GENES INDUCED BY ALUMINUM TOXICITY

IN WHEAT (*Triticum aestivum* L.)

By

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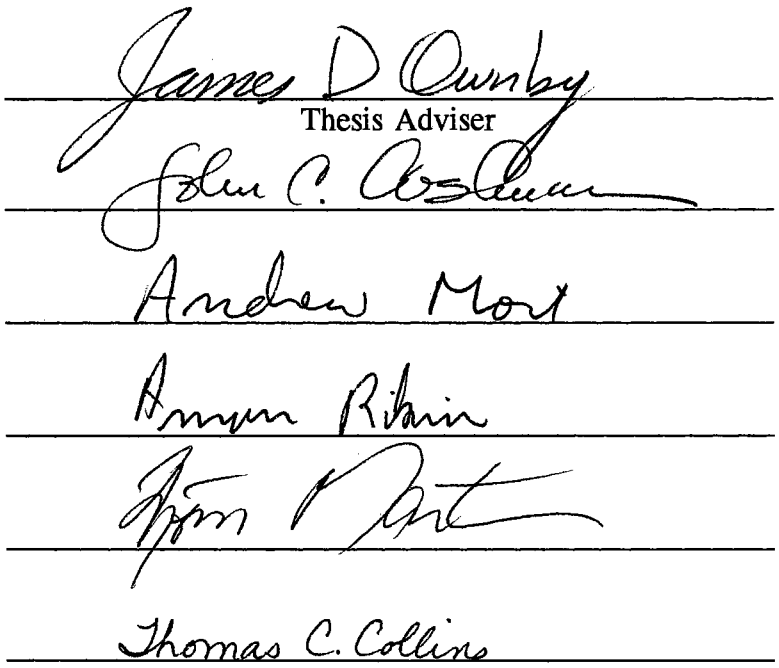

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INTRODUCTION

Plants as sessile organisms are vulnerable to various and diverse environmental stresses. Of these, aluminum toxicity is a major growth limiting factor in soils whose pH is below 5.0 (Foy et al. 1978). In spite of much research on Al toxicity, there is no consensus on the physiological mechanism(s) of Al toxicity or tolerance in plants. Like other stresses, Al induces changes in the pattern of gene expression. In wheat roots proteins (Ownby and Hruschka, 1991; Rincón and Gonzalez, 1991; Delhaize et al. 1991; Basu et al. 1994a) and cDNA clones (Snowden and Gardner, 1993; Richards et al. 1994) have been identified that are up-regulated by Al toxicity. Further characterization of the Al-induced proteins, and identification of additional genes regulated by Al, is necessary in order to gain a better understanding of the mechanisms of Al toxicity in plants.

Triticum aestivum L, or common wheat, is an important crop plant that is sensitive to Al in acid soils. Forage and grain yields are reduced by Al toxicity when soil pH drops below 5.0 (Westerman et al. 1992). Al-tolerant wheat varieties are needed. Identification of genes that confer tolerance to Al toxicity would be an important aid for further biotechnological studies and improvement of Al tolerant wheat varieties.

The present study had as its main objective the molecular characterization of TAl-18, a protein induced by Al toxicity in wheat roots whenever root growth was

arrested (Cruz-Ortega and Ownby, 1993). To accomplish this objective, a cDNA library made from wheat (cv Victory) roots treated with toxic levels of Al was screened. For the first screen, a degenerate oligonucleotide, made from the partial amino acid sequence of TAl-18, was used as probe. None of the seven positive clones selected corresponded to TAl-18. However, two interesting cDNA clones were obtained. The first corresponded to the full length cDNA clone that codes for a novel 1,3-ß-glucanase in wheat. These glucanases are pathogenesis-related proteins (PR) which can be induced by different stress factors (Simmons, 1994). The second partial cDNA clone was similar to the cytoskeletal proteins, fimbrin and plastin, that have been characterized in animals and fungi. These proteins are involved in the three-dimensional organization of the cytoskeleton.

A second screening of the cDNA library was done using the full-length cDNA clone of pathogenesis-related PR2 from parsley as a probe (Somssich et al. 1988), to which TAl-18 amino acid sequence has similarity. None of the positive clones selected were similar to PR2 or any of the pathogenesis-related proteins. Two of five cDNA clones sequenced were similar to cytochrome P-450 from *Zea mays*. This result was of interest because P-450 enzymes are involved in the synthesis of secondary metabolites, many of which protect the plant against a variety of pests and pathogens (Frey et al. 1995).

This dissertation is divided into four chapters: Chapter I presents an overview of the fundamentals of Al toxicity and pathogenesis-related proteins. Chapter II describes the different attempts made to characterize TAl-18 gene. Chapter III

describes the characterization of 1,3-ß-glucanase cDNA clone, and its expression under Al toxicity. Chapter IV describes the cDNA clone encoding a cytoskeleton protein and its expression under Al toxicity. The importance and significance of the genes which were expressed in Al-intoxicated roots are discussed.

CHAPTER I

LITERATURE REVIEW

A. Environmental Stress and Plant Gene Responses

Plants, like all living organisms, are subjected to both biotic and abiotic environmental stresses. They have evolved different physiological mechanisms to help them to cope with these adverse conditions. Among these mechanisms, alteration of the normal pattern of gene expression is an important part of the ability of the plant to respond to the environment (Sachs and Ho, 1986). During the last decades, many groups have studied the response of plants to stress with emphasis on the analysis of gene expression (For review see Basra, 1994). These studies have been focused on the identification of genes that may confer, or are related to, stress tolerance. The characterization, function, and regulation of these genes are the bases for future goals in biotechnology for improvement of genotypes in agronomic species.

Environmental stresses such as pathogens, temperature stress, salinity, water stress, and metal toxicity have all been shown to induce changes in plant gene expression and therefore in the pattern of proteins. Examples of stress-induced genes are those that code for heat shock proteins (HSP) (Vierling, 1991; Waters, 1995); LEA proteins (Bray, 1993); dehydrins (Serrano and Gaxiola, 1994); Pathogenesis-related proteins (PR)(Van Loon et al. 1994); COR proteins (Dure, 1993)), among others.

Aluminum toxicity, as an environmental stress, also induces alteration in gene expression. Since this study concerns an Al-induced protein, TAl-18, that has similarity with PR proteins, the purpose of this chapter is to review major aspects of the physiology of Al toxicity, as well as a brief description of PR proteins.

B. Aluminum Toxicity

1. Aluminum, acid soils and site of aluminum toxicity.

Aluminum is the most abundant metal in the earth's crust. In soils where pH is neutral or mildly acidic (5.5 to 6.0) aluminum is in the form of insoluble aluminosilicates or oxides, or bound to inorganic ligands such as PO_4^{3-}, SO_4^{2-}, F^{1-}, or to organic acids such as citrate and malate (Kinrade, 1991). When the pH of the soils falls below pH 5.5, solubilization of aluminum occurs and phytotoxic species, such as Al^{3+}, are released into the soil solution to levels that affect plant growth (Foy et al. 1978). Aluminum toxicity has been recognized as a primary factor limiting crop production on acid soils, which comprise 30 to 40% of the arable world's land, particularly in the tropics and subtropics (for review see Foy, 1988, Delhaize and Ryan, 1995, Kochian, 1995; and Von Uexkull and Mutert, 1995).

The first and major symptom of aluminum toxicity is a rapid inhibition of root elongation (Foy et al. 1978) which can occur within the first and second hour after exposure to aluminum (Ownby and Popham, 1989). The root apex (root cap, meristem, and elongation zone) accumulates more Al and experiences greater physical damage than the mature tissues. Ryan et al. (1993) have shown that only the apical 2 to 3 mm of maize roots, which includes the meristem and root cap, need to be

exposed to aluminum to cause inhibition of root growth. They showed that root growth is unaffected when aluminum is selectively applied to the elongation zone or to the entire root (except the root apex), and that aluminum inhibition of root growth was unaltered in decapped maize roots. Kochian (1995) asserts that although the last assumption argues against a major role for the root cap in Al-toxicity or Al tolerance, the research on the mechanisms of Al toxicity or modes of action should be directed to Al interaction within the root apex and not to the entire root system.

2. Mechanisms of Aluminum toxicity.

How does aluminum interfere with plant cellular processes? Must it enter the symplasm to be toxic? Which aluminum species are present inside the cytoplasm? These are questions that are still unresolved. There are many different mechanisms of aluminum toxicity that have been proposed. These mechanisms include:

a. Aluminum interactions with the root cell wall. Foy et al. (1978) proposed that, through the displacement of Ca^{2+} from cell wall pectic acids, Al can produce a strong adhesion between cell walls, thus inhibiting cell elongation. Such a reaction could also reduce the movement of water and mineral nutrients through the cell wall interstices (Blamey et al. 1993). Recently Ostatek-Boczynski et al. (1995), in a study comparing the binding capacity of Ca-pectate and pectin for Al, found that only 29% of free Al remained in solution in the presence of Ca-pectate, while 54% remained when pectin was present. They suggested that Ca-pectate, rather than pectin itself, is responsible for binding Al in root cell walls. In this study, however, it was not determined if calcium was displaced by aluminum.

b. Aluminum disruption of the plasma membrane and transport processes.

Proposed effects of aluminum on membrane integrity and function include binding of Al to membrane lipids (Haug and Shi, 1991), inhibition of ATPase activity (Matsumoto and Yamaya, 1986), NADH-linked electron transfer (Loper et al. 1993), and disruption of ion channel functions (Rengel and Elliott, 1992). However, various electrophysiological studies have shown that cells in Al-treated wheat root maintain reasonable electrical potentials, H^+ efflux, and K^+ uptake, similar to those of the control roots, indicating a relatively intact, functional plasma membrane (Nichol et al. 1991). Studies on Al inhibition of root apical ion transport processes have shown that Ca^{2+} influx is inhibited at the root apex (the critical site for toxicity) through the inhibition or blockage of Ca^{2+} channels in the root-cell plasma membrane (Huang et al. 1992). Hypothetically, Rengel and Elliot (1992) proposed that Al may affect Ca^{2+} fluxes through the plasma membrane by: 1. affecting Ca^{2+} channels either directly or indirectly by interfering with the action of inositol-1-4,5-triphosphate and GTP-binding proteins, and 2. affecting Ca^{2+}-translocating ATPases. In the same study, the authors showed that Al and Ca^{2+} channel blockers (verapamil and bepridil) decreased net $^{45}Ca^{2+}$ uptake in *Amaranthus tricolor* protoplasts. This decrease in Ca^{2+} uptake was more pronounced when Al and bepridil were both present in the media. This additive effect of Al was not detected in the case of verapamil. The authors suggested that Al might bind to the verapamil-binding sites. In spite of Rengel and Elliott (1992) studies, there is still some controversy over whether or not Al inhibits Ca^{2+} uptake. Huang et al. (1993) observed that Al could inhibit the

uptake of Ca^{2+} in wheat seedlings, but Ryan et al. (1994) found that low concentrations of Al inhibited root growth without inhibiting Ca^{2+} uptake. More recently, Huang et al. (1994) and Piñeros and Tester (1993) have characterized a Ca^{2+} channel in the wheat root plasma membrane and have shown that micromolar Al concentrations effectively blocked this channel. This effect however, was the same in Al sensitive and tolerant wheat cultivars. Although the correlation between rapid Al-induced inhibition of root apical Ca^{2+} influx and Al sensitivity to phytotoxicity provides an attractive and speculative framework for a possible Al toxicity mechanism, it is not clear how blockage of Ca^{2+} channels would cause a rapid inhibition of root growth. However, a fundamental role of Ca^{2+} has been recognized for polarly growing cells such as rhizoids of fern and algae, fungal hyphae, and root hairs (references in Herrmann and Felle, 1995). Schiefelbein et al. (1992) showed that root-hair length in *Arabidopsis thaliana* was dependent on the concentration of Ca^{2+} in the growth medium, with a maximum length achieved at $[Ca^{2+}]$ of 0.3-3.0 mM. When root hairs were exposed to the Ca^{2+}-channel blocker nifedipine, tip growth stopped. Using a non-intrusive calcium-specific vibrating microelectrode, an extracellular Ca^{2+} gradient was detected at the tips of individual growing root-hair cells. The precise role of the Ca^{2+} taken up by the growing root hairs is not understood. The same authors speculate that possible intracellular roles of Ca^{2+} include: Ca^{2+} regulation of vesicle secretion; directed organelle transport; cytoskeleton arrangement and Ca^{2+}-and calmodulin-dependent enzyme activities.

c. Aluminum interactions with symplasmic constituents. In a study using secondary-ion mass spectrometry, Lazof et al. (1994) showed that Al can be detected in the symplasm of soybean (*Glycine max*) roots after 30 min of exposure to Al. This suggests that a symplasmic site of Al toxicity is possible. In the cytoplasm with a pH of 6.5 to 7.5, with an abundance of potential ligands, the concentration of Al^{3+} (toxic species) ions has been estimated to be in the picomolar to nanomolar range (Martin, 1988). Despite the apparent low concentration of toxic Al in the cytoplasm, there are studies that suggest that Al could cause considerable damage in the symplasm due to its high binding affinity for many metabolically important molecules (Martin, 1988; Haug et al. 1994).

Specific cytoplasmic processes that could be affected by Al include i) signal transduction processes; ii) interaction with the cytoskeleton, and iii) interaction with calmodulin:

i. Signal transduction processes. It is well documented in the medical field that aluminum toxicity can involve interactions between Al^{3+} and components of the phosphoinositide signal transduction pathway (Haug et al. 1994). A recent study in plants has shown that Al interferes with this pathway by inhibiting the signal-induced rise in cytosolic IP_3 (inositol triphosphate). Jones and Kochian (1995) found in wheat that inhibitory levels of Al (50 μM) reduced the levels of IP_3, and inhibited phospholipase C. From the medical research it appears that Al either binds a Gp (GTP/Guanine nucleotide-binding protein) probably at the Mg-binding site, or interacts directly with the membrane-receptor phospholipase C (Haug et al. 1994).

ii. Interaction with the cytoskeleton. Aluminum may also disrupts the organization of the cytoskeleton network, with an immediate consequence for growth-related activities. Studies on humans and animals have shown that Al strongly promoted tubulin assembly into microtubules and inhibited subsequent Ca-induced depolymerization of the microtubules (Macdonald et al. 1987). In plants, Grabski and Schindler (1995) showed that aluminum induces rigor within the actin network of soybean cells (see also Chapter IV). They hypothesized that this rigor may result from the formation of nonhydrolyzable [Al^{3+}-ADP] or [Al^{3+}-ATP] complexes whose binding to actin/myosin can modify contraction.

iii. Interaction with calmodulin. Much controversy exits over whether or not Al interacts with the Ca^{2+}-binding protein calmodulin. Siegel and Haug (1983) proposed that Al induced specific changes in calmodulin structure, which were reflected in reduced formation of the membrane potential when assayed with the fluorescent potential probe oxionol VI. They reported that Al^{3+} binds stoichiometrically to the Ca^{2+}-binding sites in calmodulin with a 10-fold higher affinity than Ca^{2+}, eliciting a dramatic conformational change in calmodulin that could inhibit its ability to activate other enzymes, such as calmodulin-activated phosphodiesterases. However, You and Nelson (1991), using EPR analysis of calmodulin spin-labeled at either methionine or tyrosine residues, found no effect of Al on calmodulin at the physiological pH of 6.5 or 5.5. Richardt et al. (1985), using flow dialysis techniques, found that the inhibition of the calmodulin-activated phosphodiesterase was due to direct effects of Al on the enzyme and not on calmodulin itself.

3. Mechanisms of Aluminum Tolerance.

Just as the mechanisms of toxicity are not completely understood, the mechanisms of tolerance are likewise not well defined. Several mechanisms have been proposed to explain the differential tolerance to Al among plants.

Native and crop species exhibit significant genetically-based variability in their responses to Al toxicity. Aniol and Gustafson (1984), using an aneuploid series of wheat cv Chinese Spring, found that Al tolerance genes are located on the short arm of chromosome 5A and the long arm of chromosome 2D and 4D. However, in some cases significant Al tolerance can be conferred by single, dominant genes. Delhaize et al. (1993a) produced near-isogenic lines that differed in Al tolerance at a single locus (*Alt1* locus). The intentional loss of other genes in the derivation of these lines avoids the possible complication of several different mechanisms contributing to the Al tolerance (Delhaize and Ryan, 1995).

Mechanisms of tolerance are described in detail in reviews by Foy, (1988); Roy et al. (1988); Haug and Shi (1991); Taylor (1991); Delhaize and Ryan, (1995); Kochian, (1995); and Carver and Ownby (1995). In general, the mechanisms of tolerance can be divided into two categories: 1). External mechanisms or exclusion of Al from the root apex. In this model Al is excluded from plant tissue, especially the symplastic portion of the root meristems, preventing Al from reaching metabolic sites. 2). Internal mechanisms, by which the plant in some way detoxifies the Al that enters the plant symplasm.

From the research over the past decade, external tolerance mechanisms have

received more experimental evidence than internal mechanisms.

1. External Tolerance Mechanisms. Several independent studies provide strong evidence that Al-tolerant genotypes of wheat exclude Al from their root apices. Rincón and Gonzalez (1992) showed that an Al-sensitive wheat cultivar (Scout) accumulates about 8-fold more Al in the root apex (critical site for Al toxicity), than the Al-tolerant wheat cultivar Atlas 66. They found no difference between the cultivars in Al content of mature root tissues. Delhaize et al. (1993b) found similar results with seedlings of near-isogenic lines that differed in tolerance at the *Alt1* locus. Specific mechanisms of Al exclusion are discussed below.

a. Immobilization of Aluminum in the cell wall region. It is generally thought that binding of Al^{3+} to charged sites on the cell surface is a prerequisite for uptake and toxicity. The binding and immobilization of Al^{3+} in the cell wall would prevent Al^{3+} from associating with the plasma membrane or entering the symplasm. Blamey et al. (1993) have provided evidence that Al^{3+} displaces Ca^{2+} from cell wall pectic acids, which reduces the movement of water and mineral nutrients through the cell wall interstices. On the other hand, Kinraide et al. (1992) have concluded that cell surface negative charges, derived from cell wall pectin as well as charge sites on membrane lipids and proteins, do not play a significant role in differential Al tolerance in wheat.

b. Exclusion of Aluminum at the plasma membrane. There is little evidence that fundamental differences in membrane organization contribute to differential Al tolerance in plants. Lindberg and Griffith (1993) found that Al toxicity in sugar beet

roots was associated with an increase in the ratio of phosphatylcholine to phosphatidylethanolamine which could increase membrane permeability. However, disruption of plasmalemma integrity and function is probably occurring during long-term effects of Al-toxicity, as part of the overall disruption of cell metabolism (Meharg, 1993).

c. Release of organic acids from the root apex. Among the different external mechanisms proposed, the release of organic acids from the root apex has the strongest experimental evidence. Miyasaka et al. (1991) provided evidence that Al tolerance in snapbeans was due to the release of citrate from the root apex of a tolerant line only. They pointed out, however, that this could have been caused by an Al-induced phosphorus deficiency. Stronger evidence has come from Delhaize et al. (1993a). They found that the amount of malate released from an Al tolerant wheat genotype (possessing the tolerant *Alt1* allele) was 5 to 10 times greater than the amount released from a near-isogenic but Al-sensitive genotype. They suggested that the excreted malate protects the plant by chelating and detoxifying Al around the root apex. Evidence that support their conclusion includes: 1. Malate efflux is triggered only by Al^{3+}, and not by La^{3+}, Sc^{3+}, Mn^{2+}, or Zn^{2+}; 2. Malate efflux is localized only in the root apex, the site of Al toxicity; 3. Addition of malate to nutrient solution with phytotoxic Al concentrations protects Al-sensitive cultivars from toxicity; and 4. high rates of malate efflux from roots co-segregate with the *Alt1* locus in population segregating for Al tolerance. Ryan et al. (1995a) screened 36 wheat genotypes for Al tolerance and for the amount of malate released from their

root apices. They found a significant correlation between relative tolerance of the genotypes to Al and the amount of malate released from their root apices. Basu et al. (1994b) have shown similar differences in malate release from several other Al-tolerant and sensitive wheat cultivars. The processes involved in Al perception and the associated organic acid release are discussed by Ryan et al. (1995b) and by Delhaize and Ryan (1995). Organic acids reside in the symplasm as anions, and release is presumed to be facilitated by anion channels. Ryan et al. (1995b) showed that Al exposure triggered an immediate release of malate in wheat that was inhibited by various antagonists of anion channels. Thus, the primary mechanism probably involves an Al-mediated opening of a malate-permeable channel. Root apices of both Al-tolerant and Al-sensitive genotypes showed similar activities of PEP carboxylase and malate dehydrogenase (Ryan et al. 1995a), two enzymes important in malate synthesis. Delhaize and Ryan (1995) proposed that since the root apices of Al-sensitive and Al-tolerant genotypes have the same capacity to synthesize malate, the difference must reside in their abilities to transport malate across the plasma membrane in response to Al.

The same authors proposed an hypothetical model to explain three ways in which Al^{3+} might trigger the opening of a putative malate-permeable channel: 1. Al interacts directly with the channel protein, causing a change in conformation that induces the channel to be opened. 2. Al might interact with a specific receptor on the membrane. This interaction would produce secondary messages, that would cause changes in channel activity, and 3. Al might enter the cytoplasm and alters channel

activity by directly binding with the channel protein or through a signal transduction pathway. This proposed model is illustrated in figure 1 taken from Delhaize and Ryan (1995).

