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Overexpression of Mitochondrial Citrate Synthase in *Arabidopsis thaliana* Improved Growth on a Phosphorus-Limited Soil

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The gene for mitochondrial citrate synthase (CS) was isolated from *Daucus carota* (*DcCS*) and introduced into *Arabidopsis thaliana* (strain WS) using *Agrobacterium tumefaciens*-mediated transformation. Characteristics of citrate excretion were compared between T3 transgenic plants, which were derived from the initial transgenic plants by self-fertilization and homozygous for *DcCS*, and the control plants that had no *DcCS*. The highest CS activity 0.78 μmol protein min^{-1} exhibited by the transgenic plants was about threefold greater than that found in the control plants (0.23–0.28 μmol protein min^{-1}). Western analysis of the transgenic plants showed two CS signals corresponding to signals obtained from both *D. carota* and *A. thaliana*. Thus, it appears that the CS polypeptides by ectopic expression of *DcCS* were processed into the mature form and localized in the mitochondria of *A. thliana*. The signal corresponding to the mature form of DcCS were greater in the transgenic plants having higher levels of CS activity. When the transgenic plants were grown in Al-phosphate media, a correlation between the levels of CS activity and the amounts of citrate excreted into the medium. The highest value (5.1 nmol per plant) was about 2.5-fold greater than that from control plants (1.9 nmol per plant). Both growth and P accumulation were greater in transgenic plants with high CS activity than that in control plants when they were grown on an acid soil where the availability of phosphate was low due to the formation of Al-phosphate. It appears that the overexpression of CS in *A. thaliana* improves the growth in phosphorous limited soil as a result of enhanced citrate excretion from the roots.

Key words: Al-phosphate — *Arabidopsis thaliana* — Citrate synthase (EC 4.1.3.7) — *Daucus carota* — Transformation.

Abbreviations: AtCS, *A. thaliana* mitochondrial CS; CS, citrate synthase; DcCS, carrot mitochondrial CS; GUS, β-glucuronidase; Hyg, hygromycin; Km, kanamycin; WT, wild-type.

Introduction

In acid soils, phosphorous supplied as fertilizer is often fixed in insoluble forms such as aluminum phosphate. The concentration of phosphate in these soil solutions is often less than 10 μM (Wright 1953, Clark 1990). Plants which have the ability to increase P_i uptake, either by extensive root growth, increased rate of P_i-uptake (Mimura et al. 1990) or a greater ability to absorb P_i due to increased root exudation (Gardner et al. 1981, Ae et al. 1990, Tadano et al. 1993) show superior growth on such soils. Since phosphorus is a severely limited and non-renewable resource (Ando 1983), improved P_i-acquisition is important to attain sustainable agriculture (von Uexüll and Mutert 1995). Genetic manipulation of crop plants is one approach to increase the ability of plants to grow on P_i-limited soils. This approach has recently been performed using a number of plant cell culture systems. Mitsukawa et al. (1997) found that the overexpression of a gene for high affinity P_i transport altered P_i uptake in tobacco cells. Koyama et al. (1999) found that overexpression of citrate synthase in carrot cells altered the growth and increased P_i-uptake from Al-phosphate. To determine if these approaches would be relevant to improving P_i-acquisition at the crop level, transgenic studies at the whole plant level are also important.

Organic acid excretion, which is thought to be a component of phosphate starvation rescue system in higher plants, is known to improve P_i-acquisition from aluminum phosphate (Gardner et al. 1981, Lipton et al. 1987). Physiological studies have suggested that altered carbohydrate metabolism plays an important role in organic acid excretion (Hoffland et al. 1992, Johnson et al. 1994, Johnson et al. 1996). Using a mutant carrot cell line [designated as insoluble phosphate growers (IPG) Koyama et al. 1990], we (Takita et al. 1999a) recently reported clear evidence for such an altered metabolism. The superior growth in comparison to wild-type (WT) cells was attributed to their ability to excrete large amounts of citrate (Koyama et al. 1992). Mutant cells had a higher activity of the mitochondrial citrate synthase (CS; EC 4.1.3.7) than WT cells (Takita et al. 1999a). Another study showed that transgenic carrot cells carrying the mitochondrial CS gene from *A. thaliana* had increased levels of CS activity, greater levels of citrate excretion, and showed better cell growth than WT cells when grown in Al-phosphate medium (Koyama et al. 1999). Transgenic tobacco plants contained a bacterial CS gene also showed improved citrate excretion from roots (de la Fuente et al. 1997). As a

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group, these results suggest that overexpression of CS alters citrate excretion from plant roots. This improves plant growth under conditions where aluminum phosphate is the major phosphorus source.

