PROPERTIES OF A PROTEIN ASSOCIATED
WITH METAL TOXICITY
IN WHEAT

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ROCIO CRUZ-ORTEGA

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Thesis Approved:

James D Ownby
Thesis Adviser

Arron Rikin

Andrew Mort

Thomas C. Collins
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Toxic levels of metals such as aluminum, and heavy metals such as copper, cadmium, and zinc, can occur naturally in some soils, or as a result of environmental pollution (Foy et al., 1978). Accumulation of these metals is often accompanied by an increase in soil acidity. This change in soil pH induces the release of normally insoluble metal ions and allows for their uptake by plants. In many of these acidic soils aluminum toxicity is a major factor limiting crop growth (for reviews see Foy, 1988; Taylor, 1988a; Roy et al., 1988). Aluminum toxicity symptoms first appear in the root meristem region. The first visual symptom of aluminum toxicity is the inhibition of the mucilage secretion by the root cap cells (Puthota et al., 1991), followed by root growth inhibition, resulting in a stubby, thickened, brittle, and discolored root system appearance (Foy et al., 1978).

The physiological and molecular basis for the effects of aluminum on plants, as well as the tolerance that some plants show to Al, remains unknown. Aluminum phytotoxicity has been related with the disruption of several physiological mechanisms (see reviews of Roy et al., 1988; Taylor, 1988a; Jackson et al., 1990). Aluminum tolerance is

genetically determined (Campbell and Lefever, 1981), and to date there are a number of different tolerance mechanisms that have been hypothesized (Foy et al., 1978; Haug, 1984; Taylor, 1988b; Marschner, 1991). These resistant mechanisms can be either by avoidance, in which the plant is protected externally from the effects of Al, or by tolerance, in which some internal mechanisms enables the plant to survive (Taylor, 1988b).

Among the internal tolerance mechanisms proposed, Aniol (1984) suggested that tolerance could be induced in wheat roots by sublethal doses of aluminum through the synthesis of Al-binding proteins or Al-sequestering proteins. Metal binding proteins, metallothioneins, have been described in animal cells for Cu, Cd, Zn, Ni, and Co detoxification (Hammer, 1986), and metal binding polypeptides, phytochelatins, have also been described in some plants for Cu and Cd tolerance (Tomsett and Thurman, 1988; Rauser, 1990).

Although an Al-binding protein has not yet been reported, there is some evidence that Al stress induces changes in the level of expression of specific cell proteins (Matlock and Ownby, 1988; Matlock, 1989; Ownby and Hruschka,1991; Rincón and Gonzáles, 1991). Recently Ownby and Hruschka (1991), described, in a quantitative analysis of 2D-PAGE gels, the effects of Al stress on protein synthesis in the root tips of two sister lines of hard red

winter wheat which differed in Al tolerance. In this study they tried to determined if changes in specific proteins are primary, protective responses to stress (Al tolerance), or simply a response of cellular damage manifested at the molecular level, and if these changes occur in an specific fraction of cell proteins. They found that Al stress affected the program of synthesis of both microsomal and cytoplasmic fraction, the latter being the most affected. From 600 proteins that were analyzed, 43 proteins were significantly altered by Al stress in both wheat lines. Among these 43 proteins whose level were altered by Al stress, three cytoplasmic proteins were induced only in the tolerant line. The authors suggest that although these proteins could represent "defense proteins" for the tolerant wheat, they do not represent a major part of changes in protein expression in response to Al toxicity. However, they reported that one of the most dramatic response for Al stress was the appearance of a small acidic cytoplasmic protein, with a pI of 5.2 and 18.6 KD molecular weight. This protein was not seen by silver-stained 2D-gels in control samples, but increased more than 30-fold in both cultivars, and became one of the five most abundant cytoplasmic proteins, during 24 h of Al stress. The appearance of this polypeptide, tentatively called alumitin, has been the most consistent response to Al stress. It has been observed in five wheat cultivars of differing

sensitivity to Al, always in response to Al levels that inhibit root growth. These observations suggest that alumitin is elicited as part of the program of Al toxicity. Because it appears only when the root growth has been arrested, it could represent a molecular biomarker for aluminum toxicity in crops and natural vegetation.

With this work as a base, and as a first step for a final goal to develop a diagnostic assay for aluminum toxicity based on the presence of alumitin and its mRNA, the objectives of the present study were:

1. To determine if alumitin, as a stress-related protein, could be elicited by other environmental stresses that also can cause growth inhibition. This objective was designed to answer the following questions:
a) Is alumitin induced by other metals such as copper or cadmium? b) Is alumitin a heat shock protein? Low molecular weight HSPs with a similar size and pI of alumitin have been described in wheat and corn leaves (Zivy, 1987; Porter et al., 1989; Vierling, 1991). c) Does low pH ($[H^+]$-toxicity) induced alumitin?. d) Because aluminum toxicity is closely related to Ca availability, is alumitin elicited when plants are grown under calcium deprivation?

2. To determine if alumitin is induced by Al toxicity in cereals other than wheat. Rye, an Al-tolerant cereal, and triticale, a hybrid of wheat and rye, were selected for this study.

3. To determine the time course of synthesis of alumitin in wheat roots.

CHAPTER II

LITERATURE REVIEW

Aluminum Toxicity

Soil Acidity and aluminum species.

Soil acidity can be defined as an excess of hydrogen ions in the soil solution. It is a major stress factor that influences the growth of many plants, in large areas throughout the world. Acid soils are located predominantly in the tropics, although some are found in temperate regions where coniferous forests are the dominant vegetation as in the eastern U.S. and Northern Europe. Approximately 40% of the world's cultivated lands, and up to 70% of the potentially arable lands, are acidic (Foy et al., 1978; Haug, 1984). On acid mineral soils, plant growth can be limited by a variety of different chemical factors and by interactions of these factors. In many acidic soils, the main growth-limiting factor is the excessive level of free and exchangeable aluminum. Aluminum toxicity occurs only in soils where the pH is below 5.5 and increases in severity when the pH drops below 5.0 (Foy et al., 1978). Aluminum toxicity is particularly serious in strongly acid subsoils that are difficult to lime, and it is being intensified by heavy applications of acid-forming nitrogenous fertilizers.

Besides the economic problems of liming these soils, it is difficult to incorporate lime deeper than 30 cm.

Although a large number of reports contain assumptions regarding the toxicity or nontoxicity of a particular Al species, the identity of the phytotoxic Al species is not yet known. Toxicity is generally correlated with Al^{3+} in soil solutions (Hue et al., 1986); Al^{3+} increases as pH decreases. However, toxicity has also been attributed to other Al species such as $Al(OH)^{2+}$, or polynuclear species such as Al_{13} (Kinraide and Parker, 1989; Kinraide and Parker, 1990; Kinraide, 1990; Kinraide, 1991).

Symptoms of Aluminum Toxicity. The symptoms of Al toxicity are not always easily identifiable. The most dramatic effects are reductions in both root and shoot growth. In some plants the foliar symptoms may resemble those of phosphorous deficiency (small, dark green leaves with late maturity, purple coloration of stems, leaves and leaf veins, and chlorosis and necrosis of leaf tips). In others, aluminum toxicity may appears as an induced calcium deficiency (curling or rolling of young leaves and collapse of growing points or petioles). Typically, roots are more affected that shoots. The roots become thickened, stubby, brown, brittle, and occasionally necrotic. The root system as a whole is coralloid in appearance with many stubby lateral roots but lacking in fine branching. These roots

are inefficient in absorbing nutrients and water. In general, young seedlings are more susceptible to aluminum than older plants (Foy et al., 1978 and Taylor, 1988a).

Aluminum uptake. Bennet et al., (1985a) in a time course study of $AlSO_4$ uptake by the primary root of Zea mays showed that the initial sites of uptake are the peripheral cells of the root cap and the mucilaginous secretion (mucilage) that covers the epidermal cells of the roots. Al^{3+} binds specifically to the mucilage, partly by exchange adsorption on negative charges of the polygalacturonic acid. Aluminum has been suggested to adsorb according to a non-metabolic process at Ca-binding sites on the cell surface (Kinraide and Parker, 1987). In the intact tissues, the major part of aluminum is bound to the pectic substances in the cell walls and also to plasma membrane and nucleic acids. When the plasma membrane, which acts as a barrier for the passive movement of aluminum, is destroyed or saturated, the metal diffuses into the cytoplasm where it can bind to the various phosphate compounds and nucleic acids, accumulating largely in the roots. Transport of Al as a polyvalent cation may follow the apoplasmic pathway through cortical cells. Wagatsuma (1984) proposed that although aluminum passes through cortical cell walls via the apoplasmic pathway, it may enter the stele through plasmalemma, without suberin lamella at the endodermis. Al

could cross the plasma membrane with phospholipids as negatively charged carriers, with organic chelates as neutral carriers, or through hydrophilic pores or protein channels (Haug, 1984).

Mechanisms of Aluminum Toxicity

Toxicity appears to be the result of several interactions, and there is no consensus on the mechanisms of aluminum toxicity in higher plants. Aluminum has been shown to has a deleterious effect on numerous aspects of the affected species' physiology. Several possible mechanisms by which aluminum may disrupt cellular function have been proposed. These include: 1) effects on root cap and mucilage; 2) effects on cell walls; 3) disruption of the plasma membrane; 4) inhibition of DNA; 5) alteration of mineral nutrition; and 6) effects on protein synthesis.

Effects on root cap and mucilage. Ultrastructural studies show that Al has a direct effect on the secretory activity of the peripherals cells. The peripheral cells of root cap of most plants typically secrete a mucilage made up of polymers of glucose, galactose, uronic acids, and fucose. In maize roots Al inhibits the synthesis of the cap mucilage through the disruption of the Golgi apparatus function (Bennet et al., 1985b). Puthota et al., (1991), in an ultrastructural and morphometric study with two lines of wheat, found that Al inhibits mucilage droplet formation by

inhibition of transport of mucilage-containing vesicles, and turnover of dictyosomes. Horst et al., (1982) demonstrated that in cowpea roots, Al accumulated in mucilage 5 to 7 times more than in the root tissue, and that physical removal of the mucilage resulted in an increase of Al uptake as well as a greater toxicity.

Effects on the cell wall. Clarkson (1967, cited in Taylor, 1988a), found that 85 to 95% of the total aluminum that accumulated in the roots of Hordeum vulgare was tightly bound to cell wall material, and suggested that interactions between aluminum and free carboxyl groups of polygalacturonic acid in the middle lamella of the cell wall could account for such binding.

Foy et al., (1978) suggested that aluminum binds competitively with Ca^{2+} to the non-esterified pectin carboxyl groups on the cell walls, producing a strong adhesion between cell walls and thus inhibiting root elongation. Aluminum may also participate in the formation of cross-links between proteins and pectin within the cell wall, making it more rigid (Foy and Campbell, 1984).

Effects on plasma membrane. Because the plasma membrane represents the ultimate barrier between the cytosol and its external environment, the effect of aluminum on membrane structure and function seems crucial to an understanding of the physiology of aluminum stress.

Evidence suggests that plasma membrane functions are impaired by aluminum. Zhao et al.,(1987), found that aluminum increased the permeability of Quercus rubra root cells to non-electrolytes (urea, methyl urea and ethyl urea) and decreased its permeability to ions and water. In the same study, calcium and aluminum had opposite effects on permeability. When applied together, they canceled each other's effects on permeability. These results suggest that aluminum alters the chemical environment of membrane lipids either directly by binding to polar regions of phospholipid or indirectly by binding to membrane proteins. Interactions of aluminum with membrane proteins may also be important in aluminum toxicity. Al also affects membrane carriers, by competitive inhibition of a Mg^{2+}-dependent, K^{+}-stimulated ATPase in plasma membrane preparations from Pisum sativum and Zea mays (Matsumoto and Yamaya, 1986). Aluminum can interfere with calmodulin-stimulated, membrane-bound ATPases in maintenance of potentials of plasma membrane-enriched vesicles of barley roots (Matsumoto and Yamaya, 1986). Siegel and Haug (1983), observed that micromolar concentrations of Al ions interfere with calmodulin-stimulated membrane bound ATPase activity. This ATPase activity plays a role in the maintenance of the membrane potential of plasma membrane-enriched vesicles isolated from barley roots. They also reported that Al induced changes in calmodulin structure which were reflected in reduced

formation of the membrane potential when assayed with a fluorescent potential probe, oxionol VI. Siegel and Haug hypothesized that Al-calmodulin complex represents a primary lesion in toxic responses of plants to this metal. However, Haug (1984) found that specificity of Al-Calmodulin interactions is low; Ga^{3+} and Sc^{3+} induced also structural changes in calmodulin that resembled those produced by aluminum.

On the other hand, Kinraide (1988) showed that wheat roots exhibiting severe aluminum toxicity symptoms had an undiminished capacity to extrude protons, that the membranes were intact, and that ATP synthesis was sufficient to supply the proton-translocating ATPases. The Al-cultured roots were severely stunted and gross anatomical lesions were apparent. Nevertheless, electron microscope measurements provided no evidence of injury to the plasma membrane. Chen et al., (1991), working with intact root cortex cells of Northern red oak, determined that 370 μM Al significantly increased membrane permeability to urea, to monoethyl urea, and decreased permeability to water. They also found that Al significantly altered the activation energy required to transport water (+32%), urea (+9%) and monoethyl urea (-7%) across cell membranes. They observed that above 9°C, Al increased the lipid partiality of the cell membranes, and below 7°C Al decreased it. Aluminum seemed to increase the temperature of the phase change to the gel state. They

concluded that aluminum affects the activation energy of permeation, the phase status, and the lipid partiality of cell membranes.

Effects on DNA and Mitosis. Early studies indicated that nucleic acids might provide adsorption sites for aluminum, which could alter DNA replication (Morimura et al., 1978), and disrupt normal functioning of the nucleus (Fiskesjo, 1983). Morimura et al., (1978) described inhibition of cell division in root apical meristems as the primary effect of aluminum. Horst et al., (1982) established that cell division in cowpea roots ceased within a few hours after the exposure of roots to aluminum. Although cell division resumed thereafter, it remained at a lower level compared to controls not exposed to aluminum.

Matsumoto et al., (1977) reported that aluminum accumulated in the epidermis and regions of active cell division in _Pisum sativum_. Within the cell, Al accumulated in the nuclei by binding specifically to DNA and not RNA or histones, and once bound, was not easily dissociated _in vivo_. Aluminum was suggested to increase the melting temperature (Tm) of DNA by binding to phosphate and increasing the rigidity of the double helix and repressing template activity (Matsumoto and Morimura, 1980). Wallace and Anderson (1984), on the other hand, found that Al treatment had a pronounced differential effect on both root

elongation and incorporation of ^{3}H-thymidine into root DNA in two lines of wheat, with effects on root elongation being the most rapid. They concluded that the inhibition of root growth was not due to an interference with DNA synthesis.

Effects on mineral nutrition. It is known that broad disruptions in patterns of mineral accumulation occur in Al-stressed plants (Taylor and Foy, 1985a). Al can interfere with uptake and distribution of calcium, phosphorus, nitrogen, magnesium, iron, and potassium (Marschner, 1986; Roy et al., 1988; Foy and Fleming, 1982; Taylor, 1988a).

Aluminum Tolerance

Just as the mechanisms of toxicity are not entirely clear, the mechanisms of tolerance are not yet well defined either. Tolerance occurs naturally in certain species and within selected ecotypes. Plant species and varieties vary widely in tolerance to excess aluminum in the growth medium (Foy et al., 1978). In several species, these differences are genetically controlled. Such variation represents an important source of germplasm for the development of plants adapted to acid soil conditions (Foy et al., 1978; Foy, 1988).

Two types of tolerance mechanisms have been described: 1) external tolerance mechanisms, avoidance or exclusion mechanisms, are those by which the plant prevents aluminum

from reaching metabolic sites, and 2) internal tolerance mechanisms, where aluminum enters the plant and tolerance is achieved by some means of detoxification (Foy et al., 1978; Foy, 1988; Taylor, 1988b).

External tolerance mechanisms. Taylor (1988b), proposed four possible mechanisms by which aluminum could be excluded from the symplasm: 1) immobilization at the cell wall; 2) selective permeability of the plasma membrane; 3) plant-induced pH barrier in the rhizosphere; and 4) exudation of chelate ligand:

1) Immobilization at the cell wall. The cell wall represents a potentially large sink for aluminum, and immobilization of Al at the cell wall could reduce uptake into the symplasm. A large portion of Al absorbed by roots is adsorbed in the apoplasm by exchange with Ca^{2+}. Cultivar tolerance to Al has been associated with root low cation exchange capacity (CEC). As the CEC of root tissue increases, there is a greater exchange adsorption of polyvalent cations in the apoplasm. Wagatsuma (1983a), showed that there is a close positive correlation between the CEC of different crop species and the aluminum content in their roots. However, Marschner (1986), states that dicots which have high CEC are not less tolerant than monocots, which have low CEC.

