IMMUNOLOGICAL STUDY FOR CHARACTERIZATION OF ALUMINUM-INDUCED PROTEINS IN WHEAT (*Triticum aestivum* L.) ROOTS

By

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CHAPTER I

INTRODUCTION

It has long been recognized that aluminum toxicity is a major factor limiting plant growth in acidic soils. At soil pH<5.0, the main factors are excessive levels of free and exchangeable aluminum (Werner, 1992). Signs of aluminum toxicity first appear in the root system, which becomes stubby as a result of the inhibition of elongation of the main axis and lateral roots. Cell division ceases within a few hours and root cap cells experience injury. The root damage inhibits the translation of nutrients to the above-ground parts and prevents water uptake and Ca and P uptake. The net result is marked inhibition of plant growth and crop yield (Hartwell & Pember, 1918; Hecht-Buchholz & Foy, 1981; Siegel & Haug, 1983; Haug, 1984; Chaudhry et al., 1987; Wagatsuma et al., 1987; Tomsett & Thurman, 1988; Puthota et al., 1991). Recently, environmental problems such as acid rain, and human activities such as mining and smelting, have been shown to mobilize or introduce many phytotoxic metals into the environment, including Al and heavy metals (Friedland, 1990). Thus, there is great interest in diagnosing and detecting phytotoxic effects of Al and heavy metals in both natural and crop vegetation.

Aluminum excess is known to damage plants but cannot be diagnosed with certainty either visually or by leaf analysis. Currently there are no assays for metal toxicity in plant tissues (Aniol & Gustafson, 1990). Most studies rely on low growth rates, visual appearance, or total contents for some heavy metals to determine toxicity. In many cases, the amount of mineral nutrients in parts of the plant exhibiting nutrient stress cannot be correlated to the level of stress. Neither does Al tissue concentration in the foliage may accurately reflect Al stress (Werner, 1992). Leaf Al concentration usually constitutes <10% of the total absorbed Al; Al tolerance per se in wheat is physiologically linked to root tissue rather than the foliage (Zhang & Taylor, 1988). In addition, Al toxicity induces critical deficiencies of P, Ca, Mg, Fe and Mn. In some cases, Al toxicity can be confused with P and Ca deficiency (Werner, 1992).

However, an assay based on the expression of specific proteins during conditions of nutrient stress would, for theoretical reasons, be more precise and more indicative of the level of stress than either measurement of the level of nutrients in the tissue or a visual examination of the stressed plants. According to the previous studies in our lab, of approximately 600 proteins in wheat roots separated by 2D-PAGE after 40 μM Al treatment for 24 h, 46 proteins were identified whose level was altered by Al. Among the 46

proteins, a small (18.6 kD), acidic protein (pI 5.2) called TAl-18 was induced in wheat roots in Al-sensitive, intermediate and Al-tolerant cultivars of wheat (Ownby & Hruschka, 1991). This protein was not detectable in Al-sensitive cultivars after 3-5 h treatment, but only appeared when growth was irreversibly arrested. Meanwhile, TAl-18 also was elicited by several different metals, such as Cu and Cd, but was not formed during growth inhibition caused by heat shock (Cruz-Ortega & Ownby, 1993). The results indicate that TAl-18 is not made as a consequence of growth inhibition per se, but may be synthesized specifically in response to growth inhibition specifically associated with toxic levels of metals, and may represent a molecular marker for metal toxicity. This suggests that antibodies raised against TAl-18 could be used in the development of a diagnostic immunoassay for both laboratory and field studies of Al and other metal toxicity stresses.

In order to develop this new tool to assay and diagnose Al toxic effect in plants, it is very important to confirm that the biomarker protein TAl-18 is as an indicator of Al stress in wheat and a variety of other plants. The objectives of this project were 1) to extract and purify TAl-18 by 2-D PAGE in wheat roots subjected to 80 μM Al treatment for 24 h; 2) to raise an antibody to purified TAl-18 in rabbit; 3) to survey TAl-18 by western-blotting in wheat under normal and Al-treatment conditions in the growth

chamber and field; 4) to determine if an antibody raised against a synthetic peptide containing the partial amino acid sequence of TAl-18 could detect TAl-18 in Al-stressed wheat roots.

Immunology techniques are very sensitive, specific and rapid, and small amounts of sample are sufficient for analysis. This research should help to form the basis of developing a new way to assay and diagnose Al toxicity in plants, to improve the method for screening Al tolerant cultivars, and to find related stress proteins and understand the mechanism of Al tolerance. The technique depends on the complete isolation of the biomarker. Thus confirming that TAl-18 can be a biomarker protein for Al toxicity is a key step.

CHAPTER II

REVIEW OF THE LITERATURE

1. Aluminum Toxicity to Plants

Aluminum is one of the most abundant elements in the earth's surface, is an important constituent in many inorganic noncarbonated soil minerals, and is too reactive an element to be found free in nature. In soil minerals most of the Al is tied up in the crystal lattice of alumino-silicate minerals, the remaining Al occurs in diverse forms, consequently its chemistry is complex (Soon, 1993). Al can be bound to negatively charged clay surfaces by electrostatic forces and thus can be freely exchangeable with other cations such as Ca^{2+}, Mg^{2+}, or K^{+}; in addition, Al also can be present as organic complexes, as noncrystalline coatings on soil constituents, as discrete amorphous phases, and as complex hydroxy polymeric compounds occupying the interstitial spaces of 2:1 clay minerals and thus can be only partially exchangeable or totally nonexchangeable to other cations (Barnhisel & Bertsch, 1982). Al activity in soils depends on pH and the amount of exchangeable metal (Haug, 1984). The solubility of Al^{3+} ions is very low in slightly acid (pH. 5.5) to neutral soils, but increases abruptly at pH values below 5.0. The concentration depends

on the Al saturation of the exchange capacity of the soil, Foy et al. (1965) reported that the concentration of Al^{3+} changes by a factor of 1,000 when the concentration of protons changes by a factor of 10 or with a change of one pH unit. An Al^{3+} concentration as low as 1 ppm in the soil solution is sufficient to suppress root growth (Bohn et al., 1979). The critical toxic levels of % saturation of Al in soil are different for different plants, rice is > 45%, wheat is 30%, soybean is 20%, alfalfa is 15%, but cotton is 10% (Fageria et al., 1988).

Al phytotoxic effects have been recognized from the early decades of the century (Hartwell & Pember, 1918). The toxicity is prevalent in acid soils and eventually becomes biologically significant (Helliwell et al. 1983). Al^{3+} and mononuclear hydroxy-Al species are more toxic to plants than other forms and apoplastic binding of Al is a requirement for the expression of Al toxicity. Al toxicity is a function of the Al saturation of apoplastic exchange sites which could be simulated by a ligand (Grauer, 1993). High Al^{3+} concentrations induce a complex of symptoms generally known as "acid damage" or "acid soil complex" symptoms (Werner, 1992). Replacement of Ca^{2+} by Al^{3+} from the external surface of the plasma membrane may enhance Ca^{2+} influx into the cytosol and subsequently induce callose formation in the apoplast, which restricts short-term Al toxicity to the rhizodermal and root cap cells (Marschner, 1991). In

addition, Al reduces mineral uptake by roots (Baligar et al., 1987), decreases activity of calmodulin-regulated enzymes (Slaski, 1989), inhibits DNA and RNA synthesis (Matsumoto & Morimura, 1980; Wallace & Anderson, 1984), disrupts Golgi apparatus in root cap cells (Bennett et al., 1987), inhibits P and Ca uptake and reduces plant growth, and decreases the production of crops largely.

2. Al Toxicity Symptoms in Roots and Diagnostic Parameters in Crops

Excess Al in the growth medium influences several physiological and biochemical processes in plants, these disorders can generally be divided into two categories: (1) long-term responses that take at least several hours to develop; and (2) short-term responses that are measurable within minutes or even seconds after exposure to Al (Rengel, 1992). The toxicity of Al to plants is first manifested in the root meristem region, the disappearance of mucilage from the root cap, and increased vacuolation of the epidermal and cortical cells, are usually among the first ultrastructural signs of Al phytotoxicity.(Hecht-Buchholz and Foy, 1981; Siegel and Haug, 1983; Haug, 1984; Chaudhry et al., 1987; Wagatsuma et al.,1987; Tomsett and Thurman, 1988; Puthota et al., 1991). In above-ground tissue, plants often appear stunted and have small and abnormally dark green leaves with a purplish shimmer; leaf-veins have a purplish tinge. Later

the leaves abscise and the plants die (Werner, 1992).

The typical visible symptoms of Al-toxicity on root structure and on leaves, biomass of plants, and Al, P, and Ca concentration in plants have been used to diagnose Al toxicity. Root length and dry matter yield in the presence of Al have been used as a tool for identifying Al tolerance. Paliwal et al. (1994) used these two parameters to survey Al tolerant cultivars of cowpea which can be recommended acidic infertile soils in the tropics. Likewise, the concentration of exchangeable Al in soil also has been used to predict Al toxicity to plants. Carr & Ritchie (1993) suggested that the ratio [Al]:[Na] is a more accurate soil test than [Al] alone for predicting Al toxicity of wheat grown on similar soils in different regions in yellow earths of western Australia. Because the variability of exchangeable [Al] was not sufficient to significantly alter interpretation of soil test values, the [Na] extracted by KCl was related to the ionic strength of the soil and provided an indication of toxic Al activity in the soil solution. In their work, the most appropriate time to test soil was be in late summer or early autumn, at which time changes in soil chemical properties was be minimal.

In techniques for screening Al tolerance, several depend on the staining in roots in different concentration of Al (Howeler, 1991). The hematoxylin staining method is a rapid, simple and repeatable technique for screening Al tolerant

cultivars. The reaction of hematoxylin with Al-stressed roots of sensitive cultivars exhibits more intense staining than tolerant cultivars along the vertical axis of the root (Polle et al., 1978). Ownby (1993) suggested that the damage of Al to root cells in sensitive cultivars leads to leakage of P into cell wall region and precipitation with Al. The $AlPO_4$ would react with hematoxylin to give the visual staining, so selective hematoxylin staining can be used to judge sensitive cultivar for wheat. Carver et al.(1988) screened 156 hard red winter wheat germplasms for Al-tolerant genotype and get the tolerant, intermediate, moderately susceptible and very susceptible Al-tolerant levels by using this method. The methylene blue staining technique has also been used for the discrimination of Al-tolerant protoplasts from the original protoplast population in several plant species (Wagatsuma et al.,1991).

Under conditions of Al stress, plants often exhibit Mg and Ca deficiency and depressed concentrations of K and NO_3^- (Keltjens & Dijkstra, 1991). In sorghum (*Sorghhum bicolor*), toxic Al levels reduced contents of all nutrient elements, especially Mg, Ca, and Zn, and decreased translocation of P from roots to shoots (Galvez & Clark, 1991). So the concentrations of these nutrient, especially P and Ca, are also used to diagnose Al toxicity in plant.

3. Al Tolerance Mechanisms in Plants

Plants can react to changes in environmental conditions by altering their gene expression, thus enable them to adapt and consequently survive. It has been shown that plant species and cultivars within species differ greatly in their tolerance to Al stress. Barley, winter wheat, tobacco, cotton are intolerant; oats, rye, sorghum are moderately tolerant; azalea, maize and tea are tolerant plants (Foy & Brown, 1964). Hence there are differences in metabolic pathways and alterations in other physiological and developmental responses for plant responding to Al toxicity in the environment.

At present, exudation of organic acids is perhaps the most promising mechanism of Al tolerance yet studied. Delhaize et al. (1993) investigated the role of organic acids in differing Al tolerant wheat seedlings, they found that there was a consistent correlation of Al tolerance with high rates of malic acid excretion stimulated by Al in a population of seedlings segregating for Al tolerance. Henderson & Ownby (1991) noted a strong correlation ($r=0.82$) between root mucilage volume and Al tolerance as determined by root growth assays. They suggested that the mucilage droplet would create a "boundary layer" in which diffusion of Al to the root surface was slowed, and where the organic acid/Al ratio would likely be much more favorable than in the rhizosphere as a whole.

