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Exploring the complexity of malate and citrate metabolism and their roles in aluminum tolerance in yeast and transgenic canola overexpressing a glyoxysomal malate synthase and a mitochondrial citrate synthase

by

Valar M Anoop ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

in

Plant Biology

Department of Biological Sciences

Edmonton, Alberta

Fall 2003

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ABSTRACT

Aluminum (Al) toxicity is a major constraint for crop production in acid soils. The potential role of malate and citrate in mediating Al resistance, and various genes involved in malate and citrate metabolism were investigated in Al-sensitive *Saccharomyces cerevisiae* (MMYO11) and *Brassica napus* (cv. Westar). No correlation between malate levels and Al sensitivity was observed in yeast mutants defective in various genes involved in malate metabolism (malate synthase, *MLS1*; isocitrate lyase, *ICL1;* and malate dehydrogenases, *MDH1, MDH2* and *MDH12*). In addition, transgenic canola lines overexpressing a glyoxysomal malate synthase (MS) gene showed enhanced MS transcript levels, but no increase in MS activity and malate content. This suggested that the complexity of malate metabolism and gene redundancy should be considered if gene manipulations are attempted in an effort to generate Al-resistant yeast or canola.

Yeast disruption mutants defective in genes encoding TCA cycle enzymes, both upstream (citrate synthase, CS) and downstream (aconitase, *ACO* and isocitrate dehydrogenase, *IDH*) of citrate, showed altered levels of Al resistance. A triple mutant of CS (Δ*cit123*) showed lower levels of citrate accumulation and reduced Al resistance, while Δ*aco1* and Δ*idh12* mutants showed higher accumulation of citrate and increased levels of Al resistance. Overexpression of a mitochondrial CS (*CIT1*) in MMYO11 yeast resulted in a 2 to 3 fold increase in citrate levels, and the transformants showed enhanced Al resistance. A gene for *Arabidopsis thaliana*

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mitochondrial CS was overexpressed in canola using an *Agrobacterium*-mediated system. Increased levels of CS gene expression and enhanced CS activity were observed in transgenic lines compared to the wild type (WT). The transgenic lines showed enhanced levels of cellular shoot citrate and a two-fold increase in citrate exudation when exposed to 150 µM Al. Root growth experiments revealed that transgenic lines had enhanced levels of Al resistance. This research suggests that modulation of different enzymes involved in citrate synthesis and turnover (*MDH, CS, ACO, IDH*) could be considered as potential targets-of-gene manipulation to understand the role of citrate metabolism in mediating Al resistance.

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LIST OF ABBREVIATIONS

A_{340}, A_{412}, A_{600}.................absorbance at 340 nm, 412 nm, 600 nm

Al.................................. aluminum

ANOVA..........................analysis of variance

BLAST..........................Basic Local Alignment Search Tool

bromophenol blue.............. 3', 3', 5', 5' -tetrabromophenolsulphonphthalein

bp................................. base pairs

°C................................. degrees celsius

cDNA............................complementary DNA

cv..................................cultivar

C....................................capacity

CoA..............................Coenzyme A

DNA..............................deoxyribonucleic acid

DTNB............................5,5'-dithio-bis (2-nitorbenzoic acid)

EDTA............................ethylene diamine tetra acetic acid

g; mg; µg........................gram, milligram, microgram

h.................................... hours

HEPES.......................... 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

Kb.................................kilo base pair(s)

KD................................kilo Dalton

L; ml; µl.........................litre, milliliter, microliter

LB................................ Luria-Bertani

µF................................ micro farad

M; mM; µM.................... molar, millimolar, micromolar

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mol; µmol; nmol.............. mole; micromole, nanomole

mRNA.......................... messenger RNA

min.............................. minute

MOPS..........................3-(N-morpholino) propanesulfonic acid

MS...............................Murashige and Skoog

N.................................normal

NAD.............................nicotinamide adenine dinucleotide

NADH .... nicotinamide adenine dinucleotide, reduced

%................................ percent

PAGE........................... polyacrylamide gel electrophoresis

PCR............................. polymerase chain reaction

PVP............................. polyvinyl pyrrolidine

R.................................resistance

RNA............................ ribonucleic acid

rpm............................. revolutions per minute

SD.............................. standard deviation of a series

SDS............................. sodium dodecyl sulphate

SE...............................standard error of the mean

Tris............................. tris (hydroxymethyl) aminomethane

V.................................voltage

v/v; v/w; w/v.................. volume per volume; volume per weight; weight per volume

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1. General Introduction

1.1. Impact of aluminum on agriculture

Aluminum (Al) is a major environmental stress that limits growth and productivity of most crops. It is the most abundant metal in the earth's crust and has a complex speciation chemistry that is affected by pH. In neutral and basic environments, Al predominantly exists as non-toxic forms, such as $Al(OH)_3$ and $Al(OH)_4^-$. As soils become more acidic (pH <5.0), Al is solubilized in soils and becomes phytotoxic. Acid soils are widely spread and comprise approximately 40% of the arable land in the world (Huag, 1984). Soils become acidic naturally in humid climates due to leaching of basic minerals and by addition of fertilizers and organic matter. In addition, acid precipitation produced by the combustion of fossil fuels accelerates the acidification of soils. A clear understanding on physiological, biochemical and genetic basis of Al toxicity and mechanisms of Al resistance in plants would aid in developing strategies to increase crop productivity in acid soils.

1.2. Phytotoxic effects of Al

1.2.1. The root apex is the primary site of Al toxicity

Plant roots and shoots exhibit an array of symptoms when exposed to Al. The root apex is considered to be the primary target of Al toxicity. Aluminum accumulates mostly at the root apex (Wagatsumo *et al.*, 1987; Delhaize *et al.*, 1993a) and exposure of the apical 2-3 mm region of the root (comprising of root cap and meristem) of *Triticum aestivum* L. to Al was sufficient to cause inhibition of root

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growth (Ryan *et al.*, 1993). The primary response of plants to Al toxicity is a reduction in root growth, due to inhibition of cell division and cell elongation (Clarkson, 1965). Typically, roots exposed to Al are discolored with stubby root tips and stunted lateral roots (Rhue and Grogan, 1977). Aluminum-induced inhibition of root growth has been associated with decreased mitotic activity, extrusion of nucleolar and nuclear material and enhanced vacuolation of root meristems (Marienfeld *et al.*, 1995; Liu and Juang, 1991). In addition, Al treatment results in increases in pectin, cellulose and hemicellulose contents of root cell walls (Le Van *et al.*, 1994), and decreases in mechanical extensibility of the cell wall (Tabuchi and Matsumoto, 2001). As a result of these cumulative damage to roots, plants become more susceptible to water and nutrient stress (Taylor, 1991; Baligar *et al.*, 1993).

1.2.2. Aluminum disrupts cellular structure and functioning

Aluminum affects several cellular processes and causes a multitude of effects (Kochian 1995). Aluminum-induced inhibition of root growth is correlated with disruption of microtubules and depolymerization of the actin network (Sasaki *et al.*, 1995; Grabski *et al.*, 1998). Aluminum also disrupts activity of the golgi apparatus (Bennet *et al.*, 1987) and reduces mitochondrial respiratory rate (de Lima and Copeland, 1995; Yamamoto *et al.*, 2002) leading to cellular dysfuntioning.

1.2.3. Aluminum forms complexes with essential cellular ligands

Aluminum has high affinity for oxygen donor ligands such as carboxylate, phosphate and sulphate groups, therefore forming complexes with organic acids,

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inorganic phosphates, polynucleotides and proteins (Kochian, 1995). Because of its strong interaction with phosphates, Al has been suggested to destabilize the DNA double helix and interfere with DNA replication, either by binding to polyphosphates in DNA (Matsumoto *et al.*, 1976) or by binding to phosphorylated proteins such as histones (McDonald and Martin, 1988).

1.2.4. The effect of Al on plasma membrane and ion homeostasis

The high affinity of Al for inorganic phosphate makes the plasma membrane a potential target for Al injury. Aluminum causes peroxidation of membrane lipids (Yamamoto *et al.*, 1997; Yoshino *et al.*, 1999) and alters the electrophysiological properties of the plasma membrane (Olivetti *et al.*, 1995). It is an effective cation channel blocker, primarily blocking K^+ and Ca^{2+} channels in root cells (Gassman and Schroeder, 1994). This disturbs the ion homeostasis of root cells due to reduced cation uptake and transport (Huang *et al.*, 1996).

Calcium metabolism, in particular, is severely affected by Al (Nichol *et al.*, 1993; Kochian, 1995). Huang *et al.* (1993) measured a rapid reduction in Ca^{2+} influx at the root apex of *Triticum aestivum* L. that was correlated with inhibition of root growth. In contrast, when roots of *T. aestivum* L., were exposed to phytotoxic concentrations of Al, root growth was severely inhibited but no changes in Ca^{2+} uptake were observed (Kinraide *et al.*, 1994). Aluminum-induced perturbations of Ca^{2+} metabolism are likely influenced by the co-existing ions, which could alter the

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electrical charge of the root surface and reduce the access of free Al species to root cells (Ryan *et al.*, 1997).

1.2.5. Aluminum inhibits the function of cellular enzymes

Aluminum affects cellular metabolism by binding to polypeptides and proteins. It interferes with the activities of a number of enzymes involved in primary metabolism. For instance, malate dehydrogenase, nitrate reductase (Mathys, 1975), glutamate dehydrogenase and aconitase (Zatta *et al.*, 2000) are severely inhibited by Al. Aluminum also inhibits glycolytic enzymes such as phosphofructokinase, hexokinase and glucokinase, by targeting their regulatory sites and rendering them inactive (Xu *et al.*, 1990). Aluminum enhances hydration of hexokinase proteins, leading to aggregate formation and affecting the binding of glucose and ATP to the enzyme (Socorro *et al.*, 2000). Aluminum also causes conformational changes in calmodulin, thereby affecting the functioning of calmodulin-regulated enzymes such as phosphodiesterase, mitogen activated protein kinase (MAPK) and Serine/Threonine phosphatase (McDonald and Martin, 1988, Kutuzov *et al.*, 2001; Sangwan *et al.*, 2002).

1.2.6. The effect of Al on signal transduction

In spite of considerable effort dedicated towards understanding the molecular and physiological basis of Al toxicity in plants, few studies have focused on specific mechanisms by which Al is perceived and the subsequent signal transduced in plant cells. Recent studies with animal systems have focused on the phosphoinositide

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pathway as a potential signal transduction cascade for Al in cells (Verstraeten and Oteiza, 2002). Phospholipase C (PLC) is an important component of a membrane protein complex, which includes guanine nucleotide-binding proteins (G proteins) and phosphatidylinositol-4, 5-diphosphate (PIP2). This protein complex is involved in signal perception/transduction for several plant stresses such as cold, drought and salt (Ruelland *et al.*, 2002; Cardinale *et al.*, 2002). A report of Al-induced inhibition of microsomal membrane PLC resulting in accumulation of phosphatidylinositol-1, 4,5-triphosphate (PIP3), has led to an interesting hypothesis that the phosphoinositide signal transduction pathway could be involved in perception of Al toxicity and transduction of a signal cascade (Jones and Kochian, 1995). Aluminum-induced inhibition of PLC has also been reported recently in roots of *Catharanthus roseus* L. (Pina-Chable and Sotomayor, 2001), but the effect of Al on this multicomponent signaling cascade is yet to be investigated in plants.

1.3. Overcoming problems of Al toxicity

Agronomic management strategies commonly used to ameliorate Al toxicity include delayed planting until soil pH increases by sufficient rainfall or irrigation; addition of organic matter to increase basic cations in the soil; application of fertilizers enriched with Mg^{2+}; or liming with $CaCO_3$ or dolomite to replace Al^{3+} with Ca^{2+} on the cation exchange sites and to increase the pH of the soil. Such farming practices are generally not economical and fail to ameliorate subsoil acidity. To

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overcome these limitations and improve production efficiency in acid soils, Al resistant crops must be developed.

Plant-based management strategies include selection of crop varieties that can tolerate Al toxicity. Many resistant cultivars have been developed and cultivated on acid soils. For example, the Brazilian *T. aestivum* cultivar BH 1146, developed more than 50 years ago, is still reported to have the highest levels of Al resistance of commercial *T. aestivum* cultivars (http://www.ars.usda.gov/). Several other wheat cultivars such as Carazinho and Maringa commonly used for their enhanced Al resistance have been developed on acid soils. Cultivars of several other crop species (for example, *Glycine max* L., *Phaseolus vulgaris* L., *Zea mays* L., *Triticale* and *Brassica napus* L.) also exhibit genetic variation in their ability to tolerate Al. Understanding the suite of physiological, biochemical and molecular mechanisms of resistance exhibited by resistant cultivars of these plants could enable us to fully comprehend the complexity of Al toxicity and Al resistance, and also to identify potential ways to improve Al resistance in plants. For instance, near-isogenic wheat lines with enhanced Al resistance developed by crossing Al-tolerant and Al-sensitive cultivars (Delhaize *et al.*, 1993, Basu *et al.*. 1994), have been extensively used by several research groups for understanding various cellular mechanisms that are potentially involved in Al resistance.

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1.4. Differential Al resistance in plants

Aluminum resistance in plants is hypothesized to be mediated by two strategies, namely the apoplasmic and symplasmic mechanisms. Apoplasmic (external) mechanisms detoxify Al in the apoplasm or exclude Al entry into Al-sensitive cellular sites. Although the cell wall is the major site of Al accumulation, several studies have demonstrated that Al can cross plasma membrane (Zhang and Taylor, 1989; McDonald-Stephens and Taylor, 1995; Taylor *et al.*, 2000). Once Al has crossed plasma membrane, symplasmic (internal) mechanisms can detoxify it by sequestration or chelation of Al (Taylor, 1988; 1991).

1.4.1. Natural Al accumulators provide evidence for internal tolerance mechanisms

A number of studies suggest that certain families of the class core eudicots (such as Hydrangeaceae, Ericaceae, Melastomataceae and Theaceae) are generally adapted to acid soils and accumulate large quantities of Al in roots or leaves without any apparent toxicity symptoms. These Al-accumulating plants may provide evidence for effective symplasmic mechanisms to detoxify Al (Rengel, 1996). Aluminum is speculated to be accumulated in the symplasm in a less toxic form, although the cellular localization and form(s) of Al in these plants are not well established (Ma, 2000). At the pH of symplasm (>7.0), the concentration of free Al is reduced due to the formation of insoluble $Al(OH)_3$ (Martin, 1988), but even low Al concentrations cannot be considered biologically inert (Taylor, 1991).

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1.4.2. Compartmentation of Al in vacuole

One potential internal mechanism of Al resistance is compartmentation of Al in the vacuole. Studies on yeast cells exposed to Al showed irregular vacuolar morphology (Ezaki *et al.*, 1998), but intracellular Al accumulation in yeast could not be associated with the vacuole based on observations of morin fluorescence (Ezaki *et al.*, 1999). In contrast, accumulation of Al was observed in vacuoles of Al-tolerant maize cultivars (Vasquez *et al.*, 1999), based on scanning transmission electron microscopy and energy-dispersive x-ray microanalysis.

Direct quantification of transport of Al across biological membranes has been difficult, due to limitations such as the complex speciation chemistry of Al, its high affinity to bind tightly to cell walls, the lack of an affordable and suitable isotope, and the lack of sensitive analytical techniques for detecting the low levels of Al associated with subcellular compartments (Taylor *et al.*, 2000). Using the ^{26}Al isotope, transport of Al across the plasma membrane was recently quantified within 30 min of exposure to Al in single cells of the alga, *Chara corallina*. Aluminum was reported to be subsequently sequestered into the vacuole (Taylor *et al.*, 2000).

1.4.3. Alteration in expression of genes and gene products

Aluminum-induced increases in transcript abundance have been reported in *T. aestivum L.* (Hamel *et al.*, 1998 and Delhaize *et al.*, 1999), *N. tabacum* (Ezaki *et al.*, 1995; 1996), *B. napus* (Basu *et al.*, 2001) and *A. thaliana* (Sugimoto and Sakamoto, 1997; Richards *et al.*, 1998). Although several genes have been characterized to be

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specifically induced by Al, most appear to be induced by general plant stresses. For example, Al-induced genes also respond to phosphate starvation (Ezaki *et al.*, 1995), pests and pathogens (Cruz-Ortega *et al.*, 1997; Hamel *et al.*, 1998), oxidative stress (Sugimoto and Sakamoto, 1997; Richards *et al.*, 1998; Basu *et al.*, 2001), heat shock (Ezaki *et al.*, 1998), osmotic stress (Hamilton *et al.*, 2002) and other metal stresses (Snowden *et al.*, 1995; Sugimoto and Sakamoto, 1997). Activities of several enzymes such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Slaski *et al.*, 1996), vacuolar H^+-ATPase (Kasai *et al.*, 1992; Hamilton *et al.*, 2001a) and mitochondrial ATPsynthase (Hamilton *et al.*, 2001a) are upregulated in Al-resistant cultivars of various crop species, probably for maintenance of cellular metabolism.

1.4.4. Plant-induced pH barrier in the rhizosphere

Since Al is phytotoxic at pH <5.0, maintenance of a relatively high pH in the root rhizosphere or apoplasm is a potential external mechanism of Al resistance. Several early studies reported a significant correlation between plant-induced pH of growth solutions and Al tolerance (Taylor and Foy, 1985a,b). Furthermore, Taylor and Foy (1985c) illustrated that pH changes in growth solutions were due to differential uptake of NH_4^+ and NO_3^-. However, a lack of correlation between plant-induced pH changes and Al tolerance was demonstrated in several studies (Taylor, 1988; Miyasaka *et al.*, 1989) and differential tolerance between Al-tolerant (Atlas-66) and Al-sensitive (Scout-66) cultivars of *T. aestivum* was not affected by the ratio of NH_4^+/NO_3^- in growth solutions (Taylor, 1987; 1988). In contrast, an Al-induced

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alkalinization of the root tip rhizosphere along with a two-fold increase in H^+ influx was observed in an Al-tolerant *Arabidopsis thaliana* mutant, alr-104 (Degenhardt *et al.*, 1998). Root apices of *Brachiaria decumbens* (signal grass) also alkalinized the rhizosphere in the presence of Al (Wenzl *et al.*, 2001). Although these recent results highlight the significance of pH changes in Al tolerance, it may not be the primary cause for Al resistance (Taylor 1991; Matsumoto, 2000).

1.5. Importance of exudates in Al resistance

Root exudates include a diverse array of substances with various functions. Some of these compounds are involved in nutrient availability (Brown *et al.*, 1991; Hoffland *et al.*, 1992; Gerke *et al.*, 2000; Keller and Romer, 2001), or play a role as allelochemicals (Einhellig *et al.*, 1993; Birkett *et al.*, 2001) or signaling molecules (Stacey *et al.*, 1995). Exudates can be classified into high and low molecular weight compounds. The high molecular weight compounds include mucilage and a variety of proteins. Mucilage is composed of a multitude of molecules such as polysaccharides, polygalacturonic acids and proteins rich in hydroxyproline (Matsumoto, 2000). Roots of cowpea showed increased susceptibility to Al injury after removal of mucilage compared to roots with intact mucilage (Horst *et al.*, 1982). Aluminum bound to mucilage accounted for 25 to 35% of non-exchangeable Al and contributed to the linear phase of Al uptake by *T. aestivum* roots (Archambault *et al.*, 1996). Aluminum was bound to low molecular mass sugars of mucilage of *Z. mays* roots, but it did not result in reduction of Al-induced inhibition of root elongation (Li *et al.*, 2000). When investigating the Al-binding capacity of the mucilage, the

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presence of other rhizodeposited substances in the mucilage (Henderson and Ownby, 1991) and differences in production of mucilage among different cultivars of crop species (Puthota *et al.*, 1991) need to be considered.

High molecular weight polypeptides exuded to the rhizosphere could potentially bind Al in the apoplasm. Putterill and Gardner (1988) demonstrated *in vitro* the potential Al binding ability of acidic polypeptides such as poly (L-aspartic acid) and poly (L-glutamic acid) and serum protein transferrin. An Al-induced (time- and dose-dependent) exudation of several polypeptides was observed from roots of Al-resistant *T.aestivum* cultivars, Atlas 66 and Maringa (Basu *et al.*, 1994c). A 23 KD, Al-induced polypeptide was purified from root exudates of the Al-resistant *T.aestivum* cultivar, Maringa (Basu *et al.*, 1999). This protein co-segregated with the Al resistance phenotype in F2 populations arising from a cross between doubled-haploid lines of Katepwa (Al-sensitive) and Alikat (Al-resistant). The protein was identified as a superoxide dismutase (SOD). A mitochondrial isoform of SOD (Mn-SOD) was overexpressed in *B. napus* and its protective role against Al-toxicity and oxidative damage has been demonstrated (Basu *et al.*, 2001).

1.6. Low molecular weight organic acids and Al resistance

One of the more compelling hypotheses proposed for Al tolerance is the chelation of Al by chelator ligands in the cytoplasm and/or apoplasm (or rhizosphere). Among the various candidates, low molecular weight organic acids such as malate, citrate or oxalate appear to play an important role in Al resistance

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(Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993a; Basu *et al.*, 1994; Ryan *et al.*, 1995a, Ma *et al.*, 1997, de la Fuente *et al.*, 1997, Zheng *et al.*, 1998a, Larsen *et al.*, 1998; Koyama *et al.* 1999).

Organic anions could potentially chelate Al within the cytoplasm and reduce its toxic effects within cells. The majority of Al accumulators (section 1.4.1) chelate Al with organic anions. In leaves of Hydrangea, Al was chelated with citrate at a 1:1 ratio, forming non-phytotoxic Al-citrate complexes (Ma *et al.*, 1997). Aluminum accumulates in the form of Al-oxalate complexes (1:3) in root and shoot tissues of buckwheat (*Fagopyrum esculentum* Moench, cv. Jianxi; Ma *et al.*, 1998). Aluminum also chelated with oxalate (1:3) in roots and leaves of buckwheat, and with citrate (1:1) in xylem tissues (Ma and Hiradate, 2000). Although Al-organic anion complexes formed in the symplasm are suggested to be non-toxic, studies on the effect of these complexes on house-keeping genes and functioning of key enzymes are still lacking (Taylor *et al.*, 1989).

Exudation of organic anions from roots of Al-tolerant cultivars of different crop species suggests the potential role of organic anions in detoxification of Al in the apoplasm. Organic anions exuded from roots could complex free monomeric Al in the apoplasm, and reduce absorption of Al across plasma membrane or protect sensitive apoplasmic sites (Taylor *et al.*, 1991). Exudation of several different organic anions has been described from roots of a variety of Al-resistant plant species (Table 1-1). Aluminum-induced exudation of organic anions is specific to Al; neither

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P deficiency nor other polyvalent cations elicit the efflux of organic anions (Ryan *et al.*, 1995a; Ma *et al.*, 1997; Zheng *et al.*, 199ba). Moreover, the amount of organic anions secreted was also dependent on Al concentration (Delhaize *et al.*, 1993b; Basu *et al.*, 1994c; Ma *et al.*, 1997).

Significant progress has been made in unraveling patterns of organic anion release from roots of Al-resistant plants. One pattern is a rapid release of organic anions without any discernible delay after exposure to Al. For example, after 15 minutes of exposure to Al, malate was released from roots of near isogenic, Al-resistant wheat lines (Delhaize *et al.*, 1993a). The rapid kinetics of this response suggests that all the necessary metabolic "machinery" is constitutively expressed in the root and that organic anion efflux is simply triggered by Al (Ma, 2000; Ryan and Delhaize, 2001). Due to the near-neutral pH of the cytoplasm, organic acids exist within the cytoplasm as dissociated anions (citrate^{3-}, malate^{2-}, oxalate^{2-}). As a result of the negative electrical potential across the plasma membrane, organic anions can passively diffuse out of cells (Ryan and Delhaize, 2001). Delhaize and Ryan (1995) demonstrated that the rapid release of malate was due to Al-triggered activation of anion channels in the plasma membrane. They also reported that antagonists of anion channels, such as niflumic acid and cycloheximide, inhibited Al-stimulated malate efflux (Ryan *et al.*, 1995a). Since then, a number of researchers have focused on anion channels that are specifically activated by extracellular Al, using protoplasts isolated from Al-resistant cultivars of wheat (Ryan *et al.*, 1997; Zhang *et al.*, 2001) and maize (Kollmeier *et al.*, 2001; Piñeros and Kochian, 2001).

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Table 1-1. Summary of Al-induced exudation of organic anions from roots of various crop species.

Plant Species	Organic anion(s) released	Organic anion(s) released from	Reference
Triticum aestivum L.	Malate	Root apex	Ryan *et al.*, 1995a
Triticale sp.	Malate and citrate	Whole roots	Ma, 2000
Secale cereale L.	Malate and citrate	Whole roots	Li *et al.*, 2000
Brassica napus L.	Malate and citrate	Whole roots	Zheng *et al.*, 1998a
Zea mays L.	Citrate	Root apex	Pellet *et al.*, 1995
Nicotiana tabacum L.	Citrate	Root apex	Delhaize *et al.*, 2001
Phaseolus vulgaris L.	Citrate	Root apex	Miyasaka and Hawes, 2001
Glycine max L.	Citrate	Whole roots	Yang *et al.*, 2000
Avena sativa L.	Citrate	Whole roots	Zheng *et al.*, 1998a
Cassia tora L.	Citrate	Whole roots	Ma *et al.*, 1997
Colocasia esculenta L.	Oxalate	Whole roots	Ma and Miyasaka, 1998
Fagopyrum esculentum Moench.	Oxalate	Whole roots	Zheng *et al.*, 1998b

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Several studies suggest that a lag phase can also exist between exposure of roots to Al and exudation of organic anions (Ma, 2000). For instance, 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 an Al-resistant wheat cv. Katepwa (Basu *et al.,* 1994c). Furthermore, Al-activated release of citrate increased with increasing exposure to Al (Pellet *et al.*, 1995). Delayed exudation of organic anions could possibly reflect alteration of cellular metabolism leading to biosynthesis and release of anions. Aluminum-induced modification of cellular metabolism could include altered expression of gene(s) related to the biosynthesis and catabolism of organic acids or anion channels in the plasma membrane and/or tonoplast (Ma *et al.*, 2000).

1.7. Linking organic acid metabolism to Al toxicity

Studies relating cellular metabolism with synthesis and exudation of organic acids will be required to understand the biochemical and molecular basis of this mechanism of Al resistance. Studies making use of cultivars that exhibit genotypic variability in organic anion concentration (in the symplasm and apoplasm) have helped in understanding the links between organic acid metabolism and Al resistance. For example, Al-tolerant rye plants showed an increase in activity of citrate synthase (CS), with no change in the activities of isocitrate dehydrogenase (IDH), phosphoenolpyruvate carboxylase (PEPC), and malate dehydrogenase (MDH) in response to Al, and these changes in enzyme activities were accompanied by changes

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in citrate exudation (Li *et al.*, 2000). In maize, an Al-dependent increase in citrate exudation was associated with an increase in root tip citrate content and a simultaneous decrease in root aconitate content, suggesting that Al-induced alterations in organic acid metabolism could also provide internal tolerance to Al (Pineros *et al.*, 2002).

Another potential strategy that can be used to investigate the association between organic acid metabolism and Al resistance is the overexpression or disruption of genes involved in organic acid metabolism. Knowledge of the biochemical pathways involved in organic acid synthesis and transport of organic anions from the site of synthesis to the site of chelation is vital in identifying the potential candidate genes for gene manipulation.

1.7.1. The predominant organic acids involved in Al resistance are malate and citrate

Among the various organic acids, malate and citrate are most commonly cited as playing an important role in detoxification of Al. They are strong chelators of Al (Matsumoto, 2000) and therefore could protect plants in both the apoplasm and symplasm (Pineros *et al*, 2002). Among a range of organic acids tested, malate and citrate conferred the greatest protection from Al toxicity in hydroponic cultures as they possess carboxyl groups that could form stable ring structure with Al^{3+} (Hue *et al.*, 1986).

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Phosphate deficiency is one of the important effects of Al stress in plants, due to the chelation of available phosphate by Al. Aluminum toxicity and phosphate deficiency produce similar symptoms and phosphate metabolism is significantly affected by Al toxicity (Jarvis and Hatch, 1986). Enhanced synthesis and exudation of malate and citrate has been suggested to be an effective mechanism in alleviating phosphorus stress in *Medicago sativa* L. (Lipton *et al.,* 1987) and *Brassica napus* L. (Hoffland *et al.,* 1989; 1992). A mutant carrot (*Daucus carota*) cell line (IPG, insoluble phosphate grower), released more citrate under conditions of Al stress and phosphate deficiency (Koyama *et al.*, 1999).

