Plant Physiol. (1996) 112: 1101–1109

Al Toxicity in Yeast[1]

A Role for Mg?

Colin W. MacDiarmid and Richard C. Gardner*

Center for Gene Technology, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

We have established conditions in which soluble Al is toxic to the yeast *Saccharomyces cerevisiae*. The major modifications to a standard synthetic medium were lowering the pH and the concentration of Mg ions. Alterations to the PO_4, Ca, or K concentration had little effect on toxicity. Organic acids known to chelate Al reduced its toxicity, suggesting that Al^{3+} is the toxic Al species. The unique ability of Mg ions to ameliorate Al toxicity led us to investigate the hypothesis that Al inhibits Mg uptake by yeast. Yeast cells accumulate Mg, Co, Zn, Ni, and Mn ions via the same transport system (G.F. Fuhrmann, A. Rothstein [1968] Biochim Biophys Acta 163: 325–330). Al^{3+} inhibited the accumulation of $^{57}Co^{2+}$ by yeast cells more effectively than Ga, La, or Mg. In addition, a mutant yeast strain with a defect in divalent cation uptake proved to be more sensitive to Al than a wild-type strain. Taken together, these results suggest that Al may cause Mg deficiency in yeast by blocking Mg transport. We discuss the relevance of yeast as a model for the study of Al toxicity in plant systems.

The cytotoxicity of Al is well documented in plants (Delhaize and Ryan, 1995; Kochian, 1995). The solubility of Al is dependent on low pH, and acidic soils often contain high concentrations of the dissolved cation. Plants grown in such soil have reduced root systems and exhibit a variety of nutrient-deficiency symptoms, with a consequent decrease in yield (Luttge and Clarkson, 1992). In many countries with naturally acidic soils, Al toxicity is a major agricultural problem, and hence has been predominantly studied in plant systems. However, the Al ion is also toxic to microorganisms such as bacteria, fungi, and green algae (Foy and Gerloff, 1972; Date and Halliday, 1979; Guida et al., 1991; Zel et al., 1993), and is detrimental to fish in acidic conditions (Baker and Schofield, 1982). Al has also been associated with several pathological states in humans, including neurological disorders such as Alzheimer's disease (Houeland, 1990) and syndromes related to long-term dialysis treatment (Arieff, 1990). Hence, the cytotoxicity of Al appears to be a general phenomenon.

Despite the economic and environmental importance of Al toxicity, there is little definitive information to suggest a mechanism of action for the ion in biological systems. Al has been reported to interact strongly with a number of organic molecules, including proteins, polynucleotides, lipids, and glycosides (Siegel et al., 1982; MacDonald et al., 1987; Akeson et al., 1989; Allan et al., 1990; Martin, 1992), but many of these experiments were performed in vitro with purified components and the interactions have not been convincingly linked to a toxic effect. Another suggestion is that Al acts to promote a lesion at the cell membrane, perhaps by interacting with membrane-associated proteins. Recent work on the phosphoinositide signal transduction pathway in plants revealed an interaction between Al and phospholipase C (Jones and Kochian, 1995), a membrane-associated enzyme involved in signal transduction in yeast and animal cells (Haug et al., 1994). Interaction of Al with the plasma membrane or membrane-intrinsic proteins could also conceivably alter cation homeostasis. In plants Al toxicity is modulated by the concentration of other cations in solution, most significantly Ca and Mg (Kinraide and Parker, 1987). Al toxicity in some plants is associated with decreased uptake and content of some cations (Rengel, 1990; Robinson and Rengel, 1991) and cation-deficiency symptoms (Foy, 1984). There is evidence to suggest that Al acts directly to inhibit specific membrane proteins responsible for cation uptake in root cell membranes (Rengel and Robinson, 1989; Rengel and Elliott, 1992; Gassmann and Schroeder, 1994; Huang et al., 1996), although it is less clear which of these cation transport processes is physiologically most important to the maintenance of root growth. Despite the large number of hypotheses put forward, there is not yet a definitive mechanism for Al toxicity.

A better understanding of the molecular basis of Al toxicity would be facilitated by the isolation of genes that contribute to Al resistance. A cloned resistance gene would also provide a valuable resource for use in the genetic manipulation of crop species. Al resistance in plants can be broadly divided into mechanisms of exclusion, in which Al is prevented from penetrating the symplasm, and mechanisms of tolerance, in which the tissue has the ability to withstand symplastic Al (Kochian, 1995). Natural variation in resistance between species and genetic analysis of resis-

[1] This research was supported by a grant from the Foundation for Research Science and Technology (AGR 06–052) to the New Zealand Pastoral Agriculture Research Institute. C.W.M. was the recipient of a University of Auckland doctoral scholarship.

* Corresponding author; e-mail r.gardner@auckland.ac.nz; fax 64–9–373–7416.

Abbreviations: LPM, low pH, low magnesium; YPD, yeast peptone dextrose.

tance in wheat (Delhaize et al., 1993; Delhaize and Ryan, 1995) indicates that single-gene resistance traits do exist in plants. However, the mapping and isolation of genes corresponding to such traits is hampered by the large genome size of most crop plants and could be accelerated by the use of a better-characterized genetic system. Recently, reported work with the model plant *Arabidopsis thaliana* has demonstrated that the generation of mutants with increased Al sensitivity is possible (Larsen et al., 1996), although molecular cloning of the genes responsible remains an arduous task.

We have been studying Al toxicity using the yeast *Saccharomyces cerevisiae*. As a genetic and physiological model for Al toxicity in eukaryotes, yeast has several advantages. Yeast and plants both use proton gradients to drive secondary transport systems, and yeast has been extensively used to identify the genetic factors that influence metal toxicity (Mehra and Winge, 1991). Acidic conditions (pH 3.5–5.0) are required for the optimal growth of yeast, making it particularly suited to investigations into the effects of Al. Yeast can also be grown in a defined minimal medium (Sherman, 1991), which can be modified to optimize metal toxicity (Bianchi et al., 1981). In addition, the entire DNA sequence of the yeast genome is now available, facilitating the rapid analysis of cloned traits.

In this paper we describe the development of a stringent selection for the isolation of Al-resistant yeast strains. The selection was achieved by lowering the pH and decreasing the Mg concentration of defined media. We suggest a possible mechanism for the toxicity of Al and other trivalent metals in yeast based on the effects of trivalent metals on the divalent cation transport system and the Al-sensitive phenotype of a previously characterized mutant strain deficient in cation uptake.

MATERIALS AND METHODS

Yeast Media

Saccharomyces cerevisiae was grown on YPD medium or on a synthetic minimal medium, LPM, which is a modification of synthetic dextrose medium (Sherman, 1991). LPM had a low pH (3.5) and a low concentration of KH_2PO_4 (25 μM) and $MgCl_2$ (200 μM) unless otherwise indicated. KCl (5 mM) was added to compensate for the reduced K caused by a decreased KH_2PO_4 concentration. To make LPM medium, solutions of the major salts, trace elements, and nutritional supplements were mixed, adjusted to pH 3.5 with dilute HCl, and autoclaved. Carbon sources (Glc or Gal) and vitamins (as a 500× concentrated solution) were filter-sterilized and added after autoclaving.