After Al eventually enters the nearly neutral cytosol,

it cannot exist as free, highly charged cation but is complexed mostly with proteins (Aniol, 1984) or various phosphate-containing compounds (Taylor, 1988). In plants, Al chelates with acidic polypeptides and low molecular weight ligands like citric acid (Suhayda & Haug, 1986; Putterill & Gardner, 1988) which may the inhibition of Al. The main organic acids for effective detoxification of Al are citric, oxalic, and tartaric followed by malic, malonic, salicylic, and succinic (Hue et al., 1986). Positive relationships between Al toxicity tolerance and organic acid accumulations and nitrate reductase activity in Al-tolerant and Al-sensitive sorghum genotypes were observed (Galvez et al., 1991). Tolerance to Al also appears to be achieved by maintenance of cytosolic Ca homeostasis through protection of plasmalemma transport proteins (Meharg, 1994). The amount of Al associated with several different polypeptides in root exudates from Al-tolerant cultivars is higher than that from sensitive cultivars, which suggests that these polypeptides may be chelators in Al resistance (Basu et al., 1994).

In adapted plants responding to a variety of environmental stresses, there are several changes in quality and quantity of proteins. These proteins are thought to be part of the plant defense system and have some protective functions that enable plants to adapt to the adverse environment. Aniol (1984) first suggested that Al induces the synthesis of An Al-binding protein in wheat roots which

sequesters and inactivates the metal. Ownby and Hruschka (1991) treated Al tolerant and sensitive cultivars of wheat and found that an acidic and cytoplasmic protein (18.6 kD) was enhanced 50-fold in both cultivars. Rincon and Gonzales (1991) noted that 50 μM Al^{3+} induced the production of three new proteins in both Al sensitive and tolerant cultivars. In alfalfa roots, Al stress results in an increase in detectable root proteins in both tolerant and sensitive cultivars, but an 18.7 kD protein was produced only in the tolerant cultivar (Campbell et al., 1994). It seems that an inducible mechanism of Al tolerance may be based on metal-binding proteins which allows the accumulation of increased amounts of Al in cellular root components without damage to their function.

In a number of wheat cultivars, Al tolerance is consistent with the presence of a major, dominant gene controlling most of the tolerance (Kerridge & Kronstad, 1968; Aniol, 1984; Larkin, 1987). Aniol (1991) suggested that in wheat, the genes involved in Al tolerance are located on the short arm of chromosome 5A and the long arm of chromosome 2D and 4D. Snowden & Gardner (1993) isolated and characterizated five genes (*wali1* to *wali5*) whose expression was induced in wheat root tips by Al treatment. *Wali1, 3, 4, 5* were induced by inhibitory levels of Al in sensitive and tolerant cultivars, but *wali2* was induced only in the sensitive cultivar; comparison of the nucleotide

sequences of these clones to those in the sequence data bases showed that *wali4* is homologous to phenylalanine ammonia-lyase and *wali1* is homologous to a group of plant proteins that are cysteine-rich and have homology to metallothioneins. *Wali2* encodes a novel protein with a repeating motif of cysteine amino acids, but *wali3* and *wali5* have no significant homology to any sequences.

There are thus several different mechanisms for Al tolerance, especially binding to organic acid chelators and to cell proteins. Other factors important in tolerance are differences in rhizosphere pH, exudation of Al chelators, selective inorganic nitrogen nutrition, calcium and phosphate nutrition, selective binding of Al to the cell wall, compartmentalization of Al within the vacuole and differences in plasmalemma function (Taylor, 1991).

4. Potential Role of Biomarker Proteins as a Diagnostic Tool

Al induced root damage inhibits the translocation of nutrients to above-ground parts and prevents water uptake, therefore, the symptoms exhibited by the above-ground parts often indicate deficiencies of other nutrients. In plant tops, Al toxicity is often characterized by symptoms resembling those of P or Ca deficiency, rendering these symptoms too nonspecific to be used as a consistent measure of Al toxicity in screening tests (Aniol and Gustafson, 1990). Al toxicity thoughtout the root system is detected

long before there are any visible symptoms of damage to aerial parts of the plant (Foy et al., 1978). Since the translocation of Al to the above-ground parts is also inhibited, Al concentrations in the leaves of healthy plants are often higher than in those with Al deficiency. There is thus no relationship between Al toxicity and Al levels in leaves and above-ground parts. There is a strong correlation between the Al content of the roots and the degree of damage caused by Al excess (Werner, 1992), however, quantitative root analysis for Al is difficult with soil-grown plants, owing to the adhering soil particles.

Among various assays that have been considered, it was found that the inhibition of mitotic divisions (anatomical symptoms), the induced changes of pH in roots rhizosphere, preferential ammonium vs. nitrate nutrition, Al uptake and accumulation, and the protein contents (physiological and biochemical symptoms), are either too difficult to measure on a mass scale or vary too much to be rapid and simple indicators for Al tolerance (Aniol and Gustafson, 1990). The precise biochemical test cannot be established until the exact target of Al ions is known. However, an assay based on the expression of specific proteins during conditions of nutrient stress would, for theoretical reasons, be more precise and more indicative of the level of stress than either measurement of the level of nutrients in the tissue or a visual examination of the stressed plants. Induction of

new proteins occurs in plants in response to a wide range of physical and environmental stress treatments. Under several stress conditions, such as water stress, salt stress, low temperature and heat shock, there are new proteins which can be identified as responses to stress and tolerance induction (Sach & Ho, 1986; Vernon & Bohnert, 1992; Close & Lammers, 1993).

Under exposed to Pb, Cu and NO_2^- condition, Przymusinski & Gwozdz (1994) found that an increased accumulation of a polypeptide occurred. They postulated that the polypeptide was a non-specific stress protein. Saradhi & Saradhi (1991) suggested that proline accumulation could be used as a marker to test the level of heavy metal pollution. P starvation induced enhanced secretion of proteins into the media, which was the result of Golgi-mediated secretion processes (Goldstein et al,. 1989). Nonprotein polypeptide phytochelatins (PCs) are thought have an important role in metal ion homeostasis and the regulation of ion concentrations within the cytoplasm, they are necessary for Cu and Cd tolerance, but have poor affinity for Zn and Al (Meharg, 1994). If a specific protein induced by metal stress can be isolated, a biomarker assay can be developed to diagnosis the symptoms of metal stress. Rao and Ownby (1993) have made some progress in this area. They found the levels of isozymes of phenolase were reduced by Cu deficiency and developed the Cu-requiring protein phenolase

as a biomarker of Cu nutrient status. The immunoassay for phenolase protein can detect phenolase in as little as 15-20 mg DW of leaf tissue, but the assay is more useful in cotton than in wheat, because in wheat there was less total phenolase, and significant increases in phenolase in response to Cu supply were noted only when the Cu supply did not markedly limit growth. This shows the potential of a biomarker protein as a tool for assessment of Cu status in certain crops.

5. Role of Immunological Techniques in Diagnosing and Studying Mechanisms of Al Tolerance

An immunoassay offers a number of advantages over more conventional assay such as TLC, HPLC, and GC and those utilizing enzymatic or biological properties. These tend to require considerable work-up and purification of the sample prior to analysis and may require the sample to be concentrated to achieve a detectable limit. In contrast, an immunoassay can be extremely sensitive and specific and seldom requires significant sample work-up (Robins, 1986). It is therefore important to consider the application of an immunoassay for the determination of a particular low-molecular-weight substance.

There are many reports of the use of an immunology techniques to study the characterization of the protein. The characterizations of nitrate reductase, ATPase, glycerol-3-

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There are many reports of the use of an immunology techniques to study the characterization of the protein. The characterizations of nitrate reductase, ATPase, glycerol-3-

phosphate acytransferase and calmodulin have been studied by using Abs raised against these proteins (Douady et al., 1990; Jablonsky et al., 1991; Ouazzani & Berville, 1991; Padidam & Johri, 1991). Meanwhile, Abs have been used to isolate and characterizate proteins induced by the conditions of heat shock (Robertson et al., 1994), water stress (Close & Lammers, 1993), and wounding (Lewinsohn et al., 1993). Houde et al. (1992) isolated and expressed a gene which is strongly induced during cold acclimation of wheat. Those authors raised Abs to this protein and used the protein as a molecular marker to select for freezing tolerance in Gramineae. These studies show that immunological methods are specific and convenient, and have advantages over other methods.

From the studies of Cruz-Ortega and Ownby (1993), aluminum, at growth-inhibiting levels, induced an acidic low molecular weight (pI 5.2, 18.6 kD) protein (TAl-18) in roots of the Al-sensitive wheat cultivar Victory. TAl-18 was also induced by other stress factors such as Cu and Cd, and by pH 3.5, but not by heat shock (37 °C, 4 h) or by Ca deprivation. ^{35}S-labelling showed that enhanced biosynthesis of TAl-18 in response to Al toxicity occurred during the period 3 to 6 h after exposure to Al, and reached a maximum after 9 to 12 h of treatment. Amino acid sequence data, as well as comparison of molecular weight and pI, indicated that TAl-18 was homologous to the family of pathogenesis

related (PR) plant proteins that were generally induced by fungi and viruses. However, there are no any visible evidence of microbial or fungal contamination of control or Al-treated root tips under a microscope examination (25x). TAl-18 thus met several criteria for a biomarker: a) abundant in Al-intoxicated roots; 2) expressed in many wheat cultivars under various states of Al stress; 3) and easily extracted from root tissue. Hence, the objective of this work was to use TAl-18 as a biomarker and develop an immunological method to detect Al toxicity and screen cereal cultivars for Al tolerance.

CHAPTER III

MATERIALS AND METHODS

1. Plant Materials

(A) Growth of Wheat Roots for Extracting TAl-18

TAl-18 is about 1.5% of the total cytoplasmic protein in Al treated wheat roots (Cruz-Ortega & Ownby, 1993). For raising the antibody, bulk amounts of protein sample were required. Therefore, wheat (*Triticum aestivum* L. cv Victory) (Al-sensitive, from Johnston Seed Company, Enid, OK, USA) roots were used to isolate the protein. Wheat seeds were spread on a large bed (29 x 32 mm) of nylon mesh supported with solid frame. This was kept in the dark with wetted paper towels support for two days to germinate the seeds. The germinated seedlings were transferred to 6.3 L of the macronutrient medium of Aniol (1984) and grown for another two days. The pH of the nutrient medium was adjusted to 4.4 daily with 0.1 N HCl or 0.1 N NaOH. Then seedlings were exposed to 80 μM AlCl, for 24 h. Throughout the experiment aeration and a light intensity (300 μmole $m^{-2}sec^{-1}$) was provided for 16 hr/day in the growth chamber. After 24 h, the roots were harvested and processed for protein extraction to isolate the required amount of TAl-18 antigen.

(B) Growth of Different Cereals For Detection of TA1-18

Wheat, rice, barley and triticale seed were obtained from the Department of Agronomy, Oklahoma State University, Stillwater. Seeds were germinated in Petri dishes (9.00 cm^2) on wetted filter paper in the dark for two days. The germinated seedlings were transferred to nylon screens, which were floated in 9.5 cm diameter pot contained 450 ml of Aniol macronutrient medium (1984). The nutrient solution pH was matained at 4.4 by adjusting with 0.1 N HCl or 0.1 N NaOH daily. After two days the seedlings were treated with different concentrations of $AlCl_3 \cdot 6H_2O$ (barley: 40, 80, 100, 200 μM; rice: 80, 100, 200, 400 μM; rye: 40, 100, 200 μM; triticale: 200 μM). At specific time intervals the seedlings were harvested for further analysis. Seedlings were grown in aerated growth medium in a growth chamber on 16 hour photoperiod conditions at 22 °C . The light intensity was about 250-300 μmoles m^2s^{-1}.

2. Purification Techniques Used to Isolate TA1-18 Protein

(A) Extraction of Proteins

Root tips (terminal 5 mm) were suspended in cold Honda medium (1966), which contains: 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; samples were frozen and ground to a power in liquid nitrogen. The homogenate was thawed and cell wall and organelles were pelleted by centrifugation at 125,000 g for

30 min at 4°C. The proteins from the supernatant, henceforth referred to as the cytoplasmic fraction, were extracted into phenol and precipitated with methanol (Hurkman & Tanaka, 1986).