In the present study, we transformed *A. thaliana* with a mtCS gene from carrot. The transgenic plants showed a high activity of CS presumably due to the high accumulation of foreign CS polypeptides in their mitochondria. These plants also excreted larger amounts of citrate from their roots than WT plants. Using an acid soil culture system (Toda et al. 1999b), we found that the transgenic plants grew very well under P_i-limiting conditions.

Materials and Methods

Enzymes and chemicals

Restriction enzymes, modification enzymes and high fidelity Taq polymerase, *Ex Taq* were purchased from Takara (Otsu, Japan). Unless otherwise indicated, all other chemicals were obtained from Wako Pure Chemical (Osaka, Japan) and Sigma (MO, U.S.A.).

Vector construction and Agrobacterium strain

Full sized cDNA encoding the mitochondrial CS isolated from carrot (*Daucus carota* L. cv. MS Yonsusn) was obtained using reverse transcription PCR as described previously (DcCS;GenBank accession number AB017159, Takita et al. 1999b). Polymerase chain reaction products were subcloned into pBluescript II KS+ (Stratagene, U.S.A.) using a TA cloning method and the nucleotide sequence was determined by the dideoxy methods of Sanger et al. (1977) using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM 377 DNA Sequencer (PE Applied Biosystems, CA, U.S.A.). The full sized CS fragments were introduced into the pIG121-Hm (Ohta et al. 1990) by replacing the intron-GUS region and designated pDcCS-Hm (Fig. 1). The pDcCS-Hm was introduced into *Agrobacterium tumefaciens* (EHA101) by tri-parental mating.

Transformation and isolation of transgenic plants

A. tumefaciens mediated transformation was performed using a vacuum infiltration procedure (Bechtold et al. 1993, Bent et al. 1994). About 100 plants of *A. thaliana* strain WS were grown on rock wool (30 cm square) until bolting occurred. Flower buds were then excised and infected by *A. tumefaciens*. After self-fertilization, the primary transformants (T1) were selected by screening seeds on an R2 nutrient agar medium (Ohira et al. 1973) in the presence of Hyg (100 μg ml^{-1}) and Km (100 μg ml^{-1}). After two generations of self-fertilization of individual primary transformants (T1), transgenic lines that were homozygous for the DcCS gene and null lines (pseudo-WT) obtained from the same primary transformants that produced transgenic plants were selected from populations of T3 plants and used for experiments. Plants were grown in a growth chamber (Koitotron, KG50-HLA, Kyoto, Japan) with 12 h light per day (PPDF: 350 μmol m^{-2} s^{-1}) at 22°C using an ARASYSTEM culture apparatus (Lehle seeds, TX, U.S.A.).

Culture of transgenic plants under aseptic conditions

Seeds were surface sterilized in 1% (w/v) hypochloride solution for 10 min, then kept in a refrigerator at 4°C to synchronize germination as described previously (Koyama et al. 1995). To estimate expression levels of the gene for DcCS, about 200 seedlings were put in a Erlenmeyer flask containing liquid MGRL nutrients (Fujiwara et al. 1992) with 1% sucrose and anhydrous Al-phosphate or orthophosphate as the phosphate source (initial pH of 5.6). Aluminum phosphate powder was prepared as described previously (Koyama et al. 1990). In preliminary experiments we found 5′,5-dithiobis (2-nitrobenzoic acid) sensitive CS activity in our seedlings suggesting the plants also contained a glyoxysomal isoenzyme of CS. This activity was minimized by growing the plants in the presence of sucrose (1%). Plants were grown on a rotary shaker (100 rpm) under illumination (PPDF: 150 μmol m^{-2} s^{-1}) at 24–26°C for 2 weeks, and then harvested by means of vacuum filtration. Plant samples were then frozen in liquid N_2 and kept at –80°C until use for measurement of CS activity.

For collection of citrate excreted from roots, 100 plants were grown hydroponically in each plastic pot [88 (diameter) × 39 (height) (mm)] using a culture apparatus made with 50 mesh nylon mesh (50 squares per inch) and a plastic photo slide mount as described previously (Toda et al. 1999a). Each pot contained 30 ml of experimental medium consisting of MGRL nutrients as described above, excluding sucrose. Plants were cultured under illumination (PPDF: 150 μmol m^{-2} s^{-1}) at 24–26°C. After 2 weeks, the culture media were collected and stored at –20°C until citrate was measured. All apparatuses and media were sterilized before use.