1.7.2. Regulation of malate and citrate efflux from roots

Malate and citrate, when produced and accumulated in cells, are generally exuded to the apoplasm to prevent cytoplasmic acidiosis (Neumann *et al.*, 1999). Genes for malate and citrate transport proteins are not thoroughly studied in plants, although several dicarboxylate- and tricarboxylate carrier proteins (DCPs and TCPs) are well characterized in animal systems (Fiermonte *et al*, 1999). The first and only citrate carrier protein identified so far in plants is from maize mitochondria (Genchi *et al.*, 1999). Using high-throughput technologies, subsets of gene families encoding plant mitochondrial carrier proteins have been identified (Millar and Haezlewood, 2003), which should pave way for understanding more about DCPs and TCPs in plants. The role of DCPs or TCPs in transporting malate or citrate as an Al resistance mechanism would be an interesting area of research.

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1.7.3. Regulation of biosynthesis of malate and citrate

Another important question is whether the overexpression or disruption of genes involved in synthesis of malate and citrate would affect malate and citrate concentration in the symplasm and their exudation to the apoplasm. These organic anions are largely synthesized in the TCA and glyoxylate cycles; and are directly or indirectly involved with many other metabolic processes. These include the assimilation of carbon and nitrogen, regulation of cytosolic pH and osmotic potential, and balancing of charges during excess cation uptake. They also serve as substrates in various biochemical reactions such as amino acid and fatty acid syntheses. For this reason, the anabolism and catabolism of citrate and malate, as well as their concentrations tend to be strictly regulated. Flux through the TCA cycle in plant tissues is highly dependent on the metabolic requirements of the tissue. For example, the activity of the TCA cycle is modulated by the presence or absence of light (Gemel and Randall, 1992), redox state of the pyridine nucleotides, NAD and NADP (Oliver and McIntosh, 1995) and ADP: ATP ratio in mitochondria (Srere, 2000).

1.8. Biochemical pathways involved in citrate and malate metabolism

1.8.1. The biochemistry of malate synthesis

Malate is an important intermediary compound in the TCA cycle and glyoxylate cycle. Regulation of malate synthesis and its distribution in plants is complex (Martinoia *et al.*, 1994). The key enzyme involved in synthesis of malate is malate dehydrogenase (MDH, enzyme position 2 in glyoxylate and TCA cycles, in

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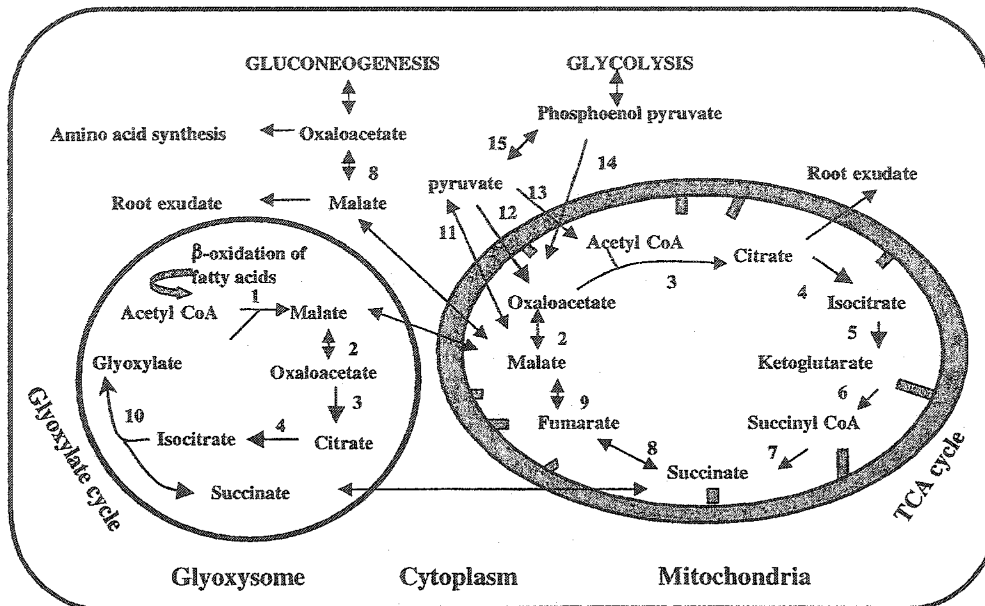

Figure 1-1. Metabolic map showing the biochemical reactions involved in the synthesis of malate and citrate in the cytosol, glyoxysome and mitochondria. (adapted from *Biochemica et Biophysica Acta*. 1992, 1100: 217-234). Enzymes involved are: 1. Malate synthase; 2. Malate dehydrogenase; 3. Citrate synthase; 4. Aconitase; 5. Isocitrate dehydrogenase; 6. a -Ketoglutarate dehydrogenase; 7. Succinate thiokinase; 8. Succinate dehydrogenase; 9. Fumarase; 10. Isocitrate lyase; 11. Malic enzyme; 12. Pyruvate carboxylase; 13. Pyruvate dehydrogenase complex; 14. Phosphoenolpyruvate carboxylase; 15. Pyruvate kinase. The arrow head(s) represent the direction of the biochemical reaction (→ unidirectional; ↔ bidirectional)

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Figure 1-1), which catalyzes the reduction of oxaloacetate (OAA) to malate. Several isoforms of MDH (cytosolic, mitochondrial and glyoxysomal) exist in plant cells. In addition, several other enzymes catalyze different reactions involved in malate synthesis. They include malate synthase (MS, enzyme position 1 in Figure 1-1), malic enzyme (ME, enzyme position 11) phosphoenolpyruvate carboxylase (PEPC, enzyme position 14) and fumarase (FUM, enzyme position 9); and the precursors involved in those reactions are glyoxylate, pyruvate, phosphoenolpyruvate (PEP) and fumarate, respectively (Figure 1-1).

Changes in levels of malate can potentially be achieved by modulating the genes mentioned above. Transgenic *Solanum tuberosum* with increased levels of PEPC showed an increase in the amount of malate in leaf tissues. Enhanced synthesis of malate in roots of *B. napus* under phosphate-starved conditions was reported to be due to increased levels of PEPC in root tissues. Since OAA is also a substrate for synthesis of citrate and amino acids like aspartate, and a precursor for gluconeogenesis, partitioning of OAA for enhanced malate production by PEPC engineering is a complex issue. Enhancing levels of malate through overexpression of FUM is a questionable strategy, since the free energy of this reaction is not high enough to drive enough substrate (succinate) towards fumarate (Figure 1-1). Enhanced synthesis of malate dehyrogenase (MDH) and malate synthase (MS) might lead to enhanced synthesis of malate. A significant increase in malate levels has been demonstrated in yeast by overexpressing a cytosolic MDH. A similar study has been

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done in *Medicago sativa*, where overexpression of a nodule-specific MDH resulted in enhanced exudation of malate from roots of transgenic plants.

Another key enzyme involved in malate biosynthesis is malate synthase (MS), which catalyzes the first reaction in the glyoxylate cycle leading to synthesis of malate from glyoxylate and acetyl CoA. This is an important biochemical reaction that facilitates conversion of fatty acids into malate and succinate required for gluconeogenesis during the early development of plants. Though a glyoxylate cycle enzyme, the presence of MS has been reported in different tissues with varying levels of activity (Turley and Trelease, 1990; Zhang *et al.*, 1994). It is induced in ripening fruits, detached leaves and roots, senescing plant organs and under various kinds of stresses (Graham *et al.*, 1992; Pua *et al.*, 2003). Reappearance of its activity generally coincides with lipid metabolism. The roles of MS in various organs involved in embryogenesis and post-germination have been thoroughly investigated. The function of MS in plants (particularly the seedling stage), under Al stress has not yet been explored. What is the role of MS in the synthesis of malate and maintaining malate pool in the apoplasm and symplasm? Can an overexpression strategy be used to enhance malate content in seedlings? Can malate levels be increased sufficiently to support malate efflux and overcome Al toxicity?

1.8.2. The biochemistry of citrate metabolism

Citrate is also an important organic anion released from roots of plants in response to Al toxicity. Increasing the level of enzymes involved in citrate synthesis

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or decreasing the activities of enzymes involved in citrate catabolism can increase citrate levels in plants. The key enzyme involved in the synthesis of citrate is citrate synthase (CS), an enzyme that plays a major role in the Krebs cycle and glyoxylate cycle by condensing oxaloacetate and acetyl CoA into citrate (enzyme position 3, Figure 1-1). Citrate synthase is the first enzyme in the TCA cycle and is also one of the rate-limiting steps in regulation of TCA cycle. Overexpressing CS in plants could enhance citrate levels in transgenic lines and thereby enhance Al resistance. In addition, decreasing the levels of enzymes involved in breakdown of citrate (aconitase and isocitrate dehydrogenase, enzyme positions 4 and 5 respectively; Figure 1-1) could also increase citrate levels in the apoplasm and symplasm.

1.8.3. Controversies behind overexpression of CS in plants

There are two isoforms of CS (glyoxysomal and mitochondrial) in eukaryotes. Targeting CS to the mitochondria might be an ideal approach to increase the synthesis and/or exudation of citrate in plants. The potential ability of CS in conferring Al resistance was first suggested by de la Fuente *et al.* (1997). They overexpressed a gene for *Pseudomonas aeroginosa* CS in the cytosol of *N. tabacum* and *Papaya* sp. and reported that the transgenic lines showed higher levels of CS activity as well as cellular and extracellular citrate content. This resulted in enhanced resistance to Al and phosphate deficiency (Lopez *et al.*, 2000). With two independent experimental systems, one with transgenic *D. carota* cell lines overexpressing an *A. thaliana* mitochondrial CS, and another with transgenic *A. thaliana* plants overexpressing a *D. carota* mitochondrial CS, Koyama *et al.* (1999; 2000) illustrated that CS activity and

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citrate excretion from transgenic lines can be enhanced by overexpressing a mitochondrial CS. They also demonstrated that these transgenic lines performed better under Al toxic and phosphate deficient conditions than the respective wild types. In contrast to these studies, Delhaize *et al.* (2001) reported that overexpression of a *P. aeroginosa* CS (similar to de la Fuente *et al.*, 1997) in the cytosol of *N. tabacum* neither resulted in enhanced exudation of citrate nor increased Al resistance.

The controversy over the role of CS in Al resistance has made this area of research an important endeavor. Is it possible to establish the role of CS in citrate synthesis and Al resistance using an overexpression strategy? Can the same strategy be applied to any other model systems? Being a rate-limiting enzyme catalyzing an irreversible reaction, is it possible to increase citrate levels by modulating other genes of TCA cycle? What are the suite of genes that could be involved in regulating levels of citrate intra- and extra-cellularly?

1.9. Experimental model systems

Addressing the questions raised in section 1.8, could improve our understanding of the complexity of synthesis and exudation of malate and citrate and their potential roles in Al resistance. Developing plants with modified organic acid metabolism through genetic engineering could be an effective approach to test whether enhanced synthesis and exudation of organic acids can render a plant Al tolerant. If this strategy could be adopted to alter levels of malate and citrate in economically important crops such as canola, wheat or rice, it would be a significant

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contribution to management strategies aimed at improving crop production in Al toxic soils.

To explore the complexity of malate and citrate metabolism, an integrated approach comprising of a suite of genes involved in metabolism of organic anions was undertaken using yeast as a model system. Genetic manipulation is relatively easy in yeast due to the availability of mutants, ease of handling and short experimental periods. Yeast (*Saccharomyces cerevisiae*) has been used as an excellent system for studies on metal toxicity and resistance. Genes involved in Al tolerance (*HSP150, SED1* and *ALU1-P*) have been identified in yeast (Ezaki *et al.*, 1998; Jo *et al.*, 1997) and the function of several plant genes (Phosphatidylserine, *BCB, NtGDI1 and ATPase*) have been elucidated by complementation of yeast mutants (Delhaize *et al.*, 1999; Ezaki *et al.*, 1999; Hamilton *et al.*, 2001b). The availability of disruption mutants defective in various 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 involved in malate and citrate metabolism in mediating Al resistance. The functions of gene and gene products of the TCA and glyoxylate cycles are likely to be conserved between yeast and plants. For example, the genes for different subunits of NAD^+-dependent IDH identified in *N. tabacum* were sufficiently conserved to sort and assemble into yeast mitochondria to allow for proper IDH function (Lancien *et al.*, 1998). Therefore the yeast TCA and glyoxylate cycle mutants should be a useful model system for understanding malate and citrate metabolism in Al resistance.

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In addition to the yeast model system, I have used an important oilseed crop *Brassica napus* L. (canola) to test whether overexpression of genes involved in synthesis of malate and citrate (MS and CS) increases Al resistance in transgenic plants. Cultivars of *B. napus* are sensitive to acid soils and Al toxicity (Clune and Copeland, 1999). The sensitivity of canola to Al stress can thus be exploited to test if enhanced expression of MS or CS will cause an enhanced exudation of malate or citrate, thereby conferring Al resistance. To ensure homozygousity and genetic purity of seeds for trangenic studies, the doubled haploid cv. Westar was used. Furthermore, development of Al-resistant *B. napus* plants through genetic engineering could provide an important management strategy to improve oilseed productivity in acid soils.

1.10. Objectives

I set out to test my hypothesis with the following objectives:

a. Analyze a collection of yeast mutants defective in TCA and glyoxylate cycle genes for their cellular and extracellular levels of malate or citrate.

b. Determine the sensitivity of selected yeast mutants to Al and establish the relationship between their cellular malate or citrate levels and Al resistance.

c. Overexpress a gene encoding citrate synthase (CS) and malate synthase (MS) with a constitutive promoter (CaMV) in *Brassica napus* L. using *Agrobacterium*-mediated transformation.

d. Characterize transgenic plants for enhanced synthesis and/or exudation of malate or citrate, and enhanced resistance to Al compared to wild type plants.

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In Chapter 2, I describe studiesconducted to investigate the role of malate synthase (MS) and malate dehydrogenase (MDH) in mediating Al resistance in yeast. The Al sensitivity of yeast mutants defective in MS and various isoforms of MDH was evaluated. Apoplasmic and symplasmic pools of malate were also determined in these mutants and correlated to their sensitivity to Al.

Since the role of MS in Al resistance has not yet been established, I developed transgenic lines of *B. napus* overexpressing a glyoxysomal MS under the control of a constitutive promoter. In Chapter 3, I describe the studies on characterization of transgenic lines overexpressing MS for enhanced levels of MS activity and malate levels. These transgenic studies with MS revealed that, even in the young seedlings where MS is highly functional, overexpression did not lead to an increase in either MS activity or cellular malate levels.

In Chapter 4, I describe the complexity of pathways involved in citrate metabolism and the potential ways to enhance citrate accumulation and exudation in yeast and canola. A collection of TCA cycle and glyoxylate cycle mutants was used to explore the role of different genes (and metabolites) in Al resistance. Citrate levels were estimated in all mutants and were associated with the Al sensitivity of these mutants. A disruption mutant defective in three isoforms of citrate synthase showed hypersensitivity to Al, while mutants with disruptions in genes downstream of citrate (aconitase and isocitrate dehydrogenase) showed reduced Al sensitivity compared to the wild type (Figure 1-1). I was also interested in determining whether

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overexpression of CS could lead to enhanced production of citrate and increased exudation out of transformed yeast cells. This study clearly revealed the importance mitochondrial CS (mtCS) in the biosynthesis of citrate in yeast. To further understand whether an overexpression approach can be successfully adopted in a plant system, an *A. thaliana* mtCS was overexpressed in *B. napus* cv. Westar. Transgenic lines were analyzed for citrate accumulation and exudation from roots, and for their resistance to Al. As observed in the yeast overexpression studies, transgenic lines overexpressing CS also showed an increase in citrate exudation and also Al resistance.

Thus, my overexpression studies with yeast and transgenic canola demonstrated that mitochondrial CS is a key component in citrate metabolism and citrate content can be increased in the apoplasm and symplasm by overexpressing mitochondrial CS. The results with yeast disruption mutants revealed that citrate metabolism is complex and mt-CS represents an important part of the complex metabolic system. This study clearly demonstrated that manipulating genes both downstream and upstream of citrate can enhance the citrate content and consequently, Al resistance.

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2. Exploring the complexity of malate metabolism in relation to aluminum resistance in *Saccharomyces cerevisiae*

2.1. Introduction

One of the compelling hypothesis proposed for Al tolerance in plants is the binding of Al by low molecular weight organic anions, either within cytoplasm or in the rhizosphere (Delhaize *et al.*, 1993; Basu *et al.*, 1994; Ryan *et al.*, 1995; Li *et al.*, 2000; Pineros *et al.*, 2002; Anoop *et al.*, 2003; Chapter 4). The synthesis and exudation of organic anions from plant roots have been linked to alterations in cellular metabolism (Neumann *et al*, 2000). For example, changes in the activities of phosphoenolpyruvate carboxylase (PEPC), citrate synthase (CS), malate dehydrogenase (MDH), aconitase (ACO) and isocitrate dehydrogenase (IDH) have been associated with synthesis and release of malate and citrate from roots (Neumann *et al.*, 2000, Masseanou *et al.*, 2001; Kihara *et al.*, 2003). Among the various low molecular weight organic anions, malate is suggested to play a significant role in alleviating Al toxicity.

The biochemical pathways involved in malate metabolism are delicately balanced since many of the intermediates in these metabolic pathways are precursors for the synthesis of various biomolecules (amino acids, nucleotides and fatty acids). The synthesis and catabolism of organic anions are therefore strictly regulated (Ryan and Delhaize, 2001; Owen *et al.*, 2002). The complex enzymology of malate synthesis, along with complicated compartmentation and transport mechanisms

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necessitates a holistic approach to be undertaken when considering the suite of genes involved in the malate metabolism.

Several studies have attempted to understand the biochemical and molecular basis of synthesis and exudation of organic anions in relation to Al tolerance using a single gene approach. For example, overexpression genes for citrate synthase (*CS*) in *Nicotiana tabacum* L. (de la Fuente *et al.*, 1997), *Arabidopsis thaliana* L. (Koyama *et al.*, 1999; 2000), *Brassica napus* L. and *Saccharomyces cerevisiae* (Anoop *et al.*, 2003; Chapter 4), have resulted in enhanced synthesis and exudation of citrate leading to an increase in Al resistance. Similarly, overexpression of malate dehydrogenase (*MDH*) in *Medicago sativa* L. resulted in increased synthesis and exudation of malate and the transgenic lines showed enhanced Al resistance (Tesfaye *et al.*, 2001). Recently, I undertook a comprehensive study with a collection of yeast disruption mutants defective in TCA and glyoxylate cycle genes and illustrated the complexity of citrate metabolism and the involvement of citrate in Al resistance (Anoop *et al.*, 2003; Chapter 4).

Aluminum-induced alterations in malate levels have been reported in several plant species (Matsumoto, 2000; Ma *et al.*, 2001). Yeast is an excellent model system to understand the role of various genes involved in modulation of malate levels by Al. Here, I present the results from my investigation of Al sensitivity of a collection of mutants defective in various TCA and glyoxylate cycle genes involved in malate synthesis (Table2-1; McCammon M T, 1996; Przybyla-Zawislak *et al.*, 1999). The

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levels of cellular and extracellular malate content were determined in these mutants, to understand the potential role of these genes in modulating the levels of malate and their involvement in Al resistance.

2.2. Materials and methods

2.2.1. Yeast strains, media and culture conditions

Yeast (*Saccharomyces cerevisiae*) strains defective in various genes of the TCA, glyoxylate and gluconeogenetic pathways, potentially involved in malate metabolism were used (Table 2-1; Figure 2-1; provided by Dr. McCammon, University of Texas Health Sceinces Center, San Antonio, Texas). These with altered malate metabolism have the genetic background of the WT yeast, MMYO11 (*Matα ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*). The single mutants were generated by the targeted-gene-disruption method using a PCR amplified linear fragment of the specific DNA containing a selectable marker (Rothstein, 1991). For example, mutant *MDH1* was developed by using a 1.2-kb *mdh1:: URA3* PCR fragment with the hybrid primers, 5'-TTGTCAAGAGTAGCTAAACGTGCGTTT TCCTCTACACTTGCCAACCCT atttttttttattcttttttttgatttcgg and 5'-CCCTATTTT TCACTCTATTTCT GATCTTGAAC AATCTA TTTAgctttttctttccaatttttttttttttcgt, where the uppercase nucleotides correspond to MDH1 and the lowercase nucleotides correspond to URA3 (Przybyla-Zawislak *et al.*, 1999). The double mutant (*MDH12*) was constructed by crossing single mutant strains (*MDH1* and *MDH2*), and screening random meiotic haploid products for *MDH12* genotype. Genotypes were confirmed

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Table 2-1. Yeast strains defective in various TCA, gluconeogenic and glyoxylate cycle genes used in studies involving malate metabolism (provided by Dr. M. McCammon, University of Texas). The genetic background of all mutants listed below is of the WT, MMYO11 (Przybyla-Zawislak *et al.*, 1999).

Strain	Genotype	Properties
MMYO11	*Matα ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*	Wild type parental strain
Δ*mls1*	*G23-1;* uncharacterized mutation but phenotype is tight	Defective in glyoxysomal malate synthase, *MLS1*
Δ*icl1*	*G37-5;* uncharacterized mutation but phenotype is tight	Defective in glyoxysomal isocitrate lyase, *ICL1*
Δ*fum1*	*fum1::LEU2*	Defective in mitochondrial fumarase, *FUM1*
Δ*mdh1*	*mdh1::TRP1*	Defective in mitochondrial malate dehydrogenase, *MDH1*
Δ*mdh2*	*mdh2::TRP1*	Defective in cytosolic (gluconeogenic) malate dehydrogenase, *MDH1*
Δ*mdh12*	*mdh1::URA3 mdh2::TRP1*	Defective in mitochondrial and cytosolic malate dehydrogenases, *MDH1* and *MDH2*

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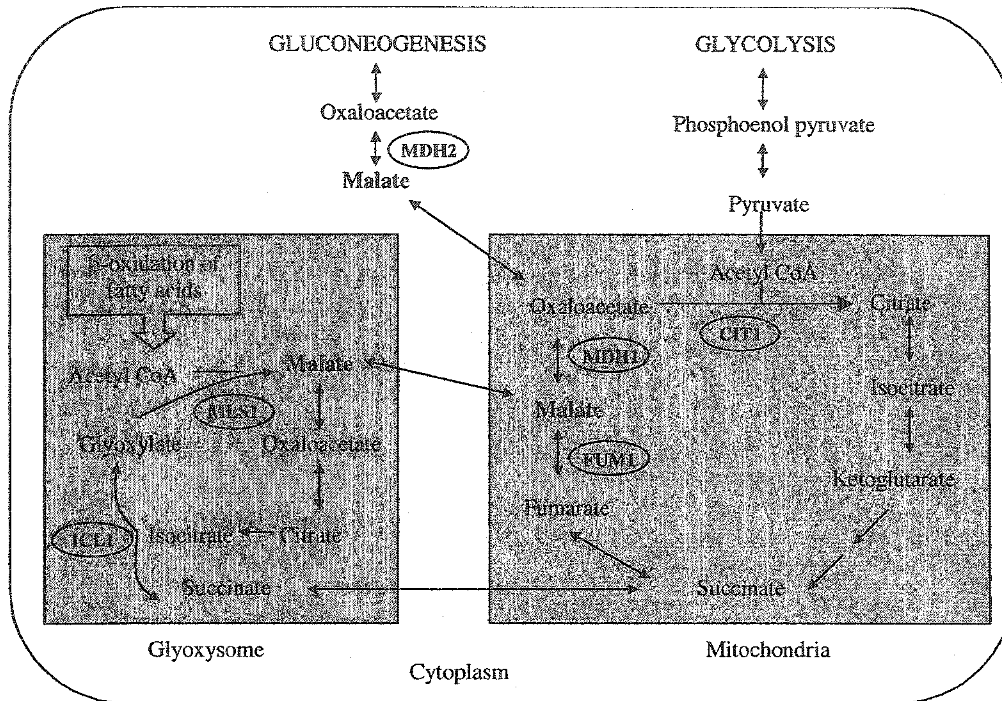

Figure 2-1. Metabolic map of TCA, glyoxylate and gluconeogenetic cycles indicating the biochemical reactions involved in malate synthesis. The collection of yeast strains disrupted for various genes of malate metabolism are also represented. Properties of these mutants are described in Table 2-1.

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by PCR using primers flanking the disruption sites, by phenotypic analysis, and by complementation analysis using marker strains (Przybyla-Zawislak *et al.*, 1999).

Seed cultures of yeast were grown for 20 h at 30°C in YPD media (1% Yeast extract, 2% Peptone, 2% D-glucose). The cells were harvested from cultures by centrifugation at 1300 g for 2 min, washed three times with sterile water and suspended in 1 ml sterile water. This cell suspension was used to inoculate the media for growing yeast cultures. An initial OD_{600} of 0.05 was used in all experiments.

2.2.2. Aluminum dose response experiments

For determining the Al sensitivity of yeast mutants with altered malate metabolism or estimating their malate levels, cultures were grown in a low phosphate medium (LPP; Anoop *et al.*, 2003; Chapter 4). This is a synthetic complete (SC) minimal media comprising of 0.67% Yeast nitrogen base (without amino acids; without phosphate) with 100 μM K_2HPO_4 and 4.5 mM KCl; and pH of the medium was reduced to 3.5 to ensure solubility of Al (Schott and Gardner, 1997). Treatment cultures (2 ml of LPP medium with 0 to 400 μM Al; starting OD_{600} of 0.05) were grown for 18 to 20 h at 30°C at 100 g in triplicates. The final OD_{600} was measured using a plate reader (μQuant, Biotek Instruments, Vermont, USA). The relative growth rate of each strain was expressed as percentage of control (0 μM Al) for that strain.

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2.2.3. Aluminum-induced changes in transcript levels

Total RNA was isolated from MMYO11 (approximately 5 X 10^7 cells) grown in LPP medium with varying concentrations of Al (0 to 400 μM) for 20 h by an enzymatic method using the QIAGEN RNAeasy mini kit (QIAGEN Inc., Canada). The RNA was separated by electrophoresis on agarose formaldehyde denaturing gels, and transferred to nitrocellulose membranes (Genescreen, NEN Research Products). Membranes were prehybridized, hybridized and washed under standard stringent conditions recommended by Genescreen. Probes for *MLS1* (MLSF-5' GTCTCAATATTCCCGCAGGGT 3' and MLSR-5' CCAGATGATAGGAGCCCCAG 3'), *ICL1* (ICLF-5' TCCATCACCTACGTCGCTTG 3' and ICLR-5'AATGTCCCGCGTCTAACAGC 3') and *MDH1* (MDHF-5'TGTACGACCTAAAGGGCGCA 3' and MDHR-5'CGATGACGTCTCTTTCGCCT 3') were amplified by PCR using yeast genomic DNA. The primers were designed using the primer-designing tool of DNAMAN software. Hybridization probes were radioactively labeled with [^{32}P]CTP using Oligolabelling kit (Amersham Biosciences, Ontario, Canada). Due to constraints in handling several samples for RNA isolation and northern blotting, the experiment was repeated twice without replicates. The data generated from each experiment was considered as replicates for statistical purpose.

2.2.4. Overexpression of *CIT1* and *MDH1* in MMYO11

A construct carrying the genomic clone for *CIT1* and *MDH1* (with their own promoter region) was previously overexpressed in wild type, MMYO11 (Anoop *et*

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al., 2003; Chapter 4) by transforming 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$^-$, Tryptophan$^-$ plates. Increase in *CIT1* and *MDH1* transcripts was confirmed by Northern analyses using yeast [^{32}P]CTP-labeled *CIT1* and *MDH1* as probes (Anoop *et al.*, 2003; Chapter 4). Cellular and extracellular malate content was estimated in the transformants as described below.

2.2.5. Sample preparation for malate estimation

Yeast cells grown in 3 ml LPP medium for 20 h were pelleted by centrifuging at 1300 g and washed three times with 3 ml of sterile water. The media and the supernatant from each wash were pooled as the extracellular fraction. Cell pellets were resuspended in 1 ml sterile water and cell density was determined by estimating OD_{600}. Cells were again pelleted (at 1300 g) and the supernatant was pooled into the extracellular fraction. It took 30 minutes to complete this procedure and throughout this time, the cells were retained on ice. Then the cells were suspended in 1 ml of boiling 80% (v/v) ethanol and vortexed vigorously to lyse the cells. The cell debris was then pelleted, the supernatant was passed through a 0.45 μm filter and the filtrate was used in the estimation of malate content in cells. The extracellular fraction was concentrated (to 1 ml) using an Evap-o-Vac (Cole Parmer, Ontario, Canada) at room temperature and pH was brought to 2.0 with 0.5 N HCl to dissociate Al-malate

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complexes. The sample was then passed through a cation exchange column with 1.0 ml resin (Bio-Rad), eluted with 10 ml deionized water and concentrated to 1.0 ml. An aliquot of 0.5 ml of this sample was used for citrate assay as described earlier.