Solid Medium

Strains were routinely cultured on YPD medium solidified with 2% Bacto agar (Difco, Detroit, MI). LPM medium was gelled with 1% agarose (type II EEO medium, Sigma). To prevent hydrolysis of agarose solutions at low pH during sterilization, the medium was prepared from separately autoclaved solutions of double-strength salts with amino acids (pH 3.5) and 2% agarose, and then mixed. When required, solutions of $Al_2(SO_4)_3$, $GaCl_3$, and $LaCl_3$ were filter-sterilized and added to the complete medium prior to pouring plates.

Growth Curves

Yeast cultures in synthetic medium at late log phase were harvested by centrifugation, washed twice with distilled water, and resuspended in 1 mL of water. The cell suspension was used to inoculate 25 mL of LPM medium to a final A_{600} of 0.01 to 0.02. After a 4-h incubation, the Al ion was added as $Al_2(SO_4)_3$, and growth of the cultures was monitored by determining the optical density of 1-mL samples (A_{600}). For amelioration experiments organic acids were added 5 min before the addition of Al.

Co Uptake Assay

Co uptake was assayed using the protocol of Conklin et al. (1993) with some modifications. The S288C derivative FY23 (Winston et al., 1995) was grown to saturation in YPD medium, and 100 μL was used to inoculate 200 mL of high-PO_4 LPM containing 200 μM $MgCl_2$ and 5 mM KH_2PO_4, pH 4.0. The culture was harvested at log phase (A_{600} = 0.5; approximately 14 h of growth), and the cells were washed twice with 50 mL of distilled water, then resuspended in 5 mM Tris-succinate buffer (pH 4.0) at a density of approximately 10^8 cells/mL. One milliliter of cells was incubated for 30 min with 2% Glc at 25°C to provide metabolic energy for transport (Norris and Kelly, 1977). The incubation was followed by the immediate addition of 0.25 μCi of $^{57}CoCl_2$ (Amersham) with cold $CoCl_2$, to give a final concentration of 100 μM total Co^{2+}. Aliquots of the suspension (150 μL) were filtered onto cellulose filters (0.45-μm pore size, Millipore), which were immediately washed twice with 10 mL of cold wash buffer (50 mM Tris-succinate buffer, pH 4.0, containing 20 mM $MgCl_2$ and 1.0 mM $CoCl_2$, at 0°C). Nonexchangeable radioactivity on the filters was quantified with a gamma counter (Wizard 1480, Wallac, Gaithersburg, MD). Controls containing no cells showed insignificant binding of the tracer to the filter. For competition experiments metal salts were mixed with the tracer before being added to the cell suspension.

RESULTS

To ensure the solubility of Al in the cell culture medium, several modifications were made to the standard synthetic medium (Sherman, 1991). The effect of these changes was monitored by the measurement of growth rates in liquid culture. GSY122 (Wagenbach et al., 1991) (see Table I) was used as a representative laboratory strain for the optimization of Al toxicity.

Growth of Yeast in Low-PO_4, Low-pH Conditions

When using Al the pH of the medium should be at 4.2 or below to ensure high activity of the Al^{3+} ion in solution (Haug, 1984). In the synthetic medium active growth was obtained with an initial pH as low as 3.5. The pH decreased during growth, consistently reaching a level of 2.9 to 3.0 at

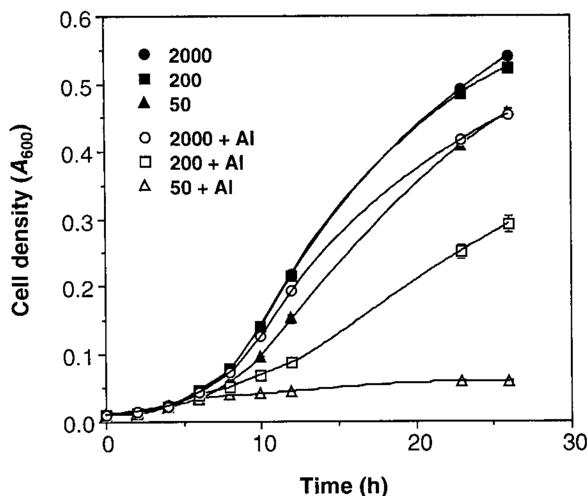

Figure 1. Effect of Mg concentration on Al toxicity. Duplicate samples of low-pH (3.5) medium containing 25 μM KH_2PO_4 and different $MgCl_2$ levels (50, 200, or 2000 μM) were inoculated with washed cells from a culture of GSY122 (grown in synthetic medium and harvested at log phase) to give an initial A_{600} of 0.01. Cultures were incubated at 30°C with shaking, and growth was monitored by spectrophotometry (for GSY122, an A_{600} of 1.0 = 3 × 10^7 cells/mL). After 4 h of incubation, Al was added to a final concentration of 50 μM (open symbols), or was not added (filled symbols). Error bars represent ±SE (n = 3).

the stationary phase (data not shown). Fermentative growth of yeast is known to reduce the medium pH (Sigler et al., 1980), and because this would not be expected to decrease the activity of Al^{3+} in solution no attempt was made to buffer the medium. In subsequent experiments the initial pH was 3.5, to maximize the activity of Al^{3+}.

PO_4 Requirements for Growth

Al and PO_4 ions have been reported to interact and precipitate in solution, reducing both the soluble Al concentration and the available PO_4^{3-} (Koyama et al., 1988). Reduction of the PO_4 concentration was essential in ensuring Al toxicity in tobacco cell culture experiments (Conner and Meredith, 1985). To determine the minimal PO_4 requirement of yeast we performed growth assays using GSY122 with a range of PO_4 concentrations from 0 to 500 μM. PO_4 limitation affected the final cell density reached at the stationary phase but did not affect the initial growth rate, and adequate growth was obtained with as little as 25 μM PO_4^{3-} (data not shown). To rule out any interaction with Al in solution, the PO_4^{3-} concentration of the liquid medium was reduced to 25 μM unless otherwise stated. In subsequent experiments with modified media, no evidence of any PO_4^{3-} amelioration of Al toxicity was seen, even with PO_4^{3-} levels up to 5 mM (data not shown). As a result, for some applications (e.g. in solid medium) the PO_4 concentration was increased to 100 μM.

Effect of Cation Concentration on Al Toxicity

When 200 μM Al as $Al_2(SO_4)_3$ was added to log-phase yeast subcultures in a low-pH, low-PO_4, synthetic medium, a decrease in the initial rate of growth was observed relative to controls and the final cell density was reduced by approximately 20%. However, higher levels of Al (up to 5 mM; data not shown) caused no additional decrease in growth rate or final cell density. This level of growth inhibition was insufficient to provide a selection using physiologically relevant (micromolar) levels of Al. For this reason we decided to modify the synthetic medium further to determine if we could increase Al toxicity.