Biochemical assays for CS and citrate

The CS activity was measured according to the method of Srere (1967) as described previously (Takita et al. 1999a). Plants extracts were prepared by grinding 2 g FW of plant tissue in an extraction buffer [50 mM HEPES-NaOH (pH 7.6), 5 M glycerol, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol] using a mortar and pestle at 4°C. Samples were then centrifuged at 28,000 × *g* for 10 min at 4°C. The supernatant was desalted with a Bio-Gel P-6 (Bio-Rad, CA, U.S.A.) column and used to measure the specific activity of CS. Soluble protein was quantified using the Bradford method (Bradford 1976).

Western blot analyses of the CS polypeptides in the extracts were performed as described previously (Koyama et al. 1999). Polypeptides in plant extracts were separated by two dimensional PAGE according to O'Farrell (1975) using Ampholine (pH 5.0–8.0: Amersham Pharmacia, U.S.A.) for isoelectric focussing, followed by SDS-PAGE using a 12.5% (w/v) gel. The CS polypeptides were then transferred onto a polyvinyliden difluoride membrane (AE-6666, ATTO, Tokyo, Japan) using electro-blotting (Horizblot, ATTO, Tokyo, Japan) and then incubated with anti-mtCS antibodies raised against carrot mtCS. An alkaline phosphatase conjugated goat anti-rabbit IgG (Promega, WI, U.S.A.) was used for visualization of the polypeptides.

To measure the amounts of citrate in the growth media, it was necessary to separate the citrate from aluminum present in the growth medium. This was achieved by passing solutions through a Dowex 50 column (H^+-form, Bio-Rad) at pH 2.0. Citrate was then quantified using an enzyme cycling method (Hampp et al. 1984, Kato et al. 1973) as described previously (Takita et al. 1999a).

Soil culture conditions, soil analysis and plant analysis

A typical nonallophanic andosol (Kawatabi andosol) containing a large amount of exchangeable Al was kindly provided by Dr. M. Saigusa and Dr. T. Ito (Kawatabi Experimental Farm of Tohoku University, Naruko, Miyagi, Japan). Culture of *A. thaliana* using on this soil was carried out by the methods described previously (Toda et al. 1999b). Amounts of exchangeable Al and pH value of pure soil were 6.5±0.2 me per 100 g soil and 4.5±0.1, respectively. Soil pH and phosphate availability were altered by adding $CaCO_3$ and NaH_2PO_4 to the basal soil that contained 0.48 g KCl, 0.36 g $MgSO_4$ and 1.32 g $(NH_4)_2SO_4$ per kg soil. Micro nutrients were supplied by adding 1 ml of 1/10 strength of a micro nutrient stock solution from the MGRL medium (Fujiwara et al. 1992) to each 6 g of soil, once a week in a plastic chamber [2 cm (W), 2 cm (L) and 4.5 cm (H)]. Four seedlings were

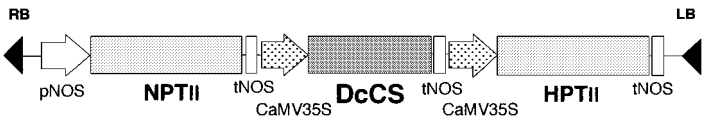

Fig. 1 Construct of the vector used for transformation of *Arabidopsis thaliana* with citrate synthase gene isolated from *Daucus carota* (DcCS). pDcCS-Hm was prepared from the plant vector pIG121-Hm (Ohta et al. 1990, *Plant Cell Physiol.* 31: 805) by replacing the intron-GUS with mitochondrial citrate synthase from *Daucus carota* Takita et al. 1999a, Takita et al. 1999b). LB; left border, RB; right border, CaMV35S; cauliflower mosaic virus 35S promoter, HPTII; hygromycin phosphotransferase II, NPTII; neomycin phosphotransferase II, pNOS; nopalin synthase gene promoter, tNOS; nopalin synthase gene terminator.

grown per vessel and cultivated under controlled environmental conditions (12 h light per day at 20,000 lux: 22°C; Koitotron, KG50-HLA, Koito, Japan). After 4 weeks, the diameter of the rosette of each plant was measured as an index of growth and shoots were harvested. The soil pH (H_2O) and the available P in soil were measured according to the methods of Shoji et al. (1964). Exchangeable Al was determined by the method described by Yuan (1959) with the minor modification as described previously (Toda et al. 1999a). Shoots were digested in H_2O_2-H_2SO_4, and then the P_i content was also measured (Koyama et al. 1992).

