

REVIEW PAPER

The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils

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Abstract

Acid soils restrict plant production around the world. One of the major limitations to plant growth on acid soils is the prevalence of soluble aluminium (Al^{3+}) ions which can inhibit root growth at micromolar concentrations. Species that show a natural resistance to Al^{3+} toxicity perform better on acid soils. Our understanding of the physiology of Al^{3+} resistance in important crop plants has increased greatly over the past 20 years, largely due to the application of genetics and molecular biology. Fourteen genes from seven different species are known to contribute to Al^{3+} tolerance and resistance and several additional candidates have been identified. Some of these genes account for genotypic variation within species and others do not. One mechanism of resistance which has now been identified in a range of species relies on the efflux of organic anions such as malate and citrate from roots. The genes controlling this trait are members of the *ALMT* and *MATE* families which encode membrane proteins that facilitate organic anion efflux across the plasma membrane. Identification of these and other resistance genes provides opportunities for enhancing the Al^{3+} resistance of plants by marker-assisted breeding and through biotechnology. Most attempts to enhance Al^{3+} resistance in plants with genetic engineering have targeted genes that are induced by Al^{3+} stress or that are likely to increase organic anion efflux. In the latter case, studies have either enhanced organic anion synthesis or increased organic anion transport across the plasma membrane. Recent developments in this area are summarized and the structure–function of the TaALMT1 protein from wheat is discussed.

Key words: Acid soils, aluminium, citrate, malate, resistance, roots, tolerance.

Introduction

Aluminium (Al^{3+}) toxicity limits plant production on acidic soils. The primary symptom of toxicity is inhibition of root growth but Al^{3+} can disrupt many other functions including root hair elongation, nutrient uptake (especially Ca and K), induce oxidative stress, disrupt the cytoskeleton and apoplastic processes, and affect intracellular transport (Kochian, 1995; Matsumoto, 2000; Sivaguru *et al.*, 2000; Yamamoto *et al.*, 2002; Kochian *et al.*, 2004; Horst *et al.*, 2010). Acid soils now encompass ~35% of arable land so Al^{3+} toxicity is a major selection pressure for adaptation. Many species have evolved mechanisms to improve their survival on acid soils. Even before these mechanisms were

fully understood they were divided into those that were likely to exclude Al^{3+} from the root (exclusion or resistance mechanisms) and those that would enable plants to accommodate Al^{3+} safely once it enters the symplast (tolerance mechanisms). In this review, tolerance mechanisms are distinguished from resistance mechanisms even though these terms are often used interchangeably in the literature. Exclusion mechanisms were predicted to depend on transport systems that export Al^{3+} from the symplast or on the exudation of ligands which bind Al^{3+} and limit its uptake into the cytosol. Tolerance mechanisms include those that chelate the aluminium entering the cytosol to

form harmless complexes or that safely store it in sub-cellular compartments. We now know that these two mechanisms exist and that both can operate in parallel. Many thorough reviews provide a detailed discussion of these areas (Taylor, 1991; Kochian *et al.*, 2004; Hiradate *et al.*, 2007; Ma, 2007; Poschenrieder *et al.*, 2008). This review will summarize recent developments and offer opinions on current research fronts.

Identification of Al³⁺ resistance genes

Genetic studies over the last 30 years have established that Al³⁺ resistance in many cereal species is a multigenic trait (Aniol and Gustafson, 1984; Luo and Dvorak, 1996; Reide and Anderson, 1996; Papernik *et al.*, 2001; Garvin and Carver, 2003; Magalhaes *et al.*, 2004; Ryan *et al.*, 2009; Shi *et al.*, 2009). Nevertheless one or two genetic loci can account for most of the phenotypic variation in some populations (Luo and Dvorak, 1996; Reide and Anderson, 1996; Garvin and Carver, 2003; Ma *et al.*, 2004; Raman *et al.*, 2005; Wang *et al.*, 2007). As explained below, a thorough understanding of both the genetics and physiology of resistance was pivotal for finally identifying the first Al³⁺ resistance genes. Not surprisingly, these first genes accounted for much of the genotypic variation in specific plant species. Genes later identified from mutational analyses are also required for resistance but do not appear to explain genotypic variation within the species (Table 1).