2.2.6. Enzymatic analysis of malate

An enzymatic malate assay was performed as described by Delhaize *et al.* (1993). The enzymatic reaction included 0.5 ml of sample or standard, with 0.6 ml of Glycine buffer (0.5 M Glycine and 0.4 M Hydrazine, pH 9.2) and 40 μl of 40 mM NAD^+. A stable initial A_{340} was recorded and the formation of NADH was followed after the addition of MDH (22 U $reaction^{-1}$). The final stable A_{340} was then recorded.

2.2.7. Experimental design and statistical analysis

All experiments included three independent replicates and statistical analyses were performed using Sigmastat statistical analysis package (Version 1.0, Jandel Scientific). 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 times to ensure reproducibility of results. Data presented here are representative of each experiment.

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2.3. Results

2.3.1. The effect of Al on transcript abundance of TCA and glyoxylate cycle genes in MMYO11

The effect of Al on the expression of TCA and glyoxylate cycle genes was characterized in wild type yeast, MMYO11 by Northern analysis (Figure 2-2). Transcript abundance was estimated based on densitometric quantification of *ICL1*, *MLS1* and *MDH1* bands relative to rRNA. The transcript level for isocitrate lyase (*ICL1*) showed a significant increase to 210% at 200 µM Al and to 317% at 400 µM Al relative to control. Although the malate synthase (*MLS1*) transcript appears to be induced at 100 µM Al in the replicate presented in Figure 2-2A, densitometric scanning of both replicates indicted that transcript abundance was not significantly affected by exposure to Al (Figure 2-2B). The *MLS1* transcript level was also reduced at 200 µM and 400 µM Al (60% and 43%, respectively) compared to its control (Figure 2-2). Aluminum also had no significant effect on the transcript level of a major mitochondrial *MDH (MDH1)* even at 400 µM Al (Figure 2-2).

2.3.2. Aluminum sensitivity of yeast mutants with modified malate metabolism

The wild type yeast, MMYO11, showed progressive reduction in its growth (OD_{600} is 0.736 ± 0.018 at 0 µM Al) when exposed to varying concentrations of Al. The growth was reduced by 48% at 400 µM Al (OD_{600} 0.421 ± 0.033). Among the glyoxylate mutants studied, Δ*icl1* was hypersensitive to 400 µM Al

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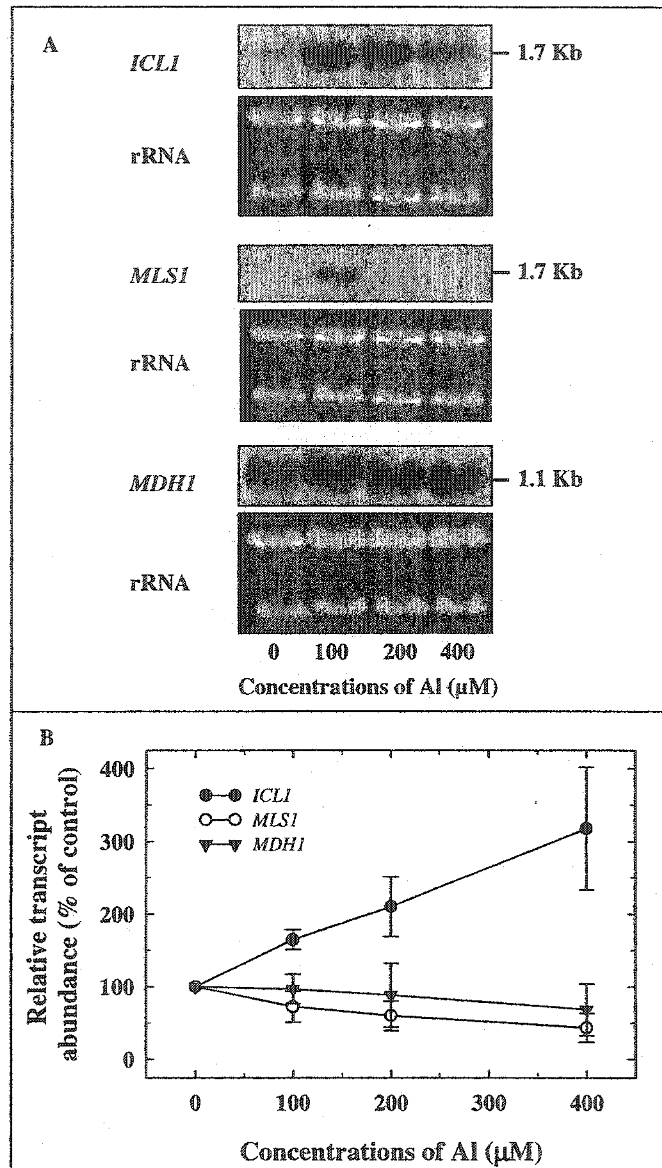

Figure 2-2. The effect of Al (0 to 400 μM) on transcript abundance in WT yeast (MMYO11). A. Transcript levels of *ICL1, MLS1* and *MDH1* in WT yeast. Total RNA isolated from yeast cells exposed to different concentrations of Al for 20 h, was probed with ^{32}P-labelled PCR amplified fragments of *MLS1, ICL1* and *MDH1*. B. Relative abundance of *ICL1, MLS1* and *MDH1* transcripts based on densitometry quantification of the RNA. Vertical bars represent standard error (n=2).

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showing 16.3% more reduction in growth compared to the WT (Figure 2-3A). Aluminum did not cause a significant reduction in the growth of Δ*mls1* mutant compared to WT (Figure 2-3A). The effect of Al on these glyoxylate cycle gene deficient mutants in LPP medium with limited sugar (0.2% glucose) or 2% glycerol as carbon source (data not shown) resulted in the same growth pattern as that of LPP medium with 2% glucose.

Among the TCA cycle mutants involved in malate synthesis, the mutants, Δ*fum1* and Δ*mdh1*, showed no enhanced sensitivity to Al compared to the WT, MMYO11. Disruption of the cytosolic isoform of *MDH* (Δ*mdh2*), resulted in a dramatic reduction (80%) in growth at 400 μM Al compared to a 48% reduction in the WT at the same Al concentration (Figure 2-3B). The OD_{600} of Δ*mdh2* was 0.819 ± 0.033 at 0 μM Al and was reduced to 0.167 ± 0.652 at 400 μM Al. The double mutant of *MDH*, Δ*mdh12* also showed enhanced sensitivity to Al (46% and 88% reduction in growth at 200 μM and 400 μM Al respectively, compared to its control) (Figure 2-3B).

2.3.3. Cellular and extracellular malate content in glyoxylate and TCA cycle disruption mutants

To determine if the differential Al sensitivity of the mutants disrupted for genes involved in malate metabolism was due to differences in their malate content, cellular and extracellular malate levels were measured. The cellular and extracellular

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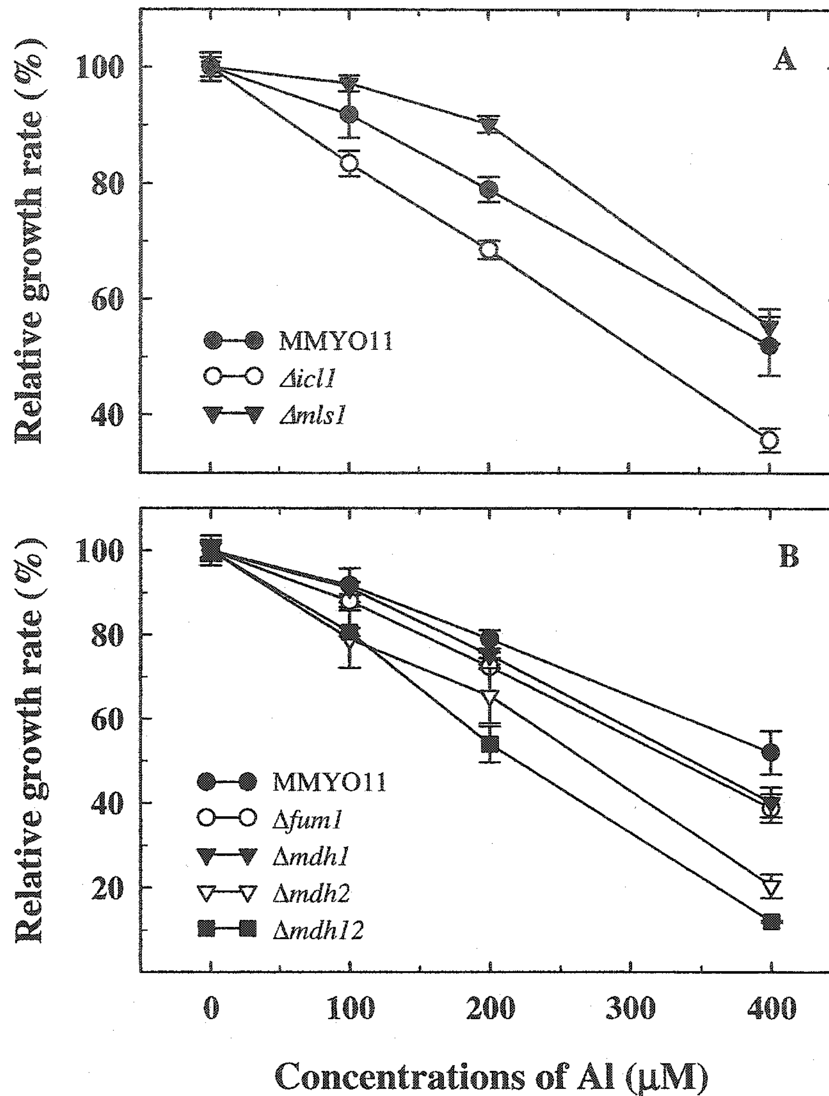

Figure 2-3. The effect of Al on relative growth rate (% of control) of WT and disruption mutants involved in malate metabolism. (A) Glyoxylate cycle mutants (Δ*mls1* and Δ*icl1*). (B) Mutants defective in fumarase and malate dehydrogenases (Δ*fum1*, Δ*mdh1*, Δ*mdh2 and* Δ*mdh12*). Cells of different genotypes were grown in LPP medium with different concentrations of Al for 18-20 h at 30°C and A_{600} was measured. Vertical bars represent standard error (n=3).

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malate content in MMYO11 was 17.3 ± 0.8 nmol 10^8 cells^{-1} and 1.6 ± 0.1 nmol 10^8 cells $^{-1}$ respectively. The mutants disrupted for glyoxylate cycle genes, the Δ*mls1* and Δ*icl1*, showed no significant difference in their malate content (cellular and extracellular) compared to the WT (Figure 2-4A). Among the mutants defective in malate dehydrogenases, Δ*mdh1* (16.8 ± 0.7 nmol 10^8 cells^{-1}) and Δ*mdh2* (20.8 ± 2.0 nmol 10^8 cells^{-1}) mutants showed no difference in cellular malate levels compared to WT. However, in the double mutant, Δ*mdh12*, cellular malate levels (12.3 ± 0.9 nmol 10^8 cells^{-1}) were significantly lower (30%) compared to the WT (Figure 4A). Interestingly, extracellular malate levels were significantly higher in Δ*mdh1* (4 fold) and Δ*mdh12* (5 fold) than in WT, but no such difference in extracellular malate content was observed in Δ*mdh2* mutant (Figure 2-4A).

In order to determine if Al affected the levels of malate in mutants with potentially altered malate metabolism, malate content was analyzed in WT and mutants grown in the presence of 400 μM Al. The cellular malate level of the WT (6.5 ± 1.2 nmol 10^8 cells^{-1}) was significantly reduced (2.6 fold) compared to its control (Figure 4B). Similarly, all mutants showed a reduction (2.2- to 2.9-fold) in cellular malate content, compared to their respective controls (Figure 2-4B). The extracellular malate content could not be measured in WT and the mutants, when grown in the presence of 400 μM Al due to interference of enzymatic assay by the sample.

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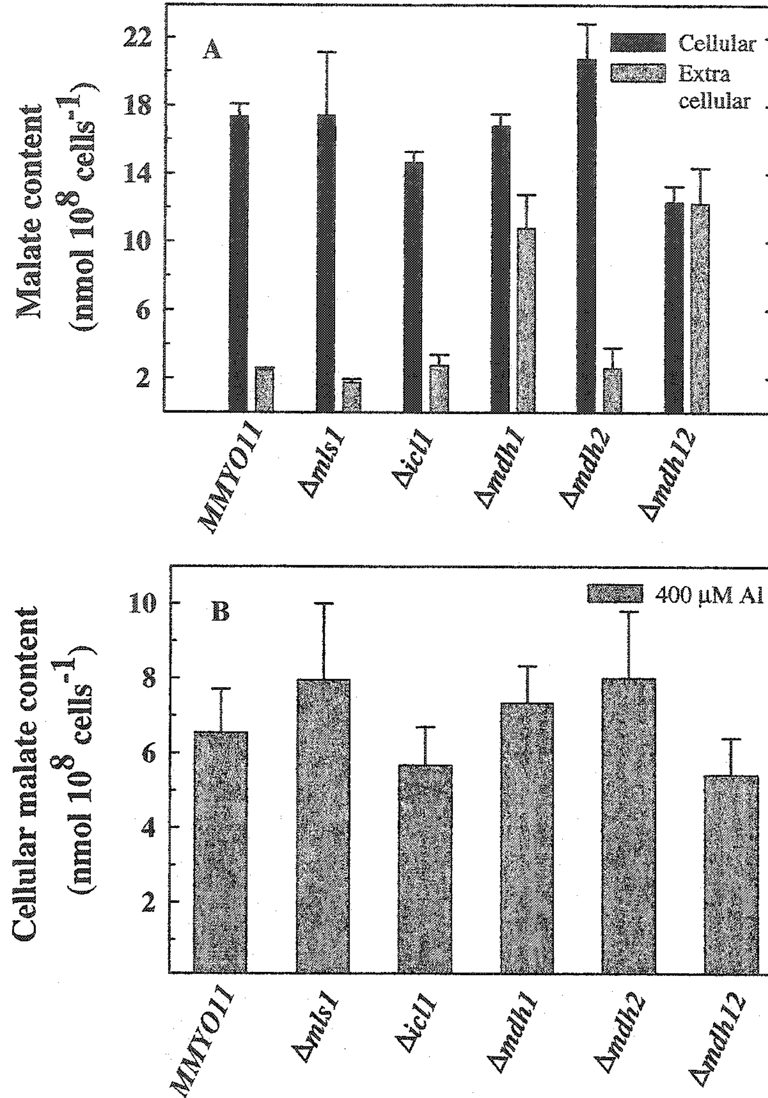

Figure 2-4. Cellular and extracellular malate levels in WT yeast and disruption mutants with altered malate metabolism. (A) Cellular and extracellular malate levels at 0 µM Al (B) Cellular malate content in the WT and mutants in the presence of 400 µM Al. Samples were prepared from cultures of specific genotypes grown in LPP medium (with 0 and 400 µM Al) for 20 h and used for estimation of malate. Vertical bars represent standard errors (n=3).

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2.3.4. Overexpression of TCA cycle genes (*CIT1* and *MDH1*) increases malate content in MMYO11

In an earlier study, I developed transformants overexpressing *MDH1* in combination with *CIT1* (MMYO11/*CIT1* + *MDH1)* and *CIT1* alone in WT yeast (MMYO11/*CIT1)*. These transformants showed enhanced Al resistance with a two-fold increase in citrate content (Anoop *et al.*, 2003; Chapter 4). In order to investigate if overexpression of genes for *CIT1* or *CIT1* + *MDH1,* leads to a simultaneous increase in malate levels (along with citrate), or to a depletion of cellular malate pool, the transformants, MMYO11/*CIT1* and MMYO11/*CIT1+MDH1* were analyzed for their malate levels. A significant increase in cellular malate levels was observed in MMYO11/CIT1 (27.3 ± 1.8 nmol 10^8 cells^{-1}) and MMYO11/CIT1 + MDH1 (40.9 ± 4.7 nmol 10^8 cells^{-1}) compared to the WT (19.4 ± 2.7 nmol 10^8 cells^{-1}; Figure 2-5B).

In the presence of 400 μM Al, the cellular malate content was significantly higher in the transformants (MMYO11/*CIT1*, (17.3 ± 2.3 nmol 10^8 cells^{-1} and MMYO11/*CIT1* + *MDH1*, 40.9 ± 4.7 nmol 10^8 cells^{-1}) than in the WT (5.4 ± 1.1 nmol 10^8 cells^{-1}) and vector controls transformed with plasmids, Yep352 and Yep352 + pRS424 (Figure 2-5). The extracellular malate content in both transformants was not significantly different from the WT (data not shown).

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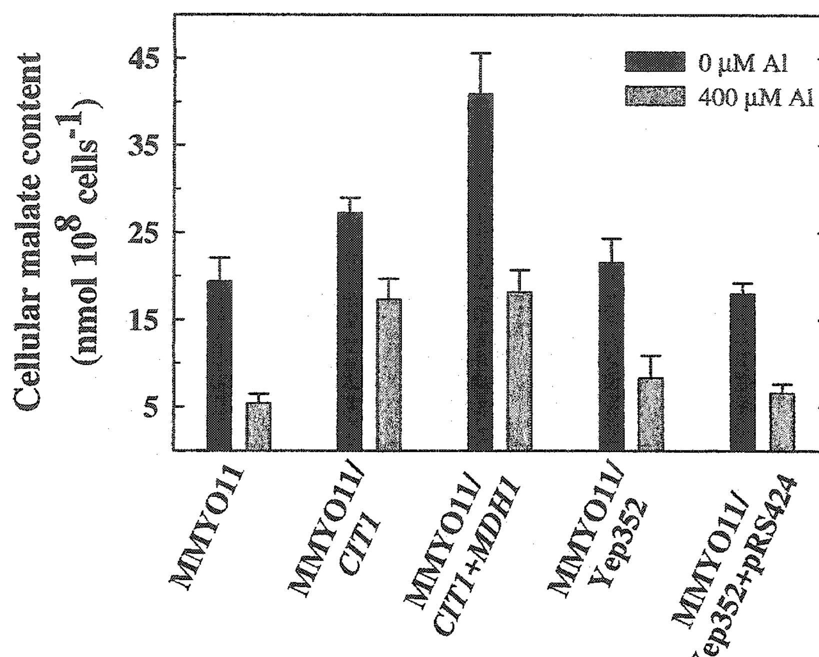

Figure 2-5. Cellular malate content in WT yeast and yeast transformants overexpressing *CIT1* (MMYO11/*CIT1*), *CIT1* and *MDH1* (MMYO11/*CIT1* + *MDH1*). Controls include transformants carrying vector plasmids, Yep352 and Yep352 + pRS424. Cultures of different genotypes were grown in LPP medium for 20 h at 30°C were used for determination of malate. Vertical bars represent standard errors (n=3).

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2.4. Discussion

Defects in TCA or glyoxylate cycle genes could alter the expression of other genes and the levels of gene products. Transcriptional profiling using microarray analysis has revealed that the expression of over 400 genes change in response to defects in one of 15 yeast TCA cycle genes and in response to altered TCA cycle metabolites such as citrate and malate (McCammon *et al.*, 2003). In order to explore the alterations in malate metabolism due to Al toxicity, yeast mutants defective in various TCA-, glyoxylate- and gluconeogenetic-pathway-genes involved in malate synthesis were used in this study (Figure 2-1 and Table 2-1).

Northern analysis of WT yeast cells exposed to varying concentrations of Al (0 to 400 μM), revealed that the abundance of *ICL1* transcript was increased up to 2-fold of the control, while the expression of *MLS1* transcript was not significantly affected by Al. Hamel and Appana (2000) observed an Al-induced increase in ICL activity in *Pseudomonas fluorescens* leading to an increase in cellular oxalate levels. No changes in MS activity or malate levels were observed in their study. In this study, disruption of glyoxylate cycle genes (Δ*mls1* and Δ*icl1*) did not alter their cellular and extracellular malate contents or the sensitivity of mutants to Al (Figure 2-3A and 2-4A). A mutant defective in fumarase (Δ*fum1*) also showed no enhanced sensitivity to Al (Figure 2-3B), possibly due lack of changes in their malate levels compared to the WT. Pines *et al.* (1996) reported that malate levels were not reduced in yeast cells lacking mitochondrial fumarase and suggested that the

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cytosolic reductive pathway leading from pyruvate via OAA to malate would be the major pathway for malate production in cells.

The single mutant, Δ*mdh1,* defective in mitochondrial *MDH*, showed no change in levels of cellular malate and in Al sensitivity compared to the WT, MMYO11. Gene redundancy and the presence of multiple isoforms of *MDH* might compensate for the loss of gene function of one isoform (Small and McAlister-Henn, 1997). In addition, malate moves freely between cellular compartments (Picault *et al.*, 2002). Thus, malate might be available for functioning of the TCA cycle, even in the absence of *MDH1*. In this study, enhanced Al sensitivity was observed in mutants lacking *MDH2* (Δ*mdh2* and Δ *mdh12*), suggesting that cytosolic *MDH* could increase Al sensitivity of yeast cells (Figure 2-3B). But this enhanced sensitivity to Al in these mutants could not be associated with their cellular malate content (Figure 2-4A and 2-4B). The mutant, Δ*mdh2* showed no reduction in malate content while Δ*mdh12* showed a slight reduction of 30% compared to the WT.

Several studies have shown that enhanced synthesis and exudation of malate from plant roots is associated with Al resistance (Basu *et al.*, 1994; Ryan *et al.*, 1995; Pellet *et al.*, 1996; Osawa and Matsumoto, 2002). In this study, however, no direct correlation between levels of cellular and extracellular malate and the Al sensitivity of mutants defective in genes for malate dehydrogenases (Δ*mdh2* and Δ*mdh12*) was observed. It is possible that disruption of *MDH2* and *MDH12*, could alter the

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expression of other gluconeogenic and TCA cycle genes, thereby affecting the synthesis and accumulation of other organic anions. This could potentially increase the Al sensitivity of these mutants. Gibson and McAlister-Henn (2003) recently reported that cytosolic *MDH* (*MDH2*) interacts with the gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, and influences the functioning of these enzymes. Metabolite profiling of these yeast mutants could help to identify the changes in other metabolite levels such as OAA, phosphoenol pyruvate and citrate that could potentially alter their Al sensitivity.

The extracellular malate content in Δ*mdh1* and Δ*mdh12* was 4 to 5 fold higher than in the WT (Figure 2-4A). The increase in extracellular malate content in Δ*mdh1* and Δ*mdh12* was not correlated with intracellular malate levels. Mutation of mitochondrial MDH (*MDH1*) impairs malate transport across mitochondrial membrane (Sandor *et al.*, 1994). If the transport of non-mitochondrial malate pool into the TCA cycle is impaired, in the mutants defective in *MDH1* (Δ*mdh1* and Δ*mdh12*), malate accumulated in the cytosol could possibly be exuded out of cells to prevent cytoplasmic acidiosis. It would be interesting to study how Al affects the malate transporters in yeast cells.

A significant Al-induced reduction in cellular malate levels was observed in WT (62% of its control) and all the mutants (54 to 66% of their controls) tested for malate metabolism (Figure 2-4B). Aluminum-induced reduction in malate, succinate and α-ketoglutarate contents has been reported in the tree species, *Melastoma*

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malabathricum (Watanabe and Osaki, 2002). Reduction in cellular malate levels upon exposure to Al (Figure 2-4B) could be due to inhibition of malate metabolism by Al. Aluminum causes serious damage to cellular metabolism, inhibiting the activities of several enzymes such as phosphofructokinase and hexokinase (Xu *et al.*, 1990; Socorro *et al.*, 2000). In this study, Al had no apparent effects on the transcript levels of mitochondrial *MDH1*, but other isoform(s) of *MDH* (cytosolic *MDH2* and peroxisomal *MDH3*) could be sensitive to Al, leading to a reduction in synthesis and accumulation of malate. The reduction in malate levels upon exposure to Al could also be due to chelation of cellular malate with Al (Al-malate complex), which could have reduced the detection of cellular malate levels by the enzymatic method. Indeed, in samples (extracellular fraction) prepared from cultures grown with Al, the extracellular malate content could not be measured due to some interference in the enzymatic assay of malate.

Sequential TCA cycle enzymes are suggested to form multi-enzyme complexes termed "metabolons" and formation of such complexes favors the kinetics of enzymes involved in the complex (Sumegi and Srere, 1984; Przybyla-Zawislak *et al.*, 1999). However, in my studies, the effect of Al on transcript abundance of CS (*CIT1*) and *MDH1* were not similar. I previously observed a 50% increase in *CIT1* expression in WT cells exposed to 400 μM Al (Anoop *et al.*, 2003; Chapter 4). In the present study, Al caused no alterations in transcript abundance of *MDH1* (Figure 2-2). Mutations in mitochondrial enzymes such as *CS* or *IDH*, affect not only the activity of the enzyme, but also the organization of other mitochondrial enzymes (Sumegi *et*

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al., 1992). Since *CIT1* and *MDH1* are two sequential enzymes in the TCA cycle metabolon, overexpression or disruption of one gene could influence the expression of the other gene or gene product, or the transport of the metabolite across membranes. For instance, disruption of genes of the immediately proximal (*FUM1*) or distal steps (*CIT1*) of the TCA cycle reactions involving malate synthesis, compromised malate transport (Sandor *et al.*, 1994). In my study, the cellular malate content (22.6 ± 4.1 nmol 10^8 cells^{-1}) of a mutant disrupted for both the sequential genes, Δ*cit1*Δ*mdh1*, was not significantly different from the WT, perhaps indicating the redundant TCA cycle gene functions.

I have previously shown that these transformants overexpressing genes for *CIT1* and *MDH1* in MMYO11 showed increased levels of citrate accumulation and Al resistance than the WT (Anoop *et al.*, 2003; Chapter 4). When these transformants were analyzed for their malate levels, their cellular malate content (1.4 and 2.1 fold increase in MMYO11/*CIT1* and MMYO11/*CIT1* + *MDH1*, respectively) was significantly higher than in the WT (Figure 2-5). This suggests that in addition to an increase in citrate levels, enhanced cellular malate levels could also have contributed to increased Al resistance in these transformants, due to the intricate relationship between the two genes of the metabolon complex. Overexpression of a cytosolic MDH in yeast has resulted in a 3-fold increase in cellular malate (Pines *et al.*, 1997). Similarly, overexpression of a nodule specific *MDH* resulted in enhanced synthesis and exudation of malate from alfalfa roots and also a significant increase in Al resistance (Tesfaye *et al.*, 2001).

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There is clear evidence that malate is a potent chelator of Al, and Al-induced increases in malate accumulation and exudation can protect plants from Al toxicity (Basu *et al.*, 1994; Ryan *et al.*, 1995; Pellet *et al.*, 1995; Osawa and Matsumoto, 2002). In my previous study, yeast transformants overexpressing mitochondrial *CIT1* alone or in combination with *MDH1*, showed enhanced Al resistance, perhaps due to increases in citrate levels. In this study, I observed increased levels of malate in these transformants, indicating that *MDH1* and *CIT1* play an important role in the synthesis of malate and in Al resistance. However, no direct correlation was observed between levels of cellular and extracellular malate and the Al sensitivity in various yeast disruption mutants tested for altered malate metabolism (*MLS1, ICL1, MDH1, MDH2* and *MDH12*). The absence of an association between malate content and Al resistance in yeast mutants suggest that the complexity of malate metabolism and the effect of gene redundancy should be taken into consideration while attempting gene manipulation of *MDH*. My studies with yeast mutants defective in genes involved in malate metabolism also revealed that Al caused a reduction in cellular malate levels in WT and all mutants tested for malate metabolism. If attempts are made to develop transgenic plants with reduced levels of malate or characterize MDH gene knockout lines, a single gene approach may not be sufficient to reduce the levels of malate appropriately, to test for their role in Al resistance.