Estimation of aluminum tolerance under hydroponic culture conditions

The aluminum tolerance of seedlings was determined according to Toda et al. (1999b). Experimental solutions (0–1 μM $AlCl_3$ in 100 μM $CaCl_2$) were prepared using a 1 mM $AlCl_3$•$6H_2O$ stock solution. The pH of the medium was adjusted to 5.2 with 0.1 M HCl. Each set consisting of 30 seedlings was grown at a vertical angle in 130 ml of test solution for 5 d using a culture apparatus as described previously (Toda et al. 1999b). Root length was measured using a TV monitor equipped with a multiple measure-unit (MC-300, Kenis) and a microscope video camera (Pico Scopeman, Kenis, Tokyo, Japan).

Results

Agrobacterium-mediated transformation of DcCS in A. thaliana

Arabidopsis thaliana (strain WS) was chosen as a host because of the high efficiency of transformation (approximately 2%). Each plant batch consisted of about 100 plants and we obtained at least one transgenic plant from each batch. Our transformation efforts resulted in 14 primary transformants (T1 plants). The vector used contained double drug resistance (Fig. 1), and the HPT II cassette was located upstream of the left border of T-DNA, so transgenic plants derived from the screening with Hyg were expected to contain *DcCS* in their genome. Using specific primers for *DcCS*, we detected the gene for *DcCS* in each transgenic line that showed resistance for Hyg (data not shown). Resistance to Hyg and Km in the T2 generation segregated at almost 3 : 1 ratio, suggesting that the transgene may be located at a single locus. In the T3 generation, we obtained 10 transgenic lines that were homozygous for *DcCS* (transgenic line: T) and 4 null lines (pseudo-wild type) that lacked *DcCS* (null line: N).

Activity of CS in transgenic A. thaliana

After 14 d of growth in Al-phosphate medium, the CS activity of null lines [0.23–0.27 μmol (mg protein)$^{-1}$ min^{-1}] was similar to that of the WT [0.2 μmol (mg protein^{-1}) min^{-1}, unit]. In contrast, the CS activity in most of transgenic lines (0.37–

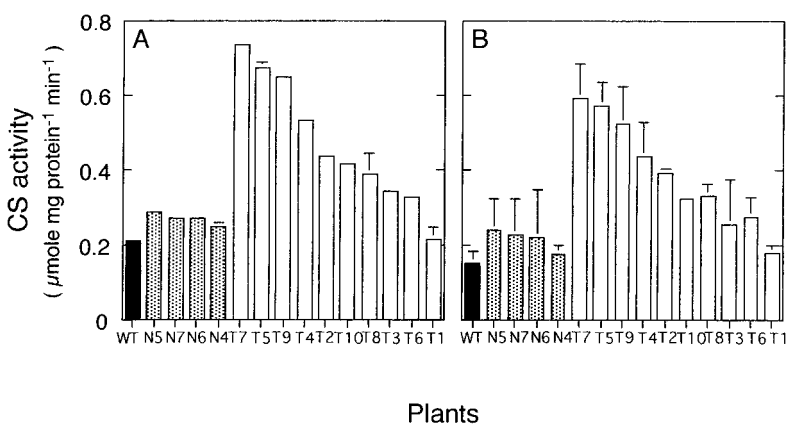

Fig. 2 Activity levels of citrate synthase in different lines of *A. thaliana* including wild-type (WT: solid bar), null plants (pseudo-wild type; N; shaded bar) and DcCS transgenic plants (T; open bar). Activity was measured in plant extracts prepared from 14-day old plants cultured in MGRL medium containing 1.75 mM of Al-phosphate (A) or 1.75 mM Na-phosphate (B). Bars represent means±SD (n=3). Null plants were obtained by self-fertilization from the same primary transformants that produced transgenic plants with the same number.