(B) Isolation of TA1-18 from Total Proteins by 2-D PAGE

To isolate TA1-18 from total proteins, two dimensional polyacrylamide gel electrophoresis (PAGE) technique was used according to O'Farrell (1975). The proteins were solubilized in a sample buffer (Hurkman & Tanaka, 1986): 9 M UREA, 4% CHAPS, 0.5% DTT, 2% ampholytes 5/7, 0.75% ampholytes 3/10. About 800 μg of the protein was loaded on each of six isoelectric focusing (IEF) tubes for the first dimension of 2-D PAGE. After running 18 h at 400 V and 2 h at 800 V, the IEF gels were removed from the tubes. The acidic part (about 5 cm) of each of 3 IEF gels was loaded onto a 12% SDS gel and run for 5.5 h at 35 mA/gel. The protein spots were visualized using 5% Coomassie blue (R-250) stain. The spots containing TA1-18 from SDS gel were cut out and stored at -25 °C.

3. Immunological Procedures Used to Raise the Polyclonal Abs Against TA1-18 and Synthetic Peptide

(A) Raising of Antibody against TA1-18 in Rabbit

After collecting 30 spots, which contained a total of about 200 μg of TA1-18 protein, an Electro eluter (Bio-Rad

Mini apparatus) was used to extract TA1-18 protein from the gel. Conjugation of haptens to carrier proteins and immunization followed the protocol of Pierce (Imject Activated Immunogen Conjugation Kit). The eluted TA1-18 protein was dissolved in 350 μl conjugation buffer (83 mM sodium phosphate buffer, 0.1 M EDTA, 0.9 M NaCl, 0.02% sodium azide, pH 7.2) and conjugated with 200 μl hapten carrier solution (dinitrophenol as the linker and keyhole limpet hemocyanin, MW 4.5 x 10^5 to 1.3 x 10^7, as the carrier). The conjugated protein was then homogenized with equal volume Freund's Complete Adjuvant and injected in the rabbit. Serum from the rabbit was collected at weekly intervals and centrifuged to remove blood cells. Serum was partially purified by addition of ammonium sulphate to 40% saturation, and centrifuged for 20 min at 10,000 xg. The precipitate was dissolved in Tris-buffered saline (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, TBS) and dialyzated against distilled water overnight at 4 °C, then collected and stored at -80 °C. The titer of anti-TA1-18 antibody in serum was confirmed using dot blot analysis: different dilutions of primary Abs were put on nitrocellulose membrane strips, and blocked with 5% milk for 2 h; incubated in differing dilutions of secondary Abs (goat anti-rabbit IgG conjugated with alkaline phosphatase) for 2 h. Color was developed by using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma Chemical Co.).

Mini apparatus) was used to extract TA1-18 protein from the gel. Conjugation of haptens to carrier proteins and immunization followed the protocol of Pierce (Imject Activated Immunogen Conjugation Kit). The eluted TA1-18 protein was dissolved in 350 μl conjugation buffer (83 mM sodium phosphate buffer, 0.1 M EDTA, 0.9 M NaCl, 0.02% sodium azide, pH 7.2) and conjugated with 200 μl hapten carrier solution (dinitrophenol as the linker and keyhole limpet hemocyanin, MW 4.5×10^5 to 1.3×10^7, as the carrier). The conjugated protein was then homogenized with equal volume Freund's Complete Adjuvant and injected in the rabbit. Serum from the rabbit was collected at weekly intervals and centrifuged to remove blood cells. Serum was partially purified by addition of ammonium sulphate to 40% saturation, and centrifuged for 20 min at 10,000 xg. The precipitate was dissolved in Tris-buffered saline (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, TBS) and dialyzated against distilled water overnight at 4 °C, then collected and stored at -80 °C. The titer of anti-TA1-18 antibody in serum was confirmed using dot blot analysis: different dilutions of primary Abs were put on nitrocellulose membrane strips, and blocked with 5% milk for 2 h; incubated in differing dilutions of secondary Abs (goat anti-rabbit IgG conjugated with alkaline phosphatase) for 2 h. Color was developed by using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma Chemical Co.).

(B) Raising of Antibody to Synthetic Peptide in Rabbit

The amino acid sequence of two peptides obtained by trypsin digestion of TAl-18 had previously been determined using Edman chemistry (Cruz-Ortega & Ownby, 1993). The results showed that peptide 1, the amino acid sequence of the C-terminal end (Lys-Ala-Ala-Glu-Ala-Tyr-Leu-Asp-Ala-Asn-Pro-Asp-Ala-Tyr-Asn) had strong homology with a pathogenesis related plant protein PR2 [identified in parsley cell cultures by Van de Locht et al.(1990)]. The possibility that TAl-18 synthesis could result from infection of roots by fungi during exposure to Al was deemed unlikely (Cruz-Ortega & Ownby, 1993). A synthetic peptide containing these 15 amino acid residues was constructed by Chiron Mimotopes (Victoria, Australia). Aliquots of the peptide (200 μg) were dissolved in 350 μl conjugation buffer, and the same procedure was used to raise antibody as for anti-TAl-18 antibody (see above). Dot-blot analysis was again used to assess the titer of anti-peptide antibody.

4. Western Blot Analysis

(A) Denatured Protein

Proteins were extracted from different tissues by grinding in Honda (1966) medium, followed by centrifugation at 5,000 x g. The concentration of protein in the supernatant was determined with a Bio-Rad protein assay kit using bovine serum albumin (BSA) as standard. The proteins

were mixed with sample buffer (4% Tris, pH 6.8, 10% SDS, 0.125% bromphenol blue, 25% glycerol, 1% DTT, 3:1 v/v), boiled at 100 °C for 10 min, transferred to ice, then separated by one-dimensional PAGE with a Bio-Rad Mini-Protean II Electrophoresis Cell. The same amount of protein was loaded in each well. SDS-PAGE was performed on 12% gels with 4% stacking gels, running voltage began at 80 v and increased to 120 v after the sample entered the separating gel at 4 °C. The separated proteins were transferred onto a nitrocellulose membrane using a Bio-Rad Mini-transblot apparatus. Prestained protein molecular mass markers were from Bio-Rad (phosphorylase B, 106 kD; BSA, 80 kD; ovalbumin, 49.5 kD; carbonic anhydrase, 32.5 kD; soybean trypsin inhibitor, 27.5 kD; lysozyme, 18.5 kD). Non-specific binding sites to the membrane were blocked with 5% powdered milk in Tris-buffered saline (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, TBS). After the blot was incubated with primary antibody at 1:1,500 dilution for TAl-18 and at 1:2,000 dilution for synthetic peptide for 3 h, it was washed 3 times for 10 min each with Tris buffer saline containing Tween-20 (TBST). The blot was then incubated in secondary antibody (alkaline phosphatase, Sigma Chemical Co.) for 2 h at 1:10,000 dilution. After 3 times washed in TBST and substrate buffer for 15 min, color development proceeded using NBT and BCIP. All Wester blotting procedures were performed at room temperature.

(B) Immunological Analysis of Non-denatured Protein Samples

Plant tissues were homogenized in 0.05 M sodium acetate buffer (pH 5.0), centrifuged at 10,000 g for 15 min (4°C) and the supernatant was collected (Shimoni, 1994). Native polyacrylamide slab gels were prepared according to Laemmli (1970) with 4% stacking gel and 7.5% separating gel. After separating the proteins, all transferring and blotting procedures were the same as those used for denatured protein.

5. Determination of Root Growth Rate

To measure root growth response in the presence of potentially toxic levels of Al, wheat seedlings [cv. Pioneer 2157 (Al sensitive) and Pioneer 2180 (Al tolerant)] were exposed to $AlCl_3$ at concentrations of 0.0, 10.0, 20.0, 30.0, and 40.0 μM. At the beginning of treatment the length of the primary root of 10 seedlings per treatment was measured. Each seedlings was numbered with tape attached to the coleoptile. After 6, 12, and 24 h of growth, the root seedlings were measured again and the increment of growth was determined.

CHAPTER IV

RESULTS

1. Dilution of Polyclonal Serum Raised against TAl-18

About 2,000 wheat seedlings (cv. Victory) were cultured in Aniol solution for 2 d, and treated against 80 μM Al for 24 h in a growth chamber. TAl-18 was then purified by 2-D PAGE. Approximately 200 μg of purified TAl-18 was injected into a rabbit on each of four occasions at about 15 day intervals. The serum was collected and the titer of antibodies was determined by using dot-blots. The dilution sequence indicated that the optimum dilution was 2,000 for primary antibody and 20,000 for the secondary antibody (Fig. 1 and Fig. 2). To get a consistent effect, the selected dilutions were 1,500 for primary antibody and 10,000 for the secondary antibody in Western-blots.

2. Cross-reaction between Anti-TAl-18 Antibody and Proteins From an Al-sensitive Cultivar

Proteins extracted from control and Al-treated wheat roots (Al-sensitive cultivar Victory) were separated by SDS-PAGE and transferred to nitrocellulose. The antibody recognized an 18 kD protein expressed in 40 and 80 μM Al treatment roots but not in the leaves after 6 h treatment

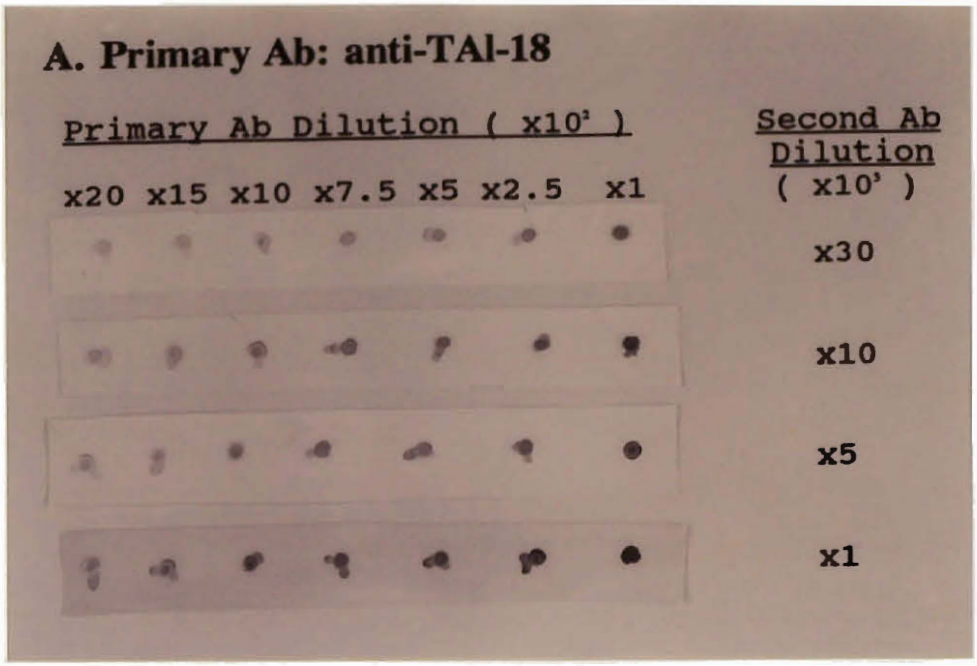

Fig. 1. Dot-blotting of Abs raised against TAl-18. 2 μl different dilutions of anti-TAl-18 Abs were loaded on nitrocellulose membrane strips.

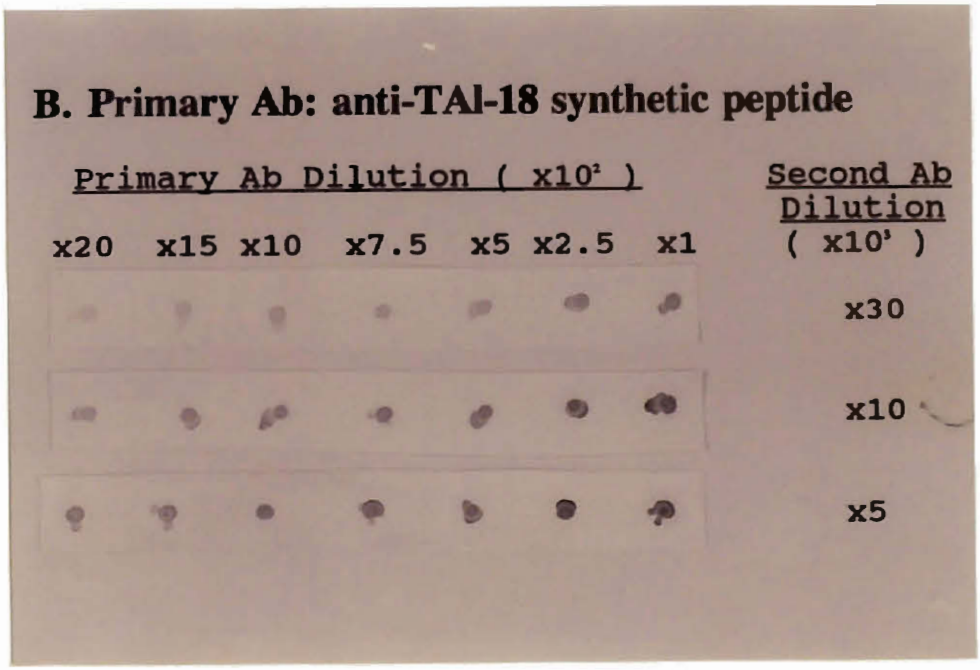

Fig. 2. Dot-blotting of Abs raised against synthetic peptide of partial amino acid sequence of TAl-18. 2 μl different dilutions of anti-peptide Abs were loaded on nitrocellulose membrane strips.

