Recent advances in aluminum toxicity and resistance in higher plants

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Aluminum toxicity is a major soil constraint to food and biomass production throughout the world. Considerable advances in the understanding of the mechanism of resistance involving exudation of organic acids have been made in recent years. However, despite intense research efforts, there are many aspects of Al toxicity that remain unclear. This article reviews the features of the chemistry of Al relevant to its toxicity followed by an examination of the mechanisms of toxicity and resistance. Emphasis, however, is given to the mechanisms of Al toxicity, since resistance has been covered recently by several reviews. Some topics which are specifically discussed in this review are: a) The possible role of cellular effects of low pH in Al toxicity, which has been largely ignored and needs to be addressed; b) The relevance of non-genotypic (cell-to-cell) variations in sensitivity to Al; c) Evidence indicating that although Al may well exert its toxic effects in the cell wall, it is highly unlikely that Al does so in a non-specific manner by mere exchangeable binding; and d) The hypothesis that the primary target of Al toxicity resides in the cell wall–plasma membrane–cytoskeleton (CW-PM-CSK) continuum has the potential to integrate and conciliate much of the apparently conflicting results in this field.
Key words: Al uptake and localization, cell wall, cytoskeleton, differential sensitivity to Al, low pH, acidity, plasma membrane.

Avanços recentes na toxicidade e resistência ao alumínio em plantas superiores: A toxicidade por Al é o principal fator limitante à produção de alimentos e biomassa no mundo. Avanços consideráveis no entendimento dos mecanismos de resistência ao Al pela exsudação de ácidos orgânicos foram obtidos nos últimos anos. No entanto, apesar da extensa literatura, muitos aspectos da toxicidade por Al permanecem obscuros. Este artigo revisa suas principais características químicas, relevantes para a manifestação de sua toxicidade, seguida por um exame dos mecanismos de toxicidade e resistência. No entanto, ênfase é dada aos mecanismos de toxicidade, já que os mecanismos de resistência já foram assunto de revisões recentes. Alguns tópicos especificamente discutidos nesta revisão são os seguintes: a) O possível papel dos efeitos celulares de pH baixo sobre a toxicidade pelo Al, o qual tem sido praticamente ignorado e que necessita ser examinado; b) A relevância de variações não genotípicas na sensibilidade ao Al; c) Evidências indicando que, apesar do Al poder exercer efeito tóxico na parede celular, é pouco provável que isso ocorra por meio de interações inespecíficas e meramente adsortivas, e d) A hipótese de que o alvo primário do Al reside no contínuo parede celular-membrana plasmática-citoesqueleto apresenta o potencial de poder integrar e conciliar grande parte dos resultados, aparentemente conflitantes, que existem nessa área.
Palavras-chave: acúmulo e localização de Al, baixo pH, acidez, citoesqueleto, membrana plasmática, parede celular, sensibilidade diferencial ao Al.

INTRODUCTION

Aluminum toxicity is the most widespread form of metal toxicity to plants and its occurrence is rivaled only by salinity. Because of its pH-dependent solubility, Al toxicity occurs only at soil pH values below 5.5 and is most severe in soils with low base saturation, poor in Ca and Mg. It is estimated that 40 % of the arable soils of the world are acidic and therefore present Al toxicity hazards (Von Uexküll and

Mutert, 1995). Even more revealing is the fact that most of these areas are located in developing countries in South America, central Africa and Southeast Asia (Wood et al., 2000), where food production can be critical. In Brazil alone, over 500 million hectares are covered by acidic soils, comprising roughly two-thirds of its total territory - the largest area of acidic soils within a single country. Soil acidity is a natural occurrence in tropical and subtropical areas, but in temperate zones, it is an increasing problem and is largely the result of acid rain in the industrial regions of the USA, Canada and Europe.

Amelioration of acid soils is complicated by the difficulty in liming subsoil layers and areas covered by perennial crops or forests, and in developing countries access to capital and machinery is frequently a further complication. The ultimate consequence is a relatively shallow root system vulnerable to drought (Foy et al., 1978). In the case of nitrogen-fixing plants, soil acidity is even more problematic since their symbiotic bacteria are also sensitive to Al and acidity (Hungria and Vargas, 2000).

Because of its unmistakable importance, understanding the mechanisms of Al toxicity and the mechanisms and genes conferring Al resistance are highly desirable and have been the focus of intense research over the past several decades. Considerable progress has been made in understanding some mechanisms and genes of Al resistance but the causes of Al toxicity are still poorly understood (Kochian et al., 2004).

Numerous hypotheses for the mechanism of Al toxicity have been advanced in the literature (Kochian, 1995; Richards et al., 1998; Barcelo and Poschenrieder, 2002). The cellular components and processes which have been proposed to be affected by Al are wide ranging and some of the most important include: cell nuclei, mitosis and cell division (Matsumoto, 2000; Silva et al., 2000), composition, physical properties and structure of the plasma membrane (Zhao et al., 1987; Wagatsuma et al., 1995; Zhang et al., 1997; Ishikawa and Wagatsuma, 1998), uptake of Ca^{2+} and other ions (Ryan and Kochian, 1993; Liu and Luan, 2001), phosphoinositide-mediated signal transduction and cytoplasmic calcium homeostasis (Haug et al., 1994; Jones and Kochian, 1995; Rengel and Zhang, 2003), oxidative stress (Boscolo et al., 2003; Yamamoto et al., 2003) cytoskeletal dynamics (Blancaflor et al., 1998; Sivaguru et al., 1999) and the cell wall - plasma membrane - cytoskeleton (CW-PM-CSK) continuum (Horst et al., 1999).