2) Exclusion of Al by the root plasma membrane. The

plasma membrane may act as a selective barrier to the passive uptake of Al into the symplasm. Wagatsuma (1983b), found that excised roots of some species showed increase uptake of Al with exposure to a range of metabolic inhibitors or to anaerobiosis. Miyasaka et al., (1989) using a microelectrode system to measure simultaneously rhizosphere pH, K^+, H^+ fluxes, and membrane potentials (Em) along the root of two wheat cultivars, found that in an Al sensitive cultivar, aluminum caused a dramatic inhibition of K^+ influx, a moderate reduction of H^+ efflux, and depolarization of the membrane potential. They suggested that Al tolerance in wheat is associated with the increase ability of the tolerant plant to maintain normal ions fluxes and membrane potentials across the plasma membrane of root cells in the presence of Al. Zhang and Taylor (1989), found that root of Al tolerant wheat cultivars treated with the protonophore, DNP (dinitrophenol), increased rates of uptake of Al. They suggested that a metabolic exclusion mechanism of Al from the symplasm of Al-tolerant cultivars occurred under normal aerobic conditions (without DNP). In contrast, they found that DNP produced minimal effect on uptake by an Al sensitive cultivar, suggesting that uptake and accumulation of Al is not as closely regulated in a direct energy-dependent process.

3) Rhizosphere pH. Control of rhizosphere pH has been

proposed as a means of Al avoidance or external mechanism, because Al solubility is pH dependent. Solubility of Al decreases rapidly in the pH range of 4 to 5.0 (Foy, 1988; Taylor, 1988b; Foy and Fleming, 1982). It is possible that plants could create a pH barrier at the root-soil interface which reduces the solubility of Al and limits its entry into the symplasm. A slight pH increase at the root surface not only reduce Al solubility but also its charge, resulting in the formation of less toxic Al species, such as $Al(OH)_3 \cdot H_2O$ that is formed at near neutral pH and is only sparingly soluble (Taylor, 1988b).

The plant-induced pH hypothesis is supported by numerous studies which show a relation between an induction of high pH in the growth medium and the Al-tolerance of cultivars of many crop species. Some Al-tolerant cultivars of wheat, barley, peas, and maize increase the pH of nutrient solutions in which they grow and thereby decrease the solubility and toxicity of Al by precipitation. Experimental evidence supporting this hypothesis was first reported by Foy et al., (1965, in Taylor, 1988b), who demonstrated that an Al-tolerant cultivar of <u>Triticum aestivum</u> maintained a higher substrate pH than an Al-sensitive cultivar, in both nutrient solutions and soils. The relationship between Al tolerance and the ability to maintain a relatively high pH in the growth medium has been demonstrated for <u>Hordeum vulgare</u>, <u>Pisum sativum</u>, <u>Secale</u>

cereale, Triticum aestivum, and XTriticosecale (Foy et al., 1974; Fleming, 1983; Taylor, 1988b). Taylor and Foy, (1985a) found that the Al tolerance of 20 winter and 20 spring cultivars of wheat was correlated with the ability of the cultivar to resist acidification of the growth solution. This pattern occur in the pH range of 3.8 to 4.5. Differences between cultivars with respect to plant-induced pH appeared to be due to differences in the relative uptake of NH_4 and NO_3. Al-sensitive plants showed a more rapid uptake of NH_4, and hence, a lower pH in the growth medium than Al-tolerant cultivars (Taylor and Foy 1985b). In contrast, Al-tolerant plants showed a less rapid uptake of NH_4, and hence, induced a higher pH in the growth solution (Taylor and Foy, 1985b). However, controversy exists over whether the observed pH differences is the cause or the effect of differential Al tolerance. Wagatsuma and Yamasaku (1985), found no possitive correlation between Al tolerance in barley and pH changes in the bulk nutrient solution induced by the plant in response to manipulation of nitrogen sources. Taylor (1987), obtained similar results for winter wheat. He found that the relative tolerance of the cultivars was unaffected by the NH_4/N ratio and by solution pH. Miyasaka et al., (1989) also found no significant difference in the rhizosphere pH between wheat cultivars.

Despite the evidence supporting the role of nitrogen nutrition and plant-induced pH in Al tolerance, this

hypothesis cannot account for all differences in Al tolerance among species and cultivars.

4) Root exudation of chelate ligands. Plants could exclude aluminum from the symplasm if chelate ligands were released into the rhizosphere and these ligands formed stable complexes with Al. A reduction in the activity of the free metal ion should affect absorption of the ion into the symplasm, and hence, its toxic effect (Taylor, 1988b). Jones (1961), proposed the hypothesis that Al-tolerant plants species contain and exude organic acids (mainly oxalic and citric) or other ligands that chelate aluminum and thereby reduce its chemical activity and toxicity. Since then, detoxification of Al by chelation has been demonstrated in many studies (Suhayda and Haug, 1986). Hue et al., (1986) suggested that Al-detoxifying capacities of organic acids correspond with relative positions of OH/COOH groups on the main C chain, that is, those favoring the formation of a stable 5 or 6 bond ring structure with Al. They showed that the addition of citric, oxalic, and tartaric acids to hydroponic solutions alleviated the inhibitory effect of Al on root extension of cotton (Gossypum hirsutum L.). Barlett and Riego (1972) found that Al complexed by citric acid or EDTA did not reduce root and shoot growth of corn plants (Zea mays L.) as did ionic Al.

A claim for Al-exclusion by organic acids was presented by Ojima and Ohira (1988). They showed that an Al-tolerant

carrot (Daucus carota L.) cell line releases more citric acid into the suspension culture medium than non selected cells. They demonstrated also that the addition of citric or malic acid into the medium could ameliorate the Al-stress. Later evidence showed that the tolerant cell line was sensitive to ionic Al because it had been selected in the presence of precipitated Al-phosphate at higher pH rather than in the presence of ionic Al at a low pH (Koyama et al., 1988). The authors concluded that excretion of organic acids by carrot cells was a response to low phosphorus availability rather than to toxic Al ions.

Exudation of organic acids into the rhizosphere in response to mineral stress especially phosphorus and iron deficiency, has been found for several plants. Lipton et al.,(1987), showed that alfalfa (Medicago sativa L.) seedlings exuded 182% more citrate under phosphorus deficiency than under conditions of sufficient phosphorus.

Some of the specific ameliorative effects of chelates have been described. Wagatsuma (1983a), found that Al supplied as an EDTA complex prevented both an accumulation of Al by excised roots of corn and an Al-induced desorption of calcium. Suhayda and Haug (1986), reported that in plasma membrane isolates from Pisum sativum, malic, glutamic and citric acids all restored K^+- stimulated Mg^{2+} dependent ATPase activity which was inhibited by Al. In Zea mays citrate had the same effect. Ownby and Popham (1989), found

that an Al-sensitive wheat cultivar resumed its growth after a 5-h pulse of Al, when the Al-free medium contained 2mM citrate. They also found that under high Ca^{2+}, a 30 min desorption with citrate after 5 h Al pulse was as effective as continuous exposure to citrate in stimulating regrowth. With lower Ca^{2+}, no regrowth was observed.

Recently Miyasaka et al. (1991), working with two cultivars of snapbeans (Phaseolus vulgaris) that differ in Al tolerance, showed that the tolerant one exuded citric acid into the rhizosphere in a concentration that was 70 times more than that of the control plants (grown with no Al), and 10 times more than that of the sensitive one grown with or without Al. They concluded that one mechanism of tolerance in snapbeans appears to be the exudation of citric acid into the rhizosphere induced either by toxic levels of Al or low phosphorus due to the precipitation of insoluble Al-phosphates. It is known that citrate enhance the availability of phosphorus from insoluble Al-phosphate (Marschner, 1986).

Internal Tolerance Mechanisms. Although the apoplasm is the major pool of Al in plants growing on Al-toxic substrates, Al does enter the symplasm. Once in the symplasm the solubility of free Al^{+3} is limited to picomolar or nanomolar level by the limited solubility of $Al(OH)_3$ and $Al(OH)_2$ H_2PO_3 at pH 7.0. However, these concentrations are

potentially phytotoxic, because of the strong affinity of Al for oxygen donor compounds as inorganic phosphates, nucleotides, RNA, DNA, proteins, carboxylic acids, phospholipids, polygalacturonic acids, among other oxygen donor ligands (Taylor, 1988b).

There is limited evidence that Al tolerance in several species is due, al least in part to an internal mechanism (Niedzella and Aniol, 1983). These tolerance mechanisms could be achieved by chelation by ligands in the cytosol, compartmentation in the vacuole, complexation by Al-binding proteins.

1) Chelation in the cytosol. The potential role of carboxylic acids in the detoxification of Al has been established in several studies. Evidence supporting detoxification of Al by chelation in the cytosol comes from *in vitro* studies. Cambraıa et al., (1983), showed that the overall response of sorghum (*Shorgum bicolor*, Moench) roots to Al was an increase in organic acid content. They found that the roots of the Al-tolerant sorghum cultivar contained higher levels of organic acids, particularly t-aconitic and malic acids, than did the Al-sensitive cultivar. On the other hand, Lee and Foy (1986), found that in the roots of snapbeans, the overall response to Al was a reduction in organic acid contents (citrate and malic acids), although the Al-tolerant snapbean cultivar maintained a higher level of organic acids than did the Al-sensitive cultivar. Foy et

al., (1987) also correlated Al-tolerance in two barley cultivars with higher concentration of organic acids in the shoots and roots of plants exposed to Al.

Matsumoto and Yamaya (1986) found that malic, glutamic, and citric acids reduced the Al-induced inhibition of a K^+-stimulated, Mg^{2+}-dependent plasma membrane ATPase in Pisum sativum. They suggested that the inhibitory effect of Al was due to the formation of an Al-ATP complex, and that amelioration by carboxylic acids was due to the formation of stable complexes with Al. Suhayda and Haug (1986), obtained the same results in plasma membrane ATPase of Zea mays. They suggested that citrate prevents a direct effect of Al on the ATPase itself rather than reducing the formation of an Al-ATPase complex. Also Suhayda and Haug (1984) found that citrate and to a lesser extent, oxalic, malic and tartaric, prevented the binding of Al to calmodulin in vitro and partially restored the native structure of calmodulin once an Al-calmodulin complex had been formed.

These results demonstrate that organic acids could protect enzyme activity internally in the plant from the deleterious effect of Al.

2) Compartmentation in the vacuole. Tolerance to Al could be achieved if Al was sequestered in sites which are insensitive to Al such as the vacuole. While compartmentation has received support as a mechanism of

tolerance to other metals, evidence supporting Al compartmentation is lacking. Taylor (1988b), pointed out that meristematic root cells, which are most affected by Al-treatment, are not vacuolated in either Al-tolerant or Al-sensitive species.

3) Aluminum tolerance and protein synthesis. Another Al-tolerance mechanism could be the induction or enhancement of proteins. It is known, for example that many environmental stress factors produce changes in the expression of specific plant cell proteins. Such stress factors include heat shock (Mansfield and Key, 1987; Vierling, 1991), water stress (Bray, 1988), low temperature (Guy and Haskel, 1987), cadmium toxicity (Delhaize et al., 1989) and salinity (Hurkman et al., 1988).

Aniol (1984), reported that a pre-treatment of wheat roots with Al at sublethal doses (0.5 μg/ml for tolerant and 0.1 μg/ml for sensitive cultivars) increased subsequent tolerance to higher levels of Al. These effects were greater in the Al-tolerant cultivar than in the Al sensitive. He also found that the Al pretreatment increased the incorporation of [^{14}C]-valine and [^{3}H]-thymidine into proteins and DNA, respectively, in wheat roots exposed to Al. The increase in tolerance was abolished by the addition of a protein synthesis inhibitor cycloheximide. He suggested that Al tolerance in wheat could involve

detoxification of Al by an inducible protein. Because the majority of Al found in root tissue was accumulated in the cytosol fraction, he suggested that Al was bound with high molecular weight compounds, probably proteins or RNA.

Ownby and Hruschcka (1991), on the other hand, in a study where they quantified the changes in cytoplasmic and microsomal proteins associated with Al-toxicity in two sister lines of wheat differing in Al-tolerance, found that from 600 proteins examined, 14 cytoplasmic and 8 microsomal proteins were induced or enhanced by Al-treatment in one or both cultivars, while 9 cytoplasmic and 12 microsomal were diminished or repressed. They concluded, that Al affected the program of formation of both cytoplasmic and microsomal proteins, but in general, changes associated with Al toxicity were more dramatic among cytoplasmic than among microsomal proteins, and that few of the observed changes could be "Al protection" proteins. The most prominent change observed was the induction of an acidic, 18.6 Kd protein, whose concentration was enhanced over 50-fold in both cultivars, becoming one of the five most abundant cytoplasmic protein during 24 h of Al stress. This protein was also elicited in other wheat cultivars after treatment with levels of Al sufficient to inhibit growth. They concluded that changes of this magnitude represents a major response to Al stress.

Copper Toxicity

Copper, an essential micronutrient, is a toxic metal ion at sufficiently high concentration. Copper enters the environment from mining and processing of copper, addition of copper-containing agrochemicals, industrial and urban activities, and addition of domestic and industrial sludge to the soil. Cu^{2+} is strongly bound in soils to humic and fulvic acids, forming copper-organic matter complexes. Cu^{2+}, because of its high affinity for ligands (amino acids, phenolic, and synthetic chelators) is rapidly complexed (For review see Jackson et al., 1990).

Copper toxicity usually induces iron deficiency and chlorosis due to the destruction of thylakoid membranes. Copper toxicity is primarily the results of its high affinity for sulphydryl groups, causing the inactivation of sulfhydryl-containing enzymes or altering their catalytic specificity. For most crop species, the critical toxicity level of copper in the leaves is considered to be above 20 to 30 μg/g dry weight (Marschner,1986).

It is know that in animals, metallothioneins are involved in copper, cadmium, nickel, cobalt, and zinc homeostasis (Hammer, 1986). Metallothioneins are low molecular weight metal-binding proteins, with high metal content, high cysteine, and no histidine or aromatic amino acids. They have an abundance of cys-X-cys sequences where

X is an amino acid other than cys. Metal ions are bound through closely spaced cysteine thiolate groups (Rauser, 1990). There is some evidence that plants, algae, and certain fungi produced copper- and cadmium-binding macromolecules, but these differ from the mammalian metallothioneins. These are sulphur-rich metal-binding peptides, termed phytochelatins. They are poly (δ-glutamyl-cysteinyl)glycine, that, like metallothioneins, bind metal ions through thiolate coordination. These peptides have higher affinity for copper than for cadmium (Jackson et al., 1985; Reese et al., 1988). Pytochelatins do not represent direct gene products. They are products of a biosynthetic pathway that consumes glutathione. Production of metal-binding polypeptides appears to be involved in the tolerance mechanisms in some species (reviewed by Tomsett and Thurman, 1988), sequestering excessive amounts of heavy metals. On the other hand, there is some evidence that Cu tolerance does not involve the synthesis of thiol-rich polypeptides (Tukendorf et al., 1984). Schultz and Hutchinson (1985) found that sulfur deficiency in the grass Deschampsia cespitosa L. reduced the amount of copper-inducible thiol-rich compounds, but did not interfere with copper tolerance. Several different biochemical mechanisms thus appear to be involved in copper tolerance in higher plants. Plants may tolerate copper by storage in cell walls, or excretion chelating compounds into the rhizosphere. Kishinami and

Widholm, (1987), found that addition of citrate and malate to Cu-sensitive cell cultures prevented copper toxicity. The mechanisms of copper tolerance are still not well understood.

Cadmium toxicity

Cadmium can enters the environment as the result of many of the same activities that are associated with the introduction of copper. Cadmium, a group IIB trace metal, is known to be harmful to human health.

The mobility of cadmium, and its availability for uptake by plants, depends on the chemical form of cadmium present,the pH of the soil, the presence of other metals ions, and the presence of ligands and adsorption sites in the soil (Jackson et al., 1989). The two main factors influencing Cd uptake by food crops are cadmium concentration and pH (Jackson et al., 1989). The toxic species is Cd^{2+}. There is evidence that Cd^{2+} interferes with respiratory carbohydrate metabolism (Reese and Roberts, 1985). Cadmium also inhibits the formation of chlorophyll, interfering with protochlorophyllide reduction and the synthesis of aminolevulinic acid (Stobart et al., 1985). Cadmium is known to irreversibly replace copper and zinc in critical metalloenzymes. Many of these enzymes are involved in RNA and DNA metabolism.

Like Al toxicity, Cd has numerous sites of action

within the plant. Therefore it is more likely that Cd tolerance will be associated with a mechanism that either excludes this toxic ion from plant tissues or sequesters it in a less toxic form.