(Fig. 3). No proteins were recognized in the control plant. When the treatment time was increased to 12 h, the putative TAl-18 was still detectable with anti-TAl-18 antibody in 40 and 80 μM Al treated roots (Fig. 4).

3. Time Course of Changes in Al Toxicity Markers in Different Organs of Wheat cv. Victory

Additional experiments confirmed that, after 6 h Al treatment, anti-TAl-18 antibody cross-reacted with TAl-18 in 40 and 80 μM Al treated roots (Fig. 5). Equal amounts of proteins separated by gel electrophoresis were used in the assays of any given sample. In addition, the anti-TAl-18 antibody also recognized the protein in sheaths (coleoptile and stem of wheat seedlings) of Al-treated seedlings, but not in the leaves. However, when the time of Al treatment was increased to 24 h, TAl-18 decreased; a 30 kD protein in Al-treated roots cross-reacted with anti-TAl-18 antibody, especially increasing in roots treated for 48 to 96 h.

4. Expression of TAl-18 in Atlas 66, an Al-Tolerant Cultivar

In the Al tolerant cultivar Atlas 66, anti-TAl-18 antibody also cross-reacted with an 18 kD protein in extracts from control and 400 μM Al-treated sheaths (Fig. 6). However, in roots exposed to Al for 6 h, anti-TAl-18 Abs recognized both an 18 kD and a 30 kD protein at the same stage. Thus, in the Al-tolerant cultivar, the 30 kD protein

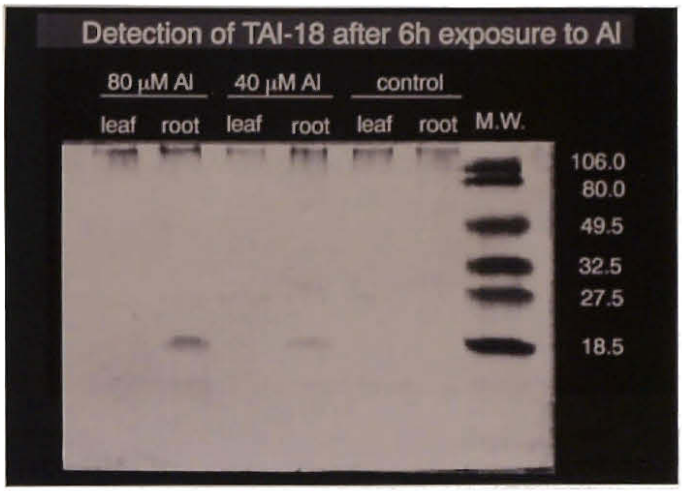

Fig. 3. Cross-reaction of Abs raised against TAl-18 with an 18 kD protein in roots of wheat cv. Victory exposed to different concentrations of Al. Crude extracts of protein (21 μg of total protein) were separated by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting.

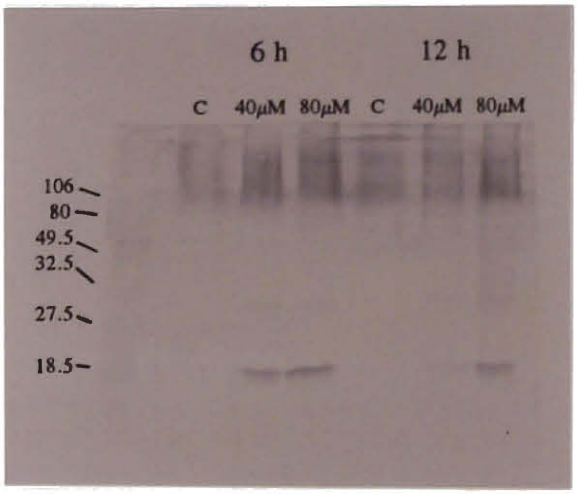

Fig. 4. Cross-reaction of Abs raised against TAl-18 with an 18 kD protein at 6 to 12 h Al treatments in Al-sensitive wheat cv. Victory. Crude extracts of protein (21 μg of total protein) were separated by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting.

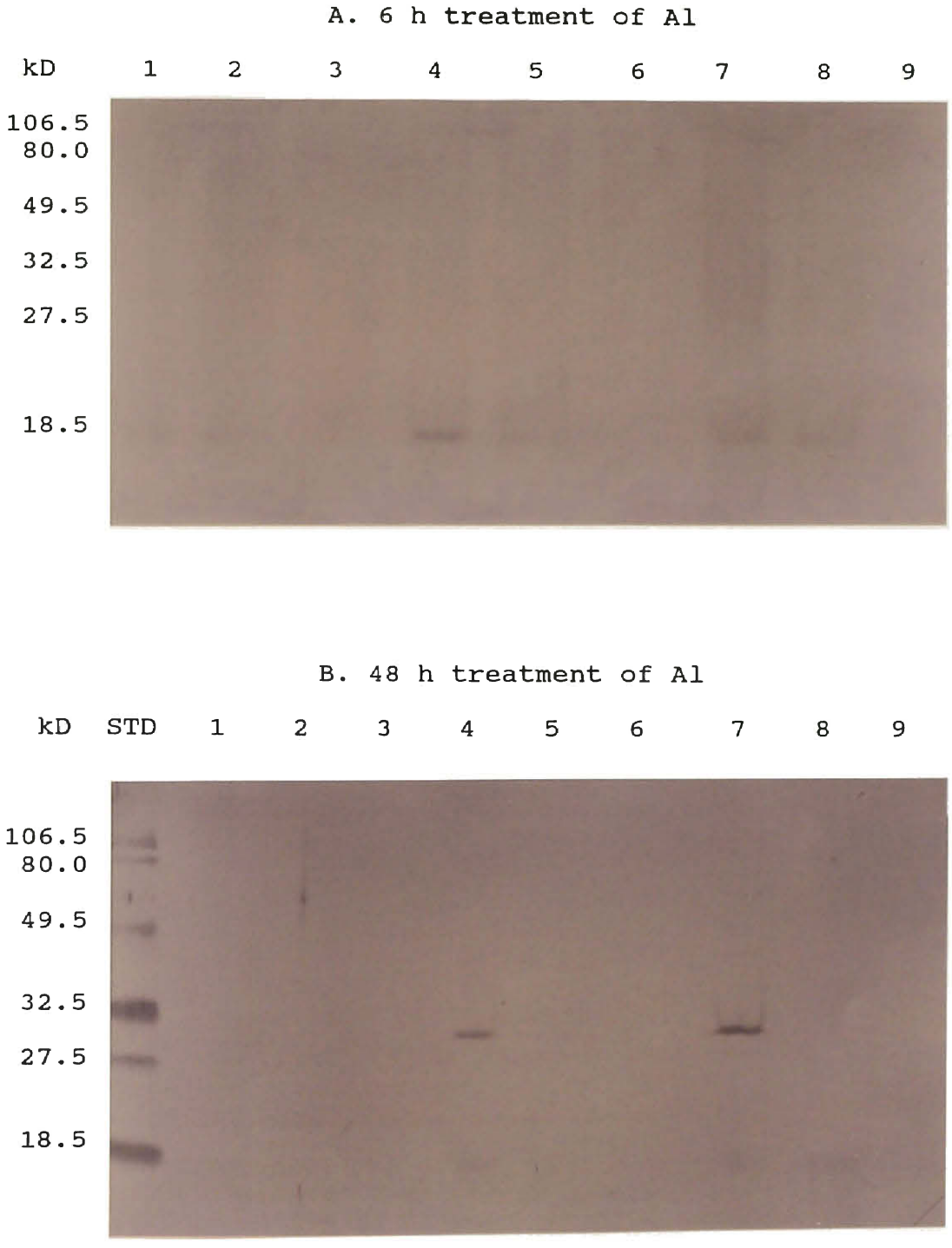

Fig. 5. Cross-reaction of Abs raised against TAl-18 with Al stress protein: time course in Al-sensitive cv. Victory. Crude extracts of protein (16.5 μg of total protein) were separated by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. Lane 1-3: control root, sheath and leaf; lane 4-6: 40 μM Al treatment root, sheath and leaf; lane 7-9: 80 μM Al treatment root, sheath and leaf.

appeared much earlier than in Al-sensitive cv. Victory.

5. Recognition of TAl-18 by Abs to a Synthetic Peptide Having the partial Sequence of TAl-18

In Atlas 66, the TAl-18 antibody recognized TAl-18 antigen in both control and Al treated sheaths. An 18 kD and a 30 kD protein cross-reacted in Al-treated roots, with 30 kD band significantly stronger than TAl-18 itself (Fig. 7). However, in the same samples, the Abs to synthetic peptide recognized only the 30 kD protein in Al-treated roots (Fig. 7). The protein amount correlated with the action of antibody with antigen. When protein levels were low (2.6, 5.4, 7.8 and 10.4 μg), anti-TAl-18 antibody recognized the 30 kD protein (Fig. 8). After the protein amount increased (about 16.5 μg), the 18 kD protein was recognized in Al-treated roots. This indicates that the antibody strongly reacts with the 30 kD protein. In sheath samples, the anti-TAl-18 Abs only cross-reacted with 18 kD protein, which was not correlated to the amount of antigen, even when the antigen amounts were increased (16.7 and 33.4 μg) (Fig. 8).

6. Root Growth Rate and Induction of Al Stress Proteins by Al in Sensitive and Tolerant Cultivars

Pioneer 2157 and Pioneer 2180 are two genetically-related wheat cultivars that differ in sensitivity to Al. To assess the relation between Al sensitivity and expression of stress

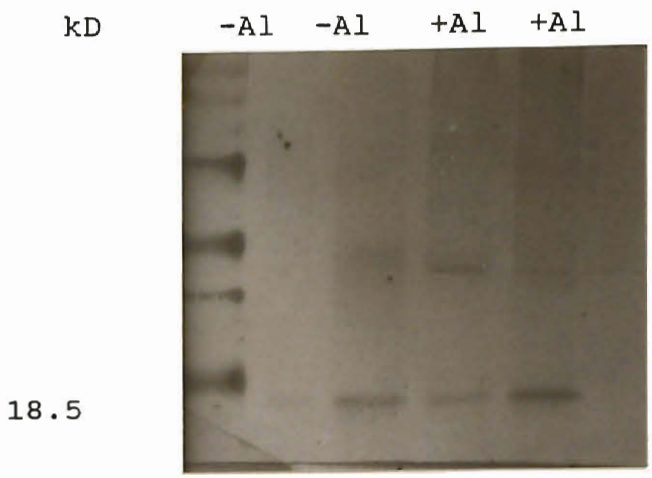

Fig. 6. Cross-reaction of Abs raised against TA1-18 with Al stress proteins in Al-tolerant cv. Atlas 66 after 6 h exposure to 400 μM Al. Crude extracts of protein (16.5 μg of total protein) were separated by in SDS-PAGE and transferred to nitrocellulose membrane. Lane 1: root; lane 2: sheath; lane 3: root; lane 4: sheath.

