

Modulation of Citrate Metabolism Alters Aluminum Tolerance in Yeast and Transgenic Canola Overexpressing a Mitochondrial Citrate Synthase¹

Valar M. Anoop*, Urmila Basu, Mark T. McCammon, Lee McAlister-Henn, and Gregory J. Taylor

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9 (V.M.A., U.B., G.J.T.); and Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas, 78229–3900 (M.T.M., L.M.-H.)

Aluminum (Al) toxicity is a major constraint for crop production in acid soils, although crop cultivars vary in their tolerance to Al. We have investigated the potential role of citrate in mediating Al tolerance in Al-sensitive yeast (*Saccharomyces cerevisiae*; MMYO11) and canola (*Brassica napus* cv Westar). Yeast disruption mutants defective in genes encoding tricarboxylic acid cycle enzymes, both upstream (citrate synthase [CS]) and downstream (aconitase [ACO] and isocitrate dehydrogenase [IDH]) of citrate, showed altered levels of Al tolerance. A triple mutant of CS ($\Delta cit123$) showed lower levels of citrate accumulation and reduced Al tolerance, whereas $\Delta aco1$ - and $\Delta idh12$ -deficient mutants showed higher accumulation of citrate and increased levels of Al tolerance. Overexpression of a mitochondrial CS (*CIT1*) in MMYO11 resulted in a 2- to 3-fold increase in citrate levels, and the transformants showed enhanced Al tolerance. A gene for Arabidopsis mitochondrial CS was overexpressed in canola using an *Agrobacterium tumefaciens*-mediated system. Increased levels of CS gene expression and enhanced CS activity were observed in transgenic lines compared with the wild type. Root growth experiments revealed that transgenic lines have enhanced levels of Al tolerance. The transgenic lines showed enhanced levels of cellular shoot citrate and a 2-fold increase in citrate exudation when exposed to 150 μM Al. Our work with yeast and transgenic canola clearly suggest that modulation of different enzymes involved in citrate synthesis and turnover (malate dehydrogenase, CS, ACO, and IDH) could be considered as potential targets of gene manipulation to understand the role of citrate metabolism in mediating Al tolerance.

Aluminum toxicity is one of the major factors limiting crop productivity in acid soils. The root apex is considered the primary site of Al-induced injury, and inhibition of root elongation is one of the most visible symptoms of Al toxicity. Although most plants are sensitive to Al, several crop species exhibit genetic variation in their ability to tolerate Al. One possible 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 low M_r organic anions such as citrate, malate, or oxalate has been reported in several crop 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 (malate²⁻) was observed in near isogenic, Al-tolerant wheat (*Triticum aestivum*) lines (Delhaize et al., 1993), several lines of evidence sug-

gest that a lag phase may also exist between exposure of roots to Al and excretion of organic anions. Citrate exudation from roots of rye was observed only after 120 h of exposure (Li et al., 2000). Similarly, an Al-induced, de novo synthesis of malate leading to enhanced malate efflux 24 h after exposure was demonstrated in Al-tolerant wheat cv Katepwa (Basu et al., 1994). Delayed exudation of organic anions could possibly be due to alteration of cellular metabolism leading to biosynthesis and release of anions.

Among the various organic acids, citrate is most commonly cited to be involved in ameliorating the toxic effects of Al. Accumulation and/or efflux of citrate can be enhanced by increased citrate production or by reduced citrate catabolism (Neumann et al., 2000). Enhanced citrate synthesis could be achieved by increasing the activities of enzymes involved in citrate synthesis such as citrate synthase (CS), malate dehydrogenase (MDH), and phosphoenolpyruvate carboxylase. Citrate turnover could be reduced by decreasing the activities of enzymes involved in breakdown of citrate such as aconitase (ACO) and isocitrate dehydrogenase (IDH; Fig. 1).

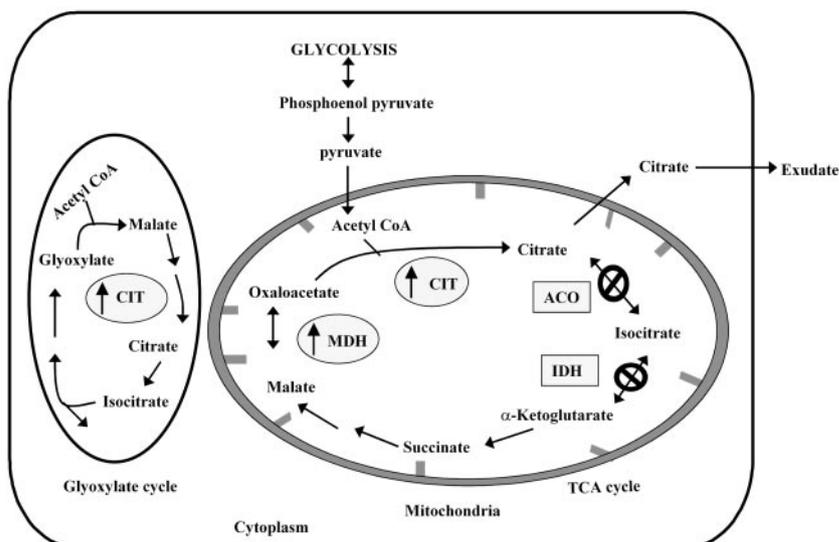
CS is a key enzyme involved in condensation of oxaloacetate (OAA) and acetyl CoA to produce citrate. This biochemical reaction plays an important

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* Corresponding author; e-mail mv@ualberta.ca; fax 780-492-9234.

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Figure 1. Metabolic map of TCA and glyoxylate cycles, indicating positions of genes that could be modulated for altering citrate metabolism. Up-regulation of genes involved in citrate synthesis (\uparrow) and down-regulation of genes involved in citrate catabolism (\circ) would enhance citrate synthesis and/or its accumulation in cells.



role in the Krebs cycle, in β -oxidation of fatty acids, and also in photo-respiratory glyoxylate pathways. As an important intermediate in various biochemical reactions, such as amino acid synthesis and fatty acid synthesis, citrate biosynthesis and catabolism are strictly regulated (Ryan and Delhaize, 2001). It is therefore important to undertake a holistic approach that considers enzymes and metabolites both upstream and downstream of citrate, while attempting to engineer citrate metabolism.

This study was conducted to investigate the role of citrate metabolism in mediating Al tolerance in both yeast and plant model systems. Yeast (*Saccharomyces cerevisiae*) is an excellent system for studies on metal toxicity and resistance (MacDiarmid and Gardner, 1998). Genes involved in Al tolerance (*HSP150*, *SED1*, and *ALU1-P*) have been identified in yeast (Jo et al., 1997; Ezaki et al., 1998), and the functions of several plant genes (*BCB*, *NtGDI1*, and *ATPase*) have been elucidated by complementation of yeast mutants (Ezaki et al., 1999; Hamilton et al., 2001). The availability of disruption mutants defective in various tricarboxylic acid (TCA) and glyoxylate cycle genes

(McCammon, 1996; Przybyla-Zawislak et al., 1999) prompted us to use yeast to test the role of specific genes and gene products in citrate metabolism and Al tolerance (Table I; Fig. 1). The function of TCA cycle genes appears to be conserved between yeast and plants. For example, the genes for different subunits of tobacco (*Nicotiana tabacum*) NAD-dependent IDH were sufficiently conserved to complement yeast IDH mutant (Lancien et al., 1998). Thus, yeast TCA cycle mutants should be a useful model system for understanding citrate metabolism in Al tolerance.

In addition, we also tested the hypothesis that increased synthesis and accumulation of citrate can mediate Al tolerance in plants using a transgenic approach. We used an important oilseed crop, canola (*Brassica napus* cv Westar), because the cultivars of this species are sensitive to acid soils and Al toxicity (Clune and Copeland, 1999). The potential use of an overexpression strategy to regulate citrate synthesis and mediate Al tolerance was first suggested by de la Fuente et al. (1997). They demonstrated that overexpression of CS from *Pseudomonas aeruginosa* in the cytoplasm of tobacco and *Papaya* sp. led to enhanced

Table I. Yeast strains defective in various TCA and glyoxylate cycle genes used in this study are listed below

The genetic background of all mutants listed below is same as the WT, MMYO11 (Przybyla-Zawislak et al., 1999).