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Reference:

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3. Investigating the potential role of malate in aluminum resistance using transgenic *Brassica napus* L. overexpressing a gene for glyoxysomal malate synthase

3.1. Introduction

Exudation of organic anions such as citrate and malate from plant roots has been proposed as one of the major Al tolerance mechanisms. Malate is suggested to protect sensitive root cells by chelating toxic Al ions in the rhizosphere, forming non-toxic complexes with Al. Malate is the predominant organic anion released from root apices of Al-tolerant wheat (*Triticum aestivum* L.) genotypes (Delhaize *et al*, 1993, Basu *et al.,* 1994; Ryan *et al.,* 1995b; Pellet *et al.,* 1996). Other crop species such as *Triticale* sp., rye (*Secale cereale* L.) and canola (*Brassica napus* L.) also exude malate in response to Al (Ma, 2000, Li *et al.*, 2000; Zheng *et al.*, 1998).

Malate exists predominantly as a divalent anion in the cytoplasm. The large negative membrane potential across the plasma membrane and the high concentration gradient between the cytosol and rhizosphere (or apoplast) facilitate movement of malate out of root cells by an energetically passive process (Kochian 1995; Ryan *et al.*, 1997). Aluminum-stimulated malate efflux in wheat has been detected within minutes after exposure to Al (Ryan *et al.*, 1995a). This rapid release was associated with Al-dependent activation of malate permeable anion channels in the plasma membrane of root apical cells (Ryan *et al.*, 1997). Several other studies have described activation of anion channels in protoplasts of wheat and maize by Al,

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leading to exudation of organic anions (Zhang *et al.*, 2001; Kollmeier *et al.*, 2001). Activation of anion channels was therefore, considered to be a key factor in Al-induced exudation of organic anions, since no apparent correlation was found between root organic anion concentration and exudation of organic anions. For example, the malate content in root tissues was not significantly different between wheat genotypes that differed in amount of malate exuded in response to Al (Ryan *et al.*, 1997).

In contrast to the results from short-term experiments, Basu *et al.* (1994) demonstrated that Al-induced exudation of malate from wheat roots after longer-term exposures (24 h), involved *de novo* synthesis of the organic anion. Other recent reports also highlight the importance of alterations in organic acid metabolism in the exudation of organic anions and their subsequent role in Al resistance. An Al-induced increase in root citrate content and an accompanying reduction in root aconitate content was observed in roots of Al-tolerant maize (Piñeros and Kochian, 2001). Furthermore, changes in gene expression and activities of enzymes involved in organic anion synthesis have been linked to enhanced exudation of organic anions. An Al-induced increase in citrate synthase (CS) mRNA expression has been reported in yeast (Anoop *et al.*, 2003; Chapter 4). Increases in CS activity have been correlated to enhanced exudation of citrate and enhanced Al resistance in several studies (Li *et al.*, 2000; Takita *et al.*, 1999). The majority of studies dealing with changes in gene expression and enzyme activities have focused on citrate metabolism. Plants that exude malate in response to Al showed no such alterations in either

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expression of genes or activities of enzymes involved in malate metabolism (Ryan *et al.*, 1995, Li *et al.*, 2000). However, the role of *MDH* in enhancing synthesis and efflux of malate, and Al resistance was demonstrated by overexpressing a gene for *MDH* in alfalfa (*Medicago sativa*; Tesfaye *et al.*, 2001).

Malate metabolism has complex enzymology due to its versatility in cellular processes such as photosynthesis (C4 and CAM), stomatal movement, nutrient uptake, respiration, nitrogen assimilation and fatty acid oxidation (Vance, 1997; Miller *et al.*, 1998). Malate is largely synthesized by the combined action of phosphoenolpyruvate carboxylase (*PEPC*; EC 4.1.1.31), which catalyzes conversion of phosphoenolpyruvate to oxaloacetate, and of malate dehydrogenase (*MDH*; EC 1.1.1.37), which causes oxidation of oxaloacetate (OAA) to form malate (Vance, 1997; Tesfaye *et al.*, 2001). Overexpression of *PEPC* and *MDH* has resulted in enhanced levels of cellular malate and OAA in transgenic lines of potato (*Solanum tuberosum*) and alfalfa (*Medicago sativa*) (Rademacher *et al.*, 2002; Tesfaye *et al.*, 2001). The *MDH* overexpressing lines have been tested for Al resistance, with transgenic alfalfa plants showing enhanced exudation of malate and increased Al resistance (Tesfaye *et al.*, 2001). Another enzyme involved in malate biosynthesis is fumarase (EC 4.2.1.2), which converts fumarate to malate. Overexpression of fumarase in yeast resulted in an increase in malate (Pines *et al.*, 1996), but this strategy has not been used to test in amelioration of Al toxicity.

Malate synthase (MS, EC 4.1.3.2) is another important enzyme catalyzing the synthesis of malate by the condensation of glyoxylate and acetyl-CoA in

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glyoxysomes. Though a glyoxylate cycle enzyme, the presence of MS has been reported in different tissues with varying levels of activity (Hock and Beevers, 1966; Wier *et al.*, 1980; Turley and Trelease, 1987). It is induced in detached leaves and roots, senescing plant organs and under different kinds of stresses (Graham *et al.*, 1992). The roles of MS in various organs involved in embryogenesis and post-germination have been thoroughly investigated. In contrast, the occurrence and function of MS in plants under aluminum stress is not well understood.

Seed germination and post-germinative growth are crucial stages in the life cycle of higher plants. During this period, the seedling must adapt its metabolic and developmental programmes to the prevailing environmental conditions to achieve photoautotrophism before its nutrient reserves become exhausted. I hypothesized that if seedlings have enhanced ability to synthesize and exude malate during the critical stages of development, it would then enable better survival of the seedlings under conditions of Al stress. Experiments carried out to test if overexpression of MS could lead to enhanced synthesis and exudation of malate from seedlings, and whether it would help the plant to overcome the phytotoxicity of Al at the seedling stage are described here.

3.2. Materials and methods

3.2.1. Plant material and growth conditions

Brassica napus L. cultivar Westar used in this study was tested for its sensitivity to Al during germination and seedling establishment. Seeds were

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germinated and grown in a controlled environment chamber at 22° C with 16 h light (300 µmol m^{-2} s^{-1}) and 8 h dark. Seedlings were grown in full nutrient solution (FNS, pH 4.2) that contained (in µM) 1000 Ca, 300 Mg, 800 K, 3300 NO_3^-, 300 NH_4^-, 100 PO_4^{3-}, 100.65 SO_4^{2-}, 34 Cl, 20.2 Na, 10 Fe, 6 B, 2 Mn, 0.5 Zn, 0.15 Cu, 0.1 Mo and 10 EDTA using the following salts $Ca(NO_3)_2$, $Mg(NO_3)_2$, NH_4NO_3, K_2HPO_4, K_2SO_4, KNO_3, $MnCl_2$, H_3BO_3, $ZnSO_4$, $CuSO_4$, Na_2MoO_4, $FeCl_3$ and Na_2EDTA. For experiments involving Al exposures, seedlings were grown in FNS containing various concentrations of $AlCl_3$ (0 to 200 µM).

3.2.2. The effect of Al on germination of canola seeds and MS mRNA

For studies on germination, seeds were germinated on sterile sand moistened with either MQ-water or with MQ-water with 150 µM Al. While seeds were germinating and growing, moisture in the sand medium was maintained by adding equal amounts of appropriate solution. The number of seeds germinated was counted three days after germination (DAG) and percent germination was expressed relative to the number of seeds germinated in control (0 µM Al).

3.2.3. Root growth elongation assay

The sensitivity of canola seedlings to Al was tested by determining root and shoot elongation in the presence or absence of Al. Seeds were germinated on a bed of sand moistened with MQ water. Seedlings (2 days old) were plated onto 1 ml graduated syringe tubes with bottoms and plungers removed, and tops filled with cotton plug to support the seedlings. Roots of seedlings grown over the syringes

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elongated in a straight line, enabling easy handling of the seedlings and accurate measurements of root lengths. Syringes were mounted onto a plastic tray and floated in a 15 L aquaria filled with 10 L of full nutrient solution (FNS) at pH 4.2.

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 and the final root lengths were recorded.

3.2.4. Plant transformation to develop transgenic lines overexpressing pHS737 and pHS737-MS

3.2.4.1. Preparation of construct carrying *Bn-MS*

The gene for MS (in pBluescribe phagemid, pBS+/-) isolated from *B. napus*, was obtained from Dr. J.J. Harada (University of California, Davis, California) (Figure 3-1A). In order to introduce appropriate restriction enzyme sites (*Eco*RI and *Kpn*I) suitable for cloning into the binary vector, the MS gene was PCR amplified using a T7 primer and a MS gene specific primer that was designed to have a restriction enzyme site for *Eco*RI at 5' primer position. This primer combination flanked the MS open reading frame of 1911bp DNA and also the restriction site for *Kpn*I upstream of the MS gene (Figure 3-1 A and 1B). All PCR reactions were done

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using Taq DNA polymerase enzyme (Qiagen, Ontario, Canada). The PCR amplification products were then electrophoresed on 1% (w/v) agarose gel in TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0) and the amplified fragment of 1.91 Kb was excised out of the gel and purified using Qiagen gel extraction kit (Qiagen, Ontario, Canada). The purified fragment was then ligated into a pBluescript-cloning vector (pBSK+/-) at *Eco*RI -*Kpn*I restriction sites and transformed into DH5α *E. coli* cells for further plasmid amplification and maintenance (Figure 3-1B).

The MS gene was excised out of plasmid, pBSK-MS, using *Eco*RI and *Kpn*I and ligated into the binary vector, pHS737 (provided by Plant Biotechnology Institute, NRC, Saskatoon, Canada), such that it was under the control of the constitutive CaMV promoter. This binary vector has a *NPT:GUS* fusion as selectable markers (coding for Kanamycin resistance and β-glucoronidase, GUS) under the control of the *Nos* promoter (Figure 3-1C). The presence of the gene insert in the resulting construct, pHS737-MS, was confirmed by PCR and also by sequencing using the following gene specific primers, *MSINF1, MSINF2, MSINR1* and *MSINR2* (positions indicated in Figure 3-1C) and with a primer from the CaMV promoter region, *35S-F* (Table 1). The nucleotide sequences were then aligned using DNAMAN software and the sequence of MS in pHS737-MS was compared with the MS of *B. napus* using NCBI-BLAST database (National Center for Biotechnology Information-Basic Local Alignment Search Tool) to confirm that no nucleotide mismatches was present in the PCR amplified MS gene fragment.

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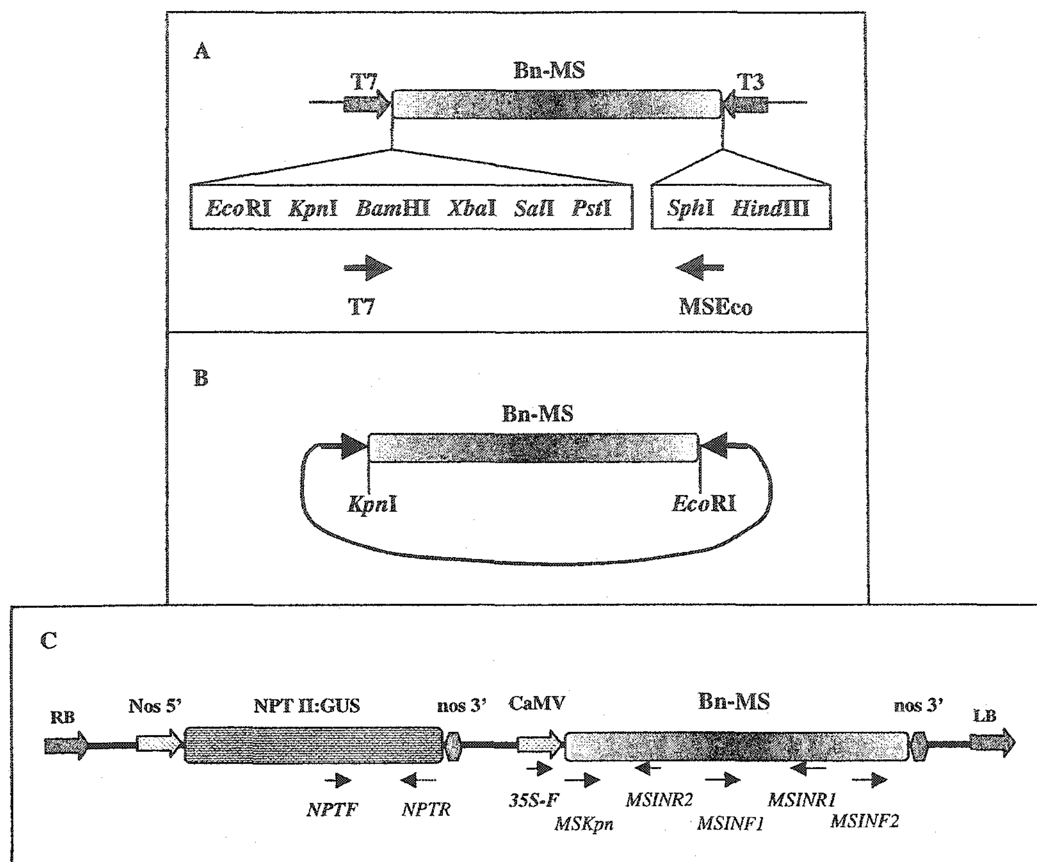

Figure 3-1. Preparation of the T-DNA construct for overexpression of a gene for *MS* (*Bn-MS*) in *B. napus*. (A) A full-length gene for *B. napus* MS in pBS, was PCR amplified with T7 and MSEco primers. (B) The PCR amplified fragment was ligated into a pSK (T-vector). The MS gene was restriction digested (*Kpn*I and *Eco*RI) and ligated into a T-DNA construct. (C) The T-DNA construct, pHS737-MS, with the MS gene under control of a constitutive cauliflower mosaic virus 35S promoter (CaMV). This T-DNA vector had *NPT II:GUS* fusion as selectable markers encoding Kanamycin resistance and β-glucoronidase respectively, which were under the control of Nos promoter. The right and left borders are represented as RB and LB. The arrows represent the positions of primers used in sequencing and genomic PCR analysis.

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Table 3-1. List of primers used in PCR for MS gene amplification, sequencing and amplification of fragments for probes used for hybridization purposes.

Primer Name	Primer sequence	Position on MS cDNA
T7	5'CGGGATATCACTCAGCATAATG3'	-
MSEco	5'GAGCTCTCAGAGCCTTGACGCTTA3'	1911 bp
MSKpn	5'GGGGTACCAAATGGAGCTAGAGACTCGGTT3'	54 bp
MSINF1	5'GGAACACTCTCGAGAAGCAA3'	743 bp
MSINF2	5'TGGAGTGGAGCTGGACGGAGA3'	1491 bp
MSINR1	5'TGCCGCCCATGCCGTGGA3'	1047 bp
MSINR2	5'CAGCACCAGAGTTGTAACGCC3'	231 bp
35S-F	5'CCACTGACGTAAGGGATGACGC3'	-
NPTF	5'GGCACAACAGACAATCGGC3'	-
NPTR	5'CGTAAAGCACGACGAGGAAGCG3'	-

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3.2.4.2. Electroporation of *Agrobacterium* with binary vectors

About 20 ng of the plasmid DNA (pHS737-MS construct and pHS737 vector) was mixed with 40 µl of thawed electrocompetent *Agrobacterium tumefaciens* (GV3101) cells in cold electroporation cuvettes (0.2 cm gap; BTX Genetronics Inc, CA). The mixture was subjected to electroporation (V =2.5 kV; R = 400 Ω; and C = 25 µF yielding a field strength of 12.5 kV cm^{-1}) using Gene Pulser II (Bio-Rad). The cells were then allowed to recover in LB medium (Luria-Bertani; 960 µl) for 2 hours at 28 °C. The electroporated cells were plated on to LB plates with Kanamycin (100 µg ml^{-1}). Controls included plating of electrocompetent cells on to selection medium as well as electrocompetent cells subjected to an electric field in the absence of Al. The presence of the construct (pHS737-MS) and the vector (pHS737) in the *Agrobacterium* was confirmed by PCR using the primers mentioned above.

3.2.4.3. Overexpression of Bn-MS in *B. napus* using an *Agrobacterium*-mediated transformation system

Seeds of *Brassica napus* L. cv. Westar (represented as WT, control plants in the transgenic studies) were surface sterilized in 15% (v/v) bleach for 15 minutes followed by 70 % (v/v) ethanol treatment for 2 minutes, and then washed thoroughly with sterile water. The sterilized seeds were plated on ½ strength MS medium (Murashige and Skoog basal medium, Sigma, Ontario, Canada) containing 0.8% (w/v) phytagar and 1% (w/v) sucrose at pH 5.8. An *Agrobacterium*-mediated plant transformation (Figure 3-2A to 2E) was carried out as described by Moloney *et al.* (1989). Cotyledonary petioles from 4-5 day-old seedlings were used as explants

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(Figure 3-2A). The *Agrobacterium* strains, GV 1031, carrying either pHS737-MS or pHS737, used to infect the explants were grown overnight in yeast extract peptone media (YEP- 2% yeast extract, 2% peptone, sodium chloride) with 100 $\mu g\ ml^{-1}$ Kanamycin.

Explants were cocultivated with *Agrobacterium* in M1 media (MS media with 3% sucrose (w/v) and 0.005% (w/v) Benzyladenine) for 2 days in the dark. They were transferred to M2 media (M1+ 300 $\mu g\ ml^{-1}$ Timentin) for a week to recover and regenerate (Figure 3-2B), and then to M3 selection media (M2 + 300 $\mu g\ ml^{-1}$ Timentin and 30 $\mu g\ ml^{-1}$ Kanamycin) for regeneration of calli to shoots (Figure 3-2C). The putative transformants with green shoots were transferred to M4 media (MS media with 0.005% (w/v) Indole butyric acid, 300 $\mu g\ ml^{-1}$ Timentin and 30 $\mu g\ ml^{-1}$ Kanamycin) for rooting (Figure 3-2D). All tissue culture materials were maintained in a controlled chamber (16 h light, 80 $\mu mol\ m^{-2}\ s^{-1}$, 20°C / 8 h dark). Presumptive positive plants remained green and rooted well into the rooting media (Figure 3-2E), while the untransformed plants turned white and did not develop further (Figure 3-2F).

Presumptive positive plants (T_1) were transferred to soil (Metromix, Apache seeds, Edmonton, Canada) and raised under controlled environment conditions (16 h light, 300 $\mu mol\ m^{-2}\ s^{-1}$ at 20°C / 8h dark at 18°C). The presence of the transgene (using genomic PCR analysis) and the level of MS gene expression (using northern analysis) was tested in 17 independent transgenic lines transformed with pHS737-MS; and 5 transgenic plants transformed with the vector, pHS737. Thirty seeds

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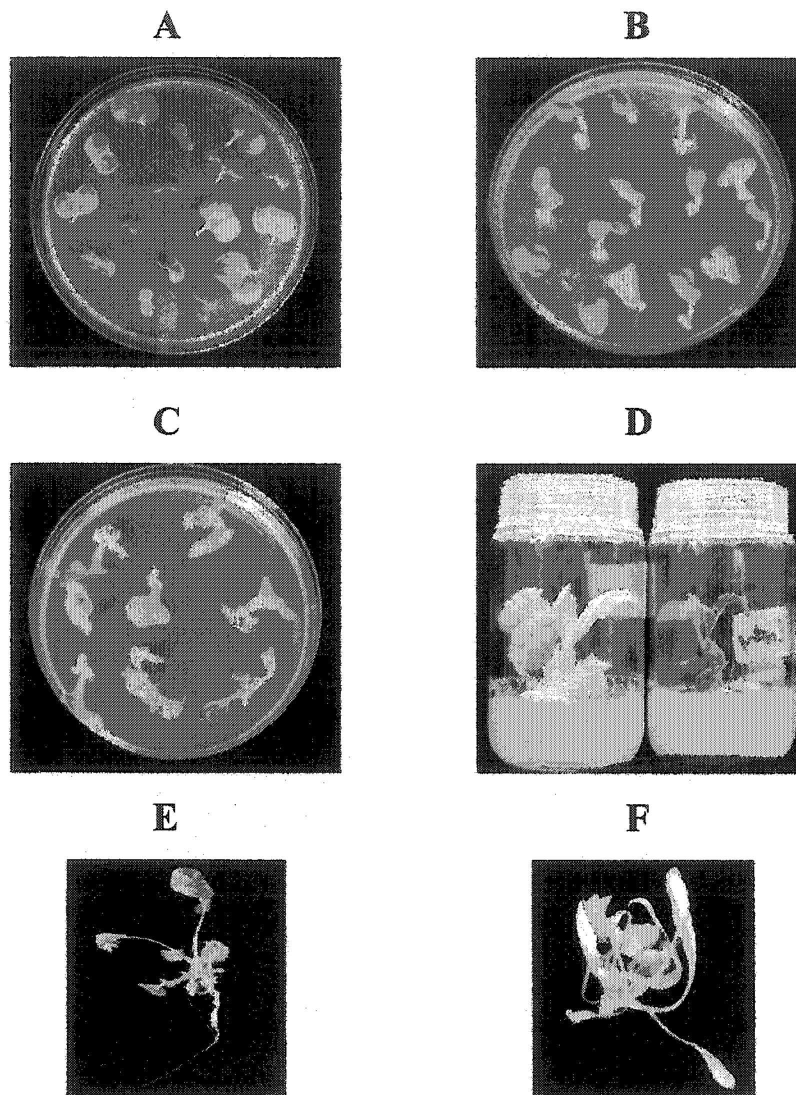

Figure 3-2. *Agrobacterium*-mediated transformation system used in developing transgenic canola plants overexpressing the pHS737-MS or pHS737 vector. (A) Cotyledonary petioles were used as explants. (B) Induction of callus and (C) regeneration of shoots from callus were done on MS media with Kanamycin (30 μg ml^{-1}). (D) Regenerated shoots were allowed to root in the rooting media with Kanamycin. (E) Presumptive positive plants remained green under antibiotic selection and (F) untransformed shoots bleached out.

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from each of the T_1 selfed-lines were surface sterilized and germinated on seed germination media with 150 µg ml^{-1} Kanamycin (Figure 3-3). Seedlings (T_2 plants), which remained green on antibiotic selection, were again tested by genomic PCR. Segregation ratios were determined based on number of green to bleached yellow seedlings. From each of the primary transformants (T_1), twenty 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.

3.2.5. Genomic DNA PCR and Southern analysis

Genomic DNA was isolated from shoots or roots of canola using QIAGEN DNeasy mini kit (Qiagen, Ontario, Canada). For genomic PCR analysis, the primers, *35S-F* and *MSINR1* were used for transgenic lines; and primers, *NPTF* and *NPTR*, were used for vector control lines primers (Table 3-1).

For southern analysis, genomic DNA (10 µg) isolated from seedlings of WT and the transgenic lines (MS3, MS5 and V2) was separated on 0.7% agarose gel at 30 V for 16 h. Hybridization probe for MS gene was prepared by PCR amplification using pBSK-MS as template DNA with MSKpn and MSINR1 (1.0 Kb). The amplified fragment was isolated using the Gel extraction kit (Qiagen, Ontario, Canada). The probe fragment was radioactively labeled with ^{32}P-CTP using Oligolabelling kit (Amersham Biosciences). Membranes were prehybridized and

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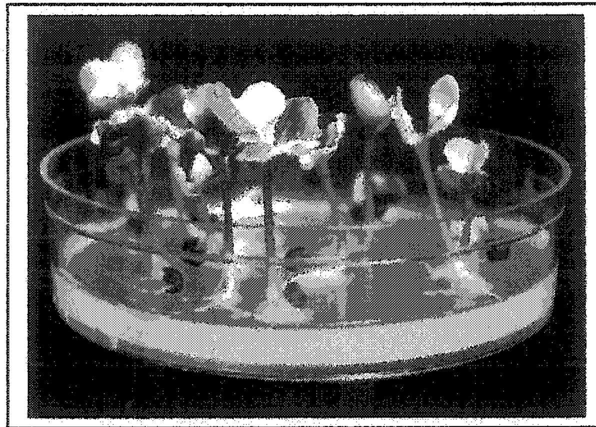
A



B

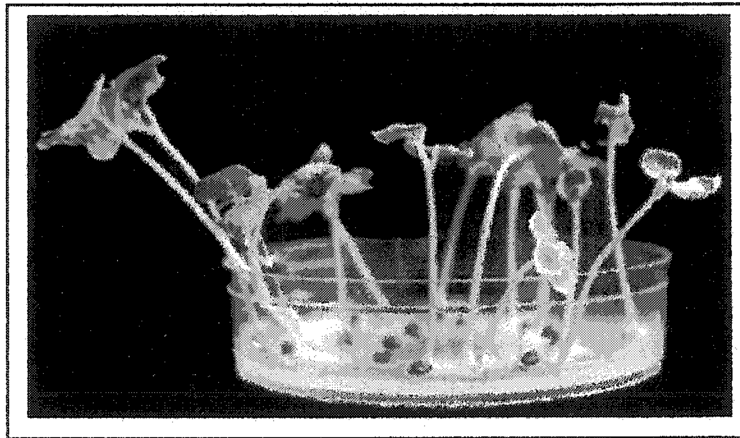


Figure 3-3. Antibiotic selection of transgenic canola seedlings on Kanamycin. Seeds of WT (A) and transgenic lines (T_1 seeds) overexpressing pHS737-MS or pHS737 (B), were surface sterilized and germinated on MS media with Kanamycin (150 μg ml^{-1}). Seedlings of the WT were bleached or purple and failed to grow further. The T_2 transgenic seedlings showed segregation into green and bleached seedlings.

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hybridized at 65°C overnight. The buffer used for prehybridization and hybridization consisted of 1% SDS, 10% dextran sulphate, 1 X Denhardt's reagent (2% Ficoll-400, 2% PVP and 2% BSA) and 2 X SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0) with denatured 100 µg salmon sperm DNA as carrier DNA. The hybridized membranes were washed under standard stringent conditions recommended by Genescreen.

3.2.6. Determining transcript abundance by Northern analysis

Total RNA was isolated from shoots and roots using the QIAGEN RNAeasy mini kit, separated by electrophoresis on agarose formaldehyde denaturing gels with MOPS solution (20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA, pH 7.0) as running buffer. The electrophoresed RNA was transferred to nitrocellulose membranes (Genescreen, NEN Research Products, Perkin Elmer Life Sciences Inc., USA). The blots were prehybridized for 2h and hybridized overnight at 42°C. The prehybridization and hybridization buffer consisted of 5 X SSPE (300 mM sodium chloride, 20 mM sodium phosphate and 2 mM EDTA), 50% (v/v) deionized formamide, 5 X Denhardt's reagent, 1% (w/v) SDS and 10% (w/v) dextran sulphate, with 100 µg salmon sperm DNA. Other standard conditions were followed as described under Southern analysis (3.2.5). For estimation of transcript abundance, the hybridized bands corresponding to appropriate transcript (MS, NPTII) and the 18S ribosomal RNA (rRNA, ethidium bromide stained) were scanned and quantified using Alpha imager (Canada).

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3.2.7. Estimation of MS enzyme activity

Seedlings were collected, frozen in liquid nitrogen, and stored at -70°C until used for enzyme assays. Approximately 200 mg tissue was ground in liquid nitrogen and homogenized with ice-cold MS homogenization buffer (100 mM K_2PO_4 and 6 mM $MgCl_2$, pH 7.0). The extract was centrifuged for 10 min at 4°C, and supernatant was collected and used in the enzyme assay. The reaction mix for MS enzyme assay consisted of 0.1 ml each of 1M Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 1 mM DTNB in Tris-Cl (pH 8.0), 100 mM glyoxylate and 0.1 ml of sample or standard. The reaction was started by the addition of 10 mM acetyl CoA (0.04 ml). When acetyl CoA and glyoxylate react in the presence of MS, CoASH is formed. DTNB reacts with the sulfhydryl compound of CoASH forming mercaptide ions which absorb light at 412 nm. Thus an increase in A_{412} due to deacetylation of acetyl CoA was measured (Srere *et al.*, 1963).

3.2.8. Estimation of malate content in transgenic lines and wild type

Seedlings (4 days old, approximately 200 mg tissue) were homogenized in boiling 80% (v/v) ethanol, vortexed thoroughly and an aliquot (100 µl) was taken out for protein estimation. The remaining mixture was vortexed again and boiled at 80°C for 10 minutes. The samples were brought to room temperature and centrifuged at 10,000 g for 5 minutes. The supernatant was collected, passed through 0.45 µm filter and the filtrate was used in malate estimation. The malate assay was performed as described by Delhaize *et al.* (1993), which consisted of 0.3 ml of sample or standard, with 0.6 ml of Glycine buffer (0.5 M glycine and 0.4 M hydrazine, pH 9.2) and 0.04

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ml of 40 mM NAD^+. A stable initial A_{340} was recorded and the final A_{340} was measured after the addition of MDH (22 U per reaction).

