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Biotechnological Solutions for Enhancing the Aluminium Resistance of Crop Plants

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1. Introduction

Acid soils limit crop yields around the world due to nutrient deficiencies and mineral toxicities. Non-adapted plants grown on acid soils typically have smaller root systems because high concentrations of soluble aluminium (Al^{3+}) inhibit root elongation. This restricts their ability to acquire water and nutrients. Plants vary widely in their capacity to tolerate acid soils and even genotypes within species show significant variation. The physiology and genetics controlling this variability have been studied for many years. The analysis of segregating populations and mutants has helped identify the mechanisms and genes controlling aluminium resistance in many species including wheat, rice, *Arabidopsis,* barley and sorghum. The release of organic anions from roots is an important mechanism of resistance in a wide variety of species. This trait is controlled by genes from two distinct gene families that encode transport proteins. Sufficient information is now available for enhancing the aluminium resistance of important crop species using biotechnology. We will review progress in understanding the mechanisms of Al^{3+} resistance in plants, the genes controlling these mechanisms and the application of genetic engineering to increase the Al^{3+} resistance of important crop plants. This approach can help maintain and even increase food production on acid soils in the future.

2. Acid soils

2.1 What is acid soil?

Soil pH is an important consideration for agriculture production (Kochian et al. 2004; von Uexkull and Mutert 1995). Some plants are sensitive to high or low pH, nutrient availability and mineral toxicities are influenced by pH and soil microbial communities are significantly affected by pH (Fierer and Jackson 2006; Osborne et al. 2011). Acid soils present multiple stresses to plants including proton toxicity, nutrient deficiencies (especially calcium, magnesium and phosphorus) and metal-ion toxicities especially aluminium and manganese. The United States Department of Agriculture classifies acid soils into five levels: *ultra acid* soils (below pH 3.5), *extremely acid* (pH 3.5 to 4.4), *very strongly acid* (pH 4.5 to 5.0), *strongly acid* (pH 5.1 to 5.5), *moderately acid* (pH 5.6 to 6.0) and *slightly acid* (pH 6.1 to 6.5). Soils with pH≤5.5 can adversely affect the production of many major food crops.

The major limitation to crop growth in acid soils is soluble aluminium. Although aluminium is the third most abundant element in the earth's crust most of it occurs in mineral forms which are harmless to plants (complexes with oxides and silicates). In acid conditions however these minerals dissolve more readily releasing aluminium into the soil solution. Soluble aluminium hydrolyses to form a range of species the prevalence of which depends on soil pH. When the pH is 4.5 or below, the Al^{3+} species predominates but as pH increases other mononuclear aluminium species are formed including $Al(OH)_2^+$ and $Al(OH)^{2+}$. The insoluble $Al(OH)_3$ (gibbsite) can also form at higher pH. Trivalent aluminium (Al^{3+}) is highly toxic to many plants but uncertainty continues regarding the relative toxicity of the hydroxyaluminium species (Alva et al. 1986; Kinraide 1997; Noble et al. 1988; Wright et al. 1987). Al^{3+} is a reactive metal ion that forms complexes with a variety of organic and inorganic ligands including carboxylates, sulphate, and phosphate and many of these complexes are less toxic to plants than free Al^{3+} (Jones 1998; Kinraide 1997; Matsumoto 2000; Takita et al. 1999).

2.2 Formation and distribution of acid soils

Acid soils can develop naturally depending on characteristics of the parent rock but human intervention can accelerate the process (Rechcigl and Sparks 1985; Vanbreemen et al. 1983). Ancient and highly-weathered soils are often acid because the basic cations (calcium, magnesium, sodium and potassium) have been leached down the profile, often with nitrate, and replaced by hydrogen (H^+). Other drivers of acidification include acid precipitation (Rechcigl and Sparks 1985; Vanbreemen et al. 1983) and nitrification. Microorganisms can also generate organic acids and nitrate from the decomposition of plant residues which also contribute the soil acidification. Conversely low pH and aluminium mobilization can affect the microbial populations (Fierer and Jackson 2006) which are required for stubble turnover and nutrient recycling.

Approximately 30% of total land area consists of acid soils, and almost 70% of the world's potentially arable lands are acidic (Vonuexkull and Mutert 1995). The two main geographical belts of acid soils include the humid northern temperate zone mainly covered by coniferous forests and the humid tropics which support savanna and tropical rain-forests.

The American continent, Asia, Africa, Europe and Australia and New Zealand account for 40.9%, 26.4%, 16.7%, 9.9% and 6.1% of the world's acid soil respectively. Most acid soils in Asia are distributed throughout Southeast Asia and the Pacific. In Africa large tracts of the acid soil cannot be used for cultivation because they are sandy, nutrient-deprived and water-limited (Vonuexkull and Mutert 1995).

Naturally acidic soils occupy about one third of Australia, but many agricultural soils in the intensive land-use regions have become more acidic as the result of removal of harvestable product, leaching of nitrate and calcium from nitrogen-producing pastures (Australia State of the Environment report, 2001), and high applications of nitrogen fertilizer (Juo et al. 1995; Matsuyama et al. 2005; Sirovy 1979). Rapid acidification associated with the overuse of nitrogen fertilizer is also an emerging problem in China (Guo et al. 2010). Extremely acid soils can mobilise and increase the bioavailability of other toxic metals such as, mercury, zinc, copper, cadmium, chromium, manganese, and vanadium. All these factors may affect plant growth as well as the ecology of soil bacteria, mosses, algae, fungi, and invertebrates.

3. Aluminium toxicity

Acid soils are often low in basic cations, prone to crusting, erosion and compaction but physical constraints and nutrient deficiencies are rarely the main reasons crop plants grow poorly on these soils. Instead, soluble Al^{3+} is the major factor limiting growth because it inhibits root growth at very low concentrations. Indeed the inhibition of root growth is the primary symptom of plant stress on acid soils (Munns 1965). There are exceptions because many plants endemic to tropical and sub-tropical regions cope well and even thrive on acid soils. The growth of these species can even be stimulated by Al^{3+} and some accumulate high concentrations in their leaves. These are discussed in more detail later.

