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EFFECT OF ALUMINUM ON GROWTH AND KINETICS OF ALUMINUM UPTAKE BY ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE CULTIVARS OF *TRITICUM AESTIVUM* L.

BY

GUICHANG ZHANG

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 PHYSIOLOGY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

FALL 1990

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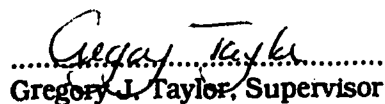
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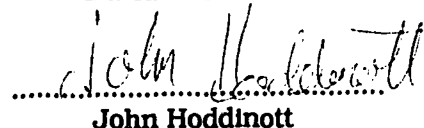
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ABSTRACT

Accumulation and distribution of aluminum (Al), kinetics of Al uptake, and the growth response of roots and leaves to added Al were investigated in Al-tolerant (Atlas 66 and PT741) and Al-sensitive (Neepawa and Scout 66) cultivars of *Triticum aestivum* L. (wheat). Tolerance to Al was a phenomenon expressed mainly in the roots. Root growth of Atlas 66 was not affected by 200 µM Al, while root growth of Scout 66 was reduced by 59 and 72% at 50 and 200 µM Al respectively. At 75 µM Al, the kinetics of Al uptake by excised roots was biphasic, with a rapid saturable phase superimposed over a linear phase. The saturable phase was removeable by 30 minute desorption with citric acid, and no distinction in the remaining linear phase was observed between Al-tolerant and Al-sensitive cultivars. Uptake of Al at 0°C suggested that a portion of the linear phase was non-metabolic, and isolation of purified cell wall material after treatment of roots with Al showed that the linear phase was composed of both symplasmic and apoplasmic compartments. The linear phase of *in vivo* Al adsorption onto the cell wall fraction appeared to be metabolism-dependent, as *in vitro* adsorption of Al by isolated cell wall material was completely removeable.

In excised roots, the rate of Al uptake during the linear phase was increased by treatment with 2,4-dinitrophenol in Al-tolerant cultivars, while uptake of Al in Al-sensitive cultivars was relatively unaffected. When excised roots were treated with gramicidin, increased uptake of Al in Al-sensitive cultivars was observed while no significant difference was found in Al-tolerant cultivars. Treatment with 2,4-dinitrophenol plus gramicidin increased rates of Al uptake synergistically in Al-tolerant cultivars and multiplicatively in Al-sensitive cultivars. These results may reflect the operation of an Al and/or chelate ligand efflux pump(s) in Al-tolerant cultivars which

act to limit entry of Al into the symplasm under normal metabolic conditions.

In conclusion, this research has demonstrated patterns of growth response of Al-tolerant and Al-sensitive cultivars to Al, characterized the kinetics of Al uptake, clarified the identity of the linear phase of uptake, and provided a hypothesis concerning the role of Al and/or chelate ligand efflux pumps as a potential mechanism of Al tolerance in *Triticum aestivum*.

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1. INTRODUCTION

Soil acidity is an important factor limiting agricultural production in large geographical regions of the world, possibly affecting 40% of the world's cultivated land and up to 70% of potentially arable lands (Osmond *et al.* 1980). In Canada, soil acidity is an agricultural concern in every province except Manitoba (Hedlin and Kraft 1984). Plants growing on acid soils suffer from nutrient deficiencies, drought intolerance and manganese toxicity (Taylor 1988b). Aluminum (Al) toxicity, however, has been identified as one of the most important growth-limiting factors on acid soils (Foy *et al.* 1978). Although decreased growth of both roots and shoots are observed, the earliest and most typical symptom of Al phytotoxicity is the reduced growth of the root apex (Tepper *et al.* 1989). Reduced growth may result from impaired structure and function of the plasma membrane, and inhibition of DNA synthesis, mitosis, and cell elongation (Taylor 1988b). The physiology and biochemistry of Al toxicity have been reviewed by several authors (Foy 1983a,1984; Haug 1984; Taylor 1988a,c; Roy *et al.* 1988; Schaedle *et al.* 1989).

Plants differ significantly in tolerance to Al toxicity, and numerous plants grow well when they are exposed to Al at low pH (Foy *et al.* 1965, 1967; Armiger *et al.* 1968; Reid *et al.* 1969; Taylor and Foy 1985a,b; Keltjens 1987; Keltjens and Ulden 1987; Baligar *et al.* 1988). In *Triticum aestivum*, root growth of Al-tolerant and Al-sensitive cultivars varied between 13 to 116% of the control with exposure to 74 μM Al (Taylor and Foy 1985a). Possible mechanisms of Al tolerance have been discussed by several authors (Foy 1983a,b, 1984; Taylor 1987, 1988a,c; Roy *et al.* 1988), and both internal and external mechanisms of Al tolerance have been suggested. Aluminum tolerance might be achieved within the cytosol by formation of Al binding proteins, chelation of Al

by organic acids, evolution of Al-tolerant enzymes, and compartmentation of Al in the vacuole. In contrast, external mechanisms, which function by preventing Al from entering the organism and reaching sensitive metabolic sites, might include selective permeability of the plasma membrane, a pH barrier in the rhizosphere, exudation of chelate ligands, and immobilization in the cell wall (Taylor 1988a,c).

Internal mechanisms of Al tolerance are suggested to operate upon entry of Al into the symplasm. Although the apoplasm has been documented as the major pool of Al in roots (Clarkson 1967; Huett and Menary 1979), Al does enter the cytosol and affects metabolism in a variety of species (Foy 1984; Haug 1984; Roy *et al.* 1988; Taylor 1988b, 1989; Schaedle *et al.* 1989). After entering the cytosol, the solubility and mobility of Al is reduced by formation of $Al(OH)_3 \cdot 3H_2O$, and free Al^{3+} is limited to less than 10^{-10} M at pH 7.0 (Martin 1986). However, low concentrations of Al in the symplasm are potentially phytotoxic because of the strong affinity of Al for oxygen donor compounds such as DNA, RNA, proteins, calmodulin, carboxylic acids, and inorganic phosphate (Matsumoto *et al.* 1976; Siegel and Haug 1983; Haug 1984; Martin 1986). Binding or chelation of Al to these metabolically active molecules causes inhibition of DNA synthesis, mitosis, cell elongation, enzyme activity and related metabolic processes (Matsumoto *et al.* 1976. 1977; Horst *et al.* 1983). On the other hand, enhanced production of ligands which bind Al, such as proteins and organic acids, could reduce the activity of Al and detoxify Al in the cytosol. For instance, Suhayda and Haug (1986) found that roots of an Al-tolerant cultivar of *Zea mays* maintained higher concentrations of malate and trans-aconitate than an Al-sensitive cultivar, and an Al-induced inhibition of root plasma membrane ATPase activity was ameliorated by treatment with these organic acids. Furthermore, roots of Al-tolerant cultivars of *Hordeum vulgare, Phaseolus vulgaris, Pisum sativum, Triticum aestivum*, and *Zea mays*

showed higher concentrations of citrate and malate than roots of Al-sensitive cultivars, both in the presence and absence of Al (Klimashevskii and Chernysheva 1980; Lee and Foy 1986). Supporting evidence was also observed from *in vitro* studies which documented reversion of Al-induced inhibition of hexokinase, plasma membrane ATPases and calmodulin by citrate, glutamate, malate, oxalate, 3-phosphoglycerate, and tartrate. These ligands may ameliorate Al toxicity by formation of stable complexes with Al (Womack and Colowick 1979; Viola *et al.* 1980; Neet *et al.* 1982; Matsumoto and Yamaya 1986) or by preventing binding of Al to metabolically active molecules such as DNA, RNA, proteins and enzymes (Suhayda and Haug 1984, 1986). While the concept that Al binding ligands are involved in Al tolerance has some experimental support, this support is equivocal. In a number of studies addressing this hypothesis, the stability constants of Al complexes with organic acids were calculated based on the trivalent cation (Al^{3+}), but it is likely that the major species of Al present in the cytosol is the neutral species $Al(OH)_3 \cdot 3H_2O$ (Taylor 1989). In order to properly estimate the potential formation of Al-organic acid complexes in the cytosol, information on the stability of $AlOH^{2+}$-, $Al(OH)_2^{+}$-, and $Al(OH)_3$-complexes are required. Thus, detoxification of Al through formation of complexes with organic acids in the cytosol as a mechanism of Al tolerance remains uncertain.

With investigations on the mechanisms of cadmium, copper, and zinc tolerances, heavy metal-binding proteins or peptides have been discovered (Grill *et al.* 1985, 1987; Lu-Kim and Rauser 1986; Steffens *et al.* 1986; Rauser 1986, 1987; Kishinami and Widholm 1987). Phytochelatins and possibly metallothioneins are thought to complex and detoxify metals in the cytosol. Recently, Al-binding proteins have also been suggested to detoxify Al in the cytosol (Aniol 1984). In *Triticum aestivum*, pretreatment with Al enhanced tolerance to subsequent Al exposures, and 71

to 74% of cytosolic Al was precipitated with trichloroacetic acid (TCA). Induction of Al tolerance was abolished by treatment with cycloheximide, suggesting most absorbed Al was bound to proteins in the cytosol (Aniol 1984). These results are consistent with inducible Al-binding proteins or peptides in the cytosol. However, it is important to recognize that the major species of Al in the cytosol at near-neutral pH will likely be the neutral species $Al(OH)_3 \cdot 3H_2O$. Thus, any protein which binds Al will likely bear little resemblance to phytochelatins or metallothioneins (Taylor 1989). Furthermore, no such Al-binding proteins have been identified, isolated, and characterized. Thus, experimental evidence supporting Al-binding protein is weak.

Evolution of Al-tolerant enzymes has also been suggested as a strategy of Al tolerance. Woolhouse (1969, 1970) found that roots of a calcareous soil (presumably Al-sensitive) ecotype of *Agrostis tenuis* showed greater inhibition of activity of acid phosphatase than an acid soil-tolerant (presumably Al-tolerant) ecotype when exposed to Al, suggesting that evolution of Al-tolerant enzymes may play a role in tolerance to Al. Since these experiments were carried out *in vivo*, however, the higher activity of acid phosphatase in the Al-tolerant ecotype may have resulted from tolerance of the roots to Al, instead of evolution of Al-tolerant enzymes. Until similar results have been observed in *in vitro* studies, the hypothesis of evolution of Al-tolerant enzymes will remain equivocal.

Finally, tolerance to Al may be achieved if Al were transported into the vacuole, which may be insensitive to the toxic effects of Al. While compartmentation in the vacuole has received support as a mechanism of tolerance to other metals (Mathys 1977; Kime *et al.* 1982; Pfeffer *et al.* 1986), evidence supporting compartmentation of Al is lacking. Furthermore, Clarkson (1969) pointed out that meristematic root cells,

those most affected by Al treatment, are not vacuolated in either Al-tolerant or Al-sensitive species. Thus, in order to reduce damage to these tissues, other mechanisms of Al tolerance are required.

While several authors have suggested that exclusion mechanisms are not important in Al tolerance (Haug and Caldwell 1985; Roy *et al.* 1988), those mechanisms have also received experimental support. For example, a large body of literature has demonstrated a correlation between Al tolerance in *Triticum aestivum* and its ability to maintain a relatively high pH in the growth medium (Dodge and Hiatt 1972; Foy 1974; Mugwira *et al.* 1976; Mugwira and Patel 1977; Mugwira and Elgawhary 1979; Foy and Fleming 1982; Fleming 1983; Taylor and Foy 1985a,b,c). Differences in plant-induced pH of the growth medium between Al-tolerant and Al-sensitive cultivars appear to be due to differences in the relative uptake of cations and anions, especially NH_4^+ and NO_3^- (Dodge and Hiatt 1972; Mugwira and Patel 1977; Fleming 1983; Taylor and Foy 1985c; Keltjens and van Ulden 1987a,b). Since the solubility of Al decreases rapidly in the range of pH 4.0 and 5.0, plants that can maintain a relatively high pH in the root apoplasm or rhizosphere may create a pH barrier at the root-soil interface that could reduce Al toxicity (Taylor and Foy 1985a,b,c).

Several studies have failed to demonstrate a relationship between plant-induced pH and tolerance to Al in cultivars of *Glycine max*, *Hordeum vulgare*, *Phaseolus vulgaris*, *x Triticosecale*, and *Zea mays* (Foy *et al.* 1972; Clark 1977; Mugwira and Elgawhary 1979; Wagatsuma and Yamasaku 1985). Also, manipulation of N source (hence, plant-induced pH) had little effect on the Al tolerance of cultivars of *Hordeum vulgare* and *Triticum aestivum* (Wagatsuma and Yamasaku 1985; Taylor 1988d). The data from *Triticum aestivum* (Taylor 1988d) are particularly damaging to the plant-induced pH

hypothesis because they deal with two benchmark cultivars used in earlier studies that supported the hypothesis. Using the same two cultivars, Miyasaka *et al.* (1989) demonstrated that differences in plant-induced pH which were observed in mixed nitrogen solution were not observed in a simple $CaSO_4$ solution. Nevertheless, differential tolerance was expressed in both solutions. Thus, the plant-induced pH barrier can not completely account for the Al tolerance, and the effect of plant-induced pH in determining cultivar tolerance to Al may be relatively small (Taylor 1987, 1988a,c, 1989; Miyasaka *et al.* 1989).

Plants could also exclude Al from symplasm by exudation of chelate ligands which form stable complexes with Al in apoplasm and/or rhizosphere, and hence, reduce activity of the free Al ion. As a result of competition with root transport sites, these exuded ligands could reduce uptake of Al into the cytosol and mitigate toxic effects (Halvorson and Lindsay 1972, 1977; Lindsay 1974). Ojima *et al.* (1984), and Ojima and Ohira (1985), found that Al-tolerant cell cultures of *Daucus carota* exuded more citrate into the growth medium than Al-sensitive cultures. However, further investigations indicated that exudation of citrate into growth medium may have been a response to phosphate starvation rather than Al toxicity (Koyama *et al.* 1988; Ojima *et al.* 1989; Koyama *et al.* 1990). Thus, consistent results demonstrating a correlation between exudation of chelate ligands and Al tolerance are lacking.

The plant cell wall may also play a role in tolerance to Al. Clarkson (1967), and Huett and Menary (1979) found that 75-90% of the total Al accumulated by roots of several species was tightly bound to cell wall material, where Al interacts with free carboxyl groups of polygalacturonic acids in the middle lamella. Furthermore, Foy *et al.* (1967), Mugwira and Elgawhary (1979), and Kennedy *et al.* (1986) found an association

between Al tolerance and root cation exchange capacity (CEC). Uptake of Al by roots of Al-tolerant (low CEC) cultivars was less than the uptake by roots of Al-sensitive (high CEC) cultivars. However, this evidence has not been supported by kinetic studies which differentiate between Al absorbed into apoplasmic and symplasmic compartments.

In other studies, it was suggested that selective permeability of the plasma membrane to Al played a role in determining tolerance to Al. Increased uptake of Al by roots of several species was observed under non-metabolic conditions (Huett and Menary 1979; Wagatsuma 1983). These data suggest that the plasma membrane acts as a barrier to the movement of Al into the cytosol, that the effectiveness of this barrier is reduced under non-metabolic conditions, and that differential permeability of the plasma membrane to Al may be involved in Al tolerance (Wagatsuma 1983). To verify this hypothesis, it would first be necessary to differentiate between Al uptake into the apoplasm and symplasm. In these experiments, evidence demonstrating that absorbed Al represents uptake of Al into the symplasm was lacking. Once again. studies of the kinetics of Al uptake are needed to clarify this confusion.

Thus, it would appear that experimental support for exclusion is incomplete; evidence which directly demonstrates differences in the rate of Al uptake between Al-tolerant and Al-sensitive cultivars of the same species have not been obtained. This information is essential for interpretation of experimental results and identification of Al tolerance mechanisms. While few studies have differentiated between uptake of Al into apoplasmic and symplasmic compartments (Taylor 1988a), several authors have attempted to relate Al tolerance to uptake of Al by comparing kinetics of uptake in different species at high concentrations of Al in the growth solution (Huett and Menary

1979; Wagatsuma 1984; Schaedle *et al.* 1986). Such experiments do provide information on Al uptake in the apoplasmic and symplasmic compartments, however, comparison of plants with such diverse genetic background using high concentrations of Al make conclusions about tolerance mechanisms speculative. In the past, use of high concentrations of Al may have been required due to lack of sensitive techniques for measuring Al. Aluminum concentrations in leaves are usually near or below the detection limit of flame atomic absorption spectrophotometry when plants are exposed to Al at physiological concentrations. If short-term kinetics of Al uptake were to be investigated with small tissue samples, accumulated Al would be undetectable by conventional flame atomic absorption spectrophotometry. Fortunately, the detection limit of flame atomic absorption spectrophotometry is no longer an experimental limitation. Graphite furnace atomic absorption is capable of measuring Al at ng ml^{-1} concentrations, a 10^3 fold improvement over the μg ml^{-1} detection limit of conventional flame atomic absorption. Thus graphite furnace atomic absorption spectrophotometry may permit short-term kinetic analysis of Al uptake which would not otherwise be feasible with conventional flame atomic absorption spectrophotometry.

With the aid of this powerful new technique, investigation of the growth response to Al, characteristics of accumulation and distribution of Al, and kinetics of Al uptake in Al-tolerant and Al-sensitive cultivars of the same species would produce valuable information with which to evaluate external mechanisms of Al tolerance. Thus, the objectives of this research were to: 1) differentiate patterns of growth response to Al toxicity between Al-tolerant and Al-sensitive cultivars of *Triticum aestivum*, 2) characterize the kinetics of Al uptake in these cultivars, and 3) determine if kinetic data are consistent with external or exclusion mechanisms of Al tolerance in terms of differences in Al uptake between Al-tolerant and Al-sensitive cultivars. The

results of my research have demonstrated distinctive patterns of growth response to Al between Al-tolerant and Al-sensitive cultivars (Chapter 2; Zhang and Taylor 1988), characterized short-term kinetics of Al uptake in excised roots and cell wall material (Chapter 3 and 4; Zhang and Taylor 1989, 1990a), clarified the identity of the linear phase of Al uptake (Chapter 3; Zhang and Taylor 1990a), and identified differences in the kinetics of Al uptake between Al-tolerant and Al-sensitive cultivars which may reflect active efflux of Al or chelate ligands as a mechanism of exclusion of Al in Al-tolerant cultivars (Chapter 3 and 5; Zhang and Taylor 1989, 1990b).

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2. GROWTH RESPONSE TO ALUMINUM AND ACCUMULATION OF ALUMINUM IN ROOTS AND SHOOTS OF ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE CULTIVARS OF *TRITICUM AESTIVUM* L.[1]

2.1 Introduction

To understand mechanisms of Al tolerance, some authors have studied the effect of Al on growth and distribution of Al in plants. Both root and leaf growth are depressed when plants are grown in solutions containing Al (Jarvis and Hatch 1985; Ohki 1985; Taylor and Foy 1985a,c) and typically, symptoms of Al toxicity are most evident on roots (Taylor and Foy 1985a,c). Absorbed Al is primarily accumulated in roots (Bartuska and Ungar 1980; Murphy *et al.* 1984; Kennedy *et al.* 1986), but root to leaf Al ratios vary with species, plant age, and environmental factors such as pH and Al concentration of culture solutions (Huett and Menary 1980; Foy and Campbell 1984; Wagatsuma 1984; Wagatsuma and Ezoe 1985). In roots, most absorbed Al is accumulated in root tips, especially in epidermal cells (Matsumoto *et al.* 1976; Huett and Menary 1980; Wagatsuma 1984). Horst *et al.* (1982, 1983), and Ojima *et al.* (1984) reported that mucilage protected roots from damage by binding Al and thus reducing uptake of Al into the meristem.

Data documenting differences in growth responses and distribution of Al within Al-tolerant and Al-sensitive cultivars are available. For example, Niedziela and Aniol (1983) reported that an Al-tolerant cultivar of wheat tolerated 5 to 7 times higher Al

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concentrations in root tips than an Al-sensitive cultivar. However, few studies have compared the relationship between growth and accumulation of Al in Al-tolerant and Al-sensitive cultivars grown over a range of concentrations which are toxic to both cultivars. Such information may help reveal Al tolerance mechanisms. In this research, an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) were grown in culture solution with a wide range of Al concentrations, and differences in growth response, distribution of Al, and the relationship between growth and accumulation of Al were investigated.

2.2 Methods

Preparation of Plant Material. Seeds of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum* L. (wheat) were surface sterilized in 1.2% sodium hypochlorite for 20 minutes, and germinated overnight in a solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were precultured on acrylic support frames in dilute nutrient solutions containing (mM) 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S, and (µM) 34 Cl, 60 Na, 10 Fe (as Fe-EDTA), 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.5). After 9 days, 12 uniform seedlings were mounted on acrylic covers of 10 liter polyethylene containers. Growth containers were covered to inhibit algal growth. Plants were grown in a controlled environment room with temperature maintained between 21 and 24°C during a 16 hour light period and between 17 and 19°C during darkness. Relative humidity was maintained between 55 and 65% during the light period and 80 and 90% during darkness. The growth room was illuminated by 12 HID mercury halide (400 W) and 4 HID high pressure sodium (400 W) lamps located 1.5 m above the plant bases. The photosynthetic photon flux

density (PPFD) was 240 ± 3 μmol m^{-2} s^{-1} at plant base level. Although not controlled, solution temperatures were 20 ± 1°C at the end of the light period and 19 ± 1°C at the end of darkness.

Treatment with Al. A randomized block, factorial design with 2 cultivars, 9 Al treatments (0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al as $AlK(SO_4)_2 \cdot 12H_2O$), and 3 replicates was used. Aluminum treatments were superimposed over the nutrient solution described above. The pH of culture solution was adjusted initially to 4.5 with HCl or KOH and monitored 3 times weekly. Patterns of pH change were similar to that found by Taylor and Foy (1985a,b).