Strain	Genotype	Properties
MMYO11	<i>Matα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1</i>	Wild-type parental strain
Δ <i>cit1</i>	<i>cit1::LEU2</i>	Defective in mitochondrial citrate synthase <i>CIT1</i>
Δ <i>cit2</i>	<i>cit2::URA3</i>	Defective in glyoxysomal citrate synthase <i>CIT2</i>
Δ <i>cit3</i>	<i>cit3::URA3</i>	Defective in mitochondrial citrate synthase <i>CIT3</i>
Δ <i>cit12</i>	<i>cit1::LEU2 cit2::URA3</i>	Defective in <i>CIT1</i> and <i>CIT2</i>
Δ <i>cit13</i>	<i>cit1::LEU2 cit1::URA3</i>	Defective in <i>CIT1</i> and <i>CIT3</i>
Δ <i>cit23</i>	<i>cit2::URA3 cit3::URA3</i>	Defective in <i>CIT2</i> and <i>CIT3</i>
Δ <i>cit123</i>	<i>cit1::LEU2 cit2::URA3 cit3::URA3</i>	Defective in <i>CIT1</i> , <i>CIT2</i> , and <i>CIT3</i>
Δ <i>aco1</i>	<i>aco1::URA3</i>	Defective in mitochondrial aconitase <i>ACO1</i>
Δ <i>idh12</i>	<i>idh1::URA3 idh2::HIS3</i>	Defective in subunits 1 and 2 of mitochondrial isocitrate dehydrogenase, <i>IDH1</i> and <i>IDH2</i>

citrate efflux from roots and thereby enhanced Al tolerance in transgenic lines. In contrast, Delhaize et al. (2001) reported that overexpression of the same *P. aeruginosa* CS in the cytosol of tobacco neither resulted in enhanced exudation of citrate nor increased Al tolerance. Koyama et al. (1999, 2000) reported similar results to those of de la Fuente et al. (1997), where overexpression of an Arabidopsis mitochondrial CS (At-mtCS) in carrot (*Daucus carota*) cell lines and a carrot mitochondrial CS in Arabidopsis lead to increased Al tolerance. Despite a growing body of literature on this subject, a clear relationship between overexpression of CS and Al tolerance is not well established. In eukaryotes, two isoforms of CS (glyoxysomal and mitochondrial) are observed. Adopting an overexpression strategy targeting CS to the mitochondria would be an ideal approach to increase citrate biosynthesis in plants, because factors influencing citrate synthesis and accumulation would be potentially well regulated in mitochondria. Hence, we overexpressed an At-mtCS in canola and evaluated the transgenic lines for levels of CS activity, citrate exudation from roots, and their performance under Al-toxic conditions.

RESULTS

Effect of Al on Growth of Wild-Type (WT) Yeast and Induction of Genes Involved in Citrate Metabolism

A progressive reduction in growth was observed in WT yeast when exposed to increasing concentrations of Al, with a 50% inhibition at 400 μM Al (Fig. 2A). Northern analysis of WT yeast exposed to Al (0–400 μM) showed that the transcript level of *CIT1* in-

creased by 40% to 50% from 100 to 300 μM Al, and no significant increase in transcript abundance was observed at 400 μM Al. Transcript abundance for *ACO1* increased by 430% to 477% of control at 100 to 400 μM Al, whereas the *IDH1* transcript level was down-regulated to 60% of control by increasing concentrations of Al (Fig. 2, B and C).

Cellular and Extracellular Citrate Content in WT and Disruption Mutants

Cellular (Fig. 3A) and extracellular (Fig. 3B) citrate contents were estimated in different yeast mutants with altered citrate metabolism. These include strains with all possible combinations of single, double, and triple mutations in three genes, *CIT1*, *CIT2*, and *CIT3*, encoding distinct CS isozymes. Two of the isozymes (CS1 and CS3) are mitochondrial, whereas the other isozyme (CS2) functions in the peroxisome. In addition, strains lacking the next two Krebs cycle isozymes were also tested. The Δaco1 strain lacked ACO1, whereas the Δidh12 strain lacked both subunits of the NAD⁺-dependent IDH. Single mutants of CS (Δcit1 , Δcit2 , and Δcit3) showed no significant reduction in cellular citrate content compared with the WT ($8.50 \pm 0.65 \text{ nmol } 10^8 \text{ cells}^{-1} \text{ mL}^{-1}$), whereas the double and triple mutants (Δcit12 , Δcit13 , Δcit23 , and Δcit123) showed a significant reduction (2-fold) compared with the WT yeast. Interestingly, the mutants disrupted for the genes downstream of citrate (Δaco1 and Δidh12) showed a significant increase (3- to 6-fold) in citrate levels compared with WT.

No significant reduction in citrate efflux from cells of single mutants of CS was observed, whereas ci-

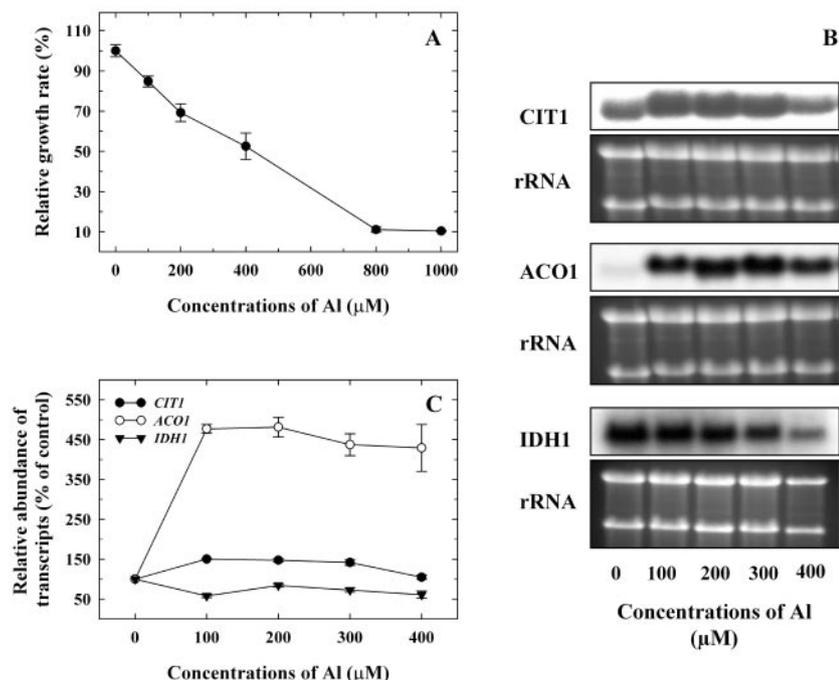


Figure 2. The effect of Al (0–1,000 μM) on WT yeast (MMYO11). A, Reduction in growth of WT yeast grown in LPP medium for 20 h, with different concentrations of Al. Vertical bars represent SE ($n = 3$). B, Transcript levels of *CIT1*, *ACO1*, and *IDH1* in WT yeast. Total RNA isolated from yeast cells exposed to different concentrations of Al for 20 h was probed with ³²P-labeled fragments of *CIT1*, *ACO1*, and *IDH1*. C, Relative abundance of *CIT1*, *ACO1*, and *IDH1* transcripts based on densitometry quantification of the RNA.