Al^{3+} can begin to inhibit root growth of wheat (*Triticum aestivum* L.) within minutes or hours in simple hydroponic solutions (Ryan et al. 1992). Longer exposures result in thickened roots, damaged root cap, and lesions in the epidermal and cortical tissues near the apices. The root system becomes small and damaged which limits water and nutrient acquisition. Root apices are the most sensitive part of the root and Al^{3+} must contact the apices directly for growth to be affected (**Figure 1**). Exposure of an entire maize root to Al^{3+} except the apical 5 mm has no affect on growth in the short term (Ryan et al. 1993). Within this region the zone between the meristematic and elongation zones (distal transition zone) appears to be the most sensitive part of the root (Sivaguru and Horst 1998). The concentration-dependent responses of root growth vary between species and even among genotypes. In some plants root growth remains unaffected at low concentrations of Al^{3+} but declines once a threshold is reached. This is called the *threshold for toxicity* response (Barcelo and Poschenrieder 2002). In other species root growth is stimulated by low concentrations of Al^{3+} but declines at higher concentrations. This *hormesis-type* response, is interpreted as Al^{3+} first alleviating H^+ toxicity at low concentrations and then becoming toxic itself at higher concentrations. A third response observed shows growth inhibition at low concentrations of Al^{3+} (or short exposures) but little or no effect at higher concentrations (or longer exposures). This is called the *threshold for tolerance* model and is indicative of an acclimation response occurring.

For many crops including the cereals, most of the Al^{3+} absorbed by roots localises to the apoplast. The fixed negative charges on the membrane surfaces and pectin in the cell walls attract and bind cations, and especially highly-charged cations like Al^{3+}. Nevertheless it is still uncertain whether this apoplastic Al^{3+} is toxic to plants or if Al^{3+} needs to enter the cytosol to cause injury. By binding to pectin in the cell walls Al^{3+} can rigidify the walls and restrict solute flow through the apoplast (Horst et al. 2010; Sivaguru et al. 2006). High concentrations of Al^{3+} in the apoplast can induce callose production (1,3 beta D-glucan) and affect membrane function by binding with lipids and proteins or by displacing calcium from critical sites on membranes (Foy et al. 1978). Al^{3+} can also directly inhibit nutrient uptake by blocking the function of ion channels involved in Ca^{2+} and K^+ influx (Gassmann and Schroeder 1994; Pineros and Tester 1993).

Cytosolic levels of free Ca^{2+} ($[Ca]_c$) are typically below 1.0 μM in most living cells but transient increases act as signals to control cellular functions and responses to hormones and stress. Ca^{2+}-sensitive fluorescent compounds have detected transient increases in $[Ca]_c$ in root cells treated with Al^{3+} (Rincon-Zachary et al. 2010). The rapidity of these responses indicate that Al^{3+} is causing damage in the apoplast and that cytosolic Ca^{2+} could signal early responses to Al^{3+} stress. Al^{3+} can interfere with another signal transduction pathway involving inositol 1,4,5-trisphosphate (Jones and Kochian 1995) as well as actin and tubulin stability (Grabski and Schindler 1995; Sivaguru et al. 2003b).

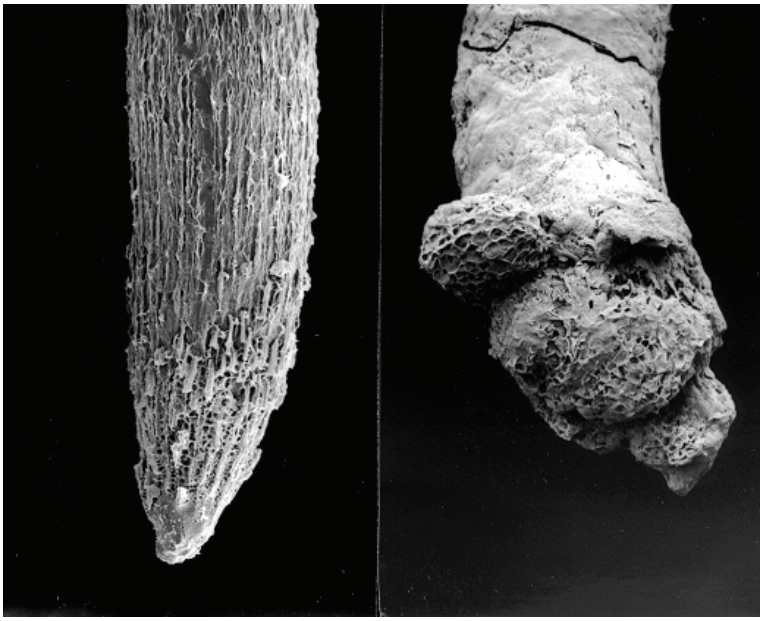

Fig. 1. Effect of Al^{3+} toxicity on roots. The scanning electronmicrographs show root apices of near-isogenic wheat plants, ET8 and ES8, that differ in Al^{3+} resistance at a single major locus. The plants were grown for 4 days growth in 0.2 mM $CaCl_2$ (pH 4.3) with 5 μM $AlCl_3$. The resistant line (ET8, on the left) is unaffected by the treatment whereas the sensitive line (ES8, on the right) shows considerable damage to its tissues. The greater Al^{3+} resistance of ET8 is controlled by the *TaAMLT1* gene on chromosome 4DL. *TaALMT1* encodes an Al^{3+}-activated anion channel that facilitates malate efflux from the root apices. (Reprinted from Delhaize and Ryan 1995).

Small but measureable amounts of Al^{3+} does enter the cytosol perhaps via non-specific cation channels (Lazof et al. 1994; Rengel and Reid 1997; Taylor et al. 2000). The combination of pH, ionic strength and availability of organic ligands in the cytosol maintain the soluble Al^{3+} concentrations to extremely low levels, perhaps less than 1 nM. However even these concentrations may cause damage because Al^{3+} can out-compete other cations like Mg^{2+} and Ca^{2+} for important binding sites and even bind with DNA (Martin 1992). Al^{3+} also triggers oxidative stress in root cells by triggering the production of reactive oxygen species (Yamamoto et al. 2001). Whether this response is induced by apoplastic Al^{3+} or symplastic Al^{3+} is unclear but these highly reactive compounds can rapidly damage membranes, proteins and nucleic acids. Oxidative stress induces callose production which in turn increases cell wall rigidity and decreases the symplastic flow of solutes via the plasmadesmata (Horst et al. 2010; Sivaguru et al. 2000).

In summary, Al^{3+} interferes with many cellular functions. The Al^{3+}-induced changes to cytosolic Ca^{2+} concentration, oxidative stress and callose production are likely to signal the early signs of Al^{3+} injury.