3.2.9. Experimental design and statistical analysis

All experiments included a minimum of 3 to 15 independent replicates. Statistical analyses were performed using Sigmastat statistical analysis package (Version 1.0, Jandel Scientific). Single- or two-factor Analysis of variance (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. For the root elongation experiments, since unequal numbers of replicates were used due to technical difficulties, Dunn's test was performed on these data. Experiments were repeated 2 to 5 times to ensure reproducibility of results.

3.3. Results

3.3.1. The effect of Al on germination of *B. napus* seeds

In order to determine if germination was affected by Al, seeds of *B. napus* cv. Westar were germinated with or without Al and the relative germination percentage was estimated three days after germination (DAG). Aluminum did not cause any visible effect on the germination of *B. napus* seeds and the seeds were able to germinate in all treatment solutions (50, 100 and 150 μM Al), similar to that of the control (0 μM Al) (Table 3-2).

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Table 3-2. The effect of Al on germination of canola (*Brassica napus* L.) seeds. Number of seeds germinated in the absence or presence of Al (50, 100, or 150 μM) were estimated and expressed as percent germination. Values represent the means ± SE of 3 replicates. The experiment was repeated twice.

Concentrations of Al (μM)	Germination %
0	52.1 ± 3.8
50	54.1 ± 5.1
100	49.4 ± 2.8
150	52.4 ± 3.5

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3.3.2. Root and shoot elongation assay on *B. napus* seedlings

Six-day-old seedlings of *B. napus* were exposed to varying concentrations of Al (0 to 200 µM) for 48 h and root and shoot elongation were measured (Figure 3-4). Root growth was not significantly reduced at 100 µM Al, while at 150 and 200 µM Al it was reduced to 53% and 6% (respectively) of control (Figure 3-4B). Shoot elongation was not significantly affected by Al at any of the concentrations (0 to 200 µM) tested in this study (Figure 3-4B).

3.3.3. The effect of Al on MS mRNA expression in *B. napus* seedlings

To estimate if Al affects MS gene expression, Northern analysis was performed on RNA isolated from *B. napus* seedlings germinated and grown in the presence or absence of Al. The MS transcript level of seeds (0 DAG) was considered as the control for comparing the alterations in MS transcript levels in the presence or absence of Al. Seedlings of 1 to 4 day-old plants showed a 3 to 5 fold increase in MS transcript abundance compared to the seeds (0 DAG; Figure 3-5A). The MS transcript progressively reduced from 4 DAG to 8 DAG and could not be visually detected from 10 DAG (Figure 3-5A; i). When seeds were germinated and grown in the presence of 150 µM Al, the MS transcript level was 7 fold higher in 1 and 2 day old seedlings compared to the seeds (0 DAG). This increase in MS transcript in 1 and 2 DAG seedlings was 2.3- and 1.3-fold higher than the MS mRNA expression in 1 and 2 DAG seedlings at 0 µM Al respectively (Figure 3-5A; i and ii). When grown at 150 µM Al, MS mRNA was observed up to 4 DAG and beyond 6 DAG, the MS transcript levels were not detected visually (Figure 3-5A; ii).

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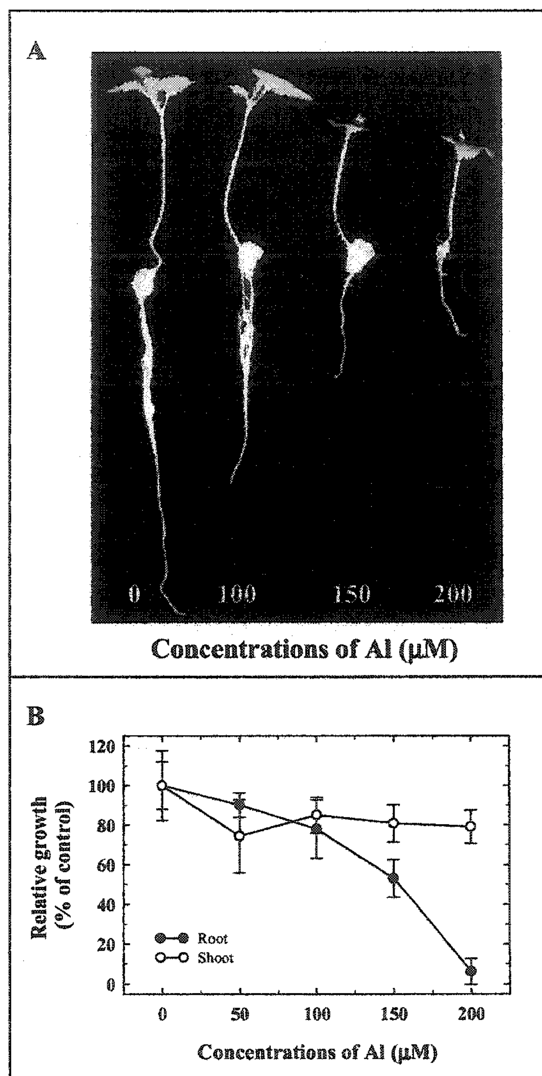

Figure 3-4. The effect on Al on growth of canola seedlings. (A) An image of whole plants exposed to varying concentrations of Al (0 to 200 μM). (B) Relative root and shoot elongation assay indicating progressive reduction in root growth in the presence of Al. Canola seedlings were exposed to 0 to 200 μM Al in FNS (pH 4.2) for 48 h. Changes in root and shoot lengths were measured before and after the treatment and expressed as relative percent of control (0 μM Al). Values represent the means ± SE (n=10-15). The experiment was repeated five times.

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3.3.4. Development of transgenic lines of *B.napus* overexpessing *Bn-MS*

Transformation of *B. napus* cv. Westar with pHS737-MS using an *Agrobacterium*-mediated system yielded 17 independent transgenic lines. The presence of the transgene, *Bn-MS* was confirmed in all these lines using genomic PCR analysis (primer positions used in genomic PCR are indicated in Figure 3-1). Amplification of an expected 1.2 Kb fragment was observed in transgenic lines and the positive control when amplified with CaMV and MSINR1 primers, but not in the WT and negative control (Figure 3-6A). Among the plants transformed with the vector construct, pHS737, five lines (V1 to V5) were selected and tested for the presence of the transgene with NPTF and NPTR primers. An expected amplification size of 0.3 Kb was observed in all vector control lines tested (Figure 3-6B).

3.3.5. Enhanced levels of mRNA expression in transgenic lines overexpressing *Bn-MS*

Northern analysis was performed on total RNA isolated from transgenic lines and WT plants to determine if the MS transcript level was enhanced in the transgenic lines when Bn-MS was overexpressed under control of the 35S promoter. The seventeen transgenic lines showed varying levels of MS transcript abundance compared to the WT (Figure 3-7A). Four independent transgenic lines, MS1, MS3, MS5 and MS 16 with different levels of MS transcript accumulation were selected for further studies and were raised till homozygosity.

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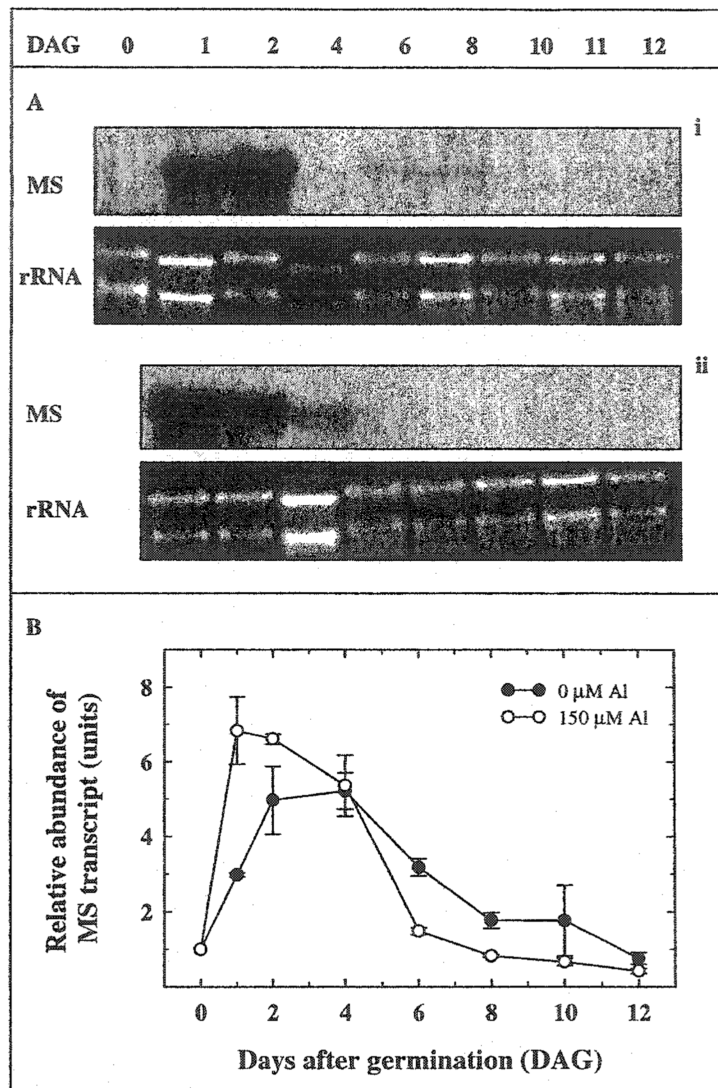

Figure 3-5. Effect of Al on transcript abundance of MS during germination and seedling development. (A) Northern blot analysis on RNA was isolated from seeds and seedlings at 0 to 12 days after germination (DAG) using radioactively labeled-MS probe. Seeds of *B. napus* were germinated in the (i) absence or (ii) presence of 150 µM Al. (B) Relative abundance of MS transcript based on densitometric quantification of RNA. The MS transcript level of seeds (0 DAG) was considered as the control for 0 and 150 µ Al. Values represent the means ± SE. The experiment was repeated two times.

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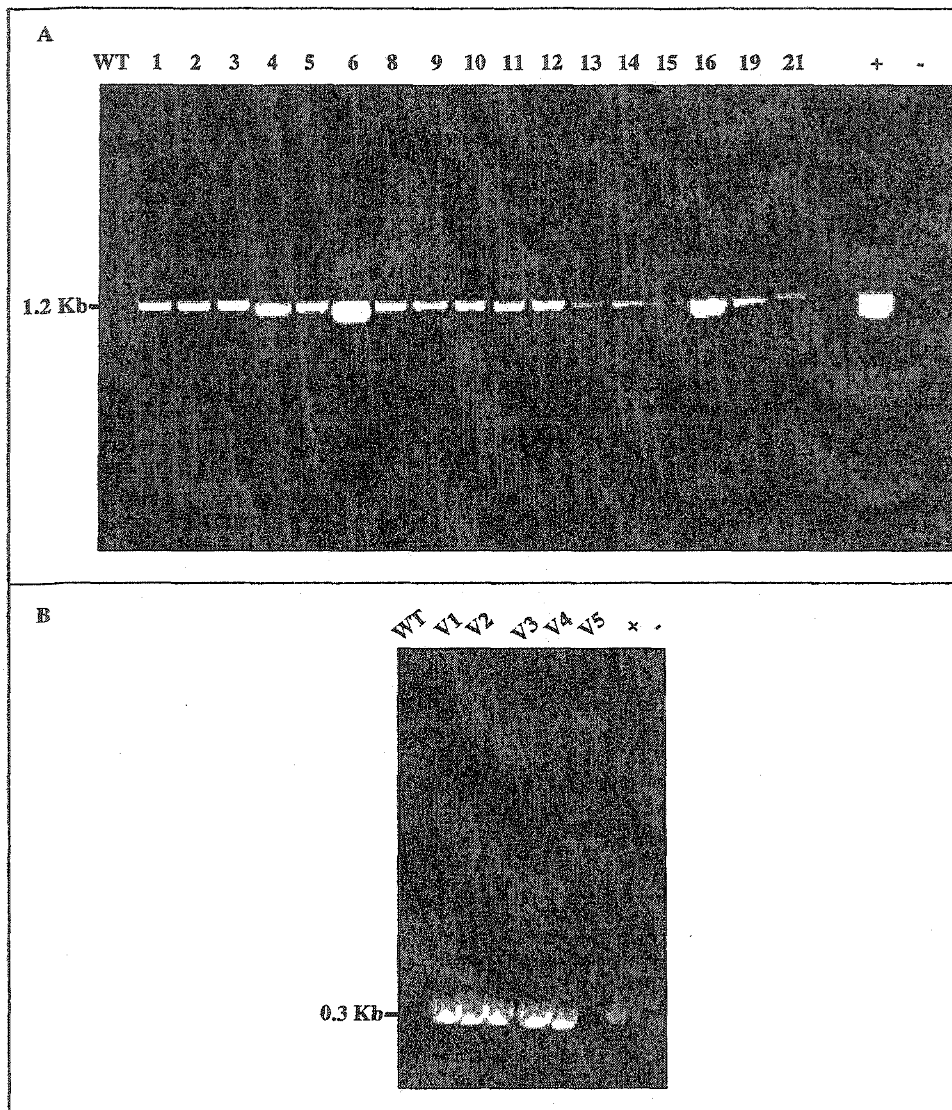

Figure 3-6. Genomic PCR analysis of transgenic canola lines overexpressing Bn-MS. (A) Genomic PCR on transgenic plants and WT using *35S-F* and *MSINR1* as primers. An expected amplification size of 1.2 Kb was observed in transgenic lines and the positive control (pHS737-MS), but not in WT and negative control. (B) Genomic PCR on vector control lines expressing pHS737 and WT using *NPTF* and *NPTR* primers. The expected amplification (0.3 Kb) was observed in only in V1 to V5 and positive control (pHS737), and not in WT and negative control.

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Transgenic lines carrying the vector (V1 to V5) were analyzed for levels of MS and NPT transcripts. The MS transcript could not be detected, while the presence of NPT transcript was confirmed in four of the lines tested (Figure 3-7B). The vector control transgenic line, V2, was raised till homozygosity.

3.3.6. Malate synthase enzyme activity and malate content are not altered in transgenic lines overexpressing *Bn-MS*

In order to determine if the specific activity of MS was enhanced in transgenic lines overexpressing *Bn-MS*, MS enzyme activity was estimated in the transgenic lines, WT and the V2 line. The MS activity in the WT was 263.6 ± 28.9 nmol mg^{-1} protein. There was no significant difference in the MS activity of transgenic lines compared to WT or vector control line. Even the transgenic line MS16, which showed highest MS transcript, had no increase in MS activity compared to the WT (Figure 3-7A and 3-8A).

The malate content in the transgenic lines (4 day old seedlings), MS1, MS3, MS5 and MS16 was not different from that of WT (0.8 ± 0.1 mmol mg^{-1} protein) and the Vec line (1.0 ± 0.1 mmol mg^{-1} protein) (Figure 8B).

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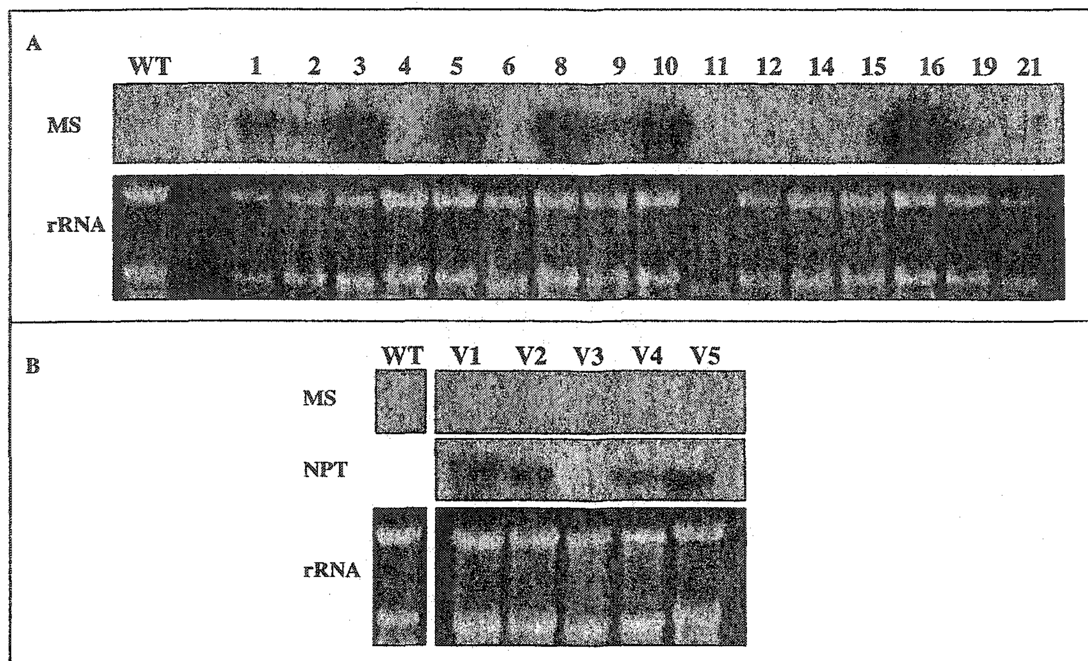

Figure 3-7. Transcript abundance of MS in transgenic *B. napus* lines overexpressing *Bn-MS* under control of the CaMV promoter. Total RNA was isolated from 6-day-old seedlings. (A) Northern analysis of transgenic plants (lines 1 to 21) overexpressing Bn-MS using ^{32}P-labeled MS as probe. Hybridization occurred at the expected size of 1.9 Kb. (B) Northern analysis of vector control transgenic plants (lines V1 to V5) using ^{32}P-labeled MS and NPT probes. The expected transcript sizes were 1.9 Kb and 2.9 Kb respectively.

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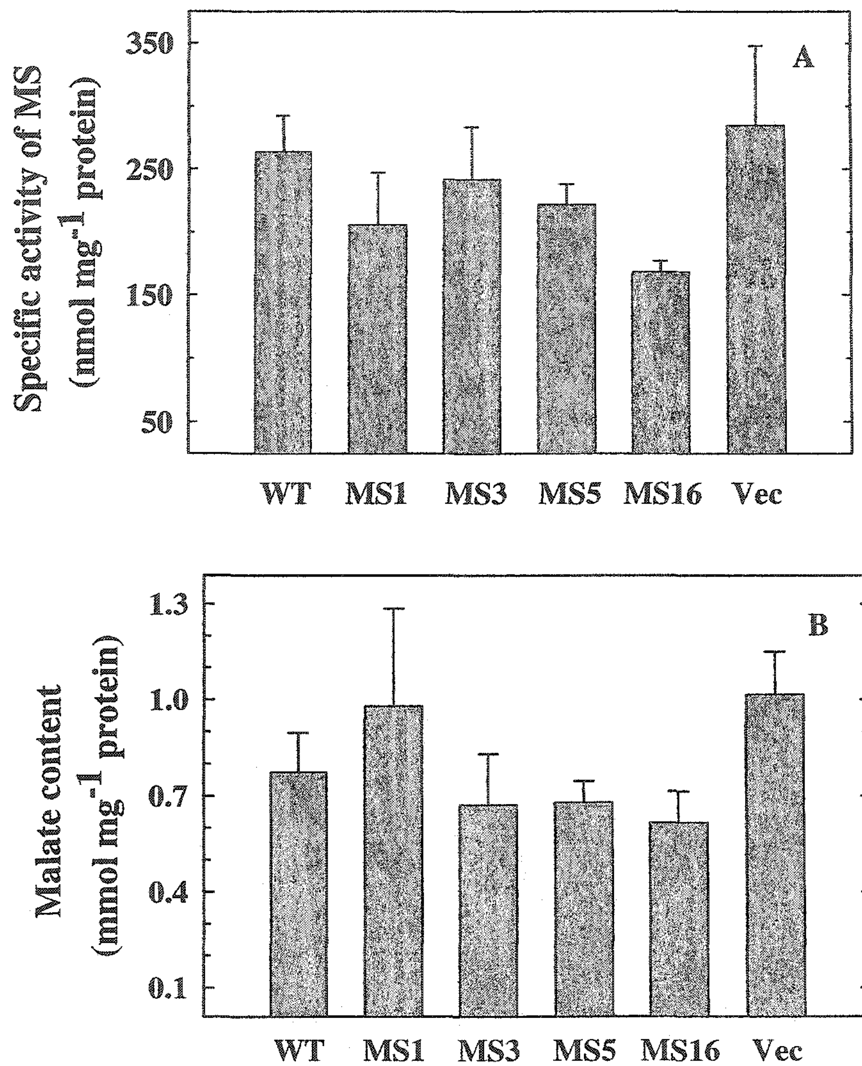

Figure 3-8. Characterization of transgenic plants for levels of MS enzyme activity and malate content. (A) Specific activity of MS and (B) malate content in transgenic lines overexpressing Bn-MS (MS1, MS3, MS5, MS16), WT and vector control line (Vec). Four-day-old seedlings were used in the enzymatic estimation of levels of MS and malate. Vertical bars represent standard errors (n=3).

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3.3.7. Immunoblotting of MS proteins in transgenic lines overexpressing *Bn-MS*

Since the transgenic lines overexpressing Bn-MS showed higher levels of MS mRNA expression, but no increase in either the MS enzyme activity or malate levels, immunoblotting was performed to determine whether enhanced MS mRNA had translated into enhanced MS protein. Western analysis on proteins isolated from transgenic plants using anti- *B. napus* MS antiserum revealed no increase in MS proteins in transgenic MS lines compared to the WT and Vec lines (Figure 3-9).

3.3.8. No enhanced Al resistance in transgenic lines overexpressing *Bn-MS*

Two of the transgenic lines, MS3 and MS16 were tested for their root growth under increasing concentrations of Al (0, 50, 100 and 200 mM Al) and compared with the root growth of WT and vector control plants. The transgenic lines showed no enhanced Al resistance compared to the WT or the vector control line (Figure 3-10).

3.4. Discussion

Brassica napus L. is an important oilseed crop and is sensitive to acidity and Al toxicity (Clune and Copeland, 1999). In this study, the effect of Al on germination of *B. napus* seeds was tested. No significant difference was observed in relative germination of seeds when grown in the presence or absence of Al (Table 3-1). de Lima and Copeland (1990) have observed a similar response when wheat seeds were tested for germination in the presence of Al. Only at concentrations greater than 1mM Al was the growth of emerging roots and shoots affected. Mobilization of

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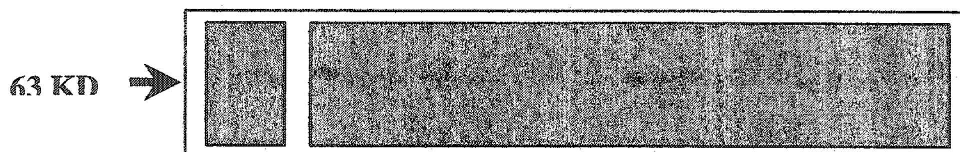

Figure 3-9. Immunoblotting of MS proteins from transgenic lines overexpressing Bn-MS, WT and a vector control line (Vec). Protein (10 μg) was isolated from 4-day old seedlings, electrophoresed on denatured SDS-PAGE. Immunoblotting was done with anti-*B. napus* MS antiserum. Malate synthase proteins (48 KD) were detected using alkaline phosphatase conjugated goat anti-rabbit IgG as secondary antibody.

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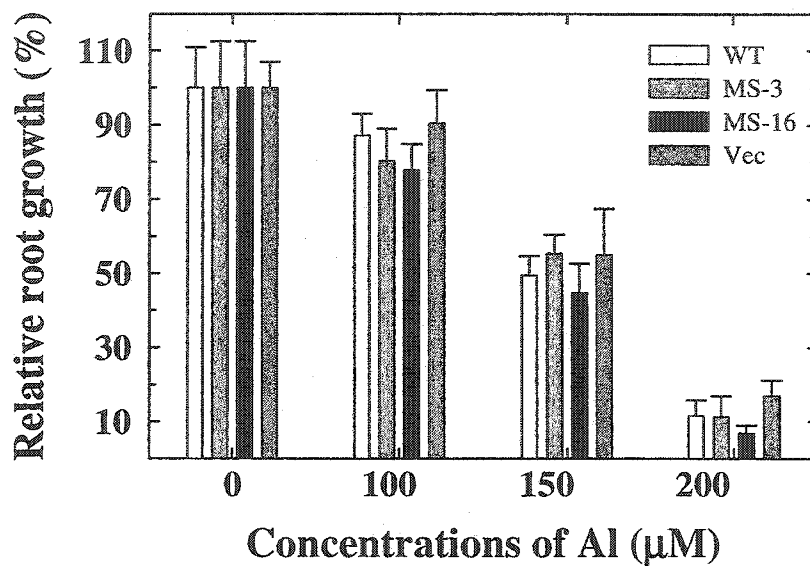

Figure 3-10. The effect of Al on relative root growth (% of control) of WT and transgenic lines overexpressing Bn-MS. Seedlings were grown in FNS and exposed to Al for 48 h. Root length (mm) was measured before and after treatment with Al (0 to 200 μM) and relative change in root length was determined. Vertical bars represent means ± SE (n=5)

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carbohydrate reserves were not affected in Al-tolerant (Carazinho) and Al-sensitive (Robin) wheat varieties (de Lima and Copeland, 1990), even at 1 to 10 mM Al.

One of the most visible symptoms of Al toxicity is a reduction in root growth. Aluminum-induced reductions in root elongation have been reported in several crop species such as wheat, maize, snapbeans and canola (Foy, 1988; Delhaize *et al.*, 1993; Pellet *et al.*, 1995; Miyaska *et al.*, 1991; Clune and Copeland, 1999). In this study, the effect of Al on root and shoot elongation of *B. napus* was determined by exposing the seedlings to various concentrations of Al (0 to 200 μM) for 48 h. No significant difference in root elongation was observed at 50 and 100 μM Al, but at higher concentrations, root elongation was significantly reduced (53 % at 150 μM Al and 6.3 % at 200 μM Al) compared to the control (Figure 3-4). No significant reduction in shoot elongation was observed when *B. napus* seedlings when grown in the presence of toxic concentrations of Al for 48 h. The majority of Al-dose response experiments have been done within 48 h exposures and the effect of Al on shoot growth has not been reported (Basu *et al.*, 1994; Ryan *et al.*, 1997; Zheng *et al.*, 1998). However, studies involving longer time exposures (more than 7 days) have reported that Al caused a significant reduction in shoot growth, shoot diameter, leaf area and shoot fresh and dry weights (Neogy *et al.*, 2002; Schaberg *et al.*, 2003)

Malate is a predominant organic anion released from roots of *B. napus* when exposed to stresses such as Al toxicity or phosphate deficiency (Zheng *et al.*, 1998 and Hoffland *et al.*, 1992). Malate synthase is a key enzyme involved in the synthesis

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of malate during early stages of seedlings growth. It is involved in the glyoxylate cycle and mobilization of seed reserves during germination and seedling development (Eggerer and Klette, 1967). In this study, MS transcript abundance was determined in *B. napus* seedlings exposed to Al. Northern analysis revealed that MS transcript levels was stimulated at 1 and 2 DAG at 150 μM Al than in the control (0 μM Al). It would be interesting to test if germinating seeds and developing seedlings exposed to Al have higher rate of β-oxidation of fatty acids and faster mobilization of seed reserves to the seedling. Aluminum caused a moderate but a significant inhibition in the MS transcript levels after 4 days of seedlings growth at 150 μM Al, while the transcript was detected up to 8 DAG in the control (0 μM Al; Figure3-5A and 3-5B). In my studies with yeast (Chapter 2), Al caused a reduction in MS transcript levels (*MLS1*). Hamel and Appana (2000) and Appana *et al.* (2003) observed no changes in MS enzyme activity when *Pseudomonas fluorescens* was grown in the presence of Al.

Since MS plays a major role in synthesis of malate in developing embryos and seedlings, developing transgenic plants overexpressing MS could be an effective strategy to enhance aluminum resistance during critical stage of plant development. An overexpression approach has been successfully adopted in experiments designed to test the role of genes involved in malate synthesis and their potential involvement in Al resistance. For example, overexpression of *MDH* in alfalfa resulted in enhanced synthesis and exudation of malate, and transgenic lines showed enhanced Al resistance (Tesfaye *et al.*, 2000). Overexpression of PEPC has also resulted in

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elevated levels of malate in the leaves of transgenic *S. tuberosum* (Rademacher *et al.*, 2002). The transgenic lines overexpressing PEPC are valuable material to test whether PEPC overexpression could enhance Al resistance either by increased cellular malate content and/or by increased exudation of malate.