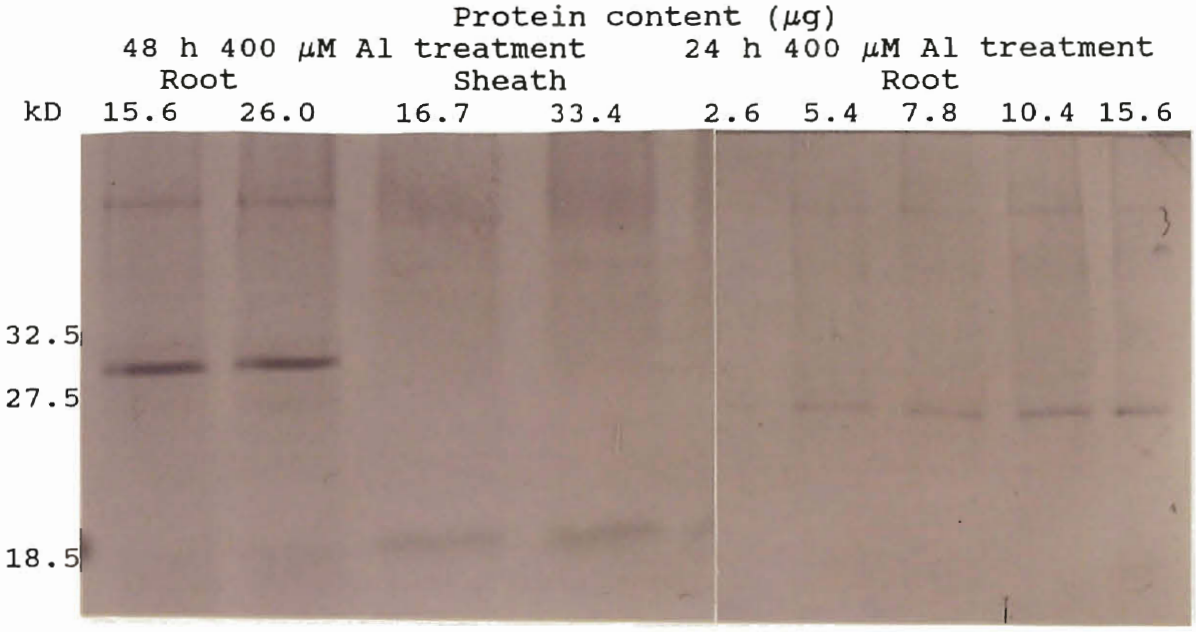

Fig. 7. Cross-reaction of Abs raised against TA1-18 with different amounts of total protein from Al-treated roots of wheat cv. Atlas 66. Proteins in the crude extracts of were separated by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting.

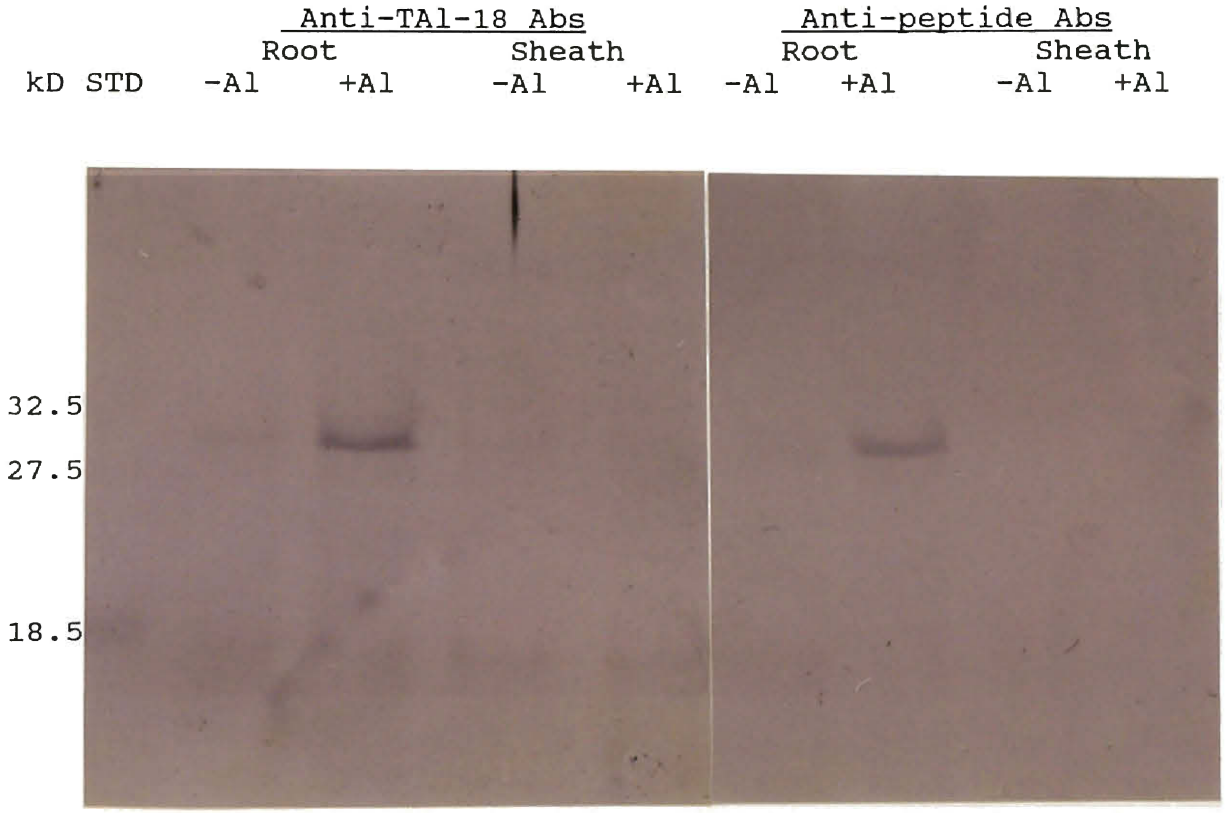

Fig. 8. Cross-reaction of Abs raised against TAl-18 and against a synthetic peptide with Al stress proteins in Al tolerant cv. Atlas 66 following 24 h exposure to 200 μM Al. Crude extracts of protein (16.5 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting.

proteins, growth response to Al was measured. During the first 6 h of exposure to various Al levels, there was similar inhibition of root growth in both cultivars (Fig. 9). During the 6 to 12 h period of Al treatment, the root growth rate in Al-tolerant cultivar Pioneer 2180 began to recover at lower Al treatments, but Al-sensitive cultivar Pioneer 2157 showed the same inhibition trend. During 12 to 24 h Al treatment, the root growth rate of Pioneer 2180 continued to be greater than in Pioneer 2157. Both cultivars thus show an initial "acute" growth inhibition, while only the Al-tolerant Pioneer 2180 has the ability to partially recover during later stages (6-24 h) of exposure.

In Western-blots, Abs to the synthetic peptide cross-reacted with different proteins in the two cultivars. In Al-sensitive Pioneer 2157, the antibody recognized several high molecular weight proteins that were expressed after 6 to 12 h of Al treatment, but not the 18 kD protein (Fig. 10). However, in Al-tolerant Pioneer 2180, the antibody cross-reacted with only one higher molecular weight protein in Al treatment condition.

7. Reaction of TAl-18 Abs with Non-denatured Proteins in Different Wheat Cultivars

Cross-reaction of anti-TAl-18 antibody with non-denatured protein was different from with denatured protein. The antibody recognized a protein in both sensitive and tolerant

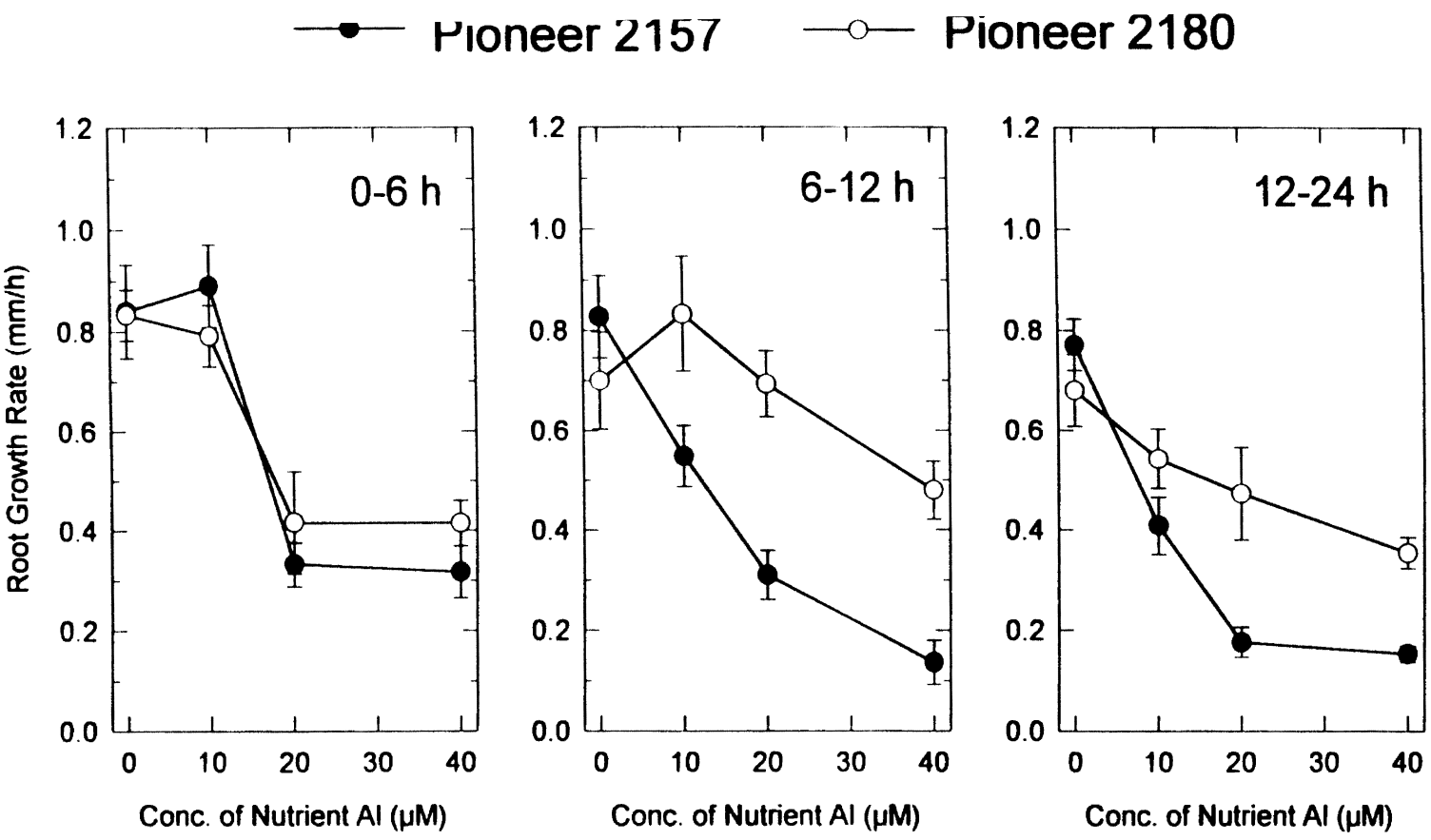

Fig. 9 . Root growth in cv. Pioneer 2157 (Al-sensitive) and cv. Pioneer 2180 (Al-tolerant) during three time after growth in normal solution for two days. Both cultivars are equally sensitive to Al duing the first 6 h, but Pioneer 2180 recovers from this "acute" Al shock and shows tolerance to Al than does Pioneer 2157.

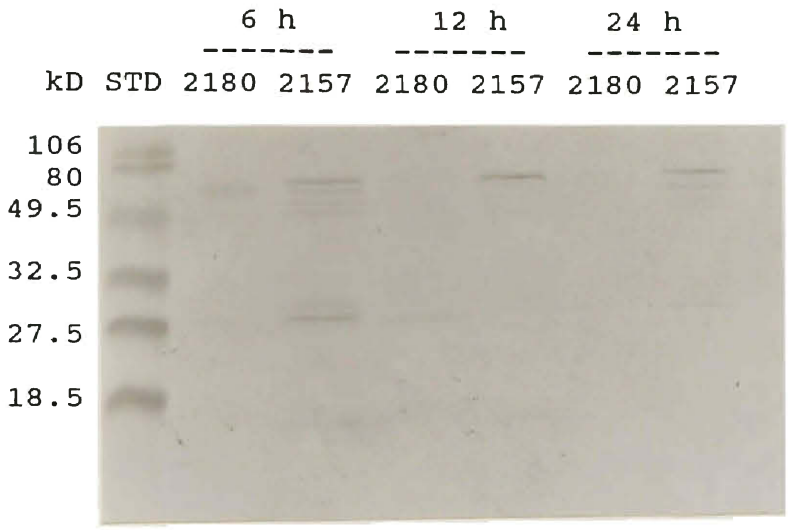

Fig. 10. Cross-reaction of Abs raised against synthetic peptide with Al stress proteins in two cultivars differing in Al tolerance after 6, 12 and 24 h exposure to 20 μM Al. Crude extracts of protein (16.5 μg) from roots were separated by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. Pioneer 2180: Al-tolerance; Pioneer 2157: Al-sensitive.