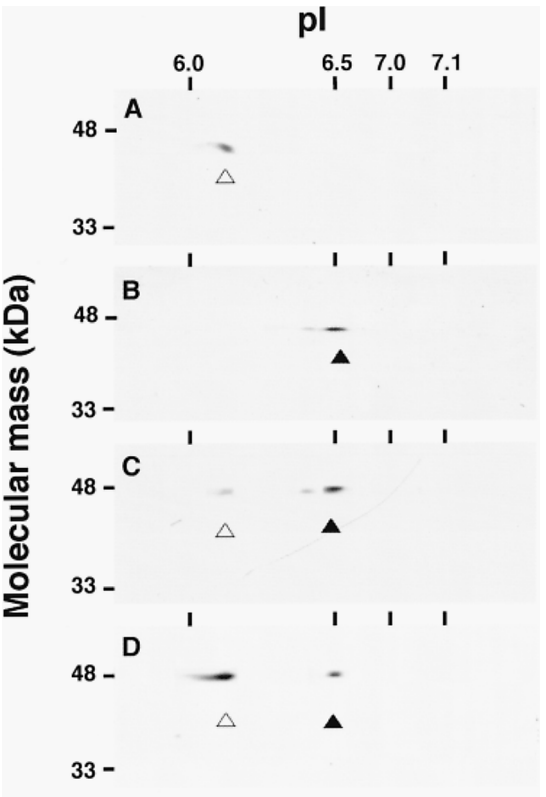

Fig. 3 Western blot analysis of mitochondrial citrate synthase. Citrate synthase polypeptides were obtained from *D. carota* (A), wild-type *A.thaliana* (B), T6 (C) and T7(D) (carrying mtCS in *D. carota*). Each protein (5 μg) was separated by 2-dimensional PAGE, electro-transferred to a polyvinylidene difluoride membrane, and incubated with anti-carrot mtCS antibodies produced from rabbit. An alkaline phosphatase conjugated goat anti-rabbit IgG was used for visualization of the separated proteins.

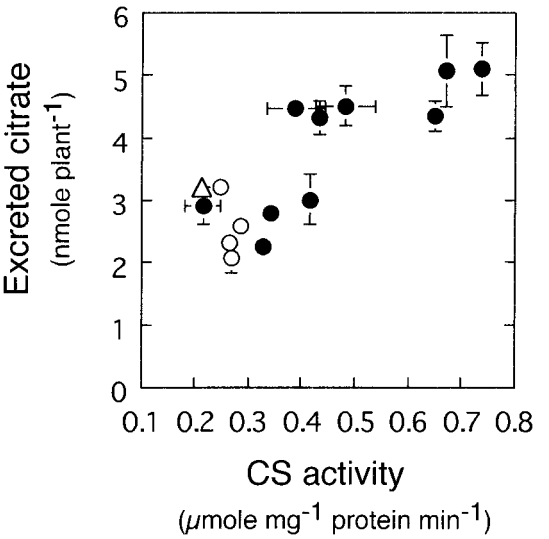

Fig. 4 Relationship between *in vitro* CS activity and the amount of citrate excreted from roots of *A. thaliana*. One hundred plants of each line were grown in Al-phosphate (1.75 mM) medium for 2 weeks using an aseptic hydroponic culture system. Citrate levels in the medium were measured for transgenic plants (closed circle), null plants (open circle) and wild type (open triangle), and plotted against the CS activity shown in Fig. 2 (n=3).

0.78 unit) that carried *DcCS* was higher than both the null lines and WT plants (Fig. 2A). To determine whether the activity of CS in transgenic plants was affected by the phosphorous source, we also measured the activity in plants were grown for 14 d in Na-phosphate medium. Although the activity of CS in plants was slightly lower in Na-phosphate medium than that found with plants in Al-phosphate medium, the transgenic plants showed higher CS activities than control plants (Fig. 2B). These results indicated that the introduction of the CS gene constitutively enhances the CS activity in the transgenic plants.

Ectopic expression of DcCS in transgenic A. thaliana

To determine if elevated levels of CS activity in transgenic lines were caused by overexpression of the DcCS gene, we determined protein levels of DcCS using Western blot analysis. As we reported previously, the polypeptides of DcCS and AtCS have different pI values (6.2 and 6.6, respectively). Thus, we could separate each CS polypeptide using isoelectric focusing (Koyama et al. 1999). We performed a Western analysis with transgenic plants having moderate CS activity (T6: Fig. 2) and high CS activity (T7), using antibody raised against DcCS polypeptide (Takita et al. 1999b). Although WT carrot and *A. thaliana* provide only one signal at the theoretical pI value for each CS polypeptide, transgenic lines each gave two signals (Fig. 3). The signal corresponding to the DcCS polypeptide was greater in T7 than in T6. Under our experimental conditions, we also found the transcript level of *DcCS* in T7 was greater than in T6 (data not shown). Thus, we speculate that the higher activity levels of CS in T7 could be caused by accumulation of DcCS polypeptides.

Citrate excretion from roots of transgenic A. thaliana

To determine whether citrate excretion may act as a tolerance mechanism against P_i-limitation in acid soils, we evaluated the ability of our plants to excrete citrate from the roots. Citrate excretion was detected in all of the transgenic lines after 14 d of culture in Al-phosphate medium. The highest concentration was observed with transgenic line, T7 (17.4 μM) and the lowest concentration was observed with null line, N6 (6.5 μM). The growth of each plant was similar under the given conditions, thus the amount of the citrate excreted was expressed as per plant (nmol per plant). A positive relationship between the amount of citrate excreted and the CS activity measured in different lines was observed (Fig. 4). All of the null lines (N4, 5, 6 and 7) and the transgenic lines that had low levels of CS activity (below 0.35 units; T1, 3 and 6) excreted levels of citrate that