3. Natural variations

Plant species vary widely in their ability to grow and yield on acid soils (Foy 1988). Mclean and Gibert (1927) investigated the relative Al^{3+} resistance of several crop plants. Sensitive crops included *Lactuca sativa* (lettuce), *Beta vulgaris* (beet), *Phleum pratense* (timothy), *Hordeum vulgare* (barley), moderately resistant crops were *Raphanus sativus* (radish), *Sorghum bicolour* (sorghum), *Capitata var. alba* L. (cabbage), *Avena sativa* (oat), *Secale cereale* (rye) and noticeably resistant species included *Zea mays* (maize), *Brassica rapa* (turnip) and *Agrostis gigantean* (redtop) (McLean and Gilbert 1927). Among the cereals rice is significantly more resistant than maize, wheat and sorghum, while barley and durum wheat are among the most sensitive cereal species (Famoso et al. 2010; Garvin and Carver 2003; Khatiwada et al. 1996).

Significant variation in Al^{3+} resistance occurs within many species as well including maize, wheat, barley, rice, sorghum, snapbean and *Arabidopsis* (Foy 1988; Foy et al. 1993; Furlani et al. 1987; Kochian et al. 2004; Koyama et al. 2003; Magalhaes et al. 2007; Ryan et al. 2011; Toda et al. 1999). This variation provides opportunities for breeders to develop new cultivars better suited to acid soils. Even barley, which is considered one of the most Al^{3+}-sensitive of the small-grained *Triticeae*, displays significant genotypic variability. A seedling-based screen of barley lines in the South and East Asian Barley Core Collection identified Kearney and Golden Promise as sensitive to Al^{3+} while Dayton and several Japanese cultivars (Honen, Ohichi and Zairai Tanbo) were among the most resistant (Moroni et al. 2010).

A greater variation occurs in hexaploid or bread wheat where differences in root growth can vary by ten-fold or more in short-term growth assays or in field screens (Bona et al. 1993; Cosic et al. 1994; Dai et al. 2009; Foy 1996; Garvin and Carver 2003; Pinto-Carnide and Guedes-Pinto 1999; Raman et al. 2008; Rengel and Jurkic 1992; Ryan et al. 1995a; Tang et al. 2003). Highly Al^{3+}-resistant genotypes of bread wheat commonly used in experiments include BH1146 and Carazinho from Brazil and Atlas 66 from the USA. In most cases enhanced Al^{3+} resistance is associated with reduced Al^{3+} accumulation in the roots. Therefore the more resistant genotypes of wheat, maize, barley, sorghum and rye are able to exclude Al^{3+} from their roots cells – especially from the root apices.

4. Genetics

The inheritance and genetics of Al^{3+} resistance have been widely studied in members of the *Triticeae*. In wheat, barley and sorghum Al^{3+} resistance is often consistent with a single genetic locus inheritance while in maize and rice it is a more complex multigenic trait.

4.1 Single or few genes: cases of simple inheritance

Crop improvement programs in Brazil and the US led to the development of highly-resistant cultivars of wheat such as BH1146 and Atlas66 which have been used for genetic mapping and quantitative trait loci (QTL) analyses. Many studies indicate a single locus controls most of the variation in Al^{3+} resistance. For instance, a population of recombinant inbred lines developed with BH 1146 and the sensitive cultivar Anahuac showed a bimodal distribution for Al tolerance, consistent with single gene inheritance. Similar results were obtained with other populations (Raman et al. 2005). The resistance locus in BH 1146, named Alt_{BH}, was mapped to chromosome 4DL and explained 85% of the phenotypic variation (Riede and Anderson 1996). The location of Alt_{BH} gene was further confirmed

using 91 recombinant inbred lines and a set of wheat deletion lines (Milla and Gustafson 2001) and Luo et al. (1996) had also linked this chromosome to Al^{3+} resistance using Chinese Spring deletion lines. More recently a major aluminium resistance gene called *TaALMT1* (**Figure 1**; see later) was mapped to the same locus on 4DL (Raman et al. 2005) while minor loci were mapped to 4BL and 3BL (Navakode et al. 2009; Ryan et al. 2009).
QTL analyses of 100 F2 barley seedlings derived from the Al^{3+}-resistant cultivar (Murasakimochi) and the Al-sensitive cultivar (Morex) identified a single Al^{3+} resistance locus on chromosome 4H which explained more than 50% of the phenotypic variation (Ma et al. 2004). The *Alp* locus was also mapped to chromosome 4H in a high-resolution map generated from genotypes Dayton and Zhepi 2 (Wang et al. 2007).
Sorghum is closely related to maize and possesses the second smallest genome among cultivated grasses (Mullet et al. 2002). Like wheat and barley the genetics indicate that a single locus, Alt_{SB}, on chromosome 3 controls most of the variation in resistance (Magalhaes et al. 2004).

4.2 Multiple genes: Cases of complex inheritance

Rye is generally regarded as a highly resistant cereal species. Unlike wheat, rye is self-incompatible, so co-segregation experiments in rye generally detect a number of Al^{3+} resistance loci. Using wheat-rye addition lines, Aniol and Gustafson (1984) identified at least three different Al^{3+} resistance loci on chromosome 6RS, *Alt2* on 3R, and *Alt3* on 4RL. Two major dominant and independent loci, *Alt1* on chromosome 6RL and *Alt3* were identified (Gallego and Benito 1997) and another on chromosome 7RS (Matos et al. 2005). More recently the 7RS locus was shown to include a cluster of *ALMT*-like genes in the resistant lines (see later) (Collins et al. 2008).
More than 30 QTLs for Al^{3+} resistance have been reported in rice using populations derived from *indica* and *japonica* cultivars as well as wild relatives like *Oryza rufiogon*. (Ma et al. 2002; Nguyen et al. 2003; Nguyen et al. 2001; Nguyen et al. 2002; Wu et al. 2000; Xue et al. 2007; Xue et al. 2006a; Xue et al. 2006b). Resistance loci on chromosomes 1, 8 and 9 were consistently identified in these studies which confirms resistance is a multigenic trait in this species. Given the conservation of genetic locations among the *Triticeae* (synteny), it will be intriguing whether orthologous loci to the resistance loci from other cereals play a similar role in rice. For instance, a major resistance locus on rice chromosome 3 is homeologous to *Triticeae* 4L where the Al^{3+} resistance loci on wheat and barley are located (Nguyen et al. 2003**)**. In maize, five QTLs on chromosomes 2, 6 and 8 contribute to Al^{3+} resistance and these explain 60% of the phenotypic variation. Dominant and additive effects were detected between these loci (Ninamango-Cardenas et al. 2003).

