Plant Cell Physiol. 40(5): 482–488 (1999)

Over Expression of Mitochondrial Citrate Synthase Gene Improves the Growth of Carrot Cells in Al-Phosphate Medium

Hiroyuki Koyama [1, 3], Eiji Takita [1], Ayako Kawamura [1], Tetsuo Hara [1] and Daisuke Shibata [2]

[1] *Laboratory of Plant Cell Technology, Faculty of Agriculture, Gifu University, 1-1 Yanagido, Gifu, 501-1193 Japan*
[2] *Mitsui Plant Biotechnology Research Institute, Sengen 2-1-6, TCI-D21, Tsukuba, Ibaraki, 305-0047 Japan*

A mitochondrial citrate synthase (CS) of *Arabidopsis thaliana* was introduced into carrot (*Daucus carota* L. cv. MS Yonsun) cells by *Agrobacterium tumefaciens*-mediated transformation. Transgenic cell lines had high CS activity, the highest value observed was 0.24 μmol (mg protein)$^{-1}$ min^{-1} which was 1.9-fold of that in wild-type cells. Transcript levels of DcCS were similar between transgenic lines, but those of AtCS were increased as the CS activity of cells was increased. Isoelectric focussing revealed that the CS polypeptide of the transgenic lines had a pI value different from that of the wild-type cells, although the molecular mass was the same. These results indicate that the CS polypeptides of *A. thaliana* were expressed and processed to the mature form in carrot cells. The growth rate and excretion was 2.2–2.8 and 2.8–4.0 fold greater in the transgenic cells than in the wild type cells, respectively. Phosphate uptake from Al-phosphate also increased in transgenic cells. It appeares, the overexpression of mitochondrial citrate synthase in carrot cells improves the growth rate in Al-phosphate medium possibly as a result of increased citrate excretion.

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

Since phosphate is a severely limited and non-renewable resource (Ando 1983), improvement in plant traits relevant to P_i-acquisition are important (von Uexüll and Mutert 1995). In most soils, the concentration of phosphate in soil solutions is lower than 10 μM and the phosphorous supplied as fertilizer is fixed in an insoluble form (Clark 1990, Wright 1953). Thus, the plants growing in such soils have to acquire P_i either by superior root growth, increased P_i-uptake rate or increased root exudation which helps P_i absorption (Ae et al. 1990, Gardner et al. 1981). One approach to introduce such traits into crop plants may be via gene manipulation. For example, the overexpression of a high-affinity P_i-transporter enhanced the cell growth of tobacco under the P_i-limiting conditions (Mitsukawa et al. 1997). Studies relevant to high-affinity P_i uptake have been reported by several authors (e.g. Mimura et al. 1990, Bun-ya et al. 1991). However, in order to fully understanding such a system, it is important to fully examine the nature of those traits using both physiological and molecular studies.

Root exudation, including acid phosphatase secretion (Goldstein et al. 1988, Tadano et al. 1993) and organic acid excretion (Lipton et al. 1987, Gardner et al. 1981), is thought to be part of the phosphate starvation rescue system found in higher plants. Physiological studies have suggested that altered carbohydrate metabolism plays a key role in organic acid excretion. This altered metabolism is typically associated with an increase in the activity of enzymes involved in the tri-carboxylic acid (TCA) cycle, as well as, phosphoenolpyruvate carboxylase (Hoffland et al. 1992, Johnson et al. 1994, 1996) These changes usually result in an increased release of citrate and malate. In previous work, we found such a typical altered metabolism in Al-phosphate utilizing cells (designated as insoluble phosphate grower, IPG; Koyama et al. 1992). These cells grew normally in anhydrous Al-phosphate medium and this growth appeared to be accompanied by enhanced citrate excretion.

Insoluble phosphate grower cells showed higher activity of citrate synthase (CS; EC 4.1.3.7) and lower activity of NADP-specific isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42) than the wild-type cells. Although iso-enzymes exist for both of enzymes, our recent work showed differences in mitochondrial CS and cytosolic NADP-ICDH between IPG and wild-type cells (Takita et al. 1999). In higher plants, mitochondrial CS mediates the first step of the TCA cycle by catalyzing the condensation of acetyl coenzyme A and oxaloacetate to form citrate and coenzyme A. Thus, the enhanced activity of this enzyme may increase the productivity of citrate in IPG cells and this in turn may increase their ability to grow normally in the presence of insoluble PO_4 source, such as Al-phosphate. To study this possibility further, the objective of this current study was to develop transgenic cell lines of carrot by gene manipulation which overexpressed mitochondrial CS, and to use this system to fully investigate the way in which increased levels of CS activity lead to in-

Abbreviations: AtCS, *A. thaliana* mitochondrial CS; Aco, aconitase; CS, citrate synthase; DcCS, carrot mitochondrial CS; GUS, β-glucuronidase; ICDH, isocitrate dehydrogenase; Hyg, hygromycin; Km, kanamycin.

[3] To whom correspondence should be addressed. e-mail, koyama @cc.gifu-u.ac.jp

creased growth on insoluble PO_4 sources.

