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Update on Environmental Stress

Aluminum Toxicity and Tolerance in Plants

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Aluminum (Al) is the most abundant metal in the earth's crust, comprising about 7% of its mass. Since many plant species are sensitive to micromolar concentrations of Al, the potential for soils to be Al toxic is considerable. Fortunately, most of the Al is bound by ligands or occurs in other nonphytotoxic forms such as aluminosilicates and precipitates. However, solubilization of this Al is enhanced by low pH and Al toxicity is a major factor limiting plant production on acid soils. Soil acidification can develop naturally when basic cations are leached from soils, but it can be accelerated by some farming practices and by acid rain (Kennedy, 1986). Strategies to maintain production on these soils include the application of lime to raise the soil pH and the use of plants that are tolerant of acid soils. Although Al toxicity has been identified as a problem of acid soils for over 70 years, our knowledge about the primary sites of toxicity and the chain of events that finally affects plant growth remains largely speculative. In this paper we review recent progress that has been made in our understanding of Al toxicity and the mechanisms of Al tolerance in plants.

ALUMINUM TOXICITY

The most easily recognized symptom of Al toxicity is the inhibition of root growth, and this has become a widely accepted measure of Al stress in plants. In simple nutrient solutions micromolar concentrations of Al can begin to inhibit root growth within 60 min. However, the inhibition of growth per se offers little information about the causes of stress that will either precede or coincide with changes in growth. To understand the mechanisms of Al toxicity, it is essential to identify the primary sites involved, both anatomical and metabolic, being mindful that Al could have diverse effects and act differently in different species. Several reviews on Al toxicity are available (see Haug, 1984; Taylor, 1988; Rengel, 1992a); here we limit our discussion to the sites of Al toxicity in higher plants and to the possible role of Ca in the primary mechanism of Al toxicity.

The Phytotoxic Form of Al

Part of the difficulty of studying Al-related processes in plants can be attributed to the complex chemistry of Al (Martin, 1988; Kinraide, 1991). Al hydrolyzes in solution such that the trivalent Al species, Al^{3+}, dominates in acid conditions (pH < 5), whereas the $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ species form as the pH increases. At near-neutral pH the solid phase $Al(OH)_3$, or gibbsite, occurs, whereas $Al(OH)_4^{-}$, or aluminate, dominates in alkaline conditions. Many of these monomeric Al cations bind to various organic and inorganic ligands such as PO_4^{3-}, SO_4^{2-}, F^{-}, organic acids, proteins, and lipids. Equilibrium constants are available for many of these reactions and these can be used to predict the relative concentrations of the monomeric Al species and other Al compounds in solution. A very toxic polynuclear Al species, Al_{13}, can also form when Al solutions are partially neutralized with a strong base (Parker and Bertsch, 1992), but its natural occurrence and contribution to soil toxicity are unknown.

Exchangeable Al has proved to be a poor indicator of Al-toxic soils and efforts to correlate some measure of plant growth (root length, yield, dry weight, etc.) with components of the soil solution are often hindered by the awkward chemistry of Al and the variability of soils. Many trivalent cations are toxic to plants and, because Al toxicity is largely restricted to acid conditions, it is generally assumed that Al^{3+} is the major phytotoxic species. However, this has been difficult to show and nearly all of the monomeric Al species listed above have been considered toxic in one study or another (see Kinraide, 1991). Even with simple, low-ionic-strength nutrient solutions, in which the concentrations of the various Al species can be predicted with more confidence, the conclusions can be confounded by the choice of equilibrium constants, the co-linearity between the concentration of certain Al species, the formation of $Al(OH)_3$ and Al_{13}, the duration of experiments, and the difficulty of separating effects of pH from Al speciation (Kinraide, 1991). Some researchers have considered the interaction between Al and the membranes of root cells (e.g. Grauer and Horst, 1992; Kinraide et al., 1992), and this approach makes sense because regardless of what is happening in the surrounding solution, it is this interaction that will ultimately determine the degree of stress. For example, by modeling the interaction between Al^{3+} and the negative surface potential on the membranes, Kinraide et al. (1992) found that root growth was more closely correlated with the predicted activity of Al^{3+} at the surface

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Abbreviation: $[Ca^{2+}]_c$, cytoplasmic Ca concentration.

of the plasma membrane than with the activity of Al^{3+} in the bathing solution.