My goal in this section of the study was to test if overexpression of MS in canola can increase malate levels and Al resistance during early seedling stages. Transgenic *B. napus* lines were developed by overexpressing a gene for *B. napus* MS (*Bn-MS*) under the control of constitutive CaMV promoter (Figure 3-6 and 3-7). The accumulation of MS transcript levels was higher in transgenic MS lines compared to the WT or the vector control, but no increase in the levels of MS activity or malate content was observed (Figure 3-8).

Gene silencing phenomena have been reported frequently in plant transformation studies where the CaMV has been used as a promoter (Neuhuber *et al.*, 1994; Mol *et al.*, 1994). Genes expressed under control of the CaMV promoter produce high levels of RNA, which are sufficient to downregulation of the expression of the endogenous gene and homologous transgene (Meins and Kunz, 1994). Plants with high transcription rates generate RNA levels that activate a cytoplasmic based, cellular process that specifically targets this overexpressed RNA for elimination. As a result, low steady-state levels of transgene mRNA are observed (Smith *et al.*, 1994; Flavel *et al.*, 1994). In this study, since a *B.napus* MS (Bn-MS) gene was used in the transformation of canola, homology-dependent co-suppression can be highly

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anticipated. Some of the transgenic lines (MS4, MS6, MS11, MS12, MS14 and MS15) showed no increase in levels of MS transcript, which could possibly be due to co-suppression resulting in high MS-RNA turnover. But, transcriptional gene inactivation was not observed in the transgenic lines used in this study (MS1, MS3, MS5 and MS16), since their MS transcript levels were higher compared to the WT (Figure 3-7).

Immunoblotting of MS proteins with anti- *B. napus* MS antiserum showed that the transgenic lines overexpressing *Bn-MS* had no increase in MS protein compared to the WT (Figure 3-9). The transgenic plants showed no increase in MS enzyme activity as well. Defects at post-transcriptional and translational stages could affect the synthesis or functioning of transgenic proteins in plants. Overexpression of a bacterial CS in *N. tabacum* leading to enhanced levels of CS transcript and CS proteins (100-fold over its WT), but with no increase in CS activity and citrate levels was suggested to reflect improper protein folding or formation of inactive protein aggregates in plants (Delhaize *et al.*, 2001). Transgenic *S. lycopersicum* lines overexpressing a gene for parthenogenesis showed an increase in transgenic mRNA levels, but had reduced transgenic protein content due to reduced translation efficiency (Pandolfini *et al.*, 2002). Desired levels of enhanced transgenic proteins by genetic manipulation at the post-transcriptional or translational level have been attempted using translational enhancers (Gallie, 2002) and by modifying the 5'-untranslated region of the transgene (Pandolfini *et al.*, 2002), to improve translation efficiency. Furthermore, substrate (glyoxylate and acetyl CoA) limitations could also

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affect the increase in MS activity in the MS overexpressing plants, particularly at the young seedling stage.

Synthesis and compartmentation of malate is considered to be a complex phenomenon (Miller *et al.*, 1998) and regulation of such organic anion metabolism is strictly controlled (Ryan and Delhaize, 2001). Overexpression of MS was hypothesized to enhance the malate content in canola seedlings so that it would increase Al resistance of young plants. But, the transgenic lines overexpressing MS did not lead to enhanced levels of MS activity and malate content. In addition, the sensitivity of these transgenic plants to Al was not increased compared to the WT. This study revealed that overexpression of a *B. napus* MS using CaMV promoter is not an effective approach and MS is not an ideal candidate gene for genetic engineering designed to increase cellular or extracellular malate levels in canola and to enhance Al resistance.

In addition to malate, citrate is another important organic anion considered to be a strong chelator of Al. Exudation of citrate from plant roots has been reported in several Al tolerant plant species (Pellet *et al.*, 1995; Zheng *et al.*, 1998a; Pineros *et al.*, 2000). In the following chapter, the role of citrate metabolism in Al resistance and the importance of various TCA and glyoxylate cycle genes in modulating the cellular and extracellular citrate levels and Al resistance is described.

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4. Modulation of citrate metabolism alters aluminum resistance in *Saccharomyces cerevisiae* and transgenic *Brassica napus* L. overexpressing a mitochondrial citrate synthase[1]

4.1. Introduction

Aluminum (Al) 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 molecular weight 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). Though a rapid release of organic anions (malate^{2-}) was observed in near isogenic, Al-tolerant wheat lines (Delhaize *et al.*, 1993), several lines of evidence suggest 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 10 h of exposure (Li *et al.*, 2000). Similarly, an Al-

[1] A version of this chapter has been published. **Anoop VM, Basu U, McCammon MT, McAlister-Henn L and Taylor GJ.** (2003) Modulation of citrate metabolism alters aluminum tolerance in *Saccharomyces cerevisiae* and transgenic *Brassica napus* overexpressing a mitochondrial citrate synthase. Plant Physiol **132:** (in press).

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induced, *de novo* synthesis of malate leading to enhanced malate efflux 24 h after exposure was demonstrated in an 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 phosphoenol pyruvate carboxylase (*PEPC*). Citrate turnover could be reduced by decreasing the activities of enzymes involved in breakdown of citrate such as aconitase (*ACO*) and isocitrate dehydrogenase (*IDH*) (Figure 4-1).

Citrate synthase (CS) is a key enzyme involved in condensation of oxaloacetate and acetyl CoA to produce citrate. This biochemical reaction plays an important role in the Krebs cycle, β-oxidation of fatty acids, and also in photo-respiratory glycolate pathways. As an important intermediate in various biochemical reactions, such as amino acid synthesis and fatty acid synthesis, citrate biosynthesis and catabolism is strictly regulated (Ryan and Delhaize, 2001). It is therefore important to undertake a comprehensive approach that considers enzymes and

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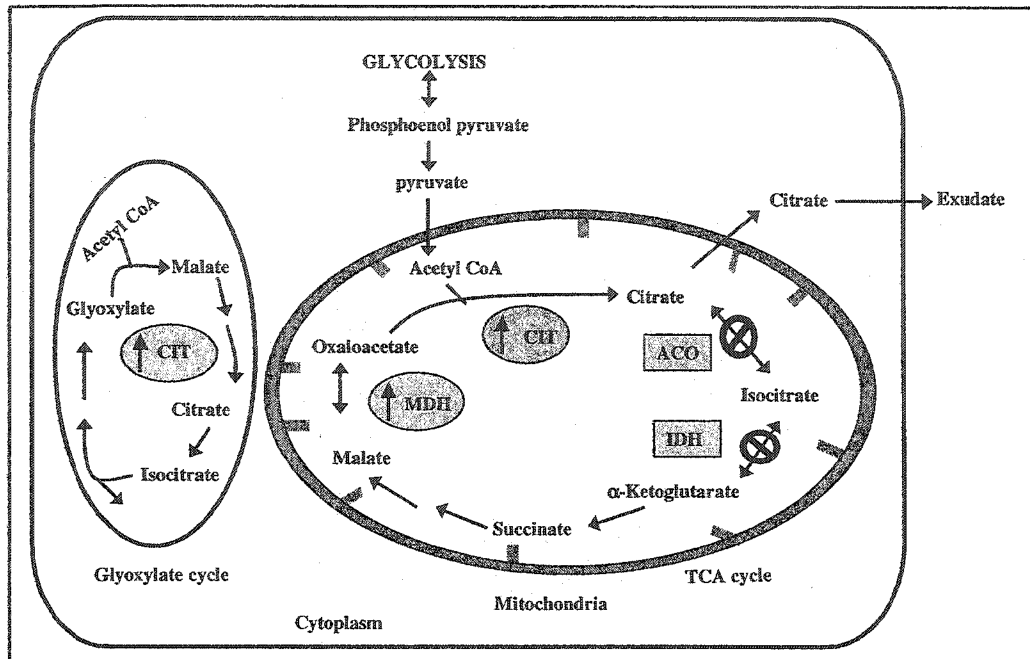

Figure 4-1. Metabolic map of TCA and glyoxylate cycles, indicating positions of genes that could be modulated for altering citrate metabolism. Upregulation of genes involved in citrate synthesis (indicated as arrows) and downregulation of genes involved in citrate catabolism (indicated as blocks) would enhance citrate synthesis and/or its accumulation in cells.

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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 resistance in both yeast and plant model systems. Yeast (*Saccharomyces cerevisiae*) is an excellent system for studies on metal toxicity and resistance. Genes involved in Al tolerance (*HSP150, SED1* and *ALU1-P*) have been identified in yeast (Ezaki *et al.,* 1998; Jo *et al.,* 1997) and the function 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 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. The function of TCA cycle genes appears to be conserved between yeast and plants. For example, the genes for different subunits of *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 resistance.

Yeast mutants potentially altered for citrate metabolism used in this study are indicated in Figure 4-1. 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 while

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Table 4-1. Yeast disruption mutants (defective in various TCA and glyoxylate cycle genes) with altered citrate metabolism (provided by Dr. McCammon, University of Texas). The genetic background of all mutants listed below is same as the WT, MMYO11. (Przybyla-Zawislak *et al.*, 1999).

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

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the other isozyme (CS2) functions in the peroxisome. In addition, mutants defective in genes downstream of CS, namely ACO1 and IDH12 were also used in this study to determine if citrate metabolism and Al resistance were affected due to defects in these genes.

In addition to yeast system, I also tested the hypothesis that increased synthesis and accumulation of citrate can mediate Al resistance in plants using a transgenic approach. An important oilseed crop, *Brassica napus* L. (cv. Westar) was used, since 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 aeroginosa* in the cytoplasm of *N. tabacum* and *Papaya* sp., led to enhanced 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. aeroginosa* CS in the cytosol of *N. tabacum* neither resulted in enhanced exudation of citrate nor increased Al tolerance. Koyama *et al.* (1999 and 2000) reported similar results to those of de la Fuente *et al.* (1997), where overexpression of an *Arabidopsis thaliana* mitochondrial CS in *Daucus carrota* cell lines and a *D. carrota* mitochondrial CS in *A. thaliana* lead to increased Al tolerance. A summary of the results from above studies is presented to compare the details of experimental techniques and observations (Table 4-2).

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Table 4-2. Comparison of various overexpression studies done in an effort to engineer CS enzyme activity, citrate levels and Al tolerance in transgenic plants.

Reference	Concentrations of Al used	Transgenic lines				Transgenic effect (% root growth compared to WT)
		Transgenic CS proteins	CS activity	Cellular citrate	Citrate efflux	
P. fluorescens CS in *N. tabacum* (de la Fuente *et al.*, 1997)	0 to 200 μM (Blaydes solution, pH 4.3)	100 to 256 fold increase	3 to 4 fold increase	10 fold increase	4 fold increase	~ 40 to 50 % better
D. carrota CS in *A. thaliana* (Koyama *et al.*, 2000)	0 to 1 μM (0.1 mM $CaCl_2$, pH 5.2)	Presence reported-not quantified	3.5 fold increase	Not determined	2.5 fold increase	20 % better
P. fluorescens CS in *N. tabacum* (Delhaize *et al.*, 2001)	25, 50 μM (modified basal solution, pH 4.3)	100 fold	No increase	No increase	No increase	No difference

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Despite a growing body of literature on this area of research, 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, since factors influencing citrate synthesis and accumulation would be potentially well regulated in mitochondria. Hence, I overexpressed an *A. thaliana* mitochondrial CS (At-mtCS) in *B. napus* and evaluated the transgenic lines for levels of CS activity, citrate exudation from roots and their performance under Al-toxic conditions.

4.2. Materials and methods

4.2.1. Yeast strains and media

Strains of *Saccharomyces cerevisiae* used in this study and their properties are listed in Table 4-1. The genetic background of all mutants listed below is same as the WT, MMYO11 (*Matα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*). Most strains harboring disruptions in different TCA cycle and glyoxylate cycle genes have been constructed previously (Przybyla-Zawislak *et al.*, 1999) as outlined in Chapter 2 (under 2.2.1). 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).

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Typically, yeast cultures were grown for 18 to 20 h at 30°C in YPD media (1% Yeast extract, 2% Peptone, 2% D-glucose). For experiments involving determination of Al response or estimation of citrate levels, cells were harvested from cultures by centrifugation at 1300 g for 2 min, washed three times with sterile water, and suspended in 1 ml sterile water. An initial OD_{600} of 0.05 was used in all experiments.

4.2.2. Overexpression of *CIT1* and *CIT1/MDH1* in MMYO11

To construct yeast overexpressing *CIT1* and *MDH1*, strain MMYO11 was transformed with Yep352-CIT1 (Kispal el 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$^-$, Tryptophan$^-$ plates. Full-length genomic clones of *CIT1* and *MDH1* have been used to generate the constructs, Yep352-*CIT1* and pRS424-*MDH1* (McCammon, 1996). Both Yep352-*CIT1* and pRS424-*MDH1* are high copy number vectors carrying 2-μ yeast episomal plasmid region. Hence transformation of MMYO11 with these constructs was anticipated to result in enhanced expression of *CIT1* and *MDH1*. Increase in *CIT1* and *MDH1* transcripts were confirmed by Northern analysis using yeast [^{32}P]CTP-labeled *CIT1* and *MDH1* as probes.

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4.2.3. Effect of Al on growth of mutants and transformants

While testing the growth response of different yeast strains to Al, a low pH, low phosphate medium (LPP) was used (Schott and Gardner, 1997). This is a synthetic complete (SC) minimal media made with 0.67% Yeast nitrogen base (without amino acids; without phosphate). In addition, K_2HPO_4 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 to 400 µM Al) with an initial OD_{600} of 0.05 were grown for 18 to 20 h at 30°C at 100 g in triplicates. The final OD_{600} was measured using a plate reader (µQuant, Biotek Instruments, Vermont, USA). The relative growth rate of each strain was expressed as a percentage of control (0 µM Al).

4.2.4. Aluminum-induced changes in transcript levels in yeast

Total RNA was isolated from 5 ml yeast cultures grown with varying concentrations of Al (0 to 300 µM) for 20 h by an enzymatic method using the QIAGEN RNAeasy mini kit (QIAGEN Inc., Canada). Northern blot analysis was done using [^{32}P]CTP-labeled *CIT1*, *ACO1* and *IDH1* probes.

4.2.5. Estimation of citrate content

Yeast cells grown in 3 ml LPP medium for 20 h were pelleted and washed three times with 3 ml of sterile water. The media and the supernatant from each wash were pooled as the extracellular fraction. The cells were resuspended in 1 ml sterile water and cell density was determined by estimating OD_{600}. Cells were again pelleted

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and the supernatant was pooled into the extracellular fraction. Cells were finally suspended in 1 ml of 80% 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 (0.1 ml) 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 ml to 1 ml) using an Evap-o-Vac (Cole Parmer, Canada) placed inside an incubator at 50°C. For cultures grown in the presence of Al, and pH was brought to 2.0 with 0.5 N HCl to dissociate Al-malate complexes. The sample was then passed through a cation exchange column with 1.0 ml resin (Bio-Rad), eluted with 10 ml deionized water and concentrated to 1.0 ml. An aliquot of 0.1 ml of this sample was used for citrate assay.

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 $ZnCl_2$, 3 U ml^{-1} MDH and 10 mM NADH. A stable initial A_{340} was noted, the reaction was started by addition of citrate lyase (Sigma). Formation of NAD^+ was followed and stable final A_{340} was recorded (Tompkins and Toffeletti, 1982; Delhaize *et al.,* 1993).

4.2.6. 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)

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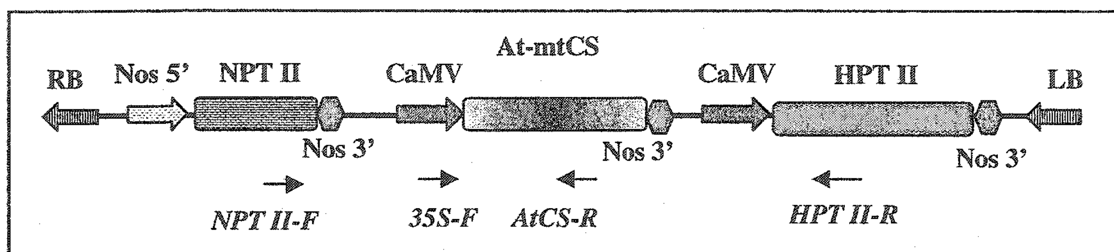

Figure 4-2. Diagrammatic representation of the construct, pACS121-Hm used in transformation of *B. napus* with At-mtCS. A full-length gene for At-mtCS was expressed under the control of a constitutive CaMV 35S promoter and a Nos terminator. The construct has selectable markers for NPT II and HPT II genes. The NPTII gene was under the control of Nos promoter while HPTII gene was under the control of CaMV promoter. The two primer pairs used in genomic DNA PCR amplification are indicated as arrows in the construct.

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and was used in the transformation of *Brassica napus* L. cv. Westar. This binary vector had a full-length cDNA for *Arabidopsis thaliana* mitochondrial CS (At-mtCS, 1.6 Kb) under the control of the cauliflower mosaic virus promoter (Figure 4-2) and two selectable marker genes encoding Kanamycin resistance (*NPT II*) and Hygromycin resistance (*HPT II*) (Koyama *et al.*, 1999). *Agrobacterium*-mediated plant transformation techniques and antibiotic selection of transgenic lines were done as described in Chapter 3 (section 3.2.). Cotyledonary petioles from *Brassica napus* L. cv. Westar (4-5 day-old seedlings) were used as explants and overnight grown cultures [in yeast extract peptone (YEP) with 50 µg ml^{-1} Kanamycin + 25 µg ml^{-1} Hygromycin] of *A. tumefaciens* strain (EHA 101 carrying pACS121-Hm) was used to infect the explants.

Presumptive positive plants (T_1) were tested for presence of the transgene 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 media with 150 µg 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 (T1), fifteen green T2 seedlings were transferred to soil and screened to identify homozygous lines. To select for homozygous lines in the T2 generation, about 20 to 30 seedlings from T2 progenies were screened by genomic PCR for the presence of transgene.

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4.2.7. Genomic DNA PCR and Northern analysis

For genomic PCR analysis, genomic DNA isolated from young tissues of canola (QIAGEN DNeasy mini kit) was used as the template. The primers used in PCR amplification include 35S-F, AtCS-R and HPT II-R (35S-F 5' CCACTGACGTAAGGGATGACG 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, USA). 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).

4.2.8. Citrate synthase enzyme assay

Roots and shoots were collected, frozen in liquid nitrogen, and stored at -70°C until used for enzyme assays. Approximately 1 g tissue was ground in liquid nitrogen and homogenized with 2 ml ice-cold extraction buffer (50 mM HEPES, 0.5% Triton-X, 1 mM EDTA, 1 mM Iodoacetamine and 10% glycerol). The extract was centrifuged for 10 min at 4°C, and the supernatant collected and desalted by passing through PD-10 columns (Bio-Rad) equilibrated with 10 mM HEPES and eluted with 2 ml of 10 mM HEPES. The reaction mix for CS enzyme assay consisted of 100 μl

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of 1 mM DTNB in Tris-Cl (pH 8.1), 40 µl of 10 mM acetyl CoA and 100 µl of the enzyme (final volume of 1.0 ml). The reaction was started by the addition of 100 µl of 10 mM oxaloacetate and increase in absorbance due to deacetylation of acetyl CoA was measured at 412 nm (Srere *et al.*, 1963).

4.2.9. Root elongation experiments

Wild type and transgenic lines (T_2 homozygous) were tested for their sensitivity to Al by the root growth elongation assay (Chapter 3, section 3.2). Seeds were germinated on a bed of wet sand. Seedlings (2 days 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 aquaria filled with 10 L of full nutrient solution (FNS) at pH 4.2. 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 seedlings were 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.

4.2.10. 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% ethanol and vortexed thoroughly. The samples were centrifuged for 2 minutes at 10,000 g and 100 µl of

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the supernatant was taken for protein estimation. The remaining portion of the samples was then vortexed and boiled at 80°C for 15 minutes. Samples were centrifuged at 10,000 g for 5 minutes, the supernatant was collected, passed through 0.45 µM filters, and 100 µl was used in citrate assay as described in section 4.2.5. (Delhaize *et al.*, 1993).

4.2.11. Estimation of extracellular citrate from root exudates

Seeds of WT and transgenic lines were surface sterilized and plated on seed germination media. Two-day-old seedlings (13 to 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 were then transferred to treatment solution (0 or 150 µM Al) under aseptic conditions and maintained for 2 days with constant shaking. The pH was checked every 8 h and adjusted to 4.2 with 0.5 N HCl. Root exudates were collected after two days and roots were harvested to obtain dry weight. The pH of root exudates were adjusted to 2.0 with 0.5 N HCl to dissociate Al-citrate complexes and passed through cation exchange columns with 1.5 ml resin (Bio-Rad). Samples were eluted with 20 ml deionized water and concentrated to 1 ml. An aliquot of 100 µl of this sample was used for citrate assay as described earlier.

4.2.12. Experimental design and statistical analysis

All experiments included 3-10 independent replicates and statistical analyses were performed using Sigmastat statistical analysis package (Version 1.0, Jandel

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Scientific). 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. For the root elongation experiments, since unequal numbers of replicates were used due to technical difficulties, Dunn's test was performed. Experiments were repeated 3 to 6 times to ensure reproducibility of results.

4.3. Results

4.3.1. Effect of Al on growth of 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 (Figure 4-3A). Northern analysis of WT yeast exposed to Al (0 to 400 μM), showed that transcript level of *CIT1* increased 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, while the *IDH1* transcript level was downregulated to 60% of control by increasing concentrations of Al (Figure 4-3B and 3C).

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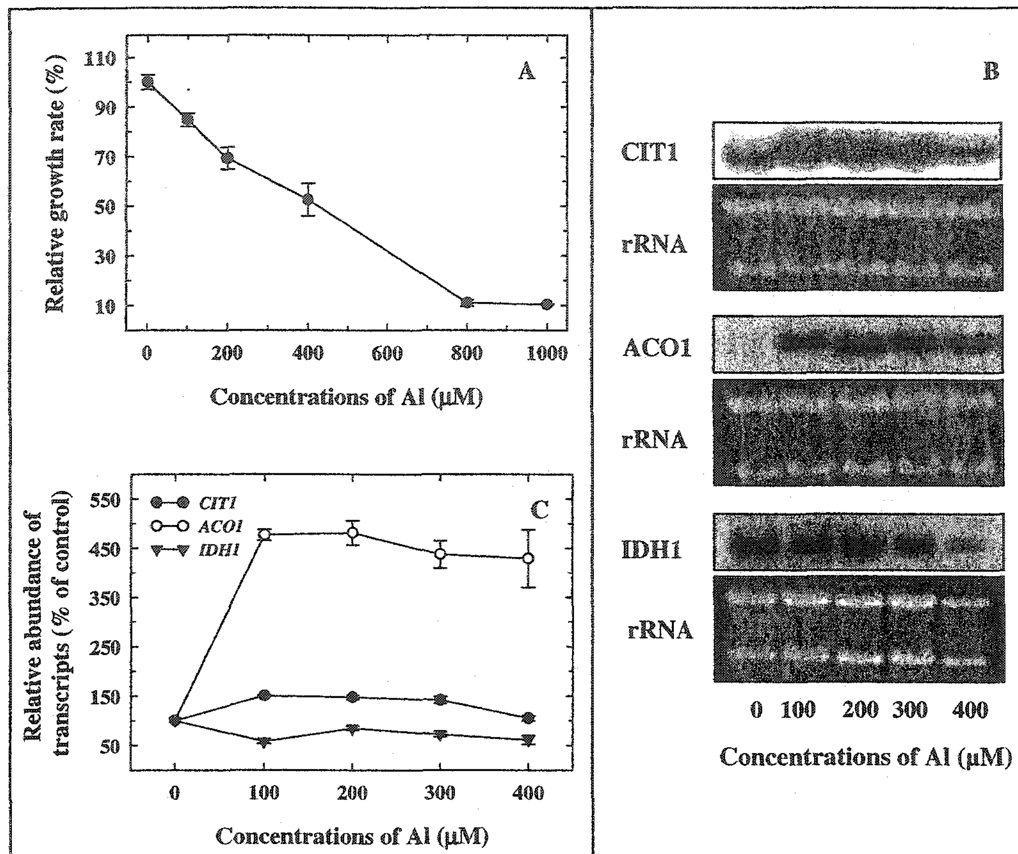

Figure 4-3. The effect of Al (0 to 1000 µM) on WT yeast (MMYO11). (A) Reduction in growth of WT grown in LPP medium for 20 h, with different concentrations of Al. Vertical bars represent standard error (n=3). (B) Transcript levels of *CIT1, ACO1* and *IDH1* in WT yeast. Total RNA isolated from yeast cells exposed to different concentrations of Al for 20 h, was probed with ^{32}P-labelled fragments of *CIT1, ACO1* and *IDH1*. (C) Relative abundance of *CIT1, ACO1* and *IDH1* transcripts based on densitometry quantification of the RNA.

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4.3.2. Effect of Al on disruption mutants of yeast

When yeast cells were challenged with Al, the single mutants (Δ*cit1,* Δ*cit2* and Δ*cit3*) did not show any significant difference in their growth compared to WT yeast (Figure 4-4A). Likewise, the double mutants, Δ*cit12,* Δ*cit13* did not show any difference in their growth response compared to WT (Figure 4-4B), whereas Δ*cit23*and the triple mutant of CS, Δ*cit123* showed increased sensitivity at 200 and 400 μM Al. At 400 μM Al, growth of Δ*cit123* was reduced by 67%, while the WT showed a reduction by 43% (Figure 4-4C). The Δ*aco1* and Δ*idh12* mutants showed reduced sensitivity to Al. The mutant disrupted for *ACO1* showed a 30% better growth, while the Δ*idh12* mutant exhibited a 19% better growth compared to WT yeast, at different concentrations of Al (Figure 4-4C).

4.3.3. Cellular citrate content in WT and disruption mutants

Cellular citrate content was determined in the yeast disruption mutants with altered citrate metabolism (Figure 4-5A). Single mutants of CS (Δ*cit1,* Δ*cit2 and* Δ*cit3*) showed no significant reduction in cellular citrate content compared to the WT (8.50 ± 0.65 nmol 10^8 cells^{-1} ml^{-1}), while the double and triple mutants (Δ*cit12,* Δ*cit13* and Δ***cit123***) showed a significant reduction (2-fold) compared to the WT yeast. Interestingly, the mutants disrupted for the genes downstream of citrate, (Δ*aco1* and Δ*idh12),* which had reduced Al sensitivity, showed a significant increase (3 to 6 fold) in citrate levels compared to WT (Figure 4-5A).