Al³⁺-resistance genes that contribute to genotypic variation

In the 1990s the genetics and physiology of Al³⁺ resistance in wheat (*Triticum aestivum* L.) was among the best characterized of any species due to previous studies with segregating populations and near-isogenic lines (Delhaize *et al.*, 1993a, b; Basu *et al.*, 1994; Ryan *et al.*, 1995). Those studies demonstrated that resistance in a wide range of genotypes relied on the Al³⁺-activated efflux of malate from root apices, a trait largely controlled by a single genetic locus (Delhaize *et al.*, 1993b; Ryan *et al.*, 1995). The model developed suggested that malate anions chelate and reduce the toxicity of the Al³⁺ cations in the apoplasm around the sensitive root apices. Sasaki *et al.* (2004) later identified a cDNA that was more highly expressed in the root apices of Al³⁺-resistant plants than sensitive plants of a pair of near-isogenic lines. The gene named *ALMT* (aluminium-activated malate transporter) belonged to a previously uncharacterized gene family and was the first Al³⁺ resistance gene identified in any plant species (Sasaki *et al.*, 2004; Delhaize *et al.*, 2007; Meyer *et al.*, 2010). The higher expression of *TaALMT1* in most Al³⁺-resistant genotypes of wheat is associated with tandemly-repeated elements in the promoter (Sasaki *et al.*, 2006; Raman *et al.*, 2008). Promoter analysis demonstrated that promoters containing these multiple repeats drive higher expression than

promoters without repeats (Ryan *et al.*, 2010). Heterologous expression of *TaALMT1* in tobacco-suspension cells (*Nicotiana tabacum* L.), barley (*Hordeum vulgare* L.) and wheat conferred Al³⁺-activated malate efflux and enhanced their resistance to Al³⁺ stress (Delhaize *et al.*, 2004; Sasaki *et al.*, 2004; Pereira *et al.*, 2010). It is demonstrated here that *TaALMT1* functions equally well in whole-plants of a dicotyledonous species. Transgenic *Arabidopsis* plants expressing *TaALMT1* show the same Al³⁺-activated malate efflux and enhanced resistance to Al³⁺ toxicity observed in the transgenic cereals (Fig. 1). *TaALMT1* is likely to be a useful tool in the future for engineering greater resistance into a wide range of crop and pasture plants.

Kinraide *et al.* (2005) modelled the diffusion of malate away from the root cells and concluded that the effectiveness of malate efflux as a resistance mechanism is unlikely to rely solely on the chelation of Al³⁺ in the rhizosphere. Their theoretical models indicated that the concentration of malate at the root surface would be too low to reduce Al³⁺ stress. Instead, they proposed that the epidermal cells near the apex are resilient to Al³⁺ stress and that malate may be more important for reducing Al³⁺ concentrations in and around the developing cortical cells of root apices.

A solid understanding of the genetics and physiology of resistance in sorghum (*Sorghum bicolor*) and barley was also pivotal in identifying the first members of a second family of resistance genes. Aluminium resistance in each of these species is controlled by a major genetic locus that segregates with the Al³⁺-dependent efflux of citrate from roots (Magalhaes *et al.*, 2004, 2007; Furukawa *et al.*, 2007; Wang *et al.*, 2007). Fine mapping of these loci identified the *SbMATE* gene in sorghum and the *HvAACT1* gene in barley, both of which belong to the multidrug and toxic compound exudation (MATE) family (Table I). The MATE family of transporter proteins is a large and diverse group present in prokaryotic and eukaryotic cells. Many appear to function as secondary carriers (mostly with protons) to remove small organic compounds from the cytosol (Omote *et al.*, 2006; Magalhaes, 2010). Heterologous expression of *SbMATE* in *Arabidopsis* plants and *HvAACT1* in *Xenopus* oocytes and tobacco plants established that they encode transport proteins which facilitate the Al³⁺-activated efflux of citrate (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007). Additional resistance genes were isolated based on their homology to these *ALMT1* and *MATE* genes and by exploiting the available information on the physiology and genetics of resistance in other species. This approach has helped identify candidate resistance genes in *Arabidopsis* (Hoekenga *et al.*, 2006), *Brassica napus* (Ligaba *et al.*, 2006), rye (*Secale cereale*) (Collins *et al.*, 2008), wheat (Ryan *et al.*, 2009), sorghum (Eticha *et al.*, 2010), and maize (Maron *et al.*, 2010). A role for some of these candidates in Al³⁺ resistance was confirmed using mutational analysis in *Arabidopsis* (Hoekenga *et al.*, 2006) and by segregation or functional analysis in rye and maize (Collins *et al.*, 2008; Maron *et al.*, 2010).