Harvesting procedures. After 12 days of treatment, plants were harvested, separated into leaves and roots, and washed immediately. The washing procedure for roots included three rinses in distilled water for a total of 5 minutes, following by desorption in 1.0 mM $CaSO_4$ for 30 minutes, and three additional rinses in distilled water for a total of 5 minutes. Leaves were rinsed three times in distilled water. Plants were dried at 60°C, and weighed. Biomass was used to express the growth response of roots and leaves to Al.

Determination of Al. For analysis of Al in roots and leaves, about 0.5 g of dried plant material was ashed at 500°C. The ash was dissolved in concentrated HNO_3 (0.4 or 1.0 ml), oxidized with 50% H_2O_2 (0.4 or 1.0 ml), and diluted to 20 or 50 ml. Aluminum was determined by flame atomic absorption spectrophotometry. Concentrations were expressed as micrograms of Al per gram dry weight (μg Al g^{-1}).

Analysis of Data. Statistical analyses of the data were performed using analysis of variance (ANOVA), simple regression, and descriptive statistics available on Statistical Graphics Corporation's statistical package, Statgraphics Version 2.0. Analyses of homogeneity of slopes were performed using ANOVA available in SAS release 5.18. Significance was defined at the 95% confidence level.

2.3 Results

The cultivars differed in their response to Al in solution. Reduced root growth was the first observed symptom of Al toxicity. After 4 days of treatment, decreased growth and browning of the root tips were observed in Scout 66 grown at 50 µM Al, while Atlas 66 showed no visual symptoms of Al toxicity at 200 µM Al in solution. Reduced growth and chlorotic spots on the upper part of primary leaves were the first observed symptoms of Al toxicity of leaves. These symptoms appeared on the leaves of Atlas 66 above 300 µM Al after 7 days of treatment, and expanded to the middle and base of the leaves with longer exposure to Al. By the end of the experiment chlorotic leaves became withered, and leaf tips became necrotic. Surprisingly, these symptoms appeared less serious on the Al-sensitive cultivar, Scout 66.

Analysis of variance for root and leaf growth showed significant main effects of Al and cultivar. Root growth of Atlas 66 was less inhibited by Al, especially at lower concentrations (Fig. 2-1), accounting for a significant interaction effect of cultivar and Al. Below 200 µM Al, root growth of Atlas 66 was not affected during the experimental period. In contrast, root growth of Scout 66 was reduced to 41 ± 1% and 29 ± 2% of control at 50 and 200 µM Al respectively. Above 200 µM Al, root growth of Atlas 66 was

decreased with an increase of Al in solution, however, root growth of Atlas 66 was always better than Scout 66. At 1000 μM Al, root growth of Atlas 66 was 53 ± 3% of control, compared to 26 ± 2% for Scout 66 (Fig. 2-1). Leaf growth of both cultivars was reduced with an increase of Al in solution in the range of 50 to 200 μM Al, although Atlas 66 showed better leaf growth than Scout 66. Above 200 μM Al, leaf growth of Scout 66 did not show further reduction, where leaf growth of Atlas 66 did not show any further reduction above 400 μM Al (Fig. 2-2).

Results of the analysis of variance for concentrations of Al in roots and leaves showed significant main effects due to Al and cultivar. Concentrations of Al in roots increased from 2450 ± 450 to 4050 ± 100 μg g^{-1} in Atlas 66, and from 1980 ± 60 to 3830 ± 20 μg g^{-1} in Scout 66 with the increase of Al in solution. Atlas 66 consistently showed 24-28% greater accumulation of Al in roots than Scout 66 (Fig. 2-3). However, the Al x cultivar interaction effect was not significant. Regression analysis also showed no statistical difference in the slopes of Al accumulation between Al-tolerant cultivar Atlas 66 (2.17 ± 0.75) and Al-sensitive cultivar Scout 66 (2.48 ± 0.75) (Fig. 2-3). Concentrations of Al in the leaves were undetectable below 200 μM Al in solution, and increased from 50 ± 2 to 180 ± 40 μg g^{-1} in Atlas 66 and from 40 ± 10 to 130 ± 20 μg g^{-1} in Scout 66 with the increase of Al in solution from 200 to 1000 μM (Fig. 2-4). Analysis of variance indicated that the Al x cultivar interaction effect was not significant, and regression analysis showed no statistical difference in the slopes of Al accumulation between Al-tolerant cultivar Atlas 66 (0.19 ± 0.02) and Al-sensitive cultivar Scout 66 (0.15 ± 0.02). Root Al to leaf Al ratios were also similar between these two cultivars (Fig. 2-5).

Analyses of the relationships between accumulation of Al and growth of both roots and leaves indicated differences between cultivars. Roots of Atlas 66 did not show reduced growth until concentration of Al in the roots reached 3030 ± 310 μg g^{-1}, while root growth of Scout 66 decreased to 41 ± 1% and 29 ± 1% of control at concentrations of 1980 ± 60 μg g^{-1} and 2265 ± 214 μg g^{-1} respectively (Fig. 2-6). Despite higher concentrations of Al, roots of Atlas 66 grew better than Scout 66. In contrast, leaf growth of Atlas 66 showed the same response to Al concentrations in the leaves as Scout 66 (Fig. 2-7).

2.4 Discussion

Patterns of growth response of roots to Al were different between Al-tolerant and Al-sensitive cultivars. Roots of Atlas 66 resisted Al toxicity and grew as well as the control below 200 μM Al in solution. The Al concentration in nutrient solution which inhibited root growth of Atlas 66 by 50% was 800 μM. In contrast, the root growth of Scout 66 was decreased by more than 50% at the lowest concentration of Al (50 μM) tested in this experiment. Compared to root growth, distinct patterns of leaf growth were not found between these two cultivars. Similar responses of root and leaf growth to Al were observed between an Al-tolerant cultivar (PT741) and an Al-sensitive cultivar (Katepwa) of *Triticum aestivum* by Briggs *et al.* (1989). These studies suggest that Al tolerance is primarily a root-related phenomenon.

Long-term uptake of Al indicated that most of the absorbed Al was accumulated in roots of both cultivars. These results are consistent with the pattern of greater accumulation of Al in roots of a variety of species (Howeler and Cadavid 1976; Bartuska

and Ungar 1980; Murphy *et al.* 1984; Kennedy *et al.* 1986). Although Al concentrations were higher in both leaves and roots in the Al-tolerant cultivar Atlas 66 than those in Al-sensitive cultivar Scout 66, no significant difference in the slopes was found in either roots or leaves. Ratios of Al concentrations between roots and leaves decreased with increasing Al concentration in nutrient solution, indicating an increased translocation of Al from roots to shoots. Similar results were also observed in some of the species from Wagatsuma's studies (1984). Increased translocation may result from a physiological and/or structural breakdown of the plasma membrane in roots (Wagatsuma 1984; Taylor 1989), however, no difference in the root Al / leaf Al ratio was found between these two cultivars over the range of concentrations of Al in solution.

Tolerance of Atlas 66 to Al toxicity was also found when Al accumulation in roots was compared to root growth. In this cultivar, growth of roots was not affected until Al concentrations in the root reached 3000 $\mu g\ g^{-1}$. The internal Al concentration which inhibited root growth by 50% was 3250 $\pm$ 380 $\mu g\ g^{-1}$. In contrast, root growth was decreased by 59% when Al concentration was 2000 $\mu M\ g^{-1}$ in the roots of Scout 66. These results highlight differences in the ability to tolerate Al between Al-tolerant and Al-sensitive cultivars. While these results may suggest involvement of internal mechanisms of Al tolerance, external mechanisms can not be excluded because compartmentation of the absorbed Al was not well defined in these experiments. Clarkson (1967), and Huett and Menary (1979) documented that 75-90% of the total absorbed Al was accumulated and tightly bound to cell wall material in roots of *Hordeum vulgare, Brassica oleracea, Lactuca sativum,* and *Pennisetum clandestinium.* If this is the case in *Triticum aestivum,* it is possible that Al in the cell wall fractions could obscure differences in Al uptake across the plasma membrane between cultivars. Thus further kinetic studies on short-term uptake of Al with complete desorption of Al from the apoplasm are necessary to

reveal the characteristics of Al uptake between Al-tolerant and Al-sensitive cultivars of *Triticum aestivum*. Such data might help to identify mechanisms of Al tolerance.

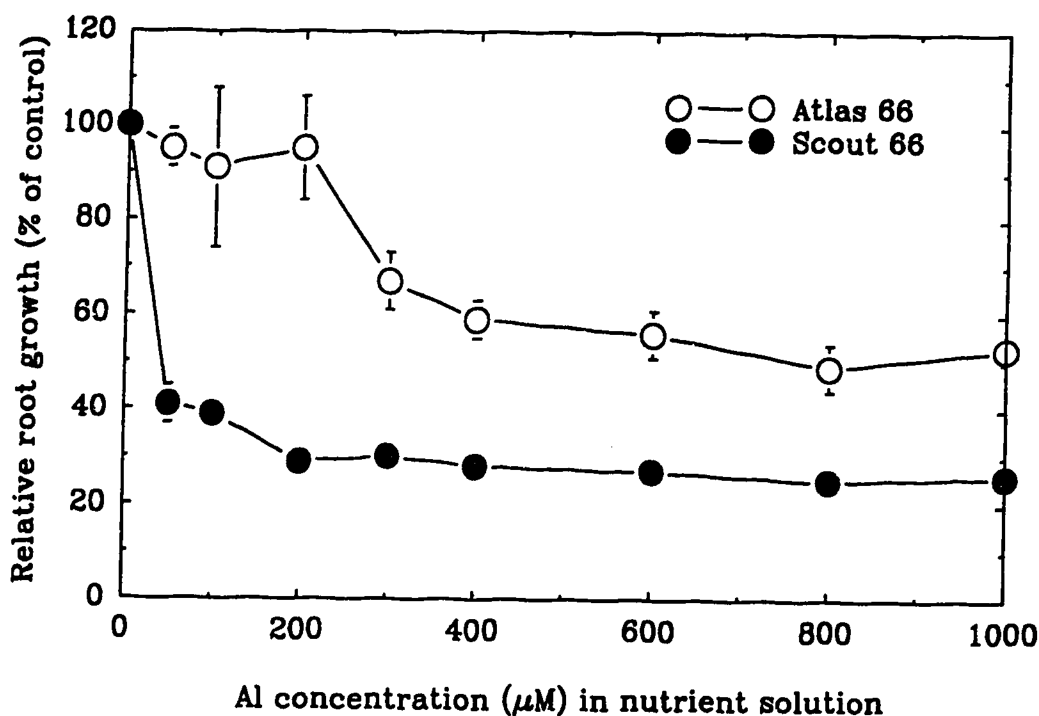

Figure 2-1. Effect of concentration of Al (μM) in the nutrient solution on relative root growth (% of control) of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum*. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al (initial pH 4.5). After 12 days, roots were separated from leaves, and washed with distilled water, 1.0 mM Ca, and distilled water again for 5, 30, and 5 minutes respectively. Values represent means of 3 replicates.

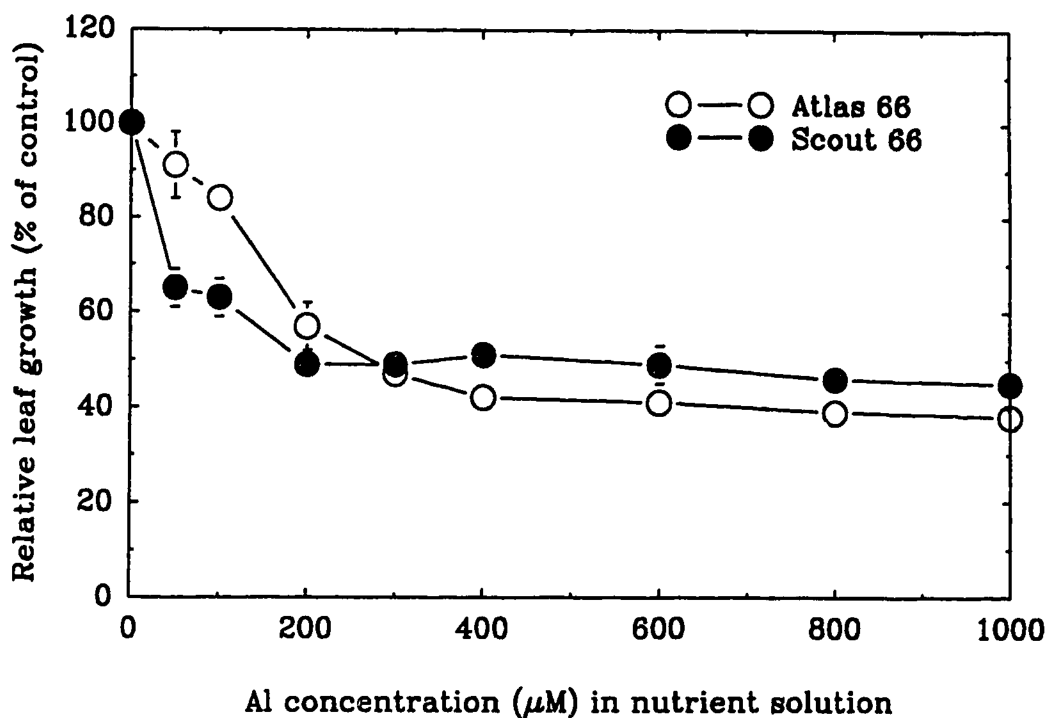

Figure 2-2. Effect of concentration of Al (μM) in the nutrient solution on relative leaf growth (% of control) of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum*. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al (initial pH 4.5). After 12 days, leaves were separated from roots, and washed three times with distilled water for 5 minutes. Values represent means of 3 replicates.

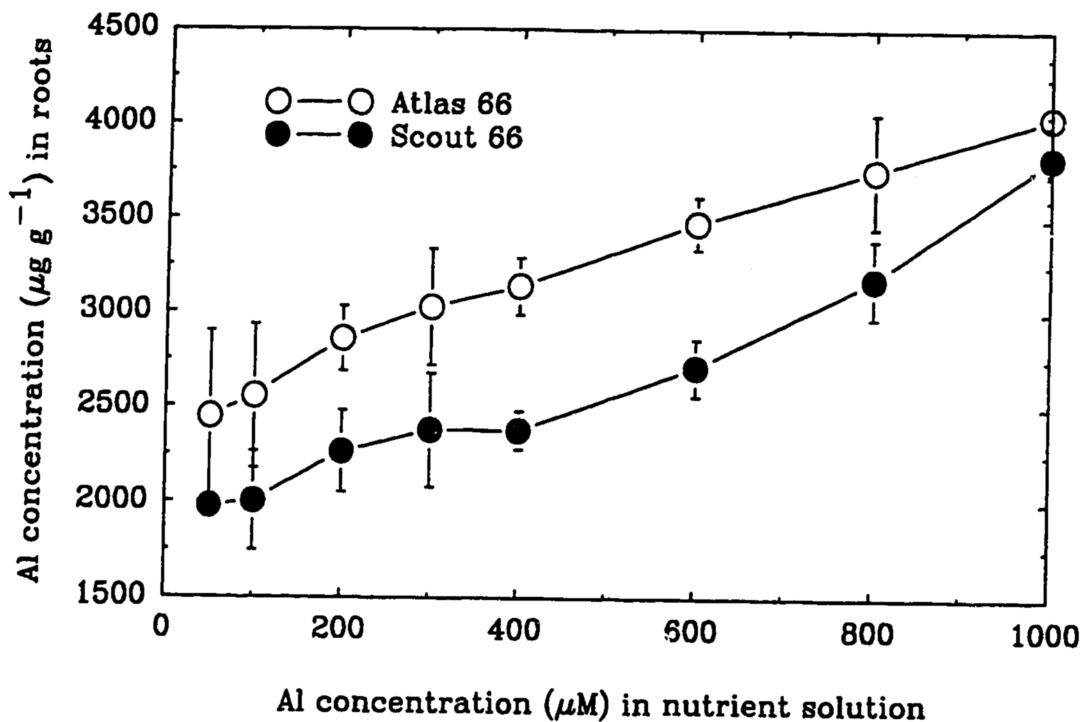

Figure 2-3. Effect of concentration of Al (μM) in the nutrient solution on concentration of Al (μg g^{-1}) in roots of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum*. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al (initial pH 4.5). After 12 days, roots were separated from leaves, and washed with distilled water, 1.0 mM Ca, and distilled water again for 5, 30, and 5 minutes respectively. Values represent means of 3 replicates.

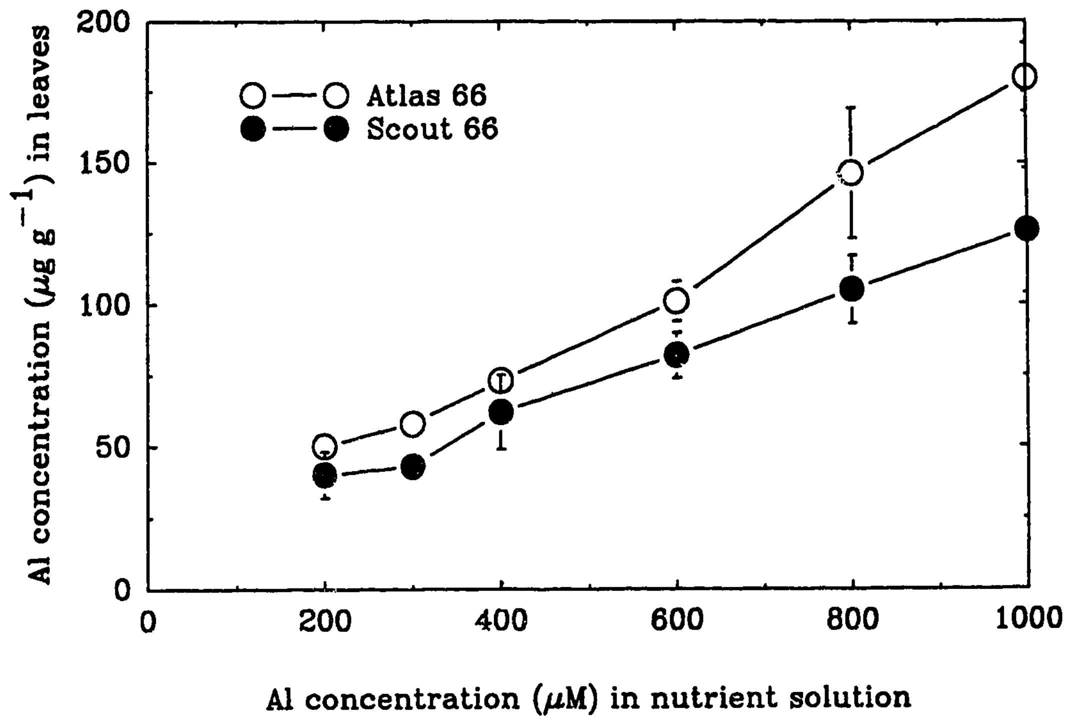

Figure 2-4. Effect of concentration of Al (μM) in the nutrient solution on concentration of Al (μg g^{-1}) in leaves of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum*. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al (initial pH 4.5). After 12 days, leaves were separated from roots, and washed three times with distilled water for 5 minutes. Values represent means of 3 replicates.

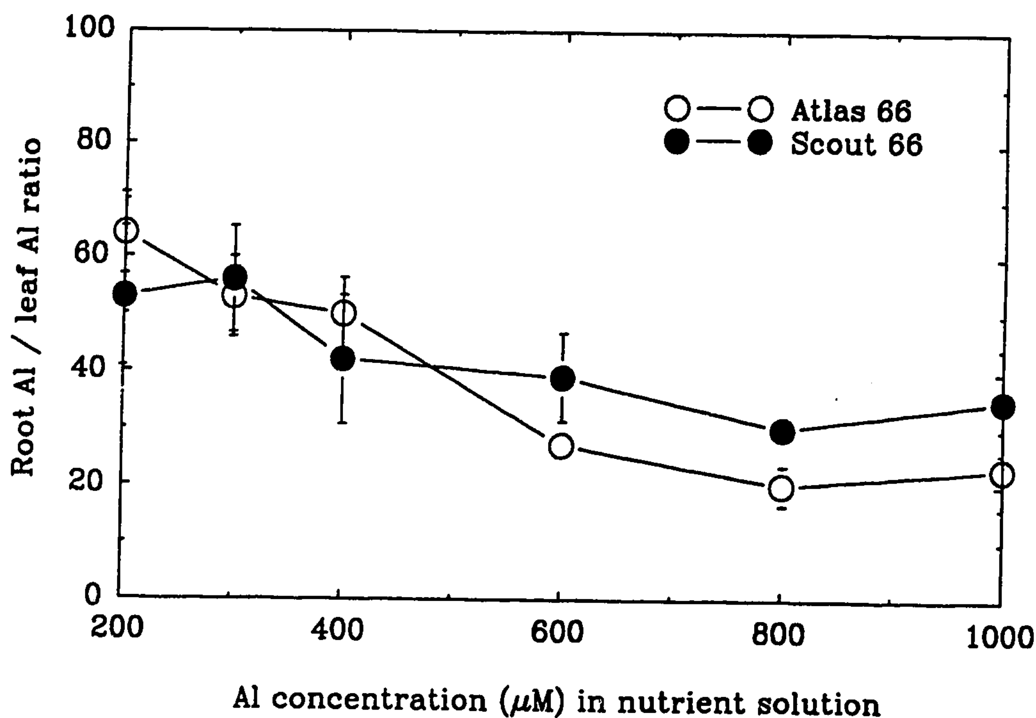

Figure 2-5. Root Al (μg g^{-1}) to leaf Al (μg g^{-1}) ratio of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum* in relation to Al concentration (μM) in the nutrient solution. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al (initial pH 4.5). After 12 days, roots were separated from leaves, and washed with distilled water, 1.0 mM Ca, and distilled water again for 5, 30, and 5 minutes respectively. Leaves were washed three times with distilled water for 5 minutes. Values represent means of 3 replicates.