Isolation of cDNAs encoding mitochondrial CS in higher plants was first established in *Arabidopsis thaliana* (Unger et al. 1989) and thereafter, in several plant species (Canel et al. 1996, la Congnata et al. 1996, Landshütze et al. 1995). Overall similarity has been found between the coding regions of these cDNAs at the amino acid and the nucleotide levels, involving mitochondrial matrix targeting peptides at the N-terminus. We isolated the entire sequence of CS cDNA from *A. thaliana* by PCR and introduced it into carrot cells by *Agrobacterium tumefaciens* mediated transformation. The transgenic lines having mature CS polypeptides may have the CS polypeptides incorporated into the mitochondria. These lines have increased levels of CS activity, greater levels of citrate excretion, and showed better cell growth than wild type cells in Al-phosphate medium.

Materials and Methods

Enzymes and chemicals—Restriction and modification enzymes for DNA and the high fidelity Taq polymerase *Ex Taq* were purchased from Takara (Otsu, Japan). Other chemicals were obtained from Wako Pure Chemical (Osaka, Japan) and Sigma (MI, U.S.A.).

Plant materials and culture conditions—A protoplast-derived callus obtained from the carrot (*Daucus carota* L. cv. MS Yonsun) cell line used in the preceding paper (Takita et al. 1999) was used as a host for transformation. Culture conditions for the estimation of growth rate and citrate excretion were the same as those described previously (Koyama et al. 1990). Cells were pre-cultured in R2 medium (Ohira et al. 1973) and 400 mg FW of these cells were inoculated in 30 ml of medium containing either 2 mM of Al-phosphate or NaH_2PO_4 as the sole phosphorous source. Cells were harvested, at appropriate times, on a Miracloth (Calbiochem-Novabiochem, CA, U.S.A.), washed with distilled water and weighed. Cells were placed in a polyethylene pouch, and frozen in liquid N_2 and kept in a deep freezer (−80°C) until enzyme activity was measured.

Vector construction and Agrobacterium strain—cDNA encoding mitochondrial CS was obtained from cDNA libraries of *Arabidopsis thaliana* (ecotype Columbia) by PCR (94°C 20 s, 55°C 30 s and 72°C 60 s, 40 cycles) using a Gene Amp 2400 system (Perkin Elmer, CA, U.S.A.). Both sense (5′-CCCCGTCTAGAGAGTTTTGGAGAATGGTGTTTTTCCGCAGCG-3′) and antisense (5′-CCCCCGATATCCTCGGTGGTGAGTTAAGCAGATGAAGC-3′) primers were prepared according to the sequence reported by Unger et al. (1989), and added *Xba*I or *Eco*RV sequences to both 5′ ends, respectively. Polymerase chain reaction products were subcloned into pBluescript II KS+ (Stratagene, U.S.A.) and the nucleotide sequence was confirmed by the dideoxy methods (Sanger et al. 1977). Then, *Xba*I-*Eco*RV fragments were introduced into the pIG121-Hm (Ohta et al. 1990) by replacing the intron-GUS region, and designated as pAtCS-Hm. The pAtCS-Hm was introduced into *Agrobacterium tumefaciens* EHA101 by a tri-parental mating.

Transformation and isolation of transgenic colonies—*Agrobacterium*-mediated transformation was carried out according to the method of Hiei et al. (1994) and Aldemita and Hodges (1996) with minor modifications. *A. tumefaciens* grown for 3 d in AB medium (Chilton et al. 1974) was collected by centrifugation (7,000 × *g*, 5 min), and then diluted until the A_{600} of the suspension reached 1.0 in R2 medium containing 20 μg ml^{-1} acetosyringone (designated as R2AS). About 400 mg FW of carrot suspension cells at the logarithmic phase of growth were collected on filter paper (No. 2, 55 mm in diameter, Advantec, Tokyo, Japan) by vacuum filtration, and then placed on a polyurethane sheet immersed in 40 ml of R2AS in a plastic petri dish. One ml of *A. tumefaciens* suspension was added to each dish, and co-cultured with the cells for 4–7 d at 25°C. Cefotaxime (500 μg ml^{-1}) was added to eliminated *A. tumufaciens* and the transformed cells were selected by addition of kanamycin (100 μg ml^{-1}) and hygromycin (50 μg ml^{-1}).

Enzyme assay and quantification of citrate in medium—Cell extracts were prepared from 1 g FW of cells by disruption with a Polytron PT-3000 (Kinematica, Switzerland) at 20,000 rpm for 1 min in extraction buffer [50 mM HEPES-NaOH (pH 7.6), 5 M glycerol, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol]. Samples were then centrifuged at 28,000 × *g* for 10 min. The supernatant was desalted with a Bio-Gel P-6 (Bio-Rad, CA, U.S.A.) column and the specific activitiy of CS was determined by the method of Srere (1967). Soluble protein was quantified using the method of Bradford (1976). Citrate in the medium was first separated from aluminum on a Dowex 50 (H^+-form, Bio-Rad) column at pH 2.0, and then quantified by the enzyme cycling method (Hampp et al. 1984, Kato et al. 1973).