Table 1. Summary of genes involved in Al³⁺ resistance or tolerance

Genes are organized into different groups: (i) demonstrated Al³⁺-resistance genes that explain some genotypic variation, (ii) genes demonstrated to be involved in Al³⁺ resistance based on mutational analysis but to date do not explain genotypic variation, and (iii) likely Al³⁺ resistance genes based on segregation analysis, homology with other known genes, and/or functional analysis.

Species	Gene	Protein function	Evidence	Reference
Al³⁺ resistance genes that explains genotypic variation				
Wheat	<i>TaALMT1</i>	Malate transport	Segregation, function	Sasaki <i>et al.</i> , 2004
<i>Arabidopsis</i>	<i>AtALMT1</i>	Malate transport	Homology, function, mutational	Hoekenga <i>et al.</i> , 2006
Sorghum	<i>SbMATE1</i>	Citrate transport	Segregation, function	Magalhaes <i>et al.</i> , 2007
Barley	<i>HvAACT1</i>	Citrate transport	Segregation, function	Furukawa <i>et al.</i> , 2007
Rye	<i>ScALMT</i> gene cluster	Malate transport	Segregation, homology	Collins <i>et al.</i> , 2008
Maize	<i>ZmMATE1</i>	Citrate transport	Segregation, function	Maron <i>et al.</i> , 2010
Al³⁺ resistance genes that do not explain genotypic variation				
<i>Arabidopsis</i>	<i>AtMATE</i>	Citrate transport—efflux	Mutational	Liu <i>et al.</i> , 2009
<i>Arabidopsis</i>	<i>AtSTOP1</i>	C2H2-type Zn finger transcription factor	Mutational	luchi <i>et al.</i> , 2007
Rice	<i>OsSTAR1</i> and <i>OsSTAR2</i>	UDP-glucose transport	Mutational	Huang <i>et al.</i> , 2009
Rice	<i>ART1</i>	C2H2-type Zn finger transcription factor	Mutational	Yamaji <i>et al.</i> , 2009
<i>Arabidopsis</i>	<i>ALS3</i>	Partial ABC protein—function unclear	Mutational	Larsen <i>et al.</i> , 2005
<i>Arabidopsis</i>	<i>ALS1</i>	Partial ABC protein—function unclear	Mutational	Larsen <i>et al.</i> , 2007
<i>Arabidopsis</i>	<i>AtSTAR1</i>	Partial ABC protein—function unclear	Mutational	Huang <i>et al.</i> , 2010
Likely Al³⁺ resistance genes				
Wheat	<i>TaMATE1</i>	Citrate transport—efflux	Segregation, homology (no mutational or functional data)	Ryan <i>et al.</i> , 2009
<i>Brassica napus</i>	<i>BnALMT1</i>			
	<i>BnALMT2</i>	Malate transport—efflux	Homology, function (no mutational or segregation data)	Ligaba <i>et al.</i> , 2006
Rye	<i>ScMATE2</i>	Citrate transport—efflux	Homology, biology (no functional or segregation data)	Yokosho <i>et al.</i> , 2010

Resistance genes that do not explain genotypic variation

Subsequently, a different set of Al³⁺ resistance genes was identified using an approach that requires no prior knowledge or assumptions regarding the genetics or mechanisms involved. In this approach, seed are mutagenized by chemical treatments, radiation or by the random insertion of a DNA fragment (T-DNA) into the genome. M₂ seedlings are screened and those that grow similarly to wild-type plants under control conditions, but are hypersensitive to Al³⁺ stress or acidity, may carry mutations in genes necessary for Al³⁺ resistance. The genes are finally identified by mapping or by obtaining sequence flanking the T-DNA and, in both cases, are verified by complementation of the mutants. These genes need not show allelic variation which means they are not necessarily responsible for natural genotypic variation within a species. At least six genes have been identified in rice and *Arabidopsis* by mutational analysis as discussed below.