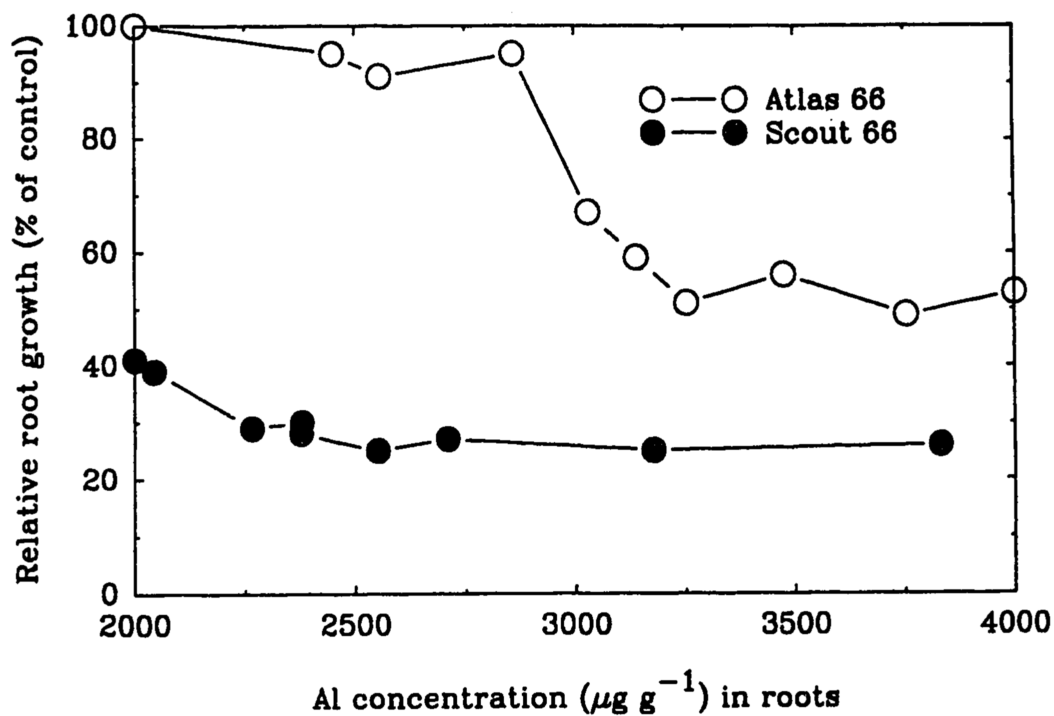

Figure 2-6. Relationship between concentration of Al ($\mu g\ g^{-1}$) in roots and relative root growth (% of control) of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum*. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 μM Al (initial pH 4.5). After 12 days, roots were separated from leaves, and washed with distilled water, 1.0 mM Ca, and distilled water again for 5, 30, and 5 minutes respectively. Values represent means of 3 replicates.

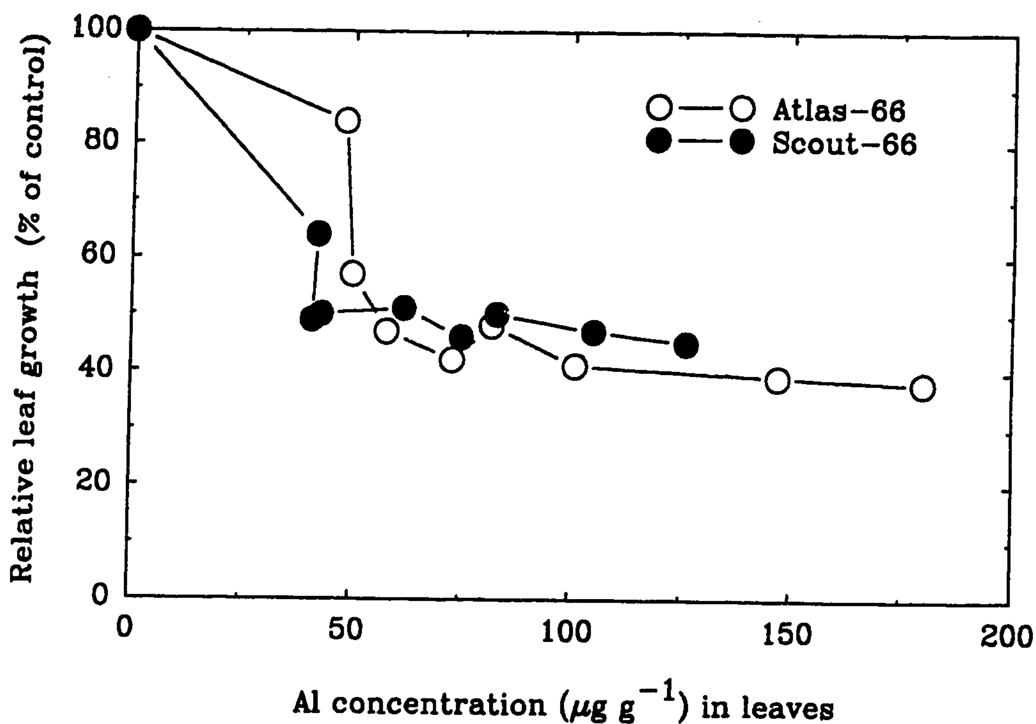

Figure 2-7. Relationship between concentration of Al (μg g^{-1}) in leaves and relative leaf growth (% of control) of an Al-tolerant cultivar (Atlas 66) and an Al-sensitive cultivar (Scout 66) of *Triticum aestivum*. Nine-day-old seedlings were treated in nutrient solutions containing 0, 50, 100, 200, 300, 400, 600, 800, and 1000 µM Al (initial pH 4.5). After 12 days, leaves were separated from roots, and washed three times with distilled water for 5 minutes. Values represent means of 3 replicates.

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3. KINETICS OF ALUMINUM UPTAKE BY EXCISED ROOTS OF ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE CULTIVARS OF *TRITICUM AESTIVUM* L.[2]

3.1 Introduction

Plants may tolerate potentially phytotoxic concentrations of Al in the growth substrate by two basic strategies (Taylor 1987, 1988a,c; Roy *et al.* 1988). One effective strategy would be to limit entry of Al into the symplasm, where it may exert its primary toxic effect (exclusion tolerance mechanisms). If exclusion mechanisms were incomplete or ineffective, tolerance might be achieved by detoxification or compartmentation of Al in the cytosol (internal tolerance mechanisms) (Taylor 1988a,c). While many authors have denied the existence of exclusion mechanisms (Haug and Caldwell 1985; Roy *et al.* 1988), Taylor (1988a,c, 1989) suggested that exclusion could be achieved by means of a pH barrier at the rhizosphere, selective permeability of plasma membrane, exudation of chelates or immobilization of Al in the cell wall. While these mechanisms of exclusion have received experimental support, this support is, nonetheless, incomplete. Few studies have differentiated between uptake of Al into apoplasmic and symplasmic compartments (Taylor 1988a). Thus, there is no evidence that shows differences in the rate of Al uptake across the plasma membrane between Al-tolerant and Al-sensitive cultivars. This information is essential for interpretation of experimental results and identification of Al tolerance mechanisms. Several authors have attempted to characterize Al uptake by comparing kinetics of Al uptake by

[2] A version of this chapter has been published in a refereed scientific journal. Zhang, G., and Taylor, G. J. 1989. Kinetics of aluminum uptake in excised roots of aluminum-tolerant and aluminum-sensitive cultivars of *Triticum aestivum* L. Plant Physiol. 91: 1094-1099.

different species at high concentrations of Al in the growth solution (either 1.0 or 1.13 mM) (Huett and Menary 1979; Wagatsuma 1984; Schaedle *et al.* 1986). While such experiments do provide information on Al uptake in the apoplasmic and symplasmic compartments, comparison of plants with such diverse genetic background using such high concentrations of Al make conclusions about tolerance mechanisms speculative.

In the present study, the kinetics of short-term uptake of Al by excised roots of Al-tolerant and Al-sensitive cultivars of *Triticum aestivum* were investigated. Use of graphite furnace atomic absorption spectrophotometry permitted uptake experiments to be performed using a physiologically relevant concentration of Al (75 μM). The results reported demonstrate uptake of Al into two distinct compartments, and suggest the involvement of an active exclusion mechanism in Al-tolerant cultivars of *Triticum aestivum*.

3.2 Methods

Preparation of Plant Material. Seeds of an Al-tolerant cultivar (Atlas 66) and an Al-tolerant pedigree (PT741: Tp//Cno/No 66/3/Bb/Cno/4/Grajo's'; see Briggs *et al.* 1989) and two Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum* L. (wheat) were surface sterilized in 1.2% sodium hypochlorite for 20 minutes, and germinated overnight in a solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were grown for 7 days on nylon mesh suspended over 16 liters of nutrient solution containing (mM) 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM) 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.5) in a growth chamber with 16 hours of light (20°C, 68% relative humidity) and 8 hours of

darkness (16^oC, 85% relative humidity). The photosynthetic photon flux density (PPFD) was 335 $\pm$ 12 $\mu mol\ m^{-2}\ s^{-1}$ at plant base level. After 5 days of growth, plants were transferred to fresh nutrient solutions.

Uptake of Al. Thirty excised root tips (2.0 cm) were placed in each of 50 "absorption tubes". Absorption tubes consisted of open-ended Plexiglas tubes (12 cm length, 2.2 cm diameter), with a nylon mesh barrier located 1.5 cm from the bottom. Four holes were cut in the tubes beneath the mesh barrier to permit circulation of absorption solution inside and outside of the tubes. During excision of roots, absorption tubes containing excised roots were placed in an aerated nutrient solution. When excision was complete (within 60 minutes), the tubes were transferred to an aerated solution of 1.0 mM $CaSO_4$ for 30 minutes. Uptake experiments were initiated by transferring the absorption tubes containing roots to 80 ml glass jars containing 50 ml of an aerated solution of 1.0 mM $CaSO_4$ and 75 μM Al as $AlK(SO_4)_2 \cdot 12H_2O$ (pH 4.5). Absorption tubes were covered at the top with nylon mesh, and the jars were incubated in a water bath at 23^oC. Five replicate tubes were removed from the absorption solutions after 0, 5, 10, 15, 20, 30, 60, 90, 120, 150, and 180 minutes of uptake. Roots were rinsed briefly with 1.0 mM $CaSO_4$ and then with deionized water (300 ml per tube), and prepared for determination of Al by graphite furnace atomic absorption spectrophotometry.

The composition of the absorption solutions were designed to eliminate potential effects of phosphate on Al solubility, nonetheless, Al will not simply be present as $Al^{3+} \cdot 6H_2O$. Speciation calculations using the modified GEOCHEM program and log K values of -5.02, -9.30, -14.99, and -23.33 for the hydrolysis of Al (Parker *et al.* 1987) suggest that Al will be present primarily as the $AlSO_4$ ion pair (22 μM), as $Al^{3+} \cdot 6H_2O$ (18 μM), and as a number of less abundant monomeric species. Aluminum might also

be present as a polynuclear species, since the ratio of $\{Al^{3+}\}/\{H^+\}^3$ in the absorption solutions will be approximately $10^{8.76}$, marginally lower than the $10^{8.88}$ threshold which Kinraide and Parker (1989) suggest is a suitable indicator for the appearance of polynuclear precipitated hydroxy-Al. It is important to note, however, that these speciation calculations apply only to the bulk phase of absorption solutions. Because of the unique physical and chemical properties of the apoplasm, the actual species which are in direct contact with the cell wall and plasma membrane are not known.

Screening of Desorption Agents. Using an Al-sensitive cultivar, Neepawa, this experiment was designed to select a desorption agent which effectively removed Al from the apoplasm. After uptake of Al for two hours as described above, absorption tubes with roots were removed from absorption solutions, rinsed with cold deionized water (4°C), and transferred to jars with 50 ml aerated desorption solution. Desorption agents included two multivalent cations, Ca^{2+} ($CaSO_4$) and Sc^{3+} ($ScCl_3$) and three effective chelators of Al, ethylenediamine tetraacetic acid (EDTA), tartaric acid and citric acid. Desorption agents were supplied at 0.5 mM, a concentration providing roughly 75 times more desorption agent than total Al absorbed by the roots at the end of uptake period. Desorption solutions were set to pH 4.5 and maintained at 0°C in an ice water bath to minimize loss of Al from the symplasm. After 0, 5, 10, 15, 20, 30, 60, 90, 120 and 180 minutes of desorption, 5 replicate absorption tubes were removed from desorption solutions, rinsed with deionized water, and prepared for determination of Al.

Determination of Desorption Time. This experiment was designed to determine if patterns of desorption with time varied between cultivars. After uptake of Al for 2 hours, roots from Atlas 66, Neepawa, PT741 and Scout 66 were removed from absorption solutions, rinsed with cold deionized water (4°C) and transferred to 0.5 mM

citric acid (pH 4.5) at 0°C for different time periods (0, 5, 10, 15, 20, 30, 60, 90, 120, 180 minutes). At the end of each desorption period, roots were rinsed with deionized water and prepared for determination of Al.

Isolation and Determination of the Linear Phase of Al Uptake. After uptake at 23°C for 0, 5, 10, 15, 20, 30, 60, 90, 120, 150 and 180 minutes, absorption tubes with roots were removed from absorption solutions, rinsed with cold deionized water (4°C), and transferred to 0.5 mM citric acid at 0°C for 30 minutes to desorb readily removeable Al from the apoplasm. At the end of desorption, roots were rinsed with deionized water, and prepared for determination of Al.

Inhibitor Studies. Excised roots were placed in absorption solutions (75 µM Al and 1.0 mM $CaSO_4$) with or without 0.1 mM 2,4-dinitrophenol (DNP). After 0, 15, 30, 60, 120 and 180 minutes of uptake, roots were removed from absorption solutions, rinsed with cold deionized water (4°C), and transferred to 0.5 mM citric acid at 0°C for 30 minutes to desorb readily removeable Al from the apoplasm. At the end of the desorption period, roots were removed, rinsed with deionized water and prepared for determination of Al.

Determination of Al. Root samples were air-dried for 12 hours at room temperature, dried to constant weight at 55°C, weighed, transferred to 50 ml borosilicate tubes, and ashed at 500°C for 24 hours. The resulting ash was dissolved in 0.2 ml concentrated HNO_3, oxidized with 0.2 ml 50% H_2O_2, and diluted to 40 ml with deionized water. Aluminum concentrations were analyzed on a Perkin-Elmer 3030 atomic absorption spectrophotometer with an HGA-500 graphite furnace attachment. Twenty microliters of diluted sample (0.6 ml sample : 1.2 ml deionized water) were mixed with 20 microliters of $Mg(NO_3)_2$ as a matrix modifier, dried at 150°C for 45 seconds, pretreated

at 1700°C for 45 seconds, and atomized at 2500°C for 5.5 seconds on a L'vov platform in a pyrolytically coated graphite tube. Concentrations were calculated by integration of peak area, and expressed as micrograms of Al per gram dry root (μg Al g^{-1}). For preparation of samples and standards for graphite furnace atomic absorption spectrophotometry, deionized water (>18 megohm/cm) and high purity reagents were used. Except for the ashing procedures, samples and standards were prepared and stored in polyethylene containers prewashed with dilute HNO_3 and deionized water.

Analysis of Data. Statistical analyses of the data were performed using analysis of variance (ANOVA), simple regression, and descriptive statistics available on Statistical Graphics Corporation's statistical package, Statgraphics Version 2.6. Analyses of homogeneity of slopes were performed using ANOVA available in SAS release 5.18. Significance was defined at the 95% confidence level.

3.3 Results

Uptake of Al by Al-tolerant and Al-sensitive cultivars showed two phases, a rapid phase in the first 30 minutes followed by a linear phase up to 180 minutes (Fig. 3-1). In the first phase, differences between Al-tolerant and Al-sensitive cultivars were small, but concentrations of Al were higher in the Al-sensitive cultivars Neepawa and Scout 66, than in the Al-tolerant cultivars Atlas 66 and PT741 after 30 minutes of uptake. In the linear phase, little difference was observed in the rate of Al uptake between Al-tolerant cultivars (1.79 ± 0.12 μg Al g^{-1} min^{-1}, Atlas 66; 1.18 ± 0.11 μg Al g^{-1} min^{-1}, PT741) and Al-sensitive cultivars (2.18 ± 0.25 μg Al g^{-1} min^{-1}, Neepawa; 1.55 ± 0.24 μg Al g^{-1} min^{-1}, Scout 66), thus concentrations of Al remained higher in roots of the Al-

sensitive cultivars (with the exception of an anomalous point at 180 minutes for Neepawa) (Fig. 3-1).

Dual kinetics similar to the pattern of Al uptake reported here have commonly been interpreted as representing uptake into the apoplasm (rapid phase) and uptake across the plasma membrane (linear phase) (Korner *et al.* 1986; Pettersson and Strid 1989). Thus, in a second experiment, an Al-sensitive cultivar (Neepawa) was used to test the effectiveness of various desorption agents for removal of Al from the putative apoplasmic compartment. As expected, desorption occurred in two phases, a rapid phase in the first 30 minutes followed by a linear phase up to 180 minutes (Fig. 3-2). During the first phase, citric acid was most effective in desorbing Al, followed by others in the order tartaric acid > EDTA > $CaSO_4$ = $ScCl_3$. By 30 minutes, 25 ± 3% of absorbed Al was removed by treatment with citric acid, while 21 ± 1, 19 ± 1, 16 ± 2 and 15 ± 4% of absorbed Al were removed by tartaric acid, EDTA, $CaSO_4$ and $ScCl_3$ respectively. After desorption of this rapidly removeable Al, the rate of desorption with time was relatively unaffected by the desorption agents used (0.09 ± 0.01, 0.07 ± 0.01, 0.08 ± 0.02, 0.04 ± 0.02 and 0.06 ± 0.03 μg Al g^{-1} min^{-1} for citric acid, tartaric acid, EDTA, $CaSO_4$ and $ScCl_3$ respectively, see Fig. 3-2). Thus, 30 minutes of desorption with citric acid appeared most effective for removal of Al from the putative apoplasmic compartment. With this treatment, all four cultivars showed a similar pattern of Al desorption; no difference was observed in the rate of desorption during the linear phase between Al-tolerant cultivars (0.06 ± 0.02 μg Al g^{-1} min^{-1}, Atlas 66; 0.10 ± 0.01 μg Al g^{-1} min^{-1}, PT741) and Al-sensitive cultivars (0.10 ± 0.02 μg Al g^{-1} min^{-1}, Neepawa; 0.06 ± 0.02 μg Al g^{-1} min^{-1}, Scout 66) (Fig. 3-3).

Uptake of Al into the linear phase was observed by monitoring Al remaining in

roots after a period of uptake (0 to 180 minutes) followed by 30 minute desorption in citric acid. For each cultivar, the rate of uptake was nearly linear. Deviation from linearity occurred primarily during the first 30 minutes of uptake, suggesting that the desorption treatment was not completely effective in removal of Al from the putative apoplasmic compartment. Incomplete desorption of the putative apoplasmic compartment was also suggested by the fact that extrapolation of the linear phase of absorption to time zero (Fig. 3-1) gave a greater estimate of the size of the apoplasmic compartment than extrapolation of the linear phase of desorption to time zero (Fig. 3-3; Table 3-1). Nevertheless, the desorption technique was largely effective in isolating the linear phase of uptake. In this phase, the rate of uptake varied between cultivars; however, no distinctive pattern of uptake distinguishing Al-tolerant from Al-sensitive cultivars was observed (2.24 + 0.11, 1.55 + 0.07, 2.20 + 0.05, 1.51 + 0.07 mg Al g-1 min-1 for Atlas 66, PT741, Neepawa, and Scout 66 respectively; see Fig. 3-4).

In the Al-sensitive cultivars, the linear phase of Al uptake was relatively insensitive to treatment with DNP. In the Al-sensitive cultivar, Neepawa, the rate of Al uptake was increased 7.0% by treatment with DNP, but this change was not statistically significant (Table 3-2). In Scout 66, a 24.7% increase was observed. In contrast, the rate of Al uptake by the Al-tolerant cultivars was strongly increased by DNP, with Atlas 66 showing a 51.9% increase and PT741 a 73.1% increase (Fig. 3-5). Similar differences in the effect of DNP on uptake of Al between Al-tolerant and Al-sensitive cultivars were also observed with an alternative experimental design in which all four cultivars were tested simultaneously, and uptake rates with and without DNP were determined by sampling after 30 and 120 minutes of absorption (data not shown).

3.4 Discussion

To my knowledge, this report and its companion paper (Zhang and Taylor 1989) are among the first to compare kinetics of Al uptake between Al-tolerant and Al-sensitive cultivars of the same species. In both Al-tolerant and Al-sensitive cultivars, uptake of Al by excised roots was biphasic. Although the identity of these two phases has not been investigated here (see Chapter 4), such kinetics have been commonly interpreted as representing uptake into the apoplasm (rapid phase) and uptake across plasma membrane (the linear phase) (Korner *et al.* 1986). If this designation is correct, uptake of Al in the apoplasm was rapid and saturated within 30 minutes (Fig. 3.1). A similar rapid phase of uptake was observed in experiments with *Brassica oleracea*, *Lactuca sativum*, and *Pennisetum clandestinium*, although saturation was not complete until 60 minutes of uptake (Huett and Menary 1979). In *Pinus taeda* and *Gleditsia triacanthus*, saturation was complete in four hours (Schaedle *et al.* 1986). Such differences between experiments may reflect differences between species, or differences in experimental conditions such as pH, Al concentration, and temperature of absorption solutions. In my experiments, the concentration of Al in absorption solutions was 75 µM, a concentration which does not affect growth of Al-tolerant cultivars of *Triticum aestivum*, but seriously reduces growth of Al-sensitive cultivars (Taylor and Foy 1985a,b; Zhang and Taylor 1988; see Chapter 2). In contrast, the experiments of Huett and Menary (1979) and Wagatsuma (1984) used 1.0, and 1.13 mM Al respectively.