Exudation of malate or other organic acids is the most promising mechanism of Al tolerance yet studied. It remains to be shown that the fluxes of malate are sufficient to protect root apices from the damage of Al. The mucilage secreted by the root cap will increase the boundary layer surrounding the root apex, helping to maintain a malate concentration sufficient to protect the root apex (Delhaize and Ryan, 1995). Henderson and Ownby (1991) have shown a significant correlation between Al-tolerance and mucilage production in various wheat cultivars differing in Al tolerance.

2. Internal Tolerance Mechanisms. There is limited evidence that Al tolerance in plants is due in part to an internal mechanism. Aniol (1984) suggested that plants could develop Al tolerance through the synthesis of proteins that bind or sequester Al and render it innocuous within the symplast. Since then there has been an intense effort by many research groups to identify proteins synthesized in tolerant but not in sensitive cultivars during Al toxicity.

Effects of Aluminum on protein and gene expression. Al induces the synthesis of a range of proteins in root apices of wheat, but there is no conclusive evidence to link these to an Al-tolerance mechanism. Many of these proteins are induced in both Al-tolerant and Al-sensitive genotypes, which argues against a role for these proteins in Al tolerance. Snowden and Gardner (1993), and Richards et al. (1994), screening

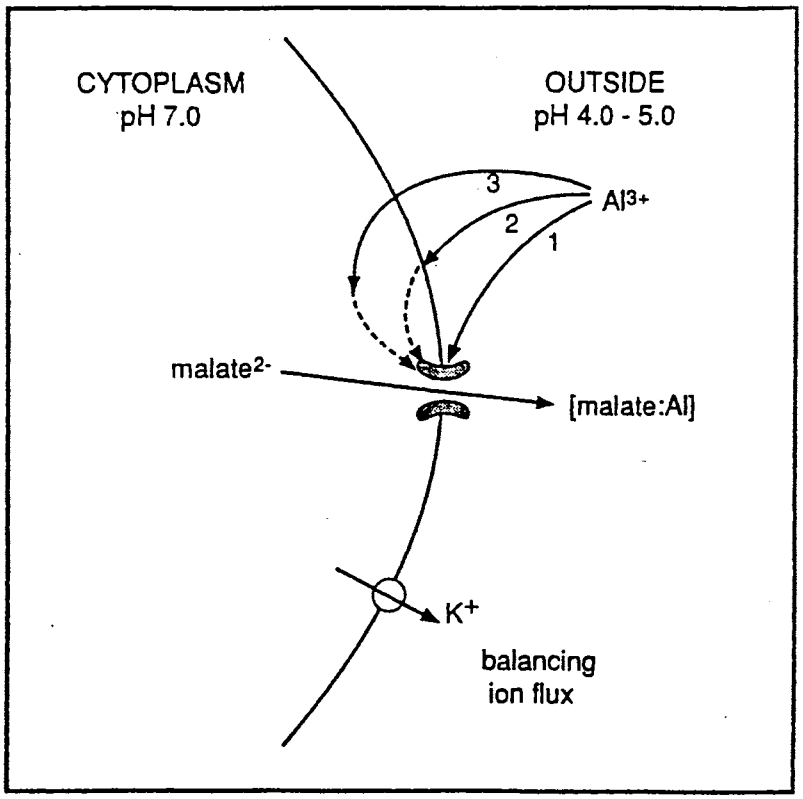


Figure 1. Hypothetical model showing how Al^{3+} interacts with a malate-permeable channel (hatched structure) in plasma membranes to stimulate malate efflux. The three numbered arrows are explained in the text. Taken from Delahaize and Ryan, (1995).

a cDNA library from an Al-sensitive wheat genotype, cloned seven cDNAs that were Al-induced. These clones were termed *wali* for wheat aluminum induced. The proteins encoded by these *wali* clones showed similarity to the metallothionein-like proteins (MLP) of plants (*wali*1), phenylalanine ammonia-lyase (PAL, *wali*4), proteinase inhibitors (*wali*3, *wali*5, and *wali*6), and part of plant asparagine synthetases (*wali*7). The *wali* genes are induced 24 to 96 h after roots are exposed to Al, and the degree of induction is related to the degree of Al stress in both Al-sensitive and Al-tolerant cultivars. None of the proteins appear to have any direct role in conferring Al tolerance to plant roots. The PAL clone was suggested to function in synthesis of flavonoids that could bind Al, and the MLPs could function in binding heavy metals (Snowden and Gardner, 1993). In a subsequent study Snowden et al. (1995) found that *wali* genes are induced by other metals (Cd, Fe. Zn, Cu, Ga, In, and La), by growth in low levels of Ca (10 μM), and by wounding. These results show that *wali* gene induction is part of a general stress response rather than Al-specific. To date there has been no unequivocal demonstration of a protein that is the product of a gene conferring Al tolerance. Picton et al. (1991), Delhaize et al. (1991), Ownby and Hruschcka (1991), and Rincón and Gonzalez (1991) have shown that Al induces the synthesis of a number of different proteins in the root apex, but in most cases these proteins are found to be induced in both Al-sensitive and Al-tolerant cultivars. On the other hand, Basu et al. (1994a) have identified two forms of a 51 kD protein, called RMP51, in the microsomal fraction extracted from the terminal 5 mm of wheat root tips. RMP51 is rapidly induced in the Al-tolerant cultivar but not

in the Al-sensitive one. It is also induced by Cd but not by heat shock or toxic levels of Mn and Cu, and turns over after Al is removed from the growth medium. Cruz-Ortega and Ownby (1993) studied the synthesis of an acidic, 18 kD protein that is induced by Al in wheat roots. This protein, named TAl-18, is also induced by other stresses such as Cu, Cd, by low levels of Ca, and low pH, but not by heat shock. Expression of TAl-18, as well as the *wali* genes, seems to be part of the general stress response of the plant.

C. Stress induced proteins: PR proteins and TAl-18 protein.

In response to pathogenic attack, some plants activate the expression of what are known as "defense-related" genes (Bowles, 1990). Pathogenesis-related or PR proteins, the products of these genes, were first detected in cultivars of tobacco (Xanthi-nc and Samsun) carrying the N-resistance gene against TMV (tobacco mosaic virus) (Van Loon et al. 1987). Since then, proteins similar to tobacco PR proteins have been identified in a wide range of both dicots and monocot plants including tomato, potato, bean, maize, and barley (Linthorst, 1991). PR proteins are induced not only by viruses, but also by fungal and bacterial pathogens, and by other agents such as ethylene and salicylic acid. In general, PR proteins are defined as: "host-encoded polypeptides which are not present in healthy plants and are synthesized only in response to pathological or related stress situations" (Linthorst, 1991). PR proteins are synthesized mainly during incompatible interactions which accompany the hypersensitive response and the establishment of the systemic acquired resistance. PR proteins are not only localized in the infected area, but are also found in uninfected

area, thus exhibiting the phenomenon of induced resistance against subsequent pathogen attack. However, direct evidence for a role of PR proteins in resistance has not yet been found (van de Rhee et al. 1994).

The PR proteins from tobacco have been the most intensively studied. Based first on serological properties and later in amino acid sequence similarities, the major tobacco PR proteins have been divided into five groups (Van Loon, 1985; Linthorst, 1991). All PR genes contain a sequence encoding a N-terminal signal peptide for transport across the membrane of the endoplasmic reticulum. The basic PR proteins have a C-terminal extension for targeting them to the vacuole. This extension is cleaved off during the translocation event. The acidic ones, which do not contain a vacuolar sorting signal, are transported to the extracellular space (Linthorst, 1991).

From the five groups of PR proteins described in the literature, group 2 (PR-2), which includes those PR proteins with 1,3-ß-glucanase activity, is important for this study and is further discussed in Chapter III.

In addition to the five groups of tobacco PR protein, another group of proteins have been described with no sequence similarity to the tobacco proteins but which are related to defense mechanisms. These proteins have a high amino acid sequence similarity to proteins in the pollen of birch trees (Breiteneder et al. 1989), which are major antigens in allergy patients. Two examples of this group are the products of parsley PR1 and PR2 genes, which are induced in parsley cell cultures by an elicitor prepared from the fungus *Phytophtora megasperma* f. sp. *glycinea* (Van de Löcht et al. 1990). The product of PR2 gene presents similarity with the Al-induced protein

TAl-18, which is described below.

TAl-18 PROTEIN. In a previous study, it was shown that aluminum toxicity induced an acidic low molecular weight (18 kD) protein in wheat roots. The expression of this protein was the most dramatic change in the pattern of proteins observed by 2D-PAGE following Al treatment of wheat roots (Ownby and Hruschka, 1991). In a subsequent study this wheat protein was named TAl-18, T from *Triticum*, Al from Aluminum, and 18 to denote its approximate molecular weight (Cruz-Ortega and Ownby, 1993). In the latter work it was observed that 1) TAl-18 protein was induced not only by aluminum toxicity, but also by other metals such as Cd and Cu, as well as by deprivation of Ca in the nutrient medium, and by low pH (3.5) but, not by heat shock. 2) Enhanced biosynthesis of TAl-18 was during the period 3 to 6 h after treatment with aluminum, and reached a maximum after 9 to 12 h of Al exposure. 3) TAl-18 partial amino acid sequence was similar to that of pathogenesis-related PR2 protein from parsley cells (Van de Löch et al. 1990). Expression of TAl-18 is apparently not related with tolerance to aluminum since it is induced in both Al-tolerant cultivars (i.e. Atlas 66) and Al-sensitive cultivars (Victory, TAM105) whenever sufficient Al is added to inhibit root growth by 70% or more. Thus, TAl-18 appears to be a stress protein whose increase levels reflect a physiological lesion resulting in root growth inhibition. On the other hand, the similarity of TAl-18 with PR proteins makes it plausible that TAl-18 is induced in wheat as part of a defense-related mechanism during periods of stress such as Al, Cu, and Cd toxicity as well as calcium deprivation. TAl-18 might have an antifungal role as do other PR proteins,

or another protective function during the stress period. The isolation and characterization of TAl-18 gene would enable us to know more about the potential function of TAl-18 protein, as well as to understand its role in aluminum toxicity. Once the gene has been isolated many of the characteristics of TAl-18 gene might be determined, such as the deduced amino acid sequence of the gene product, the conditions required for accumulation of its mRNA, and its *in situ* localization within the wheat root. The following chapter describes various attempts to isolate and characterized TAl-18 gene. Attempts to isolate the gene were based on the screening of a cDNA library made from mRNA of aluminum-intoxicated wheat roots at the time of the highest expression of TAl-18 protein.

CHAPTER II

CHARACTERIZATION OF THE GENE(S) ENCODING TAI-18, AN ALUMINUM-INDUCED PROTEIN IN WHEAT ROOTS (*Triticum aestivum* L)

INTRODUCTION

The purpose of this chapter is to described three different attempts made to characterize TAI-18 gene. The first attempt was the screening of a cDNA library made from poly A^+ RNA isolated from Al-intoxicated wheat roots (Al-sensitive cv Victory). The library was screened with a degenerate oligonucleotide designed from the partial amino acid sequence of TAI-18. A second attempt used RT-PCR (Reverse Transcription and PCR amplification). Total RNA from wheat roots was reverse transcribed using oligo dT as a primer. PCR amplification of cDNA was done using as sense primer a degenerate oligonucleotide designed from the most conserved amino acid sequence of the NH_2-terminal part of PR proteins related to PR2 and an antisense primer TAI-18 degenerate oligonucleotide. The third attempt was the screening of the cDNA library with the 800 bp full-length cDNA clone from parsley PR2 protein as a probe. In the following section the three attempts are described briefly as well as the results of each.

MATERIALS AND METHODS

1. Screening of the cDNA library with a degenerate oligonucleotide: Total RNA was extracted by the method of Chirgwin et al. (1979) and Kopeczynski et al. (1986) from root tips of intact wheat cv Victory seedlings exposed to 80 μM Al for 12 hours. Poly A^+ RNA was prepared using the FAST TRACK mRNA isolation kit from INVITROGEN Corporation (San Diego, CA). A custom cDNA library was constructed in lambda-ZAPII (Stratagene Inc., La Jolla, CA), with an insert size of $\geq$300 bp, and EcoRI as the cloning site. The cDNA was screened with a non-radioactively labelled (Lightsmith I, Promega Corporation, Madison WI) degenerate oligonucleotide probe (5' GTC TGG GTT G/C GC GTC G/CAG/A G/A T 3'). This 21-mer was designed from the partial amino acid sequence of TAl-18. The amino acid sequence Tyr, Leu, Asp, Ala, Asn, Pro, and Asp was chosen to design the oligonucleotide. The underlined amino acids are identical in PR2 protein in a region close to the C-terminal part of the protein. From back-translation this oligonucleotide has 2048 fold degeneracy. To reduce degeneracy a table of frequency (per 1000) of codon usages for different organisms was used (Brown, 1991). As an example, the codon for the amino acid proline may be CCA, CCU, CCG, or CCC. From the table of codon usages, the codon CCA has a frequency in wheat of 71.2 per 1000, compared to CCU with only 10.3 per 1000. Therefore, the codon chosen for proline in the oligonucleotide was CCA, which in the antisense sequence corresponds to TGG (second triplet in the oligonucleotide).

Approximately 160,000 pfu (plaques forming unit) were transferred onto nylon

membranes (MSI, Westboro, MA). Hybridization and washing conditions were according to the Lightsmith I (Promega Corporation, Madison, WI) manual instructions. The hybridization temperature was 50°C with 150 fmol/ml of labelled probe. After three cycles of plating and re-screening, seven positive clones were selected. DNA from these selected plaques was rescued as BlueScript SK-phagemids by co-infection with R408 helper phage (Stratagene Inc., La Jolla, CA), and used to transfect *E.coli* XL-1 Blue cells. Insert DNA was isolated using the modified mini alkaline-lysis/PEG precipitation procedure. Plasmids were excised by EcoRI digestion and analyzed on agarose gels (Sambrook et al. 1989).

DNA Sequencing Analysis: The nucleotide sequence of the positive clones was determined on an ABI 373A automated DNA sequencer using the Prism Ready Reaction Dyedeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with M13 reverse and T7 as primers. Data base searches were conducted using the National Center for Biotechnology Information BLAST E-mail (Altschul et al. 1990), and the NBRF protein sequence data, FASTA program (Pearson and Lipman, 1988).

2. RT-PCR using degenerate primers from most conserved amino acid sequence of PR-proteins and TAI-18 oligonucleotide. Total RNA from wheat roots treated with 80 μM Al for 12 h was used to synthesize first strand cDNA. The primer oligo(dT) (AAAAAA$(A)_n$ (Gibco-BRL) was used to select for poly A^+ RNA. 1 μg total RNA in 11.75 μl of DEPC (Diethyl Pyrocarbonate)-water was denatured at 65°C for 10 min, then 8.25 μl of reaction mixture was added. The reaction mixture

contained 4 μl of 5X Reverse Transcriptase buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA) (Gibco-BRL), 2 μl 100 mM DTT (Gibco-BRL), 1 μl 10 mM dNTPs mix (10mM each) (Gibco-BRL), 0.25 μl (40U/μl) RNasin (Promega), 0.5 μl oligo(dT) (500 mg/ml)(Gibco-BRL), and 0.5 μl (200U/μl) SuperScript Reverse Transcriptase (RNaseH-: Gibco-BRL)). The 20 μl reaction mixture was incubated at 42°C for 1 h. To terminate the reaction, the mixture was heated at 65°C for 10 min. Then 1 μl (2U/μl) RNase H (Gibco-BRL) was added and incubated at 55°C for 10 min. To remove primer and RNA degradation products a PCR-purification kit (QIAGEN) was used. For the amplification of the target cDNA by PCR, the sense primer 5'GGI A/CAT/C GGI GGI CCI GGI ACI A/GT C/AAA 3' (designed from most conserved region of PR proteins), and the antisense primer (5' GTC TGG GTT G/C GC GTC G/CAG/A G/A T 3') (oligonucleotide from TAI-18) were used. The PCR reaction mixture contained: 5 μl of 10X Taq polymerase buffer (Perkin Elmer-Cetus), 2 μl cDNA, 2 μl 25 mM $MgCl_2$, 8 μl 1.25 mM each of the four deoxyribonucleotides (dNTPs), 1 μl (100ng/μl) sense primer, 1 μl (150 ng/μl) antisense primer, 30.5 μl H_2O, and 0.5 μl Taq polymerase (Perkin Elmer-Cetus). The 50 μl mixture was overlayered with 50 μl of mineral oil and incubated in a MJ Thermocycler for a total of 30 cycles. The thermocycler program consisted of a denaturing step at 95°C for 1 min; 30 cycles that include 94°C for 45 sec, 50°C (annealing temperature) for 1 min, and 72°C (extension temperature) for 1.5 min; and an extra extension step at 72°C for 5 min. The expected PCR product (300 bp) was purified on a 3% agarose-1X TBE buffer (89 mM Tris, 89 mM borate, and 2 mM

EDTA) gel. The 300 bp band was cloned in PCRII vector using TA Vector Cloning kit (Promega). The plasmid was sequenced, using SP6 and T7 primers.

3. Screening of the cDNA library with the full-length PR2 cDNA clone as an heterologous probe. The same cDNA library as was used in the first attempt was screened using the 0.8 kb full-length PR2 cDNA clone from parsley cells (kindly donated by Dr I.E. Somssich, Max Planck-Institut für Züchtungsforschung, Germany). The PR2 cDNA clone was excised from the vector pUC9 with PstI and digoxygenin-labeled using the random priming method. Approximately a total of 160,000 pfu were transferred onto nylon membranes (MSI, Westboro, MA). Hybridization and washing conditions were according to the non-radioactive Genius System (Boehringer Mannheim), with a hybridization temperature of 55°C. After three cycles of plaque hybridization, 20 positive clones were selected, excised, and propagated as plasmids according to the manufacturer's instructions (Stratagene). Plasmids were excised by EcoRI or HindIII/BamHI and analyzed on agarose gels. The nucleotide sequence of five positive clones was determined as described above.

RESULTS

cDNA clones from first cDNA library screening. Seven positive clones were selected after three rounds of plaque hybridization. They were excised and propagated as plasmids, then restricted with BamHI and HindIII for insert analysis. The sequence of clones 1, 2 and 6, with size of 2.1 kb, exhibited high similarity to plant 1,3-ß-glucanases in the Gen Bank at NCBI. One of these clones was fully sequenced and is described in Chapter III. The sequence of clones 3, 4, and 7, with

size of 1.8 kb showed similarity with genes encoding cytoskeleton-like proteins. One of these clones was fully sequenced and is described in Chapter IV. Clone 5 gave no significant similarity to any sequence in the Data base bank.

RT-PCR approach. In order to obtain a larger probe to screen the cDNA library, RT-PCR technique was used to amplify TAl-18 mRNA. The PCR amplification gave a 264 bp product, which was purified by agarose gel and cloned into the PCRII vector and sequenced. The nucleotide sequence of this PCR product in the Data base showed no similarity with any PR gene or any other gene in the Gen Bank at NCBI.

cDNA library screening with heterologous probe. The cDNA library was screened with the full-length PR2 cDNA clone. 20 positive clones were selected after three rounds of plaque hybridization. These were excised and propagated as plasmids and then restricted with BamHI and HindIII for insert analysis. Clones 4, 5, 15, 16, and 20, whose insert size was around 1 kb were sequenced. The initial sequence of clones 15 and 20 were similar to cytochrome P-450 from *Zea mays* (Frey et al. 1995). Clone 16 was similar to 18S ribosomal RNA from different plants, and cDNA clones from *Arabidopsis thaliana*. Clone 4 and clone 5 were not significantly similar with any gene in the Gen Bank. No further attempts were made to sequence more clones since most of them had the same insert size as those already chosen.

DISCUSSION

Although the attempts to characterize TAl-18 gene were unsuccessful, the screening of the cDNA library provided evidence that during aluminum toxicity genes

such as the 1,3-ß-glucanase can be expressed. The 1,3-ß-glucanase cDNA clone was selected during the screening of the cDNA library with the degenerate oligonucleotide made from the partial amino acid sequence of TAl-18. Although 1,3-ß-glucanases belong to the family of PR proteins, no similarity was found between this 1,3-ß-glucanase cDNA clone and PR2 protein or any other low-molecular-weight PR protein. However, 13 out of the 21 base pairs from the oligonucleotide probe could have hybridized from position 858 to 879 of the complimentary strand of the cDNA clone, under the stringency conditions used. 1,3-ß-glucanases are considered to be PR or defense-related proteins because they are often associated with pathogen infection, particularly fungal infection, but they also can be induced by viral and bacterial pathogens (Dong et al. 1991), and by metal ions such as cobalt, barium, manganese, and mercury and silver ions (Fink et al. 1990). Thus, it is plausible that a cDNA library made from Al-stressed wheat roots might be enriched in messages for this stress-related enzyme. The estimated pI of the 1,3-ß-glucanase is 6.0, which correspond to an acidic protein. Generally it has been reported that the acidic isoforms of ß-glucanases are secreted to the intercellular spaces and play an important role in the defense against fungal attack in both dicots and monocots plants (Simmons, 1994, see also Chapter III). While a function for these ß-glucanases in protection against fungal attack seems credible, it is unknown why they are expressed during viral and bacterial attack, or during toxicity induced by metals such as aluminum. It is known that Al toxicity involves several different mechanism not well understood so far (see Chapter I). The primary response to Al toxicity in wheat is in roots,

involving inhibition of cell elongation and the swelling or thickening of the root tip cells. Bennet (1985) observed in *Zea mays* that cortical cells of Al damaged roots were swollen, with apparent distorted wall along with the rupture of the cells of the epidermis and outer cortex. This observation and the fact that Al toxicity affects the integrity of the plasmalemma and may render plant cells more leaky (Haug and Shi, 1991), suggests that during Al toxicity the root cells are more susceptible to pathogen attack. Under these vulnerable conditions the plant might induce the synthesis of stress-related proteins such as TAl-18 and 1,3 ß-glucanases as a protective mechanism against further attack by fungi or other plant pathogens.