Table 1 Growth and P_i content of transgenic *A. thaliana* [Transgenic lines (T); null lines (N); wild type (WT)] on Kawatabi andosol with different P supply

Plant	Diameter of rosette (cm)		P content in leaves [mg P (g DW)$^{-1}$][a]
	Amount of supplied P (NaH_2PO_4: g per 100 g soil)		
	0.1	0.25	
WT	1.17±0.23	1.86±0.21	7.21±1.06
N5	1.08±0.15	1.64±0.12	7.52±1.32
N6	1.28±0.34	1.71±0.07	7.83±0.20
N7	1.39±0.13	1.76±0.10	7.83±0.36
T5	1.65±0.04	2.07±0.22	9.11±0.31
T6	1.18±0.17	1.64±0.20	7.40±0.62
T7	1.87±0.02	2.38±0.04	9.88±0.19

Means ± SD from four plants are shown.

[a] P contents in leaves was measured in plants grown on soils that were supplied with 0.25 g NaH_2PO_4 per 100 g soil.

were similar to that of WT plants (3.2 nmol per plant). In contrast, transgenic lines that showed high levels of CS activity excreted more citrate (>4 nmol per plant) to the medium. In these transgenic lines, the amount of citrate excreted into the medium increased as the level of CS activity increased. The highest value was 5.1 nmol per plant (T7), corresponding to a 1.6 times increase from the level found in the WT. Under our experimental conditions, citrate content in transgenic plants was also higher than in the WT and null plants. Following 14 d culture in Al-phosphate medium, citrate content in transgenic plants was up to 2.5 times higher than in the WT and null plants (data not shown). From these results, we conclude that overexpression of *DcCS* gene in *A. thaliana* directly enhanced the ability of the plants to excrete citrate from roots as a result of their high ability to produce citrate.

Growth response of transgenic A. thaliana on phosphorous limited soil

To evaluate the ability of the transgenic plants to grow on P_i-limited soil, our transgenic plants were cultured on a typical acidic andosol (Kawatabi andosol; Shoji et al. 1985). This andosol contains a high amount of exchangeable Al, and also shows a high capacity to absorb P_i by forming Al-phosphate (Saigusa et al. 1980, Toma and Saigusa 1997). As we reported previously, growth of *A. thaliana* on this Kawatabi soil is severely inhibited without liming due to Al toxicity (Toda et al. 1999b). In addition, *A. thaliana* is also highly sensitive to proton rhizotoxicity, and thus growth may be inhibited under conditions of the low pH found in the Kawatabi soils (Koyama et al. 1995). When we added 0.05–2.5 g of $CaCO_3$ per 100 g dried soil, exchangeable Al was reduced to less than 2 me per 100 g soil and the soil pH was increased above 5.4 (Fig. 5). Under these conditions, the soil showed a high capacity to immobilize any P supplied and a maximum of 50% of supplied P was available. After 4 weeks, the transgenic lines cultured on this soil showed better growth at lower levels of supplied P than the WT plants (Fig. 5).

To determine if other variations that occurred during gene manipulation affected growth, we compared the rosette diameter of transgenic lines (T5, 6 and 7) to that of their correspond-

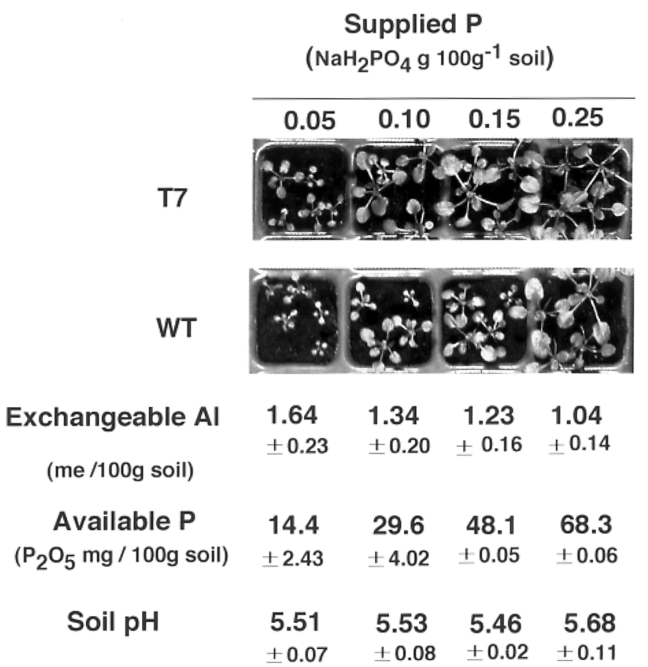

Fig. 5 Growth comparison of a DcCS transgenic plants (T7) and wild type *A. thaliana.* Plants were grown for 4 weeks on a typical Japanese acidic andosol (type Kawatabi) with a range of P-levels (0.05–0.25 g NaH_2PO_4 per 100 g soil) in the presence of 0.25 g $CaCO_3$. Exchangeable Al, available P and pH for each supplied P levels are also shown. Values represent means and SD of three replications.