Western blotting analysis—Polypeptides of mtCS in cell extracts were separated by 2 dimensional PAGE according to O'Farrell (1975) with minor modifications. Isoelectric focusing, was performed with Ampholine (pH 5.0–8.0: Amersham Pharmacia, U.S.A.), and followed by SDS-PAGE using a 12.5% (w/v) gel. Separated polypeptides were then transferred from the gel onto a polyvinyliden difluoride membrane (AE-6666, ATTO, Tokyo, Japan) by electro-blotting (Horizblot, ATTO, Tokyo, Japan) using the instructions provided by the manufacturer 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. Molecular weight and pI markers were both purchased from Bio-Rad.

Quantification of CS-gene transcripts by quantitative RT-PCR—The quantitative RT-PCR for mtCS was carried out according to the method of Canel et al. (1996) with minor modifications. Total RNA was prepared from each cell line by the method of Shirzadegan et al. (1991) and then quantified using absorbance (A_{260}). Equal amounts of total RNA (1 μg) from each cell line were reverse transcribed into DNA and then subjected to appropriate cycles of PCR using primers for DcCS (DcCSqF1: 5′-GTTTGAAAGTTACACGATGTGTACG-3′, DcCSqR1: 5′-CCTCAAACTATGCATGTCCC-3′) and AtCS (AtCSqF1: 5′-CGTCGGCCAACAATCTTCA-3′, AtCSqR1: 5′-AAGCCTCCAGACTGGGCAGTA-3′). Polymerase chain reaction products having a length of 251 bp (carrot) and 312 bp (*A. thaliana*) were visualized by Southern blotting using the same PCR products for specific proves. Probe-labeling (digoxigenin), hybridization, and detection (chemiluminescent) were carried out using a kit (DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed for 20 h in hybridization buffer (DIG Easy Hyb, Boehringer Mannheim) at 42°C. Membranes were twice washed in 2 × SSC and 0.1% SDS at room temperature for 5 min, and then twice in 0.1 × SSC and 0.1% SDS at 68°C for 15 min each time. To evaluate the efficiency of reactions, transcript levels of actin were

determined using specific primers for carrot actin (AcF1: 5′-GTGACGTTGATATCAGAAAGG-3′, AcR1: 5′-CGTCATATTCACCCTTCGAG-3′) prepared according to the reported sequence (CAc1, GenBank accession number: X17526).

$^{32}PO_4$ uptake rate of trangenic cell lines from alminum phosphate—Each cell line was grown in 0.5 mM NaH_2PO_4 medium for 10 d. Cells (2.5 g FW) were then incubated in 50 ml of medium containing colloidal Al-phosphate (2 mM $AlCl_3$, 1 mM NaH_2PO_4, and specific activity of ^{32}P was 30 μCi per mol) as the sole phosphorous source for 4 h at 25°C. The pH of medium was adjusted to 5.6 in the presence of 30 mM of MES using 1 M NaOH. Cells were collected on Miracloth and incubated in 1 mM citrate at 4°C for 1 h to eliminate absorbed Al-phosphate. Cells were then harvested on a Miracloth, rinsed with distilled water and digested in H_2O_2-H_2SO_4 as described previously (Koyama et al. 1992). Incorporated ^{32}P was measured using Cerenkov radiation as described previously (Koyama et al. 1992).

Results

Isolation of transfomed cell lines carrying AtCS—Since plant cultured cells are known to show a spontaneous variation even within the same population (Oono 1984), we used a protoplast-derived population as the host for transformation to minimize variation between our transgenic cell lines. To increase efficiency of the transformation, we used a high density of *A. tumefaciens* in the presence of acetosyringone during co-culture according to recent publications (e.g. Hiei et al. 1994). Since the carrot cells showed moderate tolerance to kanamycin, we used a vector containing double drug resistance (Fig. 1), and kanamycin and hygromycin were used for screening. HPT II cassette was locates upstream of the left border of T-DNA, so cells derived from the first round of screening with hygromycin were expected to contain AtCS in their genome. As expected, the colonies derived from screening with hygromycin contained AtCS gene as judged by genomic PCR using specific primers for AtCS (data not shown). We obtained more than 50 transformants carrying the AtCS gene and used 10 lines were randomly for the following experiments. As a control of transformation, we carried out transformation using pIG121-Hm which contained the intron-GUS reporter instead of AtCS.

Activity of CS in transgenic cell lines—Specific activity of CS was determined for 10 transformant cell lines carrying AtCS, 3 control cell lines having the intron-GUS and the parental host cell line (wild-type; WT), respectively. Following a 7-day culture of cells in Al-phosphate medium, the CS activity of the control lines carrying intron-GUS (0.11–0.14 μmol (mg protein)$^{-1}$ min^{-1}, unit) was similar to that of the WT cells (0.12 μmol (mg protein)$^{-1}$ min^{-1}, unit). However, the most of CS activity in transgenic lines carrying AtCS was higher than those in the control and WT cells (Fig. 2). We found that the CS activity of the WT cells lower than 0.15 units during 9 d when they grow in Al-phosphate medium (Takita et al. 1999). Most of the transgenic cell lines carrying AtCS showed more than 0.15 units activity with the highest activity being 0.24 unit (T8 line) (Fig. 2A).