Root Apices Are a Target for Al Toxicity

The root apex (root cap, meristem, and elongation zone) accumulates more Al and attracts greater physical damage than the mature root tissues (Fig. 1). Indeed, only the apical 2 to 3 mm of maize roots (root cap and meristem) need be exposed to Al for growth to be inhibited (Ryan et al., 1993). When Al is selectively applied to the elongation zone or to all of the root except the apex, growth is unaffected (Ryan et al., 1993). Bennet and Breen (1991) described a number of changes to the ultrastructure of cap cells in maize roots after a 2-h treatment with Al and suggested that Al might inhibit root growth indirectly, via a signal-response pathway involving the root cap, hormones, and secondary messengers. This was an attractive hypothesis and consistent with the known involvement of the root cap in signal perception and hormone distribution. However, it was later shown that both the onset and extent of inhibition of root growth by Al was the same in intact and decapped maize roots (Ryan et al., 1993). These results argue against a major role for the root cap in Al toxicity or tolerance but highlight the importance of the meristem.

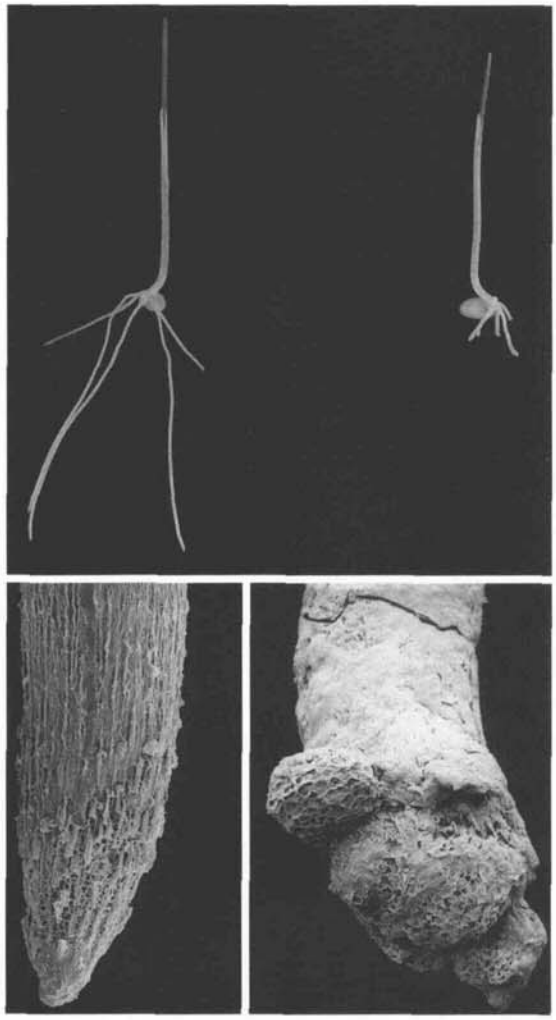

Figure 1. Effect of Al on root growth (upper panel) and structure of the root apex (lower panels) of near-isogenic wheat seedlings (Al-tolerant on left; Al-sensitive on right) that differ at the *Alt1* locus. The seedlings were grown for 4 d in a solution that contained 5 μM $AlCl_3$ in 200 μM $CaCl_2$ at pH 4.3.

Does Al Have to Enter the Symplasm to Be Toxic?