Although this review will cover general aspects related to Al toxicity and resistance, it is our intent to focus especially on the mechanisms of toxicity and to point out some aspects which have been largely ignored in this field. In particular we emphasize the possible role of cellular effects of low pH in Al toxicity, the importance of examining non-genotypic variations in sensitivity to Al and discuss evidence supporting the possibility that Al interacts with components of the CW-PM-CSK continuum. The mechanisms of Al resistance have been the focus of several recent reviews and are referred for a more detailed account (Ma and Furukawa, 2003; Samac and Tesfaye, 2003; Kochian et al., 2004).

CHEMISTRY AND BIOCHEMISTRY OF Al RELEVANT TO TOXICITY

Aluminum is the most abundant metal and the third most common element in the earth's crust. Mineral soils contain large amounts of Al, most of which is locked in aluminosilicates or Al oxides of the clay fraction and does not pose a toxicity hazard. Upon soil acidification, a fraction of this Al becomes soluble and potentially toxic to plants. Thus, acidic mineral soils are practically synonymous with Al toxicity.

Aluminum has a high ionic charge and small ionic radius, resulting in the second largest charge-to-radius ratio (z/r = 5.9). Because of this, Al strongly polarizes the water molecules in its hydration shell. Aluminum is coordinated by six water molecules in an octahedral configuration. The high degree of polarization of the O-H bond can result, depending on the pH of the medium, in the dissociation of one or more protons:

$$Al(H_2O)_6^{3+} - Al(H_2O)_5(OH)^{2+} + H^+$$

Aluminum therefore undergoes a well-known pH-dependent hydrolysis series (Orvig, 1993). For simplification, these forms of Al are represented without designating the associated water molecules, as in Al^{3+} or $Al(OH)_n^{3-n}$.

The neutral Al hydroxide species, $Al(OH)_3^0$, is the predominant form of Al at neutral and slightly acidic pH values. Thus, Al is largely insoluble under these conditions. At pH values above 7.5, $Al(OH)^{4-}$ is formed and Al is soluble again. Under conditions with a high Al/OH^- ratio, polynuclear species can form from $Al(OH)^{2+}$, of which the "Al_{13}" tridecameric polycation is probably the most important (Parker and Bertsch, 1992). In addition, hydrolysis and solubility of Al can be greatly affected by chelation. A more detailed treatment of the chemistry and speciation of Al in soils can be found in Hiradate (2004).

The general principles of the chemistry of Al relevant to its biological interactions have been established for some time, nonetheless, the complexity of its speciation along with

severe methodological limitations in its study (e.g. lack of an adequate radioisotope and limited resolution and sensitivity of X-ray microanalytical techniques) have contributed to make this a challenging field.

Despite its ubiquitous nature, Al is not known to be used by any organism. Some possible reasons for this were outlined by Williams (1999). With the exception of those cations that can undergo changes in valence, such as Fe and Co, biological systems are apparently incapable of effectively handling free trivalent cations. The two factors that apparently determine this are the small size of these cations, which places obvious limitations upon the stereochemistry of complexation and the slow ligand exchange rates of these metals (Williams, 1999).

The chemical species of Al that are toxic are presumably Al^{3+} and the mononuclear hydroxides, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ (Kinraide, 1991). Although few studies have been performed, high toxicity has also been attributed to the "Al_{13}" tridecameric polycation.

Aluminum is a highly reactive cation with a high ratio of ionic to covalent character and is thus classified as a class *a* or hard (i.e. non-polarizable) cation according to the classification scheme of Nieboer and Richardson (1980). Accordingly, Al binds preferentially to hard negative donor groups. Fluoride, the most electronegative of the anions, is the preferred inorganic monodentate ligand. However, since it is not incorporated into multidentate ligands, it is oxygen containing moieties of multidentate molecules which bind Al most intensely (Orvig, 1993). The most important ones are carboxyl (-COOH), hydroxyl (-OH), carbonyl (-CO) and phosphate (-PO_3) groups. Amines are usually important binders of Al only when part of multidentate ligands such as nitrilotriacetic acid (NTA) and ethylendiaminetetraacetic acid (EDTA) (Martin, 1992), while sulfhydryl groups do not bind Al strongly even when part of a chelate ring (Toth et al., 1984). This is a major reason why phytochelatins and metallothioneins are apparently of little importance for Al toxicity.

The size of cations, rather than charge, is the most important factor in metal ion substitution (Martin, 1988; Williams, 2002). Of the biological elements, the Al ion (r = 0.054 nm) is closest in size to Mg^{2+} (r = 0.072 nm) and Fe^{3+} (r = 0.065 nm). Aluminum can bind to nucleoside triphosphates with an association constant 10^7 times that of Mg^{2+}. Due to its small size, steric hindrance is also an important factor in determining the selectivity of Al binding. Therefore, Al is relatively more competitive in the formation of complexes with small ligands.

A final property of importance to binding is the fact that Al has a slow rate of exchange in and out of its coordination sphere (Orvig, 1993). Ligand exchange rates for Al are of the order of 1.3 s^{-1}, 10^5-fold slower than for Mg^{2+} (Martin, 1992).

After having examined the binding properties of Al, it is crucial to examine the relative concentrations of cations and ligands in biological systems and how this may determine the fate of Al. At pH 7.3, the free ion concentration of Al is limited to about 10^{-10} M, whereas typical free concentrations of Mg, Ca and Fe in a plant cytoplasm are around 10^{-3}, 10^{-7}, and 10^{-17} M, respectively (adapted from Williams, 2002). Therefore, in the cytoplasm, binding of Al to ligands can become limited due to competition with other cations. However, it is important to realize that pH is critical in determining the competitiveness of Al for ligands when compared to other cations such as Mg and Ca, which do not alter their solubility with pH.