The second clone selected during the cDNA screening corresponds to a cytoskeleton-like protein (described in Chapter IV). The search of the nucleotide sequence in the Gen Bank showed similarity with fimbrin/plastin proteins from chicken, human, yeast, and slime mold (de Arruda et al. 1990; Lin et al. 1993; Adams et al. 1991). Fimbrin/plastin proteins belong to the group of actin-bundling proteins (see Chapter IV). They have not yet been described in plants, thus there is no information concerning the induction of these proteins during stress conditions in plants. Details on the expression of these proteins in Al-stressed wheat roots are given in Chapter IV.

When the cDNA library was screened with the full-length cDNA of PR2 protein, two clones were selected that were similar to cytochrome P450 from maize (*Zea mays* L.). Cytochrome P450 is a membrane-bound, heme-containing enzyme implicated in several biosynthetic reactions. In plants it is involved in hydroxylation and

demethylation reactions of secondary metabolites (Bolwell, et at. 1994; Frey, et al. 1995), including hormones, phytoalexins, phenylpropanoids, flavonoids, terpenoids, alkaloids, and cyanogenic glycosides. All are related to defense mechanisms against pathogenetic microorganisms and wounding. Snowden and Gardner (1993) reported a cDNA clone (*wali4*), from Al-treated wheat roots that has similarity to PAL (phenylalanine ammonia lyase) enzyme. PAL enzyme catalyzes the first step of the phenylpropanoid pathway involved in the synthesis of secondary metabolites such as tannins, anthocyanins, flavonoids, and lignin. They hypothesized that the reduction in the effective cellular concentration of one or more of these compounds due to complexion with Al may lead to the induction of PAL. The wheat cDNA clone similar to maize cytochrome P450 has not been completely sequenced, and no effect of Al on its expression has been determined.

It is not clear why these three different attempts to characterize TAl-18 gene failed. However, the following can be considered as possibilities: 1. TAl-18 mRNA in the cDNA library was not abundant enough to hybridize to the probe (oligonucleotide or PR2-clone). The cDNA library was made from mRNA of wheat roots stressed for 12 h with Al, the time of highest level of synthesis of TAl-18, (Cruz-Ortega and Ownby, 1993). The observed increase in the synthesis of the protein might not reflect an increase at the level of the transcript. 2. The oligonucleotide used as a probe was not sufficiently specific. It may be that the preferred codons for wheat TAl-18 were not similar to those chosen, and that more degeneracy should have been added to the designed oligonucleotide. 3. The low

stringency conditions (50°C and .5X SSC), along with the GC content of the probe (45.5%) may have favored hybridization to other clones with similar GC content whose levels of transcripts were higher than those of TAl-18.

In summary, although the efforts made to characterize TAl-18 gene were unsuccessful, the identification of three different wheat cDNA clones, encoding 1,3-ß-glucanase, actin-bundling protein, and cytochrome P450, illustrate that during Al toxicity the plant induces a general stress-response system, turning on genes whose products may help the plant to cope with a range of environmental stresses. The *wali* genes identified by Snowden and Gardner (1993) can support this conclusion since these genes are also induced in response to factors such as toxic levels of other metals than Al, low Ca treatment, and physical wounding (Snowden et al. 1995).

In the following Chapters, the characterization of both 1,3-ß-glucanase cDNA clone, and the partial cytoskeleton-actin-binding cDNA clones are described. These two clones were characterized further in order to have a better understanding of their expression during Al-toxicity, and to contribute to our understanding the function of these genes in wheat.

CHAPTER III

NUCLEOTIDE SEQUENCE AND EXPRESSION OF A cDNA CLONE THAT ENCODES A 1,3-ß-GLUCANASE ASSOCIATED WITH ALUMINUM TOXICITY IN WHEAT ROOTS

INTRODUCTION

Endo 1,3-ß-glucanases, or 1,3-ß-D-glucan glucanohydrolases (E.C. 3.2.1.39), are hydrolytic enzymes that are widely distributed in higher plants. They exist in several structural isoforms that differ in size, isolectric point, and location in cellular compartments. These enzymes are implicated in many different physiological processes in plants. In normal plant growth and development they appear to be involved in cell wall turnover during germination, cell growth, pollen tube growth, microsporogenesis, flowering, fruit and seed maturation, abscission and senescence (Hoj and Fincher, 1995; Hird et al. 1993; for review see Simmons, 1994). As a members of the "pathogenesis-related " (PR-2) group of proteins they have shown to be very important in the plant's defenses against potentially pathogenic microorganisms (Kauffmann et al. 1987).

Several cDNAs and genomic clones of glucanases have been isolated and characterized in both dicot and monocot plants. These ß-glucanase genes have a great diversity. The entire family exhibits about 44 to 49 % identity in amino acid

sequence. The structural diversity of these ß-glucanases is about as great between monocots and dicots (about 44% amino acid identity) as it is within either monocots (about 44%) or dicots (about 52%). However, the monocot enzymes are somewhat more diverse (Simmons, 1994). Hoj and Fincher (1995) have pointed out that this percent of amino acid identity may reflect the evolutionary relationships of these enzymes. The monocot 1,3 ß-glucanases genes typically have a higher G+C content and strong codon bias. Dicot 1,3-ß-glucanases do not have this codon bias and normally are slightly rich in A+T (Simmons, 1994).

In monocots, as in dicots, 1,3-ß-glucanase expression appears to be a common response to attack by a variety of fungal pathogens. Stress-related glucanases that have been described in agronomically important cereals are described below:

In oats (*Avena sativa*), a 1,3-ß-glucanase is induced when plants are infected with the non-pathogenic rust fungus *Puccinia recondita* f.sp. *tritici* or *P. graminis* f.sp. *tritici* (Fink et al. 1990). In barley (*Hordeum vulgare*) six 1,3-ß-glucanases (GI to GVI) have been described and sequenced. Only enzyme GII has been shown clearly to exhibit stress-related regulation, it is induced in barley leaves after powdery mildew infection (*Erysiphe graminis* f.sp. *hordii*) (Xu et al. 1992). The role in barley plants of other forms of glucanase (GI, GIII, GIV, GV, and GVI) enzymes is thus far not clear.

In rice (*Oryza sativa*) a 1,3-ß-glucanase gene, *GNS1*, is expressed at higher levels in roots, at low levels in panicles, germinating seeds, shoots, and flowers (Simmons et al. 1992). The expression of *GNS1* is induced in shoots after treatment with

ethylene, kinetin, salicylic acid, wounding, heat stress, fungal elicitors, and by infection of the fungal pathogen *Sclerotium oryzae*. This gene has high structural similarity to the barley germination 1,3;1,4-ß-glucanase (EC 3.2.1.73) gene (EI gene), although, it exhibits a stress related role, as a 1,3-ß-glucanase (Simmons et al. 1992).

In maize (*Zea mays*) leaves, 1,3-ß-glucanase activity increased in relation with resistance to corn leaf blight caused by *Exserohilum turcicum*, by bromegrass mosaic virus, and to mercuric chloride treatment (Jondle et al. 1989 and Nasser et al. 1990). Recently, Wu et al. (1994) have characterized a cDNA clone for an acidic 1,3-ß-glucanase from mercuric chloride-treated maize seedlings. They used as a probe an amplified fragment of the 1,3-ß-glucanase from barley, GII. The expression of this enzyme is induced by mercuric chloride, ethephon, salicylic acid, and cycloheximide.

In wheat (*Triticum aestivum*) the only two glucanases identified so far correspond to 1,3;1,4-ß-glucanases. Two cDNA clones, lambda-LW2 and lambda-LW1 were characterized from germinated wheat grain (Lai et al. 1993). These clones were isolated from a cDNA library made from gibberellic acid-treated wheat aleurone layers, and using as a probe a 1,3;1,4-ß-glucanase cDNA from barley.

The 1,3;1,4-ß-glucanases best studied are EI and EII, from germinated barley grain. Slakeski and Fincher (1992) have shown that EI and EII expression is regulated by the hormones gibberellic acid and IAA and occurs in both aleurone and scutellar epithelium. However, EI isoenzyme has also been found in vegetative tissue, roots and leaves. From this latter results, the authors suggested that EI and

EII could have a function in barley germination and also in cell wall metabolism. So far 1,3;1,4-ß-glucanases have not been related with any defense mechanisms against pathogens or induction by any environmental stress as the 1,3-ß-glucanases are.

This chapter describes the characterization of a cDNA clone that codes for a novel 1,3-ß-glucanase selected from the cDNA library made from mRNA of wheat root tips treated with Al and screened with the oligonucleotide probe from TAl-18 (Chapter II). This cDNA clone represents a gene not previously described in wheat plants. Since 1,3-ß-glucanases are stress-related proteins induced by several biotic and abiotic factors, the purpose of this work was to further characterize this wheat cDNA clone to gain a better understanding of its role during Al toxicity.

MATERIALS AND METHODS

Plant Material. Seed of wheat (*Triticum aestivum* L. cv. Victory) (Al- sensitive) were obtained from Johnston Seed Company, Enid, OK, USA. Seed were germinated in Petri dishes on Whatman No. 4 filter paper wetted with deionized water. After 2 days, seedlings were transferred to nylon screens and floated in 450 ml of the nutrient solution described by Aniol (1984). The pH of the nutrient medium was adjusted to 4.40 with either 0.1 M HCl or 0.1 M NaOH. All seedlings were grown in vigorously aerated nutrient solutions in a growth chamber. The seedlings were grown on a 16-h photoperiod at 26°C (day) and 22°C (night) with an illumination of 350 μmol m^{-2} s^{-1}. Four-day-old wheat seedlings were stressed by the addition of 80 μM $AlCl_3 \cdot 6H_2O$ to the Aniol solution. Leaves and roots were harvested at 6, 12 and 24 hours of Al-stress treatment and immediately frozen under liquid N_2 and kept at -

70°C until used.

cDNA library and screening. The cDNA library screening and the isolation of poly A^+ RNA are described in Chapter II (pages 23 and 24).

DNA Sequencing Analysis. The nucleotide sequence of the cDNA clone and the Data base searches were determined as previously described in Chapter II (page 24). The full-length cDNA clone was sequenced by designing 6 synthetic primers for each strand to complete the sequencing in both strands. DNA sequence data were analyzed using the MacVector/AssemblyLign sequence analysis program (Eastman Kodak). Multiple sequence alignments were conducted using the Clustal Multiple-alignment program (Higgins and Sharp, 1989).

Northern and Southern Blot Analysis. Total RNA was extracted as previously described (page 23) from root tips exposed to 80 μM Al for 6, 12, and 24 hours. For Northern blot analysis 20 μg of total RNA were electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde. Equal loading of RNA was verified by ethidium bromide staining. The gels were blotted onto nylon membranes (Magna, Micron Separations, Inc., Westboro, MA.) by capillary transfer. Membranes were UV-crosslinked (254 nm) (Stratalinker, Stratagene La Jolla, CA), and prehybridized in hybridization buffer (50% formamide, 5X SSC, 2% blocking reagent [Boehringer-Mannheim], 20 mM sodium maleate, 0.1% N-laurylsarcosine, 0.2% SDS) for 2 hours at 42°C. Membranes were hybridized at 42°C overnight with the full-length 1,3-ß-glucanase cDNA clone that was digoxygenin labeled using the random priming method. Northern blots were processed according to the manufacturer's specifications

(Boehringer-Mannheim). After hybridization, membranes were washed once for 15 min in 2X SSC, 0.1% SDS and twice for 15 min in 0.1X SSC, 0.1% SDS at 65°C. Membranes were then blocked with 2% blocking reagent in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) and exposed to antidigoxygenin IgG Fab conjugated to alkaline phosphatase. Membranes were washed in maleate buffer and exposed to Lumiphos 530 (Boehringer-Mannheim) for chemiluminescent reaction. Hybrids were visualized after exposure to x-ray film (RX, Fuji, Japan) at room temperature for 12 hours.

Total genomic DNA was isolated from leaves according to the method of Dellaporta (1983). For Southern analysis, 10 μg of DNA were digested with the enzymes EcoRI, HindIII, XbaI, XhoI, and BamHI. After restriction digestion and separation on 0.8% agarose gels, DNA was blotted onto nylon membranes (Magna, Micron Separations, Inc. Westboro MA). Membranes were UV-crosslinked (250 nm) (Stratalinker, Stratagene), and prehybridized in 5X SSC, 1% blocking reagent (Bowhringer Mannheim), 0.1% N-laurylsarcosine, 0.2% SDS for 1 hour at 65°C. The same digoxygenin-labeled full-length probe described for Northern analysis was used on the Southern blots, except that the probe was diluted in Southern prehybridization solution before use. Membranes were hybridized overnight at 65°C. After hybridization, membranes were washed for 15 min in 2X SSC, 0.1% SDS at room temperature and then twice for 15 min in 0.1X SSC, 0.1%SDS at 65°C. Detection of digoxigenin-labeled probe was carried out as describe above for Northern analysis.

Western blot analysis. Total protein was extracted from roots and leaves from 4-day-old wheat (cv Victory) seedlings treated for 6, 12 and 24 hours with 80 μM Al. Wheat roots of cv Atlas 66 (Al-tolerant) cultivar was treated with 10, 20, 40, 80, 200, and 400 μM Al. Root growth was measured before and after 24 h of Al treatment. Relative growth was calculated with respect to the control root growth rate. Frozen tissue was homogenized in 50 mM KPO_4 (K_2HPO_4 and KH_2PO_4) pH 6.8, 1 mM phenylmethylsulphonyl fluoride extraction buffer (1:4 w/v tissue) (Hird et al. 1993). This extract was centrifuged at 12,000 g for 20 min at 4°C. Protein concentration in the supernatant (crude extract) was determined by the method of Bradford (1976). Total soluble protein (50 μg, roots or 100 μg, leaves/lane) was denatured (100°C for 3 min) in preheated SDS sample buffer (60°C for 3 min) (125 mM Tris-HCl, pH 6.8; 2% (v/v) SDS, 0.02% DTT; 10% (v/v) glycerol, and 0.02% (w/v) Bromophenol Blue as tracking dye) and resolved by 12% SDS-PAGE (Laemmli, 1970). After electrophoresis, proteins were electroblotted to nitrocellulose (Trans-Blot, Bio-Rad Laboratories, Hercules, CA), using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad). The transfer was performed for 3 h at 90 volts in a transfer buffer consisting of 20 mM Tris, 192 mM glycine, and 20 % (v/v) methanol, pH 8.3. The blot was blocked with 5% non-fat milk in TBS (10 mM Tris and 150 mM NaCl, pH 7.5) for 2 h or overnight. After blocking the blots were washed with TBST (TBS with 0.5% Tween 20) thrice for 10 min, and washed once with TBS. The blots were incubated with rabbit anti *Brassica napus* A6 protein polyclonal antibody, kindly donated by Diane Hird and Rod Scott from the University

of Leicester, Leicester, UK., (1:1000 dilution) in TBS, 0.1% non-fat milk, 0.02% sodium azide for 3-4 hours. After incubation, blots were washed thrice in TBST for 10 min, and once in TBS for 10 min. Then the blots were incubated in 5% non-fat milk in TBST for 5 min, and then in secondary antibody (alkaline phosphatase-conjugate goat anti-rabbit IgG, Sigma Chem. Co., St Louis, USA) in TBS with 0.5% non-fat milk (1:20,000 dilution) for 2 hours. After washing in TBST as before, the blots were developed by immersing in a solution of 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 0.3 mg/ml nitroblue tetrazolium (NBT) in Tris-HCl, pH 9.5, 1 mM $MgCl_2$. The reaction was stopped by washing with distilled water. All treatments were carried at room temperature, except that incubation with antibody was overnight at 4°C.

RESULTS

Isolation and nucleotide sequence determination of 1,3 ß-glucanase of wheat roots: The nucleotide sequence of three of seven positive clones selected from screening a wheat root cDNA library showed high similarity to plant 1,3-ß-glucanases. The restriction analysis of insert length showed that these clones had an insert size of 2.1 kb. One clone was completely sequenced in both strands and found to be a full length clone. The nucleotide sequence and the deduced amino acid sequence of this clone are shown in Figure 2. This cDNA clone is 2072 nucleotides long, with a 36-nucleotide 5' untranslated leader sequence and a 650-nucleotide 3' untranslated trailer sequence. Two polyadenylation signals having the AATAAA consensus sequence are located at the 1745 and 1807 nucleotide position (Figure 1,

underlined). An open reading frame of 1385 bp begins with an ATG initiation codon at position 36, and ends with a TGA termination codon at position 1419 as shown in Figure 1. This wheat cDNA has a strong G+C bias, as do many genes encoding monocot 1,3-ß-glucanases. The deduced polypeptide contains 461 amino acids, the first 25 of which represent a putative signal peptide that is enriched in neutral and hydrophobic amino acids typical of eukaryotic signal sequences (Von Heije, 1983). It also contains a C-terminal extension of 112 amino acids with three N-acetyl glycosylation consensus sequences (asn-xxx-ser/thr) at amino acids 336, 340, and 361, counting from the beginning of the mature polypeptide. Figure 3A and B shows the main characteristics of the wheat root 1,3 ß-glucanase protein. It has a predicted molecular weight of 46.3 kD (without the signal peptide of 25 amino acids) and an estimated pI of 6.0. If the C-terminal sequence is removed during the last step of processing, the final product will be similar in size to others monocots 1,3-ß-glucanases, which are typically 30 kD to 36 kD. Figure 3C represents the hydrophobicity plot of the wheat 1,3-ß-glucanase. The upper part of the plot shows the regions of the protein that contain hydrophilic amino acids (surface of the protein) and the lower part represents the hydrophobic region (inside of the protein). The first highly hydrophobic region (1-25 amino acids) corresponds to the signal peptide, rich in hydrophobic amino acids (SP in figure 3C).

Amino acid sequence alignment with other 1,3-ß-glucanases. Figure 4 shows the alignment of the wheat 1,3-ß-glucanase with other plant glucanases. From the Data base search using the National Center for Biotechnology Information BLAST E-

mail and FASTA E-mail server, this protein exhibits similarity to the *Hordeum vulgare* neutral GV and to the basic GII 1,3-ß-glucanases (35 and 37% amino acid identity, respectively) (Xu et al. 1992 and Malehorn et al. 1993); with two acidic tobacco (*Nicotiana tabaccum*) 1,3-ß-glucanases (32% amino acid identity) (Linthorst, 1991); and with the slightly basic pea (*Pisum sativum*) 1,3-ß-glucanase (32% identity)(Chang, M-M, 1992). The wheat protein is also similar to two basic 1,3-ß-glucanases from *Brassica napus* and *Arabidopsis thaliana* (33% identity)(Hird et al. 1993) in both size and in the presence of the long amino acid extension at the C-terminal end. Figure 4 also shows the most conserved amino acid region in plant 1,3-ß-glucanases. Regions "a" and "b" represent the conserved residues thought to participate in enzymatic activity. The amino acid sequence alignment of the wheat 1,3-ß-glucanase with only the 1,3-ß-glucanases from *B. napus* and *A. thaliana* (A6 clone) is shown in Figure 5; this alignment emphasizes the strong similarity between wheat 1,3-ß-glucanase and *Brassica* and *Arabidopsis* A6 clone in size and in the long C-terminal.

Expression of 1,3-ß-glucanase transcript levels. Northern blot analysis was used to examine the expression of the wheat 1,3-ß-glucanase under aluminum toxicity. Total RNA was isolated from roots of four-day-old wheat seedlings treated with 80 μM Al^{3+} for 6, 12, and 24 hours. The full length cDNA clone was used as a probe. Figure 6 shows two bands, of around 2.0 to 2.2 kb. The size of the transcript(s) corresponded to the size of the cDNA clone, which confirmed that it was a full-length clone. These transcripts were detectable in control roots, in agreement with the

general observation that acidic 1,3-ß-glucanases transcripts are expressed constitutively at very low levels in healthy tissues (Simmons, 1994). An increase in the signal was observed after 6 h of exposure to Al, which coincides with the period of growth inhibition. Synthesis of 1,3-ß-glucanase transcript reached its maximum level after 12 h of Al exposure, and by 24 h had begun to decline but remained higher than in control roots. These results suggest that under Al toxicity stress, genes such as 1,3-ß-glucanases may be induced as a mechanism of defense under general stress conditions.