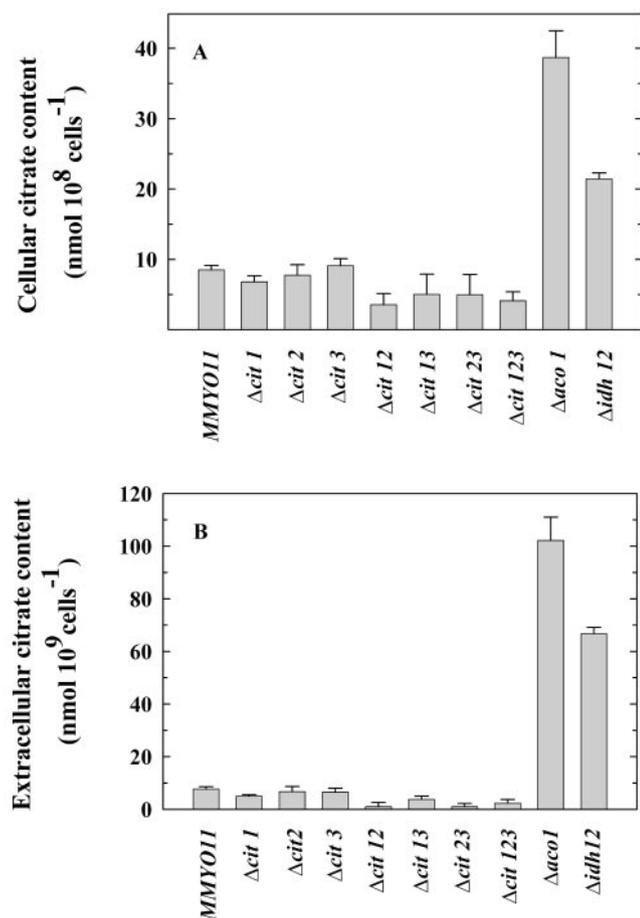


Figure 3. Cellular (A) and extracellular (B) citrate content in WT yeast and disruption mutants with altered citrate metabolism. Cultures of different genotypes grown in LPP medium for 20 h were used for estimation of citrate. Vertical bars represent SE ($n = 3$).

trate exuded from double and triple mutants of CS was reduced 2-fold compared with the WT yeast ($7.7 \pm 0.9 \text{ nmol } 10^9 \text{ cells}^{-1} \text{ mL}^{-1}$). Citrate released from cells of $\Delta aco1$ and $\Delta idh12$ was significantly higher (7- to 10-fold) compared with WT.

Effect of Al on Disruption Mutants of Yeast

When yeast cells were challenged with Al, the single mutants ($\Delta cit1$, $\Delta cit2$, and $\Delta cit3$) did not show any significant difference in their growth compared with WT yeast (Fig. 4A). Likewise, the double mutants, $\Delta cit12$, $\Delta cit13$ did not show any difference in their growth response compared with WT (Fig. 4B), whereas $\Delta cit23$ and the triple mutant of CS, $\Delta cit123$, showed increased sensitivity at 200 and 400 μM Al. At 400 μM Al, growth of $\Delta cit123$ was reduced by 67%, whereas the WT showed a reduction by 43% (Fig. 4C). The $\Delta aco1$ and $\Delta idh12$ mutants, which had higher levels of cellular and extracellular citrate accumulation, showed reduced sensitivity to Al. The mutant disrupted for ACO1 showed a 30% better growth, whereas $\Delta idh12$ mutant showed a 19% better growth

compared with WT yeast, at different concentrations of Al (Fig. 4C).

Effect of Al on Growth of CIT1-Overexpressing Transformants and Their Citrate Levels

The gene encoding the major mitochondrial isoform of CS, *CIT1*, was overexpressed alone and with mitochondrial MDH gene, *MDH1*, in WT yeast. MDH is the enzyme involved in synthesizing OAA from malate. We therefore postulated that overexpression

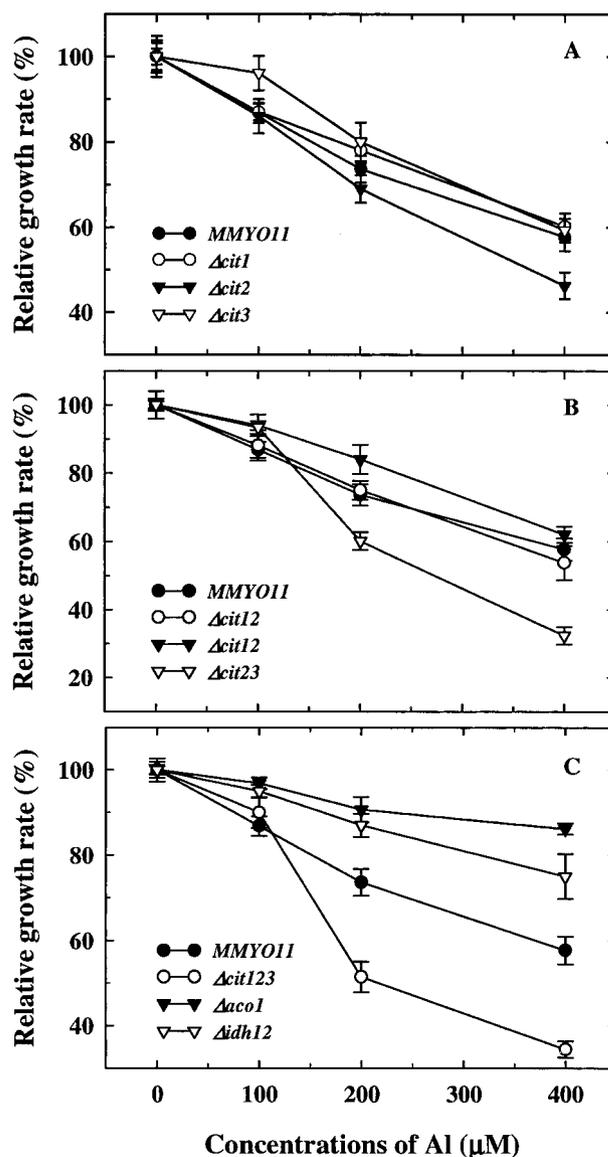


Figure 4. Effect of Al on relative growth rate (percentage of control) of WT and disruption mutants with altered citrate metabolism. A, Single CS mutants ($\Delta cit1$, $\Delta cit2$, and $\Delta cit3$). B, Double CS mutants ($\Delta cit12$, $\Delta cit13$, and $\Delta cit23$). C, Triple mutant of CS, and ACO- and IDH-deficient mutants ($\Delta cit123$, $\Delta aco1$, and $\Delta idh12$). Cells of the specific genotypes were grown in LPP medium with different concentrations of Al for 18 to 20 h at 30°C, and A_{600} was measured. Vertical bars represent SE ($n = 3$).

of MDH might supply additional OAA for citrate synthesis. Northern analysis of yeast transformants overexpressing *CIT1* (MMYO11/*CIT1*) and *CIT1* + *MDH1* (MMYO11/*CIT1* + *MDH1*) showed similar levels of increase in *CIT1* transcript (Fig. 5A). When challenged with 500 μM Al, the transformants MMYO11/*CIT1* and MMYO11/*CIT1* + *MDH1* showed significantly better growth (20% and 30%, respectively) compared with the WT and vector controls (Fig. 5B). Also, a significant increase (2.5- to 3-fold) in both cellular and extracellular citrate levels was observed in MMYO11/*CIT1* and MMYO11/*CIT1* + *MDH1* transformants relative to the WT (Fig. 6, A and B). Interestingly, citrate levels were not higher with simultaneous overexpression of *CIT1*/*MDH1* as in MMYO11/*CIT1* + *MDH1* compared with MMYO11/*CIT1*.

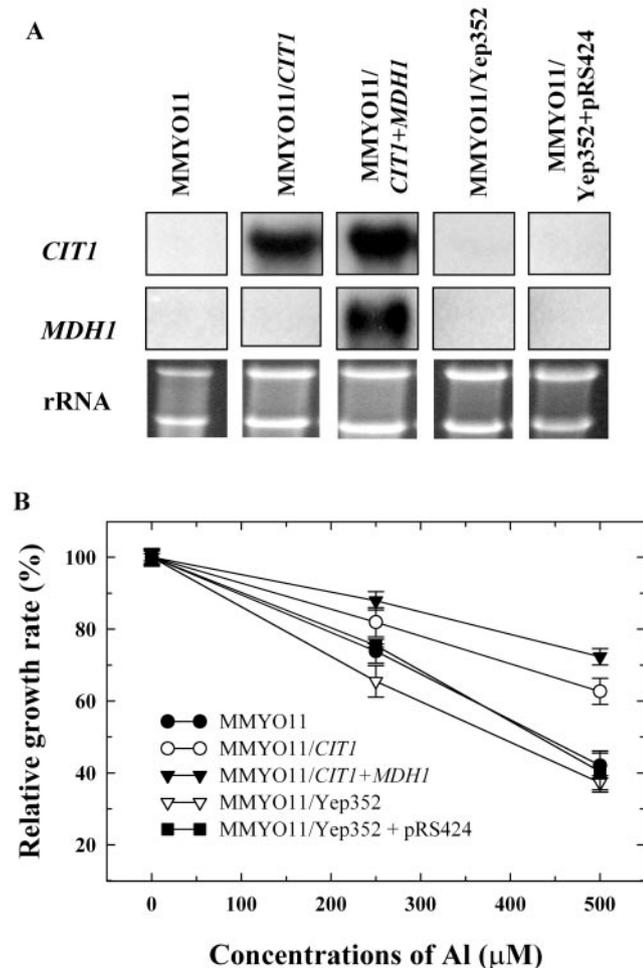


Figure 5. Overexpression of mitochondrial CS, *CIT1* (MMYO11/*CIT1*), and MDH, *MDH1* (MMYO11/*CIT1* + *MDH1*), in WT yeast. A, Northern-blot analysis of total RNA isolated from WT and yeast transformants overexpressing *CIT1* and *CIT1* + *MDH1* and vector plasmids, Yep352 and Yep352 + pRS424 (probed with radiolabeled ^{32}P -labeled *CIT1* and *MDH1* fragments). B, Effect of Al on growth of WT yeast and yeast transformants. Vertical bars represent SE ($n = 3$).