5. Mechanisms of Al^{3+} resistance

Some plants have evolved mechanisms that enable them to tolerate Al^{3+} toxicity and acid soils better than others. The identification and characterization of these mechanisms has been the focus of considerable research. Some very resistant species like tea (*Camelia sinensis*) and *Hydragenea* sp accumulate high concentrations of Al^{3+} in their roots and leaves while others, such as resistant members of the *Triticeae*, exclude Al^{3+} from their root and shoots. For instance the concentration of Al^{3+} in the root apices of an Al^{3+}-sensitive wheat cultivar after 24 h in 50 µM Al^{3+} was 10-fold greater than a resistant cultivar (Rincon and Gonzales

1992) and similar results were reported in closely-related wheat lines that differed in Al^{3+} resistance (Delhaize et al. 1993a). Therefore two main mechanisms have been proposed to account for resistance: exclusion mechanisms and tolerance mechanisms, and evidence is now available for both of these. Exclusion mechanisms prevent Al^{3+} from entering the cytosol and minimize harmful interactions from occurring in the apoplast. Tolerance mechanisms allow plants to safely take-up and accumulate Al^{3+} within their cells. Both mechanisms may be operating in the same plant.

5.1 Mechanisms of Al^{3+} exclusion

There are several ways Al^{3+} could be prevented from accumulating in apoplastic and symplastic fractions of root tissues. Cell wall chemistry could affect Al^{3+} binding, the maintenance of a slightly higher rhizosphere pH could shift the hydrolysis of soluble aluminium from Al^{3+} to $Al(OH)^{2+}$ which would reduce accumulation in the cell wall, compounds could be released from the root which bind the harmful Al^{3+} and limit other more damaging interactions from occurring and Al^{3+} could be actively exuded from the root cell by some active transport process. Charged residues on cell wall pectin will attract and accumulate cations but pectin content is not consistently correlated with either Al^{3+} sensitivity or resistance (Horst et al. 2010). Recent studies showing that methylation of the pectin residues is correlated with reduced Al^{3+} accumulation in the wall support the idea that modifications to cell walls can increase Al^{3+} resistance.

Currently there are no examples of resistance based on Al^{3+} exudation and nor are there convincing cases linking higher rhizospheric pH to genotypic variation in resistance despite detailed studies in wheat and maize (Pineros et al. 2005). However there are claims of an Al^{3+}-resistant *Arabidopsis* mutant (*alr-104*) showing a pH dependent increase in resistance (Degenhardt et al. 1998). Measurements with micro-pH electrodes detected a 0.15 unit higher pH at the root surface of *alr-104* plants compared to wildtype plants and subsequent experiments indicated this relatively small pH change could explain the increased resistance. The molecular biology of the *alr-104* mutation has not been characterised in detail.

The importance of Al^{3+} exclusion to the very high resistance of rice was confirmed after characterizing two Al^{3+}-sensitive mutations, *als1* and *c68,* because both of these recessive mutations lead to increased accumulation of Al^{3+} in the roots (Huang et al. 2009; Ma et al. 2005). *als1* carries a mutation in a gene encoding part of an ATP binding cassette (ABC) transporter (see later) while the *c68* mutation remains uncharacterized at the genetic level.

The exclusion mechanism for which most supporting evidence is available is the release of organic anions from roots (Delhaize et al. 2007; Ma et al. 2001; Ryan et al. 2001). Malate and citrate are the two anions most commonly reported but oxalate efflux occurs from a few species. Once these anions are released from root cells they bind the Al^{3+} and prevent it from accumulating in the apoplast, damaging the cells and being absorbed by the roots. Efflux is largely restricted to the root apices and in nearly all cases it does not occur continuously but is activated by exposure to Al^{3+}. The effectiveness of these anions in reducing Al^{3+} toxicity is demonstrated by adding them to solutions containing toxic concentrations of Al^{3+}. Root growth improves as the anion concentration increases. This occurs for malate, citrate and oxalate additions but not for anions, such as succinate and acetate, which have lower stability constants for Al^{3+} (Ryan et al. 2001). This exclusion mechanism has now been reported in species from the Poaceae (e.g. wheat, barley, sorghum, maize and rye), Araceae (e.g. taro), Polygonaceae (e.g. buckwheat), Brassicaceae (e.g. *Arabidopsis*) and the Fabaceae (e.g. soybean, snapbean, common bean, *Cassia tora*).

The first study linking organic anion efflux with Al^{3+} resistance was described by Miyasaka et al. (1991). They showed that Al^{3+} activated citrate exudation from snapbean roots and that the efflux from a resistant cultivar was 10-fold greater than efflux from a sensitive cultivar. Another example was reported soon after in wheat by Delhaize et al.(1993b) and Ryan et al. (1995a) using near-isogenic wheat lines differing in Al^{3+} resistance. These studies showed that addition of Al^{3+} to a nutrient solution rapidly stimulated malate release from the root apices of the resistant iso-line but not from the sensitive line. This rapid activation of efflux is termed a Type I response (**Figure 2**). Type I responses are interpreted as Al^{3+} activating a transport protein already present in the plasma membrane so little or no delay occurs (Ma et al. 2001). An F_2 population generated from these near-isogenic lines demonstrated that resistance co-segregates with malate efflux. Subsequent analyses revealed a strong positive correlation between malate efflux and Al^{3+} resistance in diverse germplasm which supports the importance of this major trait in wheat (Raman et al. 1995a, b; Raman et al. 2005). Al^{3+} resistance in barley is correlated with citrate efflux from roots. Organic anion efflux does not appear to be important contributor to the high resistance of rice but it does appear to be a minor contributor in maize. Several maize genotypes display an Al^{3+}-activated efflux of citrate but the efflux is delayed by several hours after Al^{3+} addition. This is referred to as a Type II response (**Figure 2**). The delay is interpreted as Al^{3+} first inducing expression of the transport protein before then activating anion efflux (Ma et al. 2001). Type II responses have also been reported for citrate efflux from *Cassia tora,* rice bean, and rye (Ma et al. 2001; Yang et al 2006). Some maize genotypes also show a slower Al^{3+}-inducible increase of citrate content suggesting that Al^{3+} resistance may also rely on internal detoxification (Pineros et al. 2002). Nevertheless no clear correlation has been established between citrate exudation and Al^{3+} resistance among a large range of maize genotypes (Pineros et al. 2002) which supports a model where several different mechanisms contribute to Al^{3+} resistance in this species.