As model species, rice and *Arabidopsis* are attractive systems for mutational analysis. Rice is an important crop worldwide with a high basal level of resistance compared with other small-grained cereals (Famoso *et al.*, 2010). The striking similarities between the resistance genes isolated from rice and *Arabidopsis* using a mutational approach is intriguing given that rice is considerably more resistant (Table I). In contrast to other cereals, Al³⁺ resistance in rice

is not explained by organic anion efflux. Indeed, the Al³⁺-activated efflux of citrate from rice roots is considerably lower than the efflux measured in rye where it does represent a major mechanism of resistance (Li *et al.*, 2000; Collins *et al.*, 2008).

A recent breakthrough in uncovering the molecular basis of Al³⁺ resistance in rice was achieved when the gene underlying an Al³⁺-sensitive mutant, *star1*, was identified (Huang *et al.*, 2009). The wild-type gene named *OsSTAR1* (sensitive to Al rhizotoxicity) encodes the nucleotide binding domain of a bacterial-type ABC-transporter. The *OsSTAR1* protein interacts with *OsSTAR2* which possesses a transmembrane domain from this same ABC-transporter family. Both *OsSTAR1* and *OsSTAR2* are predominantly expressed in roots and expression of both is specifically induced by Al³⁺ treatment. The *OsSTAR1:OsSTAR2* complex localizes to vesicular membranes and transports UDP-glucose, but it is not clear how this function confers resistance. The *OsSTAR* proteins may release UDP-glucose to the apoplast by exocytosis and provide protection by modifying the cell walls. It is also plausible that the *OsSTAR* proteins confer Al³⁺ ‘tolerance’ rather than ‘resistance’ by performing other functions in the cytosol. Neither of the *OsSTAR* genes underlies QTLs for Al³⁺ resistance in rice, but they are possibly responsible for its high basal level of resistance (Huang *et al.*, 2009). Subsequently, Yamaji *et al.* (2009) identified a zinc-finger transcription factor (*ART1*) that regulates the Al³⁺-induced

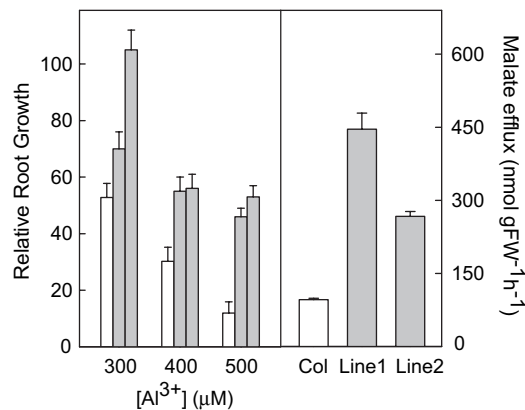


Fig. 1. Expression of the wheat gene *TaALMT1* enhances the Al³⁺ resistance of *Arabidopsis*. (A) Al³⁺ resistance of wild-type *Arabidopsis* (Col) (white bars) and T₂ homozygous transgenic lines (lines 1 and 3) expressing *TaALMT1* (grey bars). Relative root growth was calculated by comparing the length of the longest root on plants grown on control plates (no Al³⁺) and on plates containing AlCl₃. Plants were grown for 14 d on agar plates containing a minimal nutrient solution with different concentrations of AlCl₃. Relative root growth was calculated as ((length+Al³⁺) × 100)/(length–Al³⁺). Data show mean and SE ($n=20-25$ plants). (B) Malate efflux from wild-type control plants (white bars) and T₂ homozygous transgenic lines 1 and 3 (grey bars). Plants were first grown over a simple nutrient solution for 2 weeks. The solution was then replaced with 0.5 mM CaCl₂ (pH 4.9), containing 100 μM AlCl₃. Malate released from the roots over 24 h was measured by enzyme assay. Data show the mean and SE ($n=3$) where one replicate represented approximately 100 seedlings. *TaALMT1* cDNA was ligated in the pPLEX502 binary vector behind the CaMV 35S promoter and transformed into *Arabidopsis* (Col) as described previously (Ryan *et al.*, 2007).

expression of both *OsSTAR1* and *OsSTAR2* along with over 30 other genes. Some of these other genes might also contribute to Al³⁺ resistance because they map to previously identified QTLs for Al³⁺ resistance on the rice genome. The observation that the *star1* mutant is as Al³⁺ sensitive as the *art1* mutant suggests that the *OsSTAR* genes control a major mechanism of Al³⁺ resistance in rice.