Ca and Mg ions are potent ameliorators of Al toxicity, both in whole plant systems (Alva et al., 1986; Kinraide and Parker, 1987) and in plant cell cultures (Conner and Meredith, 1985). We performed experiments to determine the yeast requirement for Ca and Mg, with the aim of reducing divalent cation concentrations if possible. When the Ca content of the medium was reduced to 1 μM the growth rate of GSY122 was not significantly affected, indicating that this strain has a very low Ca requirement (data not shown). This is consistent with the results of Iida et al. (1990), who found that the addition of Ca chelators and Ca ionophores was required to arrest yeast growth in Ca-deficient medium. Reducing the Ca concentration to 1 μM (in a medium with 2.0 mM Mg) did not increase Al toxicity (data not shown).

In contrast, growth of GSY122 was dependent on available Mg. The lowest concentration of Mg that did not affect the growth rate was 200 μM (Fig. 1). Moreover, Al toxicity significantly increased with decreasing Mg concentration. In a medium with 200 μM Mg, the addition of 50 μM Al strongly inhibited growth (Fig. 1). Increasing the Mg concentration to 500 μM or above efficiently ameliorated the Al toxicity, so we lowered the Mg concentration of the synthetic medium to 200 μM for subsequent experiments. At this Mg concentration, Al was strongly inhibitory to growth at concentrations of 50 to 250 μM (Fig. 2). The effect of lowered Ca concentration on Al toxicity was re-determined in a

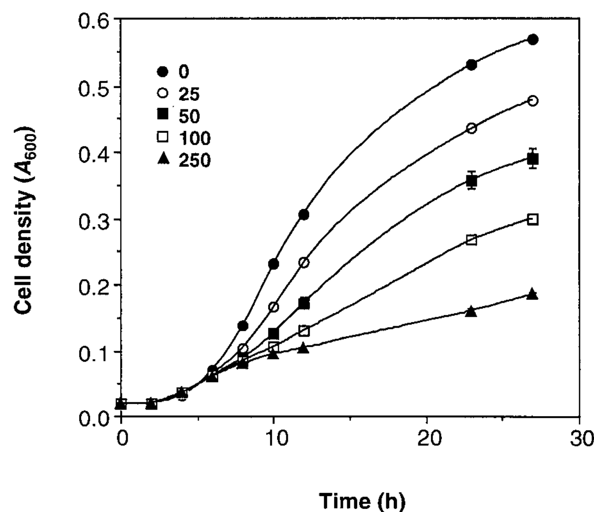

Figure 2. Effect of Al on growth in LPM medium. A low-pH (3.5) medium containing 25 μM KH_2PO_4 and 200 μM $MgCl_2$ was inoculated with GSY122 as before (see Fig. 1). Cultures were incubated for 4 h, and Al was added to give the final concentrations shown (0–250 μM). Error bars represent ±SE (n = 3).

Table I. *Yeast strains*

Strain	Genotype	Source
GSY122	*ura3-52 leu2-3,112 pep4::HIS3 prb-1D1.6R leu2::hisG can1 cir8 MATα*	P. Bergquist
CYP520	*ura3-52 leu2-3,112 his4-539 MATα*	D. Conklin
CYP522	*cot2-Δ1::LEU2 ura3-52 leu2-3,112 his4-539 MATα*	D. Conklin
FY23	*ura3-52 trp1Δ63 leu2Δ1 MATα*	ATCC[a]
SH2332	*pho3-1 pho4::HIS3 his3-532 leu2-3 leu2-112 ura3-1,2 trp1-289 ade2 MATα*	S. Harashima
CG379	*ade5 can1 leu2-3 leu2-112 trp1-289$_a$ ura3-52 gal2 [Kil-0] MATα*	YGSC[b]

[a] ATCC, American Type Culture Collection. [b] YGSC, Yeast Genetic Stock Center (Berkeley, CA).

medium with 200 μM Mg, and was found to be negligible (data not shown).

K is another cation that ameliorates Al toxicity in plants, albeit more weakly than Mg and Ca (Kinraide and Parker, 1987). GSY122 was very sensitive to K concentration, and growth was strongly reduced at less than 2.5 mM K. However, lowering the K concentration of the medium did not significantly increase Al toxicity (data not shown).

Al Selection on Solid Medium

The addition of Al to LPM gelled with agarose (1%) provided a selection sufficiently stringent for routine use. The use of agarose was necessary due to the high Mg content of commercial agar preparations (data not shown). Several yeast strains, including CG379, SH2332, FY23, and CYP520 (Table I), were tested for sensitivity to Al in solid LPM medium. Although some differences in sensitivity were seen, growth of all of the strains was strongly inhibited by Al concentrations in the range of 100 to 200 μM (data not shown; see Fig. 4 for example).

Effect of Organic Acids on Al Toxicity

A variety of substances have been shown to chelate Al in vitro, including the carboxylic acids malic and citric acid (Jackson, 1982). Strong Al-acid complexes are nontoxic to cells, and the secretion of organic acids from roots has been suggested as a possible mechanism for Al exclusion in plants (Suhayda and Haug, 1986; Miyasaka et al., 1991; Delhaize et al., 1993). We examined the effect of citric and malic acids on Al toxicity in LPM. As shown in Figure 3A, citric acid in 10-fold excess over Al allowed growth almost to control levels. Malic acid was less effective, but still significantly decreased toxicity at 100-fold excess over Al (Fig. 3B).

Toxicity of Trivalent Cations in LPM

We were interested to see if the lowered Mg concentration in LPM affected the toxicity of other trivalent metal cations. The growth of four different yeast strains was compared on low pH medium with added Al, Ga, or La and two different levels of Mg (2.0 mM and 100 μM; Fig. 4). For all four strains the toxicity of Al and Ga but not La was modulated by the Mg concentration; compare the overall growth and colony size in the upper and lower plates of Figure 4. Note that for the two most tolerant strains, CG379 (C) and GSY122 (A), the Ga sensitivity was manifested as differences in colony size. Higher levels of Ga gave a total growth dependence on the Mg concentration similar to that seen for the two more sensitive strains shown in Figure 4 (data not shown). In the LPM medium the trivalent metals were toxic in the order Al > Ga > La.

Soluble Al Inhibits Divalent Cation Uptake in Yeast

Since Al toxicity in yeast appeared to be related to Mg availability, we investigated the effect of Al on the yeast divalent cation transport system using $^{57}Co^{2+}$ as a tracer.