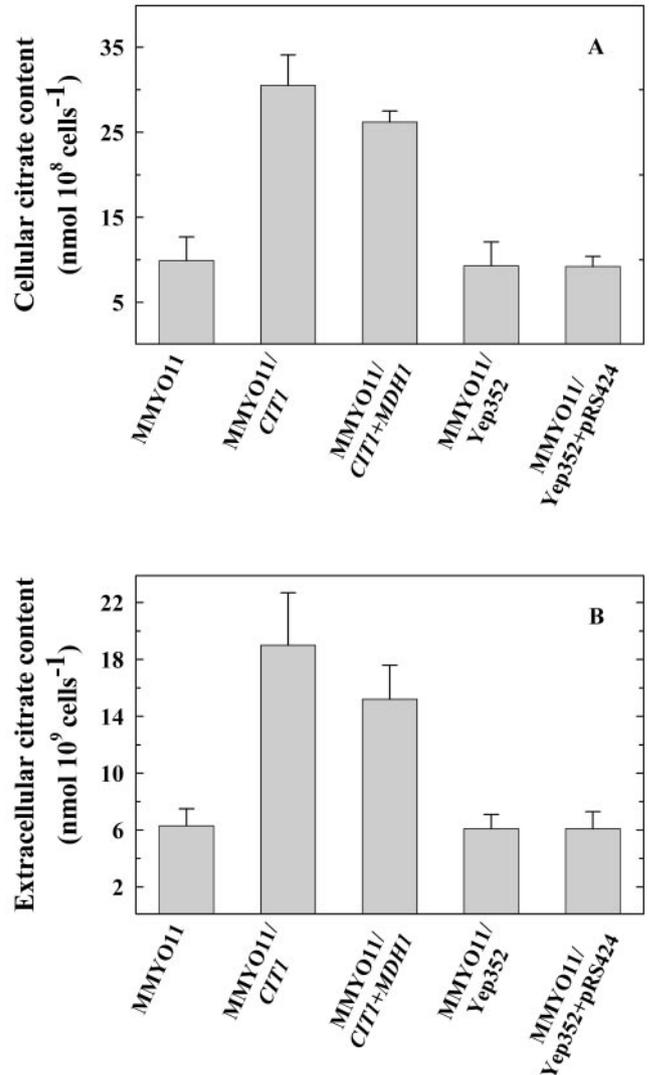
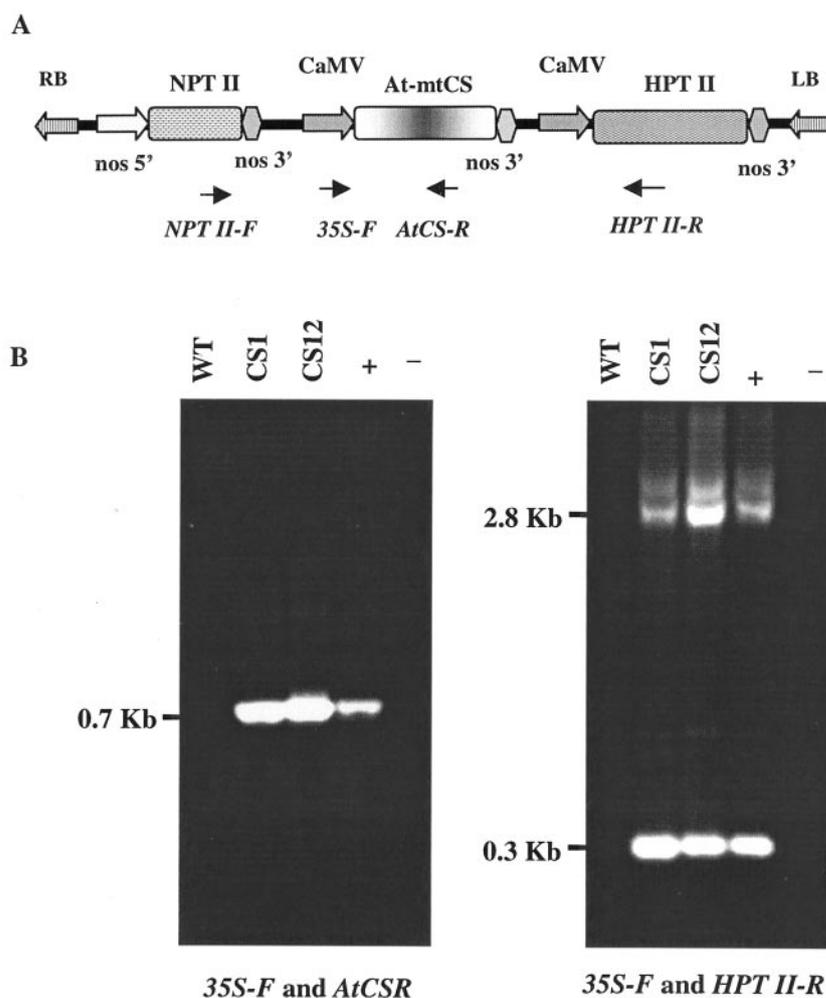


Figure 6. Cellular (A) and extracellular (B) citrate content in WT yeast and yeast transformants overexpressing *CIT1*, *CIT1* + *MDH1*, and vector plasmids, Yep352 and Yep352 + pRS424. Cultures of different genotypes grown in LPP medium for 20 h at 30°C were used for estimation of citrate. Vertical bars represent SE ($n = 3$).

Development of Transgenic Plants Overexpressing an At-mtCS

Transformation of canola cv Westar with pACS121-Hm (Fig. 7A) using an *Agrobacterium tumefaciens*-mediated system yielded 12 independent transgenic lines. The presence of the transgene, At-mtCS, was confirmed in four of these lines using genomic PCR analysis (primer positions used in genomic PCR are indicated in Fig. 7A). Amplification of an expected approximately 0.7-kb fragment was observed in transgenic lines and positive control when amplified with cauliflower mosaic virus (CaMV)-F and AtCS-R primers, but not in the WT and negative controls. When amplified with CaMV-F and HPTII-R primers, two bands of expected sizes (approximately 0.3 and 2.8 kb) were observed in transgenic lines and

Figure 7. Construction of transgenic plants overexpressing CS. Details of the construct (A), genomic DNA PCR analysis (B) used in transformation of canola with At-mtCS. A, Diagrammatic representation of pACS121-Hm. The gene, At-mtCS, is expressed under the control of a constitutive 35S promoter. The construct has selectable markers for NPT II and HPT II genes. The two primer pairs used in genomic DNA PCR amplification are indicated as arrows in the construct. B, Genomic DNA PCR analysis of transformants confirming the presence of At-mtCS transgene. Genomic DNA was isolated from WT and two independent transgenic lines (CS1 and CS12) and was used as template for PCR with two primer pairs indicated above. 35S-F/AtCS-R, Expected amplification size of 0.7 kb; 35S/HPT II-R, expected amplification sizes of 0.3 and 2.8 kb. Positive (+) control includes plasmid DNA (pACS121-Hm), and negative (-) control includes PCR reactions with no DNA.



the positive control. Transgenic lines CS1 and CS12 were fertile and were raised to obtain homozygous seeds for further studies (Fig. 7B).