AtALS1 and *AtALS3* are Al³⁺-resistance genes isolated by mutational analysis of *Arabidopsis*. They also encode ‘half’ ABC transporters with *AtALS1* having significant sequence similarity to *OsSTAR2* (Larsen *et al.*, 2005, 2007). Nevertheless Larsen (2009) speculated that the AtALS proteins transport aluminium, possibly in a chelated form, between cells (*AtALS3*) or to the vacuole (*AtALS1*). A zinc finger transcription factor, *AtSTOP1*, was initially identified from a pH-sensitive *Arabidopsis* mutant. *AtSTOP1* is related to *OsART1* from rice but regulates a different range of genes including the known resistance genes *AtALMT1*, *AtMATE1*, and *ALS3* (Sawaki *et al.*, 2009) and several genes associated with proton tolerance (Iuchi *et al.*, 2007). More recently, the closest homologue to *OsSTAR1* in *Arabidopsis*, named *AtSTAR1*, was shown to contribute to Al³⁺ tolerance as well because plants carrying a knockout

mutation in *AtSTAR1* were hypersensitive to Al³⁺ stress (Huang *et al.*, 2010). Furthermore, some evidence suggests *AtSTAR1* may be interacting with *AtALS3* (Huang *et al.*, 2010).

Other genes were isolated by a strategy that identified mutations able to suppress the hypersensitivity of *Arabidopsis als3-1* mutants (Gabrielson *et al.*, 2006). *als3-1* seeds were further mutagenized and M₂ seedlings screened for mutations that recovered Al tolerance. A description of *AtATR* (*Ataxia telangiectasia-mutated and RAD3-related*) and other genes identified in this way can be found elsewhere (Gabrielson *et al.*, 2006; Larsen, 2009). Not all of these genes can be considered Al³⁺-resistance genes in the strict sense. For instance, a mutation in *AtATR* improved root growth on Al³⁺ toxic media by altering fundamental processes of the cell cycle that detect DNA damage and these may carry other costs for the plant in the absence of Al. Interestingly, those studies led Larsen and colleagues to radically re-interpret the mechanism of Al³⁺ toxicity. Instead of root growth being inhibited by a series of lesions in the symplasm and apoplasm that disrupt cell function, they proposed that growth is actually inhibited by the plant’s own responses to the stress rather than by the stress itself (Larsen, 2009).

Transgenic approaches for increasing Al resistance: Why bother?

By 2050 world population will have increased by two billion people. Crop production will need to increase by 50% or more to meet the added demand for food, fibre, and animal feed. Most of these gains will have to be met by increasing yields and cropping intensity, but additional land, perhaps previously considered unsuitable for farming, will also need to be cultivated. Acid soils are widespread in sub-Saharan Africa, Asia, and other regions likely to see the largest increases in population. While the application of lime (calcium carbonate) is the most efficient way of ameliorating soil acidity and improving its suitability for agriculture, it is not practical or common in developing countries that rely on small subsistence farms for food production. A combination of Al³⁺-resistant germplasm with the application of lime or some other ameliorant, where possible, is a common management strategy. Some crops perform adequately on acid soils and others can be improved with conventional breeding strategies. However, for crops that possess insufficient natural variation to breed for Al³⁺ resistance, other solutions are required. Demands on our production systems from population growth and climate change require all options to be considered, including transgenic strategies (Hoisington, 2002; Bhalla, 2006).

Once the role of organic anion efflux in Al resistance was understood, many researchers tried genetically engineering this trait into model species. The two main strategies were to increase organic anion synthesis with the hope that this leads to greater efflux, and to increase organic anion transport across the plasma membrane. Some argued that the transport step was likely to be the rate-limiting step for efflux because the capacity to synthesize citrate and malate

is probably in excess in wild-type cells or is tightly regulated (Ratledge, 2000; Ryan *et al.*, 2001). Furthermore, the large electrochemical gradient across the plasma membrane favours anion release from cells indicating that malate and citrate efflux should occur spontaneously once a pathway is provided. Nevertheless, early attempts to increase resistance targeted organic anion synthesis because the genes encoding the transport proteins were not available at the time.