As in the case of Cu, Cd tolerance is associated with the ability to produce large amounts of small, heat-stable cysteine-rich polypeptides. These are cytoplasmic thiol-rich cadmium complexes, containing Cd and poly (δ-glutamylcysteinyl) glycine, known as cadystins, phytochelatins and Class III metallothioneins (reviewed by Rauser, 1990). Reese and Wagner (1987), found that when they grew cultured tobacco cells treated with Cd, Zn, or Cu in presence of buthionine sulfoximine, which inhibits the synthesis of δ-glutamylcysteine, glutathione, and phytochelatins, this leads to cadmium sensitivity in normally tolerant cell cultures. Delhaize et al., (1989) found that a Cd tolerant cell line of _Datura_ _innoxia_, in the presence of Cd, possesses two abundant mRNAs which are normally produced. These mRNAs encode proteins of low molecular weight (11 KD) and are either present at low level or absent in Cd-sensitive cells. They also found that Cd-induced proteins are also induced by heat shock, however a subset of mRNAs were induced specifically by Cd, while other mRNA were heat-shock specific.

In summary, the mechanisms of Cd tolerance and response of plants to this toxic metal are quite complex and cannot

be explained by the production of only one class of molecules (Jackson et al., 1990).

Heat Shock

Plants and other organisms are subjected to sudden and drastic changes in their natural environments. One of the most characterized response in all organisms is that produced by heat shock. The heat shock response occurs in response to an 8-12°C shift up from the normal growing temperature. It is characterized by a rapid induction of heat shock gene transcription coupled with a precipitous decline in the transcription of most other genes (Kimpel et al., 1990). This response is enhanced further by the selective translation of heat shock mRNAs at heat shock temperature (or a rapid turnover of non-heat shock mRNAs) giving as a result a selective and rapid accumulation of a group of proteins called heat shock proteins or HSPs (see reviews of Schlesinger, et al., 1990; Lindquist and Craig, 1988; Vierling, 1991). HSPs occur in most if not all the organisms, ranging from bacteria and lower eukaryotes to mammals and plants. Plant species adapted to temperate environments, including some crop plants such as corn, soybean, pea, and wheat, begin to synthesize HSPs when tissue temperature exceeds 32-33°C (Cooper and Ho, 1983; Kimpel and Key, 1985a; Necchi et al., 1987). The heat shock response of plants shares many parameters with the responses

to heat shock that have been described for other organisms such as _Drosophila_.

HSPs in plants are categorized into two major groups based on their molecular weight: high molecular weight (HMW) (65 to 110 KD), and low molecular weight (LMW) (15 to 27 KD) (Kimpel and Key, 1985b). LMW HSPs, in the range of 15 to 18 KD, appear to be unique to higher plants (Sachs and Ho, 1986; Vierling, 1991). LMW HSPs belong to four multi-gene families. Two of these families encode proteins that are primarily localized in the cytoplasm, another encodes LMW HSPs in the chloroplast, and the last one appears to encode an endomembrane protein (Vierling, 1991).

The precise function of HSPs is not well known. One function assigned to these proteins to date is their possible role in modulating the expression of thermotolerance. In soybean seedlings it has been demonstrated that there is a correlation between the synthesis and accumulation of HSPs and their ability to survive short heat treatments at otherwise lethal temperatures (Lin et al., 1984). HSPs have been suggested to protect the cell by allowing normal protein synthesis for a longer period than may occur without HSPs (Altschuller and Mascarenhas, 1981). Orzech and Burke (1988) found that heat shock can provide protection against metal toxicity (Al, Cd, Fe, and to a lesser extent to Cu, and Zn) in wheat leaves. HSPs may also be involved in reparıng proteins damaged by

high temperatures to keep them from unfolding, or even to help dispose of unneeded proteins, via the ubiquitin proteolytic pathway (Ferguson et al., 1990).

Heat is not the only stress treatment that leads to elevated expression of many HSPs. Ethanol, arsenite, heavy metals, amino acid analogues, glucose starvation, calcium ionophores, and a number of other treatments affect the synthesis of many HSPs in different organisms (Brodl, 1990; Czarnecka et al., 1984; Edelman et al., 1988). For these reason, HSPs have also been referred to more generally as "stress proteins".

Genetic Aspects of Aluminum Toxicity in Wheat, Rye, and Triticale

Triticale is a synthetic cereal species that is a hybrid of wheat (*Triticum* spp L.) and rye (*Secale cereale* L.). X*Triticosecale* Wittmack is the generic name that is accepted.

The early hybrids were mainly octoploids (genome: AABBDDRR) resulting from crosses of hexaploid wheat (genome: AABBDD) and diploid rye (genome: RR). Hexaploid triticale (genome: AABBRR) was derived from hybridization of the tetraploid wheat *T. durum* (AABB) with rye (RR). These hexaploid triticales have proven to be more stable and agronomically more satisfactory for commercial use than the earlier octaploid types.

The three commercially important polyploid species of wheat are:

1. *T. aestivum* L., a hexaploid (AABBDD), with four classes for marketing purposes: hard red winter, hard red spring, soft red winter, and white wheat.

2. *T. compactum* Horst., a second hexaploid (AABBDD), also known as club wheat, and

3. T. *durum*, the only tetraploid (AABB).

Two genomes or chromosomes groups of *T. aestivum* and *T. compactum,* designated A and B genomes, are common with the two genomes of *T. durum*. The third genome, D, of the two hexaploid species has some homology with the R genome that comprises rye. Therefore, the hexaploid triticale resulting from the hybridization of *T. durum* (AABB) and rye (RR) has an AABBRR genomic make-up.

Cereals differ in response to Al, with rye being one of the most tolerant and wheat generally less tolerant. Many triticales have some degree of tolerance to Al, but not as much as rye itself. Aniol and Gustafson (1984), in a study to locate the chromosomes carrying Al tolerance genes in wheat and rye, found that genes for aluminum tolerance in the medium-tolerant wheat variety Chinese spring were found in chromosome arms 6AL, 7AS, 3DL, 4DL and 4BL and on chromosome 7D. Genes for tolerance in rye seemed to be located on 3R and 6RS and on 4R. They observed that Al tolerance in rye was mainly due to chromosome 6R and was

significantly affected by the wheat background. In other words, chromosomes from a tolerant rye incorporated into an Al-sensitive wheat variety caused little increase in Al tolerance, while substitutions involving a tolerant wheat cultivar (i.e., Atlas 66) resulted in a higher level of tolerance above that observed in the tolerant wheat. They concluded that the genes involved in Al tolerance are mainly located on the A and D chromosomes in hexaploid wheat, and that Al tolerance of the wheat parent used for development of triticale is of great importance in breeding for Al-tolerant triticale varieties.

CHAPTER III

MATERIALS AND METHODS

Plant Material

Seeds of Al-sensitive wheat (Triticum aestivum L.) cultivar Victory obtained from Johnston Seed Company, Enid, Oklahoma, were used in this study. Seeds were germinated in Petri dishes on filter paper (Whatman #4) wetted with deionized water. After 2 days, seedlings were transferred to nylon screens, when they were floated in a 9.5 cm diameter pot contained 450 ml of nutrient medium. The composition of the macronutrient medium was (in mM): 0.40 $CaCl_2$; 0.65 KNO_3; 0.25 $MgCl_2$ $6H_2O$; 0.01 $MgSO_4$; 5.5 10^{-4} H_3BO_3 at pH 4.4 (Aniol, 1984). The pH of the nutrient medium was adjusted daily to 4.4 with 0.1N HCl or 0.1N NaOH. Four-day-old seedlings were exposed to the following stress factors: aluminum, copper, cadmium, low pH (3.5), calcium deficiency and 4 and 24 hours heat shock at 37°C. Stress-treated seedlings were either used for root-growth experiments or harvested for protein extraction and purification by 2D-PAGE. All seedlings were grown under vigorous aeration in a growth chamber on a 16 hour photoperiod at 26°C (day) and

22°C (night), with an illumination of 350 μmoles $m^{-2}s^{-1}$.

Root-growth experiments.

Metal-Toxicity

To determine the level of aluminum, copper, cadmium, required to inhibit root growth in cv Victory, 4-day-old seedlings were treated with different concentrations of each metal. The concentrations used for the three metals were: for Al (in μM) 1, 5, 10, 20, and 40. For Cu (in μM): 0.16, 0.80, 1.60, 4.8, and 8.0. For Cd (μM): 2, 8, 20, and 100. The three metals were provided as their chloride salt ($AlCl_3$ $6H_2O$; $CuCl_2$ $2H_2O$; $CdCl_2$ $\frac{1}{2}H_2O$, respectively). At the time of treatment the length of the primary root of 8 seedlings per treatment was measured. Each seedling was labelled by numbering it with a tape attached to the coleoptile of each seedling. After 24 hour of growth, the root seedlings were measured again and the increment of growth was determined. These experiments were repeated at least two times.

Calcium deficiency.

Seedlings were grown in the same nutrient medium described above but with no $CaCl_2$; 6 mM EGTA was added to the nutrient medium to complex tissue calcium. The level of EGTA required to inhibit growth was determined in preliminary experiments.

Heat shock.

The seedlings were exposed to heat shock treatment by placing them in a 37°C water bath for 4 or 24 hours. After heat shock treatment, one set of 4 h and 24 h heat shock seedlings was treated with 80 μM Al. After 24 h of aluminum treatment, the seedlings were transferred to nutrient solution with no aluminum to follow recovery. Root measurements were done before and after heat shock treatments and during recovery time.

pH experiments.

Four-day-old seedlings growing in nutrient medium at pH 4.4 were transferred to the same nutrient medium but with a pH range of 4.4, 4.1, 3.8, and 3.5, for 24 hours. The pH was adjusted with 0.1 N HCl. During the experiment the pH was monitored every day and maintained with either 0.1 N HCl or 0.1 N NaOH. Root growth measurements were done as described previously for metal toxicity.

Extraction of proteins

Four-day-old seedlings treated for 24 h with 80 μM Al, 0.8 μM Cu, 100 μM Cd, or pH 3.5 were used for protein extraction and purification. The terminal 1 cm of the primary root and 2 seminal roots were excised from about 250 seedlings per treatment. The root tips were collected in

cold nutrient medium and subsequently suspended in 700 μl (in a ratio g/v 1:2) of cold Honda medium, which contained: 5% (w/v) Dextran 40; 2.5% (w/v) Ficoll 400; 250 mM sucrose; 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.4, made to 2% (v/v) PMSF[1] (Honda et al., 1966, Dunham and Bryant, 1983). The roots were placed in a cold petri dish and chopped with a razor blade for 5 min. After the tissue was minced, it was homogenized in a cold mortar and pestle. The homogenate was filtered through two layers of miracloth (wetted with Honda medium) and centrifuged at 300 g for 10 minutes to pellet the nuclear fraction. The supernatant was then centrifuged at 125,000 g for 30 min at 4°C. Proteins from the supernatant, hereinafter referred to as the cytoplasmic fraction, were precipitated as described by Hurkman and Tanaka (1986) by adding an equal volume of water-saturated phenol to the supernatant. After 10 to 15 min with shaking at room temperature, the organic and aqueous phases were separated by centrifugation at 3000 g for 5 min. Proteins in the phenol phase were precipitated by addition of 5 volumes of ice-cold 0.1 M ammonium acetate in methanol and incubated at -20°C overnight. The precipitate was centrifuged for 5 min at 3000 g and then washed twice with cold methanol, and then twice with cold acetone. The pellet was redissolved in solubilization buffer that contained: 9 M

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[1] PMSF, phenylmethylsulfonyl flouride.

urea; 4% CHAPS[2] (Perdew et al., 1983), 0.5% DTT[3] and 2% 5-7 ampholytes and 0.5% 3-10 ampholytes. This sample was incubated for 2-3 hours at room temperature and the insoluble material was removed by centrifugation. Total cytoplasmic protein was determined by the method of Bradford (Bradford, 1984; BioRad, 1984), following precipitation in 10% (w/v) TCA[4] and resolubilization in 0.1 N NaOH as described in Hurkman and Tanaka (1986).

Two-dimensional gel electrophoresis (2D-PAGE).

2D-PAGE was performed according to O'Farrell (1975). For the first dimension, the IEF tubes had an I.D. of 3 mm. The monomer solution to cast the IEF tubes contained 9 M urea, 4% acrylamide-bis, 0.05% (w/v) CHAPS, with an ampholyte ratio of 5:2 (pH 5-7 to pH 3-10 respectively). Samples containing approximately 40 to 50 μg of protein were loaded at the basic end of the focusing gels, and then overlaid with 5 M urea and 5% of 5-7 ampholytes and 2% 3-10 ampholytes. The upper (cathode) buffer was 0.1 M NaOH, and the lower (anode) buffer was 0.05% (v/v) H_3PO_4. Isoelectric focusing was conducted for 18 hours at 400 V, followed by 2

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[2] CHAPS, 3-[(chloramido-propyl)dimethylammonio]-1-propanesulfonate.

[3] DTT, dithiothreitol

[4] TCA, trichloroacetic acid

hours at 800 V, with no pre-run. After extrusion, the gels were either frozen at -70°C or loaded onto a second dimension gel. The frozen IEF gels were equilibrated twice for 5 min with reducing buffer (62.5 M Tris-HCl, pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 0.02% DTT and 0.05% (w/v) bromophenol blue as tracking dye), and then loaded onto a 12% polyacrylamide resolving gel (3.75 mM Tris-HCl, pH 8.8; 0.1% SDS; 12% acrylamide-bis, 0.05% ammonium persulfate and 0.05% TEMED), having a thickness of 1.5 mm for running in the second dimension. No stacking gel was used. The running buffer was 25 mM Tris, 0.192 mM glycine, and 1% (w/v) SDS, pH approximately 8.3. Electrophoresis was done at 35 mAmp/gel, constant current, until the tracking dye reached the bottom of the gel, usually about 5h. The 2D-gels were then removed, fixed, and silver stained according with Morrisey (1981).

Al Toxicity in Triticale and Rye

Seeds and root-growth. Seed of triticale (XTriticosecale, Witmack) cv T-23 and seed of rye (Secale cereale L.) cv Elbon were obtained from Johnston Seed Company, Enid, Ok. They were germinated and grown as described for wheat seed, using the same macronutrient medium at pH adjusted at pH 4.4 and checked periodically during the experiments. To determine the level of aluminum required to inhibit root growth of both triticale and rye,

four-day-old seedlings were treated for 24-h to 4 μM, 20 μM, 40 μM, and 100 μM Al provided as $AlCl_3$ $6H_2O$. Root growth measurements were done as described before for wheat experiments. Proteins were extracted from root tips of four-day seedlings treated with a 24-h pulse 100 μM Al, and were separated and analyzed by 2D-PAGE as described for root tip wheat proteins.

Radiolabelling and Flourography.

In order to know the time of synthesis of alumitin and its turnover, four-day-old seedlings were grown in the presence of 80 μM Al for 0, 6, 12, and 24 hours. During the last 3 hours of each treatment, 611 μCi of ^{35}S (Trans^{35}S-Label, contained 70% L-Methionine, [^{35}S], 15% L-Cysteine, [^{35}S], and various non-labeled amino-acids, with an specific activity of >1000 Ci/mole, from ICN) was added to the nutrient medium. Ten μg/ml chloramphenicol was also added to the nutrient medium to reduce uptake of label by bacteria. After the 3h pulse, protein extraction was done as described before.

Incorporation of Trans [^{35}S]-label into proteins was determined by precipitation in 10% TCA. Aliquots of 20 μl each of total homogenate, nuclear, cytoplasmic, and microsomal fractions were place onto nitrocellulose filters and counted in a liquid Scintillation counter (Beckman LS 7500).

Protein having a radioactivity of to $1.75 \cdot 10^5$ cpm was separated by 2D-PAGE as described above. The gels were prepared for fluorography as described by Chamberlain (1979): the 2D-gels were fixed with 50 % methanol overnight; they were then washed twice with distilled water for 30 min each, followed by 1M sodium salycilate, as the fluor enhancer, for 30 min. The gels were dried for 2 hours in a gel dryer, and then exposed to Kodak X-OMAT-AR film at -70° for 3 days. Fluorographs were developed for 6 min in Kodak developer, 30 sec in 3% acetic acid, and 4 min in Kodak fixer.