At the end of the first phase of uptake, roots of the Al-sensitive cultivars (Neepawa and Scout 66) showed higher concentrations of Al than roots of the Al-tolerant cultivars (Atlas 66 and PT741) (Fig. 3-1). While these differences were small, they could reflect a lower cation exchange capacity of the cell wall material in the Al-

tolerant cultivars (Mugwira and Elgawhary 1979; Kennedy *et al.* 1986), which might contribute to Al tolerance by altering membrane selectivity and nutrient absorption (Taylor 1988a,b). Extrapolation of the linear phase of uptake to time zero indicated that less than 50% of absorbed Al was localized in the putative apoplasmic compartment in each of the four cultivars (Table 3-1), a value well below the 75-95% reported to be associated with cell wall material in roots of *Hordeum vulgare*, *Brassica oleracea*, *Lactuca sativum*, and *Pennisetum clandestinium* (Clarkson 1967; Huett and Menary 1979). While such differences may result from variation of properties of cell wall material and characteristics of metabolism between different species, it is also possible that the linear phase of uptake included both symplasmic and apoplasmic accumulation of Al. Precipitation of Al phosphate compounds or formation of insoluble polynuclear Al species could account for immobilization of Al in the apoplasm during the linear phase of uptake. If accumulation of Al in the apoplasm occurs during the linear phase, then extrapolation of the linear phase to time zero may underestimate apoplasmic uptake. This possibility has been further investigated in Chapter 4.

Although the four cultivars showed similar rates of Al uptake during the linear phase, further investigations of this phase of uptake were conducted using experiments designed to remove readily removeable Al from the apoplasm. Of various desorption agents tested, citric acid was most effective in desorption of Al from the putative apoplasmic compartment. The effectiveness of citric acid was consistent with its ability to protect plant cells from Al injury (Woolhouse 1983; Haug and Caldwell 1985; Suhayda and Hang 1986); citric acid is a tridentate chelator with chelation through two terminal carboxyl groups and a central hydroxyl group (Jackson 1982), resulting in a high-stability constant (about 10^8) for 1:1 Al-citrate chelates (Kragten 1978). Aluminum-EDTA and Al-tartrate complexes are less stable, possibly accounting for the

less effective nature of these desorption agents. The relative inability of Ca^{2+} and Sc^{3+} to desorb Al from the putative apoplasmic compartment was surprising, and suggests that Al uptake and desorption from the apoplasm may not be solely an ion exchange phenomenon. Thirty minute desorption in citric acid appeared sufficient for completion of the rapid desorption phase (Fig. 3-2). The biphasic pattern of desorption of Al from all four cultivars (Fig. 3-3) was similar to desorption of Al from roots of *Brassica oleracea, Lactuca sativum,* and *Pennisetum clandestinium* (Huett and Menary 1979).

While desorption with citric acid was largely effective in isolating the linear phase of uptake, uptake of Al into this fraction deviated from linearity during the first 30 minutes of uptake (Figs. 3-4 and 3-5), and extrapolation of uptake to time zero indicated some Al remained in the apoplasm of all four cultivars (Fig. 3-4). Differences in the estimated size of the apoplasmic compartment based upon extrapolation of the linear phase of uptake and the linear phase of desorption also suggested incomplete desorption of Al from the apoplasm (Table 3-1). Incomplete desorption of the apoplasmic compartment has been reported in other kinetic studies. For example, a small fraction of nonexchangeable ^{63}Ni in cell walls of *Hordeum vulgare* and ^{109}Cd in cell walls of *Glycine max* was reported in experiments using 1.0 mM EDTA (Korner *et al.* 1986) and 0.5 mM $CaCl_2$ or 0.4 to 10 mM $CdCl_2$ (Cataldo *et al.* 1983) as desorption agents, respectively.

If the rapid and linear phases of Al uptake reported in this study reflect uptake into the apoplasmic and symplasmic compartments respectively, then the pattern of uptake into the linear phase was inconsistent with the operation of an exclusion mechanism in Al-tolerant cultivars. If exclusion were important, different rates of uptake should have distinguished Al-tolerant from Al-sensitive cultivars. This was not

observed. I have, however, questioned the identity of the linear phase of uptake. This phase may include progressive accumulation of tightly bound Al in both the apoplasm and symplasm (see Chapter 4). Thus, the failure to detect differences between tolerant and sensitive cultivars in the rate of uptake across the plasma membrane (one component of the linear phase) may have been due to differences in the rate of accumulation of tightly bound Al in the apoplasm (the remaining component of the linear phase). This interpretation is consistent with Wagatsuma and Ezoe's (1985) suggestion that plants that effectively exclude Al at the plasma membrane may promote polymerization and accumulation of hydroxy Al in the apoplasm, thus contributing to detoxification of Al. Further investigation of the cellular localization of Al in the two phases of uptake will be needed to clarify this question.

While Haug and Caldwell (1985), and Roy *et al.* (1988) suggested that exclusion mechanisms are not important in Al tolerance, the potential operation of an exclusion mechanism in Al-tolerant cultivars of *Triticum aestivum* was suggested in this study by uptake experiments using DNP. Increased rates of uptake of Al by roots of Al-tolerant cultivars treated with DNP (Table 3-2) suggested that metabolic exclusion of Al from the symplasm of Al-tolerant cultivars occurred under normal aerobic conditions (without respiratory inhibitor). In contrast, the minimal effect of DNP on uptake by Al-sensitive cultivars suggests that uptake and accumulation of Al is not as closely regulated in a direct energy-dependent fashion (Table 3-2). Increased uptake of Al in roots treated with DNP was also reported in experiments with several species by Huett and Menary, who suggested that DNP increased permeability of the plasma membrane to Al (Huett and Menary 1979).

Since DNP is reported to uncouple oxidative phosphorylation, impair membrane structure and permeability, and disrupt the proton gradient across the plasma membrane, the way which DNP affected uptake of Al in Al-tolerant and Al-sensitive cultivars can not be identified. However, metabolic exclusion of Al might be achieved by means of an active efflux of Al into the apoplasm, by metabolic maintenance of plasma membrane structure and function in the face of Al stress (repair), or by enhanced exudation of Al chelators such as citric acid and tartaric acid in the cell wall space of Al-tolerant cultivars. Treatment of roots with DNP could inhibit these mechanisms by inhibiting ATP synthesis and/or reducing the driving force for efflux of organic anions. Characteristics of the effects of other metabolic inhibitors on kinetics of Al uptake and more definitive information on the localization of Al during the linear phase of uptake may help to identify possible exclusion mechanisms. Experiments designed to address these questions are reported in Chapter 4 and 5.

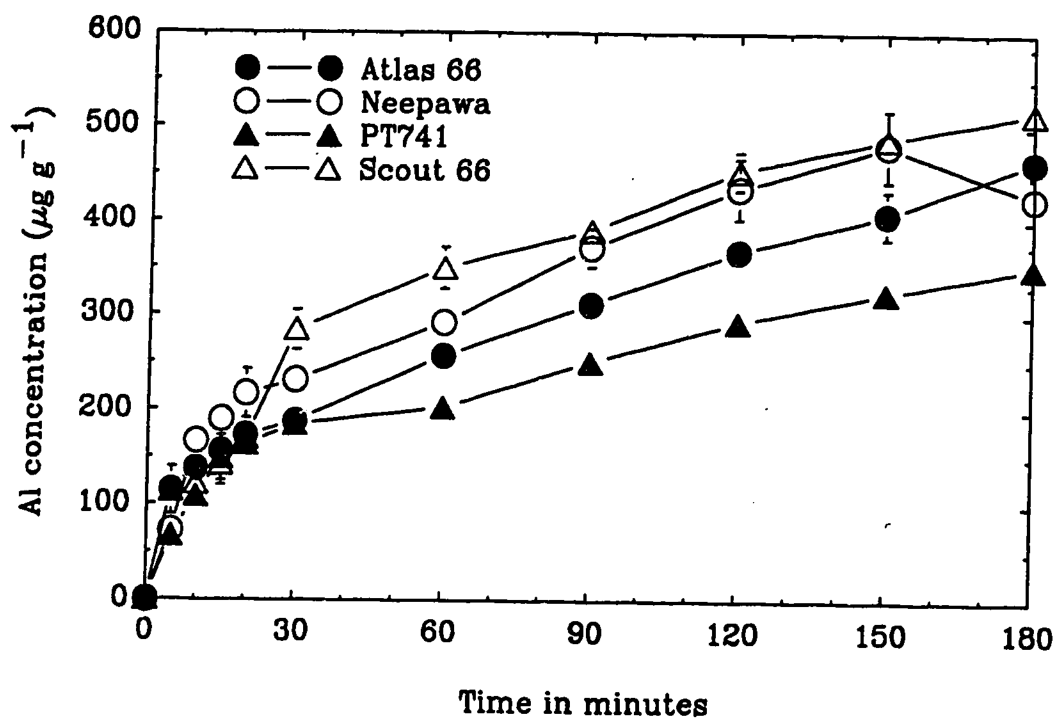

Figure 3-1. Uptake of Al ($\mu g\ g^{-1}$) by excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum*. Excised roots were exposed to 75 µM Al and 1.0 mM $CaSO_4$ (pH 4.5, 23°C) for 0, 5, 10, 15, 20, 30, 60, 90, 120, 150 and 180 minutes, followed by brief rinses with 1.0 mM $CaSO_4$ and deionized water. Values represent means of 5 replicates.

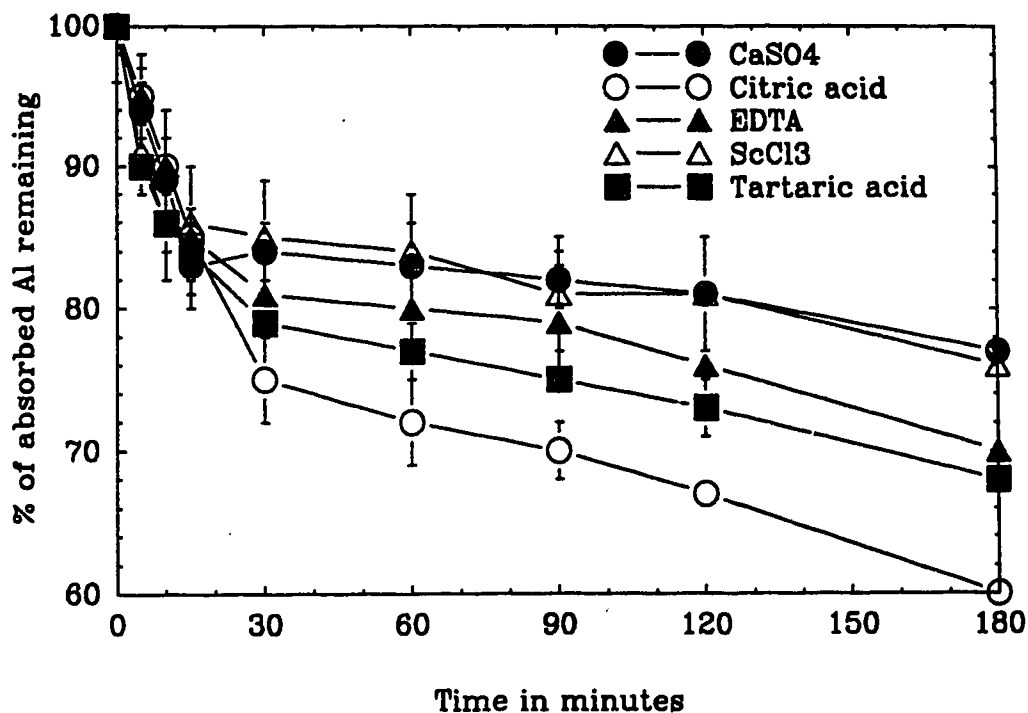

Figure 3-2. Desorption of Al from excised roots of an Al-sensitive cultivar (Neepawa) of *Triticum aestivum* by $CaSO_4$, $ScCl_3$, EDTA, citric acid and tartaric acid. Excised roots were treated with 75 μM Al and 1.0 mM $CaSO_4$ (pH 4.5, 23°C) for 120 minutes, followed by desorption in 0.5 mM $CaSO_4$, $ScCl_3$, EDTA, citric acid, or tartaric acid (pH 4.5, 0°C). Values represent means of 5 replicates.

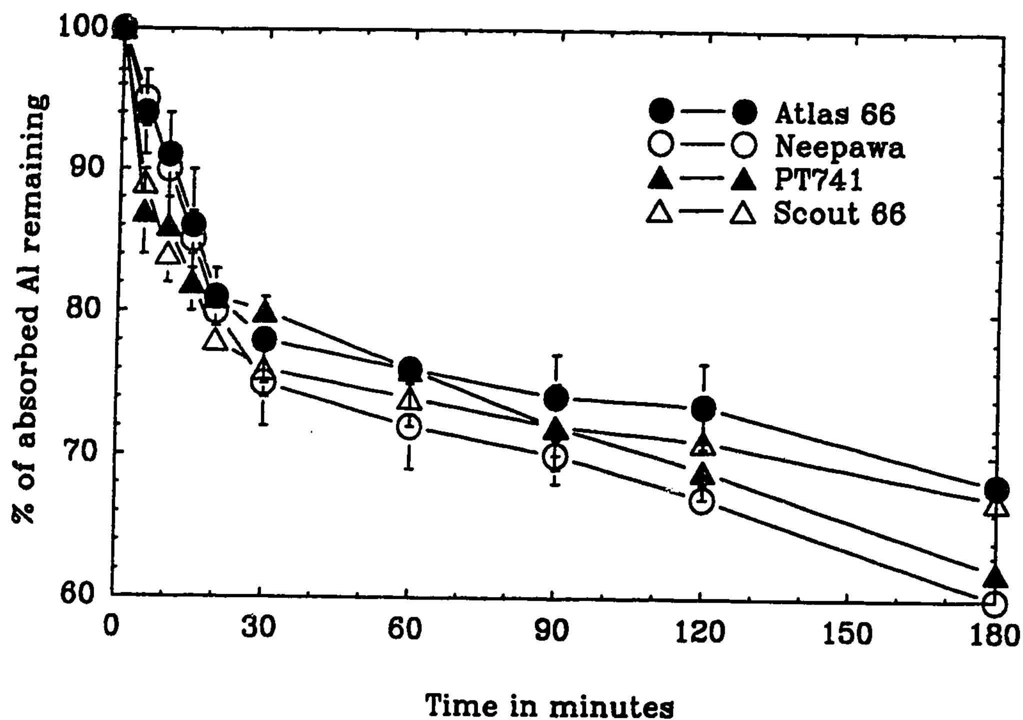

Figure 3-3. Desorption of Al from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum* by citric acid. Excised roots were treated with 75 μM Al and 1.0 mM $CaSO_4$ (pH 4.5, 23°C) for 120 minutes, followed by desorption in 0.5 mM citric acid (pH 4.5, 0°C). Values represent means of 5 replicates.

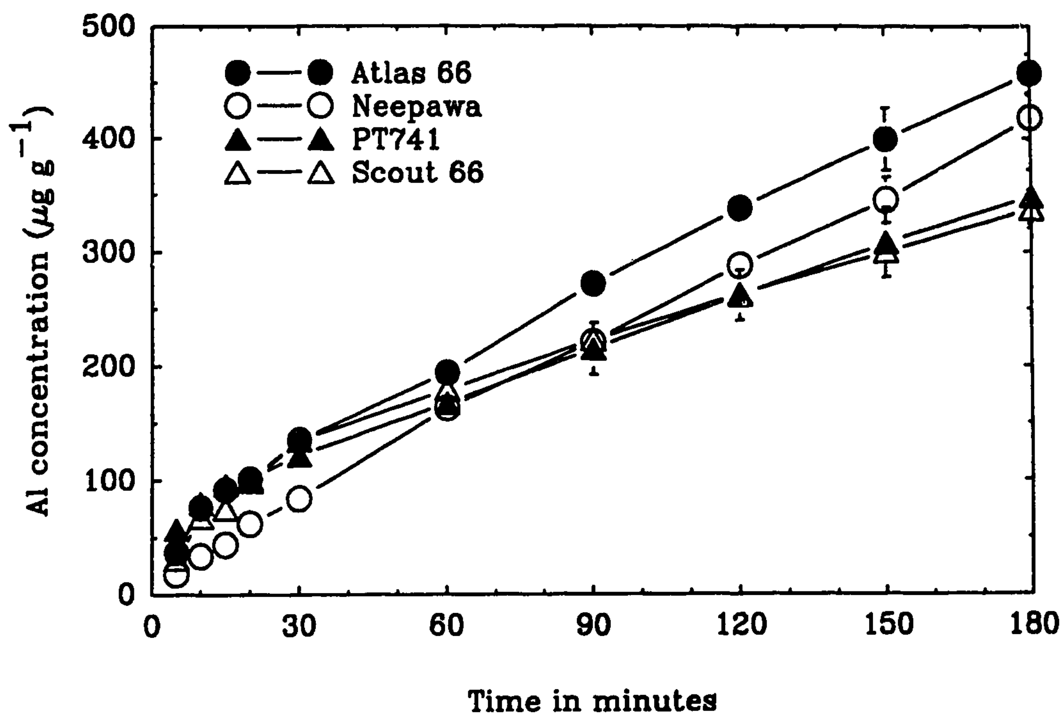

Figure 3-4. Uptake of Al (μg g^{-1}) into the linear phase by excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum*. Excised roots were treated with 75 μM Al and 1.0 mM $CaSO_4$ (pH 4.5, 23°C) for 0, 5, 10, 15, 20, 30, 60, 90, 120, 150 and 180 minutes, followed by desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 5 replicates.

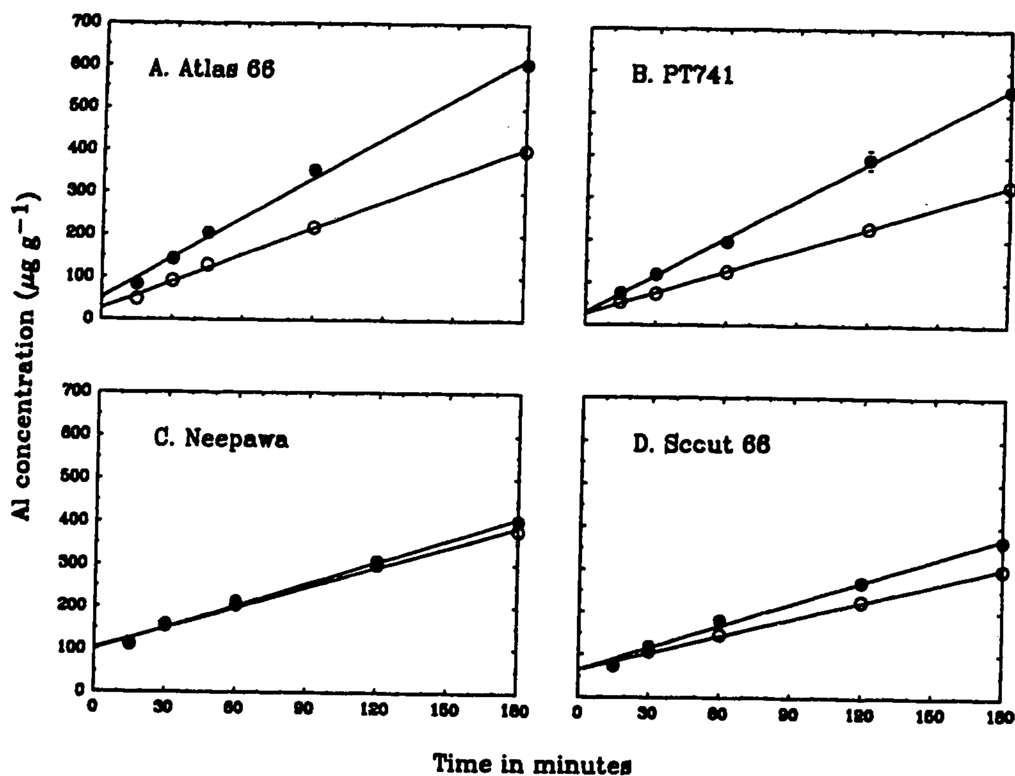

Figure 3-5. Uptake of Al (μg g^{-1}) by excised roots of Al-tolerant cultivars Atlas 66 (A) and PT741 (B), and Al-sensitive cultivars Neepawa (C) and Scout 66 (D) of *Triticum aestivum* with or without DNP. Excised roots were treated with 75 μM Al and 1.0 mM $CaSO_4$ (pH 4.5, 23^oC) with (●) or without (O) 0.1 mM DNP for 15, 30, 60, 120 and 180 minutes, followed by desorption in 0.5 mM citric acid (pH 4.5, 0^oC) for 30 minutes. Values represent means of 5 replicates.

Table 3-1. *Estimated contribution of the apoplasmic compartment to total uptake of Al in Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Values were calculated by extrapolation of the linear phase of Al uptake (Fig. 3-1) and the linear phase of Al desorption (Fig. 3-3) to time zero, and are expressed as a percent of total Al uptake.

