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Plant improvement for tolerance to aluminum in acid soils – a review

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Abstract

Development of acid soils that limit crop production is an increasing problem worldwide. Many factors contribute to phytotoxicity of these soils, however, in acid soils with a high mineral content, aluminum (Al) is the major cause of toxicity. The target of Al toxicity is the root tip, in which Al exposure causes inhibition of cell elongation and cell division, leading to root stunting accompanied by reduced water and nutrient uptake. Natural variation for Al tolerance has been identified in many crop species and in some crops tolerance to Al has been introduced into productive, well-adapted varieties. Aluminum tolerance appears to be a complex multigenic trait. Selection methodology remains a limiting factor in variety development as all methods have particular drawbacks. Molecular markers have been associated with Al tolerance genes or quantitative trait loci in *Arabidopsis* and in several crops, which should facilitate development of additional tolerant varieties. A variety of genes have been identified that are induced or repressed upon Al exposure. Most induced genes characterized so far are not specific to Al exposure but are also induced by other stress conditions. Ectopic over-expression of some of these genes has resulted in enhanced Al tolerance. Additionally, expression of genes involved in organic acid synthesis has resulted in enhanced production of organic acids and an associated increase in Al tolerance. This review summarizes the three main approaches that have been taken to develop crops with Al tolerance: recurrent selection and breeding, development of Al tolerant somaclonal variants and ectopic expression of transgenes to reduce Al uptake or limit damage to cells by Al.

Introduction

Acid soils that limit crop production are found throughout the world. An estimated 30–40% of the world's arable soils have a pH below 5.5 (von Uexküll and Mutert, 1995). A northern belt of acid soils occurring in the humid northern temperate zone is comprised of predominantly organic acid soils and supports coniferous forests. A southern belt of mineral acid soils occurs in the humid tropics. Currently approximately 12% of land in crop production is acidic (von Uexküll and Mutert, 1995), however, the extent of acid soil is increasing world-wide. Mineral acid soils result from parent materials that are acidic and naturally low in the basic cations (Ca^{2+} , Mg^{2+} , K^+ and Na^+), or because these elements are leached

from the soil, reducing pH and the buffering capacity of the soil. As soil pH decreases, aluminum (Al) is solubilized and the proportion of phytotoxic aluminum ions increases in the soil solution. In most mineral soils there is sufficient Al present to buffer the soil to around pH 4. Organic acid soils, consisting of large amounts of humic acids and partially decomposed plant matter, typically have little Al buffering and the pH of these soils can fall well below pH 4 (Kidd and Proctor, 2001). Superimposing agricultural production on an ecosystem, especially ammonium fertilization, accelerates soil acidification due to nitrification. In the US, this has become a serious problem in large wheat-producing areas of Oklahoma, Texas and Kansas, where N fertilization and removal of forage and grain has caused soil acidification (John-

son et al., 1997). Also, acid soils can be problematic in intensively managed pasture systems, where nitrogen fixation by legumes acidifies the rhizosphere due to excess cation uptake (Haynes, 1983; Bolan et al., 1991). Furthermore, acid rain, containing nitric and sulfuric acids, is increasing the rate of soil acidification in many locations worldwide. As many areas with acid soils also have human populations with increasing demands for crop production, it is imperative to continue to develop crop species with acid soil tolerance.

A number of factors contribute to acid soil toxicity depending on soil composition. In acid soils with a high mineral content, the primary factor limiting plant growth is Al toxicity. The Al released from soil minerals under acid conditions occurs as $\text{Al}(\text{OH})_2^+$, $\text{Al}(\text{OH})^{2+}$ and $\text{Al}(\text{H}_2\text{O})^{3+}$, the latter commonly referred to as Al^{3+} (Kinraide, 1991). For most agriculturally important plants, Al ions rapidly inhibit root growth at micromolar concentrations. In wheat the most toxic ion is Al^{3+} , while in dicots the more toxic ions appear to be $\text{Al}(\text{OH})_2^+$ and $\text{Al}(\text{OH})^{2+}$ (for review, see Delhaize and Ryan, 1995; Kochian, 1995). In some acid mineral soils, Mn toxicity may be more important than Al toxicity in limiting crop production (Sumner et al., 1991). Plants in acid soils also suffer from deficiencies in phosphorus, nitrogen, calcium, magnesium and potassium. For legumes, acid soils pose an additional challenge because their symbiotic rhizobia are acid sensitive (Hartel and Bouton, 1989). In organic acid soils, H^+ ions dominate in the soil solution. Although organic acid soils make up a large proportion of acid soils worldwide, the effect of H^+ ions on plant growth has not been well studied. In contrast to the large amount of literature on Al tolerance mechanisms in plants, little is known about how plants manage high H^+ ion concentrations. Limited studies indicate that adaptation to Al and H^+ are controlled by separate mechanisms (Lazof and Holland, 1999; Kidd and Proctor, 2001). Clearly, additional research on H^+ adaptation is needed.

The primary target of Al toxicity is the root apex. Aluminum affects a host of different cellular functions, frustrating attempts to identify the principal effect(s) of Al toxicity. Exposure to Al causes stunting of the primary root and inhibition of lateral root formation. Affected root tips are stubby due to inhibition of cell elongation and cell division. The resulting restricted root system is impaired in nutrient and water uptake, making the plant more susceptible to drought stress. Plants sensitive to Al toxicity have greatly reduced yield and crop quality.

Several strategies have been pursued to manage acid soils. The primary method in North America and Europe has been application of lime (calcium carbonate), to raise soil pH and cause conversion of Al to less toxic forms and application of phosphorus (P). Acid soils often have low levels of P in forms available for plant uptake and these soils can also absorb large amounts of added P by forming complexes with Al and iron hydrous oxides. However, these soil amendments are not practical in many locations, such as highly erodible slopes, nor are they economical where large areas require amendment or where transportation costs are prohibitive. In addition, soil pH below the plow layer is raised very slowly by liming (Dall'Agnol et al., 1996). Organic soil amendments have been shown to have a temporary ameliorative effect on Al toxicity. Humic molecules and organic acids complex with Al in the soil solution rendering it non-toxic. Incorporation of organic residues also causes a short-term increase soil pH, due to complexing of protons with organic acids and consumption of protons with decarboxylation of organic acids (Yan and Schubert, 2000; Haynes and Mokolobate, 2001). In many crop species, a range of Al tolerance has been identified and selective breeding programs have produced crop varieties with increased Al tolerance. Physiological and molecular characterization of these plants has led to a better understanding of the mechanisms of Al tolerance.

This review will focus on the techniques employed to increase Al tolerance in crop plants and the results of these efforts. Review topics include: screening methods to identify Al-tolerant germplasm, selection *in vitro* of Al tolerant cells and plants, identification of Al tolerance genes and genetic modification of plants with genes to confer enhanced Al tolerance. The effects of Al on plant metabolism and mechanisms of Al tolerance will be covered briefly, as there are many excellent recent reviews on these topics (Carver and Ownby, 1995; Delhaize and Ryan, 1995; Kochian, 1995; Kochian and Jones, 1997; de la Fuente-Martínez and Herrera-Estrella, 1999; López-Bucio et al., 2000; Matsumoto, 2000; Ma et al., 2001; Rout et al., 2001; Ryan et al., 2001; Barceló and Poschenrieder, 2002; Čiamporová, 2002; Kochian et al., 2002; Watanabe and Osaki, 2002).

Effects of aluminum on plant roots

Aluminum rapidly affects a number of cellular functions. Accordingly, it has been difficult to distinguish

the primary effect of Al on plant cells and unravel the causes from the consequences of toxicity. Indeed, the principal cause of toxicity has not been unambiguously identified. An understanding of Al toxicity and mechanisms of tolerance is important for developing appropriate tests for selecting tolerant germplasm and for developing plants with enhanced performance in acid soils.