The simple answer to this question is that no one knows. Ions, and especially polyvalent ions such as Al^{3+}, are virtually insoluble in lipid bilayers, so the plasma membrane is a barrier to Al entry. Yet, not only does some Al cross the plasma membrane (probably as a neutral Al ligand, by endocytosis, through membrane-bound proteins, or via stress-related lesions), but surprisingly up to one-half of the total Al present in the root apex may be located in the symplasm (see Tice et al., 1992). We do know that root apices of Al-tolerant wheat (*Triticum aestivum* L.) accumulate less Al than Al-sensitive genotypes (see "Aluminum Tolerance"). We also know that relatively short exposures to Al (<60 min) inhibit root growth, but whether Al moves into the symplasm quickly enough or in sufficient quantity to cause this response has been difficult to determine. Reliable short-term measurements of Al influx have been hindered by the inability to resolve the symplasmic and apoplasmic fractions of Al. However, in a recent study using secondary-ion MS, Lazof et al. (1994) detected Al in the symplasm of soybean (*Glycine max*) roots after only 30 min of exposure to Al. This demonstrates that entry of Al into cells can occur before root growth is inhibited and suggests that a symplasmic site of Al toxicity is possible. Upon entering the symplasm, the prevailing pH (pH 6.5–7.5) and abundance of potential ligands will maintain the concentration of Al^{3+} ions at a very low level. There is little doubt that Al could cause considerable damage in the symplasm, even at low concentrations, due to its high binding affinities for many metabolically important molecules (Haug, 1984; Martin, 1988; Haug et al., 1994). Therefore, if Al needs to enter the symplasm to be toxic, we can surmise that the primary causes of toxicity result from the formation of an Al-ligand complex. Either Al inhibits the vital function of the ligand that binds it (e.g. enzymes, calmodulin, tubulin, ATP, GTP, DNA), or the Al-ligand complex itself poisons other metabolic processes.

Although the rate at which Al enters the symplasm is only now being measured reliably, there is no doubt that Al has easy and rapid access to the apoplasm. Interactions with the cell wall and cell membranes will necessarily precede any transport into the symplasm and many of these interactions are potentially harmful. For example, Al could bind to the pectic residues or proteins in the cell wall and decrease extensibility or hydraulic conductivity, displace other ions from critical sites on the cell wall or membranes, bind to the lipid bilayer or membrane-bound proteins to inhibit nutrient transport, or possibly disrupt intracellular metabolism from the apoplasm by triggering

secondary-messenger pathways (Haug, 1984; Taylor, 1988; Bennet and Breen, 1991; Rengel, 1992a; Haug et al., 1994). Once again, it is difficult to determine whether any of these interactions cause toxicity, and the evidence that has been presented for an apoplasmic site of toxicity is equivocal. For example, Ownby and Popham (1989) showed that the recovery of root growth after an Al treatment is enhanced by a 30-min wash in citrate, a strong chelator of Al. Because citrate is equally effective at pH 4.0 and pH 6.0 (conditions where the diffusion of citric acid into the symplasm would be very different), they concluded that the removal of Al from the apoplasm is sufficient to alleviate the inhibition of root growth. However, an alternative explanation is that the removal of apoplasmic Al reduces the transport of Al into the root cells.

A Role for Ca in Al Toxicity?

It is not surprising that an Al-Ca interaction of some kind has been implicated in Al toxicity. In early studies it was noted that the symptoms of severe Al toxicity in the field resembled Ca deficiency and that application of Ca as gypsum ($CaSO_4$) or lime ($CaCO_3$) alleviated Al stress. Although the Al-induced inhibition of root growth occurs too quickly to be explained by a systemic deficiency of Ca, other interactions between Al and Ca could cause stress. The growing awareness of Ca's pivotal role in metabolism has spurred a flurry of activity in this area, causing old ideas to be resurrected and many new ones to be proposed. The following discussion summarizes some of these studies by focusing on three such interactions: (a) inhibition of Ca uptake; (b) displacement of Ca from the apoplasm; and (c) disruption of Ca homeostasis in the cytoplasm.