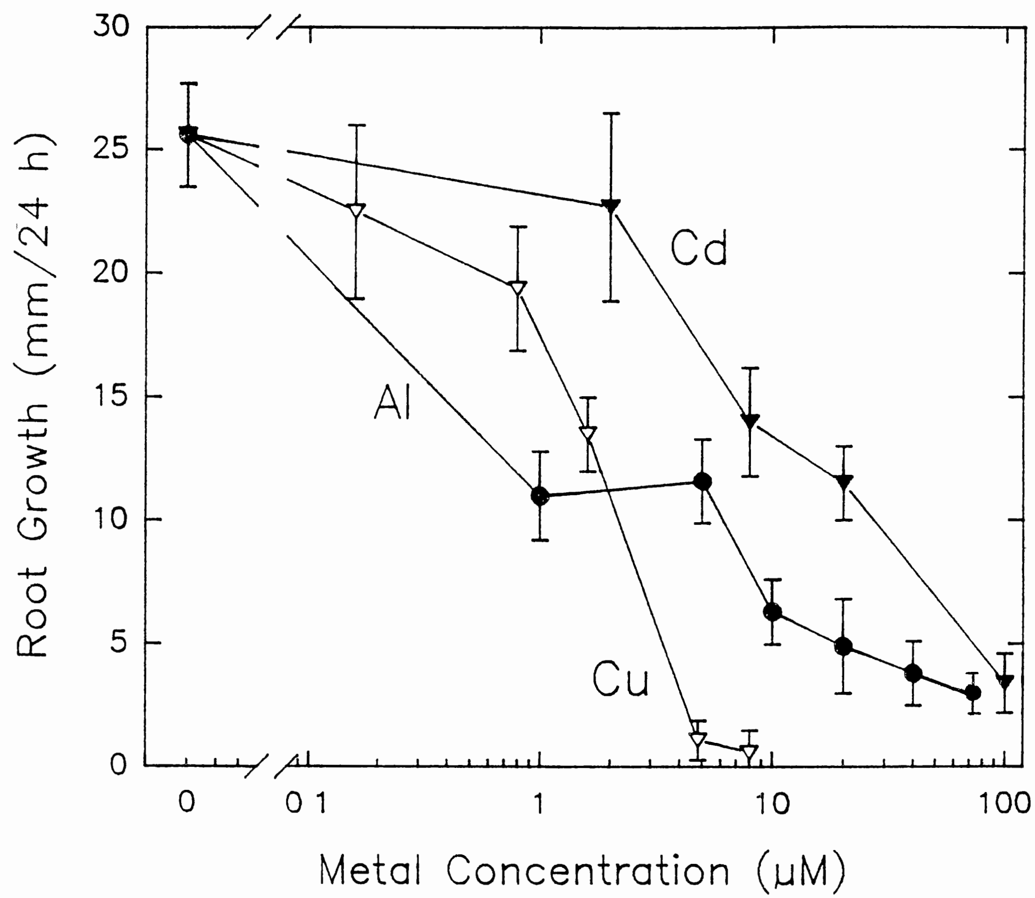
CHAPTER IV

RESULTS

Aluminum, Copper and Cadmium as stress factors

Previous studies have shown that the cytoplasmic protein alumitin is induced in roots of wheat cultivars by levels of Al sufficient to arrest growth (Ownby and Hruschka, 1991). The effects of different concentrations of Al, Cu, and Cd on the root growth of cv. Victory four-day-old seedlings are shown in Figure 1. Primary root growth was inhibited more than 90% by a 24-h pulse of 4.8 μM and 8.0 μM Cu. A 24-h pulse of 100 μM Cd produced 85% inhibition of the primary root. Some inhibition was also observed with 8 μM and 20 μM Cd. Aluminum significantly inhibited the root growth of Al-sensitive cv Victory, 40 μM and 80 μM Al producing 75% inhibition. The results with Al were similar to those reported by Matlock (1989), and Ownby and Popham (1989). Base on these experiments, seedlings were exposed to 8 μM Cu, 100 μM Cd, to determine if alumitin was induced by metals other than aluminum. Proteins were extracted and separated by 2D-PAGE from root tips of four-day-old cv Victory seedlings exposed to 24-h 8 μM Cu, 100 μM Cd, or 80 μM Al.

Figure 1. Determination of the level of Al, Cd, and Cu required to inhibit root growth of wheat cv Victory. Seedlings were grown for 4 days in the macronutrient medium, then exposed to various concentrations of metals for 24 h. Each value represents the mean ± S.D. of 8 seedlings whose primary root length was measured at the beginning and again at the end of the exposure to the metals.



Root Growth (mm/24 h)
30
25
20
15
10
5
0
Cd
Al
Cu
0
0.1
1
10
100
Metal Concentration (µM)

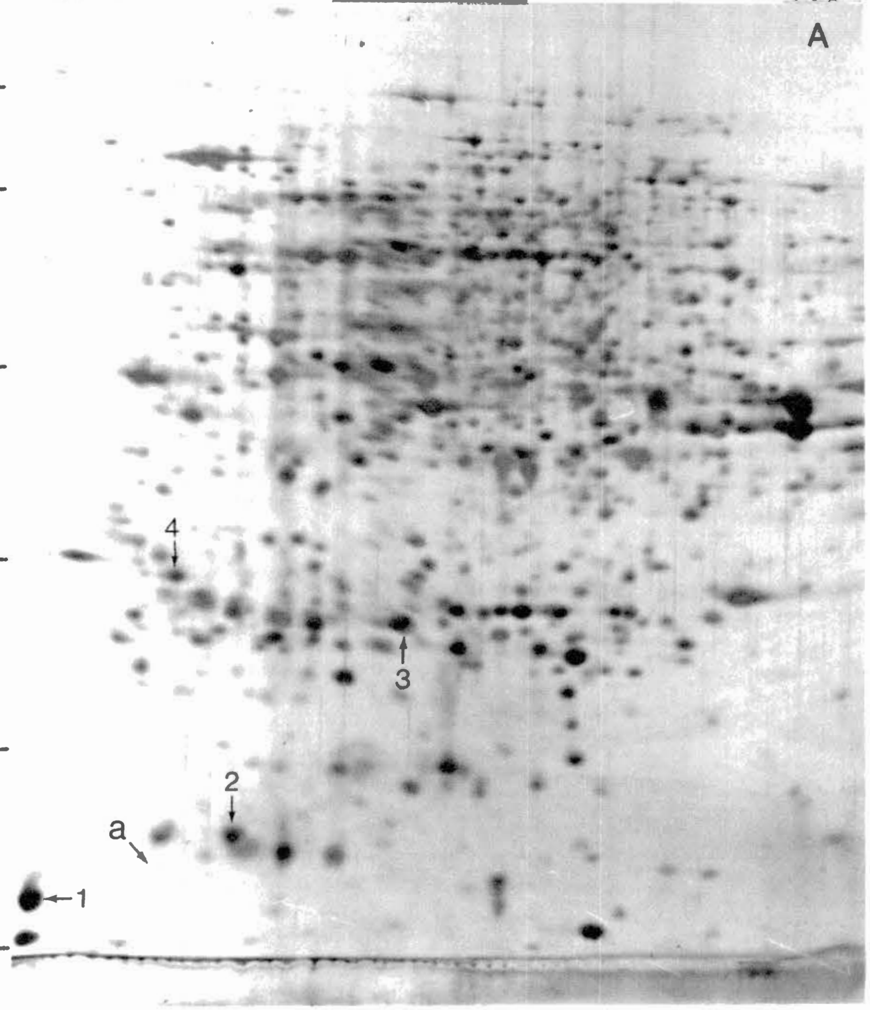
Presence of alumitin in Cu and Cd treated seedlings

Silver-stained 2D-gels of Al-treated cytoplasmic root tip proteins of cv Victory showed the induction of alumitin, which has a pI of 5.2 and a molecular weight of 18.5 kD (Figure 2). Alumitin is identified by the lower case letter a in the acidic, low molecular weight region of the gels. Proteins labeled with the numbers 1,2,3, and 4 are used as a reference proteins to help to locate alumitin. Figures 3A and 3B show that a protein with the same pI and molecular weight of alumitin was induced after 24-h pulse of either 8 μM Cu or 100 μM Cd. Cadmium treatment also induced other acidic low molecular weight proteins (Figure 3B). These could represent low molecular weight HSPs induced by cadmium (Edelman et al., 1988). On the other hand, copper treatment seems to cause a decrease or disappearance of many proteins in this region of the gel (Figure 3A).

Low pH experiments

To determine the [H^+] concentration sufficient to inhibit wheat root growth, four-day-old seedlings were grown for 24h in nutrient medium in which the pH was adjusted to 4.1, 3.8 and 3.5. At pH 4.1 and 3.8 no inhibition of root growth was observed (Figure 4A). [H^+]-toxicity was evident when the pH of the nutrient solution was 3.5; the primary root was inhibited by 88% compared to seedlings growing at

Figure 2. 2D-PAGE gels showing that a major new protein, tentatively called alumitin, is elicited in the cytoplasmic fraction of roots of wheat cv Victory during aluminum toxicity. (A) Control; (B) treatment for 24 h with 80 μM Al. The letter "a" denotes alumitin protein in the acidic, low molecular weight region of the gel. Numbers 1,2,3, and 4 identify reference proteins.


4.8
IEF
7.5
A
97.4
66.2
41.0
31.0
21.5
14.4
Mr
4
3
2
a
1

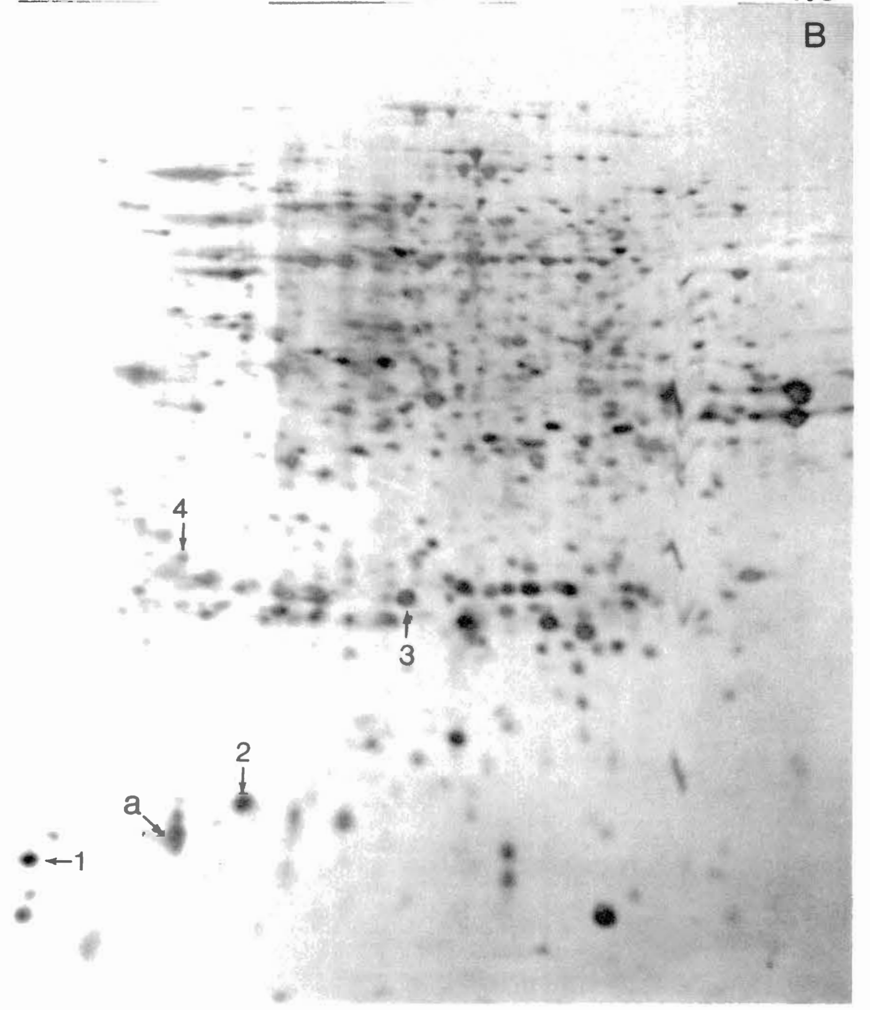
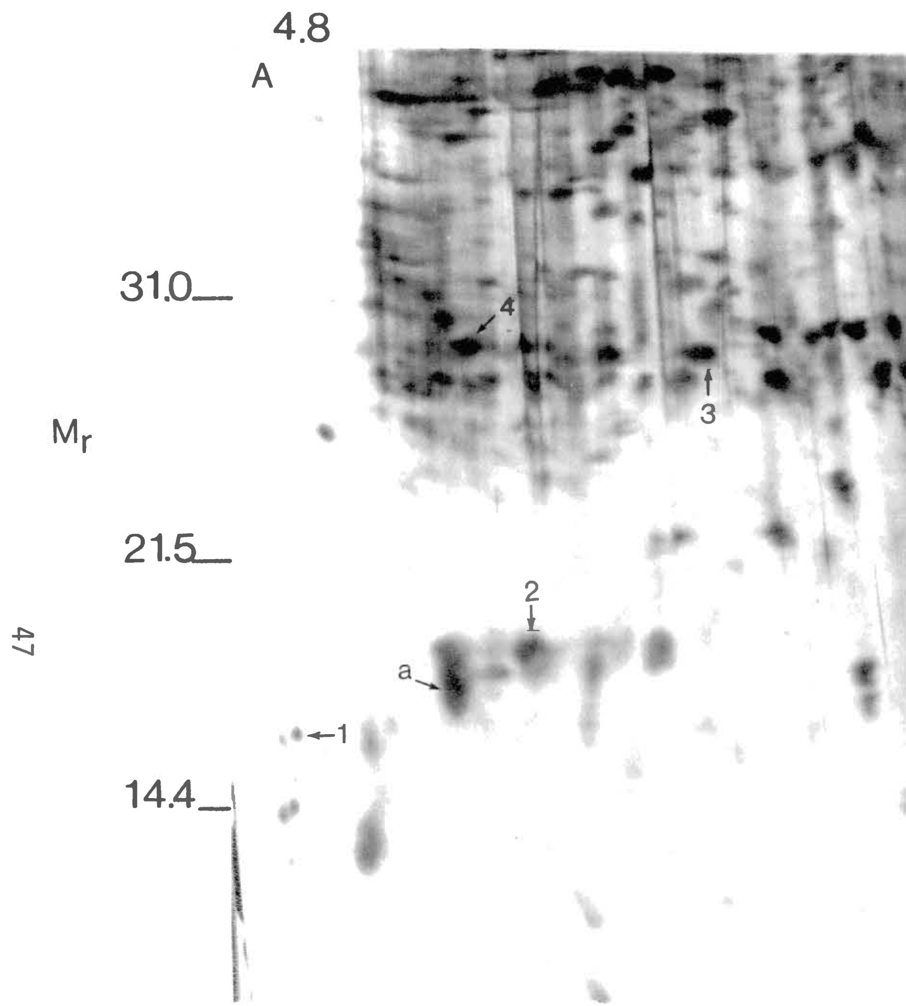
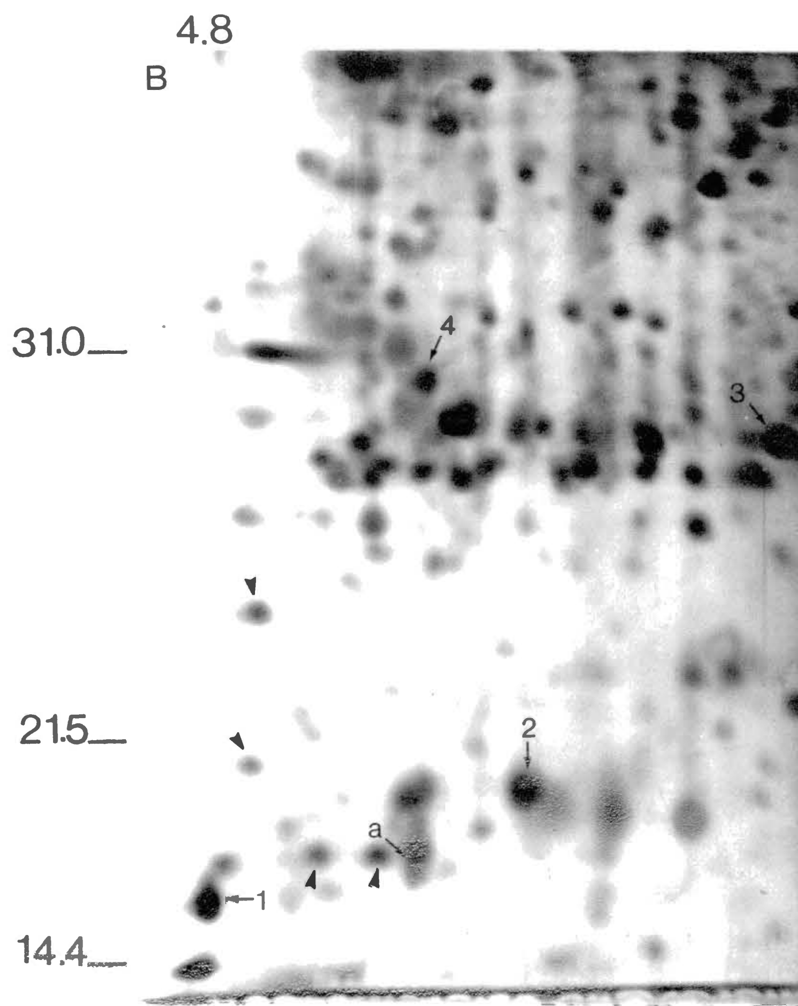

4.8
IEF
7.5
B
4
3
2
a
1

Figure 3. 2D-gels showing the effects of 24 h of 8 μM Cu and 100 μM Cd on the appearance of alumitin in roots of 4-day-old seedlings of wheat cv Victory. (A) Copper; (B) Cadmium. The letter "a" identifies alumitin in each gel. Numbers denote reference proteins used in locating alumitin. Triangles in (B) denote putative Cd induced proteins. See Figure 2A for example of a gel showing proteins from control seedlings.