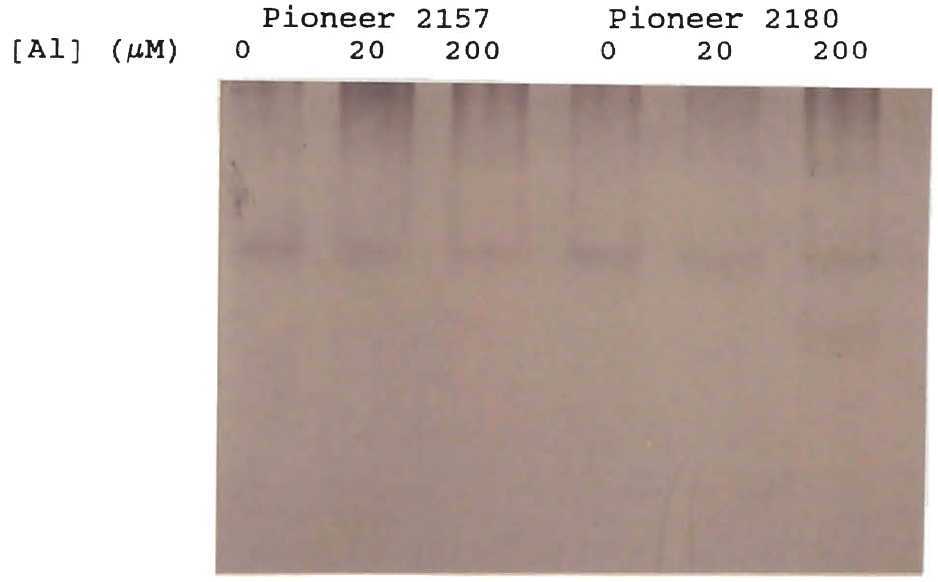

Fig. 11. Cross-reaction of Abs raised against TAl-18 with non-denatured proteins in two cultivars differing in Al tolerance after 24 h Al treatment. Crude extracts of protein (25 μg) from roots were electrophoresed in native gel and transferred to nitrocellulose membrane for Western blotting. Pioneer 2157, Al-sensitive cultivar; Pioneer 2180, Al-tolerant cultivar.

cultivars in both control and Al treatment conditions (Fig. 11). This protein was thus not induced by Al stress. After 24 h of 200 μM Al treatment, another two proteins were recognized by anti-TAl-18 antibody in the tolerant cultivar Pioneer 2180. However, the anti-synthetic peptide antibody did not recognize any non-denatured proteins in either cultivar in control or Al-treatment. This suggests that the cross-reaction between the antibody and the stress proteins is conformation-dependent.

8. Detection of TAl-18 and Other Al-induced Proteins in Different Plants

Based on the above studies with TAl-18 and other Al-stressed proteins, we reasoned that if these proteins are a broadly distributed class of Al-stress proteins in other plants, then the antibodies used for the TAl-18 studies might also be useful for demonstrating the existence of the protein in other plants by Al stress. The results of this a study showed that anti-TAl-18 antibody did not recognize any protein in the other cereals. Anti-peptide antibody recognized several higher molecular weight proteins, but not TAl-18, in both control and Al-treated root samples of barley, rice, rye and triticale, which were treated with 200 μM Al for 24 h. There were no any specific proteins induced only by Al toxicity. It seems that the two antibodies thus cannot be used to diagnose Al toxicity in these plants. This

result warrants a further search for Al-stress proteins in other plant species.

CHAPTER V

DISCUSSION

TAl-18 is an acidic, 18 kD protein induced during Al toxicity in wheat roots in both sensitive and tolerant cultivars (Cruz-Ortega & Ownby, 1993). They hypothesized that TAl-18 might serve as a biomarker for screening wheat cultivars for Al toxicity and for detection of Al stress in field work. Hence if an antibody raised against the biomarker can be produced, then an immunoassay to diagnose Al toxicity in plants can be developed. In this work, polyclonal Abs against both TAl-18 induced during Al toxicity in wheat and a synthetic peptide based on the partial amino acid sequence of TAl-18 were produced and partially purified. The antibodies made against purified TAl-18 as well as the synthetic peptide (containing 15 amino acid residues from the C-terminal end of TAl-18) had high titers and both could be diluted to the same extent for Western-blots (Fig. 1 & 2). Results showed that anti-TAl-18 antibody cross-reacted with an 18 kD protein in Al-treated roots, confirming that the TAl-18 was induced by Al toxicity and recognized by anti-TAl-18 antibody (Fig. 3 & 4). Thus studies with antibodies have the potential for characterization of TAl-18, analyses of its function, and

detection of other Al-stress proteins.

Expression of TAl-18 was different among different organs of wheat seedlings (Fig.5). As a biomarker indicator, the preparation of materials and detection of TAl-18 would be easier and more convenient if it present in leaves. However, TAl-18 was produced under Al-toxic conditions only in wheat roots. It did not appear in leaves in sensitive and tolerant cultivars, which indicates that TAl-18 is induced by Al toxicity only in damaged roots. Thus, only the roots can be used as test materials. Interestingly the protein was expressed constitutively in the sheath in both control and Al treatment conditions. It seems that the protein is not associated with Al toxicity in the sheath, it is possible that the protein has different functions in different organs. Similarly, some dehydrins are present in some tissues whether or not seedlings are stressed (close et al., 1993). But the protein is immediately synthesized in roots in response to Al toxicity after detection of the Al signal.

The distinction between primary (short-term) and secondary (long-term) plant responses to Al stress depends on the time-course of Al uptake into the apoplasm and the symplasm (Rengel, 1992). In Al sensitive cultivar cv. Victory seedlings, the amount of TAl-18 was changed with Al treatment time. During 6 to 24 h Al treatment (primary stage), the level of TAl-18 was maintained in roots; but

after 24 h (secondary stage) a 30 kD protein was expressed, and the TAl-18 decreased in roots. Is the 30 kD protein synthesized in response to Al stress? In tolerant cultivar cv. Atlas 66, the 30 kD protein was induced in roots after only 6 h Al treatment, and the 30 kD protein was not present in sheath in either cultivar. It should be emphasized that the 30 kD protein was specifically induced by Al toxicity, unlike the non-specific bands or the background in many of the Western-blots. This raises the question of how it was possible that an antibody raised against TAl-18 could recognize a 30 kD protein?

Generally, a complete antigen (Ag) possesses several epitopes. A specific polyclonal antiserum might contain a collection of specific Abs directed against different epitopes of an immunizing Ag. A particular antigenic epitope might interact with a number of structurally similar binding sites, displayed by different Abs. It might react more strongly with some than with others (Coleman et al., 1992). McElwain & Spiker (1992) also found that the antiserum to high mobility group chromosomal protein cross-reacted with the low molecular weight heat-shock proteins which was not the Ag for raising Ab. They concluded that there was a possibility of sharing a common epitope. Thus it is possible that there are similar epitopes among the 30 kD and 18 kD proteins. If the two proteins belong to the same family, the anti-TAl-18 antibody could thus recognize both

proteins. The 18 kD and 30 kD proteins may have different properties. The 30 kD protein is clearly a stress protein and could be related to Al toxicity tolerance.

Al toxicity exists in many soils throughout the world, a vast area of land that can be made much more productive if Al toxicity were reduced. Perhaps the best solution for overcoming Al toxicity would be a combination of both liming and selection of Al tolerant plant species or cultivars (Fageria et al., 1988). Environmental stress is widely implicated in altered gene expression resulting in the release of stress-induced proteins that allow plants to adjust to stressful conditions (Sachs & Ho, 1986). Aniol (1984) suggested that proteins play an important role in the mechanism of Al tolerance in wheat. He hypothesized that the synthesis of Al-binding proteins could protect cellular components from Al damage. In both the Al-sensitive cultivar Tam105 and the intermediate-tolerant cultivar Bounty 203-A, Al induced several proteins (18.5, 32, and 37 kD) synthesized at Al concentrations that inhibited root growth (Rincon & Gonzales, 1991). Picton et al. (1991) indicated that five proteins were specific to the tolerant cultivar in the absence of Al. The same five were induced in response to Al in sensitive and tolerant cultivars in wheat. There are some proteins synthesized as indicators in sensitive and tolerant cultivars for responding to Al toxicity, such as TAl-18. However some other proteins that appear more rapidly

and earlier in a tolerant cultivar, such as the 30 kD protein, may play a function in Al tolerance.

Thus, we have presented evidence that TAl-18 is a biomarker for the primary response to Al toxicity in wheat root. The 30 kD protein appears useful as a biomarker for the secondary response to Al toxicity in wheat root, with functions possibly related to Al tolerance.

TAl-18 is about 1.5% of total cytoplasmic proteins in Al-stressed roots (Cruz-Ortega & Ownby, 1993). Production and purification of TAl-18 is not easy and requires much time and materials. From previous work on TAl-18, the partial amino acid sequence of TAl-18 is already known (Cruz-Ortega & Ownby, 1993). A large quantity of a synthetic peptide representing a portion of TAl-18 can be easily made on a commercial scale. As an example of how this technique can be used, Close et al. (1993) described procedures for generating anti-dehydrin consensus region Abs. The Abs against the synthetic peptide which is near the C-terminus of partial dehydrins (15 amino acid residues) recognized dehydrins in a wide range of plants and sizes ranging from about 15 to 150 kD. The C-terminal peptide seems to be relatively constant in these proteins (Close et al., 1993). In other work, Abs raised against the N-terminal (residues 6-51), the C-terminal (residues 851-949), and central (residues 340-650) domains of *Arabidopsis thaliana* plasma membrane H^+-ATPase (expressed as the domains of cloned

ATPase genes) have been used to characterize ATPase (Sekler & Pick, 1993). The results showed that the central hydrophilic domain containing the catalytic site is more conserved than the C- and N- terminal ends. However, in the study described here, the peptide synthesized contained only the 15 amino acids of the C-terminal end of TA1-18. Its conformation in conjugation buffer was not known. Also unknown was whether the partial amino acid residue includes the epitope in TA1-18 or if the epitope recognized by anti-TA1-18 is in the N-terminal end or central part of TA1-18. These questions need to be addressed.

Western blots showed that both antibodies recognized a 30 kD protein in roots, however anti-TA1-18 antibody also recognized an 18 kD protein, even though the signal was actually weaker than the 30 kD protein (Fig. 7). The results of experiments using different protein amounts indicated that anti-TA1-18 antibody recognized the 30 kD protein first with low levels of protein extract (Fig. 8), and began to recognize 18 kD protein with increasing amounts of protein. It seems likely that there are more epitopes in the 30 kD protein than in TA1-18 and this protein thus shows a stronger signal. The antibody to synthetic peptide apparently recognized the same epitopes on the 30 kD protein as did TA1-18 Abs. Since anti-peptide antibody, raised against the C-terminal end of TA1-18 never recognized TA1-18 in Western blots, the epitope for the 18 kD protein most

likely is in the N-terminal or central part of the protein. The C-terminal end must be hidden in TAl-18, thus anti-TAl-18 antibody is recognizing epitopes in the N-terminal end or central part rather than the C-terminal end. In summary, the anti-TAl-18 Abs can recognize TAl-18 and a 30 kD protein, but anti-peptide Abs only recognizes the 30 kD protein that must have exposed epitopes similar to that of the synthetic peptide.