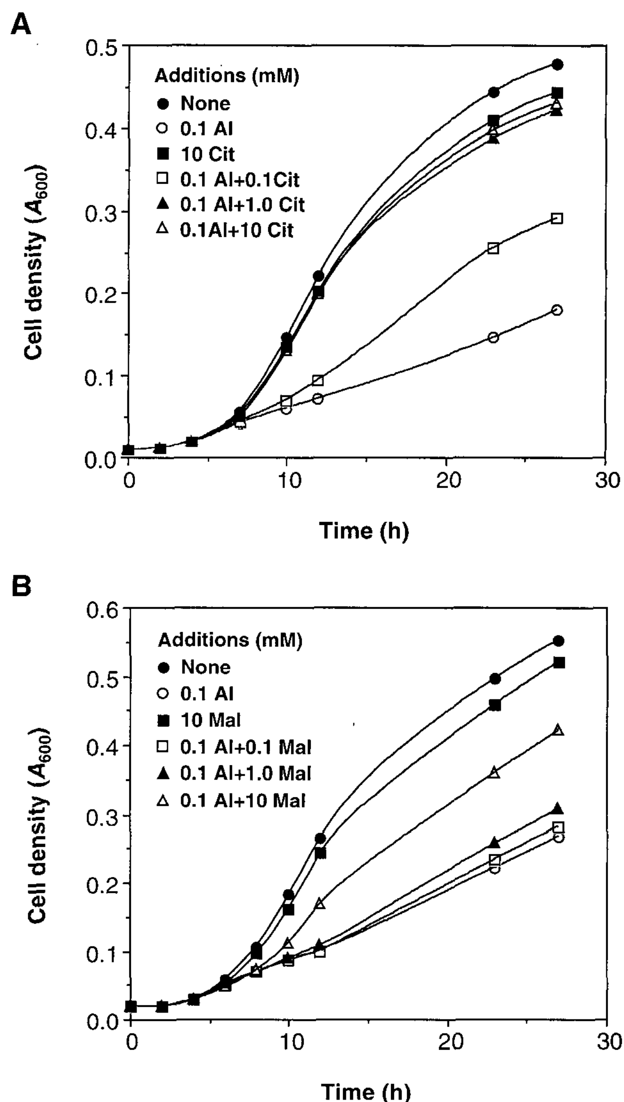

Figure 3. Amelioration of Al toxicity by organic acids. LPM medium (200 μM Mg) was inoculated with GSY122 (see Fig. 1). After 4 h of growth, citric acid (A), malic acid (B), and Al were added to give the indicated concentrations. Error bars represent ±SE (n = 3) and are smaller than the data points.

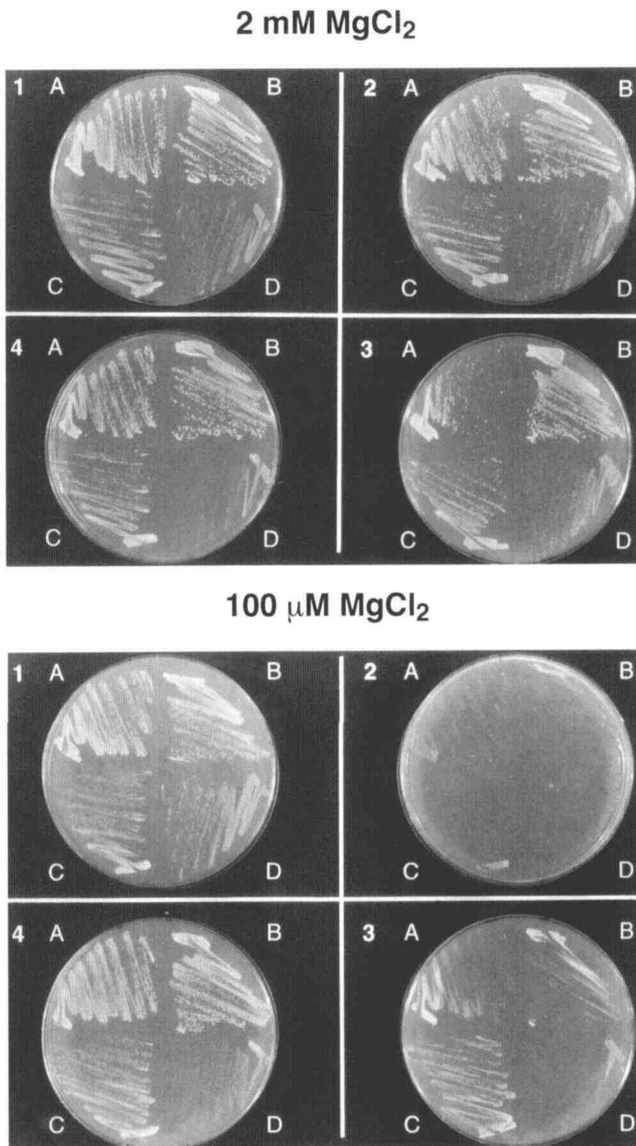

Figure 4. Comparative toxicity of trivalent metals to yeast. LPM plates (100 μM PO_4, pH 3.8) contained either 2 mM or 100 μM $MgCl_2$ as indicated. Trivalent metal solutions were added to a final concentration of 100 μM. 1, No metal; 2, $Al_2(SO_4)_3$; 3, $GaCl_3$; and 4, $LaCl_3$. Strains were incubated at 30°C for 3 d. A, GSY122; B, FY23; C, CG379; and D, SH2332.

Divalent cations such as Mg, Co, and Mn are transported into the yeast cell by the same energy-dependent system, the activity of which is coupled to Glc availability (Fuhrmann and Rothstein, 1968; Norris and Kelly, 1977; Borst-Pauwels, 1981). Cells of the FY23 strain were grown to mid-log phase, harvested, and resuspended in 5 mM Tris-succinate buffer, pH 4.0. The low pH of the buffer ensured the solubility of Al. Although succinate as a dicarboxylate anion could potentially bind Al, Al-succinate complexes are relatively weak compared with Al-citrate complexes (Jackson, 1982; Jackson and Cosgrove, 1982). With 100 μM Co uptake was very rapid and essentially complete after 35 min (Fig. 5A). When Al was added to the cells along with the Co tracer, uptake was strongly inhibited. Al lowered both the initial rate of uptake and the final Co content of the yeast cells.

Using the same assay we compared Co uptake in the presence of Al^{3+}, Ga^{3+}, La^{3+}, and Mg^{2+} ions (Fig. 5B). La and Mg had previously been reported to inhibit divalent cation uptake in yeast (Norris and Kelly, 1977; Okorokov et al., 1977), whereas Ga is toxic in LPM medium (Fig. 4 and data not shown). When values for final uptake (after 2 h) were compared Al was the most effective inhibitor, followed by Ga (Fig. 5B). Although La reduced the initial rate of uptake as effectively as Ga, final values of Co accumu-