In seedlings, it is clear that the root tip is the most Al-sensitive part of the root. Experiments in which maize seedling roots were placed in divided chambers showed that root growth is inhibited only when the apical 2–3 mm of the root is exposed to Al. Application of Al to any other portion of the root does not affect root growth (Ryan et al., 1993). The root tip also accumulates more Al than other portions of the root (for review, see Kochian, 1995; Čiamporová, 2002). Whether Al targets cell division or cell elongation has been a matter of controversy. Several lines of evidence support inhibition of root growth due to interference with cell elongation. A number of studies have shown that inhibition of root growth occurs rapidly (minutes to hours) after exposure to Al, while inhibition of cell division requires 6–24 h to occur (for review, see Čiamporová, 2002). The cells in the distal portion of the transition zone (DTZ) of the maize root apex, which have recently divided but not yet started to elongate, are the most active in Al uptake and the most sensitive to Al toxicity (Sivaguru and Horst, 1998; Kollmeier et al., 2000). Walls of the cells in this zone accumulate Al much more rapidly than older cells, due to a higher proportion of pectin in the cell walls (Čiamporová, 2002). Aluminum binding to root cell walls displaces calcium in the pectic fraction, which inhibits wall extension (Carver and Ownby, 1995; Blamey et al., 1997; Rengel and Reid, 1997; Ryan et al., 1997). Al treatment has also been shown to rapidly inhibit auxin flow in cells of the DTZ of Al-sensitive maize roots, which was associated with inhibition of root elongation (Kollmeier et al., 2000). Some plants respond to Al by increasing synthesis of hemicellulose, cellulose and pectin (Tabuchi and Matsumoto, 2001; Teraoka et al., 2002). Although these carbohydrates may help to 'trap' Al in the apoplasm, they may further interfere with cell elongation. In addition, Al-induced inhibition of K^+ uptake by blocking inward K^+ channels would interfere with turgor-driven cell elongation (Liu and Luan, 2001). Finally, in response to aluminum, developing root cells accumulate callose, suggesting that the inhibition of root elongation is due in part to use of sugar substrates for callose formation instead of cellu-

lose formation (Kaneko et al., 1999). Aluminum-induced callose has been shown to be deposited in plasmodesmata, which could block cell-to-cell movement of molecules and interfere with intercellular communication (Sivaguru et al., 2000).

In addition to rapid accumulation in cell walls and the apoplasm of roots, aluminum rapidly accumulates in the plasma membrane and the symplasm of sensitive plants, affecting many processes involved in root growth (for review, see Delhaize and Ryan, 1995; Kochian, 1995; Kochian and Jones, 1997; Rout et al., 2001; Čiamporová, 2002). The plasma membrane has been proposed as the primary site of Al toxicity (Huang et al., 1995; Sasaki et al., 1995; Wagatsuma et al., 1995; Horst et al., 1999). Aluminum binds strongly to the plasma membrane and may cause membrane depolarization which triggers a cascade of events (Blancaflor et al., 1998; Sivaguru et al., 1999). In sensitive maize roots, depolarization of the plasma membrane potential of the outer cortex cells of the DTZ occurs upon contact with Al. How Al enters the cytoplasm is not known although transport across the membrane by a calcium channel has been suggested (Liu and Luan, 2001). Al has been shown to accumulate in the symplast (Lazof et al., 1996) and nuclei of root tip cells within 30 min of Al treatment of a sensitive genotype (Silva et al., 2000). Within 1 h of exposure, microtubules of the cytoskeleton in these cells disintegrate and callose is deposited in cell walls (Sivaguru et al., 1999). Aluminum treatment also rapidly affects cytosolic free calcium concentrations (Jones et al., 1998; Plieth et al., 1999). Sivaguru et al. (1999) suggest that disruptions in calcium homeostasis and Al inhibition of phospholipase C activity may affect the cytoskeleton structure and function. Inhibition of phospholipase C may also disrupt the inositol 1,4,5-triphosphate signal transduction pathway (Jones and Kochian, 1995).

Aluminum toxicity is associated with alterations in a number of physiological processes and biochemical pathways after cessation of cell elongation. Aluminum is very reactive, with a strong binding affinity for proteins, inorganic phosphate, nucleotides, RNA, DNA, carboxylic acids, phospholipids, flavonoids and anthocyanins (Taylor, 1991; Delhaize and Ryan, 1995). Later stages of Al toxicity are associated with lipid peroxidation (Yamamoto et al., 2001) and oxidative stress. Mitochondrial activity was repressed in cultured tobacco cells and pea roots treated with Al followed by inhibition of respiration, depletion of ATP and production of reactive oxygen species at later stages (Yamamoto et al., 2002). Due to the many

cellular responses to Al treatment, a number of changes in gene expression have been associated with Al treatment including genes associated with oxidative stress and pathogen invasion (Cruz-Ortega et al., 1997; Hamel et al., 1998; Richards et al., 1998; Rodriguez Milla et al., 2002). Because pathogen invasion leads to production of active oxygen species and general oxidative stress, it is perhaps not surprising that disease defense response genes are expressed in response to Al treatment.

Tolerance mechanisms

The genetic and physiological basis of Al-tolerance has been investigated in several crop and model plant species in which both Al sensitivity and tolerance has been observed. As proposed by Taylor (1991), the tolerance strategies identified can be separated into those involved in exclusion of Al from the root apex and mechanisms that allow the plant to tolerate Al within cells. A wealth of studies provide very strong evidence that Al-tolerant genotypes of wheat, corn, sunflower, soybean and common bean, among others, exclude Al from roots by excretion of organic acids that chelate Al (for review, see Jones, 1998; López-Bucio et al., 2000; Ma, 2000; Ma et al., 2001; Ryan et al., 2001; Kochian et al., 2002; Watanabe and Osaki, 2002). Of the organic acids, citrate has the highest binding activity for Al followed by oxalate, malate and succinate (Hue et al., 1986). Activation of organic acid efflux occurs rapidly without any measurable delay after exposure to Al in several plant species, including wheat, in which it has been very well studied (Ryan et al., 1995; for review, see Ryan et al., 2001). In some other species, a lag in efflux is observed, suggesting a requirement for gene induction and protein synthesis (Ma et al., 2001; Ryan et al., 2001; Piñeros et al., 2002). There is strong evidence that malate exudation from wheat and citrate exudation from corn roots in response to Al occurs by activation of an anion channel located in the plasma membrane (Piñeros and Kochian, 2001; Ryan et al., 2001; Zhang et al., 2001).

Although organic acids have been shown to have a central role in exclusion of Al, additional exclusion mechanisms have been identified. Exposure of the Al-tolerant *Arabidopsis* mutant *alr-104* to Al induces a 2-fold increase in H⁺ influx at root tips resulting in a net increase in the root surface pH (Degenhardt et al., 1998), which could precipitate Al from the soil solu-

tion, inhibiting it from entering roots, or may shift the equilibrium to less toxic Al ions. In cowpea, root cap mucilage was shown to bind Al and removal of mucilage increased the sensitivity of roots to Al (Horst et al., 1982). The mucilage of wheat roots also binds Al (Archambault et al., 1996). Henderson and Ownby (1991) correlated the amount of mucilage produced by wheat roots to Al tolerance and suggested that mucilage aided in forming a diffusion barrier to Al or concentrated organic acids that chelated Al. The mucilage from maize roots has been shown to bind Al (Li et al., 2000) but did not protect roots from Al toxicity in hydroponic assays. They suggest that lack of protection is due to the distance between the root cap, the site of mucilage production, and the Al-sensitive cells in the DTZ. The role of mucilage in exclusion of Al from roots in soil requires further investigation. Exclusion of Al from tolerant wheat roots by localized excretion of phosphate at the root apex to precipitate Al was proposed by Pellet et al. (1995, 1997). Recent studies indicate that phosphate efflux does not play a significant role in Al tolerance in wheat (Tang et al., 2002). Other mechanisms of Al exclusion include binding of Al by secreted proteins (Basu et al., 1999) and selective permeability of the plasma membrane as a barrier to movement of Al into the cytosol (Archambault et al., 1997).

