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REVIEW: PART OF A HIGHLIGHT SECTION ON PLANT–SOIL INTERACTIONS AT LOW PH

The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review

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• *Background* Aluminium (Al) toxicity is the most important soil constraint for plant growth and development in acid soils. The mechanism of Al-induced inhibition of root elongation is still not well understood, and it is a matter of debate whether the primary lesions of Al toxicity are apoplastic or symplastic.
• *Scope* The present review focuses on the role of the apoplast in Al toxicity and resistance, summarizing evidence from our own experimental work and other evidence published since 1995.
• *Conclusions* The binding of Al in the cell wall particularly to the pectic matrix and to the apoplastic face of the plasma membrane in the most Al-sensitive root zone of the root apex thus impairing apoplastic and symplastic cell functions is a major factor leading to Al-induced inhibition of root elongation. Although symplastic lesions of Al toxicity cannot be excluded, the protection of the root apoplast appears to be a prerequisite for Al resistance in both Al-tolerant and Al-accumulating plant species. In many plant species the release of organic acid anions complexing Al, thus protecting the root apoplast from Al binding, is a most important Al resistance mechanism. However, there is increasing physiological, biochemical and, most recently also, molecular evidence showing that the modification of the binding properties of the root apoplast contributes to Al resistance. A further in-depth characterization of the Al-induced apoplastic reaction in the most Al-sensitive zone of the root apex is urgently required, particularly to understand the Al resistance of the most Al-resistant plant species.

Key words: Aluminium, aluminum, resistance, apoplast, cell wall, pectin, root elongation.

INTRODUCTION

It has been estimated that soils of 30 % of the ice-free land area of the world are acid, where crop productivity is limited by a range of growth-limiting factors related to soil acidity (von Uexküll and Mutert, 1995). Aluminium (Al) toxicity is the most important soil constraint for plant growth and development in acid soils. It is now well understood that the toxicity of Al in aquatic and terrestrial systems is not correlated with total Al concentrations (7 % of mineral soils), but is a function of the concentration of the biologically active fraction in solution (Lewis, 1989). Among the soluble Al species in the soil solution, the inorganic monomeric forms, particularly Al^{3+}, are considered the most important (Kinraide *et al.*, 1992). Organic and inorganic Al complexes are regarded as not phytotoxic, or considerably less so (Kerven *et al.*, 1989).

As early as 1918, Al toxicity was implicated as the main cause of the inhibition of root growth of barley (*Hordeum vulgare*) and rye (*Secale cereale*) in an acid soil (Hartwell and Pember, 1918). Aluminium-induced inhibition of root elongation can be measured within hours or less after the roots have been exposed to excess Al supply (Llugany *et al.*, 1995; Blamey *et al.*, 2004, 2005). Ryan *et al.* (1993) were the first who unequivocally demonstrated the role of the root apex in the perception of Al toxicity in maize (*Zea mays*). They also clearly refuted the hypothesis put forward by Bennet *et al.* (1985) that the root cap plays a decisive role in the expression of Al-induced inhibition of root elongation. In a more refined methodological approach, Sivaguru and Horst (1998) presented evidence that in the Al-sensitive maize cultivar 'Lixis' the distal part of the transition zone (DTZ, 1–2 mm) is the most Al-sensitive apical root zone in maize. Application of Al only to the DTZ reduced cell elongation in the elongation zone (EZ) to the same extent as application to the entire 10 mm root apex. However, application of Al only to the EZ did not inhibit root elongation, needing signal transduction as proposed by Bennet *et al.* (1985) between the DTZ and the EZ (Kollmeier *et al.*, 2000). These authors also provided evidence that basipetal auxin transport might be implicated in the signalling. The role of ethylene-mediated inhibition of polar auxin transport in Al-induced inhibition of root elongation has recently been substantiated in *Arabidopsis* (Sun *et al.*, 2010). The importance of the TZ (1–2 mm) as a main target of Al was also confirmed in common bean (*Phaseolus vulgaris*) by Rangel *et al.* (2007). However, in contrast to maize, in common bean Al also reduced root elongation when applied only to the EZ, the zone initially impacted by Al in a digital microscopy study with mungbean (*Vigna radiata*) (Blamey *et al.*, 2004).

Treatment with Al as well as with other metals causes transverse ruptures to develop in sub-apical regions of the root through the breaking and separation of the rhizodermis and outer cortical layers from the inner cortical cell layers (Blamey *et al.*, 2004; Kopittke *et al.*, 2008). It was proposed that these ruptures relate to the binding of Al to the cell wall thus increasing cell wall rigidity and decreasing elasticity.

The differences between metals could be related to the strength with which they bind to the cell wall (Kopittke *et al.*, 2009) in agreement with Kinraide (2009). However, the relationship between these ruptures and inhibition of root elongation is not well understood to date (Ryan *et al.*, 1993; Kopittke *et al.*, 2008).

Another sensitive indicator of Al injury in roots is the induction of callose synthesis (Wissemeier *et al.*, 1987; Staß and Horst, 2009), particularly in the root apex (Wissemeier and Horst 1995; Sivaguru *et al.*, 2006). Al-induced callose formation is an indicator of Al sensitivity and a reliable parameter for the classification of genotypes of different plant species in terms of Al resistance (Wissenmeier *et al.*, 1992; Horst *et al.*, 1997). Collet and Horst (2001) developed a rapid non-destructive screening procedure for maize cultivars for adaptation to acid soils with high Al supply, and Eticha *et al.* (2005*c*) successfully used Al-induced callose formation to study inheritance of Al resistance and adaptation to an acid, Al-toxic soil, using a 13 × 13 diallel of maize cultivars of largely different origin.

Much progress has been made during recent years in the physiological and molecular understanding of Al exclusion (for reviews, see Matsumoto 2000; Kochian *et al.*, 2004; Zheng and Yang, 2005; Delhaize *et al.*, 2007; Ma, 2007; Panda and Matsumoto, 2007). In agreement with Ryan and Delhaize (2010), in this review we use the expression 'Al resistance' consistently as a plant property which allows a plant to grow with little or no injury with elevated Al supplies. The plant mechanism conferring resistance may be exclusion from binding to and uptake by the roots (Al exclusion) or Al tolerance (Al binding and uptake without Al injury). In spite of the progress made, the Al exclusion mechanisms of some of the most Al-resistant plant species such as rice (*Oryza sativa*) (Yang *et al.*, 2008; Huang *et al.*, 2009), rye (Shi *et al.*, 2009) and signalgrass (*Brachiaria decumbens*) (Wenzl *et al.*, 2001; Watanabe *et al.*, 2006) are still far from being understood. Furthermore, the primary target site of Al phytotoxicity leading to inhibition of root elongation is still not well defined. Indeed, the relative importance of symplastic vs. apoplastic lesions of Al toxicity remains a matter of debate, and the role of cell wall properties in Al resistance is not widely acknowledged. Horst (1995), Rengel (1996) and Blamey (2003) focused their attention on the role of the apoplast in Al toxicity and Al resistance. The additional experimental evidence since then justifies an updated consideration of the interactions of Al with apoplastic components. This review summarizes the current understanding of the role of the root apoplast in Al-induced inhibition of root elongation and in Al resistance of plants.