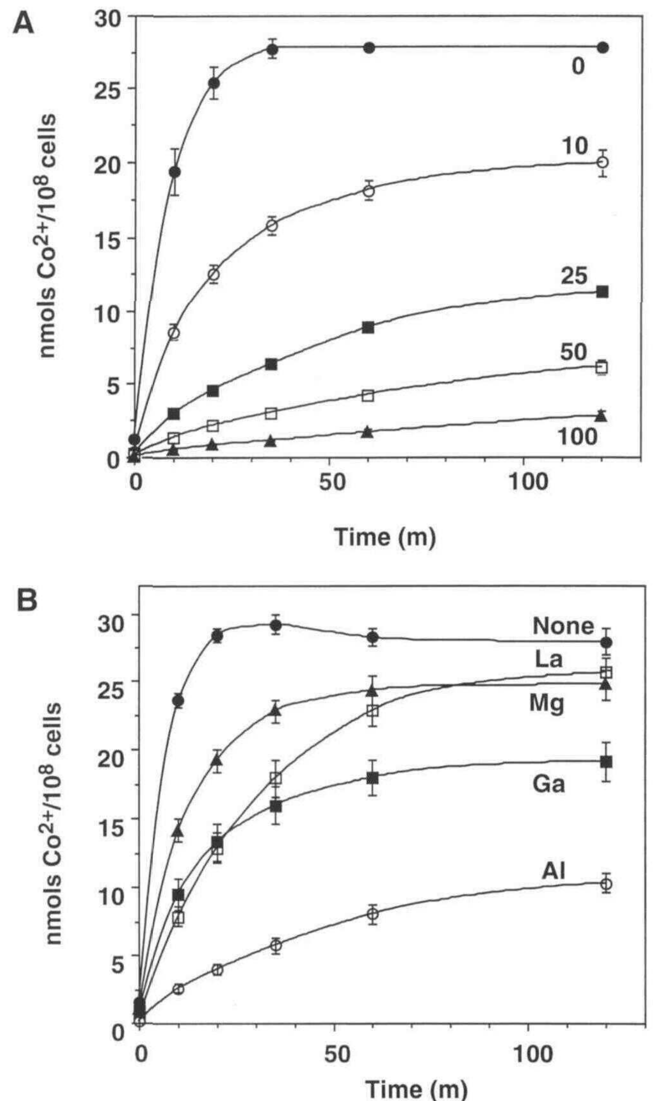

Figure 5. Effect of Al and other metal ions on Co uptake. Error bars represent ±SE (n = 4). A, Effect of Al concentration on Co uptake. Time course of $^{57}Co^{2+}$ uptake by strain FY23 is shown without Al, and with the addition of $Al_2(SO_4)_3$, to give 10, 25, 50, and 100 μM final Al^{3+} concentration. B, $^{57}Co^{2+}$ uptake in the presence of different metal ions. Conditions were as before with no metal added and with 12.5 μM $Al_2(SO_4)_3$, 25 μM $GaCl_3$, 25 μM $LaCl_3$, and 25 μM Mg Cl_2.

lated were comparable with inhibition by Mg and significantly higher than with Ga.

Analysis of the *COT2* Mutant under Al Stress

In yeast the *COT2* (*GRR1*) gene product controls the activity of the divalent cation transport system by coupling its regulation to the Glc growth signal (Conklin et al., 1993). When grown in Glc *cot2* mutant strains exhibited reduced Co uptake (approximately 10% of wild-type levels; Conklin et al., 1993). Growth of a *cot2::LEU2* (CYP522) strain and an isogenic wild-type strain (CYP520) were compared in LPM medium with 2% Glc. Without Al CYP522 grew more slowly than CYP520 during the log phase (data not shown). This effect of the *cot2/grr1* mutation on growth rate in Glc media is well documented, and is believed to result from a decreased capacity for Glc transport (Conklin et al., 1993; Vallier et al., 1994). When the two strains were grown using Gal as the carbon source, there was little difference in growth rate, because Gal uptake is unaffected by the *cot2* mutation (Vallier et al., 1994). Therefore, we compared the effect of Al on growth of the two strains in Gal medium. The results showed that the *cot2* strain CYP522 was significantly more affected by Al than was the wild type (Fig. 6). The Al sensitivity of CYP522 was also seen when the strains were compared on a solid LPM plus Al medium with Gal (data not shown). Since the *cot2* mutation probably affects the uptake of Mg we also examined the growth of both strains in a Mg-deficient medium. Trace amounts of Al could be toxic to yeast at very low Mg concentrations, so 50 μM citric acid was added to the medium to chelate any Al present. No consistent difference in growth rate was seen between the two strains at Mg concentrations as low as 5 to 10 μM (data not shown).

Effect of Al Stress and Lowered Mg on the Morphology of *cot2* Cells

The *cot2* mutation has previously been reported to be associated with altered budding and morphology of yeast cells (Conklin et al., 1993). We also observed a budding defect when the *cot2* strain CYP522 was grown in YPD or LPM media (200 μM Mg) (Fig. 7B). A much more severe morphological defect was noted when the same strain was grown under Al stress (Fig. 7H). The defect appeared to result from unipolar bud elongation and incomplete cell division, and was more extreme than that previously reported to result from growth of *cot2* strains on sporulation medium (Conklin et al., 1993). Addition of equimolar quantities of citrate to LPM plus Al medium restored growth and reduced the filamentous morphology of the CYP522 strain (data not shown), indicating that the growth defect was related to Al toxicity. Filamentous growth was not seen when the wild-type strain was treated with Al; instead the cells became highly vacuolated (Fig. 7G), appeared to have weak cell walls, and exhibited swelling and bursting when exposed to hypotonic solutions. The effects of Al on the morphology of both strains could be duplicated by growth in citrate-containing medium with a low Mg concentration (0 μM Mg) (Fig. 7, E and F). For CYP522 the distortion became progressively more extreme as Mg concentrations decreased (compare 200 μM with 25 μM and no $MgCl_2$; Fig. 7, B, D, and F). The effects of Mg starvation and Al treatment on morphology and budding in the CYP strains could not be duplicated by the reduction of PO_4, Ca, or K concentrations in the medium (data not shown).

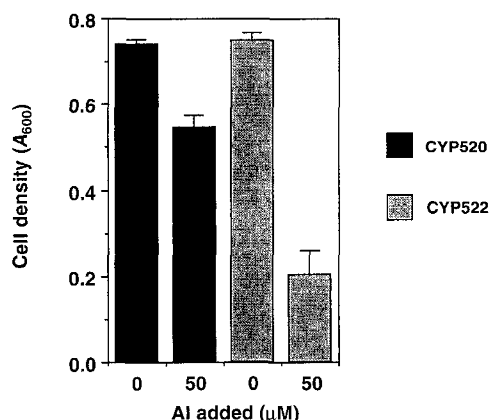

Figure 6. Effect of the *cot2* mutation on growth under Al stress. Aliquots (25 mL) of LPM medium (200 μM Mg, 2% Gal, pH 3.8) were inoculated with log-phase cells of either CYP520 (*COT2*) or CYP522 (*cot2*) to give an initial A_{600} of 0.01. After 4 h of growth, Al was added (50 μM). After 40 h of growth at 30°C, A_{600} of the cultures was recorded. Error bars represent ±SE (n = 3).