The first influential study of this type was from de la Fuente *et al.* (1997) who over-expressed a bacterial citrate synthase gene in tobacco. They reported greater citrate efflux and enhanced Al³⁺ resistance in several transgenic lines. Perhaps expressing a bacterial gene in plants helped the enzyme avoid normal regulatory mechanisms. In any case this publication triggered a flurry of activity as groups tried to repeat this study and perform similar experiments using other genes involved in anion synthesis (e.g. citrate synthase, malate dehydrogenase, isocitrate hydrogenase). A number of reports claimed success (Table 2) while others were unable to repeat the original study of de la Fuente and colleagues (Delhaize *et al.*, 2001). However, even when successful, the gains in resistance were relatively modest. Rarely is relative root growth more than 3-fold greater than the controls (Table 2).

Other studies enhanced Al³⁺ resistance in plants by over-expressing genes whose expression is induced by Al³⁺ treatment, particularly those associated with oxidative stress (Ezaki *et al.*, 2000, 2005). For instance, increasing the expression of glutathione *S*-transferase, peroxidase, GDP-dissociation inhibitor, and a blue copper protein in *Arabidopsis* increased relative root growth by 1.5–2.5-fold compared with the controls. These and other studies (Basu *et al.*, 2001) demonstrate that boosting a plant's normal defences to oxidative stress can also enhance Al³⁺ tolerance. Once again, the benefits were small, with most improving relative root growth by less than 2-fold.

Modest gains in the tolerance of transgenic plants were also obtained using an approach that assumed no prior knowledge of genetics or physiology. Candidate genes were first identified by screening plant cDNA libraries in yeast cells grown on agar plates containing sufficient Al³⁺ to suppress the growth of wild-type cells (Delhaize *et al.*, 1999; Ryan *et al.*, 2007). cDNAs that allowed yeast cells to grow were isolated and then systematically expressed in plants. A $\Delta 8$ sphingolipid desaturase isolated in this way slightly increased the Al³⁺ resistance of *Arabidopsis* by modifying the composition of cell lipids (Ryan *et al.*, 2007).

By far the largest increases in Al³⁺ resistance have been achieved by over-expressing organic anion transport proteins (Table 2). These studies targeted the known Al³⁺ resistance genes discussed above (*TaALMT1*, *AtALMT1*, *SbMATE*) or related genes that performed different functions (e.g. *Frd3* from *Arabidopsis* and *HvALMT1* from barley). The most striking examples to date have resulted from over-expressing *TaALMT1* in barley, wheat, and *Arabidopsis*. These plants showed greater relative root growth by 20, 8, and 4-fold, respectively (Table 2). Several important conclusions can be drawn from these studies. The

first is that the transport of organic anions across the plasma membrane is a more important bottleneck to efflux than their synthesis. These studies also explain why Al³⁺-resistance relies on malate efflux in some species and citrate efflux in others. This difference is determined primarily by the substrate specificity of the transport protein in the membrane, and not by the metabolism of the cells (Ryan and Delhaize, 2010). For example, Al³⁺ resistance in wild-type barley relies on citrate efflux from roots (mediated by HvAACT) yet transgenic plants expressing the wheat *TaALMT1* gene also release malate anions indicating that barley is capable of synthesizing and releasing malate provided a transport pathway is present (Delhaize *et al.*, 2004).

The isolation of genes from mutational studies provides another avenue for engineering Al³⁺ resistance. To date, these genes have been used successfully to complement their respective mutants, but in at least two instances (*AtALS3* from *Arabidopsis* and *OsART1* from rice), their overexpression did not enhance Al³⁺ resistance in transgenic plants (Larsen *et al.*, 2007; Yamaji *et al.*, 2009). Nevertheless, other genes, such as those encoding the OsSTAR proteins, need to be tested to determine whether they confer high levels of resistance if expressed in more Al³⁺-sensitive species.

Focus topic: structure–function of TaALMT

It is now clear that many Al³⁺-tolerance and resistance mechanisms operate in plants and these are controlled by numerous genes. Our understanding remains rudimentary in almost all aspects of their physiology and molecular biology. For instance, much of the plant research has been carried out on young seedlings, often grown in nutrient solution, so the behaviour of these mechanisms in older plants and in different parts of a mature root system is unknown. It is also possible that Al³⁺ resistance genes contribute to other stress pathways, nutrition acquisition, or even pathogen responses (Pineros *et al.*, 2008b; Rudrappa *et al.*, 2008; Delhaize *et al.*, 2009). This section reviews recent studies on the structure of *TaALMT1* from wheat which are beginning to reveal details of its function.

Does TaALMT1 function as an anion channel?