AL INTERACTIONS WITH APOPLASTIC BINDING SITES LEAD TO Al INJURY

Cell wall

Aluminium is accumulated by roots, with a rapid initial phase and a lower rate thereafter (Zhang and Taylor, 1989, 1990). The rapid initial phase reflects the binding of Al in the apoplast, which has been demonstrated using fractionated extraction methods (Wang *et al.*, 2004; Rangel *et al.*, 2009*a*), surgical separation of the cell wall and symplast in the giant algae *Chara corallina* (Rengel and Reid, 1997; Taylor *et al.*, 2000) and also *in situ* localization techniques such as X-ray microanalysis (Marienfeld and Stelzer, 1993) and secondary ion mass spectrometry (SIMS) (Horst *et al.*, 2007). The primary binding site of Al^{3+} in the apoplast is probably the pectic matrix, with its negatively charged carboxylic groups having a particularly high affinity for Al^{3+} (Blamey *et al.*, 1990; Chang *et al.*, 1999). Short-term Al accumulation by roots is closely related to the pectin content and may explain the differences in Al contents between apical root sections of maize and faba bean (*Vicia faba*) (Fig. 1). It appears that the Al content and thus the binding of Al to the pectic matrix are closely positively correlated to Al-induced callose formation and thus Al sensitivity (Horst *et al.*, 1999).

The role of the pectin content in Al accumulation and Al sensitivity has been further substantiated using different approaches modifying the pectin content of intact maize plants (Horst *et al.*, 1999) and maize cell suspension cultures

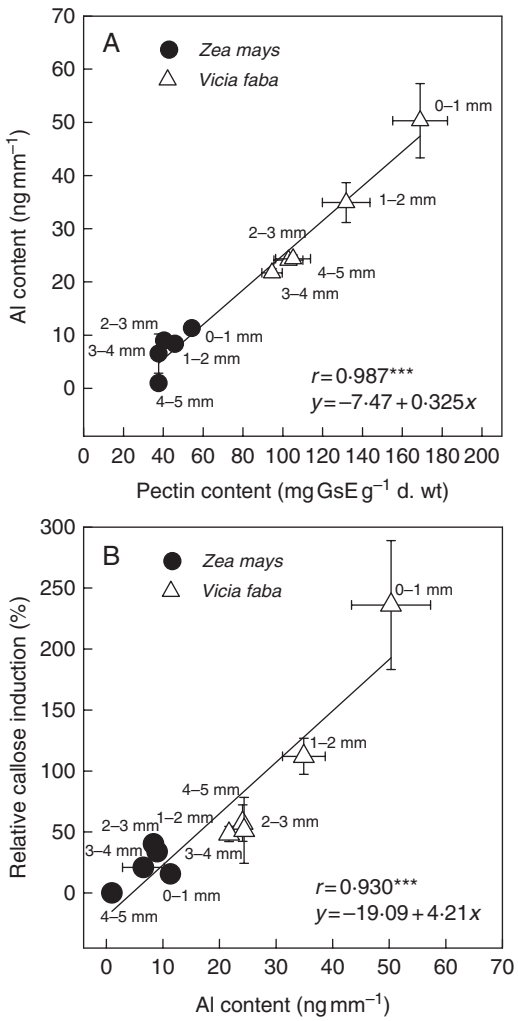

FIG. 1. Relationships between (A) pectin and Al content, and (B) Al content and relative callose induction (digitonin = 100) of root sections of maize (*Zea mays*) and faba bean (*Vicia faba*). Roots were incubated for 3 h in nutrient solution ± 50 μM Al or 10 μM digitonin at pH 4·3. ***Significant at $P < 0{\cdot}001$. From Horst *et al.* (2007).

(Schmohl and Horst, 2000). In fact, the factor responsible for Al binding to pectin is not the pectin content alone but its negative charge determined by its degree of methylation (DM) (Eticha *et al.*, 2005*a*), which is controlled by pectin methylesterase (PME) (Bordenave, 1996; Gerendás, 2007). Schmohl *et al.* (2000) provided evidence that Al accumulation and Al sensitivity of maize cell suspension cells is modulated by the DM of their cell walls. Also, short-term treatment of intact maize roots with PME enhanced Al accumulation and Al-induced inhibition of root elongation (Horst *et al.*, 2007).

Accumulation of Al in the cell wall can also be expected on the basis of the measured low rates of transport of Al through the plasma membrane into the symplast of the model plant *C. corallina* (Rengel and Reid, 1997; Taylor *et al.*, 2000). However, both studies as well as those by Marienfeld *et al.* (2000) in maize also show that a rapid transfer of Al from the apoplast to the symplast does occur. Using different techniques Tice *et al.* (1992), Lazof *et al.* (1994) and Vázques *et al.* (1999) demonstrated the accumulation of Al in the symplast in wheat (*Triticum aestivum*), soybean (*Glycine max*) and maize, respectively, leading to a rather uniform cellular distribution of Al or even accumulation of Al in the symplast at the expense of the cell wall. However, we have challenged the results based on cellular Al localization using morin, because morin is unable to bind to cell wall-bound Al (Eticha *et al.*, 2005*b*). The rapid uptake of Al into the symplast (Ma *et al.*, 1998; B. Klug and W. J. Horst, Leibniz University Hannover, Germany, unpubl. res.), transfer to the central cylinder, xylem transport to the shoots and accumulation of Al in the vacuoles of the leaves are a typical feature of Al accumulator plant species such as hortensia (*Hydrangea macrophylla*) (Ma *et al.*, 1997; Naumann and Horst, 2003), buckwheat (*Fygopyrum esculentum*) (Ma *et al.*, 1998) and tea (*Camellia sinensis*) (Carr *et al.*, 2003). The reasons for the difference in mobility of Al between Al excluders (most plant species) and includers are not yet understood (Jansen *et al.*, 2002; Klug and Horst, 2010) and the role of Al tolerance in Al resistance is still unresolved (see below).

Cell elongation requires cell turgor pressure driving expansion, the release of cell wall components from the symplast to the apoplast for cell wall synthesis, and the formation and cleavage of Ca bonds with the pectic matrix controlling cell wall extensibility (Boyer, 2009). It has been shown that Al treatment reduces root cell wall extensibility (Tabuchi and Matsumoto, 2001; Ma *et al.*, 2004). Strong binding of Al (and other metals) to the pectic matrix may prevent cell wall extension physically and/or physiologically by decreasing the effectiveness of cell wall-loosening enzymes (Wehr *et al.*, 2004).