5.2 Mechanisms of Al^{3+} tolerance

Instead of excluding Al^{3+} from their tissues, many highly-tolerant species absorb Al^{3+} and store it in their leaves sometimes to concentrations exceeding 3000 mg/kg. This relies on quite different processes involving complexation, detoxification and transport of aluminium within the plant. Aluminium accumulator species are defined as those with 1000 mg/kg aluminium or more in their leaves. Some of these species include tea (*Camelia sinensis*), *Hydrangea sp* and buckwheat (*Fagopyrum esculentum*) as well as a range of tree and shrub species (Haridasan and Dearaujo 1988). Most of the aluminium in tea leaves resides in the apoplast (Tolra et al. 2011) whereas in the leaves of *Hydrangea* and buckwheat the aluminium is bound in vacuoles by citrate and oxalate anions, respectively. *Hydrangea* is an ornamental plant that changes the colour of its flowers from pink to blue when grown in acid soils with high Al^{3+} availability (Ma et al. 1997). This colour change is caused by the formation of aluminium delphinidin complexes or aluminium caffeolylquinate complexes (Takeda et al. 1985). Buckwheat can accumulate 15,000 mg/kg aluminium in its leaves when grown in acid soils (Ma et al. 1997).

High shoot accumulation of aluminium implies soluble aluminium is transported through the xylem and then stored safely in leaf vacuoles or in the apoplast. To protect the plant cells from damage aluminium is bound by organic ligands as it is transported throughout the plant. ^{27}Al NMR studies identified aluminium oxalate complexes (1:3) in buckwheat leaves

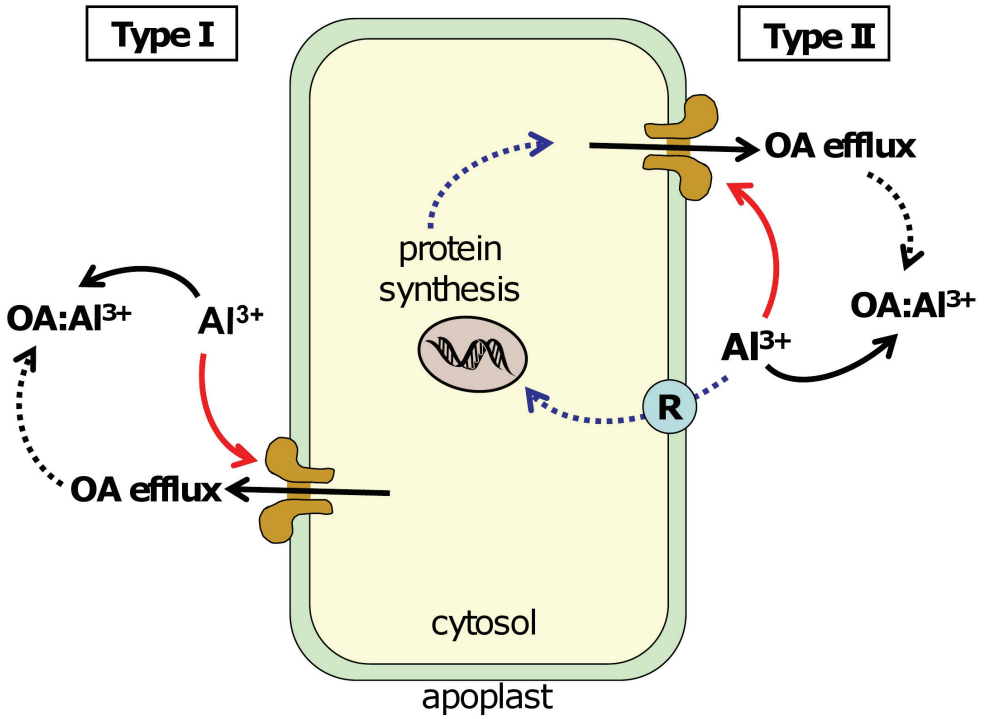

Fig. 2. Al^{3+}-activated organic anion efflux. The Type I response illustrates the rapid activation of organic anion efflux in species such as wheat where the anion channel is constitutively expressed. Al^{3+} is able to rapidly activate efflux by interacting directly with the pre-existing proteins (red arrows). The Type II response occurs in maize and rye and shows a delay between the addition of Al^{3+} and the start of organic anion efflux. This delay is interpreted as Al^{3+} first inducing the expression of the transport protein via a signal transduction pathway possibly involving a specific receptor ("R")(blue arrows). Once synthesized and inserted in the plasma membrane, Al^{3+} is thought to interact with the protein to activate efflux of organic anion (OA).

(Ma et al. 2001), but aluminium citrate complexes in the xylem (Ma and Hiradate 2000). It appears that aluminium undergoes a ligand exchange with oxalate and citrate depending on whether it is transported into xylem or being sequestered in the leaves.

6. Identification of Al^{3+}-resistance genes in plants

Several Al^{3+} resistance genes have now been mapped and cloned from a range of species (**Table 1**). Ryan et al. (2011) classifies these resistance genes into three groups: (1) those isolated by analysing segregating populations and therefore explain genotypic variation, (2) those identified from mutant analysis and therefore do not necessarily explain genotypic variation, and (3) likely resistance genes which require additional supporting information.

Species	Genes	Protein Function	Reference
Organic transporters			
Wheat	*TaALMT1*	Malate transporter	(Sasaki et al. 2004)
Arabidopsis	*AtALMT1*	Malate transporter	(Hoekenga et al. 2006)
Sorghum	*SbMATE*	Citrate transporter	(Magalhaes et al. 2007)
Barley	*HvAACT1*	Citrate transporter	(Furukawa et al. 2007)
Rye	*ScALMT1 gene cluster*	Malate transporter	(Collins et al. 2008)
Arabidopsis	*AtMATE1*	Citrate transporter	(Liu et al. 2009)
Maize	*ZmMATE1*	Citrate transporter	(Maron et al. 2010)
ABC transporters and other proteins			
Arabidopsis	*AtSTOP1*	C_2H_2-type Zn finger transcription factor	(Iuchi et al. 2007)
Arabidopsis	*AtSTAR1*	ABC transporter-basic detoxification of Al	(Huang et al. 2010)
Arabidopsis	*ALS1*	Half ABC transporter	(Larsen et al. 2007)
Arabidopsis	*ALS3*	Half ABC transporter	(Larsen et al. 2005)
Rice	*ART1*	C_2H_2-type Zn finger transcription factor	(Yamaji et al. 2009)
Rice	*STAR1,STAR2*	ABC transporter-UDP-glucose transport	(Huang et al. 2009)
Likely Al^{3+} resistance gene			
Wheat	*TaMATE1*	Citrate transporter	(Ryan et al. 2009)
Rye	*ScMATE2*	Citrate transporter	(Yokosho et al. 2010)
Brassica napus	*BnALMT1*	Malate transporter	(Ligaba et al. 2006)
	BnALMT2	Malate transporter	