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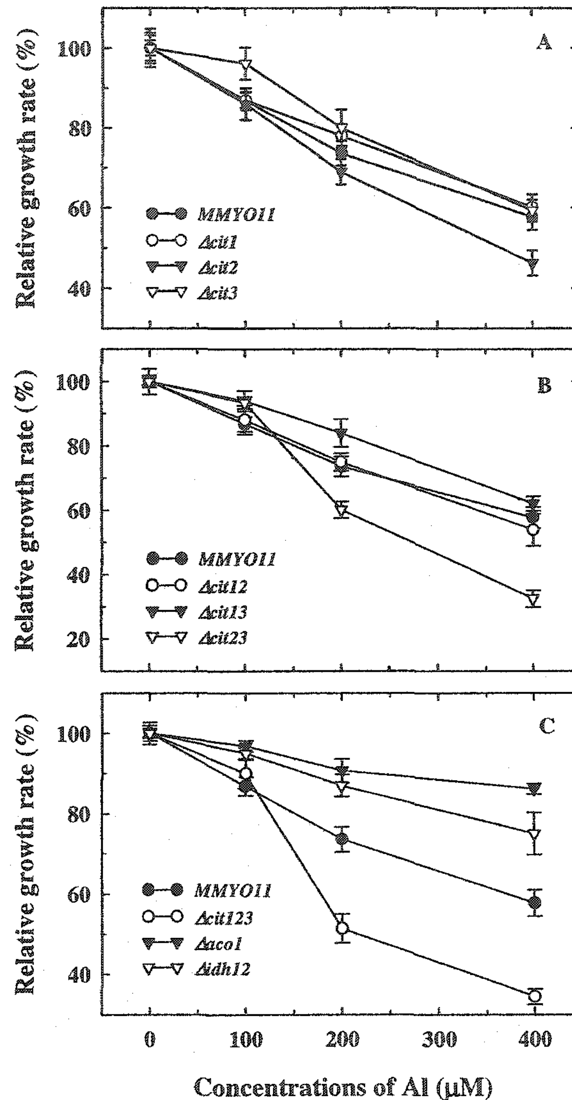

Figure 4-4. The effect of Al on relative growth rate (% of control) of WT and disruption mutants with altered citrate metabolism. (A) Single CS mutants (Δ*cit1*, Δ*cit2* Δ*cit3*). (B) Double CS mutants (Δ*cit12*, Δ*cit13*, Δ*cit23*). (C) Triple mutant of citrate synthase, and aconitase and isocitrate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

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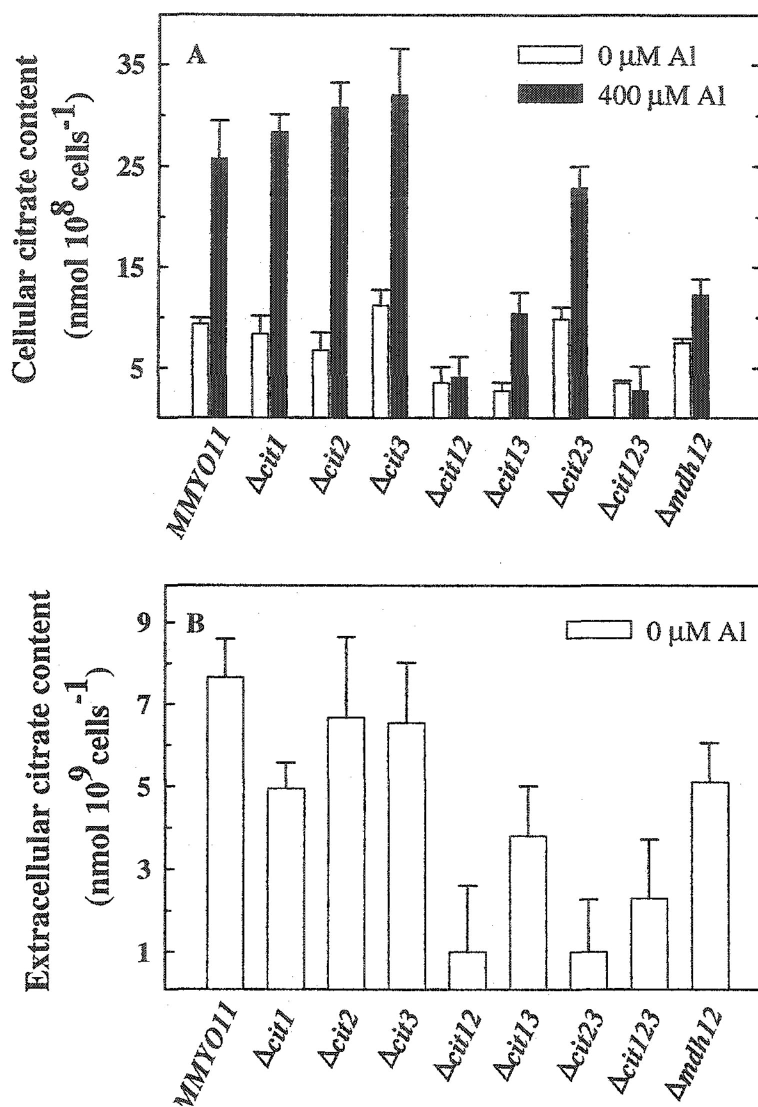

Figure 4-5. Citrate content in WT and disruption mutants defective in genes upstream of citrate. Cellular (A) and extracellular (B) citrate content was estimated in MMYO11 and disruption mutants defective in citrate synthases. Cultures of different genotypes grown in LPP medium for 20 h were used for estimation of citrate. Vertical bars represent standard errors (n=3).

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4.3.4. Effect of Al on cellular citrate levels in yeast disruption mutants

When yeast cells were exposed to 400 μM Al, there was a significant increase in cellular citrate levels in many strains (except Δ*aco1*). The WT and single mutants of CS (Δ*cit1*, Δ*cit2* and Δ*cit3*) showed a marked increase in cellular citrate levels by 27 to 45% in the presence of Al compared to their corresponding controls (0 μM Al) (Figure 4-5A). Among the double mutants of CS, Δ*cit12* did not show an increase in citrate levels when exposed to Al, but Δ*cit13* and Δ*cit23* mutants showed a significant increase in citrate content compared to their citrate levels at 0 μM Al. Unlike the single and double mutants of CS, the triple mutant, Δ*cit123,* showed no increase in cellular citrate levels when exposed to Al. The cellular citrate level of Δ*aco1* mutant was significantly reduced at 400 μM Al, compared to its control. In contrast, when Δ*idh12* was grown in the presence of Al, a slight but significant increase in citrate content was observed compared to its citrate content at 0 μM Al (24.5 ± 1.2 nmol 10^8 cells^{-1}). The Δ*mdh12* mutant showed no significant difference in cellular citrate levels when exposed to Al, but the citrate content was significantly reduced (2 fold) compared to the WT at 400 μM Al.

4.3.5. Extracellular citrate content in WT and yeast disruption mutants

The amount of citrate exuded out of cells was estimated in mutants with modified citrate metabolism (Figure 4-5B). No significant reduction in citrate efflux from cells of single mutants of CS was observed, while citrate exuded from double and triple mutants of CS were reduced approximately a two fold compared to the WT

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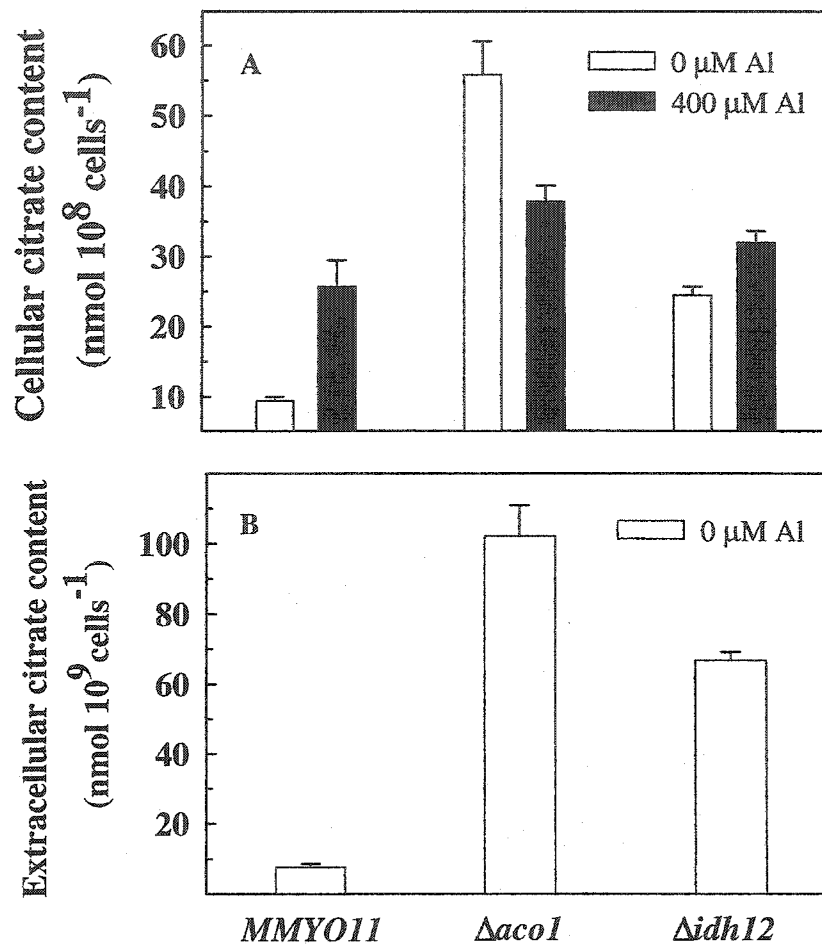

Figure 4-6. Citrate content in WT and disruption mutants defective in genes downstream of citrate. Cellular (A) and extracellular (B) citrate content was estimated in MMYO11 and mutants defective in *ACO1* and *IDH12*. Cultures of different genotypes grown in LPP medium for 20 h were used for estimation of citrate. Vertical bars represent standard errors (n=3).

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yeast (7.7 ± 0.9 nmol 10^9 cells^{-1} ml^{-1}). Citrate released from cells of Δ*aco1* and Δ*idh12* were significantly higher (7 to 10 fold) compared to WT.

The extracellular citrate content was undetectable in the samples prepared from cultures grown in the presence of Al, due to interference in the enzymatic assay. This could be due to loss of citrate during the processing of the samples when passed through the cation-exchange columns. At 400 µM Al, extracellular citrate levels could be measured only in mutants Δ*aco1* and Δ*idh12* (9.3 ± 1.3 nmol 10^9 cells^{-1} and 13.4 ± 1.4 nmol 10^9 cells^{-1}, respectively) where the citrate levels were generally 7 to 10 fold higher compared to the WT and other mutants under control conditions.

4.3.6. Effect of Al on growth of *CIT1* overexpressing transformants and their citrate levels

The gene encoding the major mitochondrial isoform of citrate synthase, *CIT1*, was overexpressed alone and with mitochondrial malate dehydrogenase gene, *MDH1*, in WT yeast. Malate dehydrogenase (MDH) is the enzyme involved in synthesizing oxaloacetate (OAA) from malate. I therefore postulated that overexpression 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 the *CIT1* transcript (Figure 4-7). To confirm the simultaneous overexpression of *MDH1* in

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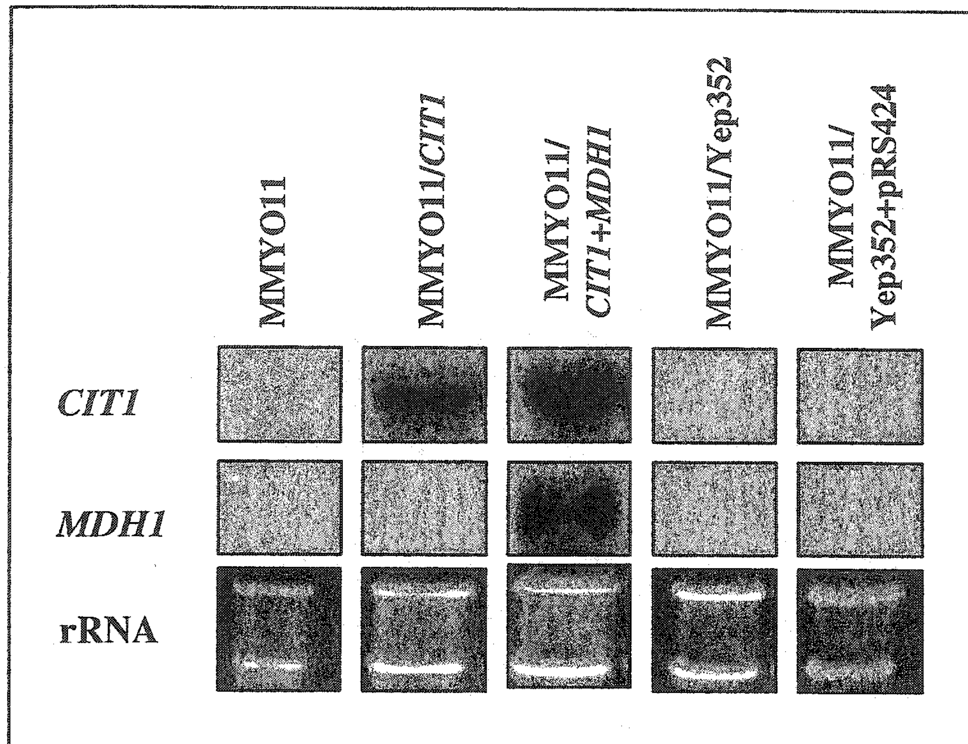

Figure 4-7. Northern analysis of yeast transformants to test enhanced mRNA expression of *CIT1* and *MDH1*. Transformation of MMYO11 with a mitochondrial citrate synthase *CIT1* (MMYO11/*CIT1*) singly and with malate dehydrogenase, *MDH1* (MMYO11/*CIT1* + *MDH1*). The vector controls included WT yeast transformed with Yep352 and Yep352 + pRS424. Hybridization probes include radiolabelled ^{32}P- *CIT1* and *MDH1* fragments.

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MMYO11/*CIT1*+ *MDH1*, the blots were probed with *MDH1* gene fragment. Enhanced level of *MDH1* transcript was observed only in MMYO11/*CIT1*+ *MDH1* and not in MMYO11/CIT1 or WT cells.

When challenged with Al (500 μM Al), the transformants MMYO11/*CIT1* and MMYO11/*CIT1*+ *MDH1*, showed significantly better growth (20% and 30%, respectively) compared to the WT and vector controls (Figure 4-8). A significant increase (2.5 to 3 fold) in both cellular and extracellular citrate levels was also observed in MMYO11/*CIT1* and MMYO11/*CIT1*+ *MDH1* transformants relative to the WT (Figure 4-9). Interestingly, citrate levels were not higher with simultaneous overexpression of *CIT1*/*MDH1* as in MMYO11/*CIT1*+*MDH1* compared to MMYO11/*CIT1*.

4.3.8. Plant transformation studies

4.3.8.1. Development of transgenic plants overexpressing an *Arabidopsis thaliana* mitochondrial CS (At-mtCS)

Transformation of *Brassica napus* L. cv. Westar with pACS121-Hm (Figure 4-2) using an *Agrobacterium*-mediated system yielded twelve 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 Figure 4- 2). Amplification of an expected ~ 0.7 Kb fragment was observed in transgenic lines and the positive control when amplified with CaMV-F and AtCS-R

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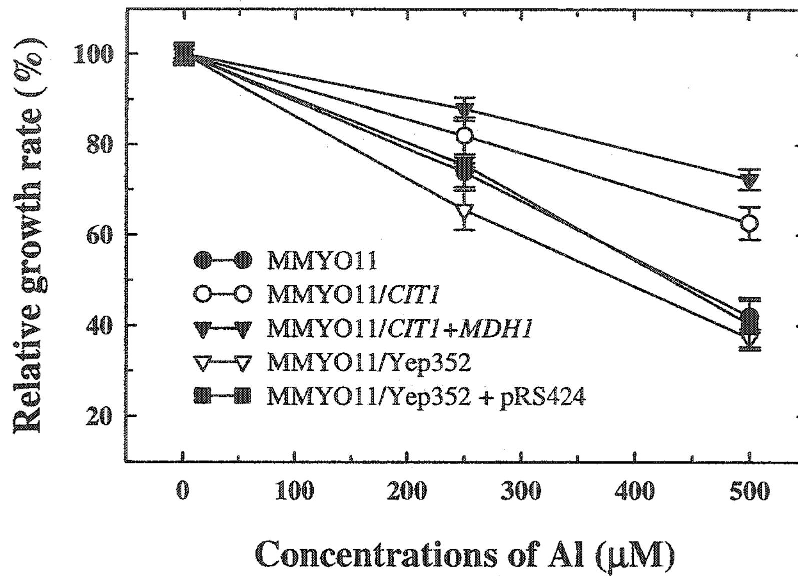

Figure 4-8. The effect of Al on relative growth rate of WT yeast and transformants overexpressing a mitochondrial citrate synthase *CIT1* (MMYO11/*CIT1*) singly and with malate dehydrogenase, *MDH1* (MMYO11/*CIT1* + *MDH1*) in WT yeast. The vector controls included WT yeast transformed with Yep352 and Yep352 + pRS424.

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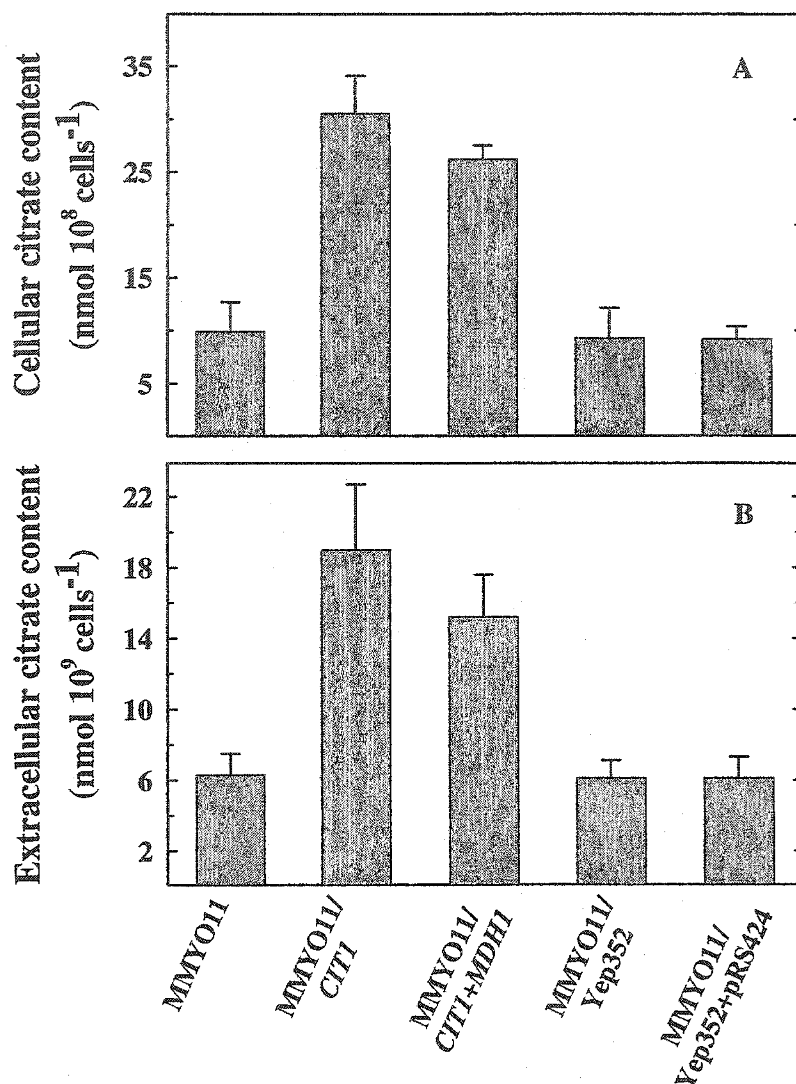

Figure 4-9. Citrate content in yeast transformants overexpressing *CIT1* and *CIT1* + *MDH1*. Cellular (A) and extracellular (B) citrate content in WT yeast and yeast transformants overexpressing *CIT1*, *CIT1* + *MDH1* and vector plasmids, Yep352 and Yep352 + pRS424. Cultures of different genotypes grown in LPP medium for 20 h at 30°C were used for estimation of citrate. Vertical bars represent standard errors (n=3).

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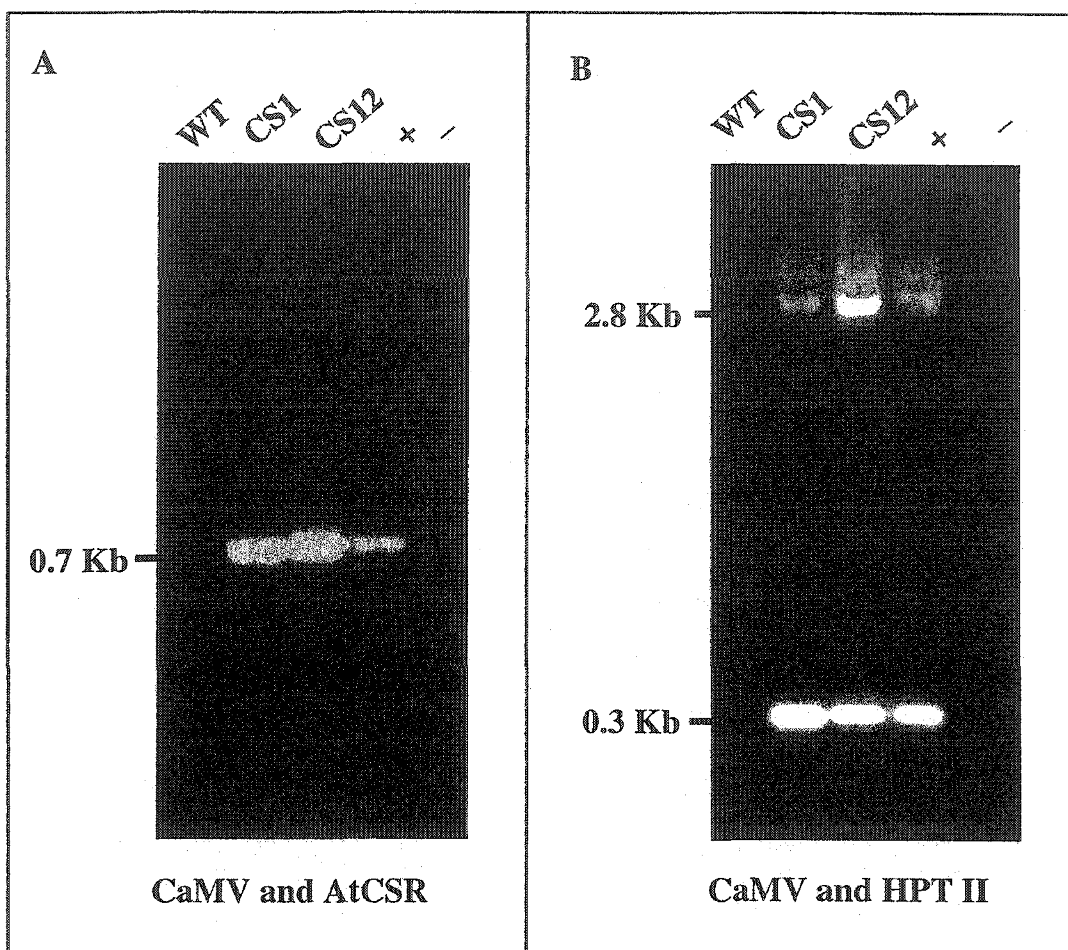

Figure 4-10. Genomic DNA PCR analysis of transformants confirming the presence of At-mtCS transgene. Genomic DNA was isolated from WT and two independent transgenic lines (CS1 and CS12), and used as template for PCR with two primer pairs indicated above. (A) CaMV/AtCS-R, expected amplification size of 0.7 Kb and (B) CaMV/HPT II-R, expected amplification sizes of 0.3 Kb and 2.8 Kb. Positive (+) control includes plasmid DNA (pACS121-Hm) and negative (-) control includes PCR reactions with no DNA.

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primers, but not in the WT and the negative control. When amplified with CaMV-F and HPTII-R primers, two bands of expected sizes (~ 0.3 Kb and 2.8 Kb) were observed in transgenic lines and the positive control. Transgenic lines CS1 and CS12, were fertile, and were raised to obtain homozygous seeds for further studies (Figure 4-10A and 9B).

4.3.8.2. Northern analysis and CS activity

Transgenic lines were analyzed for enhanced levels of the CS transcript (Figure 4-11A and 10B) 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 (~1.6 Kb) in roots and up to 3.5 to 3.8-fold increase in shoots, compared to the WT. The At-mtCS probe hybridizes to the mt-CS of both *A. thaliana* and *B. napus*. Due to the short exposure time used (2 h), the endogenous CS transcript in the WT was only visible as a faint band, while the band intensity was stronger in CS1 and CS1 for the same exposure time.

When the same membrane was stripped and reprobed with HPT II, 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).

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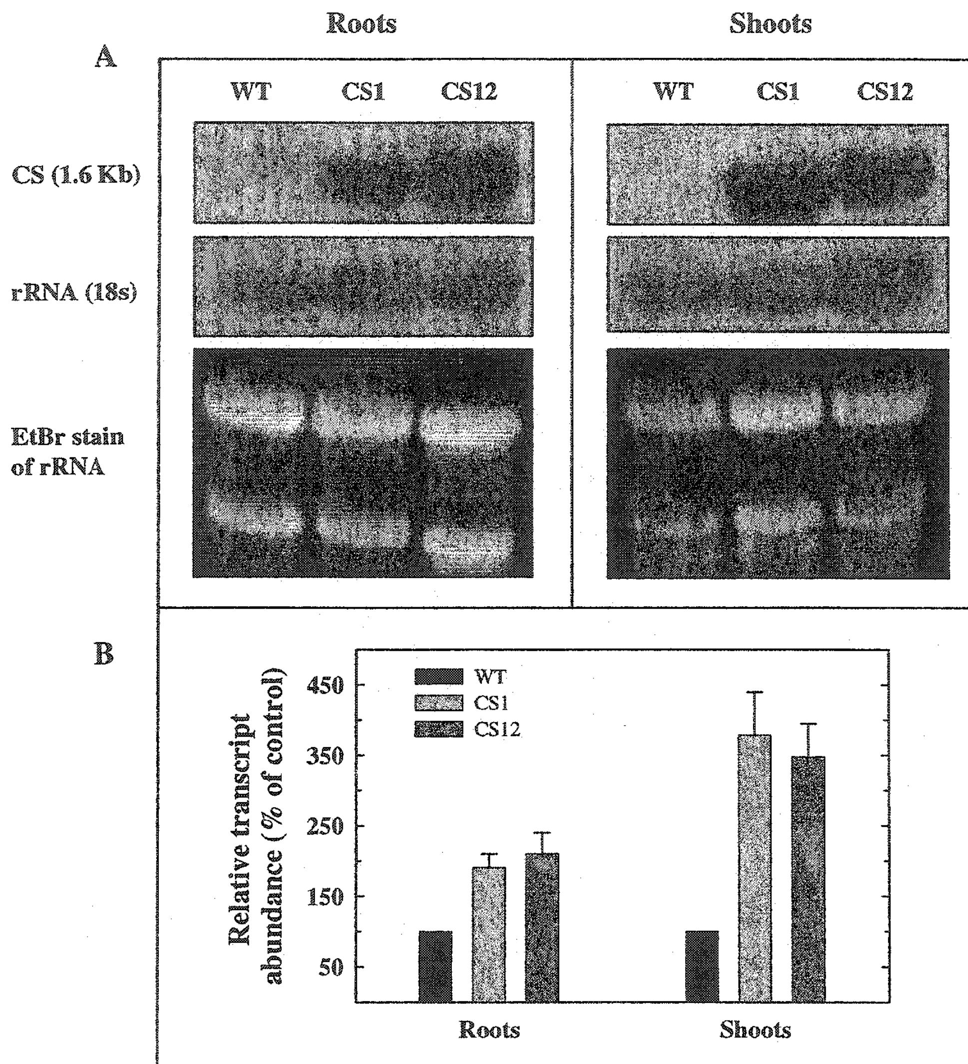

Figure 4-11. Analysis of CS transcript and transcript abundance in WT and transgenic lines overexpressing At-mtCS. Northern analysis of total RNA isolated from young roots and leaves (A and B) of WT, CS1 and CS12. RNA blots were hybridized with ^{32}P-dCTP labeled CS probe (1.6 Kb). Ethidium bromide staining (EtBr) and hybridization with rRNA probe (18S) are included as control. C) Transcript abundance of CS was estimated in transgenic lines relative to the CS transcript level in WT. Vertical bars represent means ± SE (n=3).

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Citrate synthase (CS) enzyme activity was measured in the WT and transgenic lines (Figure 4-12). 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 ± 0.1 µmol CoA utilized min^{-1} mg^{-1} protein respectively) compared to the WT (0.8 ± 0.003 µmol CoA utilized min^{-1} mg^{-1} protein).

4.3.8.3. Enhanced 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 (Figure 4-13). At 50 µM, a stimulatory effect of Al on root elongation was observed in CS1 and CS12 lines, while a growth reduction of 10% was observed in WT. At 100 µM Al, no significant change in root elongation was observed in the transgenic lines compared to their controls (0 µM Al), while the WT showed a reduction of 9% in relative root growth. At higher concentrations of Al, root elongation was greater in transgenic lines than in the WT. At 150 µM Al, root growth was reduced by 50% in WT, while 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), while the root growth of WT was reduced by 85% compared to its control.