Plasma membrane

Aluminium rapidly affects the properties not only of the cell wall but also those of the plasma membrane (Ishikawa and Wagatsuma, 1998). Interaction of Al with membrane lipids and proteins (Akeson *et al.*, 1989; Caldwell, 1989; Jones and Kochian, 1997) induces modifications of its structural properties such as fluidity and permeability (Vierstra and Haug, 1978; Wagatsuma *et al.*, 2005*a*; Khan *et al.*, 2009). Such a structural change in membrane properties is one of the prerequisites, in addition to an increase in the cytosolic Ca^{2+} activity, for the induction of callose synthesis (Kauss *et al.*, 1989), the most sensitive response of root apices to Al (see above). Binding of Al to the plasma membrane alters its surface negativity (Kinraide *et al.*, 1992; Kinraide, 2006), as shown by Ahn *et al.* (2001, 2004) in squash (*Cucurbita pepo*) and wheat. Also, in most studies, Al supply rapidly induced membrane depolarization (Lindberg *et al.*, 1991; Olivetti *et al.*, 1995) specifically in the most Al-sensitive root zone (the DTZ) (Sivaguru *et al.*, 1999*a*). This may be related to inhibition of the H^+-ATPase activity (Ahn *et al.* 2001) which in turn may lead to a disturbance of the H^+ homeostasis in the cytosol (Lindberg and Strid, 1997; Plieth *et al.*, 1999). These changes in plasma membrane properties by Al affect its ion transport properties. In soybean, Al treatment led to a rapid decrease of K^+ efflux without changing K^+ influx (Horst *et al.*, 1992; Staß and Horst, 1995). In wheat the Al-enhanced release of malate was charge-balanced by a release of K^+ (Ryan *et al.*, 1995), which is in agreement with our results in maize where Al-induced citrate release through an anion channel was observed without affecting the K^+ outward rectifier (Kollmeier *et al.*, 2001).

Aluminium-induced impairment of membrane functions may be related to Al-enhanced oxidative stress through the formation of reactive oxygen species (ROS) leading to lipid peroxidation (Yamamoto *et al.*, 1997, 2003; Jones *et al.*, 2006) and protein oxidation (Boscolo *et al.*, 2003). Oxidative stress genes are among the identified genes that are particularly expressed after Al treatment (Richards *et al.*, 1998; Ezaki *et al.*, 2005). Transformation of *Arabidopsis thaliana* with such genes conferred Al resistance (Ezaki *et al.*, 2001). However, oxidative stress in roots appears not to be the primary cause for Al-induced inhibition of root elongation (Yamamoto *et al.*, 2001), because, in most cases, it could be observed only after prolonged Al treatment (Cakmak and Horst, 1991; Maltais and Houde, 2002; Boscolo *et al.*, 2003; Liu *et al.*, 2008). However, sustained Al resistance may require protection mechanisms against oxidative stress.

In spite of these changes in plasma membrane structure and function it needs to be stressed that there is no indication that at physiological Al concentrations a severe disruption of plasma membrane functions is a prerequisite for inhibition of root elongation and callose formation (Horst *et al.*, 1992). It appears that Al triggers signal transduction pathways leading to the observed symplastic physiological disorders. In this regard, the effect of Al on cytosolic Ca^{2+} seems to play a crucial role (Rengel and Zhang, 2003; Jones *et al.*, 2006). An increase in cytosolic Ca^{2+} as an immediate response to Al treatment has been demonstrated in a range of plant species (Jones *et al.*, 1998; Zhang and Rengel, 1999; Ma *et al.*, 2002). The source of Ca^{2+} is probably the apoplastic Ca^{2+} pool, because Ca^{2+} bound in the apoplast is liberated by Al^{3+} and the change of the plasma membrane potential results in an activation of Ca^{2+} channels. However, the triggering by Al of a release of Ca^{2+} from symplastic Ca^{2+} pools cannot be excluded (Rengel and Zhang, 2003). Increasing cytosolic Ca^{2+} can explain two cellular distortions: callose formation and disorganization of the cytoskeleton (Rengel and Zhang, 2003). An increase in cytosolic Ca^{2+} is one of the prerequisites for the induction of callose synthesis by

different elicitors (Kauss *et al.*, 1989; Staß and Horst, 2009). Aluminium-induced alterations of the cytoskeleton have been reported by Blancaflor *et al.* (1998), Sivaguru *et al.* (1999*b*), Schwarzerova *et al.* (2002) and Frantzios *et al.* (2005). Amenós *et al.* (2009) also observed that Al resulted in disorganized arrangements of actin filaments in the stele cells of the TZ of maize root. Similarly, gene expression of actin and profilin was inhibited by Al (Zhang *et al.*, 2007). Profilin, an actin-binding protein, regulates the polymerization of actin filaments and plays a significant role in cell elongation (Ramachandran *et al.*, 2000). Although a direct effect of cytosolic Al on the cytoskeleton cannot be ruled out, an interaction of apoplastic Al with the cell wall–plasma membrane–cytoskeleton continuum appears more likely (Horst *et al.*, 1999; Sivaguru *et al.*, 2000*b*).