Inhibition of Ca Uptake by Al

Transport of Ca into cells is energetically passive and is probably mediated by membrane-spanning channels. Many polyvalent cations (e.g. La^{3+}, Ga^{3+}, and Gd^{3+}) inhibit Ca transport, and the ability of Al to reduce Ca uptake and translocation in plants is well documented (Huang et al., 1992; Rengel, 1992a). In view of the important role of Ca in metabolism, it was reasonable to propose that Al toxicity is directly related to this antagonism. The hypothesis seemed all the more credible when it was shown that Al inhibited Ca uptake in Al-sensitive wheat lines significantly more than in Al-tolerant lines (Huang et al., 1992). Those studies established a correlation between Al toxicity and the inhibition of Ca uptake that met all the criteria for a primary cause of toxicity: the effect is measurable within minutes, it involves the root apex (the critical site for toxicity), and it is consistent with the long-term symptoms of Ca deficiency. Despite its promise, it was later shown that this hypothesis could not account for all cases of Al toxicity. In a recent study using wheat seedlings, Ryan et al. (1994) found that low concentrations of Al could inhibit root growth without inhibiting Ca uptake, and that the addition of other cations (e.g. Na, Mg) to an Al treatment improves root growth while at the same time inhibiting Ca uptake. The reverse would be expected if Al toxicity was caused by the inhibition of Ca transport. Therefore, although some concentrations of Al reduce Ca uptake and perhaps contribute to Al stress in the process, the inhibition of root growth by low concentrations of Al appears to be caused by other interactions.

Displacement of Apoplasmic Ca by Al

A large proportion of the total Ca in root tissue resides in the apoplasm, where it is required for membrane stability and normal cell development (Kauss, 1987). Al can displace apoplasmic Ca by competing for ligands (Rengel, 1992a) or by reducing the negative potential difference on the membrane surface (Kinraide et al., 1992). Therefore, an alternative to the above hypothesis is that Al toxicity is caused by the displacement of Ca from critical binding sites in the apoplasm. This hypothesis provides a rapid interaction between Al and Ca and could explain the known phytotoxicity of many other cations. However, there are some theoretical problems with the Ca-displacement hypothesis. In short-term growth studies, Mg^{2+}, Sr^{2+}, and Ca^{2+} are equally effective in alleviating Al toxicity (Kinraide et al., 1992). Indeed, many different cations (including protons and trivalent cations) alleviate Al stress by a mechanism that is independent of changes in ionic strength. These observations are inconsistent with the Ca-displacement hypothesis because rather than increasing the Ca content of the apoplasm, addition of these extra cations to an Al solution is more likely to further decrease the Ca content of the apoplasm. Furthermore, root growth does not correlate with the predicted activity of Ca at the membrane surface, as would be expected from this hypothesis (Kinraide et al., 1994). Although these arguments do not disprove the Ca-displacement hypothesis, they do emphasize the need for further work.

Cytoplasmic Ca

The resting concentration of free Ca in the cytoplasm, $[Ca^{2+}]_c$, is usually maintained at less than 200 nM, but transient increases in $[Ca^{2+}]_c$ are vital for cell growth by acting as a "secondary messenger" to initiate and regulate metabolic processes (Kauss, 1987; Coté and Crain, 1993). These transients are caused by the influx of Ca across the plasma membrane or by its release from cellular stores, and there is increasing evidence that the latter process is triggered by a pathway involving GTP-binding proteins, protein kinase C, and phosphatidylinositides (Coté and Crain, 1993). The idea that Al could disturb cellular metabolism by disrupting Ca homeostasis developed from the known antagonism between Al and Ca and from the growing awareness of Ca's role in metabolism (Haug, 1984; Rengel, 1992a, 1992b; Haug et al., 1994). Direct interactions between Al and the phosphatidylinositide transduction pathway have been reported in animal systems using permeabilized neuroblastoma cells (Haug et al., 1994), prompting the question of whether Al can inhibit Ca-dependent metabolism in plants by a similar process. By binding to the intermediates of the pathway or by triggering secondary-

messenger signals from the apoplasm, Al might disrupt Ca-dependent metabolism by maintaining higher-than-normal Ca^{2+} levels in the cytoplasm or by preventing Ca transients from occurring altogether. The evidence supporting this hypothesis is indirect at best. For instance, callose (1–3-β-glucan) synthesis in plants requires an increase in $[Ca^{2+}]_c$, and several polyvalent metal cations, including Al, are known to induce callose synthesis in roots within 30 min (Rengel, 1992a). This provides a rapid link between Al stress and changes in $[Ca^{2+}]_c$. Alternatively, Rengel (1992b) reasoned that the internal stores of Ca in cells of the root apex might be inadequate to service the normal Ca signals when influx is blocked by Al. Until the short-term effects of Al on Ca homeostasis are measured in Al-sensitive and Al-tolerant genotypes and related to root growth, the direct involvement of this interaction in Al toxicity and tolerance will remain uncertain.