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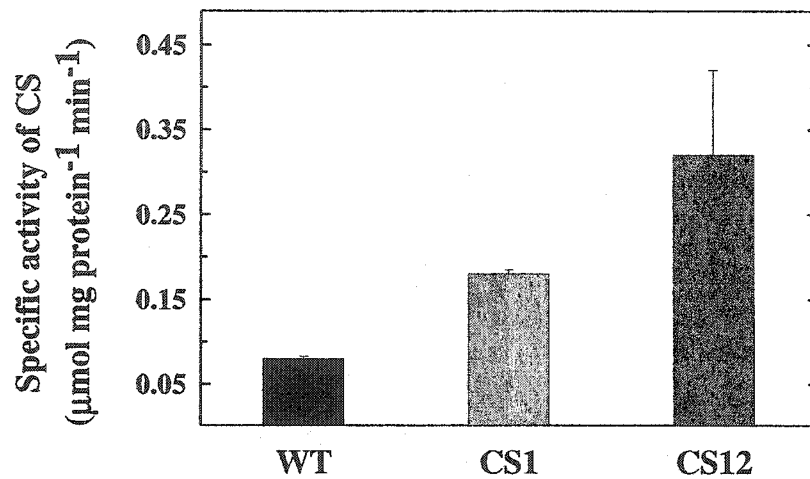

Figure 4-12. Citrate synthase enzyme activity in WT and transgenic lines, CS1 and CS12. The specific activity of CS was measured in young leaves of 10 days old seedlings. Vertical bars represent means ± SE (n=3). The experiment was repeated three times.

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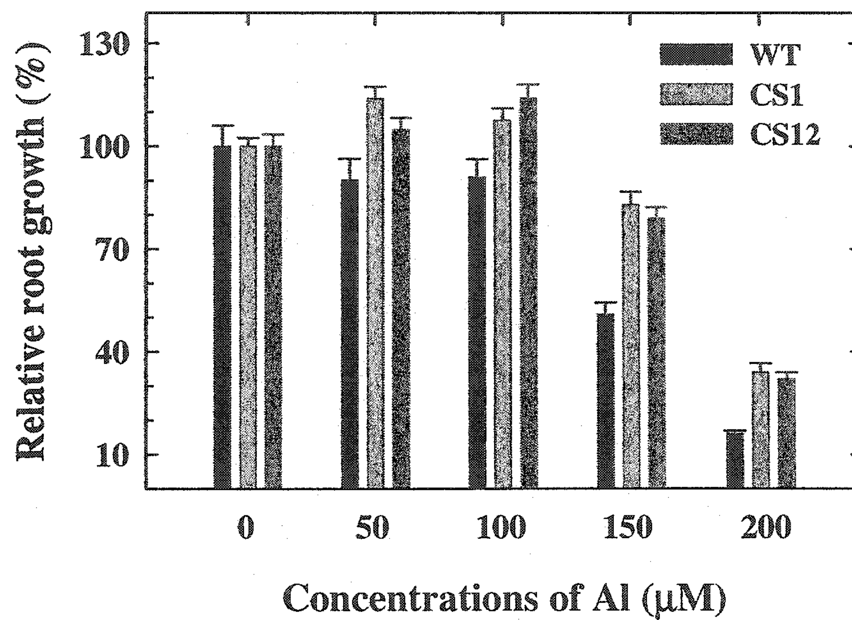

Figure 4-13. The effect of Al on relative root growth (% of 0 μM Al) of WT and transgenic lines exposed to varying concentrations (0 to 200 μM) of Al. Seedlings were grown in FNS and treated with Al for 48 h. Root length (cm) was measured before and after treatment and relative change in root length was determined. Vertical bars represent means ± SE (n=10).

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4.3.8.4. Increased levels of cellular citrate and exudation

Since the transgenic lines had the Atmt-CS expressed under control of the CaMV promoter, I expected higher levels of cellular citrate content and citrate exudation from roots of CS1 and CS12 under control conditions. But, the amount of citrate in roots, shoots and root exudates in these lines were not significantly different from that of the WT at 0 μM Al (Figure 4-14A and B). When exposed to 150 μM Al the citrate content of shoots was significantly increased (a 1.7 to 1.9 fold) in CS1 and CS12 compared to the WT (2.3 ± 0.2 μmol/mg protein) (Figure 4-14A). However, the root citrate level was not different between WT and transgenic lines even in the presence of Al (Figure 4-14B).

The amount of citrate exuded from the roots of CS1 (1.7 ± 0.2 μmol g^{-1} dry wt) and CS12 (1.2 ± 0.2 μmol g^{-1} dry wt) was 1.7 to 2.4 fold higher than WT (0.7 ± 0.2 μmol g^{-1} dry wt) in the presence of 150 μM Al (Figure 4-15). These results indicate that overexpression of mitochondrial CS in canola results in an Al-induced increase in exudation of citrate and Al tolerance in the transgenic lines.

4.4. 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 (Neumann and Martinoia, 2002; Hoffland *et al.*, 1992; Neumann *et al.*, 2000; Zatta *et al.*, 2000). In this study, exposure of yeast, MMYO11 to Al resulted in induction of *CIT1* and

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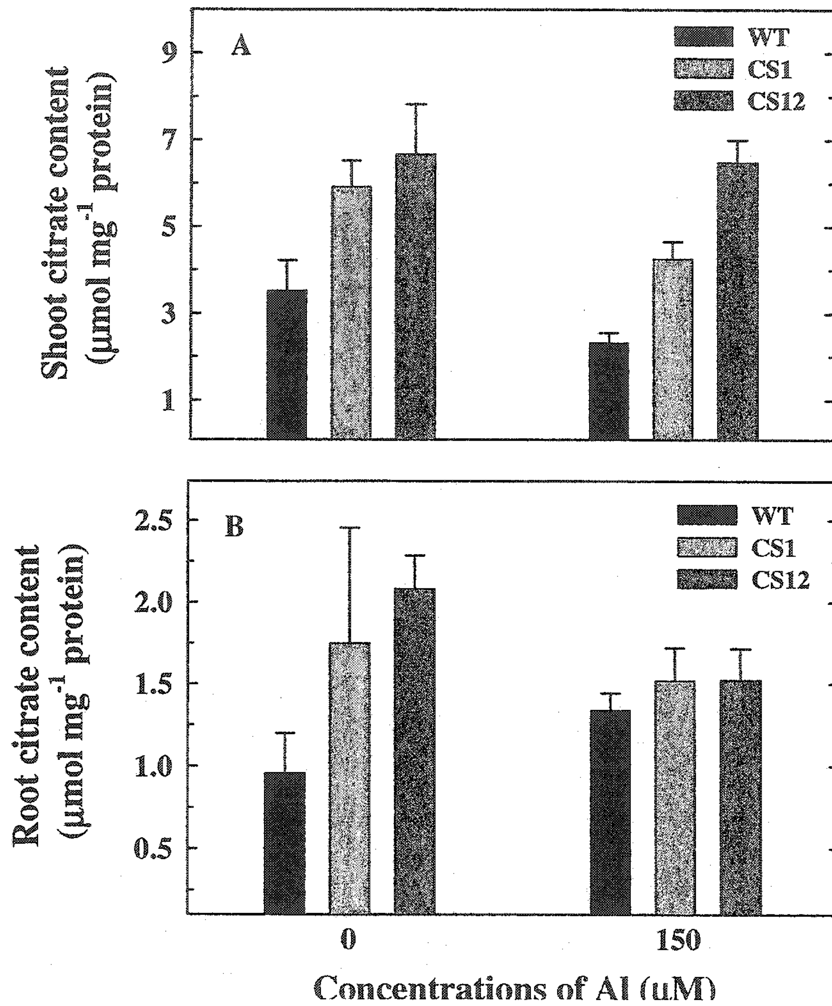

Figure 4-14. Cellular citrate content in tissues of WT and transgenic lines, CS1 and CS12. (A) Citrate levels in shoots and (B) roots of WT and transgenic lines. Shoot and root tissues from WT and homozygous seedlings of transgenic lines were collected and used in citrate estimation. Vertical bars means ± SE (n=3).

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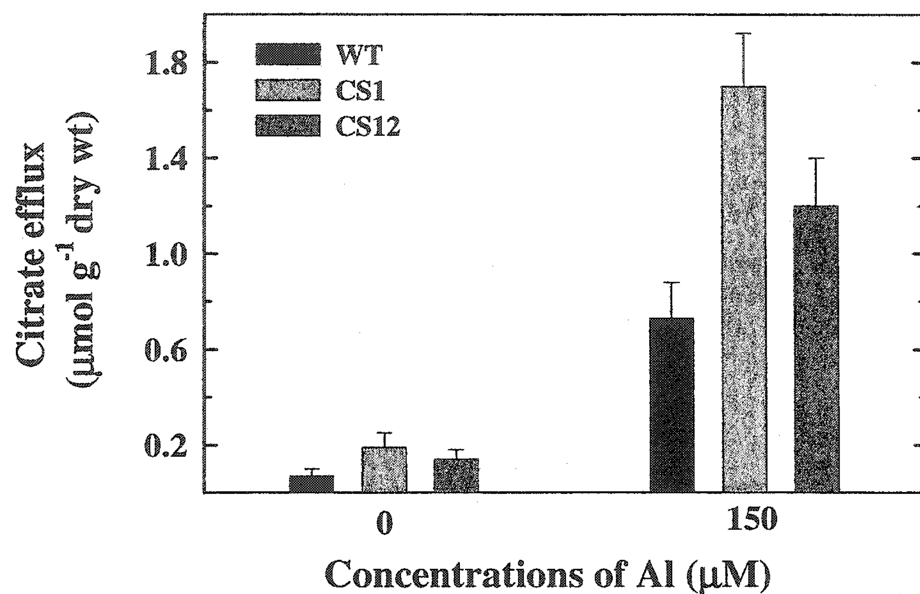

Figure 4-15. Citrate efflux from roots of WT and transgenic lines overexpressing At-mtCS. Seedlings were raised under sterile condition with or without Al (150 µM Al). Root exudates were collected and the amounts of citrate released from the roots of WT, CS1 and CS12 were analyzed. Vertical bars represent means ± SE (n=3).

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ACO1 and downregulation of *IDH1* transcript levels (Figure 4-3B,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 resistance.

Genetic manipulation is relatively easy in yeast due to the availability of mutants, ease of handling and short experimental periods. The data from yeast mutants with altered citrate metabolism (defective in TCA and glyoxylate cycles genes) revealed that accumulation of citrate in cellular and extra-cellular pools can mediate Al resistance. When measuring metabolite levels in yeast, it is important to measure both cellular and extracellular levels, since 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 acidiosis (Massonneau *et al.*, 2001).

In the present study, cellular and extracellular citrate levels were not significantly different in single mutants (Δ*cit1,* Δ*cit2* and Δ*cit3*) compared to WT. These mutants were not hypersensitive to Al, based on their growth performance compared to 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

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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 this study, Al-induced increase in citrate levels was observed in these single mutants of CS, which could have helped alleviate Al toxicity in these mutants.

In my experiments with the double mutants of citrate synthase (*Δcit12, Δcit13, Δcit23),* significant reductions were observed in their citrate content (cellular and extracellular) compared to the WT when grown under control conditions. Aluminum caused an increase in cellular citrate content in *Δcit13* and *Δcit23,* but no such increase was observed in the *Δcit12* mutant. However, no differences were observed in the growth of *Δcit12* and *Δcit13,* while *Δcit23* showed increased sensitivity to Al. No direct correlation could be made between the levels of citrate and the Al sensitivity in the double mutants. Unfortunately, the reason for this discrepancy could not be explained from the 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). 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 to WT. Unlike, the single mutants of CS and double mutants, *Δcit13* and *Δcit23,* Al-induced increase in cellular citrate content was not observed in *Δcit123,*

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indicating that enhanced sensitivity of the triple mutant could be due to complete absence of gene machinery for citrate synthesis.

Increased accumulation of citrate can also be mediated by reduced activity of enzymes involved in citrate turnover (Massonneau *et al.*, 2001). In this study, Δ*aco1* and Δ*idh12* 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 aconitase leads to increased exudation of citrate in Al tolerant maize cv. Cateto-Columbia (Pineros *et al.*, 2002) and from lupin roots under phosphate deficient conditions (Neumann *et al.*, 1999). Reduction of isocitrate dehydrogenase activities in mutant carrot cell lines capable of growing under phosphate deficiency also showed enhanced citrate exudation (Takita *et al.*, 1999 and Kihara *et al.*, 2003). It would be interesting to investigate what other organic anions accumulate in Δ*aco1* and Δ*idh12* mutants that might contribute to improved Al resistance observed in my studies.

Under control conditions, the extracellular citrate content was only a small fraction of the cellular citrate content (1 to 14%) in WT and the yeast mutants defective in citrate synthases. In contrast, Δ*aco1* and Δ*idh12* mutants exuded 18 % and 27% of their cellular level (note differing scales for citrate content in Figure 4-5 and 4-6). This indicated that yeast strains with higher cellular citrate content also

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showed higher levels of extracellular citrate. In the presence of 400 µM Al, the extracellular citrate content was not detectable in WT and mutants defective in citrate synthases, and trace amounts of citrate was measured in Δ*aco1* and Δ*idh12* mutants. Since only very small proportion of cellular citrate is effluxed as extracellular fraction, this extracellular pool could be too low to be detected or could have been lost during the processing of samples for citrate estimation.

Results from my 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 two to three fold increase in citrate content and reduced Al sensitivity compared to WT yeast. MMYO11/*CIT1*+ *MDH1*, was expected to synthesize and exude more citrate than MMYO11/*CIT1* (I postulated reduced substrate limitations). However, citrate contents in both transformants were not significantly different. The difference in sensitivity of MMYO11/*CIT1*+ *MDH1* compared to MMYO11/*CIT1*, again suggests that there could be other metabolites (perhaps malate) that attributed for the enhanced Al resistance.

Using high copy number plasmids (Yep352 and pRS424) for transformation of MMYO11 with genomic clones of *CIT1* and *MDH1*, cellular and extracellular citrate content was increased by 2 to 3-fold. These transformants did not show any difference in growth or development under control conditions compared to the WT

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yeast cells. Since CIT1 and MDH1 are house-keeping genes, if overexpression of these genes had resulted in defects in growth or development of the yeast transformants, I would have used an inducible promoter such as MET3, GAL10 or CUP1 to regulate the synthesis and transport of citrate by the alternative promoters.

Enhanced levels of organic acid synthesis or efflux is often accompanied by reduced growth under control conditions, or require 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, (Δ*aco1* and Δ*idh12*) nor the yeast overexpressing transformants (MMYO11/*CIT1* and MMYO11/*CIT1+MDH1*), revealed any apparent growth defects when grown in LPP medium. My investigation with yeast disruption mutants and yeast overexpressing a mitochondrial citrate synthase, clearly illustrate the role of citrate in alleviating Al stress and indicate 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 over 400 genes 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 genes expression may be responding.

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Hence, these metabolites may be exerting physiological effects both 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 ten-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 resistance and cellular citrate levels.

I also overexpressed a mitochondrial CS from *Arabidopsis thaliana* (At-mtCS*)* in *B. napus* 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: 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 two fold reduced activity compared to the enzyme complex, mt-CS/mt-MDH. The interaction of mt-CS was specific only to mt-MDH

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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 (shoot) 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). A quantitative relationship between the cellular citrate content and the amount of citrate efflux could not be established due to the destructive sampling technique adopted for citrate efflux experiment, in which roots were dried after the collection of root exudates. The cellular citrate content was estimated from the root and shoot tissues in separate experiments and expressed in $\mu mol\ mg^{-1}$ protein (Figure 4-14), while the citrate efflux was expressed in $\mu mol\ mg^{-1}$ dry weight (Figure 4-15).

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, 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 were not significantly different, citrate exudation was significantly higher in CS1 than in CS12. The data suggest that citrate produced in the transgenic lines are exuded out of the

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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 the transgenic lines did not have any difference in growth and development compared to 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 as the transgenic potato plants had deformed ovules (Landschutze *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 to 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 to the WT. Enhanced Al resistance due to Al-induced increase in citrate exudation in transgenic canola, clearly indicate that mitochondrial CS can play a major role in citrate metabolism and resistance to Al.

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

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generated transgenic lines by overexpressing the same *P. aeroginosa* CS gene in the cytosol of *N. tabacum*. 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. aeroginosa* 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 to 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 overexpressing a nodule specific *MDH* showed enhanced exudation of organic anions from their roots and enhanced Al resistance (Tesfaye *et al.*, 2001). Koyama *et al.*, (1999) indicated the importance of *CS* by overexpressing an *A. thaliana* mtCS in *D. carrota* cells. In addition, Koyama *et al.* (2001) showed that transgenic *Arabidopsis* lines overexpressing a *D. carrota* 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.

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In spite of a number of published studies on the topic, a holistic approach to understand citrate metabolism in relation to Al resistance has not yet been undertaken. My work with yeast and transgenic canola overexpressing At-mtCS, has helped to explore the complexity of citrate metabolism. My 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, IDH*) can be potentially used to increase citrate synthesis and its accumulation in cells (Figure 4-1). Citrate levels could be modulated by upregulation of *MDH* and *CS* by an overexpression strategy, or by downregulation 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 resistance in plants. Since cellular metabolism involves an array of enzymes that are interrelated and function in coordination with each other, metabolic profiling of these transformants could help to further understand the coordinated synthesis of other organic anions.

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5. GENERAL DISCUSSION

One of the hypothesized Al resistance mechanisms in plants is chelation of Al by organic anions in the apoplasm or symplasm, thereby protecting root cells from Al toxicity. Aluminum-induced exudation of organic anions such as malate and citrate is suggested to be related to an increased Al tolerance in several crop species (Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993; Pellet *et al.*, 1995). Exogenous application of malate and citrate to the growth medium restores root elongation to control levels in Al-sensitive cultivars of wheat (Ownby and Popham, 1989; Basu *et al.*, 1994), suggesting that these organic anions play a key role in Al resistance. Hence, I hypothesized that overexpression of genes involved in synthesis of malate and citrate can enhance the synthesis and exudation of malate and citrate respectively, and lead to enhanced Al resistance in transgenic canola plants. Regulation of organic anion synthesis and exudation, however, is a complex phenomenon. These organic anions are directly or indirectly involved in several metabolic processes including the assimilation of carbon and nitrogen, regulation of cytosolic pH and osmotic potential, and balancing of charges during excess cation uptake. In addition, they are important intermediates in various biochemical reactions, such as amino acid synthesis and fatty acid synthesis. Therefore, a comprehensive approach including a suite of genes involved in malate and citrate metabolism was undertaken using yeast as a model system, to explore the complexity of malate and citrate metabolism and their potential role in Al resistance.

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Malate metabolism

For studies involving malate metabolism, yeast mutants defective in genes involved in malate synthesis (*MLS1, ICL1, MDH1, MDH2* and *MDH12*) were used (Chapter 2). Mutants defective in glyoxylate cycle genes (*MLS1, ICL1*) showed no reduction in malate levels and no enhanced sensitivity to Al compared to WT. Among the mutants defective in various malate dehydrogenases (*MDH*), Δ*mdh1* showed no increased sensitivity to Al, while Δ*mdh2* and Δ*mdh12* were hypersensitive to Al. Mutations in *MDH1* and *MDH2* did not alter cellular malate levels in Δ*mdh1* and Δ*mdh2* mutants, while the Δ*mdh12* showed a significant reduction in malate content compared to the WT yeast. The extracellular malate content in Δ*mdh1* and Δ*mdh12* were approximately 4 to 5 fold higher than in WT, but no clear association was observed between the malate content and Al sensitivity in these mutants. Aluminum caused a reduction in cellular malate levels in WT and all mutants tested for malate metabolism. Aluminum is reported to seriously damage cellular metabolism, inhibiting the activities of several glycolytic, TCA, and gluconeogenetic enzymes (Xu *et al.*, 1990; Socorro *et al.*, 2000). This study suggested that complexity of malate metabolism and the effect of gene redundancy should be taken into account while attempting genetic manipulation of *MDH*. If efforts are made to reduce malate levels in plants by an antisense approach or by characterizing MDH gene knockout lines, a single gene approach may not be sufficient to reduce the levels of malate appropriately.

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My research with malate metabolism also involved testing if an association between malate metabolism and Al resistance could be established by overexpression of a gene involved in malate synthesis (Chapter 3). I used an important oilseed crop, canola. to test this hypothesis. Cultivars of canola are sensitive to acid soils and Al toxicity (Clune and Copeland, 1999). Hence their sensitivity to Al was exploited in an effort to develop Al-resistant transgenic plants by overexpressing the gene of interest.

Seed germination and post-germinative growth are crucial stages in the life cycle of higher plants. In this study, Al did not have any apparent effect on germination of canola seeds. But, MS transcript levels were moderately (but, significantly) inhibited when germinated in the presence of Al. Northern analysis on seeds germinated in the presence of Al showed that MS transcript was inhibited after 4 days of seedlings growth in the presence of Al, while the transcript was detected up to 8 DAG in the control (0 μM Al). In addition, canola seedlings showed significant reductions in growth, when exposed to increasing concentrations of Al (0 to 200 μM). Hence, I hypothesized that if seedlings have enhanced ability to synthesize and exude malate during the critical stages of development, this would enable them to better survive under conditions of Al stress. The key enzyme involved in the synthesis of malate during early stages of seedling growth is malate synthase (MS). Hence, a gene for MS was overexpressed in canola under the control of a constitutive promoter (CaMV). The MS transgenic plants showed an increase in MS transcript levels, but no increase in MS activity and malate content. Aluminum resistance studies are

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underway with these transgenic plants, although an Al tolerant phenotype is not expected in these lines. This study revealed that overexpression of a *B. napus* MS using CaMV promoter may not be an effective approach and MS is not an ideal candidate gene for genetic engineering designed to increase cellular or extracellular malate levels.

Citrate metabolism

My research also included testing whether Al resistance can be increased by enhancing citrate synthesis and exudation, through overexpression of a gene encoding citrate synthase (CS) in yeast and canola. I investigated a collection of yeast disruption mutants defective in genes encoding TCA cycle enzymes, both upstream (citrate synthase, CS) and downstream (aconitase, *ACO* and isocitrate dehydrogenase, *IDH*) of citrate for their citrate levels and Al sensitivity. The single mutants of CS (Δ*cit1*, Δ*cit2* and Δ*cit3*) did not show increased sensitivity to Al compared to WT yeast and their cellular and extracellular citrate levels were not different from WT. This could be due to metabolic plasticity resulting in compensation of other isoforms on the loss of another isoform of CS. The triple mutant of CS (*Δcit123*) showed lower levels of citrate accumulation and reduced Al resistance. Disruption of genes involved in citrate catabolism (*ACO1* and *IDH*) resulted in enhanced cellular and extracellular accumulation of citrate and increased Al resistance in the *Δaco1* and *Δidh12* mutants.

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I also determined the role of CS in enhancing citrate content and Al resistance by overexpression of a mitochondrial CS (*CIT1*) in WT yeast, MMYO11. Among the various isoforms of CS, I selected the mitochondrial isoform of CS 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 and allosteric inhibition of CS by ATP is low in mitochondria due to the low ATP: ADP ratio typically maintained in this organelle. Moreover, compartmentation of TCA cycle enzymes within the mitochondria ensures the availability and physical proximity of substrates required for proper functioning of CS in mitochondria. The yeast transformants overexpressing *CIT1* alone (MMYO11/*CIT1*) and with *MDH1* (MMYO11/*CIT1* + *MDH1*), showed a 2 to 3 fold increase in citrate level, and the transformants showed enhanced Al resistance (Chapter 4). In addition to citrate, the transformants showed increased levels of malate, indicating that enhanced Al resistance in these transformants could also be due to the coordinated increase in malate and citrate contents (Chapter 2).

In order to test if the overexpression strategy could be adopted to enhance levels of cellular and extracellular citrate and Al resistance in plants, a gene for *Arabidopsis thaliana* mitochondrial CS (At-mtCS) was overexpressed in canola using an *Agrobacterium*-mediated system. Transgenic canola lines overexpressing At-mtCS showed increased levels of CS gene expression and enhanced CS activity compared to the wild type (WT). Although a constitutive promoter (CaMV) was used, the transgenic lines showed no increase in citrate content (cellular and

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exudation) under control conditions. In the presence of 150 μM Al, the cellular shoot citrate content was significantly higher in transgenic lines compared to the WT plants. In contrast, no significant difference in root citrate content was observed among the transgenic lines and WT in the presence of Al. Exudation of citrate was increased two fold in transgenic lines compared to the WT, when exposed to 150 μM Al. Root growth experiments revealed that transgenic lines have enhanced levels of Al resistance compared to the WT at higher concentrations of Al (150 and 200 μM). The studies with transgenic canola revealed that overexpression of mt-CS can lead to increase in citrate levels and Al resistance. Thus, my work with citrate metabolism in yeast and transgenic canola overexpressing a mt-CS, clearly suggest that modulation of different enzymes involved in citrate synthesis and turnover (*MDH, CS, ACO, IDH*) can be considered for gene manipulation in order to alter citrate levels and Al resistance.

Future prospects

The transgenic canola lines and yeast transformants overexpressing mt-CS that I have developed and used in this research are valuable materials to test whether organic anion(s) other than citrate have contributed to the observed increase in Al resistance. Since cellular metabolism involves an array of enzymes that are interrelated and function in coordination with each other, metabolic profiling of these transformants could help to further understand the coordinated synthesis of organic anion(s) other than citrate.

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My studies with the yeast system clearly revealed overexpression of genes involved in synthesis of citrate and downregulation of genes involved in catabolism of citrate could also enhance levels of citrate accumulation and exudation. Hence, my hypothesis of enhancing Al resistance by increasing citrate levels in plants, could be tested by downregulating the levels of *ACO* or *IDH*, either by an antisense approach or characterizing specific gene knock-out mutants. A thorough knowledge of gene redundancy and the biochemistry of these metabolites are important before attempts are made to downregulate these housekeeping genes.

A concerted increase in organic acid biosynthesis and the ability to transport these anions into apoplasm may be essential for enhancing Al resistance. Genes encoding malate and citrate transport proteins are not thoroughly studied in plants, although several dicarboxylate- and tricarboxylate carrier proteins (DCPs and TCPs) are well characterized in animal systems (Fiermonte *et al*, 1999). The role of DCPs or TCPs in transporting malate or citrate as an Al resistance mechanism is an important area of research that would add to our understanding on organic anion exudation. Yeast mutants defective in a malate and citrate carrier proteins (Palmieri *et al.*, 1999; Kaplan *et al.*, 1996), can be used as model systems to test the importance of these transport protein in Al resistance. In addition, T-DNA mutants of *A. thaliana* knocked-out for genes homologous to malate and citrate carrier proteins of yeast and bacteria are available at Salk Institute Genomic Analysis Laboratory (California, USA). These SIGnAL T-DNA mutants could be tested for their sensitivity to Al, to explore the possible role of these genes in Al resistance. Is

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overexpression of gene(s) encoding malate or citrate transport proteins alone sufficient to enhance exudation of organic anions? Do plants that tolerate Al toxicity in acid soils require enhanced exudation of organic anion(s) from their roots continuously? Generating and characterizing transgenic lines overexpressing genes involved in both the biosynthesis and transport of organic anions would provide a means of testing if continuous and enhanced exudation of organic anions is essential for increased Al resistance. It is important to analyze the metabolic consequences of these strategies to determine if it is feasible as well as sustainable in these engineered plants. Perhaps, overexpressing the genes under the control of an inducible promoter could avoid the energy constraints caused by loss of carbon from the cells that exude organic anions (Taylor, 1991). These approaches would provide great insights into our understanding on organic acid metabolism and Al resistance in plants.

5.1. Conclusion

An important crop mangement strategy in overcoming low crop productivity on Al-toxic, acid soils is to improve the Al resistance of crop plants. Enhanced synthesis and exudation of organic anions such as malate and citrate have been associated with Al resistance in several plant species. A thorough knowledge of metabolic pathways by which plants produce and export these organic acids to the rhizosphere, identifcation of the enzymes that catalyze these biochemical reactions, and manipulatation of the genes that encode these enzymes will assist in improvement of Al resistance in plants. In this research, I have undertaken a holistic approach to

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understand malate and citrate metabolism in relation to Al resistance in yeast and canola.

The studies with yeast mutants with altered malate metabolism revealed no clear association between malate levels and Al resistance. My work with transgenic canola overexpressing a gene for malate synthase (MS) showed that MS might not be as ideal candidate gene for enhancing levels of malate and Al resistance in young seedlings.

Studies with yeast transformants and transgenic canola overexpressing At-mtCS, has helped me explore the complexity of citrate metabolism. My results with yeast mutants with altered citrate metabolism clearly demonstrated that *CS* represents an important component of the complex system and that genetic manipulation of several enzymes involved in citrate metabolism (such as *MDH, CS, ACO, IDH*) can be potentially used to increase synthesis, accumulation and exudation of citrate from cells (Chapter 4, Figure 4-1). My studies with transgenic *Brassica napus* lines revealed that overexpressing a gene for mt-CS can lead to an increase in citrate content (cellular and exudation) and Al resistance. Thus, metabolic engineering aimed at enhancing citrate levels is potentially an effective strategy to significantly improve Al-resistance in crop species.

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