Early physiological studies suggested that malate efflux from wheat roots was mediated by anion channels. The electrochemical gradient for malate across the plasma membrane is consistent with the involvement of a channel and known antagonists of anion channels (niflumate and anthracene-9-carboxylate) reduced the Al³⁺-activated malate efflux (Ryan *et al.*, 1995). A series of electrophysiological studies on protoplasts isolated from wheat roots (Ryan *et al.*, 1997; Zhang *et al.*, 2001), transgenic *Xenopus* oocytes (Sasaki *et al.*, 2004; Pineros *et al.*, 2008a) and tobacco suspension cells (Zhang *et al.*, 2008) found that *TaALMT1* facilitated Al³⁺-activated inward and outward currents. The inward currents were generated by organic and inorganic anion efflux that were inhibited by niflumate.

Table 2. Studies that have enhanced Al³⁺ resistance by genetic engineering

Shown are studies that have enhanced the Al³⁺ resistance of whole plants by genetic engineering. Listed are the transgenes, their species of origin, and the species that were transformed with the transgenes. Also included are estimates for the fold-increases in relative root growth compared with control plants (wild-type plants or null segregants) as well as the proposed mechanism for the phenotype. Relative root growth refers to net root growth in the presence of Al³⁺ divided by net root growth in the absence of Al³⁺.

Gene function	Transgenic strategy	Phenotype (RRG)	Proposed mechanism	Reference
Organic anion metabolism				
Citrate synthase	<i>Pseudomonas aeruginosa</i> gene expressed in tobacco	2-fold	Enhanced organic acid efflux	de la Fuente <i>et al.</i> , 1997
Malate dehydrogenase	Nodule-enhanced gene expressed in alfalfa	2.0-fold	Enhanced organic acid efflux	Tesfaye <i>et al.</i> , 2001
Citrate synthase	Mitochondrial cDNA from carrot was expressed in <i>Arabidopsis</i>	1.3-fold	Enhanced citrate efflux	Koyama <i>et al.</i> , 2000
Citrate synthase	Mitochondrial cDNA from <i>Arabidopsis</i> expressed in <i>Brassica napus</i>	2.0-fold	Enhanced citrate efflux	Anoop <i>et al.</i> , 2003
Citrate synthase	<i>Pseudomonas aeruginosa</i> gene expressed in alfalfa	2.5-fold		Barone <i>et al.</i> , 2008
Pyruvate phosphate dikinase	<i>Mesembryanthemum crystallinum</i> gene expressed in tobacco	1.2-fold	Enhanced malate and citrate efflux	Trejo-Tellez <i>et al.</i> , 2010
Malate dehydrogenase	<i>Arabidopsis</i> and <i>E.coli</i> genes expressed in tobacco	2.4-fold	Increase in malate efflux	Wang <i>et al.</i> , 2010
Stress responsive				
Glutathione S-transferase	Tobacco gene expressed in <i>Arabidopsis</i>	1.5-fold	Enhanced oxidative stress tolerance	Ezaki <i>et al.</i> , 2000
Peroxidase	Tobacco gene expressed in <i>Arabidopsis</i>	1.5-fold	Protection from oxidative stress	Ezaki <i>et al.</i> , 2000
GDP-dissociation inhibitor	Tobacco gene expressed in <i>Arabidopsis</i>	1.5-fold	Enhanced stress tolerance	Ezaki <i>et al.</i> , 2000
Blue copper protein	<i>Arabidopsis</i> gene expressed in <i>Arabidopsis</i>	1.5-fold	Protection from oxidative stress	Ezaki <i>et al.</i> , 2000
Dehydroascorbate reductase	<i>Arabidopsis</i> gene expressed in tobacco	1.5-fold	Higher ascorbic acid protects from oxidative stress	Yin <i>et al.</i> , 2010
Manganese superoxide dismutase	Wheat gene expressed in <i>Brassica napus</i>	2.5-fold	Protection from oxidative stress	Basu <i>et al.</i> , 2001
Transport				
Al ³⁺ -activated malate transporter (<i>TaALMT1</i>)	Wheat gene expressed in barley	20-fold	Enhanced malate efflux	Delhaize <i>et al.</i> , 2004
Multidrug and toxic compound efflux gene (<i>MATE</i>) called <i>Frd3</i>	<i>Arabidopsis</i> gene expressed in <i>Arabidopsis</i>	3.0-fold	Enhanced citrate efflux	Durrett <i>et al.</i> , 2007
Al ³⁺ -activated malate transporter (<i>TaALMT1</i>)	Wheat gene expressed in wheat	8-fold	Enhanced malate efflux	Pereira <i>et al.</i> , 2010
Al ³⁺ -activated malate transporter (<i>TaALMT1</i>)	Wheat gene expressed in <i>Arabidopsis</i>	4-fold	Enhanced malate efflux	Fig. 1
Multidrug and toxic compound efflux gene (<i>HvAACT1</i>)	Barley gene expressed in tobacco plants	2.3-fold	Enhanced citrate efflux	Furukawa <i>et al.</i> , 2007
Al ³⁺ -activated malate transporter (<i>AtALMT1</i>)	<i>Arabidopsis</i> gene expressed in <i>Arabidopsis</i>	3-fold	Enhanced malate efflux	Ryan PR, Dong B, DelhaizeE, Hoekenga OAKochian LV (unpublished results)
H ⁺ -pyrophosphatase AVP1	Over-expression of endogenous genes in <i>Arabidopsis</i> , tomato, and rice	1.8-fold	Enhanced organic acid efflux	Yang <i>et al.</i> , 2007
Multidrug and toxic compound efflux gene (<i>SbMATE</i>)	Sorghum gene expressed in the <i>Arabidopsis Atalmt1</i> mutant	2.5-fold	Enhanced citrate efflux	Magalhaes <i>et al.</i> , 2007
Al ³⁺ -activated malate transporter family (<i>HvALMT1</i>)	Barley gene expressed in barley.	3.4-fold	Enhanced malate efflux	Gruber, 2009
Multidrug and toxic compound efflux gene (<i>ZmMATE1</i>)	Maize gene expressed in <i>Arabidopsis</i>	3-fold	Enhanced citrate efflux	Maron <i>et al.</i> , 2010
Other				
Cell wall associated receptor kinase	<i>Arabidopsis WAK1</i> gene expressed in <i>Arabidopsis</i>	3-fold	Enhances stress responsiveness	Sivaguru <i>et al.</i> , 2003
Auxilin-like protein	Over-expression of endogenous <i>Arabidopsis</i> gene	3-fold	Reduction in endocytosis	Ezaki <i>et al.</i> , 2007
Δ8 sphingolipid desaturase	<i>Stylosanthes</i> gene expressed in <i>Arabidopsis</i>	2-fold	Changes to membrane lipid composition	Ryan <i>et al.</i> , 2007
Ced-2 (Bcl2 homologue)	<i>Caenorhabditis elegans</i> gene expressed in tobacco	2-fold	Reduced Al ³⁺ -induced programmed cell death	Wang <i>et al.</i> , 2009