DISCUSSION

Development of an Al Selection for Yeast

The key factor in obtaining Al-selective conditions in synthetic medium appears to be the Mg concentration, which was reduced to 200 μM to obtain useful growth inhibition at micromolar Al levels. Reduction of the pH of the medium to 3.5 ensured the solubility of Al, and, because yeast are tolerant to low pH and actually reduce their pH during growth, Al can be reliably maintained in solution without the use of buffers, which may chelate Al. The PO_4^{3-} content of LPM was set at a low level, which, together with the low pH of the medium, effectively eliminated any amelioration of Al toxicity by PO_4^{3-} ions.

Mechanism of Al Toxicity in Yeast

We hypothesize that in yeast Al acts to prevent the uptake of Mg, and that Al toxicity in LPM medium results from Mg deficiency for the following reasons: (a) Al is less toxic when more Mg is available. This effect was specific to Mg, since variation in Ca, K, and PO_4^{3-} concentrations had little effect on Al toxicity. (b) Al effectively blocked the uptake of Co into yeast cells, and hence probably blocks uptake of Mg via inhibition of the nonspecific divalent cation transport system. Although previous studies reported that the transport system has a high affinity for Mg, Al was far more effective than Mg as an inhibitor of Co uptake. (c) The *cot2* mutation in yeast, which decreases the activity of the divalent cation uptake system, also results in increased sensitivity to Al. (d) In both wild-type and *cot2* strains of yeast, Al toxicity and Mg deficiency produced similar morphological changes in growing cells.

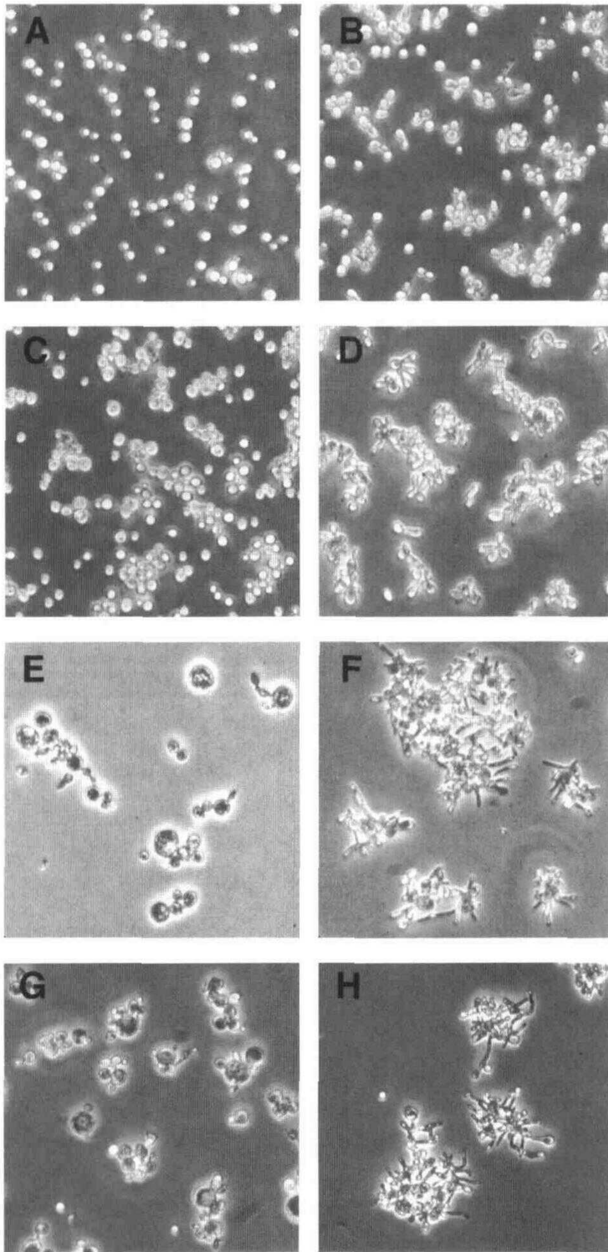

Figure 7. Morphology of the CYP522 strain changes under Al stress and Mg deficiency. Washed cells of CYP520 and CYP522 harvested from cultures at the exponential phase were subcultured into LPM and 2% Gal media, then grown for 24 h under the conditions given and examined using a phase-contrast microscope (model IMT-2, Olympus). The left column shows CYP520 (*COT2*); the right column shows CYP522 (*cot2*). A and B, LPM with 200 μM $MgCl_2$. C and D, LPM with 25 μM $MgCl_2$ and 25 μM citrate (pH 3.8). E and F, LPM with no $MgCl_2$, and 25 μM citrate. G and H, LPM with 200 μM Mg and 100 μM Al (added after 4 h of growth).

We propose that the effect of Al on Mg transport is most likely to result from competitive inhibition, although an indirect effect of Al on cation influx cannot be excluded. One explanation for the effectiveness of Al as an inhibitor of Mg uptake in yeast may lie in the similar size of hydrated Al and Mg ions (Martin, 1992). It has been previously suggested that this size similarity may allow the substitution of Al for divalent cations at Mg-binding sites of enzymes and structural proteins (e.g. tubulin [MacDonald et al., 1987]). An analogous reaction could occur if trivalent Al ions act as a substrate for the Mg transporter in yeast. However, we cannot say if Al blocks the transporter by irreversibly binding to the active site (and is not itself a substrate for transport), or simply competes more effectively than Mg for the transporter. Genetic evidence obtained using the Al selection may clarify this issue, as may further kinetic studies on the yeast transport system.

Toxic Action of Other Trivalent Ions

The relative effectiveness of three trivalent metals as inhibitors of Co accumulation corresponded to their toxicity in LPM medium. Like Al toxicity, Ga toxicity was affected by Mg availability. Ga also strongly reduced the final Co uptake after 2 h of incubation (Fig. 5B). These results are consistent with the idea that Ga also inhibits Mg transport. In contrast, although La effectively reduced the *initial rate of Co uptake, the ion was a relatively ineffective* inhibitor of Co accumulation, and La toxicity was not significantly affected by Mg concentration. The La ion is more similar in size to Ca than Mg, and may affect yeast growth via a different physiological mechanism.

Yeast as a Model for Al Toxicity in Plants

Although these results were obtained using yeast, several reports implicate the inhibition of Mg transport as a possible mechanism of Al action in plant systems. Comprehensive surveys of nutrient changes in plants in response to long-term Al treatment have shown that there are no consistent changes in dicotyledonous species (Wheeler et al., 1992c; Wheeler and Dodd, 1995), but that reductions in Mg content are the major feature of Al treatment in both cereals (Clark, 1977; Tan and Keltjens, 1990; Tan et al., 1991; Wheeler et al., 1992a) and grass species (Wheeler et al., 1992b). Greater Mg uptake has been associated with Al resistance in some plants (Foy, 1984; Huang and Grunes, 1992). Rengel and Robinson (1989) showed that Al competitively inhibited Mg uptake and concluded that this inhibition was most likely due to binding of Al to Mg-specific sites on transport proteins, rather than to Al-induced damage to the root system. Inhibition of Mg uptake occurred within 30 min, well before inhibition of root growth occurred, suggesting that the block of uptake could be the primary cause of Al toxicity (Robinson and Rengel, 1991).