BIOLOGY AND MECHANISMS OF ALUMINUM TOXICITY

General effects and symptoms of Al toxicity in plants

The most prominent symptom of Al toxicity is inhibition of root growth, which can usually be detected within 30 min to 2 hrs, even at micromolar concentrations of Al (Barcelo and Poschenrieder, 2002). However, the mechanisms of this inhibition are not well understood. Aluminum-injured roots become stubby and frequently acquire a brownish coloration. Fine branching and root hairs are reduced and the root system often takes on a "corraloid" appearance. In the root apex, cracks can easily be observed in the epidermis. Uneven and radial expansion of cells of the cortex cause root thickening and mechanical stress on the epidermis (Ciamporova, 2002).

Cells which have been reported to be affected by Al are the root cap, meristem, elongating cells, root hairs and branch initials (Foy et al., 1978; Rengel, 1996). Root tips are the most Al-sensitive region, as has been demonstrated by exposing only certain regions of the root to Al (Ryan et al., 1993). In a more detailed examination, the distal region of the transition zone (DTZ) was shown to be the most Al-sensitive root apical region (Sivaguru and Horst, 1998). Inhibition of root growth is considered to be primarily the result of inhibited cell elongation, at least in early stages of toxicity, while reduced cell division can obviously affect growth in later stages (Kochian, 1995; Barcelo and Poschenrieder, 2002; Ciamporova, 2002).

Although symptoms of Al toxicity are also manifested in the shoots, these are usually regarded as a consequence of injuries to the root system. The most common responses in shoots to Al toxicity are cellular and ultrastructural modifications in leaves, reduced stomatal opening, decreased photosynthetic activity, chlorosis and foliar necrosis. Long-term exposure to Al and inhibition of root growth generally lead to nutrient deficiencies, mainly of P, K, Ca and Mg (Haug and Vitorello, 1996). The ultimate consequence is reduced plant biomass. With the exception of Al-accumulating plants (e.g. tea plants and hydrangea) (Jansen et al., 2002; Watanabe and Osaki, 2002), little Al is transported into the shoot.

Major factors affecting severity of Al toxicity to roots are the concentrations of Ca^{2+} and other cations in the external solution, the ionic strength of solutions, temperature, the presence of chelators, cell type and plant genotype (Foy et al., 1978; Kinraide and Parker, 1987).

Interactions with low pH – a case of superimposed stresses

The solubility of Al is appreciable only at pH values below 5.5. Thus, toxicity to plants occurs only at these low pH values, with the possible exception of the toxicity of $Al(OH)_3^{4-}$ at higher pH values (Kinraide, 1990). Despite this, and in contrast to the large amount of literature on Al toxicity, very little attention has been given to H^+ toxicity, even though the latter is well known to be directly detrimental to root growth (Kidd and Proctor, 2001; Koyama et al., 2001). As in Al toxicity, H^+ toxicity is most severe in solutions of low ionic strength and low cation concentrations, and increasing the concentration of Ca^{2+} and other cations in the external solution reduces or even abolishes the detrimental effects of acidity (Marschner, 1991). An evaluation of these low-pH effects is necessary for greater understanding and correct interpretation in studies of Al toxicity, but this is rarely undertaken (Lazof and Holland, 1999; Samac and Tesfaye, 2003).

At the cellular level, low pH has detrimental and distinct effects on the plasma membrane, notably enhanced permeability (Zsoldos and Erdei, 1981; Yan et al., 1992; Koyama et al., 2001). These membrane effects are in part responsible for altered patterns of nutrient accumulation at low pH (Marschner, 1991). Although K^+ permeates the plasma membrane more readily at low pH, enhancement of efflux is greater, resulting in reduced net uptake (Zsoldos and Erdei, 1981). Low pH-induced membrane permeability can be alleviated by Ca^{2+} and other cations (Marschner, 1991; Kinraide, 1998). Several studies have indicated that Al-induced stimulation of root growth results from amelioration of proton toxicity and consequent reduction in membrane permeability (Kinraide, 1993; Llugany et al., 1995).

Aluminum toxicity is usually evaluated by comparing elongation of roots exposed to Al at a low pH to controls without Al but at the same low pH. There are potential problems in this approach, both for screening of Al-resistant plants and in Al toxicity studies (Lazof and Holland, 1999; Samac and Tesfaye, 2003). As pointed out by Lazof and Holland (1999), Al-resistance may be underestimated in plants sensitive to H^+, since they may show little further inhibition to Al, or it may be overestimated in plants showing Al growth-enhancement due to alleviation of H^+ toxicity. Obviously, the outcome of results and evaluations depends on several factors such as pH, the activity of Al and the sensitivity of the biological specimen. Limited evidence suggests that plants can be adapted to H^+ or Al independently (Lazof and Holland, 1999; Kidd and Proctor, 2001), but more studies are clearly needed.

Nevertheless, there are several reasons, based both on experimental evidence and theoretical considerations, which suggest that an interaction between Al toxicity and the effects of low pH is likely. Taking this a step further, certain biological effects of high H^+ concentrations may actually play a role in the establishment of Al toxicity.