MODIFICATION OF APOPLASTIC AL BINDING MODULATES Al TOXICITY

Aluminium accumulated in the root apoplast modifies cell wall composition and properties. Cellulose synthesis was inhibited in favour of callose synthesis in barley (Teraoka *et al.*, 2002), and Al stress has been demonstrated to increase cell wall pectin content in a number of plant species such as squash (Van *et al.*, 1994), maize (Eticha *et al.*, 2005*a*), rice (Yang *et al.*, 2008) and common bean (Rangel *et al.*, 2009*a*). Increased pectin content caused by Al treatment has been interpreted by some researchers as a tolerance mechanism (Van *et al.*, 1994), since the pectic substances bind or chelate Al^{3+} ions via their free carboxyl groups, resulting in cross-linking of pectin molecules (Klimashevskii and Dedov, 1975) and leading to detoxification of Al. However, there is increasing evidence that binding of Al to pectins appears to be more closely related to Al sensitivity since the Al-induced increase in pectin content of Al-sensitive cultivars was greater than that of Al-resistant cultivars (Eticha *et al.*, 2005*a*; Yang *et al.*, 2008). Also, in an attempt to explain the recovery from the initial (4 h treatment) Al-induced inhibition of root elongation in an Al-resistant common bean cultivar, Rangel *et al.* (2009*a*) related different cellular Al fractions to Al-induced cell elongation. They showed that recovery of root growth was closely negatively related to free apolastic and particularly strongly bound cell wall Al (Fig. 2). This suggests that the strong binding of Al to the pectic matrix of the cell wall is a main factor in Al toxicity and not a resistance mechanism in common bean. In contrast to the stably bound cell wall Al fraction, there was no indication that the labile bound (citrate-exchangeable) Al fraction was related to Al-induced inhibition of root elongation. This was unexpected, because in maize this fraction appeared to contribute to explaining silicon (Si)-mediated amelioration of Al toxicity (Wang *et al.*, 2004). However, there seems to be a principal difference between monocots and dicots in Al binding to cell walls, which is not surprising given the difference in cell wall composition (Carpita and Gibeaut, 1993; Sarkar *et al.*, 2009). This is well illustrated by the fact that treatment of cell walls with 50 mM $BaCl_2$ removed about 20 % of the cell wall-bound Al in maize (Wang *et al.*, 2004), and nearly all Al adsorbed on wheat cell walls could be exchanged with 2·5 mM $CaCl_2$ (Zheng *et al.*, 2004). In contrast, $BaCl_2$ was unable to exchange any Al in common bean even after only short-term Al treatment (Staß *et al.*, 2007). Overall, this clearly supports the view that the main role of Al-induced release of organic acid anions into the apoplast is the prevention of Al from binding to the pectic matrix. Wehr *et al.* (2003) showed that citrate and malate were able to remove Al from artificial Al–pectate gels, suggesting that exudation of organic acids would remove Al bound to pectin and this could alleviate toxicity. However, the decrease in the Al content of the stably bound cell wall Al fraction with increasing Al treatment duration, as shown in Fig. 2, by root-released citrate appears to be improbable because this fraction is defined as citrate non-exchangeable. It thus appears that once Al is firmly bound it is unlikely to be released by the citrate exuded from the cells, unless the citrate concentration in the apoplast is much higher than the concentration used for the exchange (33 mM). Therefore, it is more likely that citrate released into the apoplast reduces the binding of Al in the apoplast by complexing Al and decreasing the strength of Al binding, thus preventing the strong binding of Al to the cell wall (Zheng *et al.*, 2004).

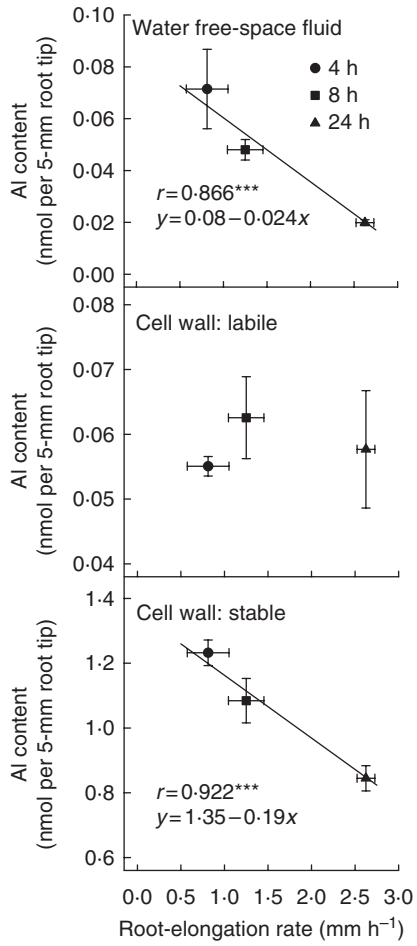

FIG. 2. Relationship between root elongation rate and the Al contents of three apoplastic fractions in 5 mm root tips of common bean genotype Quimbaya (Al-resistant) grown in a simplified nutrient solution containing 0·5 mM $CaCl_2$, 0·5 mM KCl, 8 μM H_3BO_3 and 20 μM Al for up to 24 h, pH 4·5. ***Significant at $P < 0·001$. From Rangel *et al.* (2009*a*).

The Al-induced modifications of structural properties of the cell wall (Schildknecht and Vidal, 2002) affect the mobility of solutes in the apoplast of the root cortex. Schmohl and Horst (2002) demonstrated that the release of proteins in general, and acid phosphatase in particular, and pectins was inhibited by Al in maize root apices and maize cell suspension cultures. These effects were attributed to a reduction of cell wall porosity by Al binding in the cell wall. However, these results can also be explained by a lower permeability of the plasma membrane for macromolecules. More convincing evidence of an inhibition of the apoplastic solute bypass flow in maize root apices was provided by Sivaguru *et al.* (2006). They showed accumulation of fluorescent probes with mol. wts of 524 (8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt), 3000, 10 000 and 40 000 (neutral dextran–Texas red conjugates) in the outer cortical cells, especially in the DTZ, and inhibition of transfer of these solutes to the xylem and finally the shoot (Fig. 3). Water flow was not affected, in contrast to the expectations expressed by Blamey *et al.* (1993) showing a strong reduction by Al of water flow through an artificial pectate membrane. Since the inhibition of callose synthesis by pre-treatment of the roots with 2-deoxy-D-glucose (DDG) prior to Al treatment partially alleviated the Al-induced inhibition of solute bypass flow (Sivaguru *et al.*, 2006), it is assumed that callose deposition contributes not only to the inhibition of cell to cell trafficking of solutes through plasmodesmata (Sivaguru *et al.*, 2000*a*) but also to the apoplastic bypass flow in root cortical cell walls.

COMPLEXATION OF AL IN THE APOPLAST CONFERS GENOTYPIC Al RESISTANCE BY REDUCING INTERACTIONS BETWEEN Al AND CELL WALL COMPONENTS

It is generally agreed that the release of Al-complexing solutes, particularly organic acid anions, in the Al-sensitive apical root zone is the most effective way to reduce the impact of Al on apoplastic functions (Ma *et al.*, 2001; Ryan *et al.*, 2001; Delhaize *et al.*, 2007). Using the patch–clamp technique, it is well established that the Al-induced release of malate in wheat (Ryan *et al.*, 1997; Zhang *et al.*, 2001) and of citrate in maize (Kollmeier *et al.*, 2001; Piñeros and Kochian, 2001) is mediated by plasma membrane anion channels. The frequency and magnitude of Al-induced anion currents are greater in Al-resistant than in Al-sensitive cultivars (Kollmeier *et al.*, 2000; Zhang *et al.*, 2001). The genes encoding these anion channel proteins have been identified and characterized in several plant species (see the review by Delhaize *et al.*, 2007).