Mechanisms of cellular tolerance have been less well studied. Both Al-tolerant and Al-sensitive plants accumulate Al when grown in acid soils rich in Al (Foy et al., 1992, 1993; Gaume et al., 2001; Jensen et al., 2002; Watanabe and Osaki, 2002). In Al-accumulating plants Al is associated with organic ligands such as catechin, phenolic acids and organic acids and these complexes are often sequestered in specialized cells such as leaf epidermal cells (for review, see Jensen et al., 2002; Watanabe and Osaki, 2002). Aluminum-tolerant varieties of buckwheat secrete oxalic acid from roots in response to Al and also accumulate a non-toxic Al-oxalate complex in leaf cells (Ma et al., 1998). Interestingly, Al is transported in the xylem as a complex with citrate (Ma and Hiradate, 2000). In a tolerant maize variety, Al accumulates in root cell vacuoles (Vásquez et al., 1999). Recently, Delisle et al. (2001) have proposed a tolerance mechanism in wheat based on accelerated turnover of root epidermal cells. Within 8 h of exposure to an Al culture solution, they observed a punctated pattern of cell death in the root epidermis in the tolerant variety. Cell death was not observed in the

sensitive variety at a concentration of Al that resulted in a similar amount of root growth inhibition. They suggest cell death is aimed at replacing epidermal cells intoxicated with Al while maintaining root growth. It is likely that plants utilize a number of mechanisms to tolerate Al, some that may be specific to Al and some that may be related to a more general stress response.

Screening methods for identifying aluminum-tolerant plants

A number of techniques have been used for identifying Al-tolerant plants. These include solution culture assays that are based on inhibition of root growth or measurement of Al accumulation within roots, or assays that evaluate biomass accumulation. Several *in vitro* assays that evaluate cell culture growth on an Al-containing medium for the selection of Al-tolerant plants or development of Al-tolerant variants have been described (see *in-vitro* methods used for enhancing Al tolerance, below).

Assays based on growth of plants in acid soil with toxic levels of exchangeable Al have been used to identify tolerance in alfalfa (Devine et al., 1976), barley (Foy, 1996a), tall fescue (Foy and Murray, 1998), sorghum (Foy et al., 1993), soybean (Foy et al., 1992) and wheat (Foy, 1996b) among others (for review, see Howeler, 1991). Typically, plants are grown in pots with acid soil for approximately 1 month or in the field for a growing season and then root dry matter, shoot dry matter and Al concentration in plant tissues is compared to plants grown in the same soil limed to a non-toxic pH. This assay can evaluate plants at a young stage when root growth is important for plant establishment and also at older stages in which nutrient deficiency and/or drought stress can effect plant growth. However, the assay may be too stringent for sensitive plant species. Foy et al. (1993) found that unlimed Al-toxic Tatum soil could not separate sorghum varieties, although tolerant varieties were later identified when soil was limed to pH 4.3. A similar situation was observed for durum wheat (Foy, 1996b). In addition, acidic soils with similar soil pH can vary tremendously in the amount of Al saturation (Johnson et al., 1997). Growth of wheat in acid soils is highly dependent on Al saturation of the soil and other toxic minerals such as Mn rather than on pH, as varieties with tolerance to Al may not show tolerance to Mn (Johnson et al., 1997).

Using acid soil limits where research can be carried out to locations with access to quantities of acid soil. Recently, Villagarcia et al. (2001) developed a method in which plants can be assayed for Al tolerance in sand culture that simulates growth in acid soil. This assay does not require access to acid soil and the amount of Al and other minerals delivered to plants can be controlled. However, it is a time-consuming method; plants are treated twice a day, once with an acidic Al solution and once with an acidic nutrient solution. Results in sand culture were comparable to results in a solution culture assay and may more accurately reflect Al tolerance in the field. A modified screening media that involves a soil-on-agar assay was described by Voigt et al. (1997) for small seeded plants that would have difficulty germinating in acid soil. The modified method requires only a small layer of acid soil to be placed on top of an agar layer. A difference in the time required to grow into the agar layer was observed among cultivars of a number of forage legumes, which is presumed to reflect a difference in Al tolerance (Voigt and Mosjidis, 2002).

Solution culture assays have been used widely to identify and test a variety of plants for Al tolerance including cereals (Riede and Anderson, 1996; Kim et al., 2001), maize (Canaado et al., 1999), rice (Sivaguru and Paliwal, 1993; Khatiwada et al., 1996), alfalfa (Baligar et al., 1989), *Arabidopsis* (Toda et al., 1999), cowpea (Paliwal et al., 1994), soybean (Villagarcia et al., 2001), tomato and rape (Luo et al., 1999), among others (Howeler, 1991). In most solution culture assays, seedling are conditioned in a low pH medium such as 0.1 mM CaCl₂ for several days and then moved to conditioning solution containing Al. Root growth is measured over several days and compared to control plants not receiving the Al treatment. Conventionally, Al tolerance is deduced by comparing the ratio of root growth in the presence of Al to root growth in the absence of Al. The drawback of this comparison is that slower growing plants may appear to be more tolerant than they actually are because the percent reduction in their root growth may be much less than the percent reduction in root growth of a faster growing plant (Dall'Agnol et al., 1996). These assays are relatively rapid to perform and many plants can be evaluated in a small space in a short amount of time. Assays are independent of soil nutritional factors and can evaluate stresses individually (i.e., pH, Al, Mn). However, the solution assay method is not as amenable for vegetatively propagated plants or plants in which Al tolerance is

manifested at the adult plant stage. In some species, considerable variation in plant-to-plant response can occur making identification of tolerant varieties problematic (Koyama et al., 1995). Because Al can form complexes with P and S at any pH, complete nutrient solutions cannot be used. Therefore, experiments are of short duration, requiring that plants use stored reserves for growth. It is important to identify an Al concentration that does not completely inhibit root growth; ideally several different Al concentrations should be tested simultaneously. The solutions need to be prepared carefully to insure the correct concentration of Al. Preparing the solutions can be time-consuming as the pH needs to be titrated slowly to avoid addition of base which can cause precipitation of Al (Kinraide and Sweeney, 2001). In addition, plant exudates can rapidly alter the pH of solutions, requiring frequent monitoring and pH adjustment or replacement of the solution. Recently, the buffer homopipes was shown to be useful for maintaining an acid pH in Al-containing assay solutions (Kinraide and Sweeney, 2001).

Solution culture assays have typically not evaluated low pH-induced root growth inhibition apart from aluminum-induced root growth inhibition. However, a number of plants are sensitive to low pH stress, including *Arabidopsis* (Koyama et al., 1995), common bean, pea and soybean (Lazof and Holland, 1999) and Al tolerance may be masked by H⁺ sensitivity. Assays in which root growth is measured during recovery from Al treatment was shown to successfully distinguish Al-tolerance in acid sensitive species (Lazof and Holland, 1999). For *Arabidopsis*, a unique assay was developed for testing small seedlings using a change in gravitropism to 'mark' the timing when the test solution was applied (Murphy and Taiz, 1995). Seeds are germinated on chromatography paper saturated with conditioning solution attached to a glass plate with a nylon mesh and held vertically. After each change in the assay solution, the plate is rotated 90° so that the change in root growth direction indicates each manipulation of the culture conditions. Dose-response of ten ecotypes to Al, Cu, Zn, Ni, Cr and Cd was characterized and plants with Cu tolerance were identified.

Several staining based procedures have been described as an alternative to root growth measurements in solution culture assays. These include hematoxylin staining and the fluorescent stains morin and lumogallion. The most widely-used assay is hematoxylin staining as an indicator of Al uptake by sensitive

plants. Typically, seedlings are treated with an acidic Al solution for 1–24 h, rinsed in water to remove unbound Al, stained in 0.2% hematoxylin with 0.02% NaIO₃ or 0.02% KI, rinsed to remove excess stain and then rated for the amount of purple coloration indicative of Al (Delhaize et al., 1993; Bona and Carver, 1998; Giaveno and Miranda, 2000). Hematoxylin staining is very sensitive and has been shown to precede root growth inhibition and detectable increases in root Al concentration (Delhaize et al., 1993). For a number of plant species, lack of hematoxylin staining correlates well with Al tolerance in solution culture assays (Canaado et al., 1999; Vásquez et al., 1999; Giaveno and Miranda, 2000) and in acid soil (Bona and Carver, 1998) indicating that it can be used successfully to select Al-tolerant genotypes. The assay is non-destructive, so tolerant plants can be selected and grown to maturity. The method is more of a qualitative measure rather than a quantitative measure of Al tolerance. In addition, not all plants demonstrate a rapid Al exclusion tolerance mechanism.