Table 1. Al^{3+} resistance genes in plants

6.1 Organic anion transporters

The genes controlling organic anion efflux from roots were the first Al^{3+} resistance genes to be isolated from plants. Those controlling malate efflux belong to the *ALMT* (aluminium activated malate transporter) family of genes and those controlling citrate efflux belong to the *MATE* (multi-drug and toxic compound extrusion) family of genes. The genes controlling oxalate efflux are still unknown. The first aluminium resistance gene cloned from plants was the wheat gene *TaALMT1* which encodes an Al^{3+}-activated malate channel (Sasaki et al. 2004). *TaALMT1* was identified by cDNA subtractive hybridization using near-isogenic wheat lines ET8 (resistant) and ES8 (sensitive). *TaALMT1* was identified for being more highly expressed in root tips of ET8 than of ES8 and its expression co-segregated with Al^{3+} resistance in a segregating population. Heterologus expression of *TaALMT1* in *Xenopus laevis* oocytes, tobacco suspension cells, barley, wheat and *Arabidopsis* all generate the same phenotype: an Al^{3+}-

activated efflux of malate (Sasaki et al. 2004; Delhaize et al. 2004; Pereira et al. 2010; Ryan et al. 2011). Al^{3+} resistance was not related to the coding alleles of *TaALMT1*, but to the level of expression (Raman et al. 2005). Polymorphisms detected in the promoter of *TaALMT1* are correlated with Al^{3+} resistance. Tandem repeats in the promoter of resistance genotypes explain the higher expression in resistant plants such that the larger the number of repeats the higher the expression and this is correlated with greater malate efflux (Ryan et al. 2010; Sasaki et al. 2006). Homologues of *TaALMT1* also control Al^{3+} resistance in *Arabidopsis* (Hoekenga et al. 2006) and rye (Collins et al. 2008). However not all *ALMT* genes confer Al^{3+} resistance because members of this family in barley and *Arabidopsis* (*HvALMT*, *AtALMT9* and *AtALMT12*) have other functions on the tonoplast of leaves and in guard cells (Gruber et al. 2011; Kovermann et al. 2007; Sasaki et al. 2010).

The first *MATE* gene involved in Al^{3+} resistance was cloned in sorghum by positional cloning (Magalhaes et al. 2007). *SbMATE* encodes a transport protein located on the plasma membrane that facilitates citrate release from the root cells. *SbMATE* is constitutively expressed in the root apices of resistant sorghum lines but Al^{3+} treatment increases expression over hours and days and this change parallels the increase in citrate efflux. Interestingly, the coding regions of *SbMATE* in the sensitive and resistant genotypes are identical, with polymorphisms in one of the introns only. It will be interesting to discover how *SbMATE* expression is controlled in sorghum.

MATE genes also control the citrate efflux from Al^{3+}-resistant barley plants and *Arabidopsis*. The *HvAACT1* gene from barley (also known as *HvMATE*) was also isolated by positional cloning (Furukawa et al. 2007). Like *TaALMT1* in wheat, *HvAACT1* is constitutively expressed in roots but Al^{3+} is still required to activate citrate efflux. Unlike *TaALMT1* and *SbMATE*, *HvAACT1* expression is higher slightly behind the root apices which may influence its effectiveness. Liu et al. (2009) showed that knock-out mutations in *AtMATE* prevent the Al^{3+}-activated efflux of citrate but the contribution of *AtMATE* to the resistance of this species is relatively small compared to the malate channel AtALMT1.

Al^{3+} resistance in maize is likely to involve several mechanisms. Nevertheless citrate efflux does contribute and Maron et al. (2010) isolated a *MATE* gene called *ZmMATE1* which co-localizes with a major QTL for Al^{3+} resistance. *ZmMATE1* is mainly expressed in roots, is up-regulated by Al^{3+} and shows higher expression in Al^{3+}-resistant genotypes. *ZmMATE1* elicits anion efflux when expressed in *Xenopus oocytes* and measurements with labeled [^{14}C]-citrate confirmed ZmMATE1 transports citrate (Maron et al. 2010).

Other candidate genes are likely to control Al^{3+} resistance but need confirmation. For instance, *TaMATE1* expression in wheat segregates with citrate efflux in some Brazillian genotypes but it needs to be demonstrated directly that TaMATE is a citrate transporter (Ryan et al. 2009). The citrate efflux from these few genotypes of wheat differs from other species in one important way: it occurs constitutively and does not require Al^{3+} to activate it. Two genes from *Brassica napus*, *BnALMT1* and *BnALMT2*, encode functional malate transporters in *Xenopus* oocytes and their expression is induced by Al^{3+} but no genetic analysis or knockout mutants have confirmed that they contrbute to Al^{3+} resistance.

6.2 Other resistance genes

A different set of Al^{3+} resistance genes was identified using mutant analysis (**Table 1**). This approach requires no prior knowledge regarding genetics or mechanisms involved. Mutagenized seed is generated by chemical treatments, radiation or the random insertion of a DNA fragment (T-DNA or transposon) into the genome. M2 seedlings are screened and those

that grow similar to wild-type plants under control conditions, but show altered responses to Al^{3+} stress, are selected for further analysis. Candidate genes can be isolated by mapping or obtaining the sequence flanking the T-DNA region and analysed further. The candidate genes can be characterized by overexpression studies, knockout studies, mutant analysis or association analysis. These genes need not show allelic variation within natural populations.

Using this approach Huang et al. (2009) cloned two genes from rice called *STAR1* and *STAR2* (sensitive to Al rhizotoxicity) which cause plants to be hypersensitive to Al^{3+} toxicity when knocked out. Both genes are expressed in roots and induced by Al^{3+} treatment. *STAR1* encodes a nucleotide binding domain of bacterial-type ATP binding cassette (ABC) transporter and *STAR2* encodes the transmembrane domain for an ABC transporter. Huang et al (2009) demonstrated that *STAR1* and *STAR2* interact to form a functional ABC transporter which localizes to vesicles of most root cells except for those in the epidermal layer of the mature zone. *Xenopus laevis* oocytes expressing *STAR1/STAR2* can transport UDP-glucose but a more recent study shows that STAR1 is also involved in nicotianamine transport, a secondary metabolite used for the long-distance transport of Fe^{3+} in plants. The role of STAR1/STAR2 in Al^{3+} resistance remains unclear but it could be involved with releasing compounds that modify the cell wall during Al^{3+} stress.