Genomic Southern Blot Analysis. To assess the complexity of the 1,3-ß-glucanase family in wheat, genomic Southern blots of DNA digested with 5 different restriction endonucleases were probed with the full-length cDNA clone insert. The probe hybridized under conditions of high stringency to at least 4 bands in genomic DNA digested with the restriction enzyme HindIII (Figure 7). It is plausible that this wheat 1,3-ß-glucanase has four gene copies, or at least 2 as the Northern blot analysis suggested (Figure 6). There is a high diversity in plant 1,3-ß-glucanases gene families. Hird et al. (1993) observed 6 bands in Southern blot analysis of *A. thaliana* and *B. napus* ß-glucanases. In tobacco, Ohme-Takagi and Shinshi (1990) observed four bands in Southern blot analysis. Xu et al. (1992) found 6 to 8 bands in barley, although some of them disappeared under higher stringency. From these different observations it is difficult to predict how many copies of the plant 1,3-ß-glucanases genes may exist in wheat. Due to the hexaploid character of wheat it would not be surprising if four copies of the 1,3-ß-glucanase gene were present in the wheat

genome.

Immunological detection of *Brassica napus* 1,3-ß-glucanase during Al toxicity in wheat roots. In order to confirm the similarity of wheat 1,3-ß-glucanase with *B.napus* A6 protein, and to determine if the C-terminal extension is cleaved or retained, Western blots analysis was done using rabbit antibody against *B.napus* A6 protein. A protein of 56 kD was detected in roots of wheat cv Victory treated for 6, 12, and 24 h with 80 μM Al (Figure 8). It was present in control roots, which means that wheat roots constitutively expressed a protein immunologically similar to *B.napus* A6 1,3-ß-glucanase. Expression of the protein was enhanced after 6 h of Al treatment, and was most abundant after 24 h Al. In wheat leaves a very faint band corresponding to the 56 kD protein was detected, suggesting that the 56 kD protein is also expressed in leaves but at very low levels. Interestingly, a second band can be observed in roots. This band is fainter and corresponds to a protein of 54 kD. This second band might correspond to an isoenzyme of this 1,3-ß-glucanase, confirming the two bands observed in Northern analysis (Figure 6). On the other hand, the fact that the protein crossreacting with the antibody to *B. napus* A6 protein had a molecular weight a 56 kD indicates that wheat 1,3-ß-glucanase may retain all the C-terminal extension.

Figure 9A shows the relative root growth of cv Atlas 66 seedlings treated with Al ranging from 0 to 400 μM Al. The concentration 10 μM Al stimulated 26% the root growth. From 40 μM Al root growth started to be inhibited, reaching 90% inhibition at 400 μM Al. Western blot analysis showed that the 56 kD protein did not

accumulate above control levels until 400 μM Al (Figure 9B), a concentration that inhibited 90% of the root growth.

DISCUSSION

A cDNA clone from wheat roots exposed to Al toxicity has been sequenced and shown to encode a protein with similarity to 1,3-ß-glucanases. As in the case with other glucanases, wheat 1,3-ß-glucanase is synthesized as a prepro-1,3-ß-glucanase. It has an N-terminal signal peptide of 25 hydrophobic or neutral amino acids (Figure 3C). According to Von Heijne (1983), a probable cleavage site exists between Histidine 25 (H25) and Isoleucine 26 (I26) of the amino acid sequence deduced from the cDNA clone. It is known that this N-terminal hydrophobic signal peptide mediates transport into the endoplasmic reticulum secretory pathway. The cleavage of this signal peptide leaves a protein of 436 amino acids with an estimated molecular weight of 46.3 kD. Interestingly, this wheat 1,3-ß-glucanase possesses a long C-terminal extension of 112 amino acids, with three potential N-glycosylation sites at positions 336, 340, and 361. Normally this C-terminal extension is present in basic 1,3-ß-glucanases. Glycosylation of 1,3-ß-glucanase during processing is thought to be involved in the transport to the vacuole (Sinshi et al.1988). Thus, although acidic 1,3-ß-glucanases are generally secreted into the intercellular space (Simmons, 1994), it seems likely that this wheat 1,3-ß-glucanase may be localized in the vacuole. In addition, Western blot analysis of wheat root proteins with anti-*B.napus* A6 antibody (Figure 8) detected an immunoreactive protein of 56 kD, suggesting that the long C-terminal of the wheat 1,3-ß-glucanase is retained and not cleaved. The fact that the

protein was 56 kD and not 46 kD as predicted may be due to the following: 1. glycosylation at one or more of the potential glycosylation sites within the wheat 1,3-ß-glucanase. In addition to the three glycosylation sites at the C-terminal, there are five more potential glycosylation sites within the protein sequence. The number of chains and the variable carbohydrate composition might account for part of the additional molecular weight. Since the carbohydrate content in a glycoprotein is variable (<1 to $>90\%$) it is difficult to say how many carbohydrates are present in the wheat protein. Doan and Fincher (1992) estimated that EII, (1,3-1,4-ß-glucanase) carries 4% by weight carbohydrate. 2. the presence of carbohydrates side chains interferes with the binding of SDS, retarding the movement of the protein in the polyacrylamide matrix, yielding a protein with a higher molecular weight (Dunbar, 1987). 3. The anti *B.napus* A6 may be crossreacting with a protein other than the product of the wheat 1,3-ß-glucanase clone. The last possibility seems unlikely because wheat 1,3-ß-glucanase shares a high similarity at the amino acid level to *B.napus* A6 clone (Figure 5). From these observations, studies on the effect of deglycosylation of wheat protein extracts are needed to clarify if the 56 kD protein corresponds to the product of the wheat cDNA clone and if the protein is targeted to the vacuole.

Plant 1,3-ß-glucanases have a great structural diversity. The entire family is related at about 44 to 49% amino acid identity, with the monocot enzymes the most diverse. Despite this great diversity, there are several conserved regions at 55, 75 and 90% of the way toward the C-terminus (Simmons, 1994). These conserved

regions are likely important for enzymatic activity. Residues thought to be critical for catalytic activity (Hoj and Fincher 1995) are conserved in the wheat 1,3-ß-glucanase sequence (Figure 4, regions "a" and "b" in bold), suggesting that wheat enzyme has all the necessary residues to function as a glucanase enzyme. Two Glutamic acid residues at positions 231 and 288 (E231 and E288) in barley 1,3-ß-glucanase (GII isoenzyme) are also present in wheat 1,3-ß-glucanase. These glutamic acid residues are thought to be involved in the catalytic activity of these enzymes (Chen et al. 1995), and they are conserved in most higher plants 1,3-ß-glucanases listed in the DNA and protein databases (Hoj and Fincher, 1995).

The amino acid comparison of the protein encoded by the wheat cDNA clone with other plant 1,3-ß-glucanases, as well as the identification of the conserved amino acid residues involved in catalytic activity, confirms that the wheat cDNA clone corresponds to a 1,3-ß-glucanase. Why was a cDNA clone coding for this hydrolytic enzyme selected from a cDNA library made from Al-stressed wheat roots? 1,3-ß-glucanases can have different physiological roles, but several studies have shown that they might play an important role in the plant defense against pathogen attack. It is also known that their expression can be induced during fungal and microbe attack as well as by other stress factors such as salicylic acid, and mercury chloride (Simmons, 1994, Wu et al. 1994). This study have shown by Northern blot analysis that wheat 1,3-ß-glucanase mRNA is up-regulated during aluminum toxicity. Western blot analysis with anti *B.napus* A6 antibody confirmed that levels of a 56 kD protein are increased during Al-toxicity in both sensitive (cv Victory) and tolerant (cv Atlas)

wheat cultivars. These results are consistent with the idea that 1,3-ß-glucanase expression is induced in wheat roots during Al stress. As it was stated in Chapter I (page 29), due to the phytotoxicity of Al, wheat roots are more vulnerable to pathogen attack, thus defense-related proteins such as 1,3-ß-glucanases may accumulate to protect the plant in this susceptible state. It is important to note that the peak of wheat 1,3-ß-glucanase transcript at 12 h of Al-stress corresponded to the same time at which poly A^+ RNA was extracted for cDNA library synthesis. This suggests that at this time of Al-stress (12 h), the cDNA library was enriched in 1,3-ß-glucanase transcript, making it more likely to be selected during the screening.

In conclusion, this part of the study described a wheat cDNA that codes for a protein highly similar to other plant 1,3-ß-glucanases, which is up-regulated during Al toxicity as part of a general defense mechanism. That Al induces the expression of a stress-related protein such as 1,3-ß-glucanase can be supported by the statement of Simmons (1994): " the most economical design, or at least the one selected through evolution of plant-defense gene activation, may have been to have all or most of the defense-related proteins coordinately expressed whether they are needed in coping with a particular stress or not".

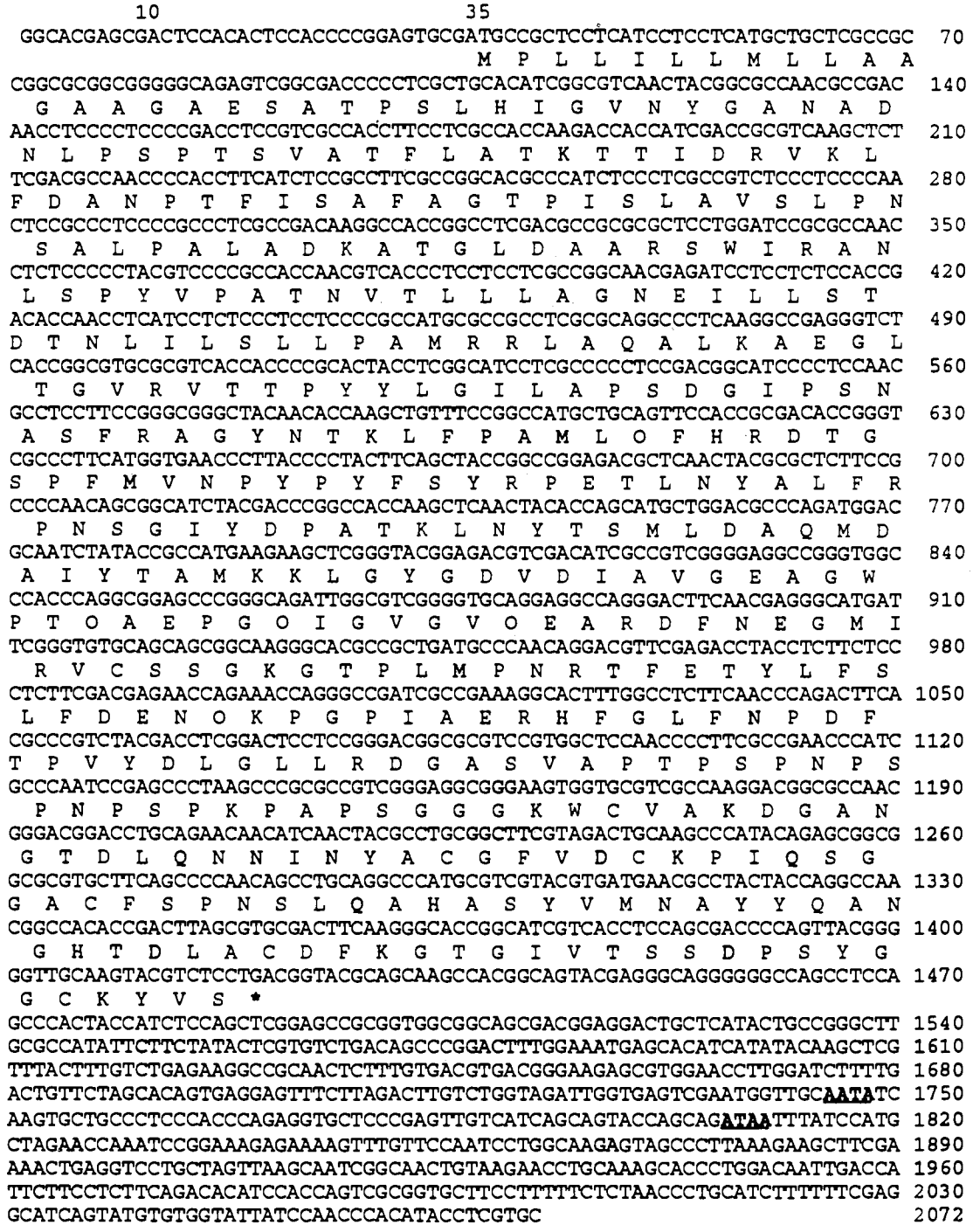
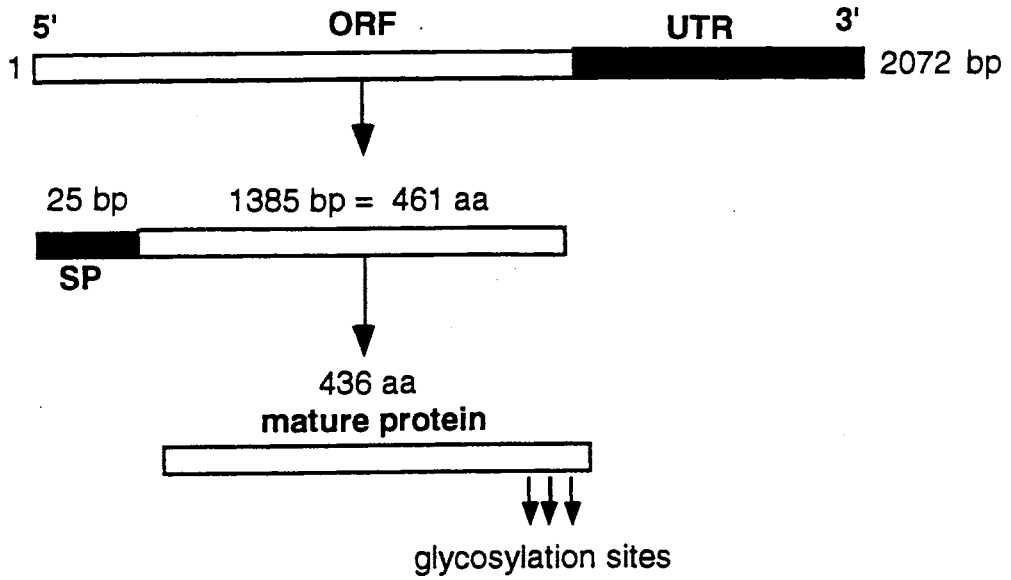


Figure 2. Nucleotide and deduced amino acid sequence of the full-length cDNA for 1,3-ß-glucanase from wheat roots (*Triticum aestivum*, cv. Victory). The deduced single-letter amino acid sequence is given below the center of each codon triplet. An asterisk (*) denotes the TGA terminator codon. The two putative polyadenylation signal consensus sequences are underlined.

A



B

Molecular weight	46.3 kD
Isoelectric point	6.0
non polar amino acids	46 %
polar amino acids	38 %
acidic amino acids	8 %
basic amino acids	8 %

C

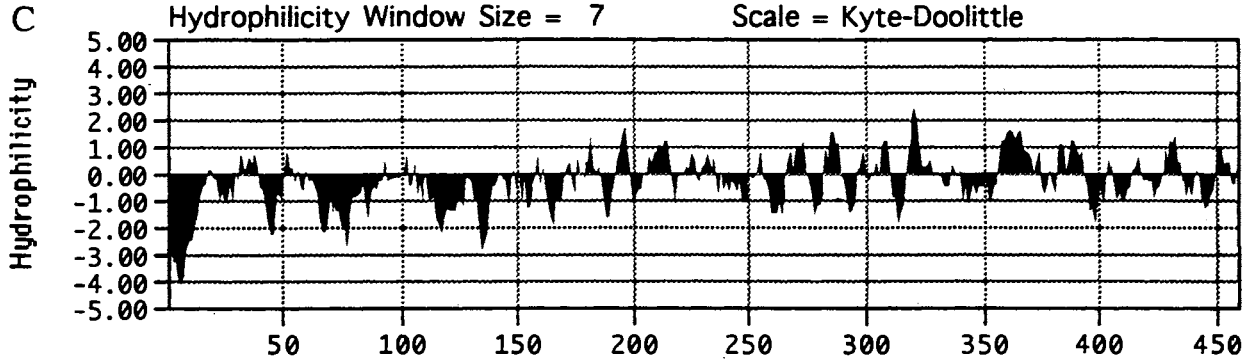


Figure 3. Summary of molecular characteristics of wheat root 1,3-ß-glucanase. (A) Schematic representation of the full length clone showing: Open Reading Frame (ORF), 3'UTR (3'untranslated region), signal peptide (SP), mature protein, and glycosylation sites. (B) Main characteristics. (C) Hydrophilicity plot (hydrophobic residues below the zero and hydrophilic residues above zero)

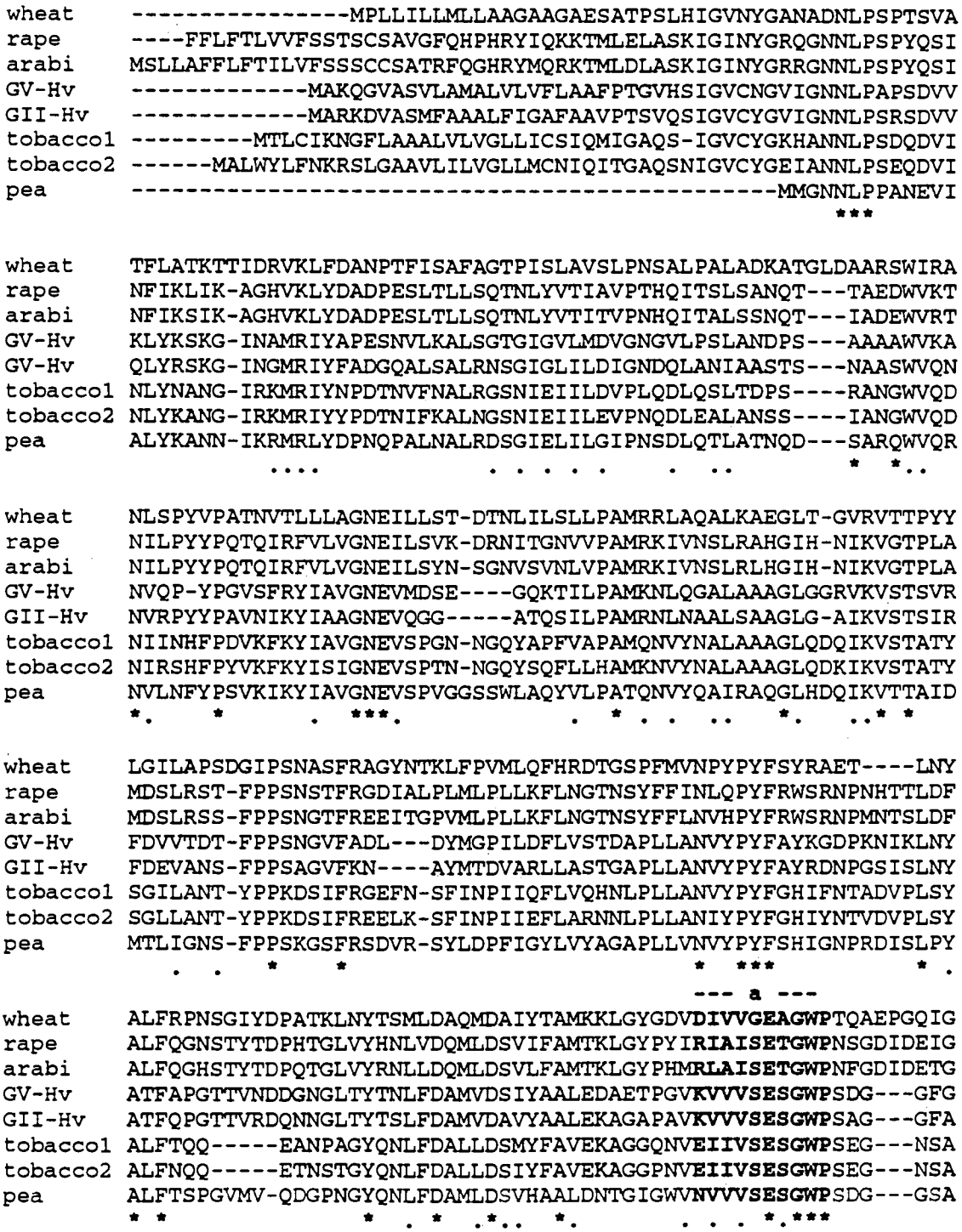


Figure 4. Comparison of the amino acid sequence of wheat 1,3-ß-glucanase with other plant 1,3-ß-glucanases. The predicted amino acid sequence of the wheat 1,3-ß-glucanase was aligned with the following 1,3-ß-glucanases: *Brassica napus* (rape), *Arabidopsis thaliana* (Arabi) (Hird et al. 1993); GV and GII from *Hordeum vulgare* (GV-Hv and GII-Hv) (Xu et al. 1992); *Nicotiana tabaccum* (tobacco 1, and 2) (Linthorst, 1991); *Pisum sativum* (pea) (Chang M-M, 1992).

```
                                                  ----- b ----
wheat     VGVQEARDFNEGMIRVCSSG--KGTPLMPNRTFETYLFSLFDENQKPGPIAERHFGLFNP
rape      ANVFNAATYNRNLIKKMTATPPIGTPARPGSPIPTFVFSLFNENKKPGSGTQRHWGILHP
arabi     ANILNAATYNRNLIKKMSASPPIGTPSRPGLPIPTFVFSLFNENQKSGSGTQRHWGILHP
GV-Hv     ATAENARAYNQGLINHVG----NGTPKRS-GPLETYVFAMFNENEKKGDPTENHFGLFNP
GII-Hv    ASAGNARTYNQGLINHVG----GGTPKKR-EALETYIFAMFNENQKTGDATERSFGLFNP
tobacco   ATIENAQTYYENLINHVKSG--AGTPKKPGKAIETYLFAMFDENNKEGDITEKHFGLFSP
tobacco   ATIENAQTYYRNLVNHVKGG--AGTPKKPGRIIETYLFAMFDENEKQGEITEKHFGLFYP
pea       TSYDNARIYLDNLIRHVG----KGTPRRP-WATEAYLFAMFDENQKS-PELEKHFGVFYP
                *  .  ...          ***         ...*..* ** *     .   *.  *

wheat     DFTPVYDLGLLRDGASVAPTPSPNPSPNPSPKPAPSGGGKWCVAKDGANGTDLQNNINYA
rape      DGTPIYDIDFTGQKPLTGFNPLPKPTNN-----VPYKGQVWCVPVEGANETELEEALRMA
arabi     DGSPIYDVDFTGQTPLTGFNPLPKPTNN-----VPYKGQVWCVPVEGANETELEETLRMA
GV-Hv     DKSPAYYMRF----------------------------------------------------
GII-Hv    DKSPAYNIQF----------------------------------------------------
tobacco   DQRAKYQLNFN---------------------------------------------------
tobacco   NRAAKYQLNFMYS--------------------------------------------------
pea       NKQKKYPFGFGGE--------------------------------------------------
                *

wheat     CG--FVDCKPIQSGGACFSPNSLQAHASYVMNAYYQANGHTDLACDFKGTGIVTSSDPSY
rape      CARSNTTCAALVPGRECYEPVSVYWHASYALNSYWAQFRSQNVQCYFNGLAHETTTNPGN
arabi     CAQSNTTCAALAPGRECYEPVSIYWHASYALNSYWAQFRNQSIQCFFNGLAHETTTNPGN
GV-Hv     ----------------------------------------------------------
GII-Hv    ----------------------------------------------------------
tobacco   ----------------------------------------------------------
tobacco   --------------DS------------------------------------------
pea       --------------RRDGEIVEGDFNGTVSLKSDM-------------------------
                    *    *                          *   *   *

wheat     GGCKYVS---
rape      DRCKFPSVTL
arabi     DRCKFPSVTL
GV-Hv     ----------
GII-Hv    ----------
tobacco   ----------
tobacco   ----------
pea       ----------
             **  *
```

Figure 4. (continued). The Clustal Multiple Alignment program was used (Higgins and Sharp, 1989). Asterisks (*) indicates consensus amino-acid identity among all organisms. Dots-positions (.) are most conserved amino acid replacements. The two catalytic sites are identified with the letters "a" and "b". The most important conserved residues in the two catalytic sites are shown in bold.