Northern Analysis and CS Activity

Transgenic lines were analyzed for enhanced levels of the CS transcript (Fig. 8A) and presence of the HPT II transcript by northern analysis. The transgenic lines showed up to a 2-fold increase in accumulation of the mt-CS transcript (approximately 1.6 kb) compared with the WT. The At-mtCS probe hybridizes to the mt-CS of both Arabidopsis and canola. Due to the short exposure time used (2 h), the endogenous CS transcript in the WT was only visible as a faint band, whereas the band intensity was stronger in CS1 and CS12 for the same exposure time. When the same membrane was stripped and reprobed with HPT II, the presence of HPT II transcript (1.8 kb) was observed only in transgenic lines (data not shown). The enhanced level of CS expression was also confirmed in the roots and shoots of T2 plants and several of T2 progenies, with At-mtCS probe in T1 and homozygous T2 generation (data not shown).

CS enzyme activity was measured in the WT and transgenic lines (Fig. 8B). Transgenic lines CS1 and CS12 showed a significant (2.5- to 4-fold) increase in CS enzyme activity (1.8 ± 0.005 and $3.2 \pm 0.1 \mu\text{mol CoA used min}^{-1} \text{mg}^{-1}$ protein respectively) compared with the WT ($0.8 \pm 0.003 \mu\text{mol CoA used min}^{-1} \text{mg}^{-1}$ protein).

Enhanced Citrate Efflux and Increased Resistance of Transgenic Canola to Al

When exposed to toxic concentrations of Al, transgenic lines CS1 and CS12 showed better performance in the presence of Al (Fig. 9A). At $50 \mu\text{M}$, a stimulatory effect of Al on root elongation was observed in CS1 and CS12 lines, whereas a growth reduction of 10% was observed in WT. At higher concentrations of Al, root elongation was greater in transgenic lines than in the WT. At $100 \mu\text{M}$ Al, no significant change in root elongation was observed in the transgenic lines compared with their controls ($0 \mu\text{M}$ Al), whereas the WT showed a reduction of 9% in relative root growth. Larger differences in root elongation were observed at 150 and $200 \mu\text{M}$ Al. At $150 \mu\text{M}$ Al,

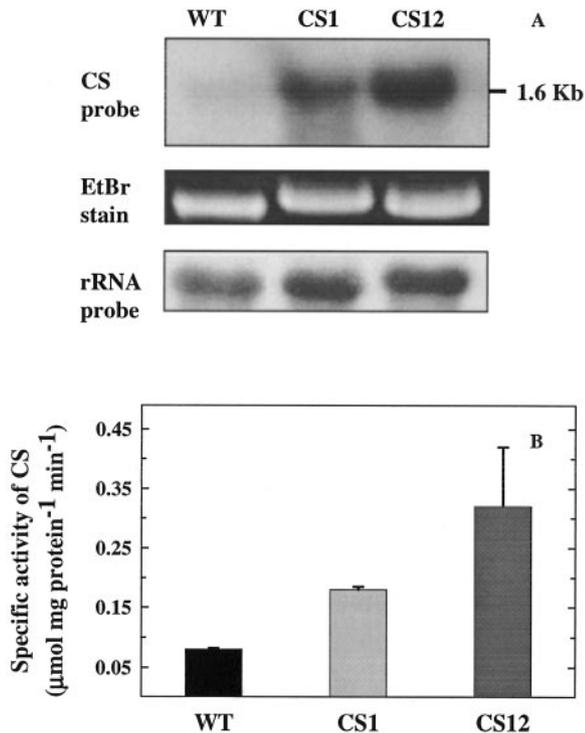


Figure 8. Analysis of transcript (A) and enzyme levels (B) in WT and transgenic lines. A, Northern analysis of total RNA isolated from young leaves of WT, CS1, and CS12. RNA blots were hybridized with [32 P]dCTP labeled CS probe (1.6 kb). Ethidium bromide staining (EtBr) and rRNA probe are included as a loading control. B, CS enzyme activity was measured in young leaves of WT and transgenic lines. Vertical bars represent SE ($n = 3$).

root growth was reduced by 50% in WT, whereas CS1 and CS12 lines showed a reduction of 17% and 20%, respectively. At 200 μM Al, the transgenic lines showed a root growth inhibition of 65% to 70% their controls (0 μM Al), whereas the root growth of WT was reduced by 85% compared with its control.

Because the transgenic lines had the *Atmt-CS* expressed under control of the *CaMV* promoter, we expected higher levels of cellular citrate content and citrate exudation from roots of CS1 and CS12 under control conditions. But the amount of citrate (shoots and root exudates) in these lines was not significantly different from that of the WT at 0 μM Al (Fig. 9, B–D). The root citrate level was not different between WT and transgenic lines at 150 μM Al, although a significant difference was observed between WT and CS12 at 0 μM Al (Fig. 9B). However, when exposed to 150 μM Al, the citrate content of shoots was significantly increased (1.7- to 1.9-fold) in CS1 and CS12 compared with the WT ($3.5 \pm 0.7 \mu\text{mol mg}^{-1} \text{protein}$; Fig. 9C). The amount of citrate exuded from the roots of CS1 ($1.7 \pm 0.2 \mu\text{mol g}^{-1} \text{dry weight}$) and CS12 ($1.2 \pm 0.2 \mu\text{mol g}^{-1} \text{dry weight}$) was 1.7- to 2.4-fold higher than WT ($0.7 \pm 0.2 \mu\text{mol g}^{-1} \text{dry weight}$; Fig. 9D). These results indicate that overexpression of mitochondrial CS in canola results in an Al-induced in-

crease in exudation of citrate levels and Al tolerance in the transgenic lines.

DISCUSSION

Several lines of evidence suggest that Al has a strong effect on the TCA cycle and glycolytic pathway. Modulation in activities of several enzymes involved in synthesis and catabolism of citrate has been reported in yeast and plants (Hoffland et al., 1992; Neumann et al., 2000; Zatta et al., 2000; Neumann and Martinoia, 2002). In our studies, exposure of yeast, MMYO11 to Al resulted in induction of *CIT1* and *ACO1* and down-regulation of *IDH1* transcript levels (Fig. 2, B and C). Metabolic engineering of biochemical pathways involved in organic acid metabolism would provide us an opportunity to understand the role of these pathways in Al tolerance.

Genetic manipulation is relatively easy in yeast due to the availability of mutants, ease of handling, and short experimental periods. Our data from yeast mutants with altered citrate metabolism (defective in TCA and glyoxylate cycles genes) revealed that accumulation of citrate in cellular and extracellular pools can mediate Al tolerance. When measuring metabolite levels in yeast, it is important to measure both cellular and extracellular levels, because these metabolites are likely to be transported out of their site of synthesis and exuded out of cells. Citrate is efficiently transported out of mitochondria by tricarboxylate carrier proteins (Barbier-Brygoo et al., 2000) and excess citrate in the cytoplasm is generally released out of cells to prevent cytoplasmic acidosis (Massonneau et al., 2001).

In the present study, cellular and extracellular citrate levels were not significantly different in single mutants (Δcit1 , Δcit2 , and Δcit3) compared with WT. These mutants were not hypersensitive to Al, based on their growth performance compared with the WT. Cellular and extracellular levels of citrate were also unaffected. This could reflect the compensatory effects from other isoforms of CS. For instance, *CIT2* mRNA levels were increased by 6- to 10-fold in *CIT1*-deficient mutant (Liao et al., 1991). Moreover, there could be alterations in levels of other metabolites in these single mutants (such as malate or succinate) that could have contributed to alleviation of Al toxicity. For example, when Kispal et al. (1988) inactivated a mitochondrial isoform of CS, increased levels of both citrate and malate were observed. In our experiment, the double mutants of CS (Δcit12 , Δcit13 , and Δcit23) showed significant reductions in their citrate content (cellular and extracellular) compared with the WT. However, no differences were observed in the growth of Δcit12 and Δcit13 , whereas Δcit23 showed increased sensitivity to Al. The reason for the discrepancy in the citrate content and Al sensitivity in the double mutants could not be explained from