A homologue of *STAR1* in *Arabidopsis*, called *AtSTAR1*, also encodes an ATP-binding domain of a bacterial-type ABC transporter (Huang et al. 2010). A line carrying a knockout mutation showed increased sensitivity to Al^{3+} and early flowering. Unlike *OsSTAR1*, *AtSTAR1* is expressed in both the roots and shoots, and its expression is not induced by Al^{3+} stress. AtSTAR1 may interact with another protein called ALS3 to form a functional ABC transporter. *ALS3* had been identified previously in similar screens in Arabidopsis because plants carrying loss-of-function mutations are more sensitive to Al^{3+} stress (Larsen et al. 2005; Larsen et al. 2007).

STOP1 (sensitive to protons) encodes a transcription factor identified by analysing *Arabidopisis* mutants which are hypersensitive to H^+ toxicity. STOP1 belongs to C_2H_2-type zinc finger family of proteins. *stop1* mutants are more sensitive to Al^{3+} but not to a range of other cations including cadmium, copper, lanthanum, manganese and sodium. STOP1 is required for the induction of a range of genes including *AtALMT1* which encodes the malate transporter. *STOP1* plays a critical role in enabling *Arabidopsis* to resist stress induced by low pH and Al^{3+} toxicity (Iuchi et al. 2007).

7. Transgenic approaches for increasing Al^{3+} resistance

The increasing demands for food from a growing world population highlight the need to overcome the major soil constraints currently limiting crop yields. For acid soils, the application of lime (calcium carbonate) can increase the soil pH but this usually only changes the surface pH in the year of application and it can take decades for acidity to be neutralized at depth. Additionally, in third world countries it can be prohibitively expensive to apply sufficient lime to neutralize soil acidity. Increasing the acid soil tolerance by conventional breeding has been successfully applied to several crop species and this complements liming practices as a way of managing acid soils. However, some species lack sufficient variation in their germplasm and genetic modification provides another avenue for increasing their acid soil tolerance. As described above the mechanisms of Al^{3+} resistance in species, such as wheat, sorghum and barley have been elucidated and the genes underlying these mechanisms have been isolated. These genes have been used to generate

transgenic plants with enhanced Al^{3+} resistance. A range of other genes, not necessarily responsible for natural variation in Al^{3+} resistance, have also been used to enhance the Al^{3+} resistance of plants. The following discussion summarises these recent attempts to enhance Al^{3+} resistance using biotechnology (see **Table 2**).

7.1 Over-expression of genes involved in organic anion biosynthesis

The important role of organic anion efflux in Al^{3+} resistance was established 20 years ago, more than a decade before the genes controlling this trait were cloned. Therefore the first attempts to increase organic anion efflux to improve Al^{3+} resistance focused on increasing organic anion synthesis because the key enzymes and genes involved in those pathways were well known (**Table 2**). This approach was based on the idea that an increased concentration of organic anions in the cytosol would result in increased organic anion transport across the plasma membrane. The underlying assumption was that transport of organic anions across the plasma membrane is not the rate-limiting step for efflux. Citrate synthase was a sensible starting point due to the known role of citrate in the Al^{3+} resistance of *Cassia tora*, maize, rye and snapbean (Ryan et al., 2001; Ma et al., 2001). Citrate synthase is the first enzyme involved in the tricarboxylic acid and glyoxylate cycles. De la Fuente et al. (1997) transformed tobacco and papaya with the citrate synthase gene (CS) from the bacterium *Pseudomonas aeruginosa* to increase the biosynthesis and efflux of citrate for enhanced Al^{3+} resistance. When homozygous lines of tobacco expressing the CS gene were analyzed they were found to accumulate up to 10 fold more citrate than the wildtype plants. Citrate efflux of the transgenics was increased four fold over wildtype and this was associated with enhanced Al^{3+} resistance. Similar results were reported for transgenic papaya expressing the same transgene. However, subsequent work by Delhaize et al. (2001) was not able to repeat these findings on the same tobacco lines or even when the gene was expressed to a much greater level. More recently other groups have reported enhanced Al^{3+} resistance when CS expression was increased in alfalfa (Barone et al. 2008), *Arabidopsis* (Koyama et al. 2000; Koyama et al. 1999) and tobacco (Han et al. 2009). In most of these cases the increases in Al^{3+} resistance were marginal except for tobacco transformed with a rice CS gene where transgenic lines showed up to 4.5-fold greater Al^{3+} resistance than the wildtype.

Malate dehydrogenase (MDH) which oxidises oxaloacetate to form malate is another enzyme involved in organic anion biosynthesis and this gene has now been over-expressed in several species. An MDH gene highly expressed in root nodules of alfalfa (*neMDH*) was over-expressed in alfalfa and this was associated with enhanced malate efflux and greater Al^{3+} resistance (Tesfaye et al. 2001). Similarly, when MDH genes from *Arabidopsis* and *Escherichia coli* were expressed in tobacco, the transgenic plants showed enhanced malate efflux and improved Al^{3+} resistance (Wang et al. 2010).

7.2 Over-expression of genes involved in organic anion transport

Once the Al^{3+} resistance genes controlling organic anion efflux were identified and cloned they were transformed into plants (**Table 2**). These genes belong to the *MATE* or *ALMT* gene families and they encode transport proteins that mediate organic anion movement across the plasma membrane to the external medium.

TaALMT1 has now been expressed in several species and in nearly all cases the transgenic plants showed Al^{3+}-activated malate efflux and enhanced Al^{3+} resistance. The one exception was rice, where *TaALMT1* expression conferred Al^{3+}-activated malate efflux but not

Gene function	Source of gene	Species transformed	Phenotype (RRG)	Reference
Organic anion metabolism				
Citrate synthase	*Pseudomonas aeruginosa*	Tobacco and papaya	2-fold	(De la Fuente et al. 1997)
Citrate synthase (*AtCS*)	*Arabidopsis*	Carrot	1.3-fold	(Koyama et al. 1999)
Citrate synthase (*DcCS*)	Carrot	*Arabidopsis*	1.2-fold	(Koyama et al. 2000)
Citrate synthase (*OsCS1*)	Rice	Tobacco	4.5-fold	(Han et al. 2009)
Citrate synthase	*Pseudomonas aeruginosa*	Alfalfa	2.5-fold	(Barone et al. 2008)
Citrate synthase (*AtmtCS*)	*Arabidopsis*	Canola	2-fold	(Anoop et al. 2003)
Malate dehydrogenase	Alfalfa	Alfalfa	2-fold	(Tesfaye et al. 2001)
Malate dehydrogenase	Arabidospis *E coli.*	Tobacco	2.4-fold	(Wang et al. 2010)
Blue-copper-binding protein gene (*AtBCB*)	*Arabidopsis*	*Arabidopsis*	1.7-fold	(Ezaki et al. 2000)
Stress response				
Glutathione S-transferase gene (*parB*)	Tobacco	*Arabidopsis*	1.7-fold	(Ezaki et al. 2000)
Peroxidase gene (*NtPox*)	Tobacco	*Arabidopsis*	1.7-fold	(Ezaki et al. 2000)
GDP-dissociation inhibitor gene (*NtGDI1*)	Tobacco	*Arabidopsis*	1.7-fold	(Ezaki et al. 2000)
Dehydroascorbate reductase	*Arabidopsis*	tobacco	1.5-fold	(Yin et al. 2010)
Manganese superoxide dismutase	wheat	*Brassica napus*	2.5-fold	(Basu et al. 2001)
Organic anion transporter				
TaALMT1	wheat	wheat	8-fold	(Pereira et al. 2010)
TaALMT1	wheat	barley	20-fold	(Delhaize et al. 2004)
TaALMT1	wheat	*Arabidopsis*	4-fold	(Ryan et al. 2011)