ALUMINUM TOLERANCE

There is considerable variability in Al tolerance within some species and this has been useful to breeders in developing Al-tolerant cultivars of various crops, as well as to researchers studying the physiology and biochemistry of Al tolerance. Wheat has proved to be particularly useful in this respect, with up to 10-fold differences in Al tolerance between genotypes. Although some wheat cultivars possess a number of major and minor genes that encode for Al tolerance (Berzonsky, 1992), near-isogenic lines developed to differ at a single Al-tolerance locus provide simplified systems for the study of Al tolerance mechanisms (Delhaize et al., 1993a; Fig. 1). The deliberate loss of other genes in the derivation of these lines avoids the possible complication of several different mechanisms contributing to the tolerance. Much of the work on Al tolerance has focused on wheat and most of the following discussion is limited to describing recent developments in our understanding of Al tolerance in this species.

Al-Tolerant Wheat Excludes Al from Root Apices

Several independent studies provide strong evidence that Al-tolerant genotypes of wheat exclude Al from their root apices. Rincón and Gonzales (1992) showed that after exposure to Al, an Al-sensitive genotype accumulates about 8-fold more Al in the root apex (terminal 2 mm of root) than an Al-tolerant genotype, whereas no differences occur in more mature root tissue. The root apex is the critical site for Al toxicity and it is in that region that genes for Al tolerance are likely to be expressed. Similar results were observed with seedlings of near-isogenic lines that differed in tolerance at the *Alt1* locus (Delhaize et al., 1993a) and with other cultivars that differed in Al tolerance (Tice et al., 1992). These studies used a range of techniques including chemical analysis of total Al in root apices, Al-binding dyes to visualize the accumulation of Al, x-ray microanalysis, and kinetic analysis of Al uptake. Al could be excluded from root apices of Al-tolerant wheat by mechanisms that excrete ligands to chelate Al^{3+}, immobilize Al in cell walls, increase the pH around root apices to precipitate Al, or actively transport Al out of the cytoplasm (Taylor, 1991).

Efflux of Malate from Root Apices as an Al-Tolerance Mechanism in Wheat

The ability of organic acids to chelate and render Al nonphytotoxic is well established, and it has been speculated for some time that Al-tolerant plants use organic acids to detoxify Al either internally or in the rhizosphere. Miyasaka et al. (1991) provided evidence that the mechanism of Al tolerance in snapbeans (*Phaseolus vulgaris* L.) involves efflux of citric acid. More recently, Delhaize et al. (1993b) showed that Al tolerance encoded by the *Alt1* locus in wheat correlates with the efflux of malate from root apices. They suggested that the excreted malate protects the plant by chelating and detoxifying Al around that critical region of the root. Evidence that supports a role for malate in Al tolerance includes: (a) malate efflux is specifically stimulated by Al; (b) malate protects Al-sensitive wheat when added to nutrient solutions that contain phytotoxic concentrations of Al; and (c) high rates of malate efflux from roots co-segregate with the *Alt1* locus in populations segregating for Al tolerance. Furthermore, the greater release of malate from Al-tolerant roots compared with Al-sensitive roots provides an explanation for the differential effects of Al on Ca influx in these genotypes (see "Inhibition of Ca Uptake by Al"). Basu et al. (1994b) showed similar differences in malate efflux from roots of several wheat cultivars differing in Al tolerance, and Ryan et al. (1995b) screened a wide range of wheat genotypes and proposed that Al-stimulated malate efflux may be a general mechanism for Al tolerance in wheat.