Lumogallion, a fluorescent stain specific for Al, is even more sensitive than hematoxylin for detecting Al in root tips (Kataoka and Nakanishi, 2001). Although this is a destructive staining technique, it is a valuable tool for following the location of Al within the root over time. In sensitive soybean roots, Al was detected in nuclei of cortical cells at 1 and 2 mm from the root tip after only 15 min of exposure to an Al solution (Kataoka and Nakanishi, 2001). In cells at 1 mm from the tip, Al accumulated in the symplasm, but Al accumulated primarily in the cell wall and cell periphery at 2 mm from the root tip. After 30 min of exposure, Al was detected in the protoxylem, suggesting that Al is transported to aerial portions of the soybean plant through the vascular tissue. Analysis by transmission electron microscopy X-ray microanalysis also shows accumulation of Al in the epidermal and cortical cells of the root tips of wheat (Delhaize et al., 1993). In maize, X-ray microanalysis revealed that Al rapidly enters root tip cells of Al-tolerant varieties which correlates with a transient Al-induced inhibition of root elongation (Vásquez et al., 1999). Root elongation recovers after 24 h of Al treatment, suggesting that Al tolerance mechanisms in maize require changes in gene expression.

Solution culture assays with or without staining procedures are efficient methods for identifying tolerance to Al. Nevertheless, in only a few cases has Al tolerance observed in solution cultures been corre-

lated with Al tolerance in acid soil. Differences between ecotypes were distinguished when the relative root growth of *Arabidopsis* plants grown hydroponically in $2.5 \mu\text{M AlCl}_3$ at pH 5.2 was measured. The same ecotypes displayed tolerance when grown in acid soil (Toda et al., 1999). Similarly, Tesfaye et al. (2001) showed that certain lines of transgenic alfalfa had increased root growth compared to control plants in solution culture and in acid soil. In contrast, rape plants grown in acid soil under field conditions had a higher relative growth rate than in solution culture. In the same experiment, tomato plants showed a similar response in solution culture and under field conditions (Luo et al., 1999). Clearly, each screening technique has distinct advantages and disadvantages and techniques also vary widely in their ease of use for screening large numbers of entries for breeding programs. With the identification of molecular markers linked with Al tolerance genes (see below), future screening for Al tolerance may be possible based on genotype or a combination of genotype and phenotype. In addition, molecular markers may be advantageous for identifying plants with tolerance mechanisms active at different plant growth stages which would be difficult or impossible to perform with phenotypic screens (Wu et al., 2000).

***In vitro* methods used for enhancing aluminum tolerance**

Plant cell culture techniques have been used in three main areas: for screening plants to select Al-tolerant genotypes, to produce and identify somaclonal variants with enhanced tolerance and to investigate cellular responses to Al toxicity. Somaclonal variation can potentially be used to increase genetic variation within a species that shows little variation for Al tolerance and to reveal previously unrecognized tolerance mechanisms. Tissue culture has also been used to generate Al-sensitive mutants from Al-tolerant germplasm in order to develop plant materials with a similar genetic background for identifying and characterizing the genes involved in tolerance (Moon et al., 1997). In order for *in vitro* selection to have a practical impact on developing Al-tolerant varieties, tolerance of cell cultures must correspond with tolerance at the whole-plant level. Several studies have shown that Al-tolerant plants can be identified by comparing growth of callus in an acidic medium with and without added Al. This suggests that similar

mechanisms of Al tolerance are active in both cell cultures and whole plants. However, cell culture selection may not necessarily be more economical or efficient than direct selection of plants in acid soil or in solution culture (Dall'Agnol et al., 1996).

Developing a plant cell culture medium in which a range of concentrations of phytotoxic Al ions can be tested is challenging due to reactions of Al with other mineral components that form insoluble precipitates. Meredith (1978) was the first to show that growth of callus cultures could be inhibited on medium prepared with Al. In the first experiments reported, standard agar-solidified culture media were supplemented with 200 or 400 $\mu\text{M Al-EDTA}$. Concentrations of free Al^{3+} ions were actually very low (nanomolar range) due to Al precipitation (Ramgareeb et al., 1999). Subsequently, a medium was formulated to more closely match Al availability in acid soil or solution culture by reducing phosphate and calcium, using unchelated iron and reducing pH to 4.0 (Conner and Meredith, 1985a). Growth of *Nicotiana plumbaginifolia* calli, placed on filter paper supported by polyurethane foam saturated with this liquid medium, was inhibited by addition of 200 $\mu\text{M Al}$. Recent analysis of the medium using the MINTEQA2 Chemical Speciation Program showed that the free Al^{3+} activity in this medium is approximately 1.5 μM (Ramgareeb et al., 1999). A range of Al^{3+} concentrations, 3–7.5 μM at pH 4, can be obtained if 1 mM SO_4^{2-} is used in standard Murashige and Skoog basal medium and EDTA is removed. This medium has sufficient nutrients for callus initiation and maintenance of a wide range of species (Ramgareeb et al., 1999) and should be useful in future selection experiments. Solidification of selection media has also been problematic as agar is hydrolyzed when autoclaved at pH 4. Use of 5–9 g l^{-1} Gelrite (Parrot and Bouton, 1990; Ramgareeb et al., 1999) or autoclaving agar separately from the acidic culture medium (Arihara et al., 1991) alleviates this difficulty. Gellan gum has also been used to solidify an acidic culture medium for identifying *Arabidopsis* mutants with increased sensitivity to Al (Larsen et al., 1996) and increased tolerance (Larsen et al., 1997).

In alfalfa, Al tolerance has been shown to be expressed at both the cell culture and whole-plant level (Parrot and Bouton, 1990; Dall'Agnol et al., 1996). Callus cultures were initiated from Al-tolerant and Al-sensitive germplasm and their growth compared on a modified acidified Blaydes medium with and without 400 $\mu\text{M Al}$. Evaluation of this medium

showed the free Al^{3+} concentration to be $7.5 \mu\text{M}$ (Ramgareeb et al., 1999). Although significant variation from plant-to-plant for Al tolerance was observed, callus from germplasm selected for Al tolerance had greater weight gains on the medium with Al than callus from unselected germplasm (Parrot and Bouton, 1990). The same media were used in a bioassay to screen alfalfa germplasm for Al tolerance (Dall'Agnol et al., 1996). Plants were selected in unlimed soil, in tissue culture for high callus growth with Al and selected in tandem for both tolerance to acid soil and callus growth. Selections were randomly intermated to develop ten populations and plants from all ten populations were evaluated for tolerance to acid soil. In addition, two experimental and two control populations were evaluated in callus culture for Al tolerance. Based on shoot and root biomass accumulation after growth in acid soil, selection in culture was as successful as selection in soil for developing acid soil tolerant germplasm. Surprisingly, callus growth of the population selected on the Al-containing medium was not greater than callus growth of the population selected on the Al-free medium. This may have been due to the large amount of variation in genotypes observed within populations. Although this bioassay is labor-intensive, increasing callus in culture allows the same plant to be tested relatively rapidly in multiple assays.

Generation of Al-tolerant somaclonal variants has been achieved using media containing Al as well as in Al-free media. In particular, variants arising in tissue culture have been used successfully in developing acid soil tolerant sorghum, a very Al-sensitive species (Foy et al., 1993). Waskom et al. (1990) initiated sorghum callus cultures from Al-sensitive varieties but did not apply Al stress during culture. Instead, regenerated plants were tested in the field in acid soil with high exchangeable Al concentrations. Of 212 plants tested, four had improved acid soil tolerance (Waskom et al., 1990) and were advanced to the R_5 generation by selfing (Miller et al., 1992). In acid soil, these four lines showed increased vigor, survival, number of panicles harvested and seed yield over the parental plants (Miller et al., 1992). Plants with good agronomic characteristics were selected and advanced to the R_7 generation in which plants with better acid soil tolerance and desirable plant phenotypes were obtained (Miller et al., 1992). Information on inheritance of the mutation(s) and the mechanism of tolerance would be useful for strategies in pyramiding tolerance in this species. In tobacco, Al tolerance in

tissue culture variants was associated with a single mutation (Conner and Meredith, 1985b). Aluminum-tolerant cell lines were selected directly by growth on acidified Al-containing medium and after rescue of cells from Al-treatment (Conner and Meredith, 1985b). Aluminum-tolerance in culture was stable in the absence of Al, as variants could be reselected on Al-containing medium. Fertile plants were regenerated from reselected cell lines and seedlings tested for Al tolerance by measuring root growth on filter paper saturated with a complete nutrient solution amended with Al. Segregation ratios of tolerant to sensitive progeny indicated that tolerance is due to the action of a single dominant mutation.