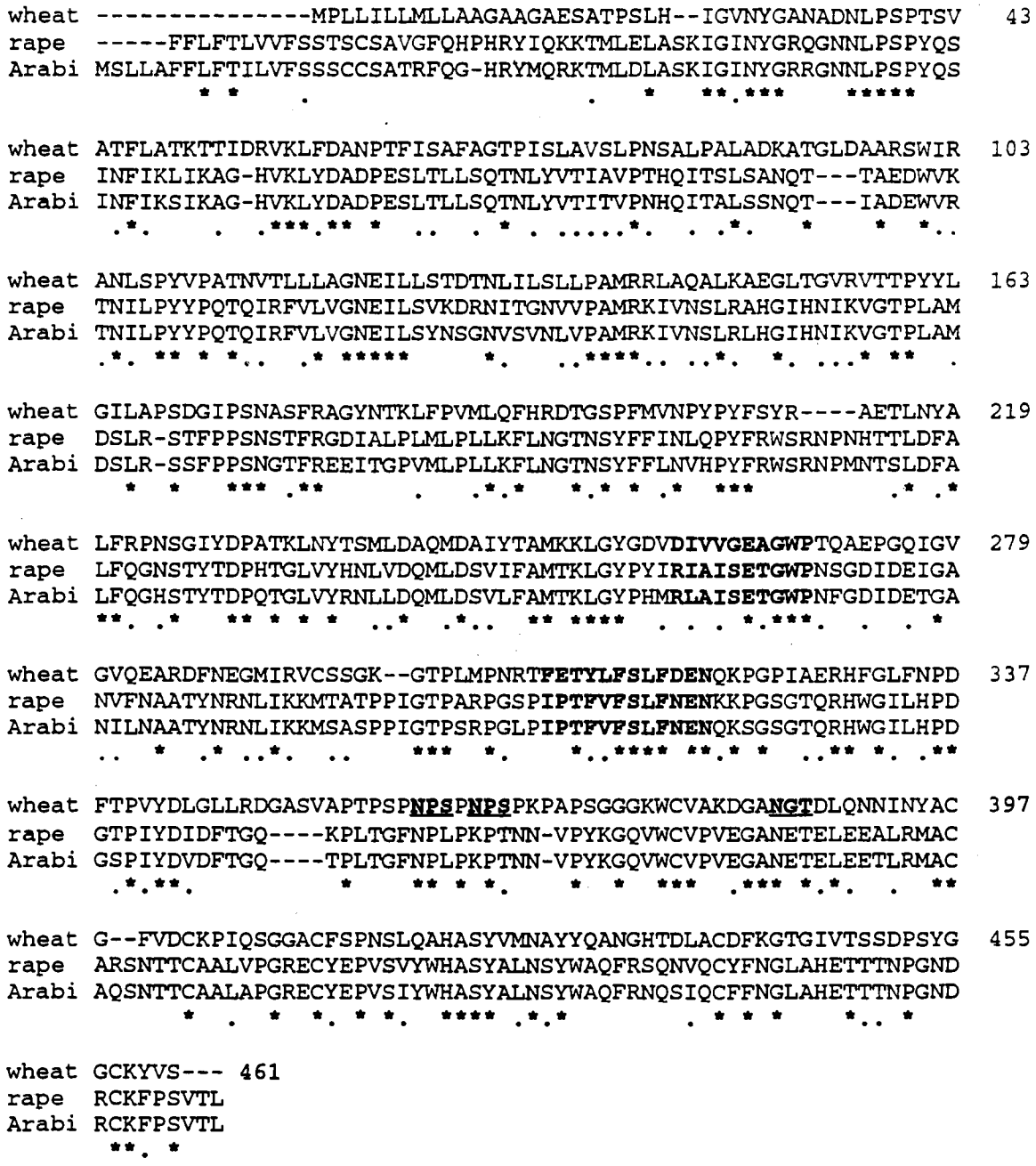
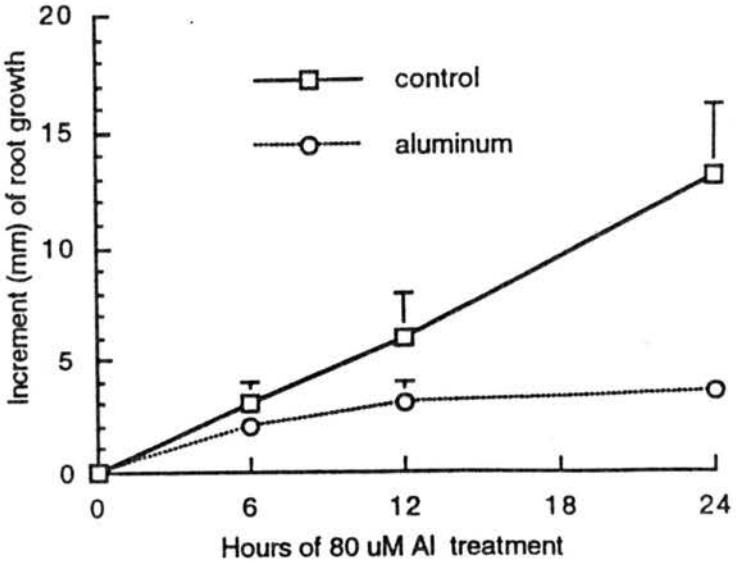


Figure 5. Amino acid sequence alignment of wheat 1,3-ß-glucanase and clone A6 from *Brassica napus* and *Arabidopsis thaliana* (Hird et al. 1993). Asterisks (*) denote consensus amino acid identity among the three glucanases. Dots-position (.) are most conserved amino acid replacements. The two consensus sequence of the catalytic sites are in bold. Potential glycosylation sites are in bold and underlined.

A



B.

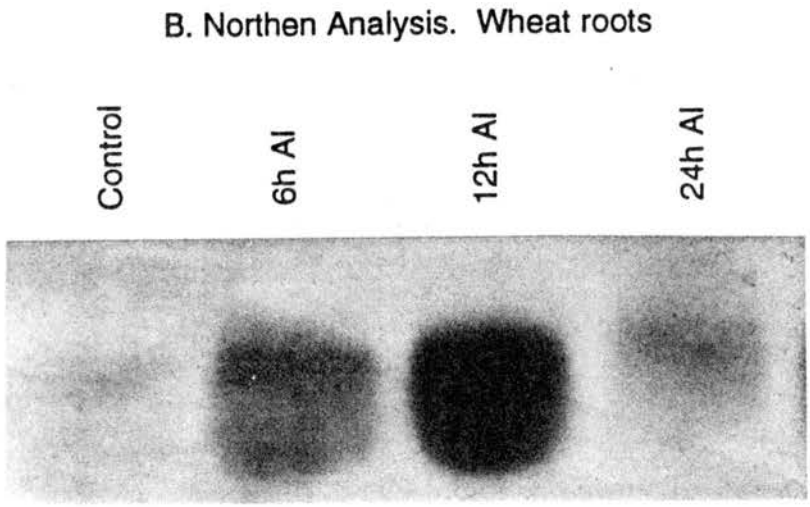


Figure 6. Expression of wheat 1,3-ß-glucanase transcript levels during aluminum toxicity in roots of wheat cv Victory. Four-day-old wheat seedlings were subjected to 80 μM $AlCl_3 \cdot 6H_2O$ for 6, 12, 24 hours. Total RNA was isolated from root tissue. 20 μg of total RNA were loaded in each lane and resolved on a 1.2% agarose gel containing formaldehyde. RNA was transferred to nylon membrane and probed with gel-purified digoxygenin-labeled full-length cDNA insert.

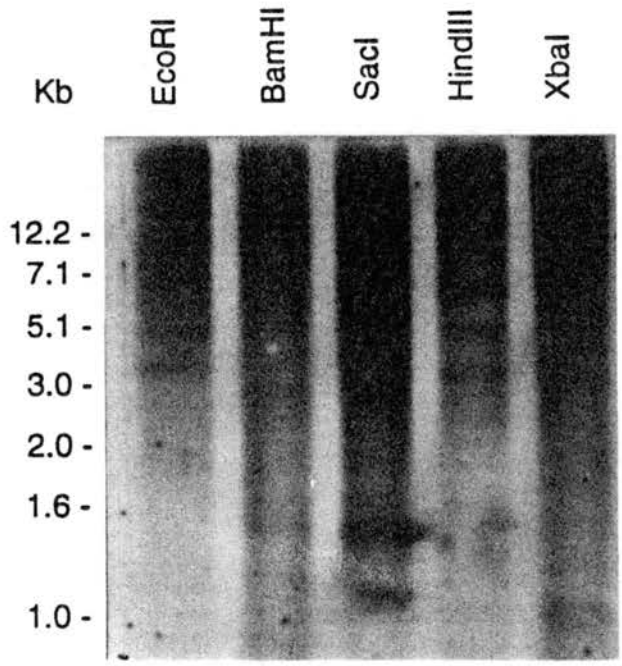


Figure 7. Genomic Southern blot analysis of 1,3-ß-glucanase gene family in wheat. Total genomic DNA isolated from wheat seedlings tissue was digested with EcoRI, BamHI, SacI, HindIII, and XbaI. The DNA samples (10 μg/lane) were separated on a 0.8% agarose gel, blotted onto nylon membranes, and hybridized with the full-length digoxygenin-labeled cDNA probe. Blots were washed under high stringency condition (0.1% SSC, 65°C), and then exposed to X-ray film.

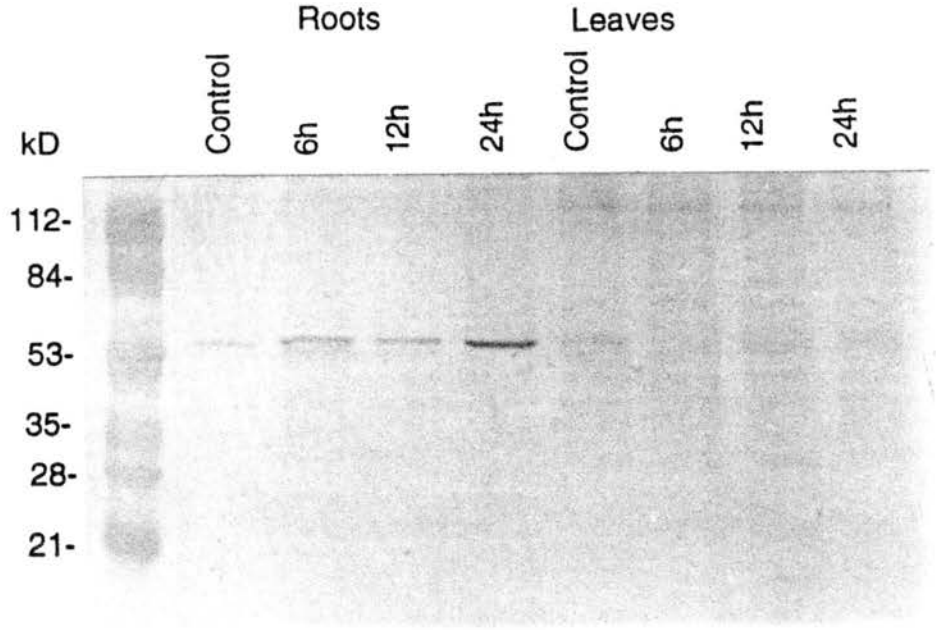
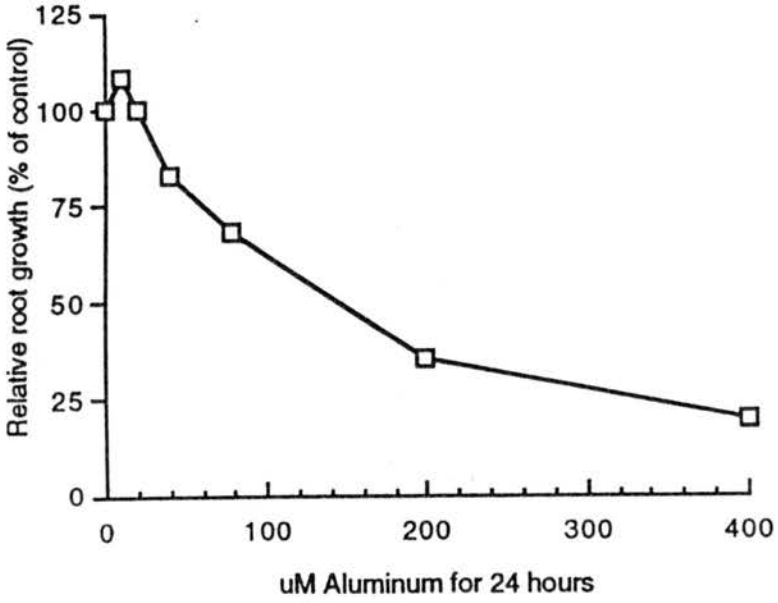


Figure 8. Immunoblot analysis of 1,3-ß-glucanase in roots and leaves of wheat cv Victory during Al toxicity. Four-day-old wheat seedlings were stressed with 80 μM $AlCl_3 \cdot 6H_2O$ for 6, 12, and 24 hours. Total soluble protein was extracted from root and leaf tissue, resolved by SDS-PAGE, and electroblotted to nitrocellulose. Each lane contained 50 μg soluble protein isolated from roots or 100 μg protein from leaves. 1,3-ß-glucanase levels were detected with anti A6 protein from *Brassica napus*.

A



B.

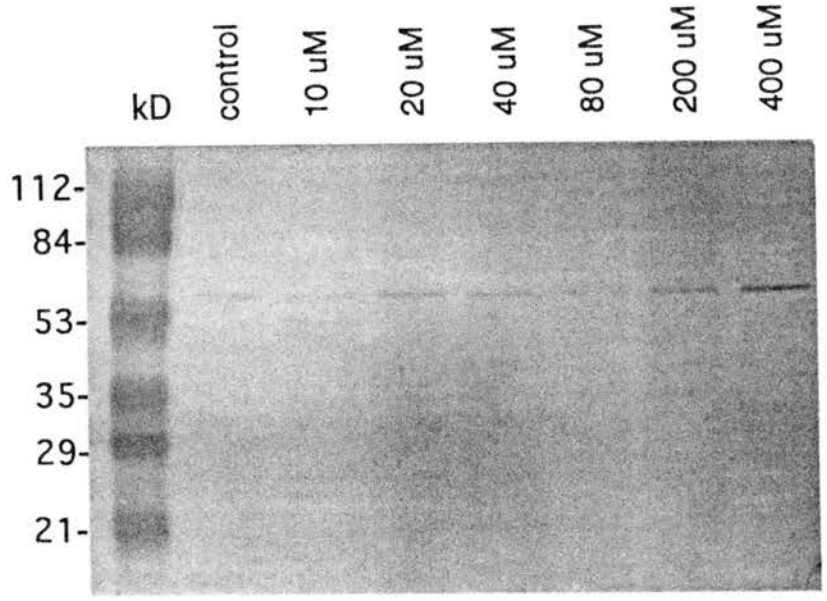


Figure 9. (A) Effect of Al on root growth in wheat cv Atlas 66 (Al-tolerant). Root length of four-day-old seedlings was measured before and after 24 h exposure to various concentrations of Al. (B) Immunoblot analysis of 1,3-ß-glucanase in roots of wheat. Four-day-old wheat seedlings were stressed for 24 h with 10, 20, 40, 80, 200, and 400 μM $AlCl_3 \cdot 6H_2O$. Total soluble protein was extracted from root tissue, resolved by SDS-PAGE, and electroblotted to nitrocellulose. Each lane contained 50 μg soluble protein. 1,3-ß-glucanase levels were detected with anti-A6 protein from *Brassica napus*.

CHAPTER IV

PARTIAL cDNA CLONE THAT ENCODES AN ACTIN-BUNDLING-LIKE PROTEIN

INTRODUCTION

The cytoskeleton, the supportive network in the cytoplasm of all eukaryotic cells, consists of two major classes of filaments: microtubules and microfilaments. Microtubules are long, hollow structures, 24 nm in diameter, that are formed of subunits of the protein tubulin. Microfilaments, on the other hand, are conserved solid structural elements, 5 to 7 nm in diameter, that are composed of the polymerized contractile protein actin. These actin filaments form the framework on which many dynamic and divergent intracellular events occur. In both plants and animal cells, actin filaments participate in processes such as cytoplasmic streaming, movement of organelles, nuclear positioning, structural integrity, and cytokinesis. In plants cells they are also involved in tip growth (see reviews of Lloyd, 1989, and Hepler et al. 1993).

The dynamics of actin microfilaments depend on the organization and functional properties of complex actin arrays (Hartwig, 1991). Actin microfilaments can exist in three spatial arrays in the cytoplasm: 1) as single filaments; 2) as meshworks or gels; and 3) as bundles. The meshworks or gels consist of an orthogonal lattice of

filaments which compose the subcortical scaffold associated with the plasma membrane (de Arruda et al. 1990). On the other hand, actin bundles are closely spaced, parallel arrays of filaments that provide structural support for fingerlike extension of the plasma membrane, as in the case of microvilli, stereocilia, and filopodia in animal cells (de Arruda et al. 1990). The formation of these actin meshworks and bundles requires the presence of crosslinking proteins. These actin-binding proteins have been shown to affect the assembly state of actin *in vitro*, but it is likely that many of them also affect the assembly state and distribution of actin *in vivo* (Adams et al. 1991). Actin-binding proteins, on the basis of their interaction with actin monomers *in vitro*, can be classified into three groups: 1) The monomer-binding proteins; 2) the "capping proteins", or end-binding proteins; and 3) the crosslinking proteins.

The monomer-binding proteins, as their name suggests, bind to the monomeric globular actin (G-actin). These proteins remove polymerizable actin from equilibrium with the filamentous actin (F-actin). Members of this protein group include: profilin, cofilin, thymosin, dextrin, and DNase I (see review of Schelicher et al. 1995, Stossel, 1993, Vandekerckhove, 1990, and Otto, 1994). The capping proteins bind to the end part of actin filaments. They inhibit further addition of monomers, keeping the actin filaments short. Examples of these cap proteins are: cap32/34, tropomodulin, gelsolin, villin, severin, fragmin, protovillin, adseverin, ß-actinin (references in Schleicher et al. 1995). The crosslinking proteins are a highly diverse family of proteins which share the ability to bind at least two actin filaments (Hartwig and

Kwiatkowski, 1991). They include those which bind along the side of the actin filaments. Three groups of crosslinking proteins can be distinguished: 1) they can be motor proteins, such as myosin I and myosin II, which slide filaments against each other causing contraction; 2) Proteins that anchor actin filaments at membranes, such as spectrin, ponticulin, and hisactophilin; and, 3) proteins that crosslink filaments and form bundles as well as three dimensional networks, such as filamin, α-actinin, and fimbrin (plastin) (Schleicher et al. 1995, Stossel, 1993, and Otto, 1994). Figure 10, taken from the review of Schleicher et al. (1995), illustrates the different actin-binding proteins and their function.