4.8
A
31.0
4
3
Mr
21.5
2
a
1
14.4


4.8
B
31.0
4
3
21.5
2
a
1
14.4

pH 4.4 (control). Proteins from root tips of seedlings grown in nutrient medium at pH 3.5 were thus extracted and separated by 2D-PAGE.

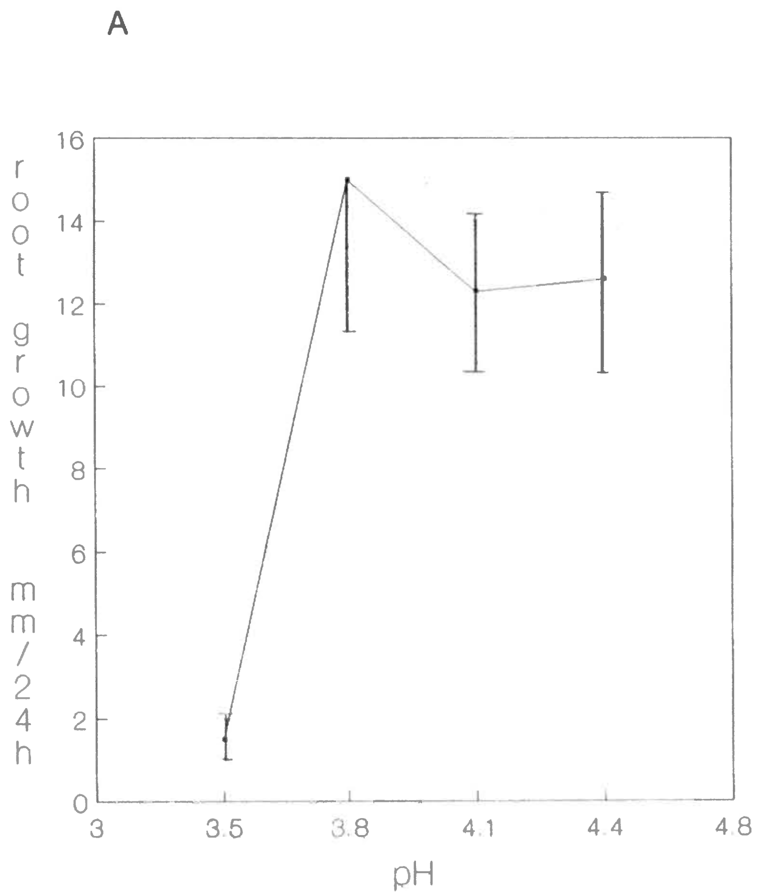
2D-PAGE showed that seedlings exposed to pH 3.5 for 24h produced a protein with the same molecular weight and pI of alumitin, although it appeared to be less abundant than in Al-treated seedlings. On the other hand, pH 3.5 caused the disappearance of several other low molecular weight proteins (Figure 4B). In another wheat cultivar, TAM-W101, alumitin was not synthesized when seedlings were grown for 24h in a pH 3.5 nutrient medium (data not shown). The results with low pH seem to represent a wheat cultivar-dependent response.

Calcium Deficiency Experiments

Elimination of calcium for 24h from the nutrient medium, did not inhibit root growth (Figure 5A). Addition of 6 mM and 8 mM of the chelating agent EGTA inhibited root growth by 60% as compared to the control plants grown with $CaCl_2$ (Figure 5A).

Root tips of seedlings grown for 24h in nutrient medium lacking calcium and containing 6mM EGTA were used for protein extraction and purification. Alumitin was not synthesized when wheat seedlings were grown in calcium deprivation (Figure 5B). As in the case of copper and low pH treatment, calcium deficiency seems to caused the

Figure 4. Effect of low pH on root growth and on appearance of alumitin in roots of wheat cv Victory. (A) Four-day-old wheat seedlings were transferred for 24 h to a nutrient medium whose pH was varied from 3.5 to 4.4 for 24 h. Each value represents the mean ± S.D. of 8 seedlings whose primary root length was measured at the beginning and end of exposure to low pH. (B) 2D-gel showing that alumitin is induced by pH 3.5. The letter "a" denotes alumitin in the acidic, low molecular weight region of the gel. See Figure 2A for example of a gel showing proteins from control seedlings (pH 4.4).


A
root growth mm/24h
16
14
12
10
8
6
4
2
0
3
3.5
3.8
4.1
4.4
4.8
pH

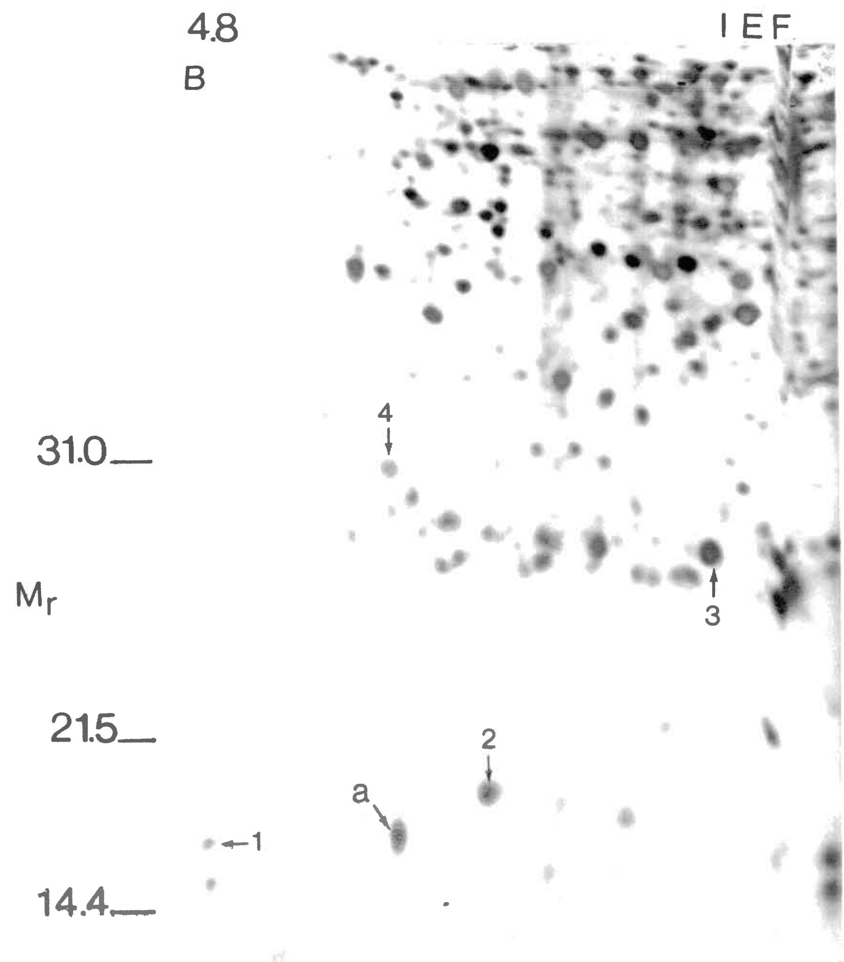
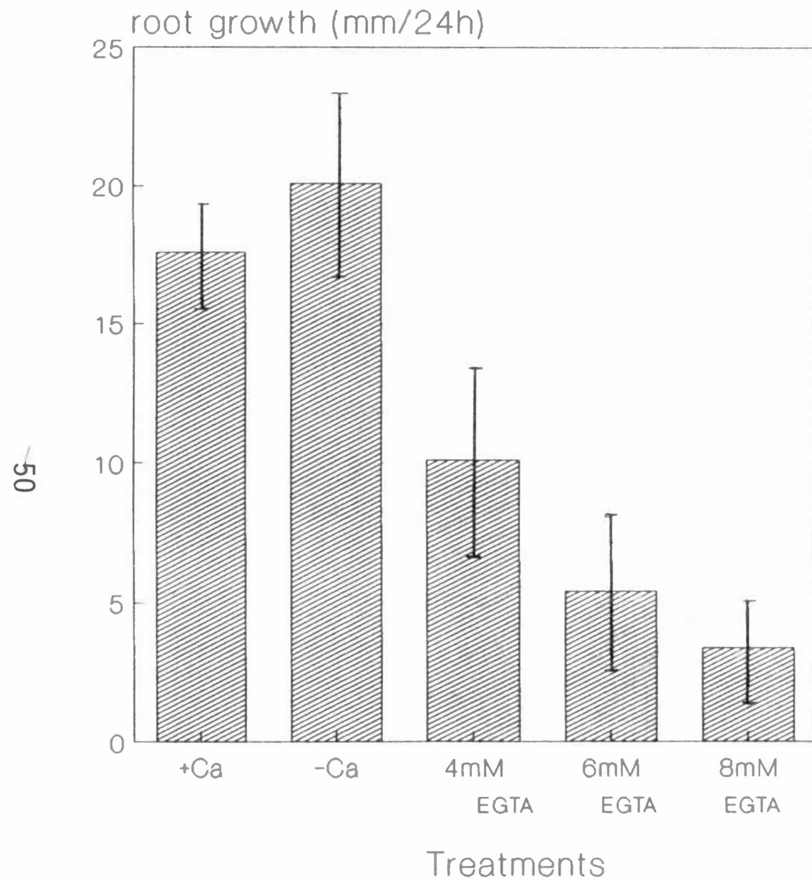
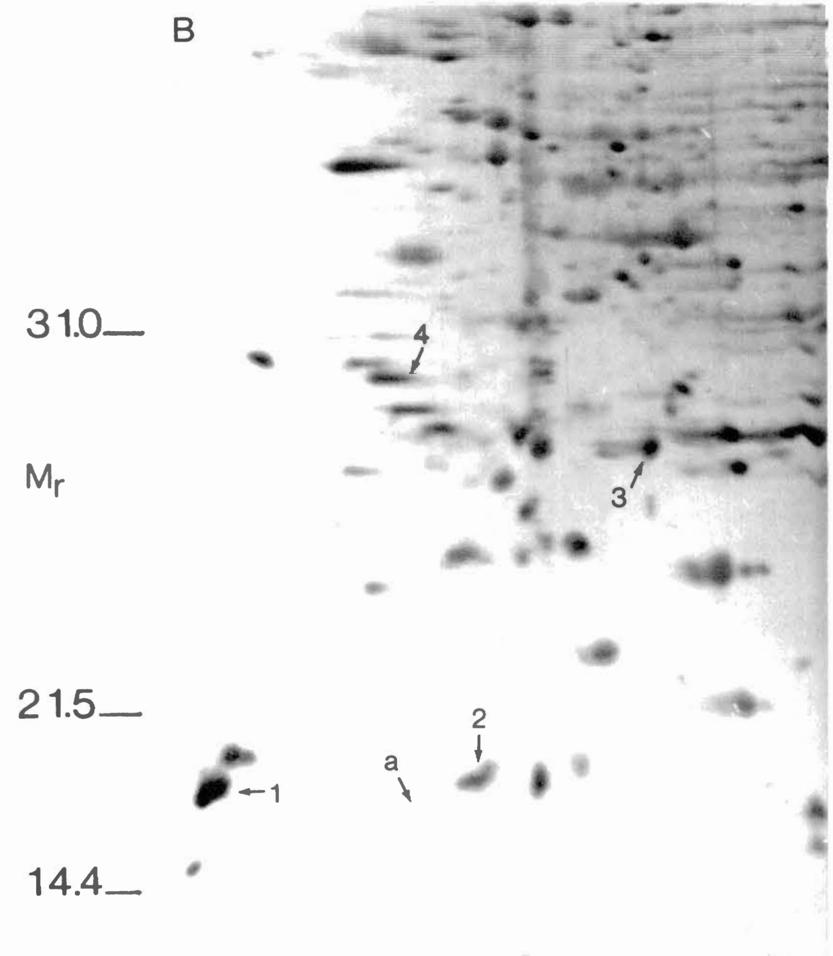

4.8
IEF
B
4
31.0
Mr
3
21.5
2
a
1
14.4

Figure 5. Effects of calcium deficiency on the root growth and on the appearance of alumitin in roots of wheat cv Victory. (A) Four-day-old seedlings were transferred to nutrient medium and grown for 24 h with no Ca and 6 mM EGTA. Each value represents the mean ± S.D. of 8 seedlings whose primary root length was measured at the beginning and end of the treatments. (B) 2D-gel showing that alumitin is not induced in roots grown in calcium starvation. See Figure 2A for example of a gel showing proteins from control seedlings (Ca-grown).


A
root growth (mm/24h)
25
20
15
10
5
0
+Ca
-Ca
4mM EGTA
6mM EGTA
8mM EGTA
Treatments


B
31.0
M_r
21.5
14.4
1
2
3
4
a

disappearance of some low molecular weigth proteins (Figure 5B).

<u>Alumitin and Heat Shock.</u>

Sublethal heat shock has been thought to provide thermotolerance (Lin and Key, 1984) and protection against metal toxicity (Orzech and Burke, 1988). A time-course root growth experiment was done to determine if heat shock at 37°C for 4h or 24h provide protection against aluminum toxicity. Table I shows that a heat shock pre-treatment did not protect wheat seedlings from Al-toxicity. Primary root growth of seedlings exposed to a 24h pulse of 80 μM Al after 4h or 24h 37°C heat shock pre-treatment were inhibited as much as the ones treated only with 80 μM Al. It should be noted that the root growth measurements in the experiments involving 24h heat shock pre-treatment were done after the 24h Al-treatment. Root regrowth was not observed at 24h or 48h of recovery with either 4h or 24h heat shock pretreatment (Table I).

To determine if heat shock induces alumitin, proteins were extracted from root tips of four-day-old seedlings exposed to 4h or 24h heat shock, and from seedlings pretreated with 4h or 24h heat shock and then exposed to a 24h pulse of 80 μM Al. There was an accumulation of a number of the acidic low molecular weight proteins induced by heat shock (see arrows in Figure 6 and Figure 7). Along

TABLE I

EFFECTS OF 4-H OR 24-H 37°C HEAT SHOCK, PRETREATMENT FOLLOWED BY 24-H PULSE OF 80 μM AL ON WHEAT CV VICTORY PRIMARY ROOT

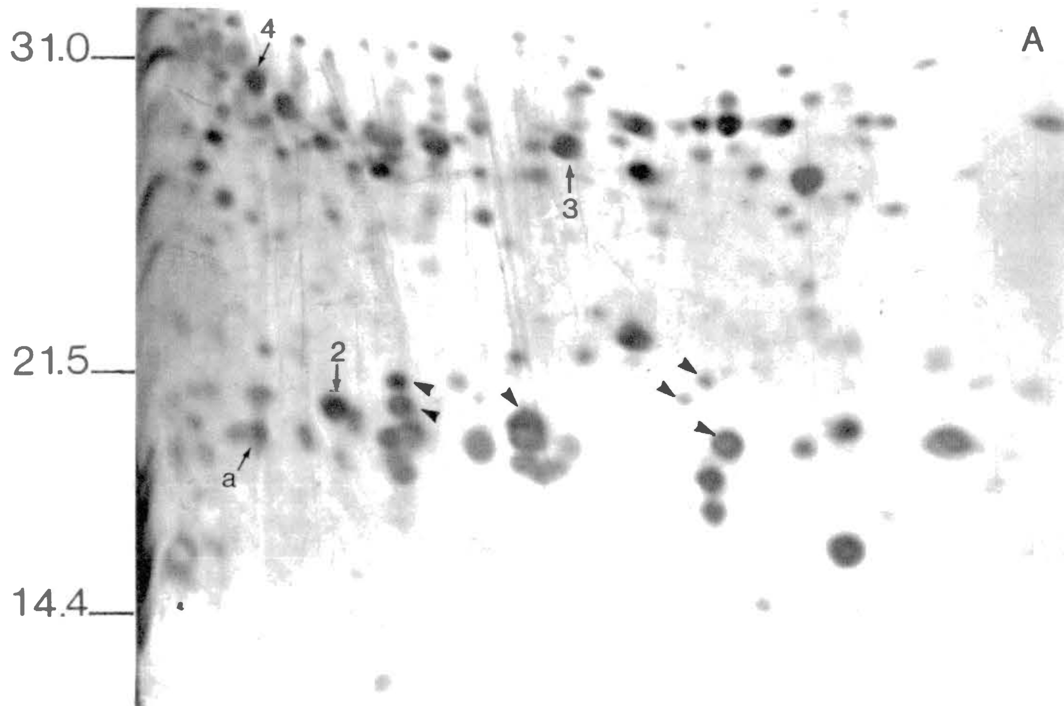
	Net Root Growth (mm)								
	24h after[b] treatment			Recovery Time[c] 24 h			48h		
HS[a]	0h	4h	24h	0h	4h	24h	0h	4h	24h
-Al	18.0±3	16.0±2	3.1±.8	18.9±2	20.6±3	9.6±1	32.3±3	35.4±4	16.6±3
+Al	4.2±.8	2.6±.9	1.4±.7	1.3±.6	0.6±.6	0.6±.5	1.9±1	2.1±.7	0.7±.5

[a] HS= heat shock pretreatment
-Al= control plants
+Al= 80 μM Al

[b] Seedlings were exposed to Al at 26°C during this 24h period.