Stable Al-tolerant carrot lines have also been selected directly on medium containing Al (Arihara et al., 1991). Al-tolerant carrot cells occurred at a frequency of approximately 6×10^{-7} when sieved suspension cell cultures were plated on an acidic Al-containing medium. Aluminum-tolerant variants were obtained after 1–2 months of culture on this medium. Fertile plants were regenerated from a number of the variant cell lines and seedling progeny tested for Al tolerance. Root lengths of seedlings were compared after growth for 72 h on filter paper with an acidic nutrient solution with or without $500 \mu\text{M}$ Al. Seedlings from several parents grew similarly with or without added Al in the solution. No data on segregation patterns for Al tolerance were reported. In a separate experiment, a carrot cell line was shown to secrete citrate into the medium when grown in the presence of precipitated AlPO_4 (Koyama et al., 1988). However, this cell line was sensitive to ionic forms of Al. Nonetheless, this suggests that somaclonal variants utilize mechanisms of Al tolerance that may be useful in generating acid soil tolerant crops and this method of generating Al tolerance warrants further development and characterization of somaclonal variants.

Identification of genes associated with aluminum tolerance

Genetic variability for Al tolerance has been exploited to develop Al-tolerant varieties of several crop species and to explore the number of genes involved in Al tolerance. Because of the availability of Al-tolerant and susceptible plant varieties, wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), soybean (*Glycine max* L.) and to a lesser

extent rice (*Oryza sativa* L.) account for the majority of the research undertakings in both classical breeding and molecular analysis of Al tolerance. Approaches taken to determine the nature and number of genes controlling Al tolerance in plants include: (a) genetic studies to identify Al tolerance loci as well as molecular mapping to identify DNA markers diagnostic of Al tolerance; (b) the isolation and characterization of genes induced during Al toxicity; (c) production and evaluation of mutant plants; and (d) the use of various transgenic plants in Al tolerance studies. All of these approaches have not only practical implications for the development of better crops suited for the large tracts of Al-containing acid soils, but also have contributed greatly to our understanding of the complexity of Al tolerance mechanisms in plants.

Wheat is the most widely studied plant species with regard to Al tolerance and detailed accounts on the genetic basis of Al tolerance in wheat genotypes can be found in other reviews (Carver and Ownby, 1995; Garvin and Carver, 2003). A comparative analysis of all wheat varieties to Al toxicity in a single experiment is lacking; except for one study that evaluated 36 wheat genotypes for Al tolerance (Ryan et al., 1995). Wheat varieties and genotypes used for determining the genetic and physiological basis of Al tolerance include Al-tolerant (Atlas 66, ET3, Waalt, BH 1146; Neepawa) and Al-sensitive (Scout 66 and ES3, Warigal, Fredrick; Anahuac) types. A few studies have attempted to correlate mechanisms of Al tolerance in wheat based on segregating populations or near isogenic lines. In general, Al tolerance in wheat appears to be attributed to the action of a single dominant gene, but several major genes have also been implicated in conditioning the degree of Al tolerance in other wheat varieties (Carver and Ownby, 1995 and references therein; Delhaize et al., 1993; Riede and Anderson, 1996). For example, Riede and Anderson (1996) generated recombinant inbred lines from a cross of the Al-tolerant variety BH 1146 and the Al-sensitive variety Anahuac. They reported single gene control of Al tolerance and identified the *Alt_{BH}* locus on the chromosome 4DL as being the major Al tolerance gene in BH 1146 (Riede and Anderson, 1996). This result substantiated an earlier finding that Al tolerance in near-isogenic Al-tolerant ET3 and Al-sensitive ES3 wheat lines segregated as a single locus, *Alt1* (Delhaize et al., 1993). RFLP mapping of F_2 generations of recombinant inbred wheat lines derived from a cross of BH 1146 \times Anahuac showed that two DNA markers, *Xbcd1230* and *Xcdo1395*,

were linked to the Al tolerance *Alt_{BH}* gene, with the marker *Xbcd1230* being more tightly linked to the Al tolerance locus and explaining 85% of the variation in root growth performance in Al-containing nutrient solutions (Riede and Anderson, 1996). In a separate experiment, seed of the Al-sensitive cultivar Anuhac was mutagenized by gamma irradiation (Camargo et al., 2000). In a hydroponic nutrient solution that contained Al, 14 mutant wheat lines showed comparable rates of root elongation and hence similar degrees of Al tolerance to the Al-tolerant wheat variety BH 1146 (Camargo et al., 2000). Each mutant line was crossed with either an Al-sensitive (cv. Anuhac) or Al-tolerant (cv. BH 1146) wild type and the population was advanced to the F_2 generation. Surprisingly, many of the Al-tolerant mutant wheat lines differed by a pair of dominant alleles from the Al-sensitive parental variety Anuhac (Camargo et al., 2000). The single pair of alleles present in the mutant wheat lines showed the same level of expression as the alleles located in the Al-tolerant variety BH 1146 (Camargo et al., 2000), substantiating the previous finding by Riede and Anderson (1996) that a single pair of dominant genes controls Al tolerance in BH 1146. Additionally, the results of Camargo et al. (2000) suggest the possibility that sensitivity to Al by the wild-type Anahuac plants may be related to the lack of expression of the Al tolerance gene, *Alt_{BH}*.

Research groups in New Zealand and Canada have identified, characterized and sequenced cDNAs that are up-regulated by Al treatment of wheat roots; *wali* genes for wheat aluminum induced or *war* genes for wheat aluminum regulated (Snowden and Gardner, 1993; Richards et al., 1994; Hamel et al., 1998). Differential screening of a root tip cDNA library of an Al-sensitive (Warigal) wheat variety revealed seven genes (*wali1* to *wali7*) that were up-regulated by Al treatment (Snowden and Gardner, 1993; Richards et al., 1994). Transcript accumulation of these genes was increased after 24 h of Al-treatment of roots of both Al-sensitive (Warigal) and Al-resistant (Waalt) wheat, although higher Al concentrations were needed for the induction of *wali* genes by roots of the Al-resistant wheat cv. Waalt (Snowden and Gardner, 1993). Four *war* cDNAs (*war4.2*, *war5.2*, *war7.2* and *war13.2*), that were up-regulated by Al were also isolated and characterized following differential screening of a root tip cDNA library from the Al-tolerant variety Atlas 66 (Hamel et al., 1998). As was the case with *wali* cDNAs, mRNA transcripts of *war* cDNAs were induced by increased concentrations of

Al in both Al-sensitive (cv. Fredirck) and Al-tolerant (cv. Atlas 66) wheat varieties (Hamel et al., 1998). Proteins encoded by *wali* and *war* cDNAs show some degree of homology to stress-related proteins in plants such as metallothionein-like proteins (*wali1*), phenylalanine–ammonia lyase (*wali4*, *war7.2*), peroxidase (*war4.2*), proteinase inhibitors and cysteine proteinase (*wali3*, *wali5*, *wali6* and *war5.2*), asparagine synthetase (*wali7*) and oxalate oxidase (*war13.2*) (Snowden and Gardner, 1993; Richards et al., 1994; Hamel et al., 1998). In a separate experiment, Snowden et al. (1995) reported enhanced accumulations of *wali1*, *wali3*, *wali4* and *wali5* mRNA transcript levels in wheat root tips after 48 h of treatments with higher concentration of metals such as Cu, Cd, Fe, Zn, Ga, In and La, as well as in mechanically wounded leaves of wheat. In Al-tolerant wheat, a 23-kDa Al-binding protein is secreted from roots in response to Al stress (Basu et al., 1999). Microsequencing of the isolated protein showed similarity to manganese superoxide dismutases (MnSOD) from a variety of sources (Basu et al., 2001). In root tips of Al-tolerant wheat, MnSOD expression is induced by Al treatment and may be involved in removal of active oxygen species associated with oxidative stress caused by Al toxicity.