Of particular interest in this study are fimbrins, a family of highly conserved actin filament-bundling proteins. These proteins are present in organisms ranging from the common yeast (*Saccharomyces cerevisiae*) (Adams et al. 1989) to humans (de Arruda et al. 1990, Lin et al. 1993). Fimbrin was first identified in chicken intestinal brush border microvilli as a 68 kD protein that crosslinked actin filaments (Bretscher, 1981). In humans, fimbrin protein homologs are referred as "plastin". So far, three human plastin isoforms have been identified: L-plastin, T-plastin, and I-plastin (Lin et al. 1988, 1993, 1994). L-plastin is a phosphoprotein whose expression is restricted to leukocytes but increases in neoplastic (transform-tumor) cells (de Arruda et al. 1990). T-plastin is expressed in a wide variety of tissues, including intestine (de Arruda et al. 1990); I-plastin is detected in small intestine, colon and kidneys (Lin et al. 1994). The three proteins are highly conserved in protein sequence, with at least 70% identity to each other and to chicken fimbrin (Lin et al. 1994). All fimbrin proteins

contain an EF-hand calcium binding domain, similar to those found in Ca^{2+}-modulated proteins such as calmodulin. The EF-hand structure has a conformation consisting of a helix, a Ca^{2+}-binding loop and a second helix. This EF-hand is followed by a pair of α-actinin-like binding domains (de Arruda et al. 1990; Lin et al. 1994) that confer the bundling property of these proteins. Namba et al. (1992) and Lin et al. (1994) have shown that Ca^{2+} affects the interaction of these proteins with actin. The binding of Ca^{2+} to the EF-hand domain may induce a conformational change which inhibits the acting-binding domain.

A yeast fimbrin protein, Sac6p, has been characterized both biochemically and genetically (Adams et al. 1989 and 1991). Sac6p bundles yeast actin filaments *in vitro*, and has 36 to 46% identity to human and chicken fimbrin (Adams et al. 1991). In subsequent studies using *sac6* null mutant cells it was shown that: Sac6p is essential for cellular morphogenesis *in vivo*; that the null mutants are defective in endocytosis; that they fail to grow at 37°C; and that human L- and T-plastin can both substitute *in vivo* for Sac6p (Adams et al. 1995). These results suggest that there is a high degree of functional conservation in the cytoskeleton proteins even between organisms as diverse as yeast and humans.

In plants, virtually nothing is known about the actin-associated proteins described above that regulate actin distribution and/or its dynamics. Recently, molecular strategies have shown that plants contain actin-associated proteins, such as profilin, which belong to the monomer-binding class (globular G-actin). The first plant profilin homologue was identified from the pollen allergen of the birch tree (Valenta

et al. 1993), while Staiger et al. (1993) have characterized profilin genes from maize and timothy grass. As was mentioned previously, the G-actin binding proteins, through their ability to bind to monomers, are responsible for regulation of actin polymer levels. Staiger et al. (1994) have shown, by microinjecting plant profilin into living cells, that profilin influences cytoplasmic streaming by disrupting filamentous actin (F-actin) strands. It is also known that profilin is a component of a signal transduction cascade that is linked to the cytoskeleton (Vojtek et al. 1991). Drobak et al. (1994) found that bean leaf plasma membrane PPI-PLC (phosphoinositide phospholipase C) is strongly and specifically inhibited by micromolar concentrations of bacterially expressed birch profilin.

This chapter describes the nucleotide sequence of a partial cDNA clone from wheat roots that has a high similarity with the fimbrin/plastin proteins. This partial cDNA clone was one of the seven positive clones selected from a cDNA library made from wheat roots exposed for 12 h to aluminum toxicity. Of the 1800 base pairs sequenced so far, the data bank search has shown 50% similarity with I-, T, and L-plastin from humans, with fimbrin from *Dictyostelium discoideum*, and with chicken fimbrin. As described before, fimbrin proteins are involved in the bundling of actin filaments in the cytoskeleton. The observation of a microfilament-bundling protein in Al-intoxicated roots is interesting because aluminum toxicity can interfere with the cytoskeleton. Puthota et al. (1991) showed in an ultrastructural study that in wheat roots Al inhibits mucilage droplet formation through the inhibition of transport of mucilage-containing vesicles, turnover of dictyosomes, and by interfering with

extracellular movement of mucilage polysacharide. Recently, Grabski and Schindler (1995) demonstrated that aluminum induces a rapid and dramatic increase in the rigidity of the actin network in soybean (*Glycine max*) root cells. They proposed that the growth-inhibitory activity of aluminum in plants may be a consequence of this rigidity of the actin network. They observed that pretreatment with cytochalasin D, which disrupts the integrity of F-actin filaments, prevents the rigidification induced by aluminum. They concluded that this rigidity within the actin network would have an immediate impact on the growth of root cells through the disorganization of associated cytoskeletal structures and promotion of altered expression of cytoskeletal proteins. Based on these previous studies, the finding of a cDNA clone encoding a cytoskeleton-bundling protein in Al-intoxicated wheat roots suggests that aluminum may interfere directly with the function of the plant cell cytoskeleton.

Although the 1.8 Kb wheat cDNA clone did not correspond to the expected size of TAl-18 gene, it was decided as an objective to characterize it because: a) it encodes a protein not previously described in plants; b) it apparently encodes a protein involved in the cytoskeleton; and c) the presence of fimbrin mRNA in Al-stressed plants suggests a possible role during Al-toxicity. The role and significance of this wheat cDNA clone is discussed.

MATERIALS AND METHODS

Plant Material. Seed material and seedling growth conditions are described in Chapter III (page 35). Seed of Atlas 66 (Al-tolerant) were the gift of Dr. Norma Ruiz, Department of Agronomy, University of Nebraska. Four-day-old Atlas 66

seedlings were stressed by addition of 80 μM and 400 μM $AlCl_3 \cdot 6H_2O$. Roots were harvested at 12 and 24 hours of aluminum-stress treatment and immediately frozen under liquid N_2 and kept at -70 C until used.

cDNA library and screening. The screening of the cDNA library and the extraction of poly A^+RNA was exactly as described in Chapter II (pages 23 and 24).

DNA sequencing analysis. The nucleotide sequence of the positive clones was determined as described for Chapter II (page 24). The 1800 bp of the cDNA clone were sequenced by designing 6 synthetic primers to complete the sequencing in both strands. DNA sequence data were analyzed as describe before (page 36).

Northern and Southern blot analysis. Total RNA was extracted as previously described (page 23). Total RNA was also extracted from root tips of the Al-tolerant wheat cv Atlas 66 after exposure of intact seedlings to 80 and 400 μM Al for 12 and 24 h. The procedure for Northern and Southern blot analysis was the same as described in Chapter II for 1,3-ß-glucanase clone (pages 36 and 37).

Rapid Amplification of 5' cDNA Ends (5'RACE): attempt to rescue the 5' missing part for the cDNA clone. In order to determine the 5' or the N-terminal part of the fimbrin-like clone a 5'RACE procedure (Frohman, 1988) was carried out. Two gene-specific primers or oligonucleotides were designed from the non-coding strand of the partial cDNA clone. Figure 11 illustrates the general 5'RACE procedure used. Total RNA from wheat roots treated with 80 μM Al for 12 h was used to synthesize the first strand cDNA. The antisense gene-specific primer 5' TTCAAGAAGTACCCATCCAT 3'(P1), located at 714 bp in the partial clone, was

used for first-strand cDNA synthesis by reverse transcriptase. 10 μg of total RNA in 10 μl DEPC (Diethyl Pyrocarbonate)-water were denatured at 65°C for 3 min, and then 10 μl of the reaction mixture was added. This reaction mixture contained: 4μl reverse transcriptase buffer (Gibco-BRL), 2 μl 100mM DTT (Gibco-BRL), 1 μl 10 mM dNTPs (Gibco-BRL), 0.25 μl (40 U/μl) RNasin (Promega), 1.5 μl (15.8 pmol) gene specific primer (P1), and 0.5 μl (200 U/μl) Superscript Reverse Transcriptase (RNaseH-:Gibco-BRL). The 20 μl reaction mixture was incubated for one hour at 42°C. To digest RNA the mixture was heated at 65°C for 15 min and then 1 μl (2 U/μl) RNase H (Gibco-BRL) was added and incubated at 55°C for 10 min. To remove excess of primer and RNA degradation products, a PCR-purification kit (QIAGEN) was used. For tailing of cDNAs, the product of the first strand of cDNA synthesis was suspended in 13 μl water and mixed with 5 μl of Terminal deoxynucleotide transferase buffer (Promega), 1 μl 5 mM dCTP (Gibco-BRL), and 1 μl Terminal deoxynucleotide transferase (TdT) (Promega). The mixture was incubated at 37°C for 5 min and then heat-denatured at 65°C for 5 min. This 20 μl mixture was brought up to 500 μl (1mM Tris-EDTA) and represented the 5'end-cDNA pool. For PCR amplification of the C-tailed first strand cDNA, a hybrid or G-anchor primer, 5'ACGCGTCGACTAGTCGGGIIGGGIIGGGIIG 3', was used as the sense primer. This anchor primer contains a mixture of G's and Inosines for annealing with the dC tail, and a SpeI restriction site (ACTAGT) for post-amplification cloning. As antisense primer the gene-specific primer (P2) 5' CATTATTCAGGTATGACTCGAC 3', located at position 674 bp in the partial

clone, was used to ensure specific amplification of the product of interest. The PCR amplification reaction contained: 27 μl cDNA pool, 5 μl Taq polymerase buffer (Perkin Elmer-Cetus), 4 μl 25 mM $MgCl_2$, 8 μl 1.25 mM each of the four deoxyribonucleotides (dNTPs), 1 μl (12.9 pmol/μl) P2-primer, 3 μl (8 pmol/μl) G-anchor primer, 0.5 μl (5U/μl) Taq polymerase (Perkin Elmer-Cetus), and 1.5 μl water. This 50 μl mixture was overlayered with 50 μl mineral oil and incubated in a MJ Thermocycler for a total of 30 cycles. The program consisted of 95°C for 1 min, 30 cycles of: 94°C for 45 s, 55°C for 1 min, and 72°C for 1.5 min; and 5 min at 72°C. The PCR product was purified by 1% agarose-1X TBE (89 mM Tris, 89 mM borate, 2 mM EDTA) gel, and sequenced as described before.

RESULTS

Nucleotide sequence determination of fimbrin/plastin like protein from wheat roots, and data base searches. A cDNA library from wheat roots treated with 80 μM Al for 12h was screened with a degenerate oligonoucleotide made from an Al-induced protein (TAl-18). Two of seven positive clones selected from the screening of the cDNA library were identified as highly similar to cytoskeleton actin-bundling proteins, or fimbrin/plastin proteins. These clones were 1.8 Kb in length, and one of them was sequenced in both strands. Figure 12 shows the 1800 nucleotides and the deduced amino acid sequence of the partial wheat cDNA clone. The data base searches using the National Center for Biotechnology Information (NCBI) BLAST e-mail and FASTA e-mail services showed that the nucleotide sequence of the wheat 1.8 Kb cDNA clone exhibits approximately 50% similarity with the human I-plastin

(Lin et al. 1994), T-plastin (Lin et al. 1993), and L-plastin (Lin et al. 1988), with the slime mold (*Dictyostelium discoideum*) fimbrin (Prassler, unpublished, 1994, from data gene bank), with the baker's yeast (*Saccharomyces cerevisiae*) *SAC6* gene encoding fimbrin (Adams et al. 1991), and with chicken (*Gallus gallus*) fimbrin (de Arruda et al. 1990). These fimbrin proteins belong to a group of actin crosslinking proteins, that bundle actin filaments *in vitro* (Adams et al. 1991). Figure 13A shows a comparison of the human, slime mold, and chicken fimbrin cDNA clones. These cDNA clones differ in size, but they have an Open Reading Frame (ORF) that codes for proteins ranging from 570 to 630 amino acids (Figure 13A). Figure 13B gives a schematic representation of the expected size of the cDNA clone, and the number of amino acids missing at the N-terminal. The 1800 bp cDNA clone has an ORF from position 2 to 1345 coding for 448 amino acids. The search in the protein sequence database indicated that wheat cDNA deduced amino acid sequence also possesses similarity with fimbrin/plastin proteins. Wheat deduced amino acid sequence was found to be 43% identical to Sac6p or fimbrin from the baker's yeast (Adams et al. 1991), 41% with L-,T-,I-plastin (Lin et al. 1988, 1993, 1994), chicken fimbrin (de Arruda et al. 1990), and with slime mold fimbrin (Prassler, unpublished, 1994).

Amino acid sequence alignments with fimbrin/plastin proteins from other organisms. The open reading frame shown in Figure 13B encodes a partial polypeptide of 448 amino acids. This deduced amino acid sequence was aligned with other fimbrin/plastin amino acid sequences from I-, L-, and T- plastin from humans, slime mold, yeast, and from chicken as shown in Figure 14. Wheat fimbrin/plastin-

like amino acid sequence aligned from residues 182 of human plastins and chicken fimbrin, and from 205 of yeast (Sac6p) fimbrin, suggesting that the partial wheat cDNA clone is missing 182 or 205 amino acids from the N-terminal region. The amino acid sequence alignment goes through the end of the proteins, which confirms that wheat fimbrin-like protein contains the C-terminal region of the fimbrin proteins. This sequence alignment for wheat fimbrin/plastin-like protein and other fimbrin/plastin proteins show that the similarities between them are extensive (Figure 14). Like other fimbrin/plastin proteins, the putative wheat fimbrin-like protein possesses the two 27 amino acid domains that are very similar to actin-binding domains identified in other proteins such as chicken fimbrin (de Arruda et al. 1990), α-actinin (Blanchard et al. 1989 in de Arruda et al. 1990), I- and T-plastin (Lin et al. 1993). These two actin-binding domains are important for the bundling activity of these proteins.

Expression of wheat fimbrin-like cDNA clone: Northern blot analysis was used to determine if Al toxicity affected the expression of the fimbrin/plastin-like cDNA clone. Four-day-old wheat (cv Victory, Al-sensitive) seedlings were exposed to 80 μM Al for 6, 12, and 24 h. In all treatments, the full-length cDNA clone used as a probe hybridized to a transcript of size 2.4 Kb. There was no difference in mRNA expression of fimbrin/like cDNA clone between the control, 6 h, and 12 h of Al exposure. At 24 h Al-exposure, the level of expression increased about 2-fold (densitometer analysis) compared to the control (Figure 15). It is important to note that 24 h of Al exposure correspond to the time when the root growth is almost

completely abolished.

To determine the expression of fimbrin-like mRNA in an Al-tolerant wheat line, a Northern blot analysis with total RNA from cv Atlas 66 was carried out. Four-day-old wheat seedlings were exposed to 80 μM and 400 μM Al for 12 and 24 h. Atlas 66 root growth was not inhibited significantly after 12 h of 80 μM Al, while a 25% inhibition was reached after 24 h. 400 μM Al inhibited Atlas 66 root growth by 90% after 12 and 24 h Al treatment (Figure 16). Figure 16B shows that the level of expression is enhanced approximately 2-fold after 12 h of 80 μM Al and remains above control levels at 24 h and also after 12 or 24 h of exposure of 400 μM Al. The increase in the level of fimbrin/plastin-like transcript suggests that the tolerant line Atlas 66 up-regulates the level of expression of fimbrin/plastin-like mRNA sooner than in the sensitive cultivar Victory.

Genomic southern blot analysis. In order to determine the complexity of the fimbrin/plastin-like cDNA clone family in wheat, genomic DNA was digested with 5 different restriction endonucleases and a Southern blot was probed with the full length fimbrin/plastin cDNA clone insert. Figure 17 shows that one band was detected in EcoRI, SacI, and XbaI. Two bands were observed with both enzymes HindIII and BamHI, however the second band could be due to the presence of internal sites for both HindIII and BamHI enzymes as determined by a restriction analysis of the partial clone using the MacVector program (data not shown) in the wheat cDNA clone. Thus, wheat fimbrin/plastin-like gene is most likely present as a single copy. This result is consistent with other observations. de Arruda et al. (1990) indicate that

chicken expresses a single fimbrin isoform; yeast fimbrin is also encoded by a single gene, *SAC6* (Adams et al. 1991). The human plastin isoforms (T-,L-, and I-plastins) are encoded by three distinct genes which display tissue-specific expression (Lin et al. 1993).

5' RACE. In order to obtain a full length clone for wheat fimbrin/plastin-like cDNA, a 5' rapid amplification of cDNAs ends (5'RACE) was attempted. Total RNA (from wheat roots treated with 80 μM Al for 12 h) and the gene specific primer (P1 in figure 11) were used to obtain the first strand cDNA by reverse transcriptase. After PCR amplification using the gene specific primer (P2) and the anchor G-primer, a single band of 1.8 kb was obtained. Since human I-plastin cDNA is 3.6 kb, it was thought that 1.8 kb was the expected size for the missing 5' of the partial fimbrin-like cDNA clone. However, sequencing of the 1.8 kb PCR band, followed by a Data base search, revealed that the sequence corresponded to wheat mitochondrial 26S rRNA gene. This result could be due to the high abundance of these 26S messeges, as well as similarity between 26S genomic DNA and the two gene-specific-primers (P1 and P2).

DISCUSSION

A partial wheat cDNA clone with high similarity to genes encoding the cytoskeleton actin-bundling proteins fimbrin/plastin has been described. The Data Bank search showed that the nucleotide sequence of this cDNA clone (1800 bp) is closely related to human fimbrin homologues L-, T-, and I-plastin (Lin et al. 1988, 1993, 1994), with yeast fimbrin, *SAC6* (Adams et al. 1991), with chicken plastin (de

Arruda et al. 1990), and with the slime mold fimbrin (*Dictyostelium discoideum*) (unpublished).

Fimbrin/plastins are a highly conserved family of actin filament-bundling proteins that are present in diverse organisms, from *Saccharomyces cerevisiae* to humans, which makes it plausible that plants would possess these proteins. The amino acid sequence alignment (Figure 14) and the 2.4 Kb band in the Northern blot analysis confirmed that wheat fimbrin/plastin cDNA clone is a partial clone. The 5' region of the cDNA clone that codes for the N-terminal region of the protein is missing. The amino acid sequence deduced from the wheat cDNA clone starts to align with other fimbrin/plastin proteins at position 182 in I-,T-, and L-plastin and at position 205 of Sac6p (yeast fimbrin). Generally, the number of amino acids in these proteins range from 570 in L-plastin (Lin et al. 1988) to 630 in chicken fimbrin (de Arruda et al. 1990). Thus, about 182 or 205 amino acids are missing from the N-terminal end of the wheat cDNA clone.

Sequence analysis of fimbrin/plastin proteins have shown they are composed of two structural domains, a headpiece and a core, each with a separate functional role. The headpiece contains an EF-hand calcium-binding domain close to the N-terminal end of the protein. Unfortunately, no comparison can be made using the putative wheat fimbrin/plastin since it is missing the region of the cDNA encoding the N terminal region of the protein. However, the core domain contains two α-actinin-like actin-binding domains, formed by a region of 27 well-conserved amino acids. These two actin-binding domains are present in wheat partial cDNA clone (Figure 14,

residues in bold). These adjacent actin-binding domains are thought to enable these proteins to cross-link actin filaments into bundles or to bind two actin microfilaments (de Arruda et al. 1990).

Thus, the percent of similarity and the alignment of the deduced amino acid sequence of wheat cDNA clone with fimbrin/plastin proteins, as well as the presence of the two putative actin-binding domains, give indirect evidence that the wheat partial cDNA clone codes for a putative homologue of fimbrin/plastin-like proteins.

There is an immediate question that arises: why was a cDNA clone that codes for a cytoskeleton-actin-bundling protein selected from a cDNA library made from Al-intoxicated roots? Northern blot analysis (Figure 15) from roots of cv Victory (Al-sensitive) showed that levels of the cDNA clone transcript did begin to increase, but not until 24 h of 80 μM Al. There was no significant difference between control, 6 h, and 12 h of Al treatment, although the transcript was present. The cDNA library was made using poly A^+ RNA from wheat roots treated with Al for 12 h, so there is no correlation in time with the highest expression of the cDNA clone transcript and the time at which the cDNA library was made. However, 14 bases out of the 21 of the degenerate oligonucleotide (5'GTC TGG GTT G/CGC GTC G/CAG/A G/ATA 3') used as a probe (designed from the TAl-18 amino acid sequence) are complementary to the region from positions 715 to 736 of the non-coding strand (715, 3'CCT ACC CAT GAA GAA CTT CAT 5', 736). These fourteen bases could have formed a stable hybrid under the stringency conditions used and thus given a positive signal. Surprisingly, no similarity was found in the Data Bank between the wheat

cDNA clone and parsley PR2 nucleotide sequence, or with any other pathogenesis-related protein gene sequence. Hence, hybridization between fimbrin/plastin cDNA clone and TAl-18 probe appears to have been a chance event. Regardless of whether or not it was serendipitous, identification of a cDNA clone encoding a cytoskeletal, actin-bundling protein suggests that Al toxicity is interfering with the functional array of the plant cytoskeleton. These results are consistent with a previous study showing that Al interferes with the function of the actin network in soybean root cells (Grabski and Schindler (1995).