First, low pH clearly affects the structure of plasma membranes. As already mentioned, one of the most common observations is increased membrane permeability, as assessed by increased solute leakage (e.g. Koyama et al., 2001). This may have profound consequences for Al toxicity, particularly regarding access to possible target sites, including the plasma membrane itself. Alterations in the structure of the plasma membrane appear to be an important factor in determining the sensitivity and uptake of Al by roots and cells (Sasaki et al., 1994; Wagatsuma et al., 1995; Vitorello and Haug, 1996; Ishikawa and Wagatsuma, 1998; Ishikawa et al., 2001; Ofei-Manu et al., 2001). In some of these studies, however, it is not clear whether these alterations were brought about by low pH or Al. In two of these studies, increased permeability of the plasma membrane was shown to be caused by low pH.

Low pH may also induce changes in the conformation of key molecules in the CW – PM - CSK continuum and which could be crucial in determining toxicity of Al. This would be somewhat analogous to a current view of Alzheimer's disease, where aberrant forms of proteins appear on the exterior surface of cells where they should not be (Williams, 1999).

A correlation between H^+ and Al tolerance was observed by Ofei-Manu et al. (2001) and a correlation between membrane permeability (K^+ efflux) and Al uptake has been observed in wheat (Sasaki et al., 1994; Souza, 1999).

Second, extracellular acidity increases the permeability of the plasma membrane to H^+ (Yan et al., 1998) and can lower intracellular pH (Plieth et al., 1999; Moseyko and Feldman, 2001). It is conceivable and perhaps even probable that these pH changes are greater in the cortical cytoplasm. In turn, lowering of intracellular pH can dramatically increase the concentrations of Al relative to other cations, thereby increasing its competiveness for cellular ligands. The structure and the binding constants of cellular ligands may also be affected.

Third, there are several commonalities between Al and H+ toxicity. Both are influenced by the ionic strength of the solution and both are alleviated by increasing concentration of cations, in the following order $M^{3+} > M^{2+} > M^+$ (Kinraide, 1993), although this may be a general property of cation toxicity. More importantly, the regions of the root which are sensitive to H^+ and Al are similar (Koyama et al., 1995, 2001) and in tobacco cell suspensions the log phase of growth is sensitive to both Al and low pH while the stationary phase is not (Vitorello and Haug, 1996). In both Al and H+ toxicities, there is a relationship between cellular growth rates and toxicity (Vitorello and Haug, 1996; Koyama et al., 2001). Finally, in both cases boron can alleviate toxicity and pectin appears to play a role in the detrimental effects of both ions (Schmohl and Horst, 2000; Koyama et al., 2001).

Al uptake and localization in plants and cells

This is an important topic since it is generally accepted and there is considerable evidence, both in roots and cell suspensions, that Al uptake is necessary for the manifestation of toxicity (Yamamoto et al., 1994; Kochian, 1995; Horst et al., 1999; Matsumoto, 2000), although apparent exceptions have been reported (Larsen et al., 1996). Therefore, localization of Al may provide information on mechanisms of toxicity.

Uptake and distribution of Al at the whole plant and root level: In most plant species, especially Al-sensitive and crop species, Al uptake is limited mainly to the root system, where it accumulates predominantly in the epidermis and in the outer cortex (Wagatsuma et al., 1987; Delhaize et al., 1993; Matsumoto et al., 1996). The endodermis possibly acts as a barrier and transport to the shoot and leaves is generally small.

However, there are many plant species that accumulate Al to a considerable extent in the shoot (Jansen et al., 2002; Watanabe and Osaki, 2002). Such plants, frequently called hyperaccumulators, are mainly woody plants from tropical or subtropical regions, such as some species native to the savannah (cerrado) region of central Brazil (Haridasan et al., 1986; Haridasan and Dearaujo, 1988). Classic examples of hyperaccumulators are the tea plant (*Camellia sinensis*), hydrangea and members of the Rubiaceae family. Unfortunately, there is not much information in the literature as to mechanisms, cellular localization and chemical forms of the Al which accumulates in these plants. In one investigation on the chemical form of Al in tea leaves, most Al was chelated to the catechin group of polyphenols, and to a lesser extent to phenolic and organic acids and as Al-F complexes (Nagata et al., 1992). In hydrangea leaves, Al was found as a complex with citrate (Ma et al., 1997) and in the hyperaccumulator *Melastoma malabathricum* it was bound to oxalate (Watanabe et al., 1998). Even less is known about the Al species formed after absorption from the soil but there is evidence that Al may be transported as Al-F species (Nagata et al., 1993).

Subcellular localization of Al: Whether Al accumulates and manifests its toxicity within the plant cell or externally, in the apoplast, has been a major topic of interest and controversy because of its implication to models of Al toxicity (Delhaize and Ryan, 1995; Kochian, 1995; Haug and Vitorello, 1996). In recent years, however, this debate seems to have subsided, perhaps because more attention has turned to the topic of Al resistance.

Aluminum has been found in the nucleus, presumably bound to DNA (Matsumoto et al., 1976), while it has been reported by others to be localized solely or predominantly in the cell wall (Marienfeld and Stelzer, 1993; Ownby, 1993; Marienfeld et al., 1995). This ambiguity is possibly in part due to differing experimental conditions, some of which may have been inadvertently flawed (Kochian, 1995). Earlier reports employed long periods of exposure to Al, which raises questions as to the integrity of the cells, and/or high concentrations of Al, which in turn raises questions as to precipitation of Al in the cell wall.

Major limitations to resolve these issues of subcellular localization have been largely methodological. Reasons for this are the complex chemistry of Al and interactions with the cell wall, the lack of a suitable Al radioisotope (Rengel, 1996) and the limited sensitivity and resolution of microanalytical

techniques, viz. X-ray microanalysis (Lazof et al., 1994). The latter is relatively poor because of the interactions of the radiation with the specimen layer and distortions in the cell samples during preparation. Finally, the effectiveness of chelator (citrate or EDTA) washes in removing Al from the cell wall has been a point of discussion (Rengel, 1996).