To determine if the activity of CS in transgenic lines was affected by phosphorous source in the growing medium, also measured the activity in 4 transgenic (T1, T3, T8 and T20), 2 control cell lines (C4 and C41) and WT cells, after 7 d growth in Na-phosphate medium. Although the activity of CS in each cell line was slightly lower in Na-phosphate medium than in Al-phosphate medium, but transgenic lines showed higher CS activities than control and WT cells. The T8 line, which had the highest CS activity in Al-phosphate medium, also had the highest CS activity (0.21 unit) in Na-phosphate medium. These results suggested that the introduction of the mtCS gene constitutively enhances the CS activity in transgenic cells.

Ectopic expression of an A. thaliana mtCS in carrot cells—To define the relationship between expression levels of AtCS gene and CS activity in transgenic lines, we measured transcripts levels of AtCS in T3 (CS activity: 0.17 unit), T8 (0.24 unit) and T20 (0.23 unit). We also measured mtCS transcripts levels in control (C4, 0.14 unit) and WT (0.13 unit) cells. Since both nucleotide sequences of AtCS and DcCS showed high homology within coding region, it

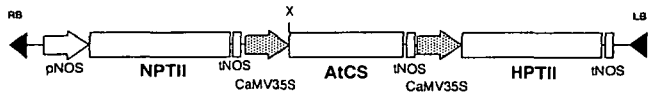

Fig. 1 Construct of vector used for transformation of citrate synthase to carrot cells. pAtCS-Hm was prepared from a plant vector pIG121-Hm (Ohta et al. 1990) by replacing intron-GUS by mitochondrial citrate synthase in *A. thaliana* (Unger et al. 1987). LB, left border; RB, right border; CaMV35S, cauliflower mosaic virus 35S promoter; HPTII, hygromycin phosphotransferase II; NPTII, neomycin phosphotransferase II; pNOS, nopalin synthase gene promoter; tNOS, nopalin synthase gene terminator; X, *Xba*I.

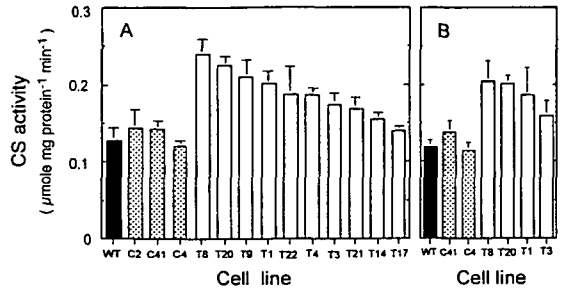

Fig. 2 Activities of citrate synthase in transgenic carrot suspension cells carrying mitochondrial citrate synthase from *A. thaliana* (T, open bar), control cells carrying intron-GUS (C, shaded bar) and parental wild-type cells (WT, solid bar) were determined using cell extracts derived from 7-day old cells cultured in Al-phosphate medium (2 mM: A) and Na-phosphate (2 mM: B). Means ± SD are indicated.

was difficult to determine transcripts levels of each CS gene by northern blotting. Thus, we performed quantitative RT-PCR analysis using specific PCR primers of both mtCS to determine the transcript levels in each cell line. In preliminary experiments, we found that the amounts of PCR products were saturated after 19 cycles. We then used 13 cycles for the quantification of transcripts of mtCS. Transcripts levels of DcCS gene in transgenic cell lines were similar to levels to found in both WT and control cells (Fig. 3A). However, the transcript levels of AtCS in transgenic cell lines which showed high CS activity (T8 and T20) were greater than transcript levels in which showed lower CS activity (T3, Fig. 3B). Although both the purity of RNA and the efficiency of reverse transcription should be important for evaluation of transcripts levels. We detected no differences between the rRNA content in the total RNA sample (Fig. 3D). The amount of RT-PCR products of actin, which was estimated by the same protocol according to the reported sequence (CAc1, GenBank accession number: X17526), were also similor for each sample (Fig. 3C). These results strongly suggested that both overexpression of AtCS in T8 and T20 is the result of a greater amount of AtCS transcripts.

To further analize of AtCS expression in transgenic lines, we used western blots to determine the presence of AtCS polypeptides in T3 and T20. Based on reported nucleotide sequences (AtCS: GenBank, X17528, DcCS: AB017159), the molecular masses of mature CS polypeptides in both carrot and *A. thaliana* are thought to be identical (48.8 kDa). However, the calculated pI values for mature CS proteins of carrot and *A. taliana* are 6.21 and 6.62, respectively. As shown in Figure 4, both carrot and *A. thaliana* each gave only 1 signal which was around the theoretical pI value. Under these conditions, both T3 and T20 gave the same levels of signals for the DcCS polypeptides, but the signal for the AtCS polypeptides in T20 was greater than in T3. Thus, we speculate that the increased CS activities in transgenic lines may be caused by the accumulation of AtCS polypeptides.