[c] Seedlings were transferred to fresh, Al-free nutrient medium at 26°C during this time.

Figure 6. 2D-gels showing the effects of 4 h of 37 °C heat shock alone, and 4 h 37 °C heat shock pretreatment followed by 24 h 80 μM Al, on the appearance of alumitin in roots of wheat cv Victory. (A) Heat shock (4 h, 37°C). (B) Heat shock pretreatment (4 h, 37°C) followed by 24 h exposure to 80 μM Al. The letter "a" denotes alumitin in each gel. Numbers are the reference proteins used to locate alumitin. Triangles denote putative heat shock proteins. See Figure 2A for comparison with a gel showing proteins from control (no heat shock or Al) seedlings.


31.0
21.5
14.4
A
4
3
2
a

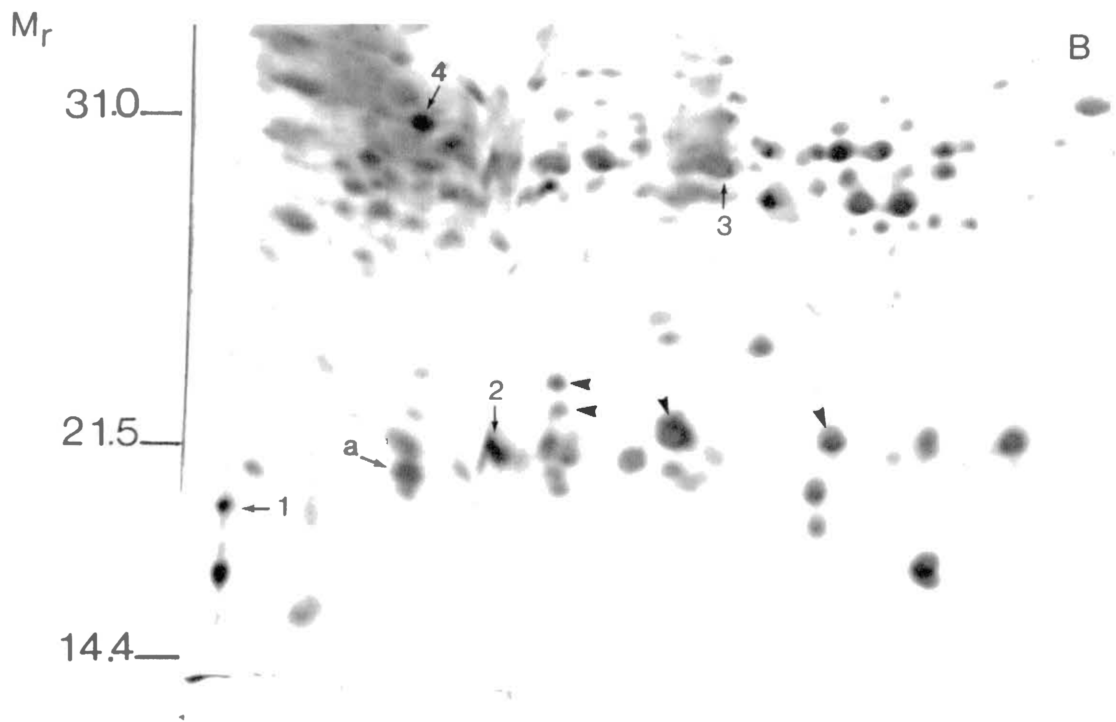
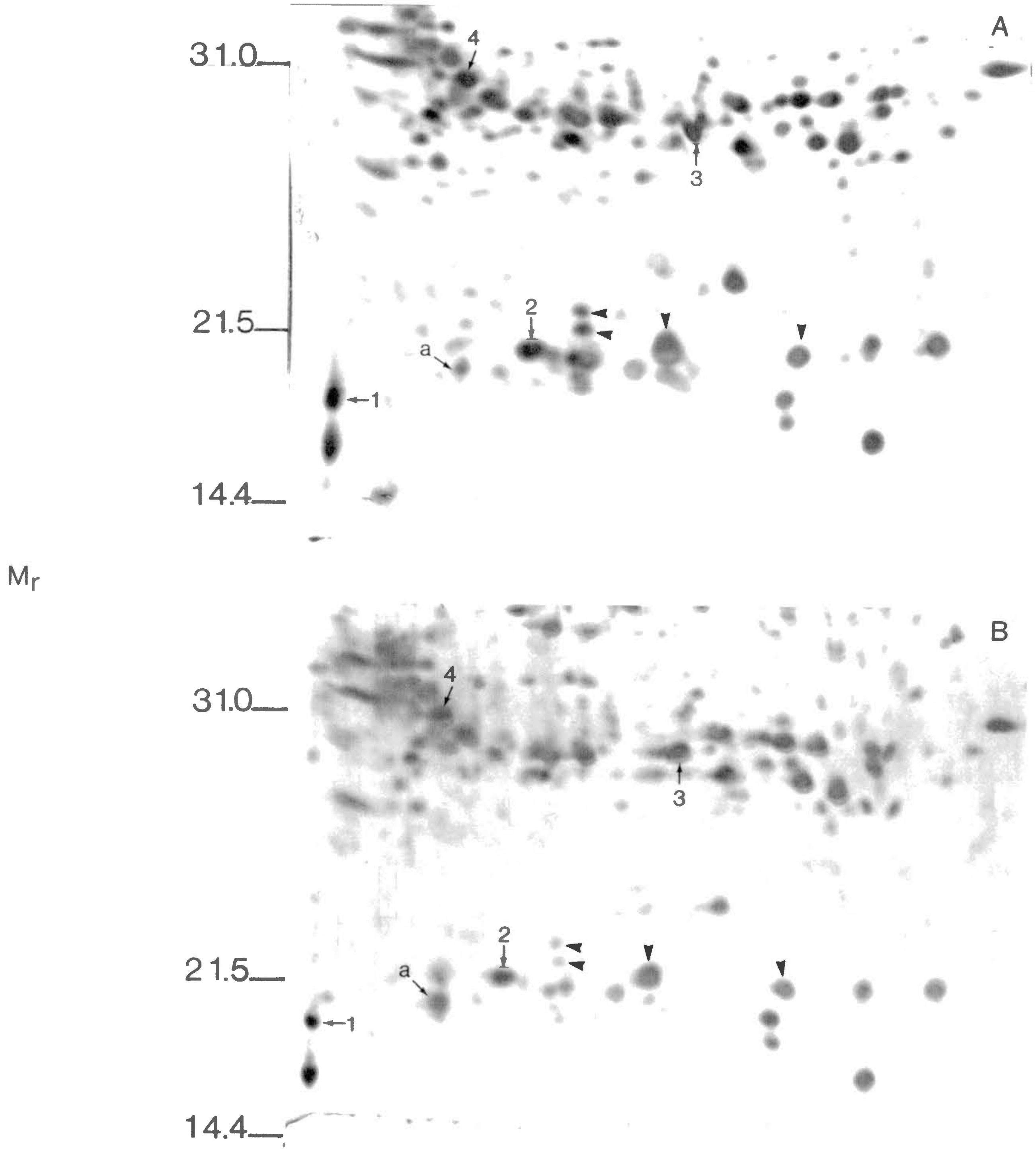

Mr
31.0
21.5
14.4
B
4
3
2
a
1

Figure 7. 2D-gels showing the effects of 24 h, 37 °C heat shock alone, and 24 h 37 °C heat shock pretreatment followed by 24 h 80 μM Al, on the appearance of alumitin in roots of wheat cv Victory. (A) Heat shock (24 h, 37 °C). (B) Heat shock pretreatment (24h, 37 °C) followed by 24 h of 80 μM Al. The letter "a" denotes alumitin in each gel. Numbers are the reference proteins used to locate alumitin. Triangles denote putative heat shock proteins. See Figure 2A for comparison with a gel showing proteins from control (no heat shock or Al) seedlings.


A
31.0
4
3
21.5
2
a
1
14.4
Mr
B
31.0
4
3
21.5
2
a
1
14.4

with these, a protein with characteristics similar to that of alumitin was visualized in both the 4 h and 24 h heat shock (Figure 6A and Figure 7A). However, alumitin is noticeably more abundant in the heat shock/aluminum treatments (Figure 6B and Figure 7B).

Al Toxicity in Triticale and Rye.

Aluminum toxicity was measured in triticale and rye to determine if alumitin is induced by levels of Al sufficient to inhibit growth in cereals other than wheat. The effects of different levels of Al on the primary root growth of both triticale and rye are shown in Figure 8. Rye and triticale have been reported as more Al-tolerant than wheat, the former being the most tolerant (Aniol and Gustafson, 1987). This observation was confirmed with the results of these experiments. Neither rye (cv Elbon) seedlings nor triticale seedlings (cv T-23) showed any inhbition with 24h 4 μM or 20 μM Al. When seedlings were exposed to 24h 40 μM Al, rye showed 17% of root growth inhibition, while root seedlings of triticale were inhibited by 53%. However, when seedlings were treated with 100 μM Al for 24h, both rye and triticale seelings showed a similar significant inhibition of 70 %.

Figure 9 shows silver-stained 2D-gels of cytoplasmic proteins from root tips of four-day-old triticale seedlings, control and after treatment with 100 μM Al for 24h. A trace of alumitin appeared to be induced by Al toxicity in

Figure 8. Determination of the level of Al required to inhibit root growth of triticale (*XTriticosecale*, cv T-23 and rye (*Secale cereale* cv Elbon). Four-day-old seedlings were treated with various Al concentrations for 24 h. Each value represents the mean ± S.D. of 8 seedlings whose primary root length was measured at the beginning and end of exposure to Al.

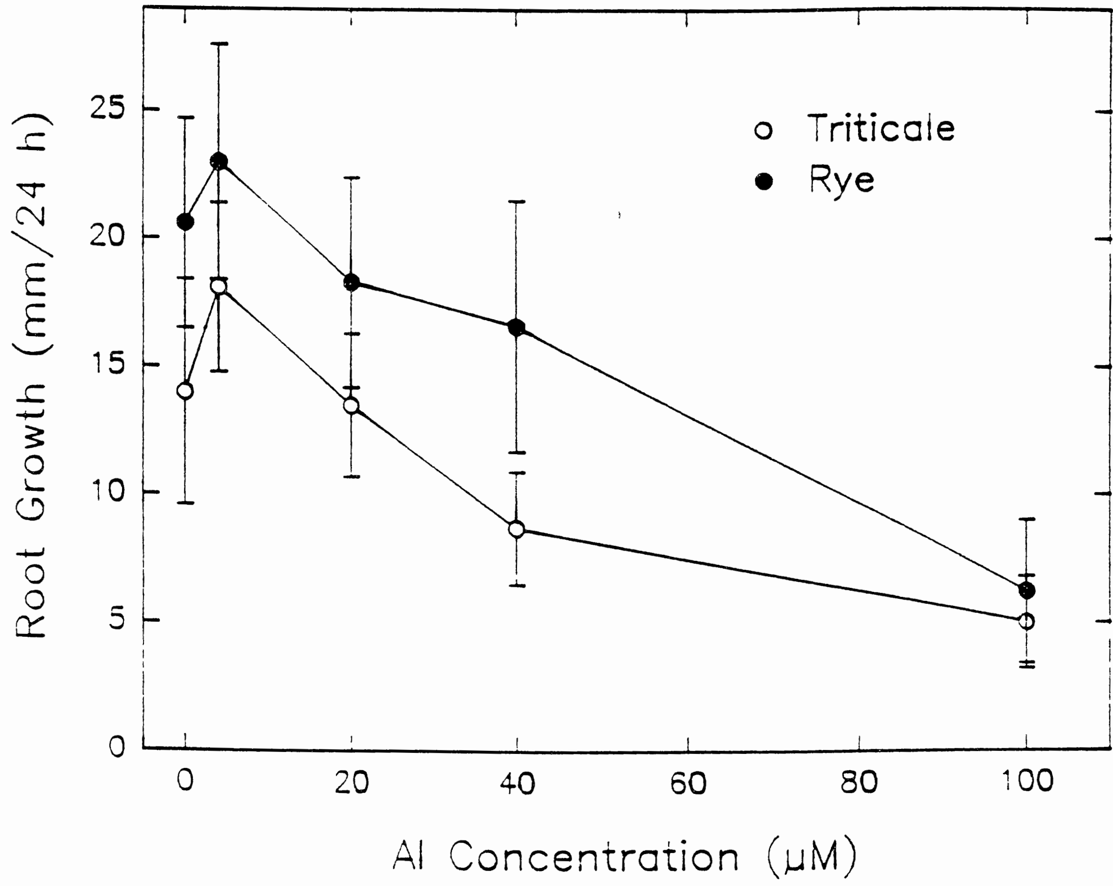
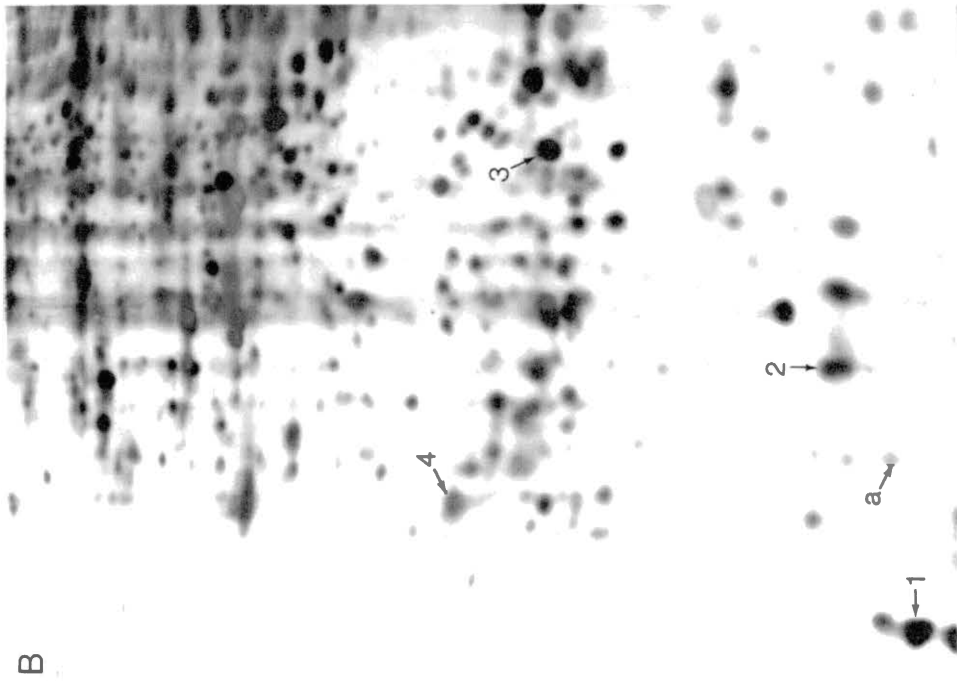

Root Growth (mm/24 h)
0
5
10
15
20
25
Triticale
Rye
0
20
40
60
80
100
Al Concentration (µM)

Figure 9. 2D-gels of cytoplasmic proteins from root tips of triticale. Four-day-old seedlings were treated with 100 μM Al for 24 h. (A) Control; (B) Al treated. The letter "a" denotes the position of alumitin protein. Numbers denote the same reference proteins identified in wheat.