Attempts to circumvent these limitations and to address the problem of Al uptake and its cellular site of accumulation have been based on various strategies. One approach, based on the kinetics of short-term Al uptake, attempted to distinguish cell wall Al from uptake across the plasma membrane based on the biphasic behavior of Al uptake (Pettersson and Bergman, 1989; Zhang and Taylor, 1989; Zhang and Taylor, 1990). The rapid, initial, non-linear phase of uptake was interpreted as being accumulation of exchangeable Al in the cell wall, and could be desorbed with citrate (Zhang and Taylor, 1990). The slower, second phase of uptake was linear and interpreted to be uptake across the plasma membrane (Pettersson and Bergman, 1989) or metabolism-dependent accumulation in the cell wall (Zhang and Taylor, 1990).

A second approach has employed a relatively new and sophisticated microanalytical technique, secondary-ion mass spectrometry, to detect Al in root tips exposed to Al. This method was estimated to have a spatial resolution of about 2 μm, which is perhaps one order of magnitude better than X-ray microanalysis (Lazof et al., 1994).

A third approach has made use of dyes which present enhanced fluorescence upon binding to Al, such as morin (Vitorello and Haug, 1997) and lumogallion (Silva et al., 2000). This approach probably offers the best resolution to date for localization of Al, it is amenable to confocal microscopy, it is simple to perform and little specimen preparation is required or even in vivo observations in the case of morin are possible (Vitorello and Haug, 1996). The major disadvantage of this approach may be the fact that it depends on complex formation with Al and thus competition with other ligands may affect results. Despite this, in the case of morin, good correlations between fluorescence signals and graphite furnace atomic absorption spectrometry (GFAAS) have been encountered (Vitorello and Haug, 1997).

Finally, a fourth approach has employed the giant alga *Chara corallina*. In these cells, separation of cell wall and symplasm can be surgically performed and therefore uptake across the plasma membrane can be assessed (Taylor et al., 2000).

In general, the various studies have made the case that Al can indeed enter the cytoplasm. However, whether this leads to toxicity is not clear, especially in view of recent studies showing a good relationship between Al binding to pectin and inhibition of cell elongation (Schmohl and Horst, 2000). As discussed below, an intermediate scenario may be more likely, where Al accumulates and binds to components of the CW-PM-CSK continuum.

It is clear that Al can indeed accumulate in the nucleus (Silva et al., 2000), even at low Al concentrations and short exposure periods. However, it must be shown that this occurs in cells before loss of viability or in which the intracellular pH has not decreased (see above section on interaction with low pH).

In the case of the hypothesis of binding to the cell wall, the mere electrostatic binding of Al to the cell wall is unlikely to be a significant mechanism of toxicity. First, several studies show a relationship between non-exchangeable Al in the cell and toxicity (e.g. Archambault et al., 1997), and it is this form of cell-associated Al which is considered in most studies of Al uptake since a chelator wash is usually performed at the end of the Al-exposure period. If Al is prevented from accumulating in cells by different chelators, it should be possible to remove Al which is complexed to the surface by washes with the same chelators. Second, several studies and observations show that Al does not accumulate in isolated cell walls in a non-exchangeable manner (Zheng et al., 2004). Indeed, Al is not observed to accumulate in the cell walls of plasmolyzed cells (Vitorello and Haug, 1996). An associated protoplasm is necessary for this to occur. Work by Zhang and Taylor (1991) is a very good example of the latter. In other words, cell-mediated (i.e. protoplasm-mediated) processes must occur for Al to accumulate in a non-exchangeable manner, including Al in the cell wall. This could be, for example, the synthesis of new cell wall material, where Al might become occluded from exchange processes.

Third, there are a number of studies and evidence indicating a role of the plasma membrane in Al uptake. Increased permeability of the plasma membrane at low pH has been correlated with Al uptake (Ishikawa et al., 2001; Ofei-Manu et al., 2001) and decreased cell turgor decreases Al uptake (Vitorello and Haug, 1996). Metabolic inhibitors, such as 2,4-dinitrophenol (DNP) or m-chlorocarbonylcyanie-phenyl hydrazone (CCCP), increase Al uptake (Wagatsuma, 1983; Zhang and Taylor, 1991; Rincon and Gonzales, 1992). This was initially proposed to be due to an energy-dependent exclusion mechanism but it could also be due to increased membrane permeability (Taylor et al., 2000).

Non-genotypic variations in cellular sensitivity to Al

An important but overlooked and underexplored realization is that cells of a same individual, organ or tissue differ in their sensitivity to Al depending on their developmental or cellular state. This is true for both plant and mammalian cells. In several cases, differences in sensitivity can be quite large even without obvious cellular changes. Thus, there are important non-genotypic factors which determine sensitivity to Al.

In plants, this has been known for some time, since symptoms are manifested mostly in root tips, however only recently has this been more appreciated and actually demonstrated experimentally (Ryan et al., 1993). Such examinations have been furthered and an important study is that of Sivaguru and Horst (1998) which established the distal part of the transition zone (DTZ) as the most Al-sensitive region of the root. Thus, sensitivity of cells to Al changes as cells transit through the root tip. Transversal differences in sensitivity probably exist likewise, the epidermis being the most sensitive (Ciamporova, 2000; Ciamporova, 2002) but this has not yet been clearly established, especially since apparent differences may be due to differences in proximity to the external Al-containing solution. Root hairs and neighboring epidermal cells also show distinct differences in sensitivity to Al (Jones et al., 1995).