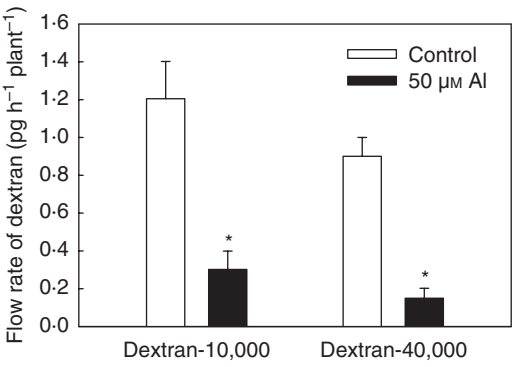

FIG. 3. Effect of Al on the flow rate of dextran-10,000-TR and dextran-40,000-TR in root xylem exudates of maize cv. Lixis. Al and fluorescent tracers were applied to the 1 cm root apex for 2 h including the 1 h exudate collection period. Values are means of 8–10 independent replicates ± s.e. Means with * indicate significant differences at $P < 0{\cdot}05$ (Tukey test). From Sivaguru *et al.* (2006).

The role of the metabolism of organic acids in Al resistance is still a matter of discussion. Most studies have shown no clear relationship between the root content and release of organic acid anions (Ryan *et al.*, 2001). Also, the activities of enzymes involved in the synthesis of organic acids did not differ significantly between Al-resistant and Al-sensitive genotypes. These findings and others led Ryan and Delhaize (2010) to suggest convergent evolution of Al resistance in Al-excluder plant species through mutation of transport proteins to organic acid anion permeases. However, based on a detailed study on release from and content of specific 1 mm apical root sections, Kollmeier and Horst (2001) showed that an Al-sensitive cultivar was not capable of maintaining the level of citrate in the apical root sections in spite of a lower citrate release rate. This was in agreement with a general trend of Al-enhanced activities of enzymes involved in citrate synthesis such as NAD-malate dehydrogenase (MDH) and phosphoenolpyruvate decarboxylase (PEPC), but not citrate synthase (CS), in the Al-resistant cultivar and of citrate-degrading aconitase in the Al-sensitive cultivar (Kollmeier and Horst, 2001). Also, sustained recovery from Al stress through citrate exudation in the Al-resistant common bean genotype Quimbaya after 24 h Al treatment relied on restoring the internal citrate pool and the constitutively high activity of CS fuelled by high PEPC activity (Rangel *et al.*, 2009*b*). In the Al-sensitive genotype VAX-1 the citrate exudation and, thus, Al exclusion and root elongation could not be maintained, resulting in an exhaustion of the internal citrate pool and decreased CS activity.

There was no difference between the genotypes in the upregulation of MATE genes coding for citrate permeases (Eticha *et al.*, 2010). Further evidence for an involvement of enhanced organic acid synthesis and reduced degradation in Al resistance comes from studies using transgenic plants with modified organic acid metabolism. Al-induced citrate exudation driven by Al-inducible expression of mitochondrial CS enhancing Al resistance in *Paraserianthes falcaria*, a leguminous tree, was reported: Al treatment increased the accumulation of mitochondrial CS transcripts, its activity and gene expression (Osawa and Kojima 2006). The over-expression of the mitochondrial CS gene from *Citrus junos* conferred Al resistance in *Nicotiana benthaminana* (Deng *et al.*, 2006). Similarly, a successful transformation of several independent tobacco lines with rice mitochondrial CS resulted in increased citrate efflux and greatly enhanced Al resistance (Han *et al.*, 2009). Not only the over-expression of CS, but also that of MDH (Tesfaye *et al.*, 2001) and PEPC (Osaki *et al.*, 2001), was reported to enhance Al resistance of plants. A role for PEPC in Al resistance of soybean genotypes through citrate exudation has also been convincingly demonstrated by Ermolayev *et al.* (2003). It thus appears that the maintenance of cytosolic

organic acid anion concentrations and their release into the root tip apoplast through activation of anion permeases are both key factors for Al resistance in some plant species.

In addition to organic acid anions the release into the apoplast of polypeptides (Basu *et al.* 1999) and phenols (Heim *et al.*, 1999; Kidd *et al.*, 2001) may also be involved in genotypic Al resistance in wheat and maize, respectively.

MODIFICATION OF Al BINDING PROPERTIES OF THE APOPLAST CONFERS GENOTYPIC Al RESISTANCE

Negativity of the root apoplast

As shown above, Al binds readily to negative binding sites of the cell wall and the plasma membrane in the most Al-sensitive zones of the root apex. Since this may lead to enhanced transport of Al into the symplast and/or to impairment of root growth and functions (see above), it cannot only be expected, but has to be postulated, that reduced binding of Al in the apoplast is a prerequisite for Al resistance. Kinraide *et al.* (1992) were able to explain inhibition of root elongation by Al^{3+} in the presence of competing cations, including protons, on the basis of the computed cation distribution on a negatively charged root membrane surface. Blamey *et al.* (1992) and Grauer and Horst (1992) came to comparable conclusions based on similar but conceptually different approaches. A lower root cation exchange capacity as a measure of cell wall negativity has been reported in plant species adapted to acid soils with high Al supply (Blamey *et al.*, 1990; Büscher *et al.*, 1990). However, across a large range of plant species a clear relationship between root cation exchange capacity and Al resistance does not exist (Grauer, 1992).

The negativity of the cell wall depends mainly on the pectin content and its DM. Across all plant species studied so far, there is no consensus on differences in constitutive pectin contents of plants with regards to Al resistance. Without Al supply, Al-resistant and Al-sensitive maize cultivars did not differ in pectin content in the 5 mm root apex (Eticha *et al.*, 2005*a*). However, in rice, the pectin content of the root apex in the Al-resistant cultivars was lower than in Al-sensitive cultivars (Yang *et al.*, 2008). In common bean the initial (4 h Al supply) high Al sensitivity and Al accumulation by roots of the Al-resistant cultivar (Quimbaya) prior to the induction of citrate exudation (Rangel *et al.*, 2009*b*) was related to a higher unmethylated pectin content in the 5 mm root tip (Rangel *et al.*, 2009*a*).

A modulating role for the DM of root cell walls in Al resistance is most convincingly supported by the comparison of potato transformants differing in the expression of PME from *Petunia inflata* (Schmohl *et al.*, 2000): transformants with higher PME expression accumulated more Al, produced more callose and were more inhibited in root growth when exposed to Al than the wild type (Fig. 4). Applying a pectin immunolocalization method to root tips, Eticha *et al.* (2005*a*) demonstrated the importance of the DM of cell wall pectin for differential Al resistance of two maize cultivars.