	Al-tolerant cultivars		Al-sensitive cultivars	
Estimated by:	Atlas 66	PT741	Neepawa	Scout 66
Linear phase of uptake	31	40	31	46
Linear phase of desorption	20	17	22	23

Table 3-2. *Rate of Al uptake (μg Al g^{-1} min^{-1}) by excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum from absorption solutions with or with*[illegible] *DNP.* Data were adapted from Figure 3-5.

	Al-tolerant cultivars		Al-sensitive cultivars	
	Atlas 66	PT741	Neepawa	Scout 66
Control	2.06 ± 0.08	1.75 ± 0.08	1.86 ± 0.12	1.50 ± 0.08
DNP treatment	3.13 ± 0.13	3.03 ± 0.11	1.99 ± 0.14	1.87 ± 0.09
% increase	51.9*	73.1*	7.0	24.7*

* Indicating significant difference in the rate of Al uptake.

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4. IDENTITY OF THE LINEAR PHASE OF ALUMINUM UPTAKE BY EXCISED ROOTS OF ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE CULTIVARS OF *TRITICUM AESTIVUM* L.[3]

4.1 Introduction

In Chapter 3, I presented data which suggested that kinetics of Al uptake in Al-tolerant cultivars and Al-sensitive cultivars differ under non-metabolic conditions. Do these differences reflect differences in the rate of membrane transport? In order to understand the physiological and biochemical basis of Al toxicity and tolerance in plants, information on the movement of Al into apoplasmic and symplasmic compartments is essential. Unfortunately, the lack of a suitable isotope for monitoring short-term transport of Al in plant tissues has hampered progress in this field, and has made interpretation of kinetic data difficult. Despite this shortcoming, a number of authors have used kinetic analysis of Al uptake to estimate the rate of movement of Al across the plasma membrane (Clarkson 1967; Wagatsuma 1983a; Schaedle *et al.* 1986; Pettersson and Strid 1989). In *Triticum aestivum*, studies on the kinetics of Al uptake by excised roots have demonstrated a biphasic pattern of Al uptake, with a rapid phase of uptake superimposed over a linear phase of uptake (Pettersson and Strid 1989; Zhang and Taylor 1989; see Chapter 3). Although direct experimental evidence is lacking, these two phases have been interpreted as representing passive accumulation in the apoplasm (rapid phase) and transport across the plasma membrane into the symplasm (linear phase) (Korner *et al.* 1986; Pettersson and Strid 1989).

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[3] A version of this chapter has been accepted for publication in a refereed scientific journal. Zhang, G., and Taylor, G. J. 1990. Kinetics of aluminum uptake in *Triticum aestivum* L. Identity of the linear phase of aluminum uptake by excised roots of aluminum-tolerant and aluminum-sensitive cultivars. Plant Physiol. 93: (in press).

Pettersson and Strid (1989), and Zhang and Taylor (1989; see Chapter 3) compared the kinetics of Al uptake by roots of Al-tolerant and Al-sensitive cultivars of *Triticum aestivum* and failed to observe differences in uptake between cultivars. While these results could suggest that Al tolerance is not linked to initial uptake of Al (Pettersson and Strid 1989), Zhang and Taylor (1989; see Chapter 3) acknowledged that the precise identity of the linear phase is still in doubt. They reported that the apparent size of the apoplasmic compartment for Al was larger when estimated by extrapolation of the linear phase of uptake to time zero than when estimated by extrapolation of the linear phase of desorption to time zero (Zhang and Taylor 1989; see Chapter 3). If the linear phase of uptake represents accumulation of Al in both symplasmic and apoplasmic compartments (not just accumulation of Al in the symplasmic compartment), then differences between Al-tolerant and Al-sensitive cultivars in the uptake of Al across the plasma membrane might be obscured by differences in accumulation of Al in the cell wall. This would be particularly true if exclusion of Al at the plasma membrane leads to increased polymerization or precipitation of Al in the cell wall.

This study was designed to determine if the linear phase of Al uptake by excised roots of Al-tolerant and Al-sensitive cultivars of *Triticum aestivum* includes accumulation of Al in the cell wall. My results support a novel view of the identity of the linear phase of Al uptake.

4.2 Methods

Preparation of Plant Material. Seeds of two Al-tolerant cultivars (Atlas 66 and PT741) and two Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum* L. (wheat) were surface sterilized in 1.2% sodium hypochlorite for 20 minutes, and germinated overnight in a solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were grown for 7 days on nylon mesh suspended over 16 liters of nutrient solution containing (mM) 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (µM) 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.5) in a growth chamber with 16 hours of light (20°C, 68% relative humidity) and 8 hours of darkness (16°C, 85% relative humidity). The photosynthetic photon flux density (PPFD) was 335 ± 12 µmol m^{-2} s^{-1} at plant base level. After 5 days of growth, plants were transferred to fresh nutrient solutions.

Uptake of Al by Excised Roots. Thirty root tips (2.0 cm) were excised and placed in each of 36 to 50 "absorption tubes" as described by Zhang and Taylor (1989; see Chapter 3). During excision of roots, absorption tubes containing excised roots were placed in an aerated nutrient solution. When excision was complete (within 60 minutes), the tubes were transferred to an aerated solution of 1.0 mM $CaSO_4$ for 30 minutes. Uptake experiments were initiated by transferring the absorption tubes containing roots to 80 ml glass jars containing 50 ml of an aerated solution of 75 µM Al as $AlK(SO_4)_2 \cdot 12H_2O$ and 1.0 mM $CaSO_4$ (pH 4.5) in a water bath at 23°C, or in an ice-water bath at 0°C. Four or five of the replicate tubes were removed from absorption solutions after 0, 15, 30, 60, 120, and 180 minutes of uptake, rinsed briefly with 1.0 mM $CaSO_4$ and deionized water (300 ml per tube), and transferred to 0.5 mM citric acid (pH 4.5) at 0°C for 30 minutes to desorb removeable Al from the apoplasm. After 30 minutes of

desorption, roots were removed, rinsed with deionized water and prepared for fractionation and/or determination of Al.

The composition of the absorption solutions was the same as described in Chapter 3, thus Al will be present primarily as the $AlSO_4$ ion pair (22 μM), $Al^{3+} \cdot 6H_2O$ (18 μM), a number of less abundant monomeric species, and possibly a polynuclear species. Again, it is important to note that speciation calculations apply only to the bulk phase of absorption solutions. Because of the unique physical and chemical properties of the apoplasm, the actual species which are in direct contact with the cell wall and plasma membrane are not known.

Crude Separation of Pellet and Supernatant. After absorption and desorption treatments as described above, roots were blotted, weighed, cut into 1 mm long segments and stored on ice. The root segments (about 0.15 g fw) were homogenized in 1.5 ml 0.5 mM tris-HCl buffer (pH 7.8) in an ice-water bath for 90 seconds and centrifuged for 20 minutes at 18,000 rpm (25,300 g) at 4°C. The pellet and supernatant were collected after two washings with buffer and deionized water.

Isolation and Desorption of Purified Cell Wall Material. Purified cell wall material was isolated using a technique adapted from Tu *et al.* (1988). After absorption and desorption treatments, roots were blotted, weighed, cut into 1 mm long segments and stored on ice. Root segments were homogenized in 1.5 ml 0.1 M Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) - Mes (2-[N-morpholino]ethanesulfonic acid) and 0.3 M sucrose buffer (pH 7.8) for 10 seconds, and placed in a Parr cell disruption bomb under nitrogen pressure (110 kg/cm^2) for 10 minutes. After extrusion to atmospheric pressure, the homogenate was sonicated in an

ice-water bath for 7 minutes at 60% output control on a 25 watt ultrasonic homogenizer. The homogenate was then filtered through a 20 μm nylon mesh. Cell wall material trapped on the mesh was rinsed with 50 ml cold deionized water (4°C). Sixteen of the 32 cell wall samples were desorbed in 10 ml 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. After desorption, the cell wall material was again trapped on the nylon mesh and rinsed with 50 ml cold deionized water (4°C). Both the cell wall material and the filtrate were collected for determination of Al.

Adsorption of Al by Isolated Cell Wall Material. In several experiments, Al was also supplied to purified cell wall material isolated from excised roots of Al-tolerant and Al-sensitive cultivars. In these experiments, cell wall material from excised roots with no prior exposure to Al was isolated as described above. During the fractionation procedure, the cell wall material was suspended in 15 ml centrifuge tubes containing 5 ml of 1.0 mM $CaSO_4$ (pH 4.5) in an ice-water bath. Before the adsorption treatment, the cell wall material was brought to the absorption temperature (23°C). The adsorption period was initiated by adding 5 ml of a solution containing 1.0 mM $CaSO_4$ and 150 μM Al (pH 4.5), which brought the final concentration of Al to 75 μM. After 0, 30, 60, 120 and 180 minute absorption, the cell wall material from 8 replicate tubes was trapped on nylon mesh and washed with 50 ml cold deionized water (4°C). Four of the 8 tubes were desorbed in an aerated 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes as described above. Purified cell wall material both with and without desorption treatment were prepared for determination of Al.

Test of Cell Wall Purity. Microscopic examination with neutral red and Even's blue showed complete cell breakage. The isolated cell wall material was free of cytosolic contamination, whereas cell contents (the filtrate fractions) showed some contamination

with cell wall fragments. Total ATPase activity and cytochrome c oxidase activity were used as cytosolic markers to test the purity of the isolated cell wall material. Total ATPase activity was determined by measuring liberation of inorganic phosphorous from ATP (Berczi and Moller 1986). Cytochrome c oxidase was determined spectrophotometrically by measuring the rate of oxidation of reduced cytochrome c at A_{550} (Kirkpatrik *et al.* 1983). These tests demonstrated that the purified cell wall material obtained was virtually free of cytosolic contamination. Only 0.6% of total ATPase activity and no detectable cytochrome c oxidase activity were observed in the cell wall preparations.

Determination of Al. Roots and cell wall material were ashed at 500°C, dissolved with concentrated HNO_3 and oxidized with H_2O_2 as described by Zhang and Taylor (1989; see Chapter 3). Filtrates were directly used for determination of Al without further processing. Aluminum concentrations in prepared samples were determined by graphite furnace atomic absorption spectrophotometry as described by Zhang and Taylor (1989; see Chapter 3). Concentrations were calculated by integration of peak area, and expressed as micrograms of Al per gram dry weight (μg Al g root dw^{-1}, as in excised roots) or fresh weight of roots (μg Al g root fw^{-1}, as in subcellular fractions).

Analysis of Data. Statistical analyses of the data were performed using analysis of variance (ANOVA), simple regression, and descriptive statistics available on Statistical Graphics Corporation's statistical package, Statgraphics Version 2.6. Analyses of homogeneity of slopes were performed using ANOVA available in SAS release 5.18. Significance was defined at the 95% confidence level.

4.3 Results

Uptake of Al by Al-tolerant and Al-sensitive cultivars at both 0°C and 23°C showed a clear linear phase, with no sign of saturation within the experimental period (Fig. 4-1). Exposure to low temperature (0°C) reduced the rates of Al uptake equally in both Al-tolerant and Al-sensitive cultivars. The rate of Al uptake was reduced by 57% (from 2.10 ± 0.17 to 0.91 ± 0.07 $\mu g\ g^{-1}\ min^{-1}$) and 72% (from 1.40 ± 0.11 to 0.39 ± 0.06 $\mu g\ g^{-1}\ min^{-1}$) in the Al-tolerant cultivars Atlas 66 and PT741, and 53% (from 1.97 ± 0.16 to 0.93 ± 0.11 $\mu g\ g^{-1}\ min^{-1}$) and 55% (from 2.03 ± 0.12 to 0.92 ± 0.11 $\mu g\ g^{-1}\ min^{-1}$) in the Al-sensitive cultivars Neepawa and Scout 66. Retention of the linear phase at 0°C and its non-removeable nature after desorption in citric acid suggests that the linear phase of uptake includes a non-metabolic component, and that this non-metabolic component is not simply an exchange/absorption phenomenon.

A crude fractionation technique was employed to determine if the linear phase of uptake could be completely accounted for by uptake of Al into the cytosol. Uptake of Al in the supernatant fraction isolated from roots pre-treated with Al clearly showed a linear component, but this component was small compared to the rate of uptake in the pellet. Uptake of Al in supernatant fractions accounted for only 9 to 15% of total uptake, with no observed differences between Al-tolerant and Al-sensitive cultivars (Table 4-1). Clearly, uptake of Al into the soluble cytosol fraction is not sufficient to account for the linear phase of uptake. Accumulation of Al in the cell wall and/or organelles must also be postulated.

Analysis of Al from purified cell wall material isolated from excised roots pre-treated with Al confirmed that the linear phase of Al uptake may include an apoplasmic

component. In all cultivars, adsorption of Al onto purified cell wall material clearly showed a linear component (Fig. 4-2). Interestingly, the rate of adsorption of Al onto cell wall material (0.36 ± 0.12 to 0.61 ± 0.05 μg g^{-1} min^{-1}) and uptake in the remaining filtrate (0.45 ± 0.03 to 0.58 ± 0.04 μg Al g^{-1} min^{-1}) occurred at similar rates in the Al-tolerant and Al-sensitive cultivars (Table 4-2). A linear phase of adsorption in purified cell wall material would be observed if the cell wall contributed to this phase *in vivo*, or if redistribution of Al from the cytosol to the cell wall occurred during fractionation. If redistribution is important, then a second desorption treatment of cell wall material after isolation should effectively remove loosely bound Al. In such an experiment, a second 30 minute desorption with citric acid following treatment of excised roots with Al, desorption with citric acid, and isolation of cell wall material, did not eliminate the linear phase in the purified cell wall (Fig. 4-3). In the absence of this second desorption treatment, rates of Al adsorption in cell wall fractions were 0.53 ± 0.09 and 0.42 ± 0.08 μg g^{-1} min^{-1} for the Al-tolerant cultivar PT741 and the Al-sensitive cultivar Neepawa. With the second desorption treatment, rates of Al adsorption in cell wall fraction were 0.28 ± 0.09 and 0.43 ± 0.06 μg g^{-1} min^{-1} for PT741 and Neepawa respectively (Table 4-3). While the rate of adsorption in the Al-tolerant cultivar PT741 appeared to decrease with the second desorption treatment, no significant difference in the rate of adsorption was detected. These results indicate that the linear phase in cell wall fraction was non-removeable and, hence, I have rejected the possibility that the linear phase resulted from redistribution of Al during fractionation. These results challenge the traditional interpretation of the linear phase of Al uptake as transport across the plasma membrane, and suggest a more complex phase of uptake including Al uptake the symplasm and adsorption in the apoplasm.

Metabolism-dependent binding of cations in the cell wall has been suggested in

several studies (Barber and Shone 1967; Ighe and Pettersson 1974). It is therefore possible that the linear phase in cell wall material may require normal functioning of the plasma membrane and continued cellular integrity. To test this hypothesis, Al adsorption by isolated cell wall material treated with Al *in vitro* was investigated. Adsorption of Al by isolated cell wall material was biphasic with a linear phase in the absence of a desorption treatment (Fig. 4-4). The rate of Al adsorption onto isolated cell wall material during the linear phase was 1.27 $\pm$ 0.23 and 1.12 $\pm$ 0.32 $\mu g\ g^{-1}\ min^{-1}$ for the Al-tolerant cultivar PT741 and the Al-sensitive cultivar Neepawa respectively (Table 4-4). In contrast to the results where excised roots were treated with Al, this linear phase was completely removeable by 30 minute desorption with citric acid. In both the Al-tolerant cultivar PT741 and the Al-sensitive cultivar Neepawa, Al accumulated in isolated cell wall material exhibited saturated kinetics after desorption, without significant slopes (Fig. 4-4; Table 4-4). The removeable nature of the linear phase in isolated cell wall material suggests that Al adsorption by purified cell wall material reflects an exchange/absorption process. In contrast, the non-removeable linear phase of *in vivo* adsorption in cell wall fraction may represent metabolism-dependent adsorption of Al into the cell wall.

4.4 Discussion

Differences in the uptake of Al between 23°C and 0°C by all four cultivars suggested that the linear phase of Al uptake is composed of two components, a non-metabolic component observed at both 0°C and 23°C, and a metabolic component observed only at 23°C. The metabolic component likely represents uptake of Al across the plasma membrane. Active transport of Al has not been reported, although

beneficial effects of Al on the growth of *Zea mays*, *Oryza sativa*, *Triticum aestivum* and *Camellia sinensis* have been suggested (Howler and Cadavid 1976; Clark 1977; Matsumoto 1977; Foy and Fleming 1978). Wagatsuma (1983a) and Pettersson *et al.* (1986) suggested that this metabolic component may represent passive diffusion of Al across plasma membrane. The reduced rate of Al uptake at low temperature suggests that the metabolic component depends on the existence of the driving force associated with metabolism. This driving force would be a membrane potential created by proton-translocating ATPases (Serrano 1985). The activity of these ATPases may be reduced at low temperature, possibly accounting for the change in Al uptake with temperature. The non-metabolic component could represent polymerization or precipitation of Al in the cell wall, or Al tightly bound to cell wall material (Clarkson 1967; Wagatsuma and Yamasaku 1985). The decrease in proton concentration outside the plasma membrane resulting from decreased activity of proton-translocating ATPases may result in an increase in apoplasmic pH. This could in turn affect the speciation, solubility, and mobility of Al in the apoplasm. Because the driving force for Al transport will not be completely eliminated by low temperature, the non-metabolic component could also include passive transport of Al across the plasma membrane with the concentration gradient and the electrical potential across the plasma membrane serving as driving forces.

The effect of low temperature on uptake of Al may vary with species. Low temperature did not affect the uptake of Al by *Brassica oleracea*, *Lactuca sativa*, *Pennisetum clandestinum*, and *Hordeum vulgare* (Clarkson 1967; Huett and Menary 1979), however, different pH (4.0 to 4.2) and Al concentrations (0.2 to 1.1 mM) in these experiments make results difficult to compare to the results presented here. In *Triticum aestivum*, decreased uptake of Al at low temperature (2°C) was also observed

by Pettersson and Strid (1989). In contrast to the present results, however, Pettersson and Strid (1989) reported a saturable phase of Al uptake at low temperature. In their experiments, roots were simply blotted dry at the end of absorption period, with no washing or desorption procedure to remove Al from cell wall exchange sites. Their saturable phase of uptake at low temperature may also have reflected a lower pH of absorption solutions (pH 4.1). Huett and Menary (1979) demonstrated that a decrease in pH of the absorption solution from 4.2 to 4.0 changed the pattern of Al uptake by *Brassica oleracea* at low temperature (1°C) from non-saturable to saturable.

Crude fractionation of roots into a supernatant and pellet fraction demonstrated that uptake of Al into both the supernatant and pellet was linear, although the relative size of the supernatant fraction was small. Aluminum uptake into the supernatant fraction accounted for less than 15% of the total absorbed Al (Table 4-1). Clarkson (1967) and Huett and Menary (1979) also suggested a minor accumulation of Al in the cytosol of *Brassica oleracea, Lactuca sativa, Pennisetum clandestinum* and *Hordeum vulgare*, emphasizing that most absorbed Al (75 to 90%) was located in cell wall. Although the supernatant fraction was not well defined here, my results are consistent with other studies which suggest that the plasma membrane and cell wall play an important role in restricting entry of Al into the cytosol (Taylor 1988).

A dual pattern of Al uptake (a rapid saturable phase superimposed over a linear phase) in excised and whole roots has been reported by several authors (Huett and Menary 1979; Pettersson and Strid 1989; Zhang and Taylor 1989; see Chapter 3), and the linear phase has been suggested to represent uptake in the symplasm (Korner *et al.* 1986; Pettersson and Strid 1989). However, this interpretation of the identity of the linear phase is not consistent with my results. While I would agree that the non-

metabolic, saturable phase of uptake in excised and whole roots represents accumulation of Al in the cell wall, the linear nature of Al adsorption in purified cell wall material suggests that the linear phase represents adsorption of Al onto the cell wall and transport into the symplasm. In comparison to the rate of Al uptake into the cytosol, the cell wall compartment made a substantial contribution to the linear phase (Fig. 4-2; Table 4-2). My suggestion that the linear phase of Al uptake includes accumulation of Al in the apoplasm is supported by the non-removeability of Al accumulated in the cell wall fraction. A second desorption treatment of the purified cell wall material after isolation did not eliminate the linear phase in cell walls isolated from roots pre-treated with Al. Thus, this phase cannot reflect redistribution of Al from the cytosol during fractionation. The nature of binding in the cell wall, however, is still unclear.

Differences in Al adsorption onto cell wall material between *in vivo* and *in vitro* experiments also supported metabolism-dependent accumulation of Al in the cell wall. In comparison to experiments in which Al was supplied to excised roots, the linear phase in isolated cell wall material exposed to Al *in vitro* was completely removed by desorption with citric acid in both the Al-sensitive cultivar Neepawa and the Al-tolerant cultivar PT741. Thus, the non-removeable nature of the linear phase in the cell wall fraction depends on the integrity of the cell and/or the plasma membrane. Once again, the precise nature of metabolism-dependent binding of Al in the cell wall fraction is not clear. It could result from formation of hydrated Al complexes associated with pectic substances (Wagatsuma 1983b) or free carboxyl groups (Clarkson 1967), or polymerization of absorbed monomeric Al in the cell wall (Matsumoto *et al.* 1977; Wagatsuma and Yamasaku 1985). If the functional relationship between the plasma membrane and cell wall is altered during homogenization, high pressure, or sonication

treatments, then the functional relationship between exclusion of Al at the plasma membrane and binding of Al by cell wall may be altered. Furthermore, loss of the integrity of the cell wall may result in changes in the physical, chemical, and biochemical properties of the surface of the cell wall, possibly causing a loss of metabolism-dependent binding.