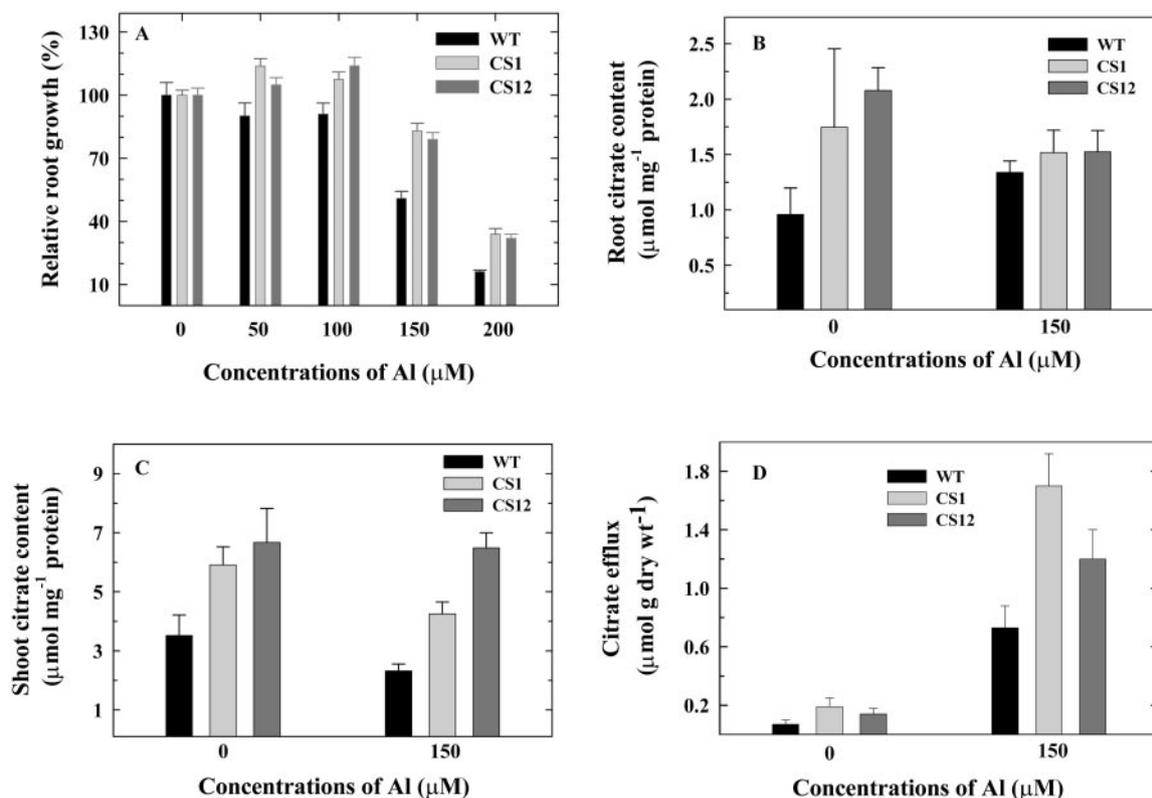


Figure 9. The effect of Al on root growth (A), cellular citrate content in roots (B) and shoots (C), and citrate efflux (D) on WT and transgenic lines overexpressing At-mtCS. A, Relative root growth (percentage of 0 μM Al) of WT and transgenic lines exposed to Al for 48 h ($n = 10$). B, Citrate content in root tissues of WT and transgenic lines, CS1 and CS12. C, Citrate levels in shoots of WT and transgenic lines. Root and shoot tissues from WT and homozygous seedlings of transgenic lines were collected and used in citrate estimation ($n = 3$). D, Root exudates from seedlings of WT, CS1, and CS12 were analyzed for levels of citrate released in the presence (150 μM Al) or absence of Al. Vertical bars represent SE. ($n = 3$).

our data. Mutants defective in CS are affected in the transport of several organic anions (citrate, isocitrate, and malate) across mitochondrial membranes (Sandor et al., 1994). Hence, alterations in metabolism involving internal compartmentation and transport of these organic anions across membranes could potentially affect the sensitivity of these mutants to Al. The triple mutant, Δcit123 , showed a significant reduction in both intracellular and extracellular citrate levels, and also showed reduced growth in the presence of Al compared with WT.

Increased accumulation of citrate can also be mediated by reduced activity of enzymes involved in citrate turnover (Massonneau et al., 2001). In our study, Δaco1 and Δidh12 mutants accumulated higher levels of citrate (cellular and extracellular) and showed improved Al resistance. These mutants showed only a 13% and 24% reduction in growth respectively, at 400 μM Al, whereas the WT showed a 43% reduction in growth at the same Al concentration (400 μM Al). Previous evidence suggested that an Al-induced reduction in levels of ACO leads to increased exudation of citrate in Al-tolerant maize (*Zea mays* cv Cateto-Columbia; Pineros et al., 2002) and from lupin roots under phosphate-deficient con-

ditions (Neumann et al., 1999). Reduction of IDH activities in mutant carrot cell lines capable of growing under phosphate deficiency also showed enhanced citrate exudation (Takita et al., 1999; Kihara et al., 2003). It would be interesting to investigate what other organic anions accumulate in Δaco1 and Δidh12 mutants that might contribute to improved Al resistance observed in our studies.

Results from our yeast overexpression studies demonstrated that cellular and extracellular citrate levels can be enhanced in yeast by overexpressing a mitochondrial *CIT1* either alone or with a mitochondrial *MDH1*. The transformants MMYO11/*CIT1* and MMYO11/*CIT1* + *MDH1* showed a 2- to 3-fold increase in citrate content and reduced Al sensitivity compared with WT yeast. MMYO11/*CIT1* + *MDH1* was expected to synthesize and exude more citrate than MMYO11/*CIT1* (we postulated reduced substrate limitations). However, citrate contents in both transformants were not significantly different. The difference in sensitivity of MMYO11/*CIT1* + *MDH1* compared with MMYO11/*CIT1* again suggests that there could be other metabolites (perhaps malate) that attributed for the enhanced Al tolerance.

Enhanced levels of organic acid synthesis or efflux is often accompanied by reduced growth under control conditions or requires specific culture conditions for enhanced synthesis and exudation to occur (Ryan and Delhaize, 2001). This could possibly be due to energy constraints caused by loss of carbon from the cells that exude organic anions (Taylor, 1991). It is important to mention that neither the yeast disruption mutants (Δaco1 and Δidh12) nor the yeast-overexpressing transformants (MMYO11/*CIT1* and MMYO11/*CIT1* + *MDH1*) revealed any apparent growth defects when grown in low phosphate medium (LPP). Our investigation with yeast disruption mutants and yeast overexpressing a mitochondrial CS clearly illustrates the role of citrate in alleviating Al stress and indicates that altering metabolic pathways involved in organic acid synthesis could enhance citrate accumulation.

Changes in TCA cycle function have wide-ranging effects. Transcriptional profiling using microarray analysis of responses to TCA cycle dysfunction (McCammon et al., 2003) has revealed that the expression of more than 400 gene changes in response to defects in one of 15 yeast TCA cycle genes. Gene expression also changes in response to altered TCA cycle metabolites. Citrate and malate are two of the TCA cycle metabolites to which nuclear gene expression may be responding. Hence, these metabolites may be exerting physiological effects intracellularly as well as extracellularly. The yeast cultures in these experiments were grown under fermentative conditions (McCammon et al., 2003) in which the TCA cycle is not essential and the genes are repressed as much as 10-fold (DeRisi et al., 1997). It will be interesting to test how oxidative growth conditions, which induce expression of the TCA cycle enzymes, affect Al tolerance and cellular citrate levels.

We also overexpressed a mitochondrial CS from *Arabidopsis* (At-mtCS) in canola cv Westar. Between the two isoforms of CS in eukaryotes (glyoxysomal and mitochondrial), modulating the mitochondrial isoform of CS might be a better strategy to test whether enhanced synthesis and exudation of citrate could be achieved by overexpressing CS. The mitochondria is the major site of function for CS in eukaryotes. Allosteric inhibition of CS by ATP would be low in mitochondria due to the low ATP to ADP ratio typically maintained in the organelle. Moreover, compartmentation of TCA cycle enzymes within the mitochondria not only ensures the availability of substrates but also physical proximity of substrates required for proper functioning of CS. A number of multi-enzyme complexes involving sequential enzymes termed "metabolons" exist in the TCA cycle, and their interactions are highly specific (Sumegi and Srere, 1984; Przybyla-Zawislak et al., 1999). Formation of such complexes affects the kinetics of enzymes involved in the complex (Datta et al., 1985). For example, in vitro studies on free enzymes

of mitochondrial MDH or CS showed 2-fold reduced activity compared with the enzyme complex, mt-CS/mt-MDH. The interaction of mt-CS was specific only to mt-MDH and not to cytosolic MDH (Igor and Srere, 1998). Cellular compartmentalization is therefore a key phenomenon to carry out orderly and controlled reactions.