Citrate excretion and growth of transgenic cell lines—Citrate excretion and growth were measured in the transgenic lines in both Al-phosphate and Na-phosphate medium. Under P_i-sufficient conditions, the growth of each transgenic (T3, 8 and 20), control (C4) and WT lines was nearly the same (Table 1), which suggests that growth rate was not affected by introducing foreign mtCS. In contrast, when cells were cultured for 9 d in Al-phosphate medium, the growth of T8 and T20 were 2–3 fold that of the WT and C4 lines. To reduce the internal P_i concentration of the cells, in this experiment, we employed the pre-culture for 10 d in a medium with only 0.5 mM P_i. Thus, the difference in cell growth in Al-phosphate medium is considered to depend on the ability of cells to utilize Al-phosphate. It has been suggested that in the presence of Al-phosphate as the sole phosphate source, growth may be regulated by citrate exudation which causes solubilization of the phosphate (Koyama et al. 1990). In fact, the concentration of citrate in the media of transgenic lines which showed su-

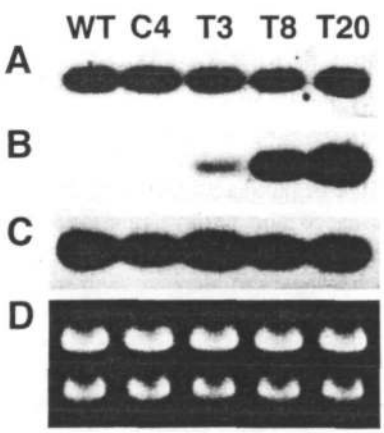

Fig. 3 Quantitative PCR analysis for mtCS gene transcripts in carrot cells. Membrane-bound PCR products at 13 cycles derived from the reverse transcripts obtained from equal amounts of total RNA (1 μg) of each cell type were visualized using digoxigenin-labeled probes. PCR products derived from reverse transcripts using specific primers for either carrot mtCS (DcCS: A), *A. thaliana* mtCS (AtCS: B) or carrot actin (Dacl: C) are shown. Gel image for ribosomal RNA in each 1 μg of total RNA (D).

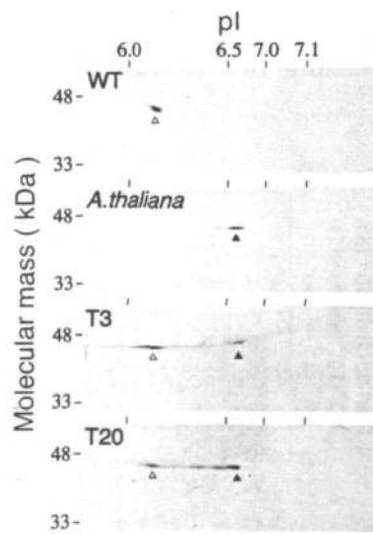

Fig. 4 Western blots of mitochondrial citrate synthase in transgenic carrot cells. CS polypeptides were obtained from wild-type cells, T20 and T3 (carrying mtCS in *A. thaliana*). Each protein (5 μg) was separated by 2-dimensional PAGE, electrotransferred to polyvinylidene difluoride membrane and incubated with anti-carrot mtCS antibodies of rabbit. An alkaline phosphatase conjugated goat anti-rabbit IgG was used for visualization of separated proteins. Protein extracts from *A. thaliana* seedlings are also indicated.

Table 1 Growth and citrate excretion of transgenic carrot cells carrying mitochondrial citrate synthase in *A. thaliana*

Cell line	Fresh weight (g flask^{-1})		Citrate in medium (mM)	
	NaH_2PO_4	$AlPO_4$	NaH_2PO_4	$AlPO_4$
WT	3.55±0.04	0.52±0.03	0.05±0.00	0.06±0.01
C4	3.38±0.12	0.65±0.08	0.07±0.01	0.06±0.02
T3	3.60±0.14	0.65±0.09	0.06±0.01	0.06±0.02
T8	3.23±0.11	1.45±0.04	0.20±0.06	0.21±0.01
T20	3.63±0.11	1.12±0.12	0.19±0.04	0.19±0.05

Cells were grown for 9 d in 30 ml of R2 medium containing $AlPO_4$ or NaH_2PO_4 as a sole source of phosphorous. WT; wild-type cells, C4; control line carrying intron-GUS, T3,T8,T20; transformant carrying AtCS. Means and SD from 3 independent assay are indicated.

perior growth (T8 and T20) was 2.5–3 times higher than that in the medium from control and wild type cells. Transgenic lines T3 showed no significant increase in growth or citrate release over that of control and wild type cells (Table 1). To further investigation the efficiency of P_i uptake from Al-phosphate by the transgenic cell lines, we measured short-term uptake of the P_i from ^{32}P-labeled Al-phosphate. After 4 h incubation, the amount of $^{32}P_i$ incorporated into the cells was similar between T3, C4 and WT. However, T8 and T20 incorporated about 2 times the amount of $^{32}P_i$ than other cell lines (Fig. 5). These results suggest that the superior growth of T8 and T20 may be caused by a more efficient uptake of P_i from Al-phosphate medium as a result of their enhanced ability to excrete citrate.