To my knowledge, this is the first report which specifically addresses the identity of the linear phase of Al uptake which has been observed in short-term kinetic studies. My data clearly do not support the interpretation of the linear phase of Al uptake as simply representing Al uptake across the plasma membrane. I believe that the linear phase of Al uptake in the excised roots represents both apoplasmic and symplasmic compartments, however, the relationship between non-metabolic and metabolism-dependent accumulation of Al in the cell wall is not clear. Further studies are needed to investigate the mechanisms of metabolism-dependent accumulation in the cell wall, the nature of non-metabolic and metabolic uptake across the plasma membrane. Furthermore, it is important to determine if differences in the kinetics of Al uptake of Al-tolerant and Al-sensitive cultivars are simply due to difference in Al accumulation in the cell wall. Experiments designed to address the latter question are reported in Chapter 5.

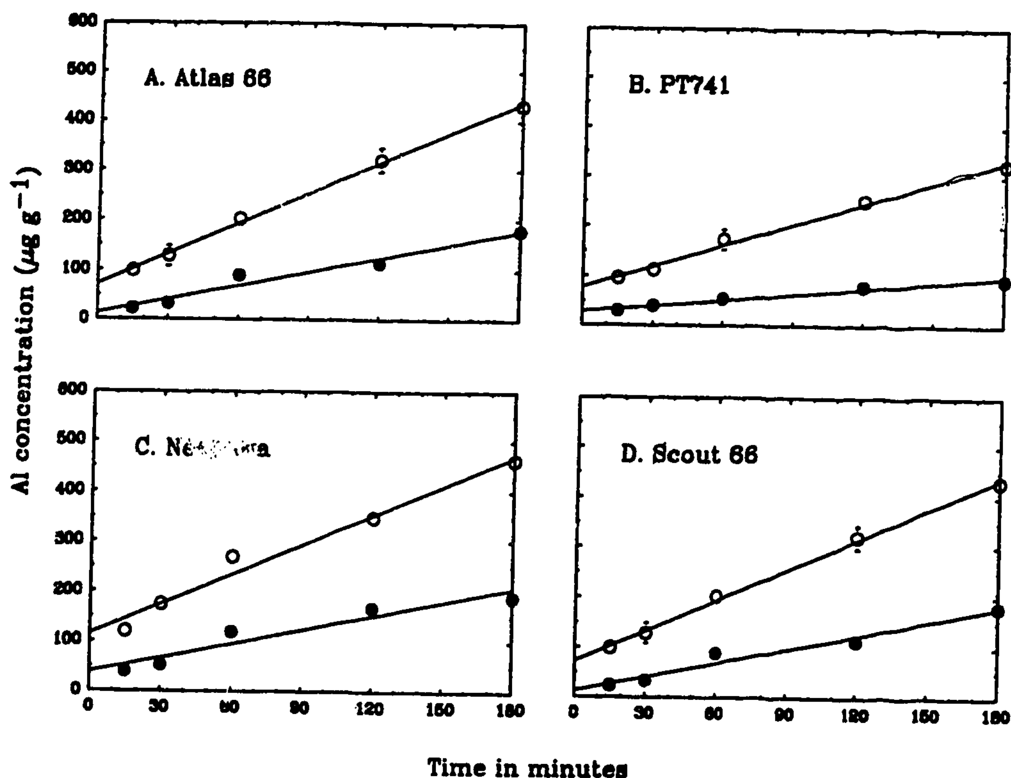

Figure 4-1. Uptake of Al (μg g root dw^{-1}) by excised roots of Al-tolerant cultivars Atlas 66 (A) and PT741 (B), and Al-sensitive cultivars Neepawa (C) and Scout 66 (D) of *Triticum aestivum* at 0°C and 23°C. Excised roots were treated with 75 μM Al and 1.0 mM Ca (pH 4.5) for 0, 15, 30, 60, 120 and 180 minutes at 0°C (●) or 23°C (O), followed by desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 5 replicates.

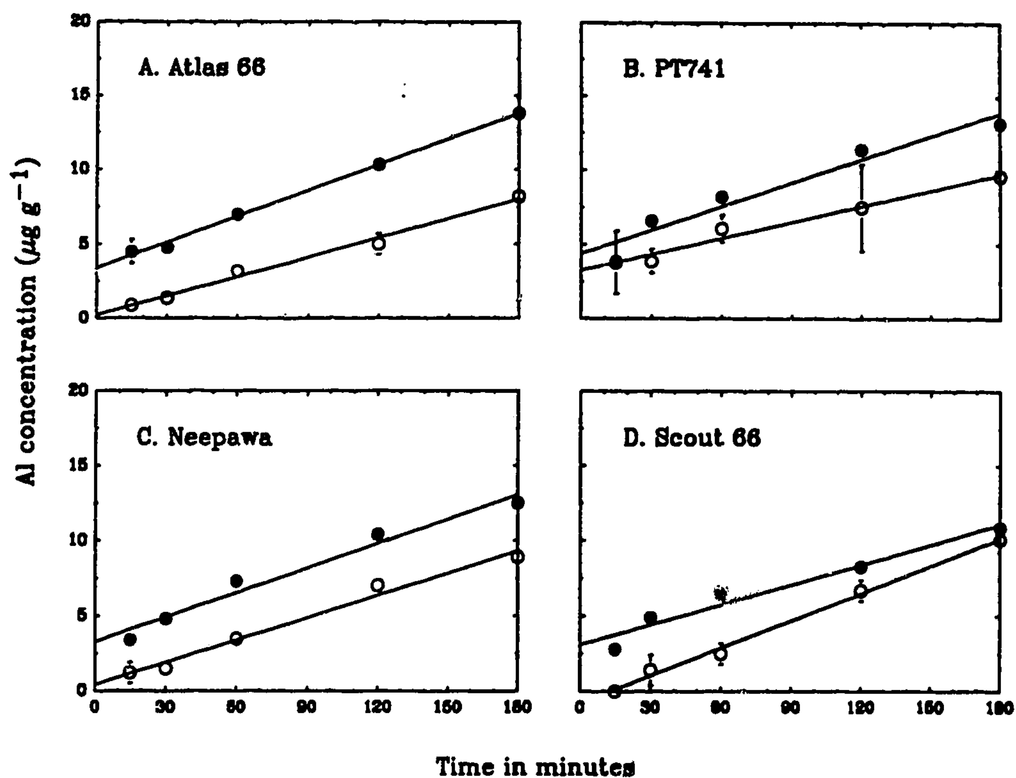

Figure 4-2. Accumulation of Al (μg g root fw^{-1}) into purified cell wall material (O) and the remaining filtrate (●) isolated from excised roots of Al-tolerant cultivars Atlas 66 (A) and PT741 (B), and Al-sensitive cultivars Neepawa (C) and Scout 66 (D) of *Triticum aestivum*. Cell wall material was isolated from excised roots pretreated with 75 μM Al and 1.0 mM Ca (pH 4.5, 23°C) followed by desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 5 replicates.

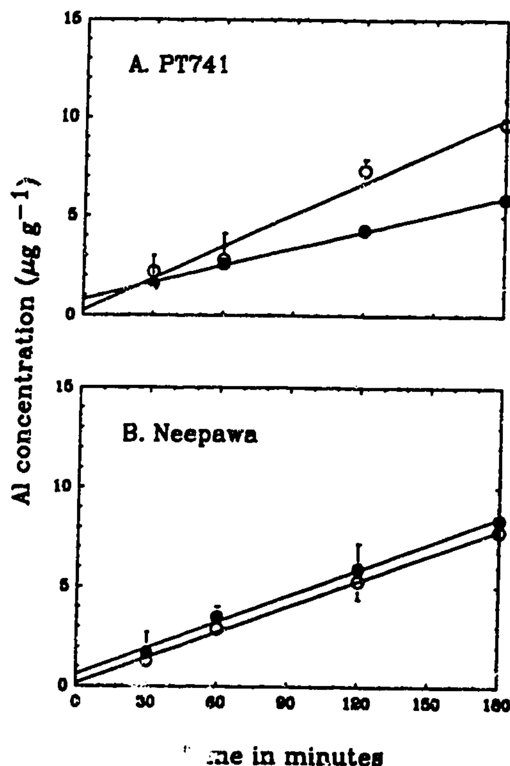

Figure 4-3. Adsorption of Al (µg g root fw^{-1}) onto purified cell wall material isolated from excised roots of an Al-tolerant cultivar PI741 (A) and an Al-sensitive cultivar Neepawa (B) of *Triticum aestivum* with (●) or without (O) a second desorption treatment. Roots were pretreated with 75 µM Al and 1.0 mM Ca (pH 4.5, 23°C) followed by desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Cell wall material was then isolated and half of the samples received a second desorption treatment with 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 4 replicates.

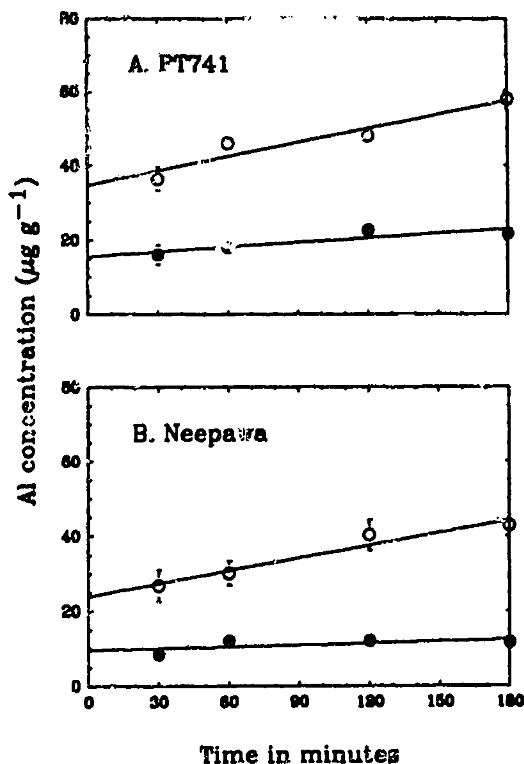

Figure 4-4. *In vitro* Adsorption of Al (µg g root fw^{-1}) onto purified cell wall material isolated from excised roots of an Al-tolerant cultivar PT741 (A) and an Al-sensitive cultivar Neepawa (B) of *Triticum aestivum* with (●) or without (O) desorption treatment. Purified cell wall material isolated from roots without Al pre-treatment, was treated in 75 µM Al and 1.0 mM Ca (pH 4.5, 23°C), followed by desorption or no desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 4 replicates.

Table 4-1. *Rate of Al uptake (μg g root fw^{-1} min^{-1}) and Al concentration (μg g root fw^{-1}) in the pellet (18,000 rpm) and supernatant fractions isolated from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Pellet and supernatant fractions were isolated from roots after an absorption period of 0, 15, 30, 60, 120 and 180 minutes in 75 μM Al and 1.0 mM Ca (pH 4.5, 23°C) followed by desorption treatment in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Concentrations were calculated from the 180 minute absorption period. Values represent means of 5 replicates.

	Rate of Uptake		Concentration	
Cultivars	Supernatant	Pellet	Supernatant (%)	Pellet (%)
Atlas 66	0.06 ± 0.03	0.39 ± 0.0[illegible]	1.5 ± 0.5 (15)	8.4 ± 1.2 (85)
PT741	0.06 ± 0.01	0.39 ± 0[illegible]	1.6 ± 0.2 (12)	11.6 ± 3.0 (88)
Neepawa	0.08 ± 0.02	0.58 ± 0.09	1.9 ± 0.1 (12)	13.9 ± 1.2 (88)
Scout 66	0.12 ± 0.02	1.01 ± 0.22	2.3 ± 0.3 (9)	23.2 ± 3.0 (91)

Table 4-2. *Rate of Al accumulation (μg g root fw^{-1} min^{-1}) and Al concentration (μg g root fw^{-1}) in purified cell wall material and remaining filtrate isolated from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Purified cell wall material was isolated from roots after an absorption period of 0, 15, 30, 60, 120 and 180 minutes in 75 μM Al and 1.0 mM Ca (pH 4.5, 23°C) followed by desorption treatment with 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Concentrations were calculated from the 180 minute absorption period. Values represent means of 5 replicates.

	Rate of Accumulation		Concentration	
Cultivars	Cell Wall	Filtrate	Cell Wall (%)	Filtrate (%)
Atlas 66	0.43 ± 0.04	0.58 ± 0.04	8.2 ± 0.6 (38)	13.8 ± 1.0 (62)
PT741	0.36 ± 0.12	0.53 ± 0.04	9.6 ± 1.1 (42)	13.2 ± 0.5 (58)
Neepawa	0.49 ± 0.04	0.55 ± 0.03	8.9 ± 0.6 (41)	12.5 ± 0.6 (59)
Scout 66	0.61 ± 0.05	0.45 ± 0.03	10.1 ± 0.7 (48)	10.9 ± 0.5 (52)

Table 4-3. *Rate of Al accumulation (µg g root fw^{-1} min^{-1}) and Al concentration (µg g root fw^{-1}) in purified cell wall material isolated from excised roots of an Al-tolerant cultivar (PT741) and an Al-sensitive cultivar (Neepawa) of Triticum aestivum with or without a second desorption treatment.* Cell wall material was isolated from roots which had been pre-treated with Al for 0, 30, 60, 120 and 180 minutes in 75 µM Al and 1.0 mM Ca (pH 4.5, 23°C) and desorbed in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. After fractionation, half of the cell wall samples received a second desorption treatment with 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Concentrations were calculated from the 180 minute absorption period. Values represent means of 4 replicates.

Cultivars	Treatments	Rate of accumulation	Concentration
PT741	Without second desorption	0.53 ± 0.09	9.8 ± 1.2
	With second desorption	0.28 ± 0.09	5.6 ± 1.4
Neepawa	Without second desorption	0.42 ± 0.08	7.8 ± 1.0
	With second desorption	0.43 ± 0.06	8.4 ± 1.4

Table 4-4. *Rate of Al adsorption (μg g root fw^{-1} min^{-1}) and Al concentration (μg g root fw^{-1}) in purified cell wall material isolated from excised roots of an Al-tolerant cultivar (PT741) and an Al-sensitive cultivar (Neepawa) of Triticum aestivum with or without desorption.* Cell wall material isolated from roots without Al pre-treatment, was treated in 75 μM Al and 1.0 mM Ca (pH 4.5, 23°C) for 0, 30, 60, 120 and 180 minutes, followed by desorption treatment (if indicated) in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Concentrations were calculated from the 180 minute absorption period. Values represent means of 4 replicates.

Cultivars	Treatments	Rate of Adsorption	Concentration
PT741	Without desorption	1.27 ± 0.23	58.0 ± 2.5
	With desorption	0.41 ± 0.19	21.8 ± 3.1
Neepawa	Without desorption	1.12 ± 0.32	42.8 ± 3.7
	With desorption	0.15 ± 0.17	11.7 ± 2.3

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5. EFFECTS OF BIOLOGICAL INHIBITORS ON KINETICS OF ALUMINUM UPTAKE BY EXCISED ROOTS AND PURIFIED CELL WALL MATERIAL OF ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE CULTIVARS OF *TRITICUM AESTIVUM* L.[4]

5.1 Introduction

Several studies using excised roots of *Triticum aestivum* have failed to demonstrate differences in kinetics of Al uptake between Al-tolerant and Al-sensitive cultivars under normal metabolic conditions (Pettersson and Strid 1989; Zhang and Taylor 1989; see Chapter 3), thus Pettersson and Strid (1989) suggested that Al tolerance was not linked to the initial uptake of Al. Zhang and Taylor (1989; see Chapter 3), however, reported that DNP increased uptake of Al in Al-tolerant cultivars and suggested that active exclusion of Al may occur in these cultivars. In both of these studies, the linear phase of uptake was used as a measure of transport of Al across the plasma membrane. However, in Chapter 4, I demonstrated that the linear phase of uptake also includes a cell wall component.

The failure to observe differences in the kinetics of Al uptake between Al-tolerant and Al-sensitive cultivars might be because the proposed exclusion mechanism(s) of Al tolerance does not exist. On the other hand, differences between cultivars in the rate of Al uptake across the plasma membrane could be obscured by the linear phase of Al accumulation in the cell wall. This latter alternative seems possible. If transport of Al

[4] A version of this chapter has been submitted for publication to a refereed scientific journal. Zhang, G., and Taylor, G. J. 1989. Effects of biological inhibitors on kinetics of aluminum uptake by excised roots of aluminum-tolerant and aluminum-sensitive cultivars of *Triticum aestivum* L. J. Plant Physiol.

across the membrane is reduced by selective permeability of the plasma membrane, then a localized build up of Al at the membrane surface could promote precipitation or polymerization of Al in the cell wall. Thus conditions which favour reduced membrane transport would favour accumulation of Al in the cell wall. Also, if half of Al uptake in the linear phase represents accumulation of Al by the cell wall fraction (Zhang and Taylor 1990; see Chapter 4), the differences in the rate of Al uptake across the plasma membrane might be difficult to estimate by the linear phase of uptake. Only large differences in the rate of uptake across the plasma membrane would be observed as changes in the rate of the linear phase of uptake. Under these circumstances, kinetic studies investigating the effect of a variety of metabolic inhibitors on Al uptake may be useful to reveal mechanisms of exclusion of Al from the symplasm. Inhibitors may intensify differences in the rate of Al uptake between Al-tolerant and Al-sensitive cultivars by inhibiting specific metabolic processes. In addition, kinetic studies in conjunction with fractionation techniques may help to determine if differences in the kinetics of Al uptake reflect metabolic exclusion from the cytosol.

In this research, kinetics of Al uptake by excised roots and cell wall material of Al-tolerant and Al-sensitive cultivars of *Triticum aestivum* were investigated with the uncoupler of oxidative phosphorylation, DNP, the channel-forming ionophore, gramicidin, the protein synthesis inhibitors, cycloheximide and chloramphenicol, and anaerobiosis (N_2). Possible mechanisms of Al tolerance were discussed.

5.2 Methods

Preparation of plant material. Seeds of two Al-tolerant cultivars (Atlas 66 and PT741) and two Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum* L. (wheat) were surface sterilized in 1.2% sodium hypochlorite for 20 minutes, and germinated overnight in a solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were grown for 7 days on nylon mesh suspended over 16 liters of nutrient solution containing (mM) 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM) 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.5) in a growth chamber with 16 hours of light (20°C, 68% relative humidity) and 8 hours of darkness (16°C, 85% relative humidity). The photosynthetic photon flux density (PPFD) was 335 ± 12 μmol m^{-2} s^{-1} at plant base level. After 5 days of growth, plants were transferred to fresh nutrient solutions.

Uptake of Al by Excised Roots. Thirty root tips (2.0 cm) were excised and placed in each of 32 to 50 "absorption tubes" as described by Zhang and Taylor (1989; see Chapter 3). During excision of roots, absorption tubes containing excised roots were placed in an aerated nutrient solution. When excision was complete (within 60 minutes), the tubes were transferred to an aerated solution of 1.0 mM $CaSO_4$ for 30 minutes. Uptake experiments were initiated by transferring the absorption tubes containing roots to 80 ml glass jars containing 50 ml of an aerated solution of 75 μM Al as $AlK(SO_4)_2.12H_2O$ and 1.0 mM $CaSO_4$, with or without 10 μM gramicidin-D, 0.1 mM DNP, 1.0 mM cycloheximide or 0.5 mM chloramphenicol (pH 4.5) in a water bath at 23°C. In the anaerobic treatment, solutions were bubbled with N_2 instead of air. Five replicate tubes were removed from absorption solutions after 0, 15, 30, 60, 120, and 180 minutes of uptake, rinsed briefly with 1.0 mM $CaSO_4$ and deionized water (300 ml per

tube), and transferred to 0.5 mM citric acid (pH 4.5) at 0°C for 30 minutes to remove loosely bound Al from the apoplasm. After 30 minutes of desorption, roots were removed, rinsed with deionized water, and prepared for isolation of cell wall material or determination of Al.

The composition of the absorption solutions was the same as described in Chapter 3, thus Al will be present primarily as the $AlSO_4$ ion pair (22 μM), $Al^{3+}\cdot6H_2O$ (18 μM), a number of less abundant monomeric species, and possibly a polynuclear species. Again, it is important to note that speciation calculations apply only to the bulk phase of absorption solutions. Because of the unique physical and chemical properties of the apoplasm, the actual species which are in direct contact with the cell wall and plasma membrane are not known.

Pretreatment with Protein Synthesis Inhibitors. Cycloheximide (1.0 mM) and chloramphenicol (0.5 mM) were added to nutrient solutions in which plants were grown. After 0, 0.5, 1, 2, 4, 8, 12, and 24 hours, plants were removed from the solution. Root tips were then excised and placed in an aerated absorption solution containing 75 μM Al and 1.0 mM $CaSO_4$ with either 1.0 mM cycloheximide or 0.5 mM chloramphenicol (pH 4.5, 23°C). After two hours, roots were removed from the absorption solution and desorbed in an aerated 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Analysis of Al uptake showed that there were no differences in the kinetics of Al uptake between the various pretreatment periods (data not shown). Thus, the results of the experiment reported here reflect uptake without pretreatment with cycloheximide and chloramphenicol.

Isolation of Cell Wall Material. Cell wall material was isolated as described by Zhang

and Taylor (1990; see Chapter 4). After absorption and desorption treatments, roots were blotted, weighed, cut into 1 mm long segments and stored on ice. Root segments were homogenized in 1.5 ml 0.1 M Hepes-Mes and 0.3 M sucrose buffer (pH 7.8) for 10 seconds, and placed in a Parr cell disruption bomb under nitrogen pressure (110 kg/cm^2) for 10 minutes. After extrusion to atmospheric pressure, the homogenate was sonicated in an ice-water bath for 7 minutes at 60% output control on a 25 watt ultrasonic homogenizer. The homogenate was then filtered through a 20 μm nylon mesh. Cell wall material trapped on the mesh was rinsed with 50 ml cold deionized water (4°C), and collected for determination of Al. Analysis of total ATPase and cytochrome c oxidase (cytosolic markers) activity showed that cell wall material isolated in this procedure was free of cytosolic contamination (Zhang and Taylor 1990; see Chapter 4).