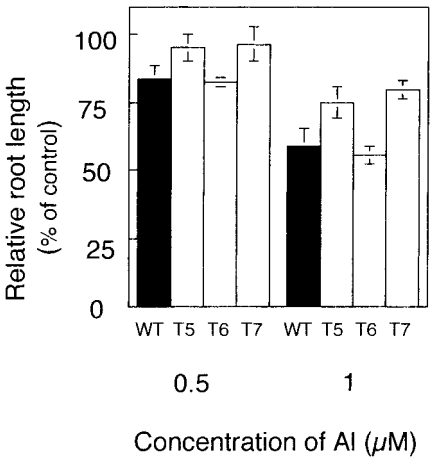

Fig. 6 Relative root elongation of DcCS transgenic *A. thaliana* grown in the presence of Al. Each of 15 seedling of *A. thaliana* was grown for 5 d in 100 μM $CaCl_2$ solution containing $AlCl_3$ (0–1 μM) at an initial pH of 5.2. Root elongation was compared to control plants (expressed as 100%) grown in the absence of Al. The absolute values in the control for WT, T5, T6 and T7 are 5.7, 5.6, 5.9 and 5.6 mm, respectively.

ing null lines (N5, 6 and 7). In the presence of 0.25 g of NaH_2PO_4, the transgenic plants with high levels of CS activity (T5 and 7; 2.07 and 2.38 cm) grew 10–25% better than the WT and null plants (N5 or 7; 1.64 and 1.76 cm) (Table 1). Phosphorous content in the leaves of transgenic plants (T5 and 7; 9.11 and 9.88 mg (g DW) $^{-1}$) was also 20–30% higher. In the presence of 0.1 g of NaH_2PO_4, the growth of transgenic plants (T5 and 7; 1.39 and 1.87 cm) was 20–30% less than in the presence of high P, but growth was still 20–50% greater than the WT and null plants. In contrast, transgenic plant T6, which had moderate levels of CS activity (Fig. 2), had a growth rate and P-content comparable to that of the WT and null plants (Table 1). Under the given conditions, all of the null lines (N5, 6 and 7) grew to a level comparable to that of the WT plants. These results suggest that the superior growth and P uptake observed in T5 and T7 are result of the overexpression of the *DcCS* gene. Thus, we speculated that the overexpression of foreign mitochondrial CS (*DcCS*) in *A. thaliana* enhanced their ability to excrete citrate from the roots which in turn allowed more efficient P_i uptake from Al-phosphate in soils.

Root elongation of the transgenic plants in Al containing solution

Since excretion of organic acids is thought to be involved in Al-tolerance, it was possible that our transgenic lines may show an increased tolerance to this toxic metal. We tested the Al-tolerance of our transgenic *A. thaliana* by measuring root elongation using a simple culture solution method which we have recently developed (Toda et al. 1999a). Although the pH of solution strongly affects the proportions of various Al-ions and, which in turn influences the Al toxicity of medium (e.g. Kinraide and Parker 1990), the root elongation of *A. thaliana* is severely inhibited in low pH solution due to the high sensitivity for low pH stress (Koyama et al. 1995, Toda et al. 1999b). Thus, we chose pH 5.2 for studying Al-tolerance of transgenic plants as a compromise between minimizing proton toxicity for enhancement of root elongation and the desire to maximize Al-toxicity in the solution. Under these conditions, the transgenic plants that had a high level of CS activity (T5 and 7) grew better in the presence of Al than WT (Fig. 6), whereas the transgenic plants with a low level of CS activity (T6) grew similar to the WT. The growth of the WT plants was inhibited by about 20% in 0.5 μM Al, but in the absence of Al, T7 and T5 grew at a similar rate to that of the WT. This indicated that overproduction of citrate altered Al-tolerance in *A. thaliana*.