Differences in the effects of Al on cells within roots were reviewed by Ciamporova (2002). In this review, Al uptake was found to differ between cell types and was largely responsible for the different effects of Al on these cells. Epidermal and cortical cells were mostly shorter and wider than the cells in control roots and within the root cortex, individual cells or a few cells of a file had severely damaged cytoplasm, in contrast to almost undisturbed cytoplasm of adjacent cells (Ciamporova, 2000). Root hairs are generally very sensitive to Al, but the degree of severity depends greatly on its physiological activity (Care, 1995; Jones et al., 1998). Root hairs which had completed their elongation were less sensitive to Al (Sattelmacher et al., 1993).

More recently, differences in Al sensitivity between tap and basal roots of common bean seedlings (Shen et al., 2004) and seminal and crown roots of rice nursery seedlings (Nagasaka et al., 2003) were examined. These differences were suggested to be due to exudation of organic acids, but this has not been demonstrated. It should be noted that differences in sensitivity to Al along the root axis cannot be explained by differences in exudation of organic acids, since exudation is most intense in the root apex, the most Al-sensitive region of the root (Delhaize et al., 1993; Pellet et al., 1995; Mariano and Keltjens, 2003), or at least not enough to justify the large differences in sensitivity between the tip and the rest of the root. Therefore, exudation of organic acids may explain differences in Al resistance between genotypes, but probably not between cell types of the same root.

The importance of these observations is that such cell status-dependent changes in Al sensitivity offer new opportunities to examine the mechanisms of Al toxicity and resistance, and may represent a new or a shift in paradigm for Al toxicity research. So far, this approach seems to have been largely underexplored. Obviously, examining these differences may be somewhat difficult considering the complexity and heterogeneous nature of these tissues (both longitudinally and transversally), in which sensitive and resistant cells may be mixed among each other. The feasibility of this approach will depend either on advances in techniques for the study of single (or few) cells or on the use of alternative experimental systems.

Differences in sensitivity to Al can also be found in plant cell cultures and may offer such an alternative system to the root. Tobacco cells in the log-phase of growth are Al-sensitive but cells in the stationary phase are not (Yamamoto et al., 1994; Vitorello and Haug, 1996; Sivaguru et al., 1999). It is also possible to alter Al sensitivity by manipulating plant cells. Cultured cells in the log phase of growth acquire Al resistance when submitted to phosphorus starvation (Yamamoto et al., 1996) or when inorganic salts are removed from the growth medium (Vitorello and Haug, 1999). Sensitivity to Al was also found to be modulated by manipulating the pectin content and pectin methylesterase activity in *Zea mays* and *Solanum tuberosum* cell cultures (Schmohl and Horst, 2000; Schmohl et al., 2000). Differences in sensitivity can also be found between mammalian cells. Undifferentiated and differentiated human neuroblastoma cells showed marked difference in Al sensitivity (Verstraeten et al., 2002).

Several reports have now established a general relationship between cellular growth and expansion and sensitivity to Al (Vitorello and Haug, 1996; Sivaguru et al., 1999) or present data that are suggestive of this (Chang et al., 1999; Vazquez et al., 1999). It is interesting to note that some of the cell types mentioned above as being sensitive to Al have high relative growth rates. Ivanov (1997) reported that cells of the DTZ have the highest relative growth rates along the root axis. Likewise, root hairs are among the fastest elongating plant cells. It is also interesting to note that localized changes

in apoplastic and cytoplasmic pH, which can be decisive for Al toxicity, are frequently observed and associated with cellular growth process, such as root hair development in *Arabidopsis thaliana* (Bibikova and Gilroy, 2002).

The relationship to cell growth and Al-sensitivity makes sense in light of some current views of the mechanisms of Al toxicity, as discussed below, but also brings about important implications for the interpretation of results in this field, particularly evaluation of Al resistance.

Primary mechanisms of Al toxicity

The search for the primary target(s) of Al injury and thus a complete understanding of the mechanisms of Al toxicity is an important area in Al toxicity studies, but this aspect still remains elusive. Many hypotheses have been advanced, but they all need to be supported by more convincing evidence. Part of the problem may be that it is possible or even likely that Al may have more than one primary target. It is difficult to adequately cover all possible mechanisms in a single review. Nonetheless, hypotheses on the mechanisms of Al toxicity can be roughly divided into those affecting phosphate and/or nucleotide metabolism, cell wall structure and function, membrane structure and function, membrane transporters, cytoskeletal dynamics, signal transduction and oxidative stress.

Aluminum is capable of binding tightly to DNA, presumably to its phosphate backbone, or alternatively to associated histones (Matsumoto, 1991) and this led to one of the earlier hypotheses of toxicity, that cell division was impaired because of interactions of Al with nuclear DNA. Aluminum also has a high binding affinity to free nucleotide triphosphates and a model for Al toxicity based on its binding to ATP in the cytoplasm was proposed (Pettersson and Bergman, 1989).

Aluminum has also been shown to alter the structure of the plasma membrane (Zhao et al., 1987) and has pronounced effects on ion fluxes across the membrane, particularly Ca^{2+} uptake (Liu and Luan, 2001). Aluminum was also shown to affect membrane physical properties in Neuroblastoma cells (Verstraeten et al., 2002).

