experimental evidence that both, de novo synthesis and activation of an anion channel, are needed for an Al-induced efflux of citrate in *Cassia tora*. It was also found that an Al-inducible citrate-transporter gene expression was associated with the induction of Al tolerance in *Sorghum bicolor* via enhanced root citrate exudation (Magalhaes et al. 2007).

Overexpression of *CS* gene from *Pseudomonas aeruginosa* in the cytoplasm of tobacco and papaya species enhanced the levels of cellular citrate and citrate exudation from roots of transgenic lines compared with wild-type plants, with a concomitant increase in Al tolerance (de la Fuente et al. 1997). Overexpression of *Arabidopsis* mitochondrial *CS* in carrot cells improved citrate excretion and growth rate in Al-phosphate medium (Koyama et al. 1999). Koyama et al. (2000) showed that transgenic *Arabidopsis* lines overexpressing a carrot mitochondrial *CS* showed 60% increase in citrate efflux, performed better under toxic concentrations of Al. In addition, overexpression of *Arabidopsis* mitochondrial *CS* in canola enhanced levels of cellular citrate and citrate exudation and the transgenic plant had a higher Al tolerance (Anoop et al. 2003). However, Delhaize et al. (2001) reported that transgenic tobacco plants overexpressing the same *P. aeruginosa CS* gene did not show an increased accumulation of citrate in roots or an increased Al-activated efflux of citrate from roots and were not Al tolerant. In other transgenic tobacco lines expressing the *CS* gene to a very high level, Delhaize et al. (2001) suggested that incorrect folding of the bacterial protein or formation of protein

aggregates may have inactivated the protein. In addition, the negative results for alfalfa obtained by Delhaize et al. (2001) may be based on the fact that they only analyzed two transgenic alfalfa events. The two events tested by Delhaize et al. (2001) might simply be not enough to capture the full extent of phenotypes possible (Barone et al. 2008). Furthermore, Delhaize et al. (2003) reported that overexpression of the endogenous mitochondrial *CS* in tobacco had no effect on internal citrate concentrations and citrate efflux from roots. Barone et al. (2008) introduced the *P. aeruginosa CS* gene controlled by the *Arabidopsis* Act2 constitutive promoter and tobacco RB7 root-specific promoter into alfalfa. Fifteen transgenic plants were assayed for internal citrate content and for shoot and root growth in either hydroponics or in soil assays. The transgenic plants did not show increased citrate content in shoot and root and the citrate efflux from the root was not measured. But, two transgenic lines showed a better Al-tolerance than non-transgenic plants in soil assays. In the present study, transgenic *N. benthamiana* plants overexpressing *CjCS* showed an increased expression and enzyme activity, resulting in an increase in levels of citrate and citrate exudation from roots of transgenic lines relative to wild-type plant. The contrasting results might be attributed to the different sources of the *CS* genes (e.g., bacterial versus plant), the use of different recipient species and different growth conditions. It was found that both internal citrate concentrations and CS activities were markedly affected by growth conditions in tobacco (Delhaize et al. 2003).

The transgenic lines showed enhanced Al tolerance. At 35, 50, and 100 μM Al, root elongation rates were significantly higher in the transgenic lines compared with the control. Enhanced Al tolerance due to Al induced increase in citrate exudation in transgenic *N. benthamiana* plants supported the idea that plant mitochondrial *CS* overexpression was a promising strategy to enhance citrate exudation and Al tolerance. Delhaize et al. (2004) demonstrated that overexpression of the *ALMT1* gene of wheat, encoding a malate transporter, conferred Al tolerance in barley through Al-activated efflux of malate. Overexpression of the MATE family gene in tobacco enhanced citrate secretion and Al resistance compared with the wild-type plants (Furukawa et al. 2007). Combining of organic acid overproduction and enhancing of its excretion might be more efficient strategy to increase Al tolerance in plants.

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