Malate exists primarily as the divalent anion in the cytoplasm, and if it is transported out of the cell in this form electroneutrality must be maintained either by an equivalent uptake of anions or by an equivalent efflux of cations. Ryan et al. (1995a) provided evidence that K^+ efflux accompanies efflux of $malate^{2-}$. A local increase in pH might be expected to occur when $malate^{2-}$ is protonated in the external solution, thereby reducing the activity of Al^{3+} by a pH effect as well as through chelation by malate. However, Miyasaka et al. (1989) used microelectrodes to show that the rhizosphere pH around the apices of Al-tolerant and Al-sensitive wheat roots is similar and not greatly affected by Al treatment. Despite the correlation between Al tolerance and malate efflux, it remains to be shown that the observed fluxes of malate are sufficient to protect root apices from Al. It is not necessary that all of the Al in solution be detoxified, but rather that the Al concentration around the root apex, or possibly just at the cell plasma membranes, be reduced. Mucilage exuded by the root cap will increase the unstirred layer around the root apex, helping to maintain a malate concentration sufficient to protect the root apex (Henderson and Ownby, 1991).

A Model to Explain Al-Stimulated Efflux of Malate

Efflux of $malate^{2-}$ from the cytoplasm to the external solution is down an electrochemical gradient and could be

mediated by channels in the plasma membrane. The rapid release of malate in response to Al and its inhibition by antagonists of anion channels are consistent with the involvement of a channel (Ryan et al., 1995a). Figure 2 outlines a hypothetical scheme for such a mechanism. In this model we suggest three ways in which Al, probably as Al^{3+}, triggers the opening of a putative malate-permeable channel. 1. Al interacts directly with the channel protein, causing a change in conformation and increasing its mean open time or conductance. 2. Al interacts with a specific receptor on the membrane surface or with the membrane itself, which, through a series of secondary messages in the cytoplasm, changes channel activity. 3. Al enters the cytoplasm and alters channel activity either directly by binding with the channel or indirectly through a signal transduction pathway.

Efflux of malate from Al-tolerant root apices is associated with de novo synthesis of malate as determined by radiolabeling experiments (Basu et al., 1994b), which is consistent with data showing that the malate content of Al-tolerant root apices is turned over four times during the initial 2 h of Al exposure (Delhaize et al., 1993b). Although root apices of Al-tolerant seedlings synthesize more malate than those from Al-sensitive seedlings in response to Al, root apices of both genotypes show similar activities of PEP carboxylase and malate dehydrogenase (Ryan et al., 1995a), two enzymes important in malate synthesis. Since the root apices of Al-sensitive and Al-tolerant genotypes have the same capacity to synthesize malate, the differences in efflux probably lie in their relative abilities to transport malate across the plasma membrane in response to Al. Therefore, the *Alt1* locus could code for a malate-permeable channel that is responsive to Al or for a component of the pathway that regulates the activity of the putative channel.

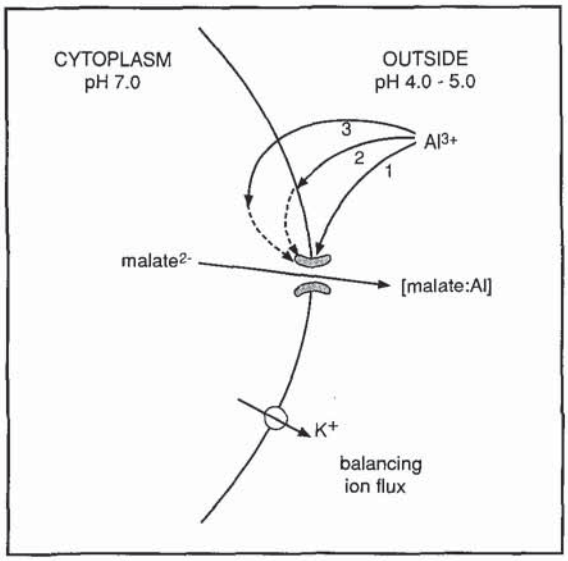

Figure 2. A hypothetical scheme showing how Al^{3+} interacts with a malate-permeable channel (hatched structure) in plasma membranes to stimulate malate efflux. The three mechanisms suggested (numbered arrows) are explained in the text. Electoneutrality is maintained by efflux of K^+.