Aluminum toxicity has frequently been linked to Ca^{2+} (Rengel, 1992; Rengel and Zhang, 2003) either because of Al-induced perturbations in cellular Ca^{2+} metabolism or because of Ca^{2+} amelioration of Al toxicity (Kinraide and Parker, 1987). Several investigations found Al-induced alterations in the structure of calmodulin, the chief mediator of intracelular Ca^{2+} signaling and initiated considerable research on the role of calmodulin in Al toxicity (Haug and Vitorello, 1996). More recently, phosphoinositide-mediated signal transduction, a pathway which also involves Ca^{2+} as an intracellular messenger, has been investigated as a primary site of Al toxicity in mammalian (Haug et al., 1994) and plant cells (Jones and Kochian, 1995; Jones et al., 1995). In both cases Al treatment presumably inhibited the activity of phospholipase C or possibly the action of the trimeric G-protein.

Considerable evidence has emerged in the literature in recent years, both in plants and animals, that Al promotes oxidative stress in cells, although perhaps certain conditions are required for this to occur. In plants, evidence for this includes the promotion of lipid peroxidation (Cakmak and Horst, 1991; Yamamoto et al., 2001), the expression of oxidative stress genes (Richards et al., 1998; Milla et al., 2002) and the amelioration of Al toxicity in plants transformed with oxidative stress genes (Ezaki et al., 2000).

However, whether Al-induced oxidative stress is a primary or secondary effect is still a matter of debate and continued investigation. Although lipid peroxidation has been frequently observed (Ono et al., 1995; Peixoto et al., 2001) and is an early symptom, Yamamoto et al. (2001) argued that it was not a primary cause of Al toxicity.

Although oxidative stress is well known to be induced by heavy metals, the pro-oxidant activity of Al, a non-redox-active metal, is intriguing. The mechanisms of this pro-oxidant activity have been reviewed by Zatta (2002) and Exley (2004). Since Al cannot induce oxidative stress directly, it must do so by its influence on the substrates of oxidation, such as membrane lipids, on other pro-oxidants, such as iron, or on the oxidant itself, such as the superoxide radical anion (Exley, 2004). It is not yet known which of these mechanisms operate in cells.

In the plant literature, Al-induced oxidative stress has been most commonly attributed to alterations in membrane structure by Al, which would then favor radical chain reactions mediated by Fe ion (Yamamoto et al., 2002). This is not surprising, given that Al is known to affect membrane structure and the presence of iron has been shown to increase membrane peroxidation induced by Al (Ono et al., 1995; Yamamoto et al., 1997). The effects of Al on the antioxidant system of the cell cannot be dismissed, and such studies are emerging in the literature (Devi et al., 2003; Guo et al., 2004), however, it must be kept in mind that activation of the cellular antioxidant system is a general stress response and may not be specific to Al toxicity.

One of the most interesting hypotheses is that the primary site of Al toxicity resides in the CW-PM-CSK continuum (Horst et al., 1999). This hypothesis is attractive because it is perhaps the only one with the potential to integrate and conciliate much of the apparently diverse and conflicting information which has accumulated on Al toxicity. Some of the reasons which favor this hypothesis are listed:

a) The current view is that the cell wall, plasma membrane and cytoskeleton are interconnected, in a manner which must still be fully elucidated, and that perturbations in one component may have profound effects on another. This may possibly accommodate the fact that there is very good evidence that Al interacts with the cell wall (Schmohl and Horst, 2000) the plasma membrane (Ishikawa and Wagatsuma, 1998) and cytoskeleton (Blancaflor et al., 1998; Sivaguru et al., 1999);

b) This hypothesis can conciliate the localization of Al at the periphery of the cell (Vitorello and Haug, 1996) with the involvement of the plasma membrane and requirement of a protoplast for the non-exchangeable uptake of Al;

c) It may also be capable of explaining the general relationship between growth rates and sensitivity to Al and also why certain cell types are more sensitive to Al, such as cells of the DTZ and root hairs.

However, the exact nature of this target is not known. Nonetheless, it would be expected to be a component that is interconnected with other components of the continuum. Unfortunately, our knowledge of this system is still poor but its components and workings are quickly emerging (Baluska et al., 2003). There are probably plenty of potential targets for Al in this system. It is at least curious that the expression of a cell wall-associated receptor kinase (WAK) was reported to be induced by Al (Sivaguru et al., 2003).

Cellular responses to Al toxicity and Al-induced genes

How do plant cells respond to Al exposure? Responses at the cellular level are wide-ranging. In principle, the more immediate the response, the more likely that it is related to the primary mechanism of toxicity or to a mechanism of resistance, thus the interest in understanding cellular responses to Al toxicity. However, the distinction between the effects of toxicity and the cellular response triggered by toxicity are not always clear. To illustrate, are changes in levels of free intracellular calcium a result of toxicity or are they the beginning of a cellular response?

One well-known Al-induced cell response is the synthesis of callose which is frequently used as an indicator for Al-induced stress (Horst et al., 1997). But it is not generally considered very specific nor do all cells synthesize this compound in response to Al.

Changes in gene expression can be, in general, regarded as a cellular response rather than an effect, although in principle that may not actually be the case. Information on Al-induced gene expression may allow the understanding of mechanisms of toxicity and resistance. Among the first studies to examine Al-induced genes are those of Gardner (Snowden and Gardner, 1993).

Al induces the synthesis of several proteins (Basu et al., 1994) and also the expression of several genes (Snowden and Gardner, 1993; Snowden et al., 1995). Some of these proteins have been identified and include phenylalanine ammonia-lyase, metallothionein-like proteins, proteinase inhibitors and asparagine synthetases (Snowden et al., 1995). Thus, in general, the proteins synthesized and the genes expressed in response to Al appear to be general stress- or wound-response proteins and genes (Sugimoto et al., 2004). But there are also other genes which may be induced by Al and which are promising for resistance, particularly transporters for organic acids (Sasaki et al., 2004).