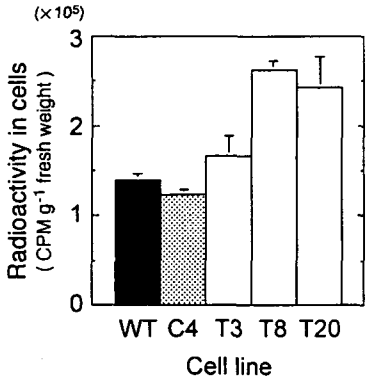

Fig. 5 Uptake of $^{32}P_i$ of transgenic carrot cells from Al-phosphate. Transgenic cell lines carrying AtCS (T3, T8 and T20) or intron-GUS (C4), and WT cells were incubated with ^{32}P labeled Al-phosphate (specific activity of $^{32}P_i$ is 30 μCi per mol) for 4 h. Prior to the determination of the incorporated $^{32}PO_4$, cells were incubated in 1 mM citrate (pH 4.5) at 4°C for 1 h to eliminate absorbed Al-phosphate, and then digested with H_2SO_4-H_2O_2. Means±SD of CPM values derived from 1 g FW of each cell is shown. (n=3)

Discussion

Since the precipitation of phosphate as Al-phosphate is thought to be one of the major problems affected the growth of plants in acid soils, an increased ability to utilize phosphate in this form would be an important trait for increased crop productivity in such soils. In this study, transformation of cells using *A. thaliana* mitochondrial CS caused the CS activity to increase by up to 1.9 fold in carrot cells (Fig. 2), and caused an increase in citrate excretion to the medium (Table 1). Growth of the transformed cells after a 9-days culture in Al-phosphate medium was 2.2–2.8 fold of that in the control and wild-type cells (Table 1). It appears that P_i is solubilized from Al-phosphate as a result of enhanced citrate excretion and then incorporated to the transgenic cells within a relatively short-term (Fig. 5). These results suggest that the gene manipulation technique used in the present study may be an effective means of producing crop plants with an enhanced ability to use Al-phosphate, i.e., to grow in acid soils.

Recent work has suggested that gene manipulation can directly alter the organic acid metabolism in higher plants. Transgenic tobacco and papaya plants, carrying bacterial CS, have been reported to have a high citrate excretion rate in roots, which appears to increase their tolerance to Al (de la Fuente 1997). However, increased tolerance to Al is not the only function thought to result from the increased excreion of citrate from plant roots. In addition, increased organic acid excretion is thought to increase tolerance to Al (e.g. Larsen et al. 1998, Ma et al. 1997) and the uptake of phosphate from insoluble phosphate sources like Al-phosphate (e.g. Koyama et al. 1990, Johnson et al. 1994). In the present study, we have shown a clear example of enhanced Al-phosphate-utilizing capacity as a result of our transformations. It appears that more detailed examination of the physiological relevance of citrate excretion appears to be important.

Modified organic acid metabolism, including increased activity of phosphoenolepyruvate carboxylase (Johnson et al. 1994) and CS (Hoffland et al. 1992), is thought to play a key role in organic acid release from plants, but molecular biological studies in this area have been limited. As mentioned previously the recent work of de la Fuente et al. (1997) is one of the first to report transgenic plants carrying high CS activities. Unfortunately, this study did not identify which isoform of CS was responsible for this increased activity. It is possible that the bacterial CS proteins used in this study may be localized in the cytosol and in plants that most abundant CS activities are believed to be in mitochondria. We have gone one step further in our study and have provided the strong experimental evidence that we are looking at the overexpression of mitochondrial CS activity as opposed to cytosolic CS activity. We used a mtCS gene for making transgenic cells which showed CS activities which were greater than activities of WT cells. Although the AtCS gene used in this study contained a mitochondrial targeting signal sequence, we wanted to further confirm of localization of CS we had manipulated in our transgenic lines. We examined CS polypeptides using western blot analysis and found that polypeptides had a molecular mass of 48.8 kDa and a pI value of 6.6 (Fig. 4). These values correspond to values associated with the theoretical values of mature AtCS polypeptide. This result suggested that AtCS polypeptides should be worked in mitochondria in our transgenic lines. These transgenic lines showed increased citrate excretion (Table 1) and P_i uptake from Al-phosphate (Fig. 5). There were no differences in activity levels for any of the other enzymes which are relevant to citrate production including aconitase, NAD-ICDH and NADP-ICDH (data not shown). It appears that the introduction of mtCS into carrot cells enhanced the CS activity and the citrate excretion in transgenic cells, but this had no effect on other enzymes involved in citrate synthesis. From these results, we speculate that an increase in mitochondrial CS activity alone could directly increase citrate excretion from plants.