Discussion

Since immobilization of phosphate in soil due to the formation of Al-phosphate is thought to be one of the major problems affecting the growth of plants on acid soils, an increased ability to utilize this form of phosphate may be an important trait for increased crop productivity in such soils. Using carrot suspension cells as an experimental model, we recently found evidence that we can improve P_i acquisition of cells from Al-phosphate by manipulation of the mitochondrial CS (mtCS) (Koyama et al. 1999). An overexpression of mtCS in carrot cells resulted in the superior growth of cells compared to WT cells due to their enhanced ability to excrete citrate. In the present study, we have gone further by investigating the possibility whether this strategy can improve growth at the whole plant level. Transformation of *A. thaliana* with *D. carota* mtCS using vector shown in Fig. 1 caused CS activity to increase by 3.5-fold in transgenic plants (Fig. 2), and also increased in the citrate excretion from the root (Fig. 4). The transgenic plants with high ability to excrete citrate from the roots (T7 and T5) grew better on P-limited soil than the wild type plants and the null plants (Fig. 5, Table 1). These transgenic plants also showed a higher phosphate content in the leaves which may have been caused by a higher P-uptake from the soil due to the solubilization of P_i via citrate excretion (Table 1). These results suggest that the genetic manipulation technique used in the present study may be an effective means of producing crop plants with an enhanced ability to use Al-phosphate and therefore an enhanced ability to grow in acid soils.

Physiological studies have suggested that CS plays a key role in organic acid release from plants (Hoffland et al. 1992, Takita et al. 1999a, Takita et al. 1999b), but molecular biological studies in this area are somewhat limited. Previous de la Fuente et al. (1997) using bacterial CS, has suggested that genetic manipulation can directly alter the citrate production in higher plants (such as tobacco and papaya) and in turn increase the citrate excretion from roots. In this study, the CS polypeptides caused by ectopic expression of the bacterial CS may be

localized in the cytosol, whereas in plants the mitochondria are believed to be the main site for CS activities. We have extended this step further in our study to evaluate the role of mitochondrial CS on citrate excretion. We used a full-size mtCS cDNA from carrot including a mitochondrial targeting signal sequence for making transgenic plants (Fig. 1). Western blot analysis revealed that the transgenic plants contained an additional CS polypeptides having theoretical pI value (pI 6.21) and molecular mass (48.8 kDa) of the mature mtCS polypeptide of *D. carota* (Fig. 3). This result suggested DcCS polypeptides should be worked in the mitochondria in our transgenic plants. The transgenic plants that had a high level of CS activity (T7) contained greater levels of the mature DcCS polypeptides than in the transgenic plants with a moderate levels of CS activity (T6) (Fig. 3), and in turn showed a superior citrate excretion from the roots (Fig. 4). These results strongly suggested that an increase in CS activity in the mitochondria could directly alter citrate excretion from plants. This possibility is supported by our recent transgenic study of carrot cells that showed superior citrate excretion with an enhanced CS activity by gene manipulation (Koyama et al. 1999).

Increased organic acid excretion is thought to be a possible Al tolerance mechanism in plants (e.g. Ma et al. 1997, Larsen et al. 1998) either by acting as an Al-exclusion mechanism (Taylor 1991) or by increasing the uptake of phosphate from insoluble phosphate sources like Al-phosphate (e.g. Koyama et al. 1990, Johnson et al. 1994). Thus, we could speculate that our transgenic plants with an enhanced citrate excretion showed both Al tolerance and superior P_i acquisition from Al-phosphate. As we expected, our transgenic plants showed both Al-tolerance (Fig. 6) and superior P uptake from P-limited soil containing Al-phosphate (Fig. 5, Table 1). Although an enhanced Al tolerance by gene manipulation technique has been reported previously (de la Fuente et al. 1997), as far as we know, our research may be the first clear example of improving a plant ability to utilize P from Al-phosphate by the genetically manipulating organic acid metabolism. Very recently, the superior P_i uptake from Ca phosphate has been reported in transgenic tobacco carrying bacterial CS (López-Bucio et al. 2000). This finding suggests that our transgenic plants may also show superior growth on alkaline soils.

According to Taylor (1991) carbon loss is thought to be a serious problem associated with organic acid excretion in terms of it being a high energetic cost. Under controlled conditions, we found no difference in the growth of transformed and WT plants of *A. thaliana* (Table 1), but one should also consider the possibility that this species has a slow growth rate. Thus, carbon loss may be a more serious problem in fast growing species such as crop plants. This needs to be examined in more detail in the future using a fast growing species before one can infer that crop growth could be improved by manipulating organic acid metabolism. Another important factor requiring investigation would be the partitioning of C sources between carbohydrate metabolism and nitrogen metabolism in transgenic plants. Recent studies on N nutrition have suggested that carbohydrate metabolism (including citrate synthesis) is directly related to the N environment for optimizing C flow to N-metabolism (Scheible et al. 1997). This factor also needs to be examined before our transgenic system is introduced in crop plants.

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