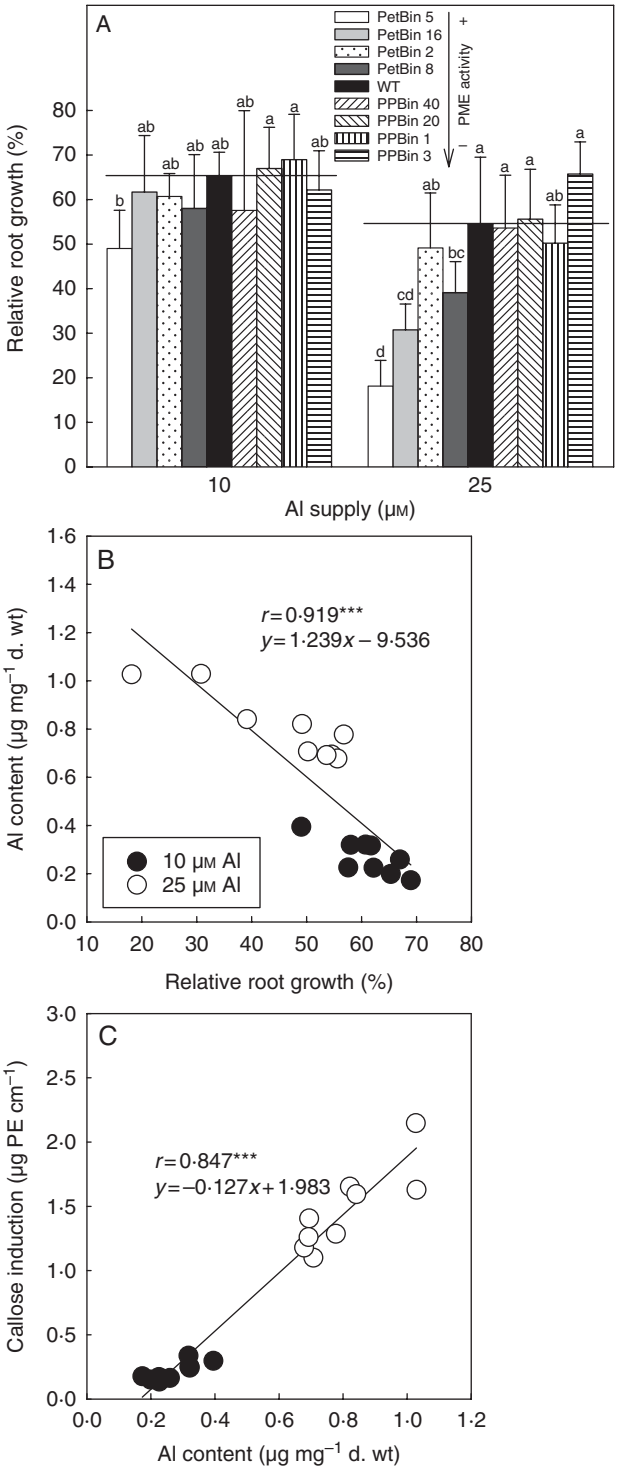

FIG. 4. (A) Effect of Al supply on relative root growth (−Al = 100 %) of transgenic potato lines that differ in expression of PME. Different letters indicate significant differences between lines at $P < 0{\cdot}05$ (Tukey test). (B) Relationship between relative root growth (−Al = 100 %) of potato genotypes (wild type and PME transformants) and Al contents, and (C) relationship between Al contents and Al-induced callose contents in root apices. Treatment of intact plants in nutrient solution for 24 h at pH 4·3. From Horst *et al.* (2007).

The cultivars did not differ in pectin content but in DM: the Al-sensitive cultivar had lower DM and consequently accumulated more Al and experienced more severe Al injury compared with the Al-resistant maize cultivar. Similarly, in rice (Yang *et al.*, 2008), root tip cell wall PME activity was constitutively higher in the Al-sensitive cultivar than in the Al-resistant cultivar. Immunolocalization of pectins showed a higher proportion of demethylated pectins in the Al-sensitive cultivar, indicating a higher proportion of free pectic acid residues in the cell walls corresponding to the higher Al contents in root tips and the cell wall. In studying Al adsorption and desorption kinetics, Yang *et al.* (2008) also confirmed that root tip cell walls of the Al-sensitive rice cultivar bound more Al, and that the bound Al was retained more tightly, compared with the Al-resistant cultivar. Using similar experimental approaches S. J. Zheng (Zhejiang University, Hangzhou, China, pers. comm.) showed that the differential Al resistance of two buckwheat genotypes could also be related to differences in the Al binding capacity of root tip cell walls.

Besides the cell wall, the plasma membrane contributes to the negativity of the apoplast and may affect the toxicity of metals (Kinraide, 2006). Wagatsuma and Akiba (1989) and Wagatsuma *et al.* (1991, 2005*b*) related differences in Al resistance between plant species to the plasma membrane negativity of protoplasts, and Yermiyahu *et al.* (1997) ascribed the higher Al sensitivity of the wheat cultivar Scout to its higher plasma membrane negativity compared with the Al-resistant cultivar Atlas. Recently, Khan *et al.* (2009) provided evidence that genotypic Al tolerance in rice was related to a lower ratio of phospholipids to Δ^5-sterols in the plasma membrane leading to a lower negativity and permeability compared with Al-sensitive cultivars. A role for the plasma membrane in Al resistance is also indicated by studies showing that the transformation of yeast and plants by a higher plant Δ^8-sphingolipid desaturase modulated Al resistance (Da Silva *et al.*, 2006; Ryan *et al.*, 2007).

Silicon nutrition

There are several reports showing that Si nutrition enhances Al resistance of plants (Hodson and Evans, 1995). Both *ex planta* and *in planta* effects are involved, but the latter effects are only poorly understood (Cocker *et al.*, 1998*a*). In maize, Kidd *et al.* (2001) provided evidence for an Al-induced enhanced release of phenolic compounds by Si-pre-treated plants, thus detoxifying Al in the apoplast. Wang *et al.* (2004), too, attributed Si-enhanced Al resistance of maize to a clear *in planta* effect. However, they could not relate Si-enhanced Al resistance to an increased release of phenols or organic acid anions, but rather to a decrease in Al binding capacity of the cell wall. Al treatment greatly enhanced Si accumulation in the cell wall fraction, reducing the mobility of apoplastic Al. In agreement with Cocker *et al.* (1998*b*), Wang *et al.* (2004) concluded from their data that Si treatment leads to the formation of hydroxyaluminum silicates (HAS) in the apoplast of the root apex, thus detoxifying Al.