In a previous study we examined a selected carrot cell line designated as insoluble phosphate grower (IPG) which showed both superior growth and higher citrate excretion than wild type cells when grew in an Al-phosphate medium. The CS activity in the IPG line was 2 times higher than that in wild type cells (Takita et al. 1999), and almost identical to the level found in our transgenic lines, T8 and T20. However, citrate excretion of transgenic cells was lower than that found in IPG cells. A previous study, which appears as an accompanying paper, showed that the activity of cytosolic NADP-ICDH, which catalyzes the conversion step of isocitrate to 2-oxoglutarate in the cytosol, was lower in IPG cells than wild type cells. Specific activities of NADP-ICDH for cells grown for 9 d on Al-phosphate medium were 0.14, 0.22 and 0.21 for IPG, WT and T8, respectively (data not shown). This may allow citrate to concentrate in the cytosol of IPG cells, and in turn allows these cells to release more citrate to the environment than the transgenic lines, despite the 2 lines having similar activity levels of mtCS. This possibility is supported by a recent study looking at an anti-sense inhibition of NADP-ICDH. In this study the concentration of citrate in leaves was very high, although location of these citrate pools was not clear (Kruse et al. 1998). To study this idea further, another set of transgenic lines would need to be made which showed modified NADP-ICDH activity. We could then run another set of experiments similar to the ones we completed for the present study to determine the role that this enzyme played in citrate excretion. Another possibility that could account for the different rates of citrate excretion between the IPG cells and the transgenic cells may be at the membrane transport level. The carriers involved in citrate transport which are most likely an anion channel (Ryan et al. 1997) might be enhanced in the membrane of IPG cells. Further analysis is clearly needed in order to define the true nature of the high ability of IPG cells to excrete citrate in large amounts.

A part of this work was supported by a grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 90234921 for HK). We wish to thank Dr. F. Suzuki and H. Suga, of Gifu University Gene Research Center for their kind technical support. The plant vector pIG121-Hm was a kind gift from Dr. K. Nakamura of Nagoya University. We also wish to thank Ms. Julie Stephens at the University of Alberta for editorial comments regarding this manuscript.

References

Ae, T., Arihara, J., Okada, K., Yoshihara, T. and Johansen, C. (1990) Phosphorus uptake by pigeon pea and its role in cropping systems of the Indian subcontinent. *Science* 248: 477–480.

Aldemita, R.R. and Hodges, T.K. (1996) *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties. *Planta* 199: 612–617.

Ando, J. (1983) Future of phosphorus resources and suggested direction for Japan. *Jpn. J. Soil. Sci. Plant Nutr.* 54: 164–169.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Bun-ya, M., Harashima, S. and Oshima, Y. (1991) The PHO84 gene of *Saccharomyces cerevisiae* encodes an inorganic phosphate transporter. *Mol. Cell Biol.* 12: 2958–2966.

Canel, C., Bailey-Serres, J.N. and Roose, M.L. (1996) Molecular characterization of the mitochondrial citrate synthase gene of an acidless pummelo (*Citrus maxima*). *Plant Mol. Biol.* 31: 143–147.

Chilton, M.D., Currier, T.C., Farand, S.K., Bendith, A.J., Gordon, M.P. and Nester, E.W. (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc. Natl. Sci. Acad. USA* 71: 3672–3676.

Clark, R. (1990) Physiology of cereals for mineral nutrient uptake, use and efficiency. *In* Crops as Enhancers of Nutrient Use. Edited by Baligar, V. and Duncan, R. pp. 131–209. Academic Press, Sandiego, CA.

de la Fuente, J.M., Ramirez-Rodrigez, V., Cabrera-Ponce, J.S. and Herrera-Esterella, L. (1997) Aluminum tolerance in transgenic plants by alteration in citrate synthesis. *Science* 276: 1566–1568.

Gardner, W.K., Parbery, D.G. and Barber, D.A. (1981) Proteoid root morphology and function in *Lupinus albus*. *Plant Soil.* 60: 143–147.

Goldstein, A.H., Baertlein, D.A. and McDaniel, R.G. (1988) Phosphate starvation inducible metabolism in *Lycopercicom esculentum*. I. Excretion of acid phosphatase by tomato plants and suspension cells. *Plant Physiol.* 87: 711–715.

Hampp, R., Goller, M. and Füllgraf, H. (1984) Determination of compartmented metabolite pools by a combination of rapid fractionation of oat mesophyll protoplasts and enzymic cycling. *Plant Physiol.* 75: 1017–1021.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficeint transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6: 271–282.

Hoffland, E., van den Boogaard, R. and Findenegg, G.R. (1992) Biosynthesis and root exudation of citric and malic acids in phosphate-starved rape plants. *New Phytol.* 122: 675–680.

Johnson, J.F., Allan, D.L., Vance, C.P. and Weiblen, G. (1996) Root carbon dioxide fixation by phosphorous deficient *Lupinus albus*. Contribution to organic acid exudation by proteoid roots. *Plant Physiol.* 112: 19–30.