Compared to wheat, the genetic basis of Al tolerance in other crop species has received limited attention. The presence of vast differences in the level of Al tolerance among barley varieties has been documented; for example, barley varieties that are known to be Al-tolerant and moderately sensitive include Dayton and Harlan Hybrid, respectively. Minnella and Sorrells (1992) evaluated Al tolerance of 37 barley genotypes and their crosses and reported a single gene for Al tolerance with multiple alleles impacting the degree of tolerance among tolerant varieties. This was subsequently substantiated by Minnella and Sorrells (1997) and Tang et al. (2000). The gene controlling Al tolerance in barley is located on chromosome 4, designated as *Phl* (Stolen and Andersen, 1978), or *Alp* (Minnella and Sorrells, 1997). RFLP analysis of F₂ mapping populations from crosses of the varieties Dayton×Harlan Hybrid revealed three DNA markers (*xcd1395*, *xbcd1117* and *Xwg464*) closely linked to the Al tolerance *Alp* gene (Tang et al., 2000). The DNA marker *xcd1395* was previously reported by Riede and Anderson (1996) to be distantly linked to the *Alt_{BH}* gene in the wheat variety BH 1146 and explained around 40% of the variation in root growth of the wheat plants tested in an Al solution assay. This suggests that Al toler-

ance genes *Alp* and *Alt_{BH}* from barley and wheat, respectively, may be orthologs.

A single gene with multiple alleles conditioning various degrees of tolerance to Al appears to be common among maize and rice varieties studied (Sibov et al., 1999; Nguyen et al., 2001). After five generations of self-pollination of tissue culture-derived maize plants, Moon et al. (1997) characterized a somaclonal variant (S1587-17) derived from a callus culture of an Al-tolerant inbred maize line Cat-100-6. The tissue culture-derived variant was identified on the basis of poor root growth in nutrient solutions containing Al that did not inhibit the root growth of the parental maize line Cat-100-6. Screening of an F₂ population of S1587-17×Cat-100-6 crosses and reciprocal backcrosses in Al-containing nutrient solutions suggested that a single semi-dominant gene, *Alm1*, may control Al tolerance in maize (Moon et al., 1997). However, genetic and molecular mapping approaches using F₂ progenies of crosses Cat-100-6×S1587-17 extended the number of Al tolerance genes to two distinct loci, *Alm1* and *Alm2*, located on chromosome 10 and chromosome 6, respectively (Sibov et al., 1999). It turns out that the *Alm1* gene has a stronger impact on Al tolerance in maize than the *Alm2* gene (Sibov et al., 1999). The most tightly linked markers to the Al tolerance genes *Alm1* and *Alm2* were clones UMC 130 and CSU 70, respectively (Sibov et al., 1999).

Nguyen et al. (2001) estimated the number of genes controlling Al tolerance in rice using RFLP analysis of F₂ populations of crosses of an Al-tolerant (var. Chiembau) and an Al-sensitive (var. Omon 269-65) rice (*Oryza sativa* subsp. indica). Approximately nine genomic regions from eight chromosomes appear to be involved in Al tolerance, although one QTL, QA1R1a, identified by DNA marker WG110 on chromosome 1, showed the greatest effect on root growth of rice plants in Al-containing nutrient solutions (Nguyen et al., 2001).

In soybeans, the cultivar Young and a soybean introduction from Japan, PI 416937, were shown to be Al-tolerant and Al-sensitive plants, respectively (Bianchi-Hall et al., 1998). Using RFLP analysis of an F₄ population from crosses of PI 416937 and Young, up to five QTLs, each with minor effects, were found to condition the degrees of Al tolerance in soybean, indicating a multigene level of control for Al tolerance (Bianchi-Hall et al., 2000).

Alfalfa is very sensitive to Al and performs poorly in acidic Al-rich soil. Extensive effort has been di-

rected to screening and selecting alfalfa for resistance to acidic Al-containing soil (Baligar et al., 1989; Bouton, 1996; Dall'Agnoletti et al., 1996). Neither plants from cultivated alfalfa varieties or plant introductions varied greatly in resistance when screened in acid soil (Bouton, 1996). Through recurrent selection and intercrossing more tolerant germplasm have been developed, however, forage yields were not significantly different than those for unselected elite varieties grown under the same acid soil conditions (Bouton and Sumner, 1983). Selection for Al tolerance in alfalfa is complex due to tetraploid inheritance, obligate outcrossing and inbreeding depression, which can mask Al tolerance. Recently, QTLs conditioning Al tolerance in diploid alfalfa have been identified using RFLP analysis of F₂ and backcross populations (Sledge et al., 2002), which should facilitate development of Al-tolerant varieties.

In many respects, large genome sizes and polyploidy have limited our understanding of the molecular-genetic and physiological basis of Al tolerance in many of the crop species studied. Additionally, few investigations have made use of isogenic germplasm with contrasting tolerances to Al, complicating genetic and molecular analyses. Nevertheless, a few research groups have been working towards the development of mutant lines using the model plant, *Arabidopsis thaliana*, which may well be a useful system for molecular genetic and physiological analysis of Al tolerance. Using EMS-mutagenized seeds of *A. thaliana* cv. Columbia, Larsen et al. (1996, 1997) generated, described and characterized several mutations with increased sensitivity to aluminum (*als*) as well as an Al-resistant mutation (*alr*). Aluminum-sensitive (*als*) mutants were identified on the basis of inability of *Arabidopsis* roots to grow in mildly inhibitory Al concentrations, while *alr* mutants showed enhanced root growth in an Al concentration that strongly inhibited root growth of the wild type *Arabidopsis* plants (Larsen et al., 1996). F₂ populations of each mutant × wild type cross were used for mapping and inheritance analysis. Eight *als* mutants were shown to be from recessive mutations representing seven unique loci, although one was a semi-dominant mutation (Larsen et al., 1997). Three *als* mutations that were chosen for mapping by Larsen et al. (1997) were located on chromosome 5. Analysis of seven confirmed *alr* mutants indicated semi-dominant mutations in all of the Al-tolerant mutants identified (Larsen et al., 1997; Degenhardt et al., 1998). Of these, five *alr* mutants were subjected to microsatellite

and RAPD-based mapping and mutations were located on chromosome 1 (*alr*-108, *alr*-128, *alr*-131, *alr*-139) and chromosome 4 (*alr*-104) (Larsen et al., 1997; Degenhardt et al., 1998) of the *Arabidopsis* genome.

Identification of additional genes for Al tolerance in *Arabidopsis* should be forthcoming as a result of the recent mapping of two QTLs and five epistatic loci for Al tolerance (Kobayashi and Koyama, 2002). The two significant single factor QTLs explained 32 and 11% of the total variation of relative root growth in Al among 100 lines of a recombinant inbred (RI) population of *Arabidopsis*. The RI population was derived from a cross of ecotypes Landsberg erecta and Columbia that were found to be Al-sensitive and Al-tolerant, respectively (Larsen et al., 1996). The two QTLs were mapped to chromosome 1 (flanked by *apx1A-ATTS0477*) and chromosome 4 (*mi51-mi204*) and were closely linked to markers *ARR4* and *mi51*, respectively (Kobayashi and Koyama, 2002). The mapping positions of the two QTLs appear to be unique, as they were located at loci different from the Al tolerance loci reported previously for *Arabidopsis* mutant lines by Larsen et al. (1996) and Degenhardt et al. (1998). In contrast, four epistatic loci were located at previously mapped positions and were associated with Al tolerance genes identified previously in EMS-mutagenized plants of *Arabidopsis* cv. Columbia (Larsen et al., 1996; Degenhardt et al., 1998), while the remaining epistatic locus identified in the RI population was unique and mapped on chromosome 2 (Kobayashi and Koyama, 2002). The availability of the complete *Arabidopsis* genome sequence and T-DNA insertion mutants will facilitate rapid identification of candidate Al tolerance genes.