Adsorpti[illegible]ated Cell Wall Material. Adsorption of Al by purified cell wall material was [illegible] according to the methods of Zhang and Taylor (1990; see Chapter 4). Purified cell wall material from excised roots with no prior exposure to Al was isolated as described above. During the fractionation procedures, the cell wall material was suspended in 15 ml centrifuge tubes containing 5 ml 1.0 mM $CaSO_4$ (pH 4.5) in an ice-water bath. Before the adsorption treatment, the cell wall material was brought to the absorption temperature (23°C). The adsorption period was initiated by adding 5 ml of a solution containing 1.0 mM CaSO4 and 150 μM Al with or without 0.2 mM DNP (pH 4.5). This brought the final concentration of Al and DNP (if added) to 75 μM and 0.1 mM respectively. After 30 and 120 minutes of absorption, the cell wall material from 3 replicate tubes of each treatment was trapped on nylon mesh, washed with 50 ml cold deionized water (4°C), and desorbed in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes as described above. At the end of desorption, the cell wall material was,

again, trapped on nylon mesh, washed with 50 ml cold deionized water, and then collected and prepared for determination of Al.

Determination of Al. Roots and cell wall material were dried, and ashed at 500°C, dissolved in concentrated HNO_3 and oxidized with H_2O_2. Aluminum concentrations were determined by graphite furnace atomic absorption spectrophotometry as described by Zhang and Taylor (1989; see Chapter 4). Concentrations were calculated by integration of peak area, and expressed as micrograms of Al per gram fresh weight of roots (µg Al g root fw^{-1}).

Analysis of Data. Statistical analyses of the data were performed using analysis of variance (ANOVA), simple regression, and descriptive statistics available on Statistical Graphics Corporation's statistical package, Statgraphics Version 2.6. Analyses of homogeneity of slopes were performed using ANOVA available in SAS release 5.18. Significance was defined at the 95% confidence level.

5.3 Results

Patterns of Al uptake which distinguished between excised roots of Al-tolerant and Al-sensitive cultivars were observed with DNP, the uncoupler of oxidative phosphorylation (Fig. 5-1, A - D). When roots were exposed to 75 µM Al with DNP, rates of Al uptake by Al-tolerant cultivars were increased by 51.2% in Atlas 66 (from 2.06 ± 0.08 to 3.13 ± 0.13 µg Al g^{-1} min^{-1}) and by 73.1% in PT741 (from 1.75 ± 0.08 to 3.03 ± 0.11 µg Al g^{-1} min^{-1}) compared to control. An increase in the rate of uptake was also observed in the Al-sensitive cultivars; however, rates of Al uptake were increased

by only 7.0% in Neepawa (from 1.86 ± 0.12 to 1.99 ± 0.14 μg Al g^{-1} min^{-1}), and by 24.7% in Scout 66 (from 1.50 ± 0.08 to 1.87 ± 0.09 μg Al g^{-1} min^{-1}). The increase in Neepawa was not statistically significant. The different response of Al-tolerant and Al-sensitive cultivars to DNP treatment suggests that metabolically active exclusion of Al may play a role in determining tolerance to Al.

Patterns of Al uptake which distinguished between excised roots of Al-tolerant and Al-sensitive cultivars were also observed with the channel-forming ionophore gramicidin (Fig. 5-1, E - H). When roots were treated with gramicidin, rates of Al uptake were increased by 60.8% and 22.0% in the Al-sensitive cultivars Neepawa (from 1.89 ± 0.16 to 3.04 ± 0.28 μg Al g^{-1} min^{-1}) and Scout 66 (from 1.50 ± 0.14 to 1.83 ± 0.15 μg Al g^{-1} min^{-1}). In contrast, rates of Al uptake by the Al-tolerant cultivars Atlas 66 and PT741 were not affected. Rates of Al uptake with and without gramicidin were 2.01 ± 0.10 and 2.01 ± 0.18 μg Al g^{-1} min^{-1} for Atlas 66, and 1.39 ± 0.09 and 1.34 ± 0.10 μg Al g^{-1} min^{-1} for PT741 respectively (Fig. 5-1, E - H).

As suggested by Zhang and Taylor (1989; see Chapter 4), the increased uptake of Al in Al-tolerant cultivars when treated with DNP could reflect the operation of an Al efflux pump and/or enhanced chelate efflux. The continued operation of such Al efflux pump or enhanced chelate efflux system in the presence of gramicidin might then account for the failure of gramicidin to stimulate uptake of Al in Al-tolerant cultivars. To test this hypothesis, the effect of DNP and gramicidin in combination was also investigated. If these efflux systems are shut down by DNP, then increased uptake by the gramicidin in Al-tolerant cultivars may become visible. In these experiments, synergistic effects of DNP and gramicidin on Al uptake were observed in Al-tolerant cultivars, while multiplicative effects were observed in Al-sensitive cultivars. Rates of Al

uptake were increased by 142% in Atlas 66 (from 2.28 ± 0.07 to 5.52 ± 0.28 μg Al g^{-1} min^{-1}), and by 138% in PT741 (from 1.11 ± 0.09 to 2.64 ± 0.19 μg Al g^{-1} min^{-1}). In contrast, rates of Al uptake with gramicidin and DNP were increased by 61% in Neepawa (from 1.48 ± 0.13 to 2.38 ± 0.20 μg Al g^{-1} min^{-1}), and by 48% in Scout 66 (from 1.82 ± 0.09 to 2.69 ± 0.14 μg Al g^{-1} min^{-1}) (Fig. 5-1, I - L). While the stimulation of Al uptake observed in Al-sensitive cultivars was similar to that expected on the basis of treatment with DNP and gramicidin alone, the stimulation of uptake in Al-tolerant cultivars was greater than expected (Table 5-1). The synergistic effects of DNP and gramicidin on the rate of Al uptake in Al-tolerant cultivars are consistent with the operation of an Al efflux pump and/or a chelate efflux system in Al-tolerant cultivars and, once again, suggest that the efflux is energy-dependent.

Experiments with cycloheximide, chloramphenicol and anaerobiosis were carried out to determine if kinetics of Al uptake were sensitive to other metabolic inhibitors. Treatment with cycloheximide decreased rates of Al uptake in Al-sensitive cultivars by 24% in Neepawa (from 1.81 ± 0.15 to 1.38 ± 0.10 mg Al g-1 min-1), and by 29% in Scout 66 (from 1.57 + 0.14 to 1.11 + 0.14 mg Al g-1 min-1). In contrast, rates of Al uptake were decreased by 8% and 5% in the Al-tolerant cultivars Atlas 66 (from 2.21 + 0.15 to 2.05 + 0.18 mg Al g-1 min-1) and PT741 (from 1.35 + 0.11 to 1.29 + 0.10 mg Al g-1 min-1) (Fig. 5-2, A - D). The differences in the Al-tolerant cultivars were not statistically significant. Rates of Al uptake in roots treated with the prokaryotic protein synthesis inhibitor chloramphenicol were not significantly different than control in both Al-tolerant and Al-sensitive cultivars (Fig. 5-2, E - H). Under anaerobic conditions, rates of Al uptake by excised roots were increased to a similar extent in both Al-tolerant and Al-sensitive cultivars. In Al-sensitive cultivars, uptake of Al was increased by 61% in Neepawa (from 1.30 + 0.07 to 2.09 + 0.17 mg Al g-1 min-1) and 26% in Scout 66

(from 1.29 + 0.10 to 1.62 + 0.10 mg Al g-1 min-1). In Al-tolerant cultivars, uptake of Al was increased by 22% in Atlas 66 (from 1.71 + 0.15 to 2.08 + 0.14 mg Al g-1 min-1), and 31% in PT741 (from 1.21 + 0.04 to 1.59 + 0.08 mg Al g-1 min-1) (Fig. 5-2, I - L).

My experiments with excised roots demonstrated that treatment with DNP, gramicidin, and DNP in conjunction with gramicidin had different effects in Al-tolerant and Al-sensitive cultivars. Because the linear phase of uptake appears to include a cell wall component (Zhang and Taylor 1990; see Chapter 4), experiments investigating the effects of these compounds on the adsorption of Al onto purified cell wall material were also performed. In these experiments, Al was supplied to excised roots both in the presence and absence of the biological inhibitors. At the end of adsorption periods, cell wall material was isolated from the excised roots. These experiments demonstrated an increase in rates of Al adsorption in cell wall fractions from both Al-tolerant and Al-sensitive cultivars when treated with DNP (Table 5-2). Compared to the control, no significant differences in rates of Al adsorption in cell wall fractions were observed with the channel-forming ionophore gramicidin in both Al-tolerant and Al-sensitive cultivars (Table 5-3). Gramicidin had no additional effect on Al adsorption in the presence of DNP (Table 5-4).

Exclusion of Al at the plasma membrane could increase concentration of Al at the membrane surface, which might promote precipitation or polymerization of Al in the cell wall. Alternatively, by inhibiting the plasma membrane proton pump, treatment with DNP could affect the pH of the cell wall free space which would affect the speciation and solubility of Al as well as its binding to the cell wall. In either case, the effect of DNP on Al uptake into purified cell wall material would depend on continued cellular integrity. To test this hypothesis, the effect of DNP on *in vitro* adsorption of Al

into cell wall material was investigated. In contrast to *in vivo* uptake experiments, *in vitro* adsorption of Al by purified cell wall material did not show a linear phase, and differences were not observed between treatments with and without DNP (Table 5-5). These results suggest that continued integrity of the cell or plasma membrane is required to maintain a linear adsorption of Al in the cell wall fraction.

5.4 Discussion

Increased uptake of Al in roots treated with DNP has been reported in *Triticum aestivum* and several other species (Huett and Menary 1979; Pettersson and Strid 1989; Zhang and Taylor 1989; see Chapter 3). While Huett and Menary (1979) suggested that DNP increased permeability of the plasma membrane to Al, Zhang and Taylor (1989; see Chapter 3) suggested that increased rates of Al uptake by excised roots of Al-tolerant cultivars treated with DNP might reflect disruption of an exclusion mechanism which operates under normal metabolic conditions. Metabolic exclusion of Al could be achieved by active efflux of Al into the apoplasm. While experimental evidence supporting the operation of metal efflux pumps has not been obtained in higher plants, energy-dependent efflux systems for metal cations have been cloned from plasmid genes and introduced into bacterial strains (Tynecka *et al.* 1981; Sensfuss and Schlegel 1986; Nies and Silver 1989; Nies *et al.* 1989; Nucifora *et al.* 1989). These efflux systems are driven by ATP or by a transmembrane gradient and are inhibited by low temperature and DNP (Nies and Silver 1989). Analogous mechanisms of exclusion may operate in higher plants and the relative effectiveness of the efflux pump could account for differential tolerance to Al. Unfortunately, the precise effect of DNP on plants is not well established, DNP is reported to uncouple oxidative phosphorylation, impair membrane

structure and permeability, and destroy the proton gradient across the plasma membrane (Huett and Menary 1979; Wagatsuma 1983; Zhang and Taylor 1989; see Chapter 3). Thus, the way which DNP affected uptake of Al in Al-tolerant and Al-sensitive cultivars has not been identified.

Metabolism-dependent exclusion of Al could also be achieved by enhanced efflux of chelate ligands such as citric or tartaric acid into the cell wall space of Al-tolerant cultivars. Such efflux could resemble chelate efflux systems which have been described in a number of species in response to phosphate deficiency (Gardner *et al.* 1981, 1983; Gardner and Parbery 1981, 1982; Koyama *et al.* 1988) and Fe deficiency (Ohfune *et al.* 1981; Sugiura *et al.* 1981; Ripperger *et al.* 1982; Mino *et al.* 1983; Takagi *et al.* 1984). Formation of Al chelate complexes in the apoplasm may decrease the activity of monomeric Al species in the apoplasm, as well as at the surface of the plasma membrane. This in turn would decrease the influx of Al. The driving force for efflux of chelate ligands may be the membrane potential which depends on normal metabolic processes, especially supply of ATP. Thus, nonmetabolic conditions (DNP treatment) could inhibit the chelate efflux.

Differences in the effects of the channel-forming ionophore gramicidin between Al-tolerant and Al-sensitive cultivars were also consistent with an active exclusion mechanism in Al-tolerant cultivars of *Triticum aestivum*. Gramicidin facilitates transport of protons (H^+) and monovalent cations (K^+, Cs^+, Rb^+, Na^+, Li^+, and NH_4^+) through the formation of transmembrane aqueous channels (4×10^{-1} nm in diameter) (Hodges *et al.* 1971; Gomez-Puyou and Gomez-Lojero 1977; Riedell and Schmid 1986) with a poor selectivity (Nicholls 1982). The extent to which trivalent cations cross the plasma membrane through these channel-forming ionophores is unknown. The radius

of Al^{3+} (0.5 x 10^{-1} nm) is smaller than those of Cs^{+} (1.69 x 10^{-1} nm), Rb^{+} (1.48 x 10^{-1} nm), K^{+} (1.33 x 10^{-1} nm), Na^{+} (0.95 x 10^{-1} nm) and Li^{+} (0.60 x 10^{-1} nm), however, the strong charge density of Al^{3+} may hinder transport across the plasma membrane due to hydration or interaction with negatively charged membrane radicals. Divalent hydroxy Al ($Al(OH)^{2+}$) and monovalent hydroxy Al ($Al(OH)_2^{+}$) were also present in the absorption solutions. Although both ($Al(OH)^{2+}$) and $Al(OH)_2^{+}$ have a lower charge than Al^{3+}, their hydrated species may still be too large to pass through the gramicidin channel. On the other hand, the facilitated uptake of Al across the plasma membrane by gramicidin could be achieved by enhanced transport through calcium channels or other nonspecific channels. Gramicidin has been shown to stimulate the activity of proton-translocating ATPases in membrane vesicles which would maintain the electrochemical gradient across the membrane (Sze 1985). A similar stimulation of the activity of proton-translocating ATPases was also reported *in vivo* by Maier and Graham (1988). They reported that gramicidin increased uptake of molybdate (MoO_4^{2-}) in *Bradyrhizobium japonicum* which has been shown to be driven by a proton gradient, not membrane potential. Installation of gramicidin channels in the plasma membrane may increase the driving force for Al transport, and in turn enhance Al transport down the electrochemical gradient. This could account for the observed increases in the rates of Al uptake into excised roots in Al-sensitive cultivars.

In contrast to results with Al-sensitive cultivars, the rate of Al uptake in Al-tolerant cultivars was unaffected by gramicidin. As I have suggested, the effect of gramicidin on Al-tolerant cultivars may be masked by continued operation of active efflux of Al or chelate efflux system. If this is true, the presence and efficiency of these efflux systems will affect the net rate of Al uptake in the presence of gramicidin. For instance, if Al-tolerant cultivars possess an Al efflux system, gramicidin-induced uptake

may be obscured by active efflux of Al. Chelate efflux could also limit the rate of membrane transport in the presence of gramicidin. Aluminum chelate complexes, by virtue of their size may be unable to pass through the gramicidin channels.

The synergistic effects of DNP and gramicidin on Al uptake in Al-tolerant cultivars provide additional evidence supporting an Al efflux pump and/or chelate efflux system. I have hypothesized that a pronounced increase in the rate of Al uptake should occur in Al-tolerant cultivars in the presence of gramicidin when the putative efflux system is shut down by DNP. When excised roots of Al-tolerant cultivars were exposed to Al with gramicidin and DNP, uptake of Al was increased synergistically. In contrast, the combined effect of gramicidin and DNP was essentially multiplicative in Al-sensitive cultivars. Thus, these results again support the existence of Al efflux pump and/or chelate efflux system in Al-tolerant cultivars, and suggest that these efflux systems may be relatively ineffective in Al-sensitive cultivars.

No differences were observed in the rate of Al uptake between treatments with and without the prokaryotic protein synthesis inhibitor chloramphenicol in both Al-tolerant and Al-sensitive cultivars. Thus, mitochondrion-encoded gene products would seem to be of little importance in determining short-term patterns of Al uptake. The effects of the eukaryotic protein synthesis inhibitor cycloheximide were relatively minor. While uptake of Al was not significantly affected by cycloheximide in Al-tolerant cultivars, a decreased rate of Al uptake was observed in Al-sensitive cultivars treated with cycloheximide. This might suggest that Al crosses the plasma membrane in proteinaceous regions which are encoded by nuclear genes. The electropositive charge of mononuclear Al species would of course favour transport across the membrane in proteinaceous regions, however, this is not *a priori* reason to expect that the membrane

mobile species is a charged ion. Protein-associated channels formed by H_{II} type phospholipids have also been suggested to provide a pathway for transport of Al across the plasma membrane (Haug 1984). In a previous study, cycloheximide increased accumulation of Al in root tips of Atlas 66 (Aniol 1984). However, long-term treatment with the protein synthesis inhibitor (up to 36 hours), high concentrations of Al (1.0 mM), low pH (4.0), and lack of desorption procedures at the end of experiments made it impossible to compare these results with my own.

Increased rates of Al uptake under anaerobic conditions (N_2) could result from increased permeability of the plasma membrane (Wagatsuma 1983). Since anaerobiosis has multiple effects on metabolism, it is not clear how anaerobic conditions act on the kinetics of Al uptake. Patterns of uptake distinguishing between Al-tolerant and Al-sensitive cultivars were not found in the present study, although higher resistance to anaerobic conditions was reported in Al-tolerant plants of other species (Wagatsuma 1983). Increased uptake of Al was also observed under anaerobic conditions in *Hordeum vulgare*, *Arictium lappa*, *Pisum sativum*, *Raphanus sativus* and *Spinacia oleracea*. In contrast, uptake of Al in *Oryza sativa*, *Asparagus officinalis*, *Cucumis sativus* and *Lagenaria sicerarla* was not affected by anaerobic treatment (Wagatsuma 1983).

My experiments with excised roots demonstrated that treatment with DNP, gramicidin, and DNP in conjunction with gramicidin had different effects in Al-tolerant and Al-sensitive cultivars. Because the linear phase of uptake appears to include a cell wall component (Zhang and Taylor 1990; see Chapter 4), experiments investigating the effects of these compounds on Al adsorption onto the purified cell wall material were also performed. Although the mechanism of binding of Al in the cell wall is not yet

clear, the nature of this binding is metabolism-dependent (Zhang and Taylor 1990; see Chapter 4). Furthermore, as my results suggested, this binding is sensitive to DNP. A DNP-induced accumulation of Al in the cell wall fraction could reflect immobilization of Al in the cell wall as a result of a DNP-induced change in pH of the apoplasm. Humphreys (1975) found that DNP induced proton influx across the plasma membrane and caused an increase in the pH of bathing solution of *Zea mays* scutellum from 3.9 to 5.5 within 30 minutes. An increase in apoplasmic pH could result in a change of Al speciation, a decrease in mobility, and hence, precipitation of Al in the cell wall. Localized changes in the concentration of Al at the plasma membrane surface could also promote polymerization or precipitation of Al as a result of exclusion at the plasma membrane (Matsumoto *et al.* 1977; Wagatsuma and Yamasaku 1985).