In this study, transgenic canola overexpressing At-mtCS showed increased CS expression at the transcript level and enhanced CS activity, leading to an increase in levels of cellular (shoots) citrate and citrate exudation from roots of transgenic lines relative to WT. Interestingly, the increase in shoot citrate content and exudation of citrate were observed only upon exposure to 150 μM Al, even though At-mtCS was expressed under the control of a constitutive promoter (CaMV). It is not clear why the root citrate level in transgenic lines, CS1 and CS12 was not increased in the presence of Al. In Al-tolerant cultivars of soybean (*Glycine max*), significant difference was observed in the citrate levels of root tips and not in roots, when exposed to Al (Silva et al., 2001). Although CS activity in CS1 and CS12 was not significantly different, citrate exudation was significantly higher in CS1 than in CS12. Our data suggest that citrate produced in the transgenic lines are exuded out of the roots upon exposure to Al but do not indicate a clear quantitative relationship between citrate synthesis and citrate export from transgenic lines.

It is important to note that our transgenic lines did not have any difference in growth and development compared with WT. A reduction in mitochondrial CS activity (up to 30% of the WT levels) in transgenic potato resulted in a normal phenotype, but this reduction in CS activity was a limiting factor during flowering because the transgenic potato plants had deformed ovules (Landschütze et al., 1995).

The transgenic canola lines overexpressing mtCS showed reduced sensitivity to Al. At 50 and 100 μM Al, root elongation rates were higher in the transgenic lines compared with the control (0 μM Al). This could be due to alleviation of H^+ toxicity by Al as suggested by Clune and Copeland (1999). At higher concentrations of Al (150 and 200 μM), the transgenic lines showed a 20% to 25% better growth compared with the WT. Enhanced Al tolerance due to Al-induced increase in citrate exudation in transgenic canola clearly indicates that mitochondrial CS can play a major role in citrate metabolism and tolerance to Al.

Overexpression of a gene for CS from *P. aeruginosa* in cytoplasm of tobacco and *Papaya* sp. resulted in enhanced levels of cellular citrate and citrate exudation from roots of transgenic lines compared with the WT, with a concomitant increase in Al tolerance (de la Fuente et al., 1997) and phosphate acquisition (López-Bucio et al., 2000) by the transgenic lines. In contrast, Delhaize et al. (2001) generated transgenic

lines by overexpressing the same *P. aeruginosa* CS gene in the cytosol of tobacco. Their transgenic lines showed up to 100-fold enhanced CS protein levels but had no increase in enzyme activity. The transgenic lines did not exhibit enhanced levels of citrate and were not Al tolerant. Delhaize et al. (2001) suggested that very high expression levels of *P. aeruginosa* CS in their transgenic lines could have inactivated the transgenic proteins through incorrect folding of the bacterial protein or formation of inactive protein aggregates in plants. Transgenic wheat lines with higher levels of luciferase (*luc*) expression in a T1 generation showed significant reduction in *luc* expression in their subsequent generations, compared with the T1 lines with moderate levels of *luc* expression (Bourdonm et al., 2002).

Several other studies have also suggested that an overexpression strategy can be used to enhance the synthesis and exudation of organic anions. For instance, transgenic alfalfa (*Medicago sativa*) overexpressing a nodule-specific MDH showed enhanced exudation of organic anions from their roots and enhanced Al tolerance (Tesfaye et al., 2001). Koyama et al. (1999) indicated the importance of CS by overexpressing an Arabidopsis mtCS in carrot cells. In addition, Koyama et al. (2000) showed that transgenic Arabidopsis lines overexpressing a carrot mtCS showed 60% increase in citrate efflux, performed better under toxic concentrations of Al, and also showed enhanced ability to grow in P-limited soils.

Despite a number of published studies on the topic, a holistic approach to understand citrate metabolism in relation to Al tolerance has not yet been undertaken. Our work with yeast and transgenic canola overexpressing At-mtCS has enabled us to explore the complexity of citrate metabolism. Our results clearly demonstrate that CS represents only a part of the complex system and that genetic manipulation of several enzymes involved in citrate metabolism (such as MDH, CS, ACO, and IDH) can be potentially used to increase citrate synthesis and its accumulation in cells (Fig. 1). Citrate levels could be modulated by up-regulation of MDH and CS by an overexpression strategy or by down-regulation of ACO and IDH using an antisense approach (perhaps, with an inducible promoter). These approaches might be adopted to generate a significant improvement in Al tolerance in plants. Because cellular metabolism involves an array of enzymes that are interrelated and function in coordination with each other, metabolic profiling of these transformants could help us to further understand the coordinated synthesis of other organic anions.

MATERIALS AND METHODS

Yeast Strains and Media

Strains of yeast (*Saccharomyces cerevisiae*) used in this study are listed in Table I. Most strains harboring disruptions in different TCA cycle and

glyoxylate cycle genes were constructed previously (Przybyla-Zawislak et al., 1999). Double and triple mutant strains were constructed by crossing single mutant strains and screening random meiotic haploid products for the appropriate genotypes. Genotypes were confirmed by PCR using primers flanking the disruption sites, by phenotypic analysis, and by complementation analysis using marker strains (Przybyla-Zawislak et al., 1999). Yeast cultures were grown for 18 to 20 h at 30°C in yeast peptone dextrose medium (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] D-Glc). For experiments involving determination of Al response or estimation of citrate levels, cells were harvested from cultures by centrifugation at 3,000 rpm for 2 min, washed three times with sterile water, and suspended in 1 mL of sterile water. An initial OD₆₀₀ of 0.05 was used in all experiments.

Overexpression of CIT1 and CIT1/MDH1 in MMYO11

To construct yeast overexpressing *CIT1* and *MDH1*, strain MMYO11 was transformed with Yep352-CIT1 (Kispal et al., 1989) singly or in combination with pRS424-MDH1 (Small and McAlister-Henn, 1997) using the lithium acetate method (Chen et al., 1992). The *CIT1*-overexpressing transformants (MMYO11/*CIT1*) were selected on Uracil⁻ plates. The *CIT1* + *MDH1*-overexpressing transformants (MMYO11/*CIT1* + *MDH1*) were selected on Uracil⁻, Trp⁻ plates. Increase in *CIT1* and *MDH1* transcripts were confirmed by northern analysis using yeast [³²P]CTP-labeled *CIT1* and *MDH1* as probes.

Effect of Al on Growth of Mutants and Transformants

While testing the growth response of different yeast strains to Al, a low phosphate medium (LPP) was used (Schott and Gardner, 1997). This is a synthetic complete minimal medium made with 0.67% (w/v) yeast nitrogen base (without amino acids; without phosphate). In addition, K₂HPO₄ is reduced to 100 μM, KCl added to 4.5 mM, and the pH of the medium is reduced to 3.5 to 3.6 to ensure solubility of Al. Treatment cultures (2 mL of LPP medium with 0–400 μM Al) with an initial OD₆₀₀ of 0.05 were grown for 18 to 20 h at 30°C at 200 rpm in triplicates. The final OD₆₀₀ was measured using a plate reader (μQuant, Biotek Instruments, Winooski, VT). The relative growth rate of each strain was expressed as a percentage of control (0 μM Al).

Al-Induced Changes in Transcript Levels in Yeast

Total RNA was isolated from 5-mL yeast cultures grown with varying concentrations of Al (0–300 μM) for 20 h by an enzymatic method using the RNeasy mini kit (Qiagen, Ontario, Canada). Northern-blot analysis was done using [³²P]CTP-labeled *CIT1*, *ACO1*, and *IDH1* probes.