SbMATE	sorghum	*Arabidopsis*	2.5-fold	(Magalhaes et al. 2007)
Frd3	*Arabidopsis*	*Arabidopsis*	2-fold	(Durrett et al. 2007)
ZmMATE1	maize	*Arabidopsis*	3-fold	(Maron et al. 2010)
HvAACT1	barley	tobacco	2-fold	(Furukawa et al. 2007)

Phenotype (RRG) shows the reported increase in Al^{3+} resistance of the transgenic plants based on measurement of relative root growth (RRG).

Table 2. Studies which have used biotechnology to increase Al^{3+} resistance in plants.

enhanced Al^{3+} resistance (Sasaki et al. 2004). The inability of *TaALMT1* to increase resistance in this case was attributed to the very high endogenous level of Al^{3+} resistance of rice.
Barley is among the most Al^{3+}-sensitive cereal crops but the small genotypic variation in resistance that does occur is correlated with low rates of citrate release, but not malate efflux (see above). Expression of *TaALMT1* in barley was associated with increased Al^{3+}-activated malate efflux and a significant increase in Al^{3+} resistance when compared to wildtype plants and null segregant lines (Delhaize et al. 2004). The transgenic barley showed enhanced Al^{3+} resistance when grown in both hydroponic culture and in acid soil. In hydroponic culture root growth of transgenics was more than 10-fold greater than wildtype (Delhaize et al. 2004). More recently it was shown that these transgenic barley had enhanced phosphorus-use efficiency and improved grain yield when grown on an acid soil (Delhaize et al. 2009).
Similarly Al^{3+}-activated malate efflux and Al^{3+} resistance were enhanced when *TaALMT1* was over-experessed in wheat (Pereira et al. 2010) and *Arabidopsis* (Ryan et al. 2011). Some of the transgenic wheat lines displayed greater Al^{3+} resistance than ET8 (the source of the *TaALMT1* gene) in both hydroponic and soil experiments (Pereira et al. 2010).
MATE genes encoding citrate transporter proteins in sorghum (*SbMATE*), barley (*HvAACT1*), maize (*ZmMATE1*) and *Arabidopsis* (*AtMATE* and *Frd3*) were transformed into Arabidopsis or tobacco plants (Durrett et al. 2007; Furukawa et al. 2007; Magalhaes et al. 2007; Maron et al. 2010). *Frd3* is not an Al^{3+}-resistance gene but it does encode a transporter which releases citrate into the xylem to assist iron movement to the shoots. In all cases these genes increased citrate efflux and enhanced Al^{3+} resistance of the transgenic plants.
These findings indicate that the *MATE* and *ALMT* genes can be effectively used to enhance the Al^{3+} resistance of not only model species, but also important crop species. The observation that organic anion efflux can be increased by expression of a transport protein suggests that biosynthesis of organic anions is not a limiting factor for many plant species. To date, the transport proteins, and TaALMT1 in particular, have provided the most effective means to increase the Al^{3+} resistance of plants.

7.3 Genes not associated with organic anions

One of the first biotechnological strategies to increase Al^{3+} resistance sought to over-express genes induced by Al^{3+} stress, and especially those involved in combating oxidative stress.
Ezaki et al. (2000) first identified a range of genes whose expression is induced by Al and then overexpressed these genes in *Arabidopsis*. They found that an *Arabidopsis* blue-copper-binding

protein gene (*AtBCB*), a tobacco glutathione S-transferase gene (*parB*), a tobacco peroxidase gene (*NtPox*) and a tobacco GDP-dissociation inhibitor gene (*NtGDI1*) conferred a degree of tolerance to Al^{3+} when over-expressed. In particular, overexpression of the *parB* gene simultaneously conferred resistance to both Al^{3+} and oxidative stresses. Other stress-related genes, such as dehydroascorbate reductase from *Arabidopsis* and manganese superoxide dismutase from wheat, were expressed in tobacco and *Brassica napus*, respectively with the transgenic plants showing enhanced Al^{3+} tolerance (Basu et al. 2001; Yin et al. 2010). Overexpression of these stress-related genes in transgenic plants exhibited a 1.5-2.5-fold increase in relative root growth compared to wildtype.

Genes encoding proteins involved in various stress responses, endocytosis, lipid biosynthesis or Al-induced programmed cell death have also conferred a degree of Al^{3+} tolerance when over-expressed in *Arabidopsis* or tobacco. These genes encode WAK1, a auxilin-like protein, a Δ8 sphingolipid desaturase and a Ced-2 protein (Ezaki et al. 2000; Ryan et al. 2007; Sivaguru et al. 2003a; Wang et al. 2009). The details of how these genes function to confer Al^{3+} resistance are not well understood and it is not yet clear that they would be sufficiently effective to enhance the Al^{3+} resistance of crop species.

8. Conclusions

Much information has been gathered on the mechanisms of Al^{3+} toxicity and tolerance over the last 20 years. Our understanding of the mechanisms involving organic anion release is more complete than other mechanisms operating in species like rice and maize. Genes belonging to the *MATE* and *ALMT* families encode organic anion transport proteins that facilitate anion efflux from the roots. Transgenic plants expressing these genes show increased organic efflux and significantly greater resistance to Al^{3+} stress. Strategies based on enhanced efflux of organic anions appear to be effective and combining them with Al^{3+} tolerance mechanisms that act within the plant could provide even greater protection from Al^{3+} toxicity. These advances pave the way for biotechnological approaches to enhance the acid-soil tolerance of important food crops through genetic engineering and by marker-assisted selection in traditional breeding programs.

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