Boron nutrition

Boron (B) is a structural component in growing plant tissues (Brown and Hu, 1997). The formation of bis-diester bonds between the rhamnogalacturonan II (RG II) subunits of pectin chains and boric acid is a main function of B in plants (Brown *et al.*, 2002; Goldbach and Wimmer, 2007). Boron-induced amelioration of Al toxicity has been reported in a number of plant species (LeNoble *et al.*, 1996*a*, *b*; Lukaszewski and Blevins, 1996; Wojcik, 2003; Hossain *et al.*, 2005; Staß *et al.*, 2007; Corrales *et al.*, 2008; Yu *et al.*, 2009). However, the picture emerging on the mechanisms responsible for B amelioration of Al toxicity is not yet clear. Lukaszewski and Blevins (1996) reported that in squash both Al toxicity and B deficiency caused a reduction in ascorbate concentrations in root apices which was correlated with reduced root growth. They proposed that Al toxicity impaired the role of B in ascorbate metabolism which could be prevented by supplemental B (Lenoble *et al.* 1996*b*) and thus enhanced anti-oxidative defence (Corrales *et al.*, 2008). However, Staß *et al.* (2007) and Yu *et al.* (2009) provided evidence that B-related changes in cell wall properties affect Al binding in root apices and thus Al toxicity. B deficiency increased the unmethylated pectin content of cell walls in common bean, which created additional binding sites for Al in the cell wall, thus enhancing Al accumulation and toxicity (Staß *et al.*, 2007). Similarly, B supplementation reduced Al uptake by pea (*Pisum sativum*) root tips and Al binding to cell walls, which resulted in less Al toxicity (Yu *et al.*, 2009). However, other studies have failed to find any ameliorative effect of B on Al toxicity (Taylor and Macfie, 1994; Corrales *et al.*, 2008). In general, dicots display a stronger response to B supply than monocots, which may be due to the higher B requirement of dicots. Enhanced Al toxicity in B-deficient dicot plant species could also be related to the pore size of the cell wall which is affected by borate ester cross-linking of the pectic polysaccharide RG II (Fleischer *et al.*, 1999). Reduced pore size of the cell wall could affect Al uptake. In a study on the interaction of Al toxicity and drought stress in common bean, Yang *et al.* (Z. Yang, Leibniz University Hannover, Germany, unpubl. res.) presented circumstantial evidence that osmotic stress induced by polyethylene glycol (PEG) treatment reduced the accumulation of cations ($Al^{3+} > La^{3+} > Sr^{2+}$) in root tips by reducing cell wall porosity in agreement with their hydrated ionic diameter (Fig. 5).

AL RESISTANCE IN Al ACCUMULATORS REQUIRES Al EXCLUSION MECHANISMS

A close relationship exists between Al exclusion from the root tip and Al resistance in Al-excluding plant species, e.g. in wheat (Delhaize *et al.*, 1993), maize (Piñeros *et al.*, 2005), triticale (Yang *et al.*, 2005), rice (Yang *et al.*, 2008) and common bean (Rangel *et al.*, 2007). In all of these plant species Al accumulation in the root tip of Al-resistant genotypes was less than that of their Al-sensitive counterparts, indicating that Al resistance is achieved through Al exclusion particularly from the apoplast where the bulk of Al accumulates. Li *et al.* (2009*b*) reported that the amount of Al adsorbed to cell walls

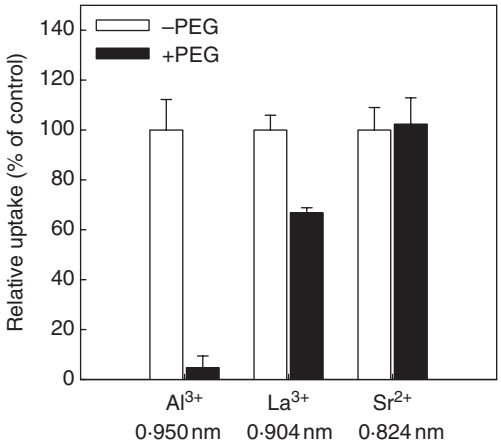

FIG. 5. Effect of polyethylene glycol (PEG) treatment on relative (no PEG = 100) Al, La and Sr accumulation of 10 mm root tips of Al-sensitive common bean genotype VAX-1. Plants were pre-treated with PEG (150 g L^{-1}) for 8 h in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 mM H_3BO_3. Then the plants were supplied with 25 mM $AlCl_3$, 5 mM $LaCl_3$ or 2·5 mM $SrCl_2$ in the absence or presence of PEG (150 g L^{-1}) in the same nutrient solution for 1 h. The hydrated ionic diameters are indicated under the ions. Bars represent means ± s.d. ($n = 4$). (Z. Yang *et al.*, Leibniz University Hannover, Germany, unpubl. res.)

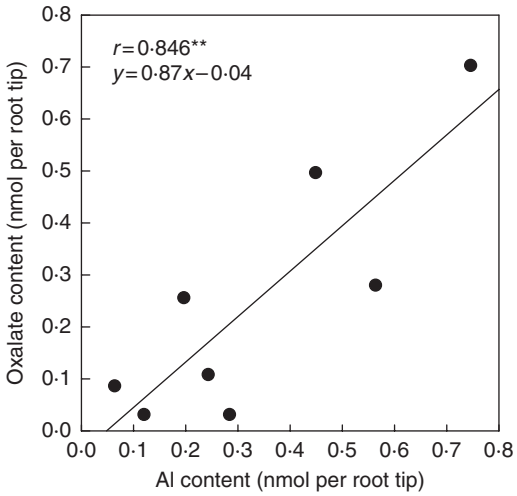

FIG. 6. Relationship between the contents of Al and oxalate in the water free-space fluid (WFSF) of adventitious buckwheat root tips. The WFSF was extracted by centrifugation of excised 10 mm root tips at 4000 *g* for 15 min. Plants were pre-treated for 0·5, 4 and 24 h at 75 μM Al in simplified nutrient solution (pH 4·3) containing 500 μM $CaCl_2$, 8 μM H_3BO_3 and 100 μM K_2SO_4. Points represent single values. **Significant at $P < 0{\cdot}01$. (B. Klug and W. J. Horst, Leibniz University Hannover, Germany, unpubl. res.)

isolated from the Al-resistant wheat line ET8 and the Al-sensitive wheat line ES8 was similar, but the latter accumulated several times more Al in root tips. This was explained by the exudation of malate by ET8 under Al stress, thereby protecting the Al-sensitive sites in cell walls from binding Al.

The additional role of Al tolerance in Al resistance of Al excluders is not yet fully resolved. Higher symplastic Al contents may be indicative of enhanced or acquired Al resistance, as suggested by Vázquez *et al.* (1999) who observed internalization of Al into the symplast contributing to Al tolerance in an Al-resistant maize genotype. Also Illes *et al.* (2006) ascribed Al internalization into endosomal or vacuolar compartments contributing to the recovery from initial Al stress in *Arabidopsis*. However, the trend of increasing symplastic Al contents with the recovery from initial Al stress and the significantly higher symplastic Al contents in the Al-resistant common bean genotype Quimbaya compared with the Al-sensitive genotype VAX-1 seems to indicate that transport of Al into the symplast is not a prerequisite for Al toxicity (Rangel *et al.*, 2009*a*). From their results it appears rather unlikely that transfer to and inactivation of Al in the symplast can explain enhanced Al resistance because of the quantitatively small Al fraction in the symplast.