Studies on yeast have revealed the importance of fimbrin/plastin protein on the functional organization of the cell cytoskeleton. Yeast mutants lacking Sac6p do not form normal actin structures. They lose asymmetry in cortical actin distribution and are defective in morphogenesis, manifested in cell shape (Adams et al. 1991). With this in mind we would expect that fimbrin/plastin proteins in plant cells might be involved also in the integrity and functional array of the actin-filaments of the cell cytoskeleton. The observation that during Al toxicity the levels of fimbrin/plastin transcript increased after 24 h of 80 μM Al might be related to inhibition of the bundling activity of fimbrin/plastin-like protein to the actin filaments, leading to an unorganized actin-filaments array. Namba et al. (1992) and Lin et al. (1994) have shown that L- and T-plastins are negatively regulated by Ca^{2+}. An increased in the cytoplasmic Ca^{2+} concentration in the micromolar range inhibits the association of L-plastin with actin filaments, through the binding of Ca^{2+} to the EF-hand calcium-binding domain. On the other hand, it is known that uptake of Al^{3+} into the cell, or

the binding of Al^{3+} to some cell surface ligand, can initiate reactions that eventually disrupt intracellular Ca^{2+} metabolism (Delhaize and Ryan, 1995 and Rengel et al. 1995). Aluminum could thus interfere with fimbrin/plastin proteins by either disrupting Ca^{2+} homeostasis, increasing the normal intracellular Ca^{2+} levels, or by displacing Ca^{2+} from the EF-hand calcium-binding domain. Either would cause inhibition of the actin-binding activity of fimbrin/plastin proteins. It can be speculated that the inhibition of the bundling-activity of fimbrin/plastin proteins might produce abnormal cytoplasmic actin cable arrays, leading also to a disruption of the microtubules as well as microfilament pattern. This disorganized cytoskeleton array would be unable to provide the framework for functions such as cell wall biosynthesis (deposition and incorporation of cellulose into the new cell wall), transport of secretory vesicles, as well as other functions. Seagull (1990) observed that the disruption of actin microfilaments by cytochalasin, which causes depolymerization of actin filaments, disrupts microtubule patterns and wall deposition in developing cotton fibers. Using a lethal actin mutant, DBY 1693 in yeast, Gabriel and Kopecká (1995) also found that the disruption of the actin cytoskeleton results in formation of an aberrant cell wall. With this scenario, the inhibition of root cell elongation observed during Al toxicity might be explained by the inability of the cytoskeleton framework to transport and deposit new cell wall material. The up-regulation of fimbrin/plastin-like transcript in cv Victory (Al-sensitive) after 24 h of 80 μM Al may be an attempt by the cell to synthesize more protein in order to maintain the integrity of the cytoskeleton after root growth has been arrested. On the other hand, the Al-tolerant

cultivar Atlas 66 has the ability to increase the level of fimbrin/plastin-like protein before root growth inhibition (Figure 16). This may be part of a tolerance mechanism that allows Atlas 66 to maintain the integrity of the cytoskeleton and therefore keep the root growing under low levels of aluminum. However, root growth was completely inhibited by 400 μM Al for 12 or 24 h despite the fact that fimbrin/plastin-like mRNA was expressed at higher levels. The increase in fimbrin/plastin-like transcript *per se* does not confer tolerance to high (400 μM) levels of aluminum. Fimbrin/plastin-like protein is probably only one of many cytoskeleton proteins whose expression and function may be affected by Al toxicity.

An alternative model or hypothesis would be that Al is interfering with fimbrin/plastin regulation by Ca^{2+} through the inhibition of Ca^{2+} uptake by blocking Ca-channels (Rengel and Elliot 1992), and keeping the intracellular Ca^{2+} levels low by altering IP_3 levels (Jones and Kochian, 1995). During Al toxicity available levels of fimbrin/plastin protein would be reduced if more bundling activity occur. If an excess of actin filaments are bundling, the cytoskeleton actin-filament array might become more rigid (Grabski and Schindler, 1995). The inhibition of root cell elongation would be explained by the rigidity of the cytoskeleton array. The increase in the levels of fimbrin/plastin transcript after 24 h of Al in cv Victory (Al-sensitive) would be to replenish the low pool of protein level and would also reflect the time of root growth inhibition. Levels of fimbrin/plastin protein should be monitored during Al toxicity to see if they are down-regulated due to an excess of actin-crosslinking-filaments.

In summary, this study has described a homologue of the fimbrin/plastin proteins in wheat plants, and their probable role during Al toxicity. The partial characterization of a novel cytoskeleton-binding protein in plants opens a new field of study on the mechanisms of aluminum toxicity at the cellular level.

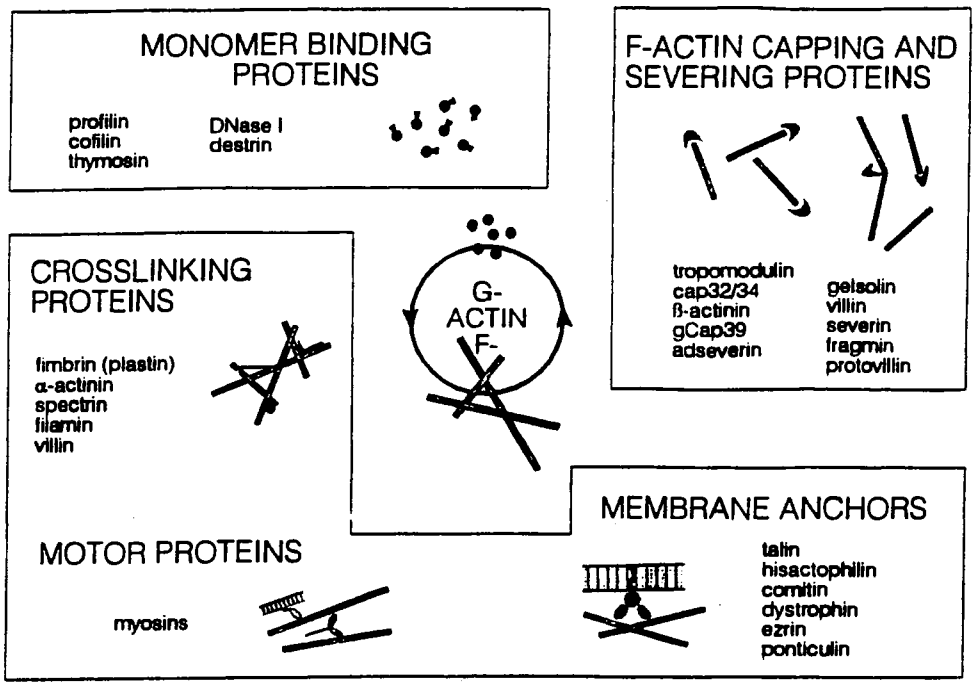


Figure 10. Actin-binding proteins and their function. The structural organization of the network of microfilaments are regulated by actin-binding proteins. Taken from Schleicher et al. (1995).

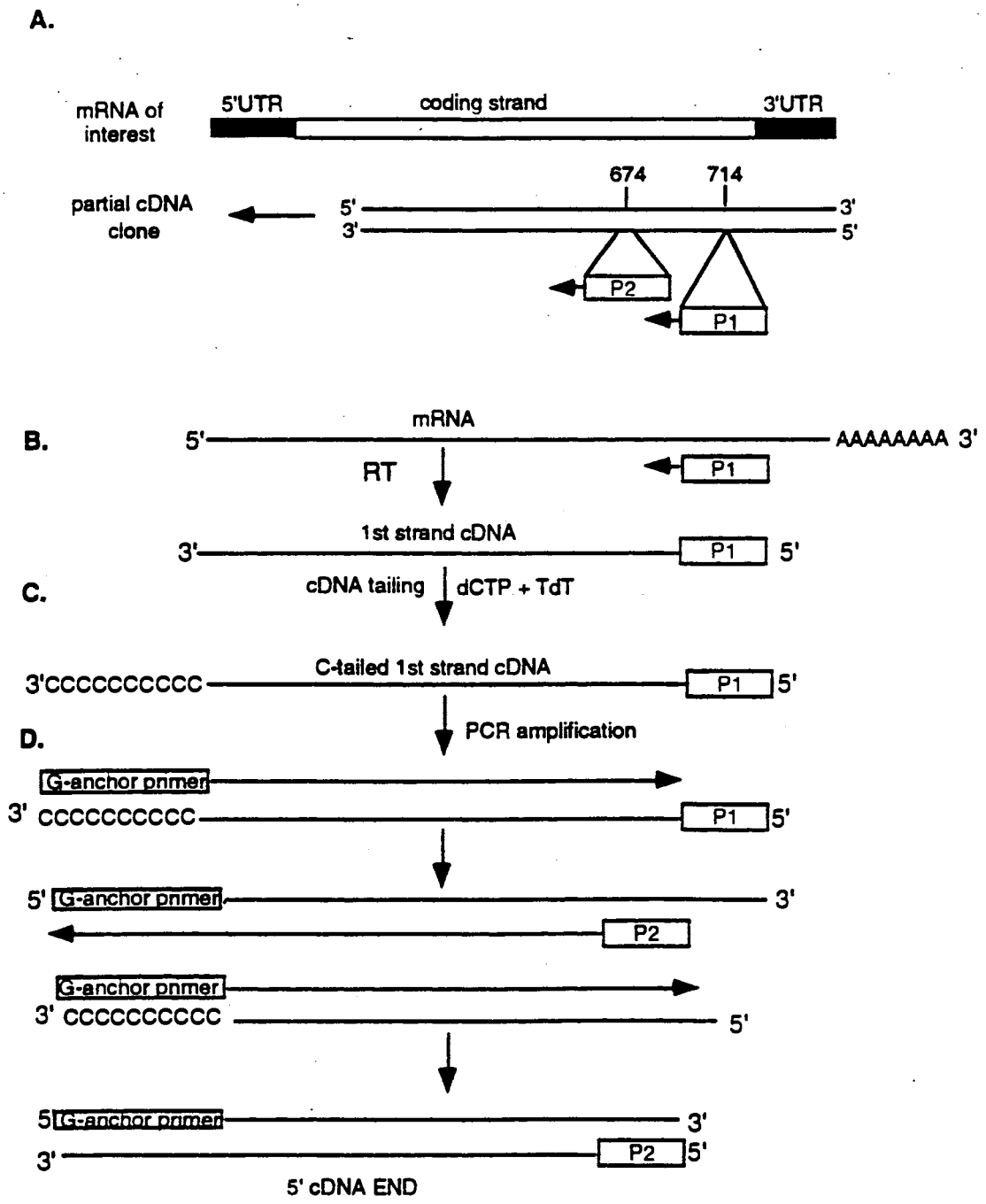


Figure 11. Schematic representation of Rapid Amplification of cDNA ends (5'RACE). (A) mRNA of interest and partial cDNA clone (fimbrin/plastin-like) showing the two gene-specific antisense primers, P1 and P2 at positions 714 and 674 bp respectively. (B) Total RNA or mRNA is reverse transcribed using P1 (5'TTCAAGAAGTACCCATCCAT 3') gene-specific primer to anneal to mRNA strand. (C) First strand cDNA is C-tailed with dCTPs and Terminal deoxynucleotide transferase (TdT). (D) PCR amplification is achieved using P2 (5'CATTATTCAGGTATGACTCGAC3') gene-specific primer and G-anchor primer as a sense primer.

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          10                  35
GGCACGAGGAGTTCTTAACCCATGGGAGAGGAATGAAAACCACACACTGTGCCTCAACTCTGCAAAGGCC   70
  A  R  G  V  L  N  P  W  E  R  N  E  N  H  T  L  C  L  N  S  A  K  A
ATTGGATGTACTGTTGTTAACATTGGCACCCAGTTTTTAGTGGAAGGAAGGCCTCATTTAGTTCTTGGAT  140
  I  G  C  T  V  V  N  I  G  T  Q  F  L  V  E  G  R  P  H  L  V  L  G
TAATATCTCAAATCATAAAGATTCAACTTTTGGCTGATCTTAATCTTAAGAAGACACCCCAGCTTGTGGA  210
L  I  S  Q  I  I  K  I  Q  L  L  A  D  L  N  L  K  K  T  P  Q  L  V  E
ATTGTTTGATGACAGTAAGGATATAGATGAGGTGTTGAGCTTGTCAACAGAAAAGATGCTGCTTCGATGG  280
  L  F  D  D  S  K  D  I  D  E  V  L  S  L  S  T  E  K  M  L  L  R  W
ATGAACCATCATCTGAAAAAGGCTGGCTACAAGAAAACTGTTAACAATTTCTCTTCGGATGTGAAGGATG  350
  M  N  H  H  L  K  K  A  G  Y  K  K  T  V  N  N  F  S  S  D  V  K  D
GTGAAGCCTATGCTTATCTTCTAAAAGCTCTTGCTCCAGAGACTTCCCCTGAAACCACACTGGAGACTAA  420
G  E  A  Y  A  Y  L  L  K  A  L  A  P  E  T  S  P  E  T  T  L  E  T  K
GAATCCTGATGAAAGGGCAAAAATGGTACTTGAACAAGCAGAGAAGTTGGACTGCAAAAGATACCTTACA  490
  N  P  D  E  R  A  K  M  V  L  E  Q  A  E  K  L  D  C  K  R  Y  L  T
CCAAAGGATATTACTGAGGGTTCTGCCAACTTGAATCTTGCATTTGTTGCACAAATATTCCAGCATCGGA  560
  P  K  D  I  T  E  G  S  A  N  L  N  L  A  F  V  A  Q  I  F  Q  H  R
ATGGTCTAACTAGTGACATTAAACAAGTTACACTCACACAGTCAGCATCACGTGATGATGTTCTAGTATC  630
N  G  L  T  S  D  I  K  Q  V  T  L  T  Q  S  A  S  R  D  D  V  L  V  S
CAGAGAAGAAAGGGCCTTCCGAATGTGGATCAACAGCCTTGGGGTCGAGTCATACCTGAATAATGTTTTT  700
  R  E  E  R  A  F  R  M  W  I  N  S  L  G  V  E  S  Y  L  N  N  V  F
GAAGATGTTCGCAATGGATGGGTACTTCTTGAAGTACTTGACAAAGTTTCTCCTGGATCTGTCAATTGGA  770
  E  D  V  R  N  G  W  V  L  L  E  V  L  D  K  V  S  P  G  S  V  N  W
AGTTGGAATCAAAACCTCCAATTAAATTGCCATTTAGGAAACTGGAGAACTGCAATCAAGTTGTCAAAAT  840
K  L  E  S  K  P  P  I  K  L  P  F  R  K  L  E  N  C  N  Q  V  V  K  I
TGGGAAGGAGTTAAAGTTTTCATTAGTAAATTTAGCTGGGAATGATATTGTTCAGGGGAAATAAGAAATT  910
  G  K  E  L  K  F  S  L  V  N  L  A  G  N  D  I  V  Q  G  N  K  K  L
GATAGTTGCACTTCTGTGGCAATTGATGAGATTTAATATCCTTCAATTGCTAAACAGACTGAGATCCCAC  980
  I  V  A  L  L  W  Q  L  R  F  N  I  L  Q  L  L  N  R  L  R  S  H  S
TCCAAAGGATCCCAAGGAAAGCAAATTACTGATGCAGATATACTGAACTGGGCAAACAGCAAAGTGAAAG 1050
K  G  S  Q  G  K  Q  I  T  D  A  D  I  L  N  W  A  N  S  K  V  K  A  S
CATCAGGAAGAACATCTCGAATGGAAAGCTTCAAGGATAAGAGCTTATCAAATGGAGTGTTCTTCCTCGA 1120
  G  R  T  S  R  M  E  S  F  K  D  K  S  L  S  N  G  V  F  F  L  E  L
ACTTCTTAGTGCAGTTCAGCCAAGGGTTGTGAACTGGAAAGTAGTTACAAAGGGAGAAGCTGACGAGGAA 1190
  L  L  S  A  V  Q  P  R  V  V  N  W  K  V  V  T  K  G  E  A  D  E  E
AAGAAGCTAAATGCTACCTACATCATTAGTGTTGCAAGAAAGCTCGGATGTTCTGTGTTTCTACTGCCAG 1260
  K  K  L  N  A  T  Y  I  I  S  V  A  R  K  L  G  C  S  V  F  L  L  P
AGGACATCATAGAGGTGAACCAGAAGATGATCCTAACTCTTACAGCTAGCATCATGTATTGGGAGCCTGC 1330
E  D  I  I  E  V  N  Q  K  M  I  L  T  L  T  A  S  I  M  Y  W  E  P  A
TGAGACAACCACAGCCTGAAATATCAGAAGCATCAGAGCCATCCAGCATGGCTTCGGACGCAACTTCCGA 1400
  E  T  T  T  A  *
CATTGGCTCGGAGGATGGTGCCTCAACAGCGGCACCGTCCGAGAGCGAAGAGGCAAACTCACTGTCCGAC 1470
AGTCTATCCACCCTGACCACAGACGACGCCACCTCAAATGCTCCACCTGCAGAAAACGGGAACGGCGCCA 1540
CCCCAGATGCTCCACCTGCAGAAAACGGGAACGACGCCACCCCAGATGCTCAACCTGCAGAAAACGGGAA 1610
CGCCGCCACCCCAGATGCCCCACCTGCAGAAAATGGGAACGATGCCACATGATCCTGATGGTTATATGTT 1680
CGTCCGTAGCGCTGTCCATCATTAGCTTCATATACAAGGGTTGGTACCCAAAGTTGCTGCTGCTACCAGA 1750
AGTGAAATTTTCTCCTGCTTTTCAGGTACTAGCATAATAGCTCGGTGCCGAATTCCCTGCAG          1812
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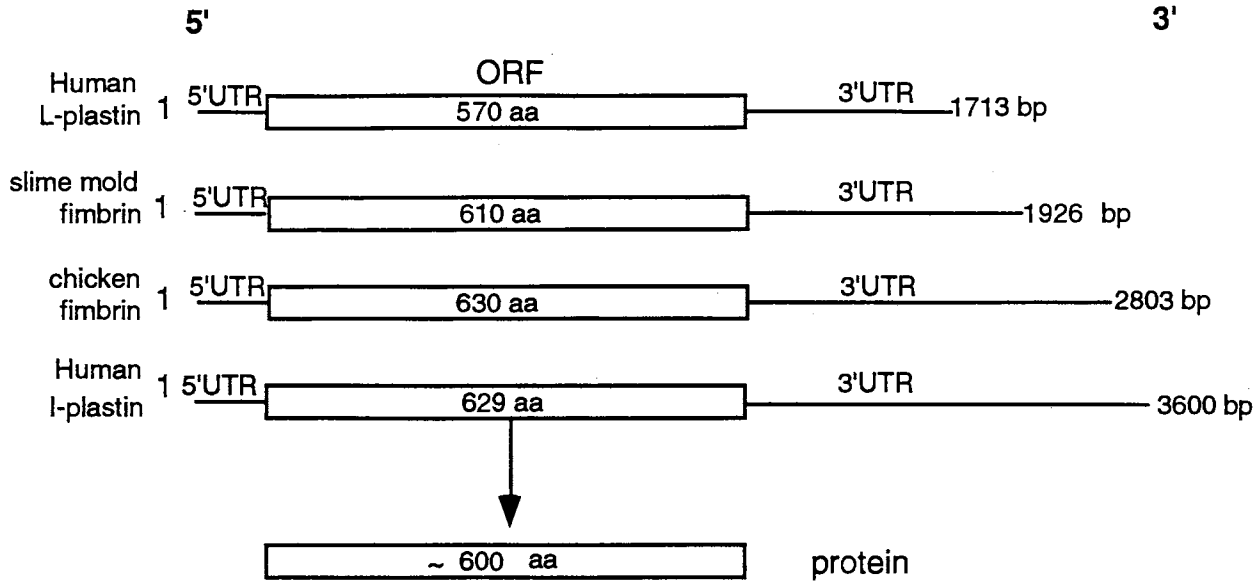
Figure 12. Nucleotide sequence and deduced amino acid sequence of partial fimbrin/plastin-like cDNA clone from wheat cDNA library. The deduced single-letter amino acid sequence is given below the center nucleotide of each codon triplet. An asterisk (*) designates the TGA terminator codon. The putative polyadenylation signal consensus sequence is underlined. The complete sequence, according to Data-Bank searches, should be around 3000 bp.

A. Fimbrin-like proteins from human (*Homo sapiens*) (L- and I- plastin), slime mold (*Dictyostelium discoideum*) and from chicken (*Gallus gallus*)



B. Fimbrin-like protein from wheat roots (*Triticum aestivum*)

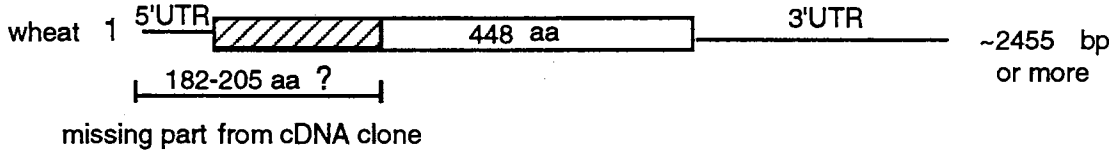


Figure 13. Schematic representation of fimbrin/plastin proteins from different organisms, all corresponding to cDNA clones. (A) ORF (Open Reading Frame), 5'UTR and 3'UTR (5'and 3' untranslated sequence respectively). (B) Partial clone of wheat fimbrin/plastin-like protein. Box with diagonal lines corresponds to missing part of ORF. Question mark (?) indicates range of the number of amino acids encoded by the missing part of the cDNA clone ORF.