Perhaps the most comprehensive analysis of Al-induced gene expression was performed by Milla et al. (2002) which expanded considerably on the 45 genes previously identified and reported to be regulated by Al. This work confirms many aspects of earlier studies, such the involvement of oxidative stress genes (Richards et al., 1998) but also confirms more recent aspects. Of particular importance is the discovery of the effect of Al on genes involved with cell division and elongation genes, oxidative stress and iron metabolism.

ALUMINUM RESISTANCE

It has been known for a long time that many plant species, including crop plants, show wide variability with respect to their resistance to Al, and this has been exploited to obtain Al-resistant varieties. The understanding of the mechanisms and genetics of Al resistance has advanced considerably over the last decade and traditional screening and breeding programs have resulted in considerable success over the years. Because of the large areas of acid soils and the importance of this constraint in Brazil, the collection of acid-soil resistant Brazilian plant varieties are among some of the best in the world.

One problem, however, is how to evaluate Al resistance. The most widely used methods use relative root growth in Al at low pH compared to growth in low pH alone. However,

as discussed above there are several problems associated with this approach (Lazof and Holland, 1999). Aluminum resistance studies should take the detrimental effect of acidity into consideration and appropriate controls designed. Screening methods for identifying Al-resistant plants and their inherent limitations were reviewed by Samac and Tesfaye (2003). They raise the point that slower growing plants tend to be selected as Al-resistant (DallAgnol et al., 1996), which is consistent with the previously mentioned link between cellular sensitivity to Al and rate of growth.

These observations raise questions as to the correlation between adaptation to soils with high levels of Al and screening for Al resistance in nutrient solutions. Thus, knowledge on the mechanisms of Al toxicity is also important because it can contribute to the development of more accurate screening procedures via improved criteria for the determination of resistance and also for the development of Al-resistant plants.

Genetics and inheritance of Al resistance

The inheritance and genetics of Al resistance has been examined mostly in cereals of the Triticeae. From these studies it was found that in some species, such as in wheat or rye, that Al resistance is determined by one or a few genes (Berzonsky, 1992), whereas in other species such as rice or maize it is multigenic and quantitative (Kochian et al., 2004). However, there is an increasing awareness that Al resistance is more likely a multigenic trait.

Until recently, no Al-resistance gene had been cloned. However, Sasaki et al. (2004) cloned a gene with properties of an Al-induced channel and subsequently transformed barley and obtained high levels of resistance (Delhaize et al., 2004). This may be the first Al resistance gene to have been cloned.

Cellular mechanisms of Al resistance

The mechanisms for resistance, like those for toxicity, are not entirely known, but at least one mechanism, the secretion of organic acids, is now reasonably well established and understood (Kochian et al., 2004). Good evidence for this mechanism initially came from three independent groups that demonstrated that malate-secretion is enhanced in Al-resistant cultivars compared to Al-sensitive ones (Delhaize et al., 1993; Basu et al., 1994; Pellet et al., 1995).

Several studies have attempted to overexpress either citrate synthase or malate dehydrogenase, with the intended purpose of increasing organic acid exudation (de la Fuente et al., 1997; Koyama et al., 2000; Tesfaye et al., 2001; Anoop et al., 2003), or general stress genes (Ezaki et al., 2000; Basu et al., 2001). However, the increases in Al resistance have been modest or results have not been reproducible (Delhaize et al., 2001). The latter is not entirely surprising given that it would not be expected that a single enzyme should change the levels of highly regulated metabolites such as organic acids. From this, it follows that if Al resistance is indeed determined by organic acid exudation then it is not surprising that multiple genes are involved.

More recently, barley plants transformed with a gene (*ALMT1*) encoding a putative malate transporter were found to be more resistant to Al (Delhaize et al., 2004). This perhaps makes more sense, given that this could increase exudation without necessarily changing cytoplasmic metabolite concentrations.

However, the results presented above must be contrasted with the fact that transformation of plants with several different general stress genes can also confer Al resistance to these plants, some of which have no obvious relation to any mechanism of Al resistance (Pellet et al., 1996; Pellet et al., 1997; Ezaki et al., 2000; Tesfaye et al., 2001; Ezaki et al., 2004). One fact that must be looked at carefully is the relation between rate of cell growth and Al toxicity and the fact that most if not all of these genes could be expected to affect cell growth rates since organic acids are important in metabolism

Despite the large number of studies in support of an organic acid mechanism of resistance, this issue is probably far from over. There are several observations that do not fit the model. The most important is that several Al-sensitive plants have high levels of organic acid secretion (Kochian et al., 2004). Rice plants also did not show increased Al resistance, despite increased organic acid efflux (Sasaki et al., 2004). How good is the correlation between organic acid exudation and resistance to Al? How well has it been quantified? These are important questions which have been discussed by Mariano and Keltjens (2003). There is of course, the case in which there are Al-resistant plants which do not show enhanced organic acid exudation, such as Signal grass (*Brachiaria decumbens*) for example (Wenzl et al., 2001). Similar results have also been found in some soybean cultivars (Nian et al., 2004). Therefore, there is clearly evidence for the existence of other resistance mechanisms also.

Unfortunately, the mechanisms of Al resistance in species native to acid soils are much less studied. Such species are

commonly divided into Al excluders and accumulators. The accumulation of Al, and thus, internal mechanisms of resistance have received more attention (Watanabe and Osaki, 2002). Exclusion mechanisms of plants native to acid-soil regions are largely unknown, although CIAT is undertaking an effort to examine this in *Brachiaria* species (Ishitani et al., 2004).

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