There was a significant difference between tolerant (Pioneer 2180) and sensitive (Pioneer 2157) cultivars in root growth rate (Fig. 9). Both cultivars were equally sensitive to Al during the first 6 h of Al treatment, but Al-tolerant Pioneer 2180 recovered from this "acute" Al shock and showed greater tolerance to Al than did sensitive Pioneer 2157 during the 6-24 h time period. This suggests that there is a specific Al tolerance mechanism in tolerant cultivar. Western-blots showed that the anti-peptide antibody recognized several higher molecular weight proteins expressed only in the sensitive cultivar and not in the tolerant cultivar. These results suggest that the anti-peptide Abs recognizes a series of Al-stress proteins that contain sequences similar to the C-terminal sequence of TAl-18 in the sensitive cultivar (Fig. 10). Comparable results have been obtained with dehydrins, another group of plant stress proteins. The anti-synthetic peptide Abs raised against the C-terminal end of partial dehydrins recognized a

wide size of dehydrins ranges from about 15 to 150 kD (Close et al., 1993). A similar situation seems to exist with our Al-stress protein. TAl-18 is homologous to PR proteins, many of whom share a homologous C-terminal sequence (Breiteneder et al.,1989). The 30 kD protein, and other proteins recognized by anti-TAl-18 Abs or anti-peptide Abs are likely to be PR-type proteins induced by Al stress.

In immunological studies of polygalacturonase, the ß-subunit protein from crude extract was identified in Western blots with anti-PG1 (polygalacturonase 1) abs (Pogson & Brady,1993). They suggested that other proteins that cross-reacted with the anti-PG1 Abs may either be precursors of the ß-subunit, distinct proteins that share a common epitope, or distinct proteins recognized by other immunoglobulins in the polyclonal serum. Hence these stress proteins induced by Al in Al-sensitive cultivar Pioneer 2157 may have homologous amino acid sequence, and function that are different from the protein induced in the tolerant cultivar.

In addition to the physiological relationship between root growth and Al toxicity that is evident in differential Al-tolerance, the immunological relationship of Al-stress protein is defined by cross-reaction with anti-peptide antibodies. Together, these relationship lead us to the interpretation that the properties of some Al-induced proteins containing similar C-terminal end amino acids in

sensitive cultivar are part of the stress reaction. The site in Al-tolerance proteins (such as 30 kD) that function in Al tolerance is possibly located in the N-terminal or central part of TAl-18.

In studies using non-denaturing conditions, anti-TAl-18 antibody recognized the same protein in all controls and treatments and both cultivars. There was cross-reaction with two more proteins expressed under 24 h 200 μM Al treatment only in Al-tolerant cultivar, Atlas 66, in native gels (Fig. 11). Gijzen et al. (1992) noted that polyclonal antibodies generated against the SDS-denatured enzyme, although excellent for specific recognition of the denatured pinene cyclase in Western blots, apparently do not recognize the native protein. Vos-Scheperkeuter et al (1989) made anti-native and anti-denatured potato branching enzyme antibodies. Their results indicate that the epitopes of native antigen are completely distinct from those exposed after SDS-denaturation. Fully denatured antigens elicit a wider range of antibodies than do native antigens. In our experiments, there may be the same conformation of partial amino acid sequence as in denatured conditions (sequences on the surface of the protein) which can be recognized by antibody raised against denatured antigen. The epitopes that were recognized by anti-peptide antibodies are likely buried in the nature protein, so the protein could not cross-react with anti-peptide Abs which only recognize the C-terminal

end. However, Al toxicity also induced changes of nature protein conformation, and made the protein epitopes exposed to outside that can be recognized by anti-TAl-18 Abs in tolerant cultivar, thus the conformation of stress proteins need to be concerned with the function of Al-tolerance. This result is consistent with the hypothesis that some epitopes, such as those presented in the C-terminal end, have been buried in the complete-sequence of TAl-18. It also suggests that Al tolerance relates not only induced protein, but also changed of protein conformation.

Although different plants had variable tolerance to Al, Al in the range of 40 to 200 μM significantly inhibited the growth of barley, rice, rye and triticale. Thus there should be a series of changes in the biochemistry and physiology of these species. However, anti-TAl-18 Abs and anti-peptide Abs could not recognize any specific Al stress protein in these plants. It seems most likely that TAl-18 is not induced in these plant and that the two antibodies are only specific for wheat. According to Cruz-Ortega and Ownby's studies (1993), after rye and triticale seedlings were exposed to 100 μM Al for 24 h (the elongation of primary roots was inhibited about 70%), trace levels of TAl-18 were induced in triticale roots, but there was no evidence of TAl-18 formation in 2D-gels of proteins from Al-stressed rye roots. Most of the changes in the level of specific proteins noted in wheat were not observed in rye and triticale. Thus, as a

result, TAl-18 appears to be a biomarker of Al toxicity for wheat, but not for barley, rice, rye and triticale. This is different from the case of dehydrin, which is present in a wide range of plant species. Specific protein that may be biomarkers of Al toxicity are needed for each of these plants.

These studies of TAl-18 and other Al-stress proteins should help us to understand their role in Al toxicity and Al tolerance. The use of antibodies represent a very important and easily accessible tool to identify specific and homologous Al-stressed proteins. The immunoassay can also provide more sensitive and specific reactions to isolate the Al-tolerant protein and the probably functional site. Thus a marker which has Al-tolerance functions can be used to screen Al-tolerant cultivars. This potential can be assessed rapidly with proteins extracted from as little as 250-500 mg fresh weigh of plant tissues. This may provide plant breeders with a simple and economical method of selection for potential Al tolerance of new cereal crops. However, the approach also has certain disadvantages. TAl-18 and other Al stress proteins occur only in roots and not in leaves. This prevents us from taking the most easily accessible plant tissue for analysis. In addition, the technique must screen roots of plants grown hydroponically. This increases the work for preparing materials. Furthermore, the antibodies only can be used in wheat and

not other species, which restricts the great potential for use these antibodies. This work also shows that in future work, if raising an antibody against peptide sequences, the best way is to select C-terminal, N-terminal and central parts of the protein. This should assure that antibodies to the synthetic peptides also recognize the complete-sequence protein in immunoassays.

CHAPTER VI

Summary and Conclusions

A small (18.6 kD) acidic, cytoplasmic protein called TAl-18 is induced by exposure of wheat roots to toxic levels of Al. TAl-18 is elicited as part of the program of Al toxicity, and appears only under conditions of Al stress sufficient to inhibit growth. As a specific physiological response in plants to metal toxicity, TAl-18 could be a biomarker and used to assay and diagnose Al toxic effects and screen Al tolerant cultivar in plants.

Sufficient TAl-18 was purified by 2-D PAGE from wheat roots subjected to 80 μM Al treatment, and Abs to TAl-18 and a synthetic peptide were raised respectively in a rabbit. The Abs were used in western blots to measure the TAl-18 in wheat tissues under normal and Al-stressed conditions. Results show that (1) TAl-18 is expressed during the first 6 h exposure to phytotoxic levels of Al; (2) TAl-18 is induced by Al only in roots, is absent from leaves, and constitutively present in the lower stem and sheath tissue; (3) from 24 to 48 h TAl-18 decreases and a 30 kD protein appears in Al intoxicated roots that cross-reacts with TAl-18 Abs; (4) the 30 kD protein is induced early and fast in Al toxic tolerant cultivar and may be a Al-tolerance

protein; (5) Abs to a synthetic peptide having the partial amino acid sequence of TAl-18 cross-reacted with higher molecular weight Al-induced protein, these proteins may be stress proteins not involved in Al-tolerance function; (6) TAl-18 is not presented in barley, rice, rye and triticale under Al stress condition.

REFERENCES

Aniol, A. (1984). Induction of aluminum tolerance in wheat seedlings by low doses of aluminum in the nutrient solution. Plant Physiol. 75, 551-555.

- (1991). Genetics of acid tolerant plant. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 1007-1017). Kluwer Academic Publishers, The Netherlands.

- & Gustafson, J. P. (1990). Genetics of tolerance in agronomic plants. In A. J. Shaw (Ed.). Heavy metal tolerance in plants: evolutionary aspects (pp. 255-267). CRC Press, Boca Raton, Florida, USA.

Baligar, V.C. Wright, R.J., Kinraide, T.B., Foy, C.D., Elgin, I.H.Jr (1987). Aluminum effects on growth, mineral uptake, and efficiency ratios in red clover cultivars. Agron. J. 79, 1038-1044.

Barnhisel, R. & Bertsch, P.M.(1982). Aluminum. In: A.L.Page, R.H.Miller & D.R. Keeney (Ed.). Methods of Soil Analysis. Part 2-Chemical and Microbiological Properties, 2nd ed., (pp. 275-300). ASA, SSSA Publisher, Wisconsin, USA.

Basu, U.; Basu, A. & Taylor, G. (1994). Differential exudation of polypeptides by roots of aluminum-resistant and aluminum-sensitive cultivars of *Triticum aestivum* L. in response to aluminum stress. Plant Physiol. 106, 151-158.

Bennett, R.J., Breen, C.M. & Fey, M.V. (1987) The effects of aluminum on root cap function and root development in *Zea Mays* L. Environ. Exp. Bot. 27, 91-104.

Bohn, H., McNeal, B.L. & O'Connor, G.A.(1979). Soil Chemistry, (pp. 195-216). J. Wiley and Sons Inc, New York, USA.

Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. & Beitenbach, M.(1989). The gene coding for the major birch pollen allergen *Betv1*, is highly homologous to a pea disease resistance response gene. EMBO J. 8, 1935-1938.

Campbell, T.A., Jackson, P.R. & Xia, Z.L. (1994). Effects of aluminum stress on alfalfa root proteins. J. Plant Nutr. 17, 461-471.

Carver, B.F., Inskeep, W.P., Wilson, N.P. & Westerman, R.L.(1988). Seedling tolerance to aluminum toxicity in hard red winter wheat germplasm. Crop Sci. 28, 463-466.

Carr, S.J. & Ritchie, G.S.P. (1993). Temporal variation in potassium chloride extractable aluminum, sodium, and soil pH, and the effect on interpretation of subsoil aluminum toxicity in yellow earths. Commun. Soil Sci. Plant Anal. 24, 513-523.

Chaudhry, M. A., Yoshida, S. & Vergara, B.S. (1987). Induced mutations for aluminum tolerance after N-methyl-N-nitrosourea treatment of fertilized egg cells in rice (Oryza sativa L.). Environ. Exp. Bot. 27, 37-43.

Clarkson, D.T. (1965). The effect of aluminum and some other trivalent metal cations on cell division in the root apices of *Allium cepa*. Ann. Bot. 29, 309-315.

Close, T.J., Fenton, R.D. & Moonan F. (1993). A view of plant dehydrins using antibodies specific to the carboxyl terminal peptide. Plant Mol. Biol. 23, 279-286.

- & Lammers, P.J. (1993). An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins. Plant Physiol. 101, 773-779.

Coleman, R.M., Londard, M.F. & Sicard, R.E. (1992). Fundamental immunology. Secondary edition, (pp. 88-115). WCB publishers, Dubuque, IA.

Cruz-Ortega, R. & Ownby, J.D. (1993). A protein similar to PR (pathogenesis-related) proteins is elicited by metal toxicity in wheat roots. Physiol. Plant. 89, 211-219.

Delhaize, E., Ryan, P.R. & Randall, P.J. (1993). Aluminum tolerance in wheat (Triticum aestivum L.) II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol. 103, 695-702.

Douady, D., Passaquet, C. & Dubacq, J-P. (1990). Immunochemical characterization and in vitro synthesis of glycerol-3-phosphate acyltransferase from pea. Plant Sci. 66, 65-72

Fageria, N.K., Baligar, V.C. & Wright, R.J. (1988). Aluminum toxicity in crop plants. J. Plant Nutr. 11, 303-319.

Foy, C.D. & Brown, J.C. (1964). Toxic factors in acid soils.II. Differential aluminum tolerance of plant species. Proc. Soil Sci. Soc. Am. 28, 27-32.

- Burns, G.R., Brown, J.C. & Fleming, A.L. (1965). Differential aluminum tolerance of two wheat varieties associated with plant-induced pH changes around their roots. Soil Sci. Soc. Am. Proc. 29, 64-67.

- Chaney, R.L. & White, M.C. (1978). The physiology of metal toxicity in plants. Annu. Rev. Plant physiol. 29, 511-566.

Friedland, A.J. (1990). The movement of metals through soils and ecosystems. In A. J. Shaw (Ed.). Heavy metal tolerance in plants: evolutionary aspects, (pp. 7-19). CRC Press, Boca Raton, Florida, USA.

Galvez, L. & Clark, R.B. (1991). Effects of silicon on growth and mineral composition of sorghum (*Sorghum bicolor*) grown with toxic levels of aluminum. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 815-823). Kluwer Academic Publishers, The Netherlands.