Effects of Al on Gene Expression

Al induces the synthesis of a range of proteins in root apices of wheat, but to date definitive evidence linking these to an Al-tolerance mechanism is lacking. 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. Seven cDNAs that code for Al-induced proteins were recently cloned from roots of an Al-sensitive genotype (termed *wali* for wheat aluminum induced; Snowden and Gardner, 1993; Richards et al., 1994). The proteins encoded by a number of these cDNAs show homology to the metallothionein-like proteins of plants (*wali1*), Phe ammonia-lyase (*wali4*), proteinase inhibitors (*wali3*, *wali5*, and *wali6*), and part of plant Asn synthetases (*wali7*). Generally, 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 genotypes. In a different study, Cruz-Ortega and Ownby (1993) showed that the synthesis of an 18-kD protein is induced by Al in wheat roots and that the protein shows homology to pathogenesis-related proteins. As with the proteins encoded by the *wali* genes, the synthesis of the 18-kD protein is induced by Al and by a range of other stresses in both Al-tolerant and Al-sensitive genotypes.

Basu et al. (1994a) identified two 51-kD microsomal proteins whose synthesis is induced by Al and to a lesser extent by Cd and Ni but not by a range of other stresses. The proteins are induced in root apices of an Al-tolerant cultivar but not in an Al-sensitive cultivar, indicating a possible role in Al tolerance. Although Ryan et al. (1995a) suggested that the induction of protein synthesis by Al was not needed for the efflux of malate, other mechanisms may well require that specific proteins be induced by Al. The Al-stimulated efflux of malate may represent a major Al-tolerance mechanism in wheat, but it does not preclude the existence of other mechanisms encoded by different genes.

Other Species and Mechanisms

The possible ways of detoxifying Al are numerous and it is likely that plants have evolved many solutions to overcome the problem of Al toxicity. Species other than wheat also appear to have developed Al tolerance mechanisms based on efflux of organic acids. An Al-tolerant cultivar of snapbean excreted about 10-fold more citric acid from its roots in response to Al treatment than did an Al-sensitive cultivar (Miyasaka et al., 1991). Recently, Pellet et al. (1995) showed that an Al-tolerant genotype of maize (*Zea mays* L.) excretes severalfold more citric acid from its root apices in response to Al than an Al-sensitive genotype. Citric acid forms a strong complex with Al^{3+} and is more effective than either succinate or malate at reversing Al toxicity (Ownby and Popham, 1989). Exudation of organic acids in

response to Al might occur in a range of species but there are also likely to be Al-tolerance mechanisms based on entirely different processes. For example, some species accumulate high concentrations of Al and must possess effective mechanisms for detoxifying the Al internally. Taylor (1991) discusses in detail a range of possible mechanisms based on properties of cell walls, pH effects to reduce the concentration of Al^{3+}, compartmentation of Al, exudation of various compounds, and development of Al-resistant proteins.

FUTURE DIRECTIONS

Progress in defining the primary sites of Al toxicity requires the further development of techniques, such as secondary-ion MS, that are capable of detecting Al uptake into the apoplasm and symplasm over the short term. In addition, plant cells such as giant algal cells, in which the symplasm can be physically separated from the cell wall, might allow Al to be measured reliably in these compartments. Techniques that measure channel activity directly, such as patch clamping, are needed to verify the presence of malate-permeable channels in plasma membranes of apical root cells of wheat. Al presumably needs to interact with some component of the plasma membrane (as discussed above) to trigger malate efflux, but at the same time the membrane needs to be protected from the toxic effects of Al. Electrophysiology studies to clarify this apparent paradox are needed and could provide information on how Al triggers the efflux of malate at the biochemical level. In addition, there is a need to clone Al-tolerance genes and to identify the proteins that they encode. Although this review has focused on Al tolerance in wheat, studies on other species more amenable to molecular genetics, such as *Arabidopsis thaliana*, may facilitate the isolation of Al-tolerance genes.

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