In contrast to Al excluder species, Al includer species which are among the most Al-resistant plant species can contain >1 mg (g d. wt)$^{-1}$ Al in their leaves (Jansen *et al.*, 2002). Thus, they can be classified as Al-tolerant. Al tolerance is attributed to the symplastic complexation of Al by organic ligands, particularly organic acids (Ma *et al.*, 1997, 1998; Morita *et al.*, 2008). Rapid transfer of Al into the symplast may contribute to keep the Al^{3+} activity lower in the apoplast. However, also in Al accumulators Al^{3+} will strongly interact with the negative binding sites of the apoplast. This assumption is supported by the results with the Al accumulator buckwheat in which a lower pectin content of the cell wall resulted in a lower Al content in the root tip of an Al-resistant compared with an Al-sensitive buckwheat genotype (S. J. Zheng, Zhejiang University, Hangzhou, China, pers. comm.). Thus Al accumulators not only symplastically complex Al but also release organic acid anions from the Al-sensitive root tips and complex Al in the root apoplast when exposed to Al. This has been shown for buckwheat (Zheng *et al.*, 1998; Klug and Horst, 2010), tea (Morita *et al.*, 2001) and hortensia (Naumann, 2001). In buckwheat, there was a close relationship between the Al and oxalate concentrations, not only in the symplast but also in the apoplast of root tips (Fig. 6). Klug and Horst conclude from their results that the formation of a 1 : 1 oxalate : Al complex in the root apoplast both protects root apoplastic binding sites from interaction with Al and is a prerequisite for rapid transport of Al into the symplast (Klug and Horst, 2010; B. Klug and W. J. Horst, Leibniz University Hannover, Germany, unpubl. res.).

MOLECULAR EVIDENCE FOR THE INVOLVEMENT OF CELL WALL PROPERTIES IN Al RESISTANCE

In recent years, more molecular information on the role of the root apoplast in Al toxicity and resistance has been accumulating. Using Al-hypersensitive mutants, Huang *et al.* (2009) discovered two genes contributing to Al resistance in rice. These genes, *STAR1* and *STAR2* (Sensitive To Al Rhizotoxicity), encode a nucleotide-binding domain and a transmembrane domain, respectively, of a bacterial-type ATP-binding cassette (ABC) transporter, and are expressed in the root particularly under Al stress. Disruption of either of these genes resulted in hypersensitivity to Al. The authors showed that the STAR1–STAR2 complex had efflux transport activity for UDP-glucose and hypothesized that UDP-glucose or glycosides derived from UDP-glucose modify the cell walls to

mask potential Al-binding sites in the apoplast, resulting in Al resistance in rice.

In line with this, the transcriptional analysis of Al resistance in maize by Maron *et al.* (2008) indicated that several genes related to cell wall structure and composition exhibit differential expression upon Al treatment. Among these, PME, the enzyme responsible for the demethylation of pectin in the cell wall, was upregulated by Al treatment in both Al-resistant and Al-sensitive maize genotypes. The PME expression was constitutively higher and its upregulation was more enhanced in the Al-sensitive genotype. These results nicely back-up the immunolocalization study on unmethylated pectins in roots of two maize cultivars differing in Al resistance by Eticha *et al.* (2005*a*) and Li *et al.* (2009*a*). The results contribute to close the gap in the understanding of genotypic Al resistance in maize which cannot be fully explained by differential exudation of citrate (Piñeros *et al.*, 2005).

In addition to PME, the expression and activity of apoplastic peroxidases were enhanced under Al stress (Kumari *et al.*, 2008; Maron *et al.*, 2008; Xue *et al.*, 2008). Plant peroxidases constitute a large group of proteins encoded by a multigene family consisting of about 138 members in rice (Passardi *et al.*, 2004) and 73 members in *Arabidopsis* (Tognolli *et al.*, 2002). These enzymes catalyse diverse reactions and are involved in a wide range of physiological processes, such as auxin catabolism, lignin and suberin formation, cross-linking of cell wall components, defence against pathogens, and cell elongation (Hiraga *et al.*, 2001). It thus may be assumed that apopalstic peroxidases are involved in both Al toxicity and Al resistance mechanisms.

In addition, Al caused changes in gene expression of a number of cell wall-modifying enzymes such as xyloglucanase, endotransglycosylases, polygalacturonases, glycosyl transferases, lipid transfer proteins and other wall-related genes in *Arabidopsis* (Kumari *et al.*, 2008), indicating the important role of dynamic changes in the apoplast for the Al stress response. Moreover, several hundreds of other Al-responsive genes have been documented through transcriptional profiling studies in *Arabidopsis* (Kumari *et al.*, 2008; Goodwin and Sutter, 2009), wheat (Guo *et al.*, 2007; Houde and Diallo, 2008), maize (Maron *et al.*, 2008) and *Medicago truncatula* (Chandran *et al.*, 2008*a*, *b*). Their possible functions in relation to Al toxicity and resistance can only be speculated at this time. Therefore, future studies should focus on elucidating the significance of these genes for Al resistance of plants.

CONCLUSIONS AND FUTURE PERSPECTIVES

The binding of Al in the cell wall and to the apoplastic face of the plasma membrane in the most Al-sensitive root tip zone impairs both apoplastic and symplastic functions, disruption of which is a major factor leading to Al-induced inhibition of root elongation. Although symplastic lesions of Al toxicity cannot be ruled out, the protection of the root apoplast appears to be a prerequisite for Al resistance in both Al-excluder and Al-accumulator plant species. The most important Al resistance mechanism in most plant species is the sustained release of organic acid anions from the root apex. These organic acid anions complex Al, thus protecting the root apoplast from Al binding. However, there is increasing physiological, biochemical and, most recently, molecular evidence showing that the modification of the binding properties of the root apoplast contributes to Al resistance.

The physiological research during the last 15 years has contributed to consolidate the role of the apoplast in Al toxicity and resistance. Whereas the role of organic acid anions in the protection of the apoplast from Al binding, including its molecular basis, are well understood, the understanding of the mechanism of Al toxicity is still mainly circumstantial. Particularly our knowledge on the molecular mechanisms governing the Al–apoplast interactions leading to inhibition of root elongation, callose formation and disruption of tissue integrity is still scarce. The recent identification of a few genes which code for proteins involved in the modification of cell wall composition, structure and functions are mostly by-products of studies searching for genes involved in organic acid anion exudation and metabolism. A more focused search for genes related to the root tip apoplast is urgently needed. This would allow the function of these genes in Al toxicity and resistance to be proved using transgenic approaches.

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