- - Klepper, L.A. & Hansen, L. (1991). Organic acid and free proline accumulation and nitrate reductase activity in sorghum (*Sorghum bicolor*) genotypes differing in aluminum tolerance. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 859-867). Kluwer Academic Publishers, The Netherlands.

Gijzen, M., Lewinsohn, E. & Croteau, R. (1992). Antigenic cross-reactivity among monoterpene cyclases from grand fir and induction of these enzymes upon stem wounding. Arch. Bioch. Biophy. 294, 670-674.

Goldstein, A.H., Mayfield, S.P., Danon, A, & Tibbot, B.K. (1989). Phosphate starvation inducible metabolism in *Lycopersicon esculentum*. III. Changes in protein secretion under nutrient stress. Plant Physiol. 91, 175-182.

Grauer, U. E. (1993). Modelling anion amelioration of aluminum phytotoxicity. Plant Soil 157, 319-331.

- & Horst, W.J. (1992). Modelling cation amelioration of aluminum phytotoxicity. Soil Sci. Soc. Am. J. 56, 166-172.

Hartwell, B.L. & Pember, F.R. (1918). The presence of aluminum as a reason for the difference in the effect of so-called acid soil on barley and rye. Soil Sci. 6, 259-281.

Haug, A. (1984). Molecular Aspects of aluminum toxicity. C.R.C. Crit. Rev. Plant Sci. 1, (pp. 345-375) CRC Press, Boca Raton, Florida, USA.

Hecht-Buchholz, C. & Foy, C.D. (1981). Effect of aluminum toxicity on root morphology of barley. Plant Soil 63, 93-95.

Helliwell, S., Bately, G.E., Florence, R.M. & Lumsden, B.C. (1983). Speciation and toxicity of aluminum in a model freshwater. Environ. Technol. Lett. 4, 141-144.

Henderson, M. & Ownby, J.D. (1991). The role of root cap mucilage secretion in aluminum tolerance in wheat. In D.D.Randall, D.G.Blevins & C.D.Miles (Ed.). Current topics in plant biochemistry and physiology Vol. 10, (pp. 134-141). University of Missouri-Columbia, MO, USA.

Honda, S.I., Hongladarom, T. & Laties, G.G. (1966). A new isolation medium for plant organelles. J. Exp. Bot. 17, 460-472.

Houde, M., Dhindsa, R.S. & Sarhan, F.(1992). A molecular marker to select for freezing tolerance in gramineae. Mol. Gen. Genet. 234, 43-48.

Howeler, R.H. (1991). Identifying plants adaptable to low pH conditions. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 885-904). Kluwer Academic Publishers, The Netherlands.

Hue, N.V. Craddock, G.R. & Adams, F. (1986). Effect of organic acids on aluminum toxicity in subsoils. Soil Sci. Soc. Am. J. 50, 28-34.

Hurkman, W.J. & Tanaka, C.K. (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. 81, 802-806.

Jablonsky, P.P., Grolig, F. Perkin, J.L. & Williamson, R.E. (1991). Properties of monoclonal antibodies to plant calmodulin. Plant Sci. 76, 175-184.

Keltjens, W.G. & Dijkstra, W.J. (1991). The role of magnesium and calcium in alleviating aluminum toxicity in wheat plants. In R.J.Wright, V.C. Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 763-768). Kluwer Academic Publishers, The Netherlands.

Kerridge, P.C. & Kronstad, W.E. (1968). Evidence of genetic resistance to aluminum toxicity in wheat *(Triticum aestivum* Vill, Host). Agron. J. 60, 710-711.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larkin, P.J. (1987). Calmodulin levels are not responsible for aluminum tolerance in wheat. Aust. J. Plant Physiol. 14, 377-385.

Lewinsohn, E., Gijzen, M., Muzika, R.M., Barton, K. & Croteau, R. (1993). Oleoresinosis in grand fir (*Abies grandis*) saplings and mature trees. Modulation of this wound response by light and water stresses. Plant Physiol. 101, 1021-1028.

Marschner, H. (1991). Mechanisms of adaption of plants to acid soils. In R.J.Wright, V.C.Baligar & R.P. Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 683-702). Kluwer Academic Publishers, The Netherlands.

Matlock, M. & Ownby, J.D. (1988). Effects of aluminum on protein synthesis in wheat root tips. Plant Physiol. S86, 357.

Matsumoto, H. & Morimura, S. (1980). Repressed templet activity of chromatin of pea roots treated by aluminum. Plant Cell Physiol. 21, 951-959.

McElwain, E.F. & Spiker, S. (1992). Molecular and physiological analysis of a heat-shock response in wheat. Plant Physiol. 99, 1455-1460.

Meharg, A.A. (1994). Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. Plant Cell Environ. 17, 989-993.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophrosis of proteins. J. Biol. Chem. 10, 4007-4021.

Ouazzani, C. & Berville, A. (1991). Isolation and antigenic characterization of sugar beet (*Beta vulgaris* L.) mitochondrial F_1-ATPase: Studies of some Beta species and of the cytoplasmic male strile Owen form. Plant Sci. 74, 53-64.

Ownby, J.D. (1993). Mechanisms of reaction of hematoxylin with aluminum-treated wheat roots. Physiol. Plant. 87, 371-380.

- & Hruschka, W. R. (1991). Quantitative changes in cytoplasmic and microsomal proteins associated with aluminum toxicity in two cultivars of winter wheat. Plant Cell Environ. 14, 303-309.

Padidam, M. & Johri, M.M. (1991). Regulation of nitrate reductase by molybdenum in chloronema cell cultures of the moss *Funaria hygrometrica*. Plant Sci. 76, 19-28.

Paliwal, K., Sivaguru, M. & Thiruselvi, A. (1994). Identification of an aluminum tolerant tropical cowpea cultivar by growth and biomass accumulation parameters. J. Plant Nutr. 17, 367-376.

Picton, S.J., Richards, K.D. & Gardner, R.C. (1991). Protein profiles in root-tips of two wheat (*Triticum aestivum* L.) cultivars with differential tolerance to aluminum. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 1063-1070). Kluwer Academic Publishers, The Netherlands.

Pogson, B.J. & Brady, C. J. (1993). Accumulation of the ß-subunit of polygalacturonase 1 in normal and mutant tomato fruit. Planta 191, 71-78.

Polle, E., Konzak, C.F. and Kittrick, J.A. (1978). Visual detection of aluminum tolerance levels in wheat by hematoxylin staining of seedling roots. Crop Sci. 18, 823-827.

Przymusinski. R. & Gwozdz, E.A. (1994). Increased accumulation of the 16 x 10' M-r polypeptide in lupin roots exposed to lead, copper and nitrite ions. Environ. Exp. Bot. 34, 63-68.

Puthota, V., Cruz-Ortega, R., Johnson, J. & Ownby, J. (1991). An ultrastructural study of the inhibition of mucilage secretion in the wheat root cap by aluminum. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 779-787). Kluwer Academic Publishers, The Netherlands.

Putterill, J.J. & Gardner, R.C. (1988). Proteins with the potential to protect plants from Al^{3+} toxicity. Biochim. Biophys. Acta 964, 137-145.

Rao, N. R. & Ownby, J. D. (1993). Development of an ELISA for estimation of the copper nutritional status of wheat and cotton. Plant Soil 155-156, 453-456.

Rengel, Z. (1992). Disturbance of cell Ca^{2+} omeostasis as a primary trigger of Al toxicity syndrome. Plant Cell Environ. 15, 931-938.

Rincon, M. & Gonzales, R.A. (1991). Induction of protein synthesis by aluminum in wheat (*Triticum aestivum* L.) root tips. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 851-858). Kluwer Academic Publishers, The Netherlands.

Robertson, A.J., Ishikawa, M., Gusta, L.V. & MacKenzie, S.L. (1994). Abscisic acid-induced heat tolerance in *Bromus inermis* leyss cell-suspension cultures. Heat-stable, abscisic acid-responsive polypeptides in combination with sucrose confer enhanced thermostability. Plant Physiol. 105, 181-190.

Robins, R. J. (1986). The measurement of low-molecular-weight, non-immunogenic compounds by immunoassay. In H. F. Linskens & J. F. Jackson (Ed.): Modern methods of plant analysis 4: Immunology in plant sciences (pp. 86-137). Springer-Verlag: Berlin Heidelberg New York.

Sachs, M.M. & Ho, T-H, D. (1986). Alteration of gene expression during environmental stress in plants. Annu. Rev. Plant Physiol. 37, 363-376.

Saradhi, A. & Saradhi, P.P. (1991). Proline accumulation under heavy metal stress. J. Plant Physiol. 138, 554-558.

Sekler, I. & Pick, U. (1993). Purification and properties of a plasm membrane H^+-ATPase from the extremely acidophilic alga *Dunaliella acidophila*. Plant Physiol. 101, 1055-1061.

Shimoni, M. (1994). A method for activity staining of peroxidase and ß-1,3-glucanase isozymes in polyacrylamide electrophoresis gels. Anal. Biochem. 220, 36-38.

Siegel, N. & Haug, A. (1983). Aluminum interaction with calmodulin: Evidence for altered structure and function from optical and enzymatic studies. Biochim. et Biophys. Acta 744, 36-45.

- & Haug, A. (1983). calmodulin-dependent formation of membrane potential in barley root plasma membrane vesicles: A biochemical model of aluminum toxicity in plants. Physiol. Plant. 59, 285-291.

Slaski, J.J. (1989). Effect of aluminum on calmodulin-dependent and calmodulin-independent NAD kinase activity in wheat (*Triticum asetivum* L.) root tips. J. Plant Physiol. 133, 696-701.

Snowden, K.C. & Gardner, R.C. (1993). Five gene induced by aluminum in wheat (*Triticum aestivum* L.) roots. Plant Physiol. 103, 855-861.

Soon, Y.K. (1993). Fractionation of extractable aluminum in acid soils: a review and a proposed procedure. Commun. Soil Sci. Plant Anal. 24, 1683-1708.

Suhayda, C.G. & Haug, A. (1986). Organic acids reduce aluminum toxicity in maize root membranes. Physiol. Plant. 68, 189-195.

Taylor, G. J. (1988). The physiology of aluminum phytotoxicity. In H. Sigel & A. Sigel (Ed.). Metal ions in biological systems. Aluminum and its role in biology vol:24 (pp. 123-163). Marcel Dekker, New York.

- (1991). Current views of the aluminum stress response; the physiological basis of tolerance. In D. D. Randall, D. G. Blevins & C. D. Miles (Ed.). Current topics in plant biochemistry and physiology vol:10 (pp. 57-93). University of Missouri-Columbia, Columbia, MO.

Tomsett, A. B. & Thurman, D. A. (1988). Molecular biology of metal tolerances of plants. Plant Cell Environ. 11, 383-394.

Van de Locht, U., Meier, I., Hahlbrock, K. & Somssich, I.E. (1990). A 125 bp promoter fragment is sufficient for strong elicitor-mediated gene activation in parsley. EMBO J. 9, 2945-2950.

Vernon, D.M. & Bohnert, H.J. (1992). A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum*. EMBO J. 11, 2077-2085.

Vos-Scheperkeuter, G.H. de Wit, J.G., Ponstein, A.S., Feenstra, W.J. & Witholt, B. (1989). Immunological comparison of the starch branching enzymes from potato tubers and maize kernels. Plant Physiol. 90, 75-84.

Wagatsuma, T., Kaneko, M. and Hayasaka, Y. (1987). Destruction process of plant root cells by aluminum. Soil Sci. Plant Nutr. 33, 161-175.

- Nakashima, T. & Tawaraya, K. (1991). identification of aluminum-tolerant protoplasts in the original root protoplast population from several plant species differing in aluminum tolerance. In R.J.Wright, V.C.Baligar & R.P.Murrmann (Ed.). Plant-Soil Interactions at Low pH, (pp. 789-793). Kluwer Academic Publishers, The Netherlands.

Wallace, S.U. & Anderson, I.C. (1984). Aluminum toxicity and DNA synthesis in wheat roots. Agronomy 76, 5-8.

Werner, B. (1992). Nutritional Disorders of Plants-Development, Visual and Analytical Diagnosis. (pp. 282-289). G. Fischer, Jena Stuttgart New York.

Zhang, G. & Taylor, G.J. (1988). Effect of aluminum on growth and distribution of aluminum in tolerant and sensitive cultivars of *Triticum aestivum* L. Comm. Soil Sci. Plant Anal. 19, 1195-1205.

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