Despite this indicative evidence, the inhibition of Mg uptake has not been a favored option in the literature as a potential mechanism for Al toxicity (see Kochian, 1995). One likely reason is that Al inhibition of root elongation can be demonstrated in a medium containing only $CaCl_2$ (see Kinraide and Parker, 1987; Ryan et al., 1994). This observation, combined with recent evidence that Al does not act by preventing Ca uptake (Ryan et al., 1994; see review by Kochian, 1995), makes it unlikely that Al inhibits

root elongation in plants by simply restricting cation uptake. However, it is clear that longer-term Mg deficiency may contribute to the overall syndrome of Al toxicity in cereals and grasses. Our results in yeast lead us to suggest a critical reassessment of the hypothesis that Al blocks Mg transport.

This work has revealed a difference in cation amelioration of Al toxicity between yeast and plants. In plants, cations in general and Ca ions in particular are effective ameliorators of Al toxicity (Kinraide and Parker, 1987; Ryan et al., 1994). Kinraide et al. (1994) suggested that this occurs by reducing the negative charge at the membrane surface and so decreasing the local activity of Al^{3+}. In contrast, we found that Ca had little effect on Al toxicity in yeast. However, yeast synthetic medium is of relatively high ionic strength (total cation concentration 47 mM) compared with those commonly used for assays of Al toxicity and cation amelioration in whole plants (e.g. 0.4–2 mM) (Kinraide et al., 1994). Although we used a wide range of Ca concentrations in our experiments (0–2 mM), it is possible that the high cation concentration of the medium may have masked any effect of Ca on Al toxicity. If the inability of Ca ions to ameliorate Al toxicity in yeast is a genuine effect, it could be attributable to variations in the ion-binding capacity of plant and yeast cell walls or to differences in the surface charge and composition of the plasma membrane.

Regardless of the mechanism of toxicity in yeast, the availability of Al-selective media for yeast provides a powerful tool for the genetic analysis of Al toxicity and resistance in both yeast and plants. Modified synthetic media have been used in our laboratory to isolate yeast mutants sensitive to Al (E. Schott and R. Gardner, unpublished results) and to identify yeast genes that provide Al resistance when overexpressed (C. MacDiarmid and R. Gardner, unpublished results). We also intend to search for plant Al resistance genes, using the heterologous expression of plant cDNA clones in a wild-type yeast strain (see Kushnir et al., 1995). A good candidate for study may be the *alt1* locus from wheat, which is associated with the Al-triggered release of malate from root tips (Delhaize et al., 1993; Delhaize and Ryan, 1995). Since malate ameliorates Al toxicity in yeast (Fig. 3), expression of the *alt1* locus may help growth on Al and hence enable the isolation of a cDNA clone encoding this trait. Such genetic investigations will shed new light on the physiology of Al toxicity and resistance in plants.

ACKNOWLEDGMENTS

We thank Douglas Conklin for the gift of the CYP strains, Rod Bieleski, Eric Schott, and Dave Wheeler for helpful discussions during this work, and Jo Putterill for her critical reading of the manuscript.

Received April 30, 1996; accepted August 14, 1996.
Copyright Clearance Center: 0032–0889/96/112/1101/09.

LITERATURE CITED

Akeson MA, Munns DN, Burau RG (1989) Absorption of Al^{3+} to phosphatidylcholine vesicles. Biochim Biophys Acta **986:** 33–40

Allan DL, Shann JR, Bertsch PM (1990) Role of root cell walls in iron deficiency of soybean (*Glycine max*) and aluminium toxicity of wheat (*Triticum aestivum*). *In* L van Beusichem, ed, Plant Nutrition—Physiology and Applications. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 345–349

Alva AK, Asher CJ, Edwards DG (1986) The role of calcium in alleviating aluminium toxicity. Aust J Agric Res **37:** 375–382

Arieff AI (1990) Aluminium and the pathogenesis of dialysis dementia. Environ Geochem Health **12:** 89–93

Baker JP, Schofield CL (1982) Aluminum toxicity to fish in acidic waters. Water Air Soil Pollut **18:** 289–309

Bianchi ME, Carbone ML, Lucchini G, Magni GE (1981) Mutants resistant to manganese in *Saccharomyces cerevisiae*. Curr Genet **4:** 215–220

Borst-Pauwels GWFH (1981) Ion transport in yeast. Biochim Biophys Acta **650:** 88–127

Clark RB (1977) Effect of aluminium on growth and mineral elements of Al-tolerant and Al-intolerant corn. Plant Soil **47:** 653–662

Conklin DS, Kung C, Culbertson MR (1993) The *COT2* gene is required for glucose-dependent divalent cation transport in *Saccharomyces cerevisiae*. Mol Cell Biol **13:** 2041–2049

Conner AJ, Meredith CP (1985) Simulating the mineral environment of aluminium toxic soils in plant cell culture. J Exp Bot **36:** 870–880

Date RA, Halliday J (1979) Selecting *Rhizobium* for acid, infertile soils of the tropics. Nature **277:** 62–64

Delhaize E, Ryan PR (1995) Aluminum toxicity and tolerance in plants. Plant Physiol **107:** 315–321

Delhaize E, Ryan PR, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol **103:** 695–702

Foy CD (1984) Physiological effects of hydrogen, aluminum, and manganese toxicities in acid soil. *In* F Adams, ed, Soil Acidity and Liming, Ed 2. American Society of Agronomy, Madison, WI, pp 57–97

Foy CD, Gerloff GC (1972) Response of *Chlorella pyrenoidosa* to aluminum and low pH. J Phycol **8:** 268–271

Fuhrmann GF, Rothstein A (1968) The transport of Zn^{2+}, Co^{2+} and Ni^{2+} into yeast cells. Biochim Biophys Acta **163:** 325–330

Gassmann W, Schroeder JI (1994) Inward rectifying K^+ channels in root hairs of wheat. A mechanism for aluminum-sensitive low affinity K^+ uptake. Plant Physiol **105:** 1399–1408

Guida L, Saidi Z, Hughes MN, Poole RK (1991) Aluminium toxicity and binding to *Escherichia coli*. Arch Microbiol **156:** 507–512

Haug A (1984) Molecular aspects of aluminum toxicity. Crit Rev Plant Sci **1:** 345–373

Haug A, Shi B, Vitorello V (1994) Aluminum interaction with phosphoinositide-associated signal transduction. Arch Toxicol **68:** 1–7

Houeland T (1990) Aluminium and Alzheimer's disease: is there a causal connection? Environ Geochem Health **12:** 173–178

Huang JW, Grunes DL (1992) Potassium/magnesium ratio effects on aluminum tolerance and mineral composition of wheat forage. Agron J **84:** 643–650