B
1
a
2
3
4

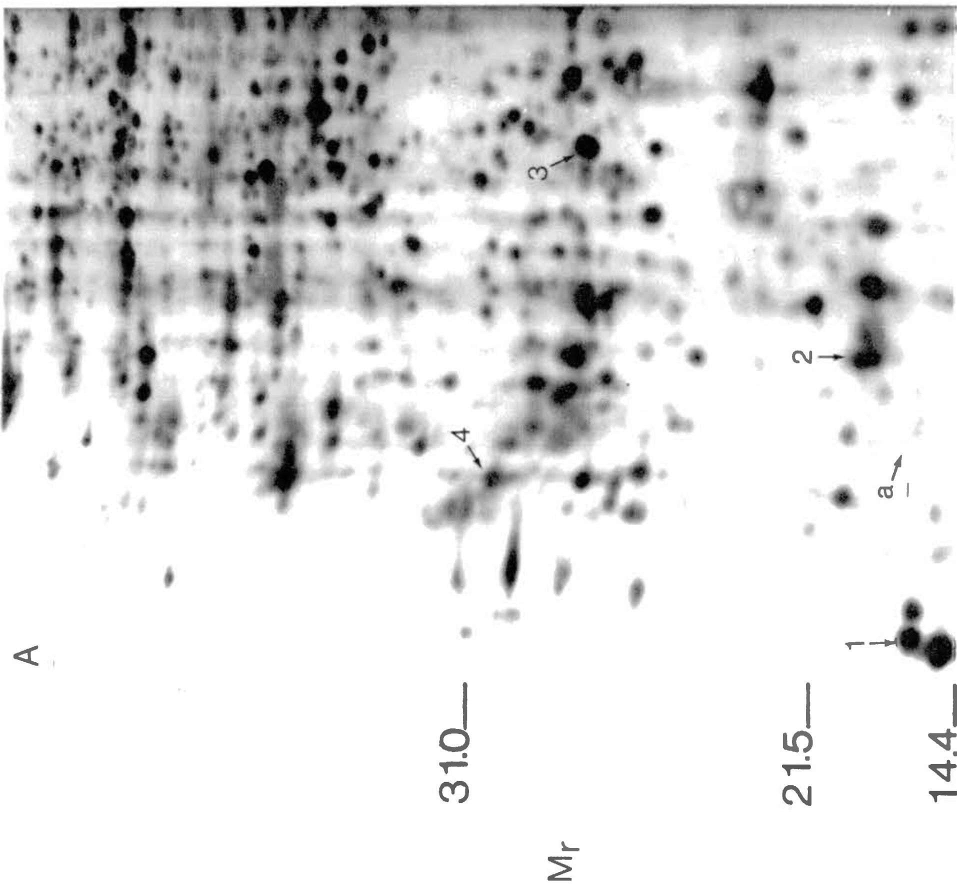
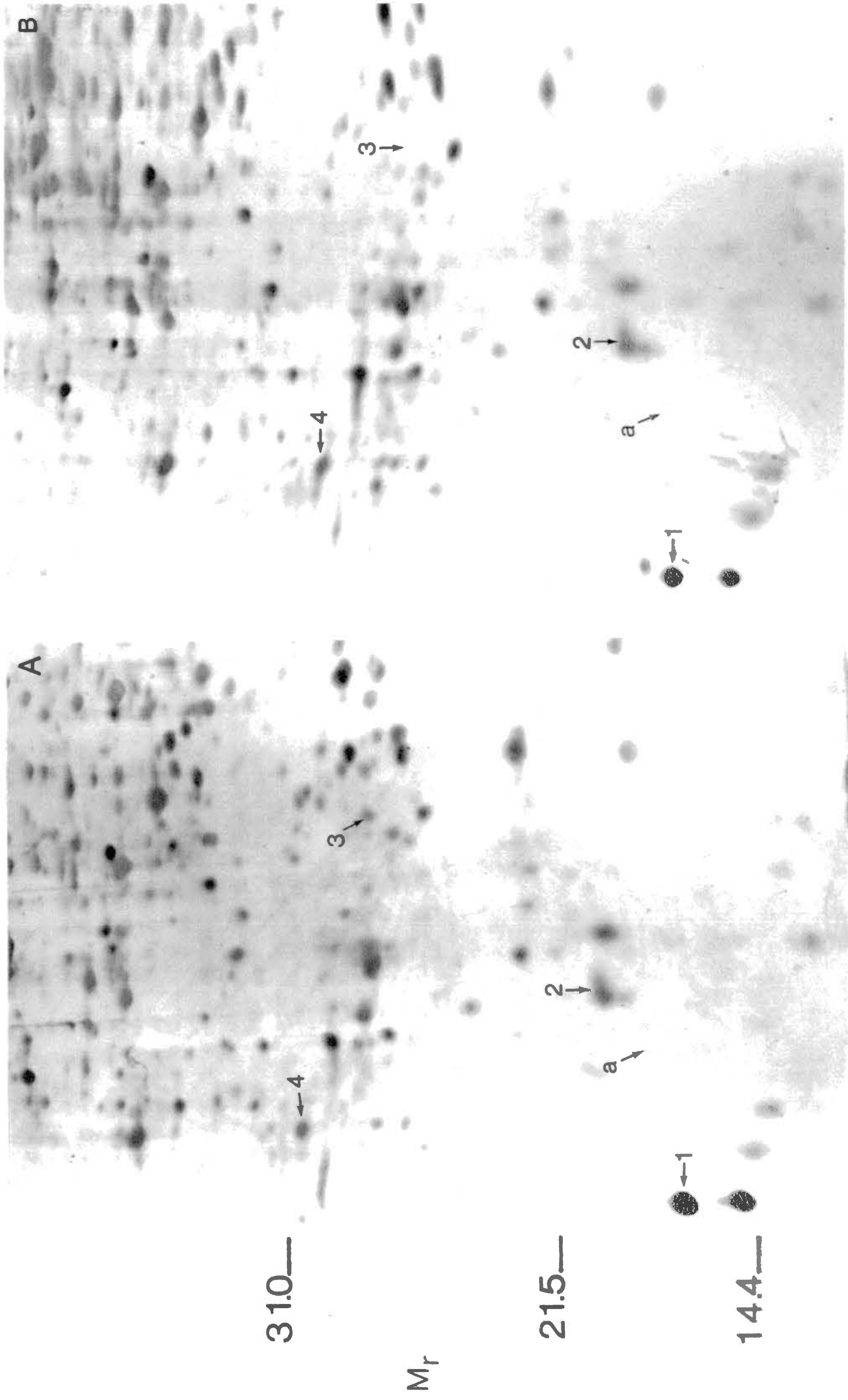

A
1
a
2
3
4
31.0
21.5
14.4
Mr

Figure 10. 2D-gels of cytoplasmic proteins from root tips of rye. Four-day-old seedlings were treated with 100 μM Al for 24 h. (A) Control; (B) Al treated. The letter "a" denotes the position of alumitin protein. Numbers denote the same reference proteins identified in wheat.


A
B
31.0
21.5
14.4
Mr
1
2
3
4
a

triticale roots (Figure 9B). Protein changes were also observed in roots treated with aluminum (see arrows in Figure 9B). On the contrary, alumitin was not induced under conditions of Al tosicity in four-day-old rye seedlings as determined by 2D-PAGE analysis (Figure 10-B).

Synthesis of alumitin

To examine the effects of aluminum on protein synthesis wheat roots seedlings, ^{35}S-Trans-label uptake and incorporation into proteins were measured in a time-course experiment. Tables II shows the total ^{35}S uptake in the different cellular fractions (nuclear, microsomal and cytoplasmic fraction), and the percent of total ^{35}S incorporation into protein cellular fractions. The highest ^{35}S uptake were observed in the cytoplasmic fraction. This ^{35}S uptake decreased when the seedlings were treated with aluminum. At 24 h aluminum treatment ^{35}S uptake was inhibited by 70% from ^{35}S uptake of the control. On the other hand, aluminum decreased the ^{35}S incorporated in cytoplasmic fraction protein, but increased the incorporation of ^{35}S into microsomal fraction proteins (fraction that contains organelles and membrane systems in general). ^{35}S incorporation were increased from 19% in the control, to 42% at 24h Al (Table II). This results are in agreement with those of Matlock and Ownby (1988). They observed also that Al stress reduced incorporation of ^{35}S into total cytoplasmic

protein, and increased the incorporation into microsomal fraction.

Fluorographs of protein synthesis in microsomal fraction show that although aluminum ehanced or decreased some proteins in this fractions (arrows in Figure 13), there was no a major change in the pattern of protein synthesis that could account for the increased in the ^{35}S-protein incorporation. The specific activity was higher in roots treated with Al for 24h (51,203 cpm/μg) compared with the specific activity of the control (15,479 cpm/μg).

Fluorographs of proteins synthesized during 0-3h, 3-6h, 6-12h, and 12-24-h after the onset of Al treatment are shown in Figures 11 and 12. Unexpectedly, alumitin appeared to be synthesized in control roots (Figure 11A) although it was not visualized in silver-stained gels. By 6h of Al treatment, alumitin began to be more evident (Figure 11B), reaching its peak of synthesis at 12h of Al-treatment (Figure 12A). At 24h the level of alumitin synthesis was similar to that of 6-h (Figure 12B).

TALE II

TOTAL ^{35}S-TRANS-LABEL AND PERCENT OF TOTAL ^{35}S INCORPORATED INTO PROTEINS IN THE THREE CELLULAR FRACTIONS

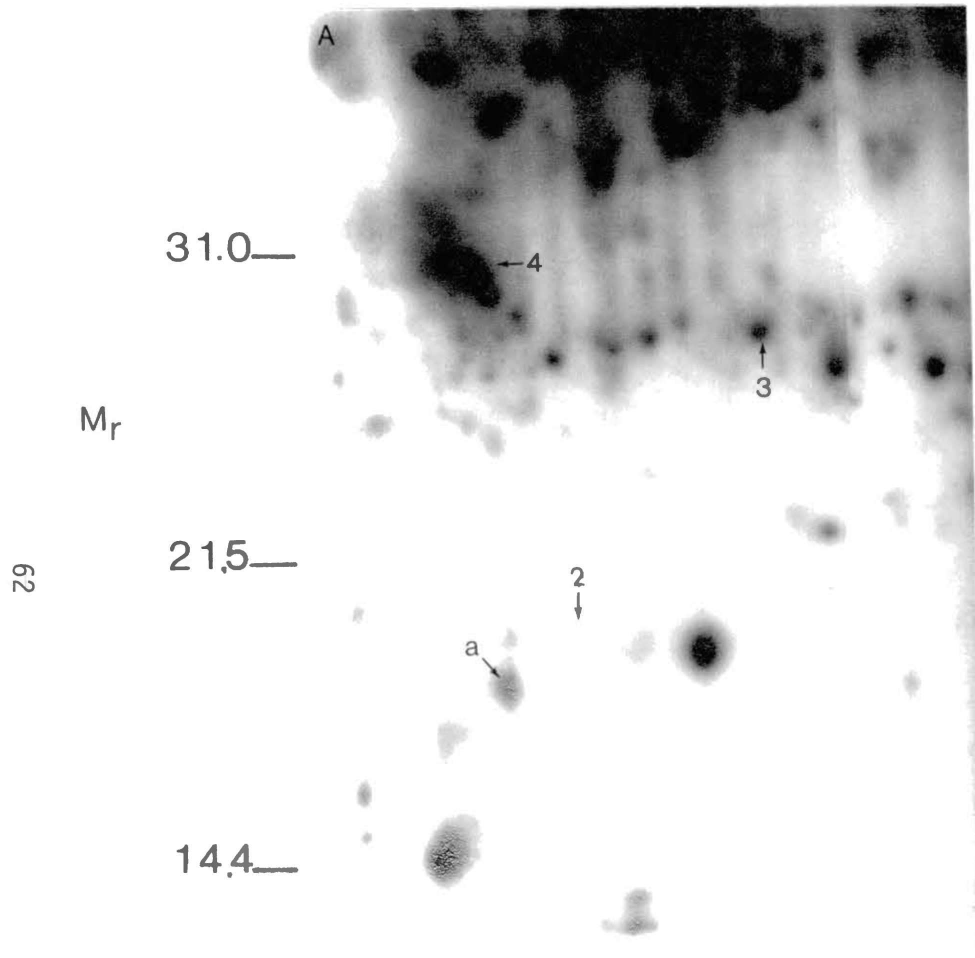
Treatments	Total ^{35}S uptake (cpm 10^{-6})				% of total ^{35}S incorporated into proteins[a]			
	Cellular fractions				Cellular fractions			
	Nuc[b]	Ms[b]	Cyt[b]	Σcpm	Nuc[b]	Ms[b]	Cyt[b]	Σcpm
Control	5.5	11.7	66.3	83.5	3.9	18.6	19.0	41.5
6h Al[c]	5.5	16.8	34.7	57.0	5.9	26.7	22.0	54.6
12h Al[c]	6.3	27.4	40.9	74.6	6.6	33.0	13.8	46.8
24h Al[c]	5.5	19.1	20.0	44.6	8.5	41.4	11.8	62.1

[a] Each value represents that fraction of total ^{35}S taken up which was incorporated into the TCA-precipitable material of each cellular fraction.

[b] Nuc: nuclear fraction; Ms: microsomal fraction; Cyt: cytoplasmic fraction.

[c] Al: 80 μM; ^{35}S label was applied during the last 3h of each Al treatment.

Figure 11. Fluorographs of proteins synthesized during a time-course of Al-treatment. Four-day-old seedlings were grown in the presence of 80 μM Al for 0, or 6 h; during the last 3 h of each treatment the seedlings were labelled with Trans^{35}S-Label. 2D-gels containing protein equivalent to $1.75 \cdot 10^5$ cpm were dried and exposed to X-ray film for 3 days. (A) Control; (B) 6h Al. The letter "a" denotes alumitin in each gel. Numbers are the same reference proteins identified in silver-stained gels.


A
31.0
21.5
14.4
M_r
4
3
2
a

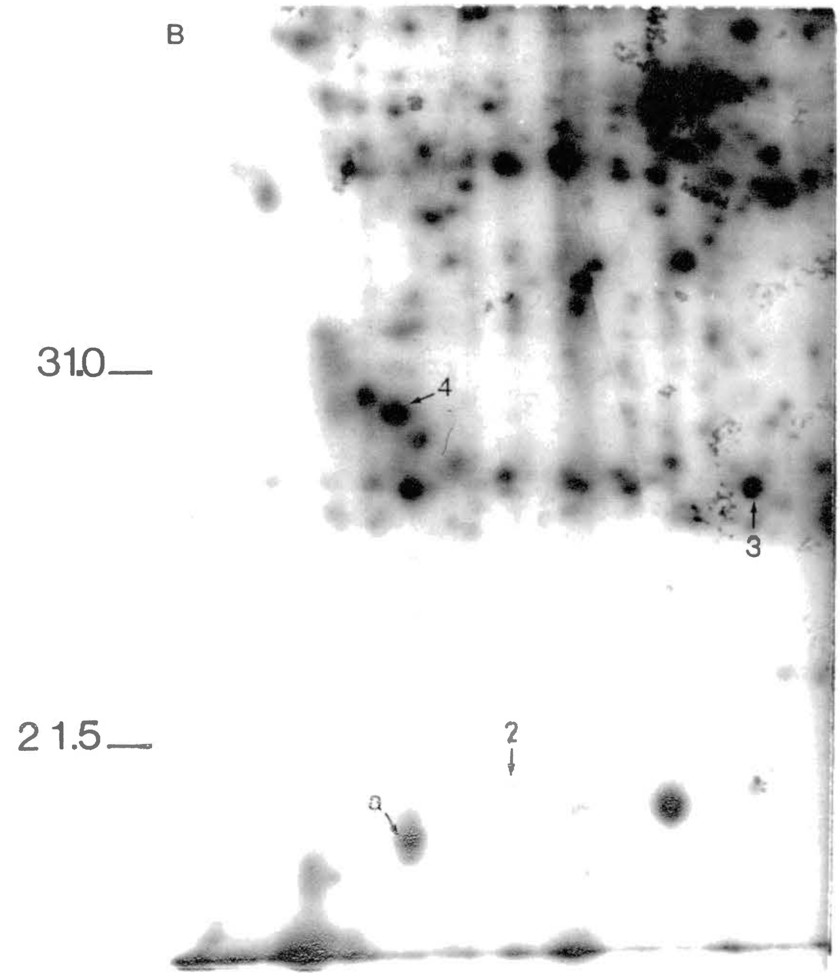
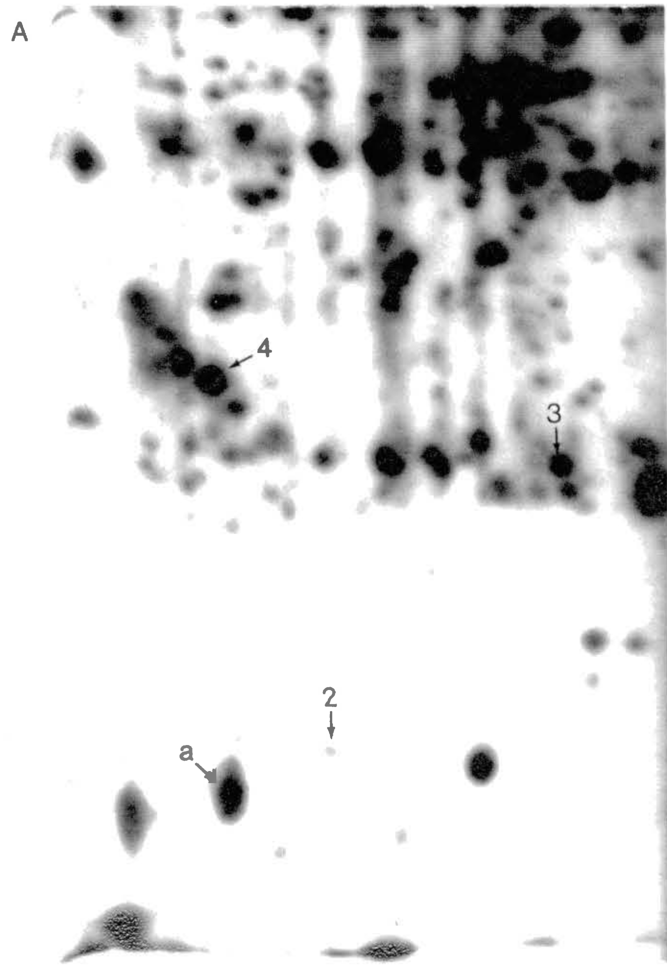

B
31.0
21.5
4
3
2
a

Figure 12. Fluorographs of proteins synthesized during a time-course of Al-treatment. Four-day-old seedlings were grown in the presence of 80 μM Al for 12 and 24 h; during the last 3 h of each treatment the seedlings were labelled with Trans^{35}S-Label. 2D-gels containing protein equivalent to $1.75 \cdot 10^5$ cpm were dried and exposed to X-ray film for 3 days. (A) 12 h; (B) 24 h Al. The letter "a" denotes alumitin in each gel. Numbers are the same reference proteins identified in silver-stained gels. See Figure 11A for comparison with a fluorograph showing proteins from control.