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CLUSTAL W(1.4) multiple sequence alignment

I-plastin       -----MENSTTTISREELEELQEAFNKIDIDNSGYVSDYELQDLFKEASLPLPGYKVREI
L-plastin       ------------------------------------------------------------
T-plastin       ------------------------------------------------------------
slime-fimb      ---------MTNFSESEISEFKASFNQFDENGDGQISALELQKILTKCGEKVTGVEVR--
yeast-fimb      MNIVKLQRKFPILTQEDLFSTIEKFRAIDLDDKGWVEKQQALEAVSKD-----GDATYDE
chicken-fi      -----MENNVTTISREELEELREAFNKIDIDNSGYVSDYELQDLFKEASLPLPGYKVREI
wheat-fimb      ------------------------------------------------------------

I-plastin       VEKILSVADSNKDGKISFEEFVSLMQELK----------------SKDISKTFRKIINK
L-plastin       ----MLDGDRNKDGKISFDEFVYIFQEVK----------------SSDIAKTFRKAINR
T-plastin       ----MATGDLDQDGRISFDEFIKIFHGLK----------------STDVAKTFRKAINK
slime-fimb      --DMIKEVDTDGNGSIDFKEFLQVMQKARQ---------------HSANASPAFASAVKK
yeast-fimb      ARETLKHVGVDASGRVELDDYVGLVAKLRESKTGAAPQTTFNVAPNSTPIVSTAATGLQH
chicken-fi      IEKIFAVTDSNKDGKINFEEFVSLIQELK----------------SKDVSKSYRKSINK
wheat-fimb      ------------------------------------------------------------

I-plastin       REGITAIGGTSTISSEGTQHSYSEEEKVAFVNWINKALENDPDCKHLIPMNPNDDSLFKS
L-plastin       KEGICALGGTSELSSEGTQHSYSEEEKYAFVNWINKALENDPDCRHVIPMNPNTDDLFKA
T-plastin       KEGICAIGGTSEQSSVGTQHSYSEEEKYAFVNWINKALENDPDCRHVIPMNPNTNDLFNA
slime-fimb      VGAVNTIGGYSGSTASGVQHSYSDEEKVAYIDWINNCLAKDVDLKSRLPIPEDGDKFFAA
yeast-fimb      KGKGTQAKIIVAGSQTGTTHTINEEERREFTKHINSVLAGDQDIGDLLPFPTDTFQLFDE
chicken-fi      KLGITALGGTSSISTEGTQHSYSEEEKVAFVNWINKALQDDPDCKHILPMNPSDASLFKS
wheat-fimb      ------------------------------------------------------------

                                                                  actin
I-plastin       LADGILLCKMINLSEPDTIDERAINK----KKLTPFTISENLNLALNSASAIGCTVVNIG
L-plastin       VGDGIVLCKMINLSVPDTIDERAINK----KKLTPFIIQENLNLALNSASAIGCHVVNIG
T-plastin       VGDGIVLCKMINLSVPDTIDERTINK----KKLTPFTIQENLNLALNSASAIGCHVVNIG
slime-fimb      CNDGLLLCKLINDAVPDTIDERVLNK----KNLNAFRINENQVLCINSAKAIGCNVVNIG
yeast-fimb      CRDGLVLSKLINDSVPDTIDTRVLNWPKKGKELNNFQASENANIVINSAKAIGCVVVNVH
chicken-fi      LADGILLCKMINFSQPDTIDERAINK----KKLTPFTISENLNLALNSASAIGCTVVNIG
wheat-fimb      --------------------------ARG-VLNPWERNENHTLCLNSAKAIGCTVVNIG
                                           *.     **  . .*** **** ***.

                -binding domain
I-plastin       ASDLKEGKPHLVLGLLWQIIKVGLFADIEISRNEALIALLNEGEELEELMKLSPEELLLR
L-plastin       AEDLRAGKPHLVLGLLWQIIKIGLFADIELSRNEALAALLRDGETLEELMKLSPEELLLR
T-plastin       AEDLKEGKPYLVLGLLWQVIKIGLFADIELSRNEALIALLREGESLEDLMKLSPEELLLR
slime-fimb      AGDLVEGRAHLIMGLTWQIIKIGLFARINLTNHPELYRLLHDGETIEDLLKLPVEEILLR
yeast-fimb      SEDIIEGREHLILGLIWQIIRRGLLSKIDIKLHPELYRLLEDDETLEQFLRLPPEQILLR
chicken-fi      SQDLQEGKPHLVLGLLWQIIKVGLFADIEISRNEALIALLNEGEELDQLMKLSPEELLLR
wheat-fimb      TQFLVEGRPHLVLGLISQIIKIQLLADLNLKKTPQLVELFDDSKDIDEVLSLSTEKMLLR
                .  .  *.  *..**  *.*.  * . . .     *  *  .   ..  . *  * .***

I-plastin       WVNYHLTNAG-WHTISNFSQDIKDSRAYFHLLNQIAPKGG-EDGPAIAIDLSGINETNDL
L-plastin       WANFHLENSG-WQKINNFSADIKDSKAYFHLLNQIAPKGQKEGEPRIDINMSGFNETDDL
T-plastin       WANYHLENAG-CNKIGNFSTDIKDSKAYYHLLEQVAPKGDEEGVPAVVIDMSGLREKDDI
slime-fimb      WFNYHLAAAGSQRRVKNFSGDIKDSECYTILLKQIAPKDA-------GVETSALNISNLD
yeast-fimb      WFNYHLKQANWNRRVTNFSKDVSDGENYTILLNQLDPALC---------SKAPLQTTDLM
chicken-fi      WVNYHLANAG-WQKISNFSQDIRDSRAYYHLLNQIAPKGDDFDEIHVEIDFSGFNDKNDL
wheat-fimb      WMNHHLKKAGYKKTVNNFSSDVKDGEAYAYLLKALAPETS---------PETTLETKNPD
                * * **  ..    . *** *. *   *  **  . *              .
```

Figure 14. Amino acid alignment of fimbrin/plastin proteins from wheat and other organisms. Fimbrin/plastin sequences from *Homo sapiens* (I-, L-, T-plastin) (Lin et al. 1988, 1991, 1994), *Dictyostelium discoideum* (slime mold) (unpublished), *Saccharomyces cerevisiae* (yeast) (Adams et al. 1991), and *Gallus gallus* (de Arruda et al. 1991) were aligned to the partial predicted amino acid sequence of

```
I-plastin       KRAGLMLQEADKLGCKQFVTPADVVSGNPKLNLAFVANLFNTYPCLHKPNNND-----ID
L-plastin       KRAESMLQQADKLGCRQFVTPADVVSGNPKLNLAFVANLFNKYPALTKPENQD-----ID
T-plastin       QRAECMLQQAERLGCRQFVTATDVVRGNPKLNLAFIANLFNRYPALHKPENQD-----ID
slime-fimb      QRAVKVLENADKLGCKKFLKPKDIVTGFQKLNLAFVANLFNTHPALEP----------VE
yeast-fimb      ERAEQVLQNAEKLDCRKYLTPSSLVAGNPKLNLAFVAHLFNTHPGLEPIQEEE--KPEIE
chicken-fi      RRAECMLQQADKLGCRQFVTPADVVAGNPKLNLAFVANLFNTYPALHKPDNSS-----YD
wheat-fimb      ERAKMVLEQAEKLDCKRYLTPKDITEGSANLNLAFVAQIFQHRNGLTSDIKQVTLTQSAS
                 **  .*. *..* *....    .  *   *****.* .*.    *

I-plastin       MNLLEGESKEERTFRNWMNSLGVNPYINHLYSDLADALVIFQLYEMIR-VPVNWSHVNKP
L-plastin       WTLLEGETREERTFRNWMNSLGVNPHVNHLYADLQDALVILQLYERIK-VPVDWSKVNKP
T-plastin       WGALEGETREERTFRNWMNSLGVNPRVNHLYSDLSDALVIFQLYEKIK-VPVDWNRVNKP
slime-fimb      DVVIIEETREEKTFRNWMNSLGVDPFVNNLYEGTYDGLILIQLFDKIYPGLVDHKKVNYP
yeast-fimb      EFDAEGE-REARVFTLWLNSLDVDPPVISLFDDLKDGLILLQAYEKVMPGAVDFKHVNKR
chicken-fi      LTLLEGESNEERTFRNWMNSLGVSPYVNHLYSDLSDALIIFQLYEMTR-VPVDWTHVNKR
wheat-fimb      RDDVLVS-REERAFRMWINSLGVESYLNNVFEDVRNGWVLLEVLDKVSPGSVNWKLASKP
                        * . *  *.*** *   .  ..      .. .  .      *
                                                    actin-binding domain
I-plastin       PYPALG-GNMKKIENCNYAVELGKNKAKFSLVGIAGQDLNERNSTLTLALVWQLMRRYTL
L-plastin       PYPKLG-ANMKKLENCNYAVELGKHPAKFSLVGIGGQDLNDGNQTLTLALVWQLMRRYTL
T-plastin       PYPKLG-GNMKKLENCNYAVELGKNQAKFSLVGIGGQDLNEGNRTLTLALIWQLMRRYTL
slime-fimb      PYKAMG-AEMKKIENCNYAIQLGKD-CKYSLVGIDGKNVYDKNKTLTLSILWQLMRGHVI
yeast-fimb      PASGAEISRFKALENTNYAVDLGRA-KGFSLVGIEGSDIVDGNKLLTLGLVWQLMRRNIS
chicken-fi      PYPLLG-GNMKKIENCNYAVELGKTKAKFSLVGIAGHDLNEGNPTLTLALIWQLMRRYTL
wheat-fimb      PIK----LPFRKLENCNQVVKIGKE-LKFSLVNLAGNDIVQGNKKLIVALLWQLMRFNIL
                *         . .**.*  . .*.    .***.. *  .   *  * . ..*****

I-plastin       NVLSDLGE------GEKVNDEIIIKWVNQTLKSANKKTSISSFKDKSISTSLPVLDLIDA
L-plastin       NVLEDLGD------GQKANDDIIVNWVNRTLSEAGKSTSIQSFKDKTISSSLAVVDLIDA
T-plastin       NILEEIGG------GQKVNDDIIVNWVNETLREAEKSSSISSFKDPKISTSLPVLDLIDA
slime-fimb      SILTALSGS-----GKPIADADIVNVANSKLSAAGKK-QISGFKDSTISTGIPILDVIEA
yeast-fimb      ITMKTLSSS-----GRDMSDSQILKWAQDQVTKGGKNSTIRSFKDQALSNAHFLLDVLNG
chicken-fi      NVLSDLGE------GEKVNDEIIIKWVNQTLANANKKTSITSFKDKSISTSLPVLDLIDA
wheat-fimb      QLLNRLRSHSKGSQGKQITDADILNWANSKVKASGRTSRMESFKDKSLSNGVFFLELLSA
                  .  .        *   *  *.  .  .   .  . ***  .*     ....

I-plastin       IAPNAVRQEMIRRENLSDEDKLNNAKYAISVARKIGARIYALPDDLVEVKPKMVMTVFAC
L-plastin       IQPGCINYDLVKSGNLTEDDKHNNAKYAVSMARRIGARVYALPEDLVEVKPKMVMTVFAC
T-plastin       IQPGSINYDLLKTENLNDDEKLNNAKYAISMARKIGARVYALPEDLVEVNPKMVMTVFAC
slime-fimb      VRPGSVDPALVATS-GSAEDNLLNAKLAVSTARKVGAVVFALPEDIVEVKPKMVLTLFAS
yeast-fimb      IAPGYVDYDLVTPG-NTEEERYANARLAISIARKLGALIWLVPEDINEVRARLIITFIAS
chicken-fi      IAPKAVRQEMVKREDLSYQDKLNNAKYAISVARKIGARIYALPDDLVEVKPKMVMTVFAC
wheat-fimb      VQPRVVNWKVVTKG-EADEEKKLNATYIISVARKLGCSVFLLPEDIIEVNQKMILTLTAS
                . *  .   ..        .   **   .* **..*. .  .*.*. **  ....*  *.

I-plastin       LMGKGLNRIK--
L-plastin       LMGRGMKRV---
T-plastin       LMGKGMKRV---
slime-fimb      LWQVEMTK----
yeast-fimb      LMTLNK------
chicken-fi      LMGRGLNKIK--
wheat-fimb      IMYWEPAETTTA
                .
```

Figure 14. (continued) fimbrin/plastin-like protein of *Triticum aestivum* (wheat). Gaps to optimize alignments are designated by dashes (-). Asterisks (*) indicate consensus amino acid identity among all organisms. Dots (.) indicate positions of conservative amino acid replacement. Residues that are thought to be involved in the actin-binding site are in bold.

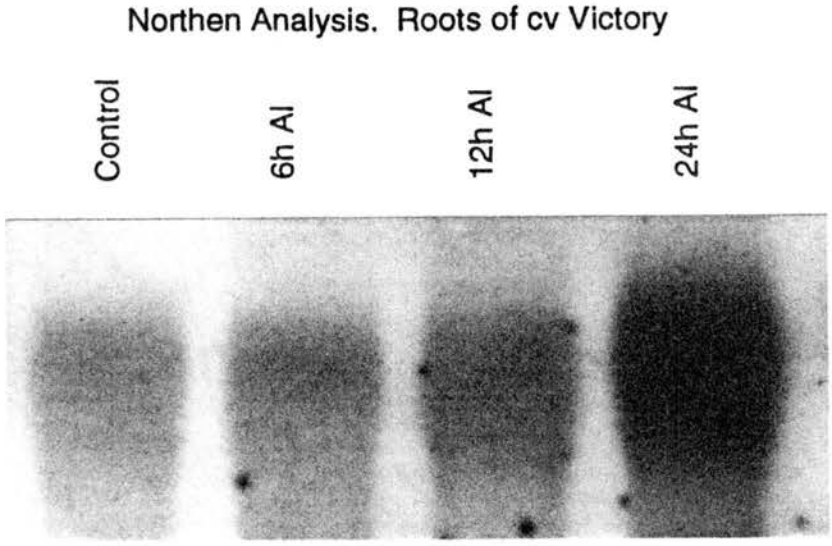
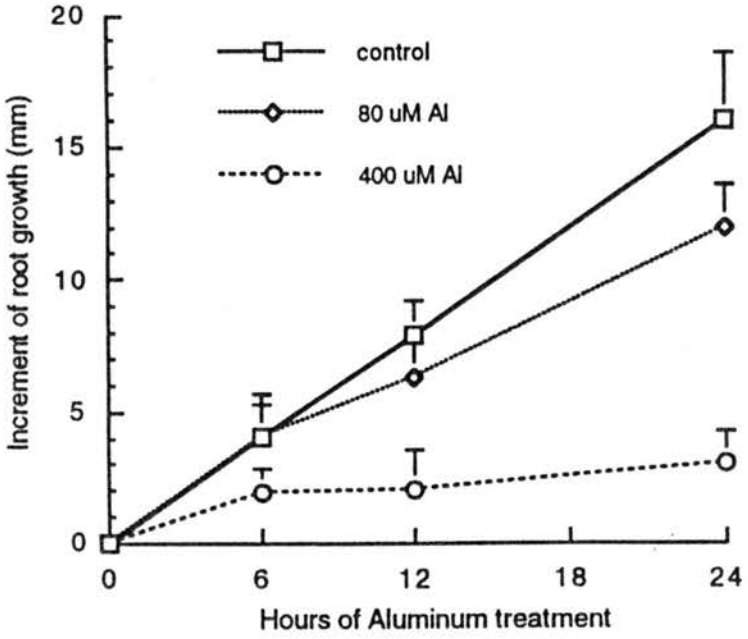


Figure 15. Expression of fimbrin/plastin mRNA in roots of wheat cv Victory (Al-sensitive) during Al toxicity. Four-day-old wheat seedlings were subjected to 80 μM $AlCl_3 \cdot 6H_2O$ for 6, 12, and 24 hours. Total RNA was isolated from root tissue. 20 μg of total RNA were loaded in each lane and resolved on a 1.2% agarose gel containing formaldehyde. RNA was transferred to nylon membrane and probed with gel-purified digoxygenin-labeled full-length fimbrin/plastin cDNA insert.

A.



B.

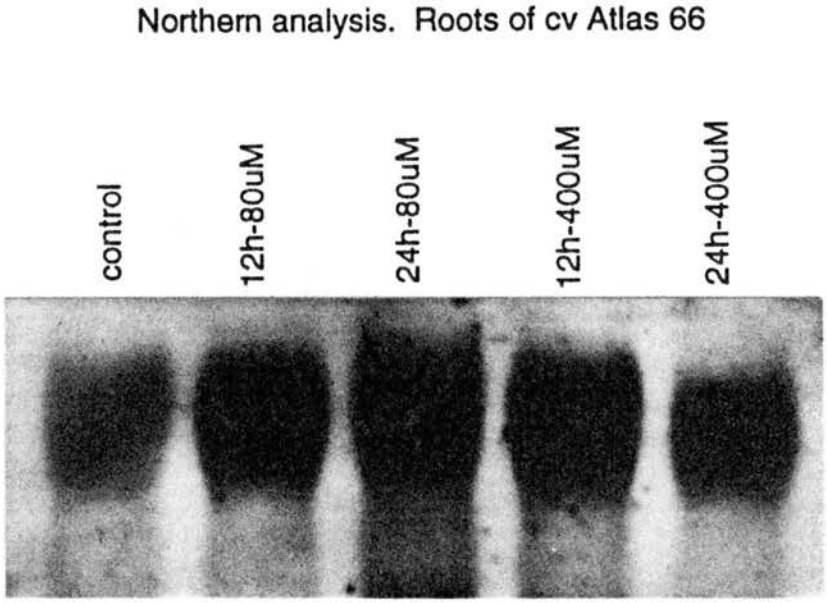


Figure 16. (A) Root growth of cv Atlas 66 exposed to 80 and 400 μM $AlCl_3 \cdot 6H_2O$ for 6, 12, and 24 hours. (B) Expression of fimbrin/plastin mRNA in roots of wheat cv Atlas 66 (Al-tolerant). Four-day-old wheat seedlings were subjected to 80 μM and 400 μM $AlCl_3 \cdot 6H_2O$ for 12 and 24 hours. 20 μg of total RNA were loaded in each lane and resolved on a 1.2% agarose gel containing formaldehyde. RNA was transferred to nylon membrane and probed with gel-purified, digoxygenin-labeled full-length cDNA insert.

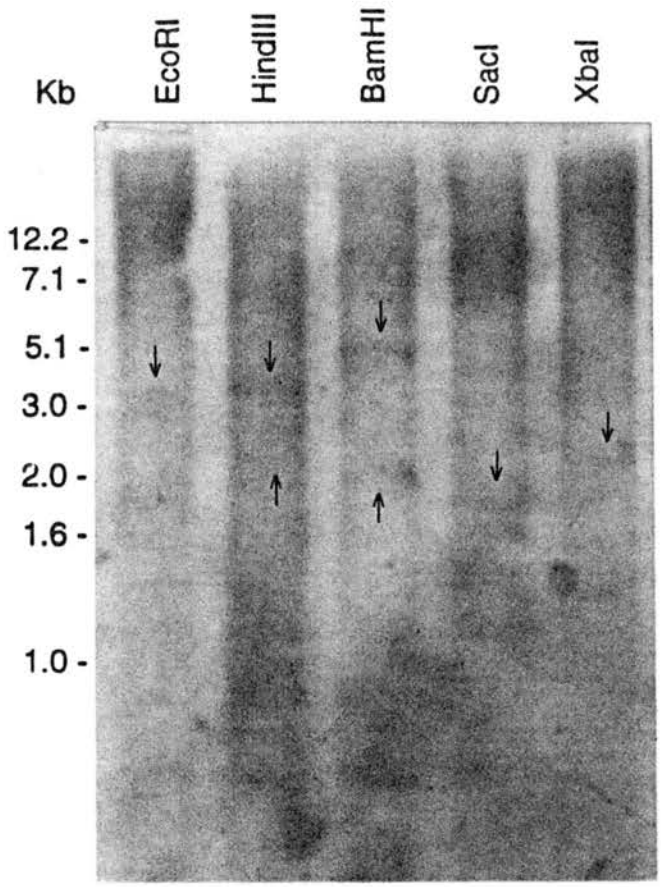


Figure 17. Genomic Southern blot analysis of the fimbrin/plastin-like gene family in wheat. Total genomic DNA isolated from wheat leaf tissue was digested with EcoRI, BamHI, HindIII, SacI, and XbaI. The DNA samples (10 μg/lane) were separated on a 0.8% agarose gel, blotted onto nylon membranes, and hybridized with a full-length digoxygenin-labeled cDNA probe. Blots were washed under high stringency conditions (0.1X SSC, 65 °C) and then exposed to X-ray film.

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