Johnson, J.F., Vance, C.P. and Allan, D.L. (1994) Phosphorous stress induced proteoid roots show alterd metabolism in *Lupinus albus*. *Plant Physiol.* 104: 657–665.

Kato, T., Berger, S.J., Carter, J.A. and Lowry, O.H. (1973) An enzymatic cycling method for nicotianamide-adenine dinucleotide with malic and alcohol dehydrogenases. *Anal. Biochem.* 53: 86–97.

Koyama, H., Ojima, K. and Yamaya, T. (1990) Utilization of anhydrous aluminum phosphate as a sole source of phosphate by a selected carrot cell line. *Plant Cell Physiol.* 31: 173–177.

Koyama, H., Ojima, K. and Yamaya, T. (1992) Characteristics of aluminum-phosphate-adapted carrot cells: uptake and utilization of phosphate. *Plant Cell Physiol.* 33: 171–176.

Kruse, A., Fieuw, S., Heineke, D. and Müller-Röber, B. (1998) Antisense inhibition of cytosolic NADP-dependent isocitrate dehydrogenase in transgenic potato plants. *Planta* 205: 82–91.

la Congnata, U., Landshütze, V., Willmitzer, L. and Müller-Röber, B. (1996) Structure and expression of mitochondrial citrate synthases from higher plants. *Plant Cell Physiol.* 37: 1022–1029.

Landshütze, V., Müller-Röber, B. and Willmitzer, L. (1995) Mitochondrial citrate synthase from potato: predominant expression in mature leaves and young flower buds. *Planta* 196: 756–764.

Larsen, P.B., Dedenharbt, J., Stenzler, L.M., Howell, S.H. and Kochian, L.V. (1998) Aluminum-resistant *Arabidopsis* mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. *Plant Physiol.* 117: 9–17.

Lipton, D.S., Blanchar, R.W. and Blevins, D.G. (1987) Citrate, malate and succinate concentrations in exudate from P-sufficient and P-stressed *Medicago sativa* L. seedlings. *Plant Physiol.* 85: 315–317.

Ma, J.F., Zheng, S.J. and Matsumoto, H. (1997) Specific secretion of citric acid induced by Al stress in *Cassia taro* L. *Plant Cell Physiol.* 38: 1019–1025.

Mimura, T., Dietz, K.J., Kaiser, W., Schramm, M., Kaiser, J. and Heber, U. (1990) Phosphate transport across bio-membranes and cytosolic phosphate homeostasis in barley leaves. *Planta* 180: 139–146.

Mitsukawa, N., Okumura, S., Shirano, Y., Sato, S., Kato, T., Harashima, S. and Shibata, D. (1997) Overexpression of an *Arabidopsis thaliana* high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limitted conditions. *Proc. Natl. Acad. Sci. USA* 94: 7098–7102.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007–4021.

Ohira, K., Ojima, K. and Fujiwara, A. (1973) Studies on the nutrition of rice cell culture. I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol.* 14: 1113–1121.

Ohta, S., Mita, S., Hattori, T. and Nakamura, K. (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron whitin the coding sequence. *Plant Cell Physiol.* 31: 805–813.

Oono, K. (1984) Genetic variability in cultured cells and their use for breeding. *Plant Tissue Cul. Lett.* 1: 2–7.

Ryan, P.R., Skerret, M., Findlay, G.P., Delheize, E. and Tyerman, S.D. (1997) Aluminum activates anion channel in the apical cells of wheat roots. *Proc. Natl. Acad. USA* 94: 6547–6552.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitor. *Proc. Natl. Acad. Sci. USA* 74: 5463–5467.

Shirzadegan, M., Christie, P. and Seemann, J.R. (1991) An efficient method for isolation of RNA from tissue cultured plant cells. *Nucl. Acids Res.* 19: 6055.

Srere, P.A. (1967) Citrate synthase. *Methods Enzymol.* 13: 3–11.

Tadano, T., Ozawa, K., Sasaki, H., Osaki, M. and Matsui, H. (1993) Secretion of acid phosphatase by the roots of crop plants under phosphorous-deficient conditions and some properties of the enzyme secreted by lupin roots. *Plant Soil.* 155/156: 95–98.

Takita, E., Koyama, H. and Hara, T. (1999) Organic acid metabolism in aluminum-phosphate utilizing cells of carrot (*Daucus carota* L.). *Plant Cell Physiol.* 40: 489–495.

Unger, E., A., Hand, J.M., Cashmore, A.R. and Vasconcelos, A.C. (1989) Isolation of a cDNA encoding mitochondrial citrate synthase from *Arabidopsis thaliana*. *Plant Mol. Biol.* 13: 411–418.

von Uexüll, H.R. and Mutert, E. (1995) Global extent, development and economic impact of acid soils. *Plant Soil.* 171: 1–15.

Wright, K.E. (1953) Aluminum toxicity studies with radioactive phosphorus. *Plant Physiol.* 28: 674–680.

(Received August 27, 1998; Accepted February 21, 1999)