Huang JW, Pellet DM, Papernik LA, Kochian LV (1996) Aluminum interactions with voltage gated calcium transport in plasma membrane vesicles isolated from roots of aluminum-sensitive and -resistant wheat cultivars. Plant Physiol **110:** 561–569

Iida H, Sakaguchi S, Yagawa Y, Anraku Y (1990) Cell cycle control by Ca^{2+} in *Saccharomyces cerevisiae*. J Biol Chem **265:** 21216–21222

Jackson GE (1982) Studies on the chelation of aluminium for biological application. Part 1. Citric acid. S Afr J Chem **35:** 89–92

Jackson GE, Cosgrove A (1982) Studies on the chelation of aluminium for biological application. Part 2. Oxalic, malonic and succinic acids. S Afr J Chem **35:** 93–95

Jones DL, Kochian LV (1995) Aluminum inhibition of the inositol 1,4,5-trisphosphate signal transduction pathway in wheat roots: a role in aluminum toxicity? Plant Cell **7:** 1913–1922

Kinraide TB, Parker DR (1987) Cation amelioration of aluminum toxicity in wheat. Plant Physiol **83:** 546–551

Kinraide TB, Ryan PR, Kochian LV (1994) Al^{3+}-Ca^{2+} interactions in aluminum rhizotoxicity. II. Evaluating the Ca^{2+}-displacement hypothesis. Planta **192:** 104–109

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol **46:** 237–260

Koyama H, Okawara R, Ojima K, Yamaya T (1988) Re-evaluation of characteristics of a carrot cell line previously selected as aluminum-tolerant cells. Physiol Plant **74:** 683–687

Kushnir S, Babiychuk E, Kampfenkel K, Belles-Boix E, Van Montagu M, Inze D (1995) Characterization of *Arabidopsis thaliana* cDNA's that render yeast tolerant toward the thiol-oxidising drug diamide. Proc Nat Acad Sci USA **92:** 10580–10584

Larsen PB, Tai C-Y, Kochian LV, Howell SH (1996) Arabidopsis mutants with increased sensitivity to aluminum. Plant Physiol **110:** 743–751

Luttge U, Clarkson DT (1992) Mineral nutrition: aluminium. Prog Bot **53:** 63–77

MacDonald TL, Humpreys WG, Martin LB (1987) Promotion of tubulin assembly by aluminum ion *in vitro*. Science **236:** 183–186

Martin RB (1992) Aluminium speciation in biology. *In* DJ Chadwick, J Whelan, eds, Aluminium in Biology and Medicine. John Wiley & Sons, New York, pp 5–25

Mehra RK, Winge DR (1991) Metal ion resistance in fungi: molecular mechanisms and their regulated expression. J Cell Biochem **45:** 30–40

Miyasaka SC, Buta JG, Howell RK, Foy CD (1991) Mechanism of aluminum tolerance in snapbeans. Root exudation of citric acid. Plant Physiol **96:** 737–743

Norris PR, Kelly DP (1977) Accumulation of cadmium and cobalt by *Saccharomyces cerevisiae*. J Gen Microbiol **99:** 317–324

Okorokov LA, Lichko LP, Kadomtseva M, Kholodenko VP, Titovsky VT, Kulaev IS (1977) Energy-dependent transport of manganese into yeast cells and distribution of accumulated ions. Eur J Biochem **75:** 373–377

Rengel Z (1990) Competitive Al^{3+} inhibition of net Mg^{2+} uptake by intact *Lolium multiflorum* roots. II. Plant age effects. Plant Physiol **93:** 1261–1267

Rengel Z, Elliott DC (1992) Mechanism of aluminum inhibition of net $^{45}Ca^{2+}$ uptake by *Amaranthus* protoplasts. Plant Physiol **98:** 632–638

Rengel Z, Robinson DL (1989) Competitive Al^{3+} inhibition of net Mg^{2+} uptake by intact *Lolium multiflorum* roots. I. Kinetics. Plant Physiol **91:** 1407–1413

Robinson DL, Rengel Z (1991) Aluminum influences on magnesium uptake and on grass tentany potential of ryegrass. Curr Top Plant Biochem Physiol **10:** 107–116

Ryan PR, Kinraide TB, Kochian LV (1994) Al^{3+}-Ca^{2+} interactions in aluminum rhizotoxicity. I. Inhibition of root growth is not caused by reduction of calcium uptake. Planta **192:** 98–103

Sherman F (1991) Getting started with yeast. Methods Enzymol **194:** 3–20

Siegel N, Suhayda C, Haug A (1982) Aluminum changes the conformation of calmodulin. Physiol Chem Phys **14:** 165–167

Sigler K, Knotkov A, Paca J, Wurst M (1980) Extrusion of metabolites from baker's yeast during glucose-induced acidification. Folia Microbiol **25:** 311–317

Suhayda CG, Haug A (1986) Organic acids reduce aluminum toxicity in maize root membranes. Physiol Plant **68:** 189–195

Tan K, Keltjens WG (1990) Effects of aluminium on growth, nutrient uptake, proton efflux and phosphorus assimilation of Al-tolerant and -sensitive sorghum genotypes. *In* ML van Beusichem, ed, Plant Nutrition—Physiology and Application. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 397–401

Tan K, Keltjens WG, Findenegg GR (1991) Role of magnesium in combination with liming in alleviating acid-soil stress with the aluminium-sensitive sorghum genotype CV323. Plant Soil **136:** 65–71

Vallier LG, Coons D, Bisson LF, Carlson M (1994) Altered regulatory responses to glucose are associated with a glucose transport defect in *grr1* mutants of *Saccharomyces cerevisiae*. Genetics **136:** 1279–1285

Wagenbach W, O'Rourke K, Vitez L, Wieczorek A, Hoffman S, Durfee S, Tedesco J, Stetler G (1991) Synthesis of wild type and mutant human hemoglobins in *Saccharomyces cerevisiae*. Biotechnology **9:** 57–61

Wheeler DM, Dodd MB (1995) Effect of aluminium on yield and plant chemical concentrations of some temperate legumes. Plant Soil **173:** 133–145

Wheeler DM, Edmeades DC, Christie RA (1992a) Effect of aluminum on relative yield and plant chemical concentrations of cereals grown in solution culture at low ionic strength. J Plant Nutr **15:** 403–418

Wheeler DM, Edmeades DC, Christie RA (1992b) Effect of aluminum on plant chemical concentrations in some temperate grasses grown in solution culture at low ionic strength. J Plant Nutr **15:** 387–402

Wheeler DM, Edmeades DC, Christie RA, Putterill J, Gardner R (1992c) Effect of aluminum and pH on relative yield and plant chemical concentrations of 7 dicotyledonous species grown in solution culture at low ionic strength. J Plant Nutr **15:** 419–433

Winston F, Dollard C, Ricupero-Hovasse SL (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast **11:** 53–55

Zel J, Svetek J, Crne H, Schara M (1993) Effects of aluminum on membrane fluidity of the mycorrhizal fungus *Amanita muscaria*. Physiol Plant **89:** 172–176