In a separate effort, Richards et al. (1998) attempted to identify and characterize genes influenced by Al in *A. thaliana* cv. Columbia. They generated a cDNA library after 2 h of treatment in an Al concentration that inhibits root growth of *Arabidopsis* completely. Differential screening of the cDNA library was performed using cDNA probes from *Arabidopsis* plants treated without or with Al for 2 h. They found nine *Arabidopsis* cDNA clones that were up-regulated, as well as two cDNA clones that were down-regulated by Al treatment. They designated the cDNA clones as pEARLI genes for early *Arabidopsis* Al-induced genes (pEARLI1, -2, -4 and pEARLI5). Similar to their previous findings for *wali* genes in wheat, some of the up-regulated clones showed strong homology to sequences that are considered plant-stress related

genes: peroxidase, blue copper-binding protein and glutathione S-transferase (Richards et al., 1998). The two cDNA clones that showed down-regulated expression after 2 h of Al treatment were considered similar to an alanine aminotransferase gene and chlorophyll *a/b* binding protein (Richards et al., 1998). Transcript analysis using RNA blots revealed that a metallothionein-like protein and proteinase inhibitors, previously identified as being induced in wheat by Al treatment (reviewed above) and an additional gene encoding for superoxide dismutase, were also up-regulated by Al treatment of *Arabidopsis* plants (Richards et al., 1998). Recently, expressed sequence tags (ESTs) from rye were identified that were differentially regulated by Al stress (Rodriguez Milla et al., 2002). Upon exposure to Al, they observed rapid down-regulation of genes encoding tonoplast aquaporins and homologs of the barley *Ids3* gene, which is involved in synthesis of phytosiderophores. Several genes involved in protection from oxidative stress and associated with pathogens were up-regulated. Similar functional genomics approaches should help to identify additional genes involved in responses to Al stress.

Genetic modification of plants for enhancing aluminum tolerance

Two different approaches have been taken to enhance Al tolerance by ectopic expression of genes in plants: expression of Al-induced plant genes and expression of genes to increase organic acid production. These approaches have met with some success. Due to the complex physiological effect of Al on plant cells, it is unlikely that altering expression of a single gene will confer high levels of Al tolerance, although transgene expression may have pleiotropic effects. Recent experiments indicate that combinations of transgenes may increase Al tolerance (Ezaki et al., 2001). In addition to plant genes, it may also be possible to identify microbial genes that confer Al tolerance in their host and enhance Al tolerance in plants.

As a means of screening previously identified Al-induced genes for their individual effect on Al tolerance, 11 plant genes were expressed in yeast and nine genes expressed in *Arabidopsis* (Ezaki et al., 2000). Two genes, one encoding a tobacco putative GDP-dissociation inhibitor and the other an *Arabidopsis* blue copper-binding protein, conferred Al tolerance to yeast. Both genes also enhanced Al tolerance in

transgenic *Arabidopsis* as did over-expression of a tobacco glutathione S-transferase gene and a tobacco peroxidase gene. Expression of the transgenes decreased the accumulation of Al in root tips and decreased oxidative stress from the Al treatment. Aluminum tolerance was measured by root elongation using the vertical mesh transfer technique and a growth medium consisting of 1/6 strength Murashige and Skoog culture medium at pH 4.0. Aluminum treatments of 200 μM were required to inhibit root growth of wild type seedlings by 60%. It is likely that added Al formed insoluble complexes with medium components and that actual free Al^{3+} was at a low concentration. In simple culture solutions, *Arabidopsis* is very sensitive to low pH and Al. For example, in a 100 μM CaCl_2 solution at pH 5.2, a 50% root growth inhibition was observed with 1 μM Al (Koyama et al., 2000). Nonetheless, in plants expressing these transgenes root growth in the 200 μM Al treatment was significantly greater than that of wild type plants (Ezaki et al., 2000). This difference was not observed with 100 or 300 μM Al treatments. The authors concluded that Al-induced genes contribute to natural polygenic variation for Al tolerance and may map to QTLs associated with Al tolerance (Ezaki et al., 2000). More recently, further characterization of plants expressing these transgenes was carried out and Al tolerance of hybrids expressing different combinations of transgenes were characterized (Ezaki et al., 2001). In plants expressing the glutathione S-transferase or the GDP-dissociation inhibitor transgene, callose deposition in root tips of Al-treated plants was reduced compared with wild type plants and plants expressing tobacco peroxidase or the *Arabidopsis* blue copper-binding protein gene had much lower accumulation of callose, indicating much reduced Al toxicity. In addition, Al content in root tips of plants expressing tobacco peroxidase or the *Arabidopsis* blue copper-binding protein transgene was significantly lower than the wild type control. In plants expressing two transgenes, an additive effect was observed with the tobacco peroxidase gene conferring the largest increase in Al tolerance. On average, root growth of plants expressing the tobacco peroxidase gene and an additional transgene in the 200 μM Al treatment was reduced by 20% while growth of roots of wild type plants was reduced by 60% (Ezaki et al., 2001). These results suggest that over-expression of certain Al-induced genes may be an effective strategy for enhancing Al tolerance in crop plants.

In efforts to identify novel genes involved in Al

tolerance, Delhaize et al. (1999) tested the ability of root tip cDNAs from Al treated Al-tolerant ET3 wheat to confer Al tolerance in yeast cells. A screen of 2 million yeast transformants identified six unique cDNAs that enhanced tolerance of yeast cells to Al. One cDNA clone had significant sequence similarity to phosphatidylserine synthase (TaPSS) and was shown to be involved in phospholipid biosynthesis. Overexpression of the TaPSS cDNA in *Arabidopsis* increased the amount of phosphatidylserine in leaves, altered phospholipid composition and resulted in stunting, necrotic lesions and leaf distortions. Although the enzyme may be involved in Al tolerance of the plasma membrane in wheat, overexpression in tobacco did not enhance Al tolerance (Delhaize et al., 1999).

Roots of Al-tolerant wheat plants secrete a 23-kDa protein that binds Al (Basu et al., 1999). The protein has similarity to a cloned wheat MnSOD cDNA. When transgenic *Brassica napus* plants overexpressing this cDNA were tested in solution culture assays, they showed enhanced Al tolerance (Basu et al., 2001). Root growth of wild type control plants in solutions containing 100 μM Al was reduced approximately 40%, while growth of the transgenic plants was decreased 10–20%. In the transgenic plants, enhanced Al tolerance was associated with decreased callose accumulation and decreased oxidative stress.

In view of the difficulties involved in identifying a single gene from an Al-tolerant plant species that will confer a high degree of Al tolerance to a sensitive species, several groups have taken the approach of increasing production of Al-chelating compounds, particularly organic acids, in plant roots. In the first report of this tactic, a citrate synthase (CS) gene from *Pseudomonas aeruginosa* was overexpressed in tobacco and papaya plants (de la Fuente et al., 1997). Citrate synthase converts oxaloacetate to citrate and is the 'pace-maker' of the tricarboxylic acid cycle, modulating synthesis of other organic acids. Of the organic acids that participate in normal respiratory metabolism, citrate has the highest binding activity for Al. However, success in increasing citrate production and exudation by overexpression of CS has been uneven. When the CS transgene was expressed in tobacco, de la Fuente et al. (1997) reported a 2–3-fold increase in CS activity in roots compared to untransformed control plants. Citrate accumulation in roots of transgenic plants was up to 10-fold greater than control plants and citrate in root exudates of the transgenic plants increased up to 4-fold. These results

have proven difficult to reproduce. Delhaize et al. (2001) found no significant increase in CS activity in tobacco plants, even with high accumulation of the *P. aeruginosa* CS protein (up to 2% of total cytosolic protein). This may indicate that most of the protein produced was not catalytically active, a problem most likely due to incorrect protein folding or aggregation of the protein in plant cells, as the gene sequence in the transgenic plants was shown to be the same as the original *P. aeruginosa* clone. Both transgenic and wild type tobacco plants responded to Al stress by exuding citrate from roots (Delhaize et al., 2001). However, the amount of citrate in roots and the amount exuded from roots was not significantly different among transgenic and wild type plants. de la Fuente et al. (1997) reported that expression of the CS transgene enhanced Al tolerance in a vertical mesh transfer assay system. They observed increasing inhibition of root growth with increasing amounts of Al in the assay solution. However, a range of Al^{3+} concentrations is not attainable in this medium (Ramgareeb et al., 1999) suggesting that plants were responding to something other than Al toxicity. Delhaize et al. (2001) reported a small significant increase in Al tolerance for two transgenic tobacco lines expressing the citrate synthase transgene when tested at 25 μM Al. No differences were observed at 50 μM Al. In addition, no change in Al tolerance was found in two transgenic alfalfa lines expressing the citrate synthase transgene. The authors suggested that the Al tolerance exhibited by transgenic tobacco may be related to a somaclonal event and concluded that expression of the bacterial CS gene is unlikely to be a robust strategy for enhancing Al tolerance in crop plants.