Estimation of Citrate Content

Yeast cells grown in 3 mL of LPP medium for 20 h were pelleted and washed three times with 3 mL of sterile water. The medium and the supernatant from each wash were pooled as the extracellular fraction. The cells were resuspended in 1 mL of sterile water, and cell density was determined by estimating OD₆₀₀. Cells were again pelleted, and the supernatant was pooled into the extracellular fraction. Cells were finally suspended in 1 mL of 80% (v/v) ethanol in 15-mL screw-capped tubes and vortexed vigorously to lyse the cells. The cell suspension was boiled at 80°C for 20 min with frequent vortexing to ensure complete lysis of cells and denaturation of proteins. The cell debris with denatured proteins was pelleted, the supernatant was passed through a 0.45-μm filter, and the filtrate was used in the estimation of citrate content in cells. To estimate citrate content in the extracellular medium, the pooled supernatant was concentrated (from 13 to 1 mL) using an Evap-o-Vac (Cole-Parmer, Ontario, Canada) placed inside an incubator at 50°C. Enzymatic analysis of citrate was conducted with 100 μL of either samples or standards. The reaction mix consisted of 100 mM Tris-Cl (pH 8.2), 0.2 mM ZnCl₂, 3 units mL⁻¹ MDH, and 10 mM NADH. A stable initial A₃₄₀ was noted, the reaction was started by the addition of citrate lyase (Sigma-Aldrich, St. Louis), and stable final A₃₄₀ was recorded (Tompkins and Toffaletti, 1982; Delhaize et al., 1993).

Plant Transformation, Growth Conditions, and Selection of Transformants

Agrobacterium tumefaciens strain EHA101 carrying a binary vector pACS121-Hm was kindly provided by Dr. Hiroyuki Koyama (Gifu University, Japan) and was used in the transformation of canola (*Brassica napus* cv Westar). This binary vector had a full-length cDNA for At-mtCS (1.6 kb) under the control of the CaMV promoter (Fig. 6A) and two selectable marker genes encoding kanamycin resistance (*NPT II*) and hygromycin resistance (*HPT II*; Koyama et al., 1999). Canola cv Westar seeds were surface-sterilized and germinated on seed germination medium (one-half strength Murashige and Skoog + 1% [w/v] Suc, pH 5.8) as described by Basu et al. (2001). Cotyledonary petioles from 4- to 5-d-old seedlings were used as explants, and *A. tumefaciens*-mediated transformation was carried out (Moloney et al., 1989). Overnight-grown cultures (in yeast extract peptone with 50 $\mu\text{g mL}^{-1}$ kanamycin + 25 $\mu\text{g mL}^{-1}$ hygromycin) of *A. tumefaciens* strain EHA 101 carrying pACS121-Hm were used to infect the explants. Explants were cocultivated with *A. tumefaciens* in M1 medium (Murashige and Skoog medium with 3% [w/v] Suc and 0.005% [w/v] benzyladenine) for 2 d in the dark. Explants were transferred to M2 medium (M1 + 300 $\mu\text{g mL}^{-1}$ timentin) for 1 week to recover and regenerate and then were transferred to M3 selection medium (M2 + 300 $\mu\text{g mL}^{-1}$ timentin and 30 $\mu\text{g mL}^{-1}$ kanamycin) for regeneration of calli to shoots. The putative transformants with green shoots were transferred to M4 medium (Murashige and Skoog medium with 0.005% [w/v] indole butyric acid, 300 $\mu\text{g mL}^{-1}$ timentin, and 30 $\mu\text{g mL}^{-1}$ kanamycin) for rooting. All tissue culture materials were maintained in a controlled chamber (16 h of light, 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 20°C/8 h of dark).

Presumptive positive plants (T_1) were transferred to soil (Metromix, Apache Seeds, Edmonton, Canada) and raised under controlled environmental conditions (16 h of light and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 20°C/8 h of dark at 18°C). The presence of the transgene was tested using PCR on genomic DNA isolated from 12 independent lines. Among the four positive lines, two of these T_1 plants were fertile. Thirty seeds from each of the T_1 selfed-lines were surface-sterilized and germinated on seed germination medium with 150 $\mu\text{g mL}^{-1}$ kanamycin. Seedlings (T_2 plants), which remained green on antibiotic selection, were again tested by genomic PCR. Segregation ratio was determined based on number of green to bleached yellow seedlings. From each of the primary transformants (T_1), 15 green T_2 seedlings were transferred to soil and screened to identify homozygous lines. To select for homozygous lines in the T_2 generation, about 20 to 30 seedlings from T_2 progenies were screened by genomic PCR for the presence of transgene.

Genomic DNA PCR and Northern Analysis

For genomic PCR and Southern analyses, genomic DNA was isolated from shoots or roots of canola using Qiagen DNeasy mini kit. For genomic PCR analysis, genomic DNA was used as template DNA, and the sequences of primers used for amplification were as follows: 35S-F, 5'-CCACTGACG-TAAGGGATGACG-3'; ATCS-R, 5'-AAGCCTCCAG ACTGGGCAGTA-3'; and HPT II-R 5'-GCCATCGGTCCAGACGGCC-3'.

RNA from shoots and roots were isolated using the Qiagen RNAeasy mini kit, separated by electrophoresis on agarose formaldehyde denaturing gels, and transferred to nitrocellulose membranes (Genescreen, NEN Research Products, Perkin Elmer Life Sciences Inc., Boston). Membranes were hybridized at 42°C overnight and washed under standard stringent conditions recommended by Genescreen. Hybridization probes were radioactively labeled with [^{32}P]CTP using Oligolabelling kit (Amersham Biosciences, Uppsala).

CS Enzyme Assay

Roots and shoots were collected, frozen in liquid nitrogen, and stored at -70°C until used for enzyme assays. Approximately 1 g of tissue 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 iodoacetamide, 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, Hercules, CA) 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).

Root Elongation Experiments

WT and transgenic lines (T_2 homozygous) were tested for their sensitivity to Al by the root growth elongation assay described by Basu et al. (2001). The seeds were germinated on a bed of wet sand. Seedlings (2 d old) were plated onto 1-mL graduated syringe tubes (with plungers removed and tops filled with cotton plug to support the seedlings). Syringes were mounted onto a plastic tray and floated in a 15-L aquarium filled with 10 L of full nutrient solution (FNS) at pH 4.2. Roots of seedlings grown over the syringes elongated in a straight line, enabling easy handling of the seedlings and accurate measurements of root lengths. After a day of recovery in FNS, syringes carrying the seedlings were transferred to FNS (pH 4.2) with or without Al. Seedlings were selected for uniform root length; initial root lengths were recorded and placed in 50 mL of treatment solution (FNS with or without Al) in a 50-mL disposable centrifuge tube. Seedlings were exposed to treatment solution for 48 h with constant shaking before the final root lengths were recorded.

Cellular Citrate Content in Shoot and Root Tissues

Shoot and root tissues (approximately 0.2 g) of WT and transgenic lines 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 13,000 rpm, and 100 μL of the supernatant was taken for protein estimation. The remaining portion of the samples was then vortexed and boiled at 80°C for 15 min. Samples were centrifuged at 13,000 rpm for 5 min, the supernatant was collected and passed through 0.45- μm filters, and 100 μL was used in citrate assay as described above (Delhaize et al., 1993).

Estimation of Extracellular Citrate from Root Exudates

Seeds of WT and transgenic lines were surface-sterilized and plated on seed germination medium. Two-day-old seedlings (13–15 seedlings per vessel, plated on a 14' mesh) were transferred to 75 mL of sterile FNS in magenta vessels (Basu et al., 1994). After a day in FNS, the mesh supporting the seedlings was then transferred to treatment solution (0 or 150 μM Al) under aseptic conditions and maintained for 2 d with constant shaking. The pH was checked every 8 h and was adjusted to 4.2 with 0.5 N HCl. Root exudates were collected after 2 d, and roots were harvested to obtain dry weight. The pH values of root exudates were adjusted to 2.0 with 0.5 N HCl to dissociate Al-citrate complexes and were passed through cation exchange columns with 1.5 mL of resin (Bio-Rad Laboratories). Samples were eluted with 20 mL of deionized water and concentrated to 1 mL. An aliquot of 100 μL of this sample was used for citrate assay as described earlier.

Experimental Design

All experiments included three to 10 independent replicates, and statistical analyses were performed using Sigmasat statistical analysis package (v1.0, Jandel Scientific, Chicago). Single- or two-factor ANOVA was performed, and a *P* value below 0.05 was considered statistically significant based on Student Newman-Keul's test or Dunnett's test. Experiments were repeated three to six times to ensure reproducibility of results.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

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