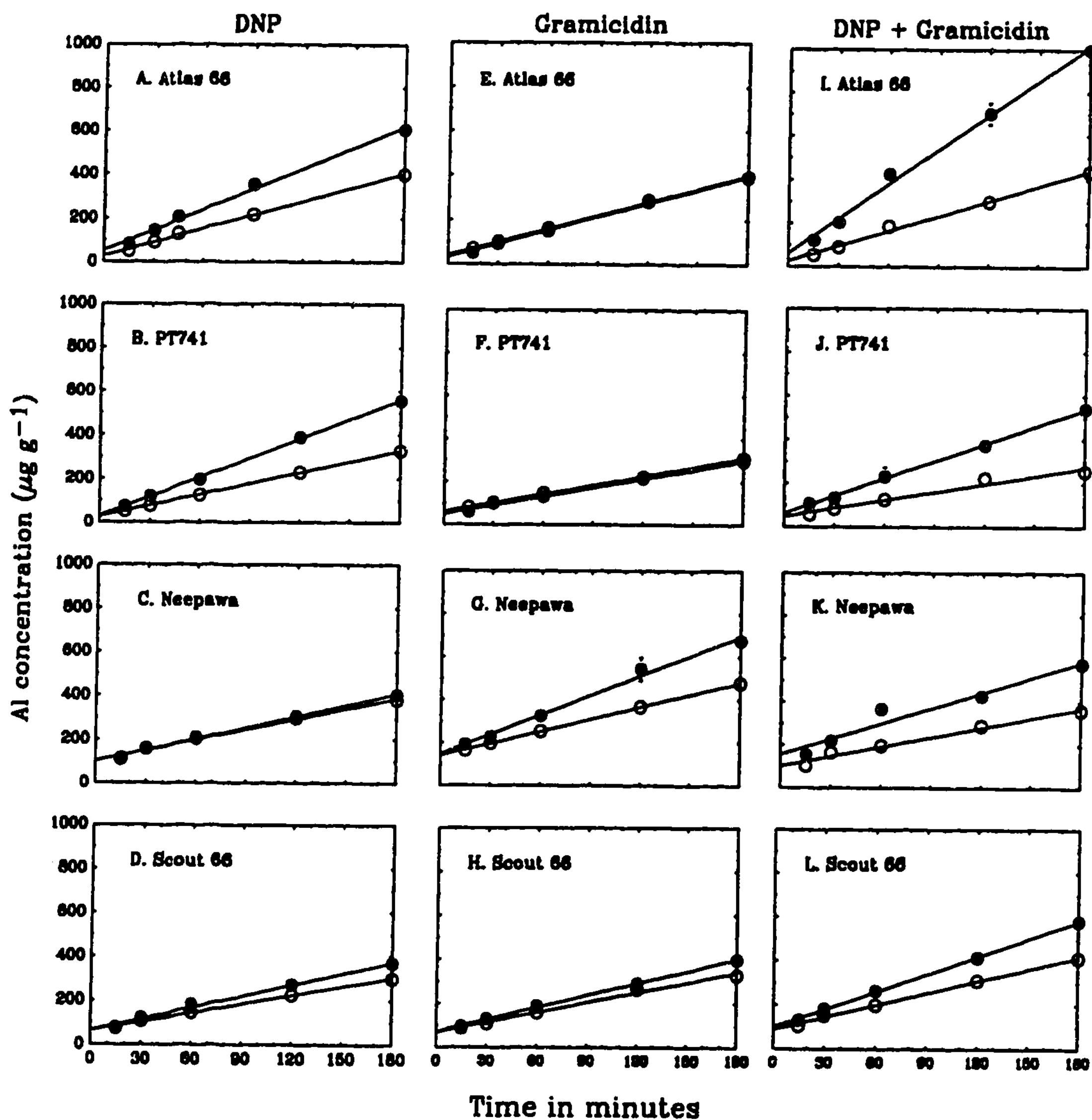
The question of how DNP-induced accumulation in cell wall material relates to the linear phase of uptake in the cell wall fraction is still not clear. However, increased accumulation of Al in the cell wall fraction can not entirely account for the DNP-induced stimulation of uptake in excised roots. This conclusion is supported by several lines of evidence. For example, increased uptake of Al was also found in the cell content fraction (data not shown). Furthermore, increased uptake which was observed in excised roots of Al-sensitive cultivars treated with gramicidin was not observed in the purified cell wall fractions. The additional effect of gramicidin (when supplied with DNP) on uptake in both Al-tolerant and Al-sensitive cultivars was also not observed in purified cell wall fractions. The failure to observe effects of gramicidin in cell wall fractions suggests that the effects of gramicidin on excised roots must be partially attributed to cell contents.

In contrast to *in vivo* accumulation of Al into cell wall material, a linear

accumulation of Al with time was not found in cell wall fractions treated with Al and DNP *in vitro*. These results were consistent with my previous report on the adsorption of Al by isolated cell wall material, and suggests that DNP-induced accumulation of Al in the cell wall fraction depends on the existence of the functional plasma membrane and/or the cell integrity.

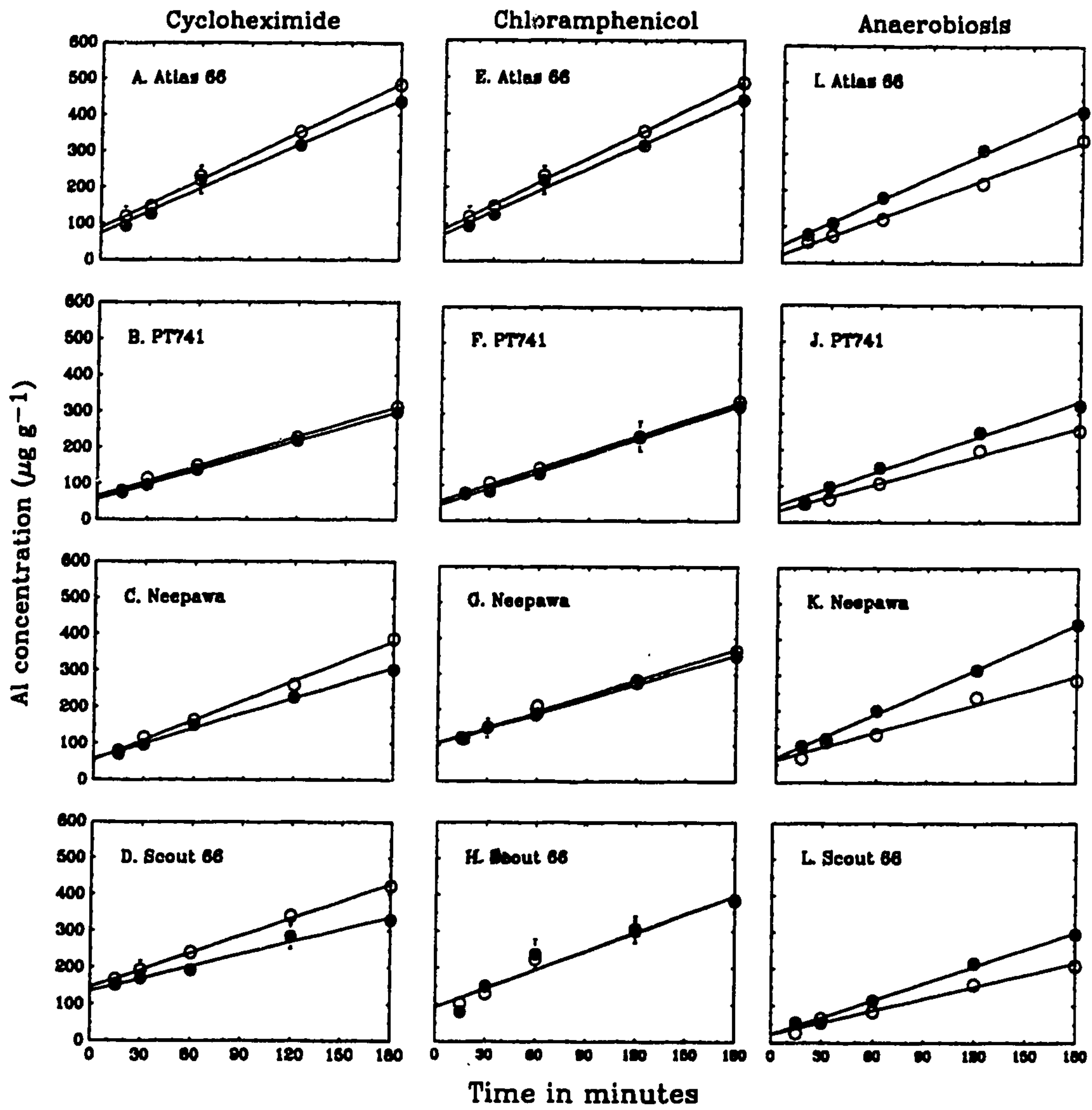
In conclusion, the effects of biological inhibitors on kinetics of Al uptake by excised roots and purified cell wall material of Al-tolerant and Al-sensitive cultivars of *Triticum aestivum* provide experimental evidence consistent with the operation of an Al efflux pump and/or chelate efflux system in the roots of Al-tolerant cultivars. In the absence of metabolic inhibitors, operation of this pump may be obscured by tight binding of Al to cell wall material. Accumulation of Al in the cell wall may result from precipitation or polymerization in the apoplasm which is sensitive to metabolic conditions (with or without DNP). This accumulation, however, depends on the existence of the functional plasma membrane and/or cell integrity. Further experiments are required to provide more substantial evidence for metabolic exclusion of Al from the cytosol.

Figure 5-1. Effects of DNP, gramicidin, and DNP plus gramicidin on uptake of Al (μg g root dw^{-1}) by excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum*. Excised roots were treated with an aerated solution containing 75 μM Al and 1.0 mM Ca, with (●) or without (O) 0.1 mM DNP (A - D), 10 μM gramicidin (E - H), or DNP plus gramicidin (I - L). Absorption at pH 4.5 and $23^{o}C$ for 0, 15, 30, 60, 120 and 180 minutes was followed by desorption in 0.5 mM citric acid (pH 4.5, $0^{o}C$) for 30 minutes. Values represent means of 5 replicates.



DNP
Gramicidin
DNP + Gramicidin
A. Atlas 66
E. Atlas 66
I. Atlas 66
B. PT741
F. PT741
J. PT741
C. Neepawa
G. Neepawa
K. Neepawa
D. Scout 66
H. Scout 66
L. Scout 66
Al concentration ($\mu g\ g^{-1}$)
Time in minutes
0
200
400
600
800
1000
0
30
60
90
120
150
180

Figure 5-2. Effects of cycloheximide, chloramphenicol, and anaerobiosis on uptake of Al (μg g root dw^{-1}) by excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of *Triticum aestivum*. Excised roots were treated with absorption solution containing 75 μM Al and 1.0 mM Ca with (●) or without (O) 1.0 mM cychloheximide, 0.5 mM chloramphenicol (aerated), or anaerobiosis (N_2). Absorption at pH 4.5 and 23°C for 0, 15, 30, 60, 120 and 180 minutes was followed by desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 5 replicates.



Cycloheximide
Chloramphenicol
Anaerobiosis
A. Atlas 66
B. PT741
C. Neepawa
D. Scout 66
E. Atlas 66
F. PT741
G. Neepawa
H. Scout 66
I. Atlas 66
J. PT741
K. Neepawa
L. Scout 66
Al concentration (μg g^{-1})
Time in minutes

Table 5-1. *Effects of DNP, gramicidin, and DNP plus gramicidin on uptake of Al by excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Excised roots were treated with an absorption solution containing 75 µM Al and 1.0 Ca (pH 4.5, 23°C) with or without 0.1 mM DNP, 10 µM gramicidin, or DNP plus gramicidin, followed by desorption treatment with 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent uptake relative to the control (1.0).

Cultivars	DNP	Gramicidin	DNP + Gramicidin	
			Expected	Observed
Atlas 66	1.52	1.00	1.52	2.42
PT741	1.73	1.00	1.73	2.38
Neepawa	1.07	1.61	1.73	1.61
Scout 66	1.25	1.22	1.53	1.48

Table 5-2. *Effect of DNP on in vivo adsorption of Al (μg g root fw^{-1} min^{-1}) onto purified cell wall material isolated from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Cell wall material was isolated from excised roots pretreated with 75 μM Al and 1.0 mM Ca (pH 4.5, 23°C) with or without 0.1 mM DNP for 0, 15, 30, 60, 120 and 180 minutes followed by desorption treatment in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 4 replicates.

	Rate of Al adsorption		DNP Effect	
Cultivars	Control	DNP	% of control	*P*
Atlas 66	0.25 ± 0.09	1.07 ± 0.10	428	0.0001
PT741	0.25 ± 0.04	1.81 ± 0.08	724	0.0001
Neepawa	0.63 ± 0.03	1.87 ± 0.12	297	0.0001
Scout 66	0.61 ± 0.15	1.46 ± 0.20	239	0.0014

Table 5-3. *Effect of gramicidin on in vivo adsorption of Al (µg g root fw^{-1} min^{-1}) onto purified cell wall material isolated from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Cell wall material was isolated from excised roots pretreated with 75 µM Al and 1.0 mM Ca (pH 4.5, 23°C) with or without 10 µM gramicidin for 0, 15, 30, 60, 120 and 180 minutes followed by desorption treatment in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 4 replicates.

	Rate of Al adsorption		Gramicidin Effect	
Cultivars	Control	Gramicidin	% of control	*P*
Atlas 66	0.25 ± 0.09	0.34 ± 0.11	136	0.5356
PT741	0.37 ± 0.11	0.50 ± 0.11	135	0.4168
Neepawa	0.49 ± 0.08	0.54 ± 0.08	110	0.6538
Scout 66	0.51 ± 0.28	0.51 ± 0.19	100	0.4930

Table 5-4. *Effect of DNP and gramicidin on in vivo adsorption of Al (µg g root fw^{-1} min^{-1}) onto purified cell wall material isolated from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Cell wall material was isolated from excised roots pretreated with 75 µM Al, 1.0 mM Ca and 0.1 mM DNP (pH 4.5, 23°C), with or without 10 µM gramicidin for 0, 15, 30, 60, 120 and 180 minutes followed by desorption treatment in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Values represent means of 4 replicates.

	Rate of Al adsorption		Gramicidin Effect	
Cultivars	DNP	DNP+Gramicidin	% of DNP	*P*
Atlas 66	1.43 ± 0.22	1.13 ± 0.18	79	0.2513
PT741	2.45 ± 0.15	2.76 ± 0.19	112	0.2170
Neepawa	1.96 ± 0.37	1.59 ± 0.24	81	0.3398
Scout 66	1.35 ± 0.08	1.54 ± 0.09	114	0.3787

Table 5-5. *Effect of DNP on in vitro adsorption of Al (µg g root fw^{-1} min^{-1}) by purified cell wall material isolated from excised roots of Al-tolerant cultivars (Atlas 66 and PT741) and Al-sensitive cultivars (Neepawa and Scout 66) of Triticum aestivum.* Purified cell wall isolated from excised roots was treated with 75 µM Al and 1.0 mM Ca (pH 4.5, 23°C) with or without 0.1 mM DNP for 30 and 120 minutes, followed by desorption treatment in 0.5 mM citric acid (pH 4.5, 0°C) for 30 minutes. Rates of Al uptake for both control and DNP treatment in all cultivars are not statistically greater than zero. Values represent means of 3 replicates.

Cultivars	Control	DNP
Atlas 66	0.40 ± 0.56	0.57 ± 0.37
PT741	0.06 ± 0.28	0.55 ± 0.65
Neepawa	0.07 ± 0.41	0.04 ± 0.84
Scout 66	0.47 ± 0.60	0.41 ± 0.82

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6. CONCLUDING DISCUSSION

In the experiments represented here, distinctive patterns of growth response to Al were observed between roots of Al-tolerant and Al-sensitive cultivars. Roots of Atlas 66 grew as well as control at 200 μM Al in solution, a concentration which reduced root growth of the Al-sensitive cultivar Scout 66 to 38% of the control. Furthermore, the relationship between relative growth and internal concentration of Al showed that roots of the Al-tolerant cultivar Atlas 66 maintained greater growth than the Al-sensitive cultivar Scout 66, despite higher accumulation of Al. In addition, most of the Al absorbed by experimental plants (>95%) was accumulated in roots, and little (<5%) was translocated to the shoots. In contrast to the effect of Al on roots, the patterns of growth response to Al between leaves of Al-tolerant and Al-sensitive cultivars were similar. These results suggest that roots, which are in direct contact with the toxic substrate (Al), are most critical in determining susceptibility or tolerance to Al and that studies on the mechanisms of Al tolerance should focus on the physiological and biochemical responses of roots to Al.

Short-term studies on Al uptake characterized the kinetics of Al uptake in *Triticum aestivum*. Kinetics of Al uptake by excised roots was biphasic, with a rapid phase in the first 30 minutes followed by a linear phase. The rapid phase was removeable by desorption in citric acid, a ligand capable of forming stable complexes with Al. This removeable nature suggested that the rapid phase was primarily located in the apoplasm. Estimation of Al in this compartment by extrapolation of the linear phase of uptake and desorption to time zero showed that less than 50% of total absorbed Al was accumulated in the apoplasm. Investigation of the linear phase under

metabolic conditions showed no distinction between Al uptake by Al-tolerant and Al-sensitive cultivars.

Although the linear phase is classically interpreted as representing the symplasmic compartment, I have questioned the identity of the linear phase. Information concerning the identity of the linear phase is important, since interpretation of experimental results depends on the nature of metabolism-dependent uptake. Different rates of Al uptake across the plasma membrane are expected between Al-tolerant and Al-sensitive cultivars if an exclusion mechanism exists and operates. Failure to observe such differences could arise if the linear phase of Al uptake included an apoplasmic compartment; apoplasmic involvement in the linear phase could obscure the differences in the apparent influx of Al across the plasma membrane. The possibility that the linear phase included both an apoplasmic and symplasmic compartment was first suggested by differences in the estimated size of apoplasmic compartment as determined by extrapolation of linear phase of uptake and desorption to time zero (Zhang and Taylor 1989; see Chapter 4). Also, the reduced rate, but non-saturable phase of uptake at $0^{o}C$ suggested a non-metabolic component of the linear phase of Al uptake. This was later confirmed with kinetic and fractionation studies. These studies showed a linear phase of adsorption in the purified cell wall fraction which was non-removeable by desorption in citric acid. Finally, *in vivo* adsorption of Al demonstrated the metabolism-dependence of the *in vivo* linear phase of Al adsorption in the cell wall fraction.

These experimental observations are inconsistent with the classical interpretation of the identity of the linear phase, and suggest that the linear phase of Al uptake is composed of both symplasmic and apoplasmic compartments. The

mechanism(s) of Al accumulation in the cell wall fraction, however, is (are) not yet clear. Tight binding of Al to cell wall material may result from formation of hydrated Al complexes associated with pectic substances (Wagatsuma 1983) or free carboxyl groups (Clarkson 1967), precipitation or polymerization of absorbed monomeric Al in the cell wall (Matsumoto *et al.* 1977; Wagatsuma and Yamasaku 1985). My work has demonstrated that accumulation of Al in cell wall fraction depends on metabolism and/or cell integrity. Metabolic activity may provide binding ligands for Al or a suitable environment that may promote tight binding to the cell wall. Such binding of Al to the cell wall may play a role in tolerance of Al (Taylor 1988a,b).

Despite difficulty in isolating the symplasmic compartment from the linear phase, kinetic patterns which distinguished between Al-tolerant and Al-sensitive cultivars were observed with DNP and gramicidin. Synergistic effects of DNP and gramicidin on the Al uptake in Al-tolerant cultivars supported the concept of exclusion of Al by an Al efflux pump and/or chelate efflux system in the plasma membrane. Such pump or system could be an important part of an integrated Al tolerance strategy. Under metabolic conditions, facilitated uptake by gramicidin channels could be obscured by an Al efflux pump and/or chelate efflux system, thus, influx in Al-tolerant cultivars might appear to be insensitive to gramicidin. When the putative efflux system was inhibited by DNP, the gramicidin induced greater electric potential across the plasma membrane in Al-tolerant cultivars may have facilitated influx of Al. Thus, uptake of Al was increased synergistically. In contrast, the efflux mechanism in Al-sensitive cultivars may be absent or relatively ineffective. In these cultivars, the effect of gramicidin could be observed in the absence of DNP, and no additional effect would be observed when DNP was added to the absorption solution containing gramicidin. This was observed in the Al-sensitive cultivars tested here.

Thus, these data are consistent with an energy-dependent efflux systems in higher plants. The putative efflux system may be analogous to efflux of metal cations in bacterial systems (Tynecka *et al.* 1981; Sensfuss and Schlegel 1986; Nies and Silver 1989; Nies *et al.* 1989; Nucifora *et al.* 1989). These efflux systems are driven by ATP or by a transmembrane gradient, and are inhibited by low temperature and DNP (Nies and Silver 1989). Alternatively, they could resemble chelate efflux systems which have been described in a number of species in response to phosphate deficiency (Gardner *et al.* 1981, 1983; Gardner and Parbery 1981, 1982; Koyama *et al.* 1988) and Fe deficiency (Ohfune *et al.* 1981; Sugiura *et al.* 1981; Ripperger *et al.* 1982; Mino *et al.* 1983; Takagi *et al.* 1984). However, supporting evidence for the putative Al or chelate efflux system is not conclusive. Although metabolism-dependent accumulation of Al appears to include uptake in both the apoplasm and symplasm, uptake into these two compartments has not been differentiated. Since DNP has multiple effects on plant metabolism, the way which metabolism-dependent accumulation of Al in these compartments relates to the effect of DNP is not clear. Furthermore, the fractionation protocol used in my studies was designed to isolate purified cell wall material; the cell content fraction was less well defined. Thus, uptake of Al across the plasma membrane can not be determined by simply measuring the concentration of Al in the filtrate fractions. Finer fractionation procedures will be required to purify the cell content fraction and give a better estimation of Al uptake across the plasma membrane. The effect of different ionophores or channel formers, such as A-23187 and X-537A which facilitate divalent cation permeability, and fusicoccin which stimulates proton-ATPase activity, inhibitors of plasma membrane ATPases and acid phosphatases, as well as calmodulin antagonists might also be used to investigate the uptake of Al.

Further investigations on several other aspects of the subject would also be useful to help verify my hypothesis. First, if the problem of recalcitrance in protoplast and suspension cultures of *Triticum aestivum* could be solved, direct evidence of exclusion could be obtained by kinetic studies with protoplasts. Such studies could eliminate incorporation of cell wall Al into the linear phase of uptake. As an alternative, *in vitro* uptake studies with plasma membrane vesicles could also be employed to ensure the existence and operation of the efflux system. Here, vesicles with inside out orientation might be used to simplify experimental procedures. If these techniques are not available, finer fractionation and purification procedure would be helpful to identify uptake of Al into the symplasm. Because the ^{26}Al isotope is prohibitively expensive, use of radioactive isotopes of other trivalent cations such as ^{46}Sc and ^{72}Ga may provide useful information on Al transport. Histochemical and cytochemical techniques with sensitive dye methods for Al may also applicable. A combination of some or all of these techniques may provide more information on the exclusion of Al from symplasm.

In conclusion, this research has: 1) differentiated patterns of growth response to Al between Al-tolerant and Al-sensitive cultivars of *Triticum aestivum* (Zhang and Taylor 1988; Chapter 1), 2) characterized the kinetics of Al uptake (Zhang and Taylor 1989; Chapter 2), 3) provided a novel interpretation of identity of the linear phase of Al uptake (Zhang and Taylor 1990a; Chapter 3), and 4) demonstrated differences between Al-tolerant and Al-sensitive cultivars in the kinetics of short-term Al uptake (Zhang and Taylor 1990b; Chapter 5). Further support for the putative Al and/or chelate ligand efflux system which has been postulated in this thesis must await further studies.

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