A plant CS gene has been expressed in transgenic *Arabidopsis* and shown to increase Al tolerance in a nutrient solution assay and in acid soil (Koyama et al., 2000). The transgene consisted of a mitochondrial CS cDNA from carrot driven by the constitutive 35S promoter. The highest CS activity in transgenic lines was approximately 3-fold greater than activity in control plants. Analysis of root exudates showed that transgenic lines secreted enhanced amounts of citrate in response to Al-P compared to controls and the amount of citrate correlated with enzyme activity. Protein immunoblots showed that the transgenic lines produced a protein specifically recognized by an antibody to the carrot CS. Whether the protein was targeted to mitochondria was not investigated. Al tolerance was tested using a simple nutrient solution

at pH 5.2. Two transgenic lines demonstrated enhanced root elongation compared to the wild type control in assay solutions with either 0.5 or 1 μM Al. Growth of transgenic lines was also greater than controls in an acidic soil with low available P and toxic amounts of Al. However, overexpression of this gene in tobacco plants did not increase citrate production or Al tolerance (Delhaize et al., 2003). In tobacco and possibly in other plants, citrate synthesis appears to be insensitive to changes in enzyme activity and other factors may influence control of citrate efflux.

Overexpression of plant genes for two other enzymes involved in organic acid synthesis, phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH), has been investigated as a means of enhancing organic acid synthesis and Al tolerance (Tesfaye et al., 2001). PEPC catalyzes the conversion of phosphoenolpyruvate and CO_2 to oxaloacetic acid (OAA) and inorganic phosphate. This reaction plays a key role in a number of non-photosynthetic processes, most notably in nitrogen-fixing root nodules of legumes where PEPC provides carbon for synthesis of malate and aspartate. The rate of synthesis of citric acid is determined by the availability of OAA and acetyl-CoA. Increasing PEPC activity and therefore substrate OAA pools, could increase synthesis of organic acids in plant roots. MDH is a ubiquitous plant enzyme that catalyzes the reversible conversion of OAA to malate. A number of forms of the enzyme occur due to its diverse roles in plant metabolism including pH balance, stomatal and pulvinal movement, respiration, β -oxidation of fatty acids and root nodule function. Five forms of MDH were cloned as cDNAs from alfalfa: a cytoplasmic form, glyoxysomal form, mitochondrial form, chloroplast form and a unique nodule-enhanced form (Miller et al., 1998). The nodule enhanced form (neMDH) has an exceptionally high turnover rate (k_{cat}) for the production of malic acid (15711/min) compared to the cytoplasmic form (560/min), evidence that the neMDH-catalyzed reaction is strongly driven towards the production of malate. The unique features of neMDH make it an attractive candidate for increasing synthesis of malate in roots.

Ten alfalfa lines containing a 35S::neMDH construct and nine alfalfa lines containing a 35S::nePEPC construct were evaluated by protein immunoblotting and enzymatic assays (Tesfaye et al., 2001). From plants with the 35S::neMDH construct, six lines with significantly increased levels of MDH activity were

identified and one line with the 35S::nePEPC construct had increased levels of PEPC activity. Accumulation of enzyme protein in root tips generally corresponded with enzyme activity. Selected transgenic plants with increased amounts of MDH and PEPC had increased amounts of organic acids in root tissues. Root tips of plants containing the neMDH transgene had enhanced amounts of citrate, oxalate, malate, succinate and acetate compared to the control untransformed line. The line containing the PEPC transgene had increased amounts of oxalate and malate compared to the control. Alfalfa responds to a low P environment by secretion of organic acids but does not secrete organic acids in response to Al (Ryan et al., 2001). When cultured in quartz sand, transgenic lines containing the neMDH transgene exuded enhanced amounts of citrate, oxalate, malate, succinate and acetate compared to the untransformed line (Tesfaye et al., 2001). The line containing the PEPC transgene did not show a significant increase in organic acids in root exudates. In acidic solution culture assays, plants expressing the neMDH or nePEPC transgene showed enhanced root elongation compared with the control untransformed line at 20, 50 and 100 μM Al. In acid soil, plants containing the neMDH transgene showed increased root and shoot growth compared with the control and the line containing PEPC. This suggests that expression of neMDH can confer broad acid soil tolerance. Ectopic expression of neMDH also shows promise for increasing Al tolerance in other crop species. In transgenic oat plants, expression of the alfalfa neMDH enhances Al tolerance in solution culture assays (Tesfaye, unpublished).

The mechanism of Al tolerance in transgenic alfalfa or oats expressing neMDH has not been investigated. Exclusion of Al from the root may be occurring due to exudation of organic acids as well as chelation of Al with organic acids in root cells. Further investigations are needed to determine if transgenic lines exclude or accumulate Al. Improved Al tolerance may also be attained by combining transgenes for enzymes involved in organic acid synthesis, such as neMDH with CS or PEPC, or combining genes involved in organic acid synthesis with gene involved in oxidative stress tolerance. To date, all experiments have utilized constitutive promoters to increase organic acid synthesis. Because the root apex is the target of Al toxicity, expression of transgenes using root tip-specific promoters may enhance Al tolerance. Further research is needed to determine the consequences of increasing

organic acid synthesis in plant roots on activity of other enzymes involved in carbon and nitrogen assimilation. Increased exudation of organic acids may also affect the populations of rhizosphere bacteria and nutrient availability. It is unlikely that increasing organic acid exudation will accelerate acid soil development as addition of organic acids to soils has been shown to increase soil pH (Yan and Schubert, 2000; Haynes and Mokolobate, 2001). In some species, organic acid exudation is associated with tolerance to other metals (Kochian et al., 2002). The deep-rooting and perennial nature of alfalfa makes it a good candidate for use in phytoremediation of contaminated soils, if additional metal tolerance can be demonstrated.

Conclusions

Acid soil tolerant plants are needed to increase alternatives for crop production in areas where liming and fertilization are impractical. Through selection and breeding strategies, Al-tolerant varieties of major crops are being developed. Although the exact mechanisms of Al toxicity are still not clearly defined, intense research efforts have succeeded in identifying the most sensitive root cells, those in the distal elongation zone and the responses of these cells to Al exposure. Designing appropriate screening methods remains the most challenging aspect for developing and characterizing Al-tolerant plants. Screening assays based on Al accumulation in roots will likely only identify plants with the ability to exclude Al from entering the root system. Assays based only on root growth in acidic Al solutions may be misleading. Highly heterotic plants with little Al tolerance may perform better in assays that measure growth or biomass accumulation than plants with less genotypic vigor expressing Al tolerance genes. Also, if plant species utilize a range of tolerance mechanisms, developing a standard screening procedure for Al tolerance becomes more difficult. With further identification of molecular markers linked with Al tolerance genes, future screening may be possible based on genotype or a combination of genotype and phenotype. Because of its major role in acid soil toxicity, Al toxicity has been investigated most intensively. However, the roles of H⁺ and Mn toxicity as well as nutrient deficiencies associated with acid soils need to be addressed in developing acid soil-tolerant crops. To date, genes specifically involved in response to Al

toxicity have not been identified. Advances in functional genomics such as microarray analysis and in proteomics may elucidate Al-specific gene expression and facilitate cloning of Al-specific genes. Characterization of tolerance genes should accelerate development of more highly tolerant, productive and well-adapted crop species. Continued identification of mutants and somaclonal variants may uncover previously unrecognized targets of toxicity and tolerance mechanisms. Finally, use of transgenes shows promise for enhancing Al tolerance, particularly in species where little natural variation for tolerance exists or to augment breeding efforts.

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