63



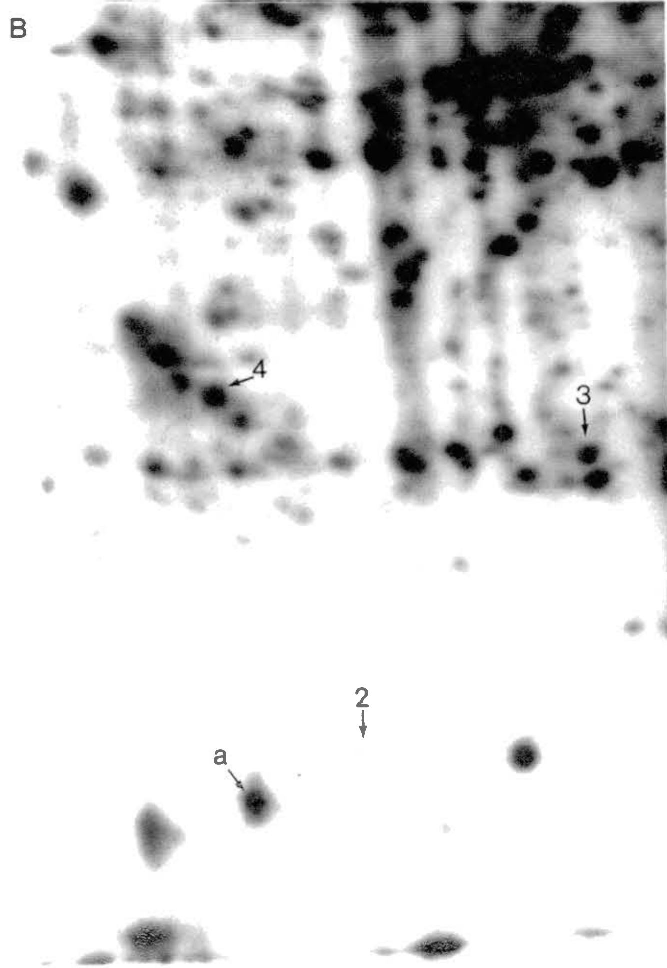


Figure 13. Fluorographs of proteins synthesized in the microsomal fraction during treatment with 80 μM Al for 24 h. (A) Control; (B) 24 h Al treatment. ^{35}S label was applied during the last 3 h of Al treatment.

A

B

CHAPTER V

DISCUSSION

Higher plants are subjected to a large number of environmental and biological stresses. These adverse stress factors produce alterations of gene expression resulting in the induction of specific new proteins, as well as enhancement and/or repression of other normally expressed proteins (Sachs and Ho, 1986). Previous studies showed that induction of alumitin protein represents the most consistent and noticeable change in the pattern of proteins synthesized in wheat roots in response to aluminum toxicity (Ownby and Hruschka, 1991). One objective of the work reported here was to determine if alumitin had properties that might enable it to be used as a biological marker for aluminum toxicity.

The results presented in this study show that: 1) alumitin is induced in wheat roots, not only by aluminum toxicity, but also by growth-limiting levels of Cu (8μM), and Cd (100μM). 2) Small amounts of alumitin are associated with low pH, and 4h or 24h heat shock, 3) it is not induced when wheat seedlings are subjected to calcium starvation. 4) It is induced in triticale but not in rye

under aluminum toxicity levels, and 5) Its peak of synthesis coincide with the time that root growth is arrested.

Results of root growth experiments showed that Al-sensitive wheat cv Victory presented differential sensitivity to aluminum, copper, and cadmium. Figure 1 shows that cv Victory was most sensitive to copper. Concentrations as small as 4.8 μM and 8.0 μM caused a 95% inhibition on the root growth, while similar levels of aluminum and cadmium (5 μM and 8 μM respectively) produced 46% of root growth inhibition. 40 μM Al and 100 μM Cd produced the same root inhibition of 85%, indicating that there is also a difference in sensitivity between Al and Cd. The observation that cv Victory differs in sensitivity to these three metals is in agreement with the study of Foy et al., (1973), where they suggested that tolerance to one metal in a given plant does not necessarily mean tolerance to another. They found that Al-sensitive wheat cv Monon was more tolerant to excess Mn than Al-tolerant cv Atlas 66.

Both heavy metals Cu and Cd at different levels induced in wheat roots a protein (Figures 3A and 3B) with the same molecular weigth and pI of alumitin that was induced by aluminum toxicity (Figure 2B). This result indicates that alumitin is not specifically induced by aluminum. The three metals, Al, Cu, and Cd are growth-limiting factors present in acidic soil. Their phytotoxicity involve a large number

of physiological and biochemical alterations within the affected cell. Thus if alumitin is induced by these three different metals, alumitin could represent a common physiological response to these stress factors. Because there are many toxicity mechanisms common to aluminum, copper, and cadmium (Taylor, 1988b; Jackson et al., 1990), it would be difficult to ascribe a particular function to alumitin.

Alumitin is induced only when root growth has been arrested. It has been observed that Al and Cu disrupt membrane structure and function (Zhao et al.,1987; Wainwright and Wouldhouse, 1973) and that Al and Cd affect DNA and RNA (Matsumoto and Morimura, 1980). Alumitin could be involved in a repair mechanism for membranes or for DNA and/or enzymes. Alternatively, since chelation of Al, Cu and Cd with organic acids (citric and malic acid) has been reported as a tolerance mechanism (Foy et al., 1987; Miyasaka et al., 1991; Kishinami and Widholm, 1987), alumitin might be involved in the biosynthesis or release of these organic acids. Alumitin could also be a metal-binding protein. The fact that it is accumulated at higher levels in response to Al stress fits with Aniol's (1984) hypothesis of an Al-binding protein, and also with the induction of phytochelatins as a cellular response to heavy metals such as Cu and Cd. Alumitin, however, is not induced at sublethal doses of aluminum in Al-tolerant wheat cultivars;

it is only induced at aluminum levels that produced root growth inhibition (Ownby, unpublished results). Thus, it seems more plausible that alumitin is not a binding protein.

Another stress factor that appearred to induce alumitin was heat shock. 4h and 24h 37 °C heat shock, which arrested growth and inhibited mucilage production by the root cap, elicited the typical pattern of low molecular weight putative HSPs, and small amounts of alumitin. In _Pisum sativum_ and _Zea mays_ two low molecular weight HSPs have been identified, having a pI of 5.96 and 5.19 and molecular weight of 18.1 kD and 17.8 kD respectively (Vierling, 1991) which are similar to alumitin. HSPs are synthesized in response to higher temperatures as a mechanism to enable the plant to cope with this stress. If alumitin is a heat shock protein then it could be a binding protein that is synthesized as part of a tolerance mechanism to protect the plant against Al, Cu, or Cd toxicity. Low molecular weight HSPs functions are not known. It has been proposed that heat shock granules (large low molecular weight HSP aggregates) are involved in protection and storage of normal cellular mRNA (Nover et al., 1978). Because of the potential of Al and heavy metals to react with nucleic acids, it is possible that alumitin may be part of such a protective mechanism.

Orzech and Burke (1988) using TTC[5] as a viability test,

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[5] 2,3,5-triphenyltetrazolium chloride

they found that heat shock can provide protection against metals such as Al, Cd, Fe, and in less extent to Cu, but not against Zn in wheat leaf tissue. This observation could not be confirmed in our experiments where we used root-regrowth as a measure of tolerance of wheat roots to aluminum. Heat shock, neither 4h nor 24h seemed to protect wheat roots from aluminum toxicity (Table I).

When wheat seedlings were grown under calcium deprivation (no $CaCl_2$ and 6mM EGTA in the nutrient medium), alumitin was not induced under conditions where inhibition of root growth was observed (Figure 5). If alumitin was induced by calcium deficiency, we might consider that aluminum is interfering with calcium uptake and/or with the physiological roles of calcium such as Ca/Calmodulin regulation. The fact that root growth was inhibited by calcium deprivation, and that alumitin was not induced under this condition indicates that alumitin is not synthesized just as a consequence of Ca deprivation and growth inhibition _per se_, but is synthesized in response to other potentially toxic metals, such as copper and cadmium, besides aluminum.

The root growth experiments with rye (cv Elbon) and triticale (T-23) confirmed that both are more Al-tolerant than wheat cv Victory. When seedlings of rye and triticale were exposed to different levels of aluminum, rye was more tolerant than triticale. As was expected, alumitin was

induced by 100 μM Al in triticale, although in small traces. Alumitin was not induced in rye, even when root growth was arrested by aluminum. Triticale is the hybrid of wheat and rye, has a genome AABBRR, where chromosomes AABB came from the tetraploid wheat parent (AABB) and RR chromosomes from rye parent. These results suggest that the alumitin-encoding gene is specific for wheat and not for other cereals such as rye. This also suggests that in the hexaploid wheat (AABBDD), the gene encoding alumitin is likely to be in chromosomes A or B of the genome.

In a previous study Matlock and Ownby (1988), found that incorporation of ^{35}S-methionine into cytoplasmic proteins of wheat cv Victory decreased from 58% to 19% of total uptake during 24h of Al treatment. They also found that the microsomal fraction showed an increased in incorporation of ^{35}S into protein from 8% to 37% of total ^{35}S-uptake. The present study confirms the results of Matlock and Ownby (1988). In the two experiments, the general observation was that ^{35}S incorporation into proteins decreased in the cytoplasmic fraction and increased in the microsomal fraction. During 24h Al treatment, incorporation of ^{35}S into microsomal proteins increased progressively from 19% to 41% (Table II). Total ^{35}S uptake, moreover, was reduced 48% at 6h Al, 40% at 12h Al and 70% at 24h Al treatment. These results showed clearly that Al inhibited ^{35}S uptake as well as incorporation into cytoplasmic

proteins. In other studies, Hurkman and Tanaka (1987) and Ramagopal (1988) reported that uptake of ^{35}S and incorporation into proteins were significantly reduced in barley roots by salt stress. One explanation of why Al inhibited ^{35}S uptake is the possibility that Al, through the disruption of membrane permeability, might be altering uptake or causing the leakiness of ^{35}S-labelled amino acids and other molecules. Alternatively, Al might be affecting the size of endogenous amino acid pools in the cells. If Al was disrupting membrane permeability, amino acid leakiness from organelles such as mitochondria or plastids could increase the endogenous amino acid cytoplasmic pool, thus less ^{35}S would be observed in proteins of this cellular fraction. Likewise, a small endogenous microsomal amino acid pool could explain the high ^{35}S incorporation into microsomal proteins. The higher incorporation does not necessarily mean more synthesis of proteins, rather it could just mean that the specific activity of the amino acid pool is higher. This could explain the observation that fluorographs of de novo synthesized microsomal proteins of control and 24 h Al treatment did not show major changes in the protein pattern (Figure 13). One interesting observation was the fact that microsomal protein patterns visualized with silver-stain did not correspond with the labelling of proteins observed with fluorography. These results suggest that proteins in the microsomal fraction

(heterogeneous membrane-containing fraction) seem to turn over at different rates. While some proteins may be turning over rapidly, others may be doing so more slowly. On the other hand, cytoplasmic proteins seem to turn over at the same time: fluorographs of labelled cytoplasmic proteins matched the pattern of silver-stained proteins.

In the study of the time course of aluminum synthesis, it was found that small amounts of alumitin were synthesized in control roots. A rapid increase in biosynthesis occurred between 3 to 6 h after the plants were treated with aluminum. The peak of synthesis was at 12 h aluminum treatment, and alumitin synthesis began to decrease at 24 h. The fact that alumitin is synthesized in control roots shows that alumitin is a protein synthesized normally, but does not accumulate at levels to be visualized by silver-stained. Some high molecular weight HSPs are likewise found at significant levels in normal, nonstressed cells; they increase their expression in response to elevated temperatures (Lindquist, 1986; Lindquist and Craig, 1988). Low molecular weight HSPs, however, are not expressed at detectable levels in leaves of plants grown at optimal temperatures (Mansfield and Key, 1987; Chen et al., 1990). Osmotin, 26 KD protein that accumulates in tobacco cells in response to osmotic adjustment, has been detected in unadapted cells, but not in cells under normal conditions. (Singh et al., 1985).

It is interesting to note that alumitin starts to accumulated at 3-6 h of Al treatment, which corresponds exactly with the time that growth inhibition starts. This indicates that alumitin biosynthesis is a rapid response to Al phytotoxicity. It has also been observed that mucilage at this time is totally inhibited in cv Victory (Matlock, 1989; Puthota et al., 1991).

In conclusion, the results presented in this study confirm that alumitin represents a stress protein that is induced significantly by aluminum, copper and cadmium, and in lesser amount by low pH and heat shock. Although alumitin is induced by other stresses, it still respresents a valuable molecular marker for aluminum toxicity for some reasons: - it is accumulated at higher levels in aluminum-stressed roots, relative to other stresses, and occurs in many different wheat cultivars, - it starts to be accumulated at the same time that root growth is inhibited. - In the field, conditions such as high levels of copper and cadmiun; or soil pH below 3.8, and soil temperature above 37°C would seldom be encountered. Thus the combination of relatively acid soils (pH 4.0 to 5.0) and the presence of alumitin would most likely be a strong indictor of specific Al toxicity in plants.

Further characterization of alumitin is necessary in order to develop it as diagnostic assay of aluminum or metal toxicity stress. This diagnostic assay could be based on

the presence of alumitin and/or its mRNA. The N-terminal sequence of alumitin is currently being determined. With a partial amino acid sequence, searches of protein sequence libraries will be conducted to resolve if alumitin is actually a known protein which has been already sequenced in other plants. Alumitin can represent a model system for using proteins as biomarkers for metal toxicity in plants.

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VITA

Rocio Cruz-Ortega

Candidate for the Degree of

Master of Science

Thesis: PROPERTIES OF A PROTEIN ASSOCIATED WITH METAL TOXICITY IN WHEAT

Major Field: Botany

Biographical:

Personal Data: Born in Mexico City, Mexico on July 24, 1957, the daughter of Jose A. Cruz and Isabel Ortega.

Education: Graduated from Vidal Castañeda y Najera High School; received Bachelor of Science degree in Biology from Universidad Autonoma de Mexico, Mexico City, Mexico in 1985; completed the requirements for the Master of Science degree at Oklahoma State University in May, 1992.

Professional Experiences: Research technician at Instituto de Fisiologia Celular, Universidad Autonoma de Mexico, Mexico City, Mexico, 1985-1989. Teaching assistant, Department of Botany, Oklahoma State University, Stillwater, Oklahoma, Spring 1990 and Spring 1991.