On the basis of those experiments it was initially concluded (see later) that TaALMT1 functions as an inwardly rectifying, ligand-gated anion channel (Ryan *et al.*, 1997; Zhang *et al.*, 2001). What does this mean? Ligand-gated refers to the requirement for Al^{3+} to activate channel function and inwardly rectifying means it passes more inward current than outward current across the voltage range. Inwardly rectifying channels would typically conduct more current as the electrical potential difference across the membrane becomes more negative (hyperpolarized). The loss of malate anions from the cell is accompanied by the release of K^+ which appears to satisfy electroneutrality (Ryan *et al.*, 1995). The model proposed that malate efflux, via TaALMT1, depolarizes the plasma membrane and triggers K^+ efflux via a voltage-sensitive, outwardly rectifying K^+ channel. However, uncertainties with this model persisted because malate-dependent, single-channel traces, diagnostic of channel involvement, were not detected in those early studies, and this cast doubt on whether TaALMT1 functioned as an anion channel or as some other type of transporter. Secondly, since Al^{3+} depolarizes the apical cell membranes of Al^{3+} -resistant wheat (Wherrett *et al.*, 2005) it was perplexing how the rapid and sustained efflux of malate could occur via a channel that was supposedly more active in hyperpolarized membranes.

Stronger evidence that TaALMT1 functions as an anion channel was later collected on protoplasts prepared from wild-type wheat roots and tobacco-suspension cells expressing TaALMT1 (Zhang *et al.*, 2008). Transient channel activity was detected in out-side out patches pulled from whole cells already activated by Al^{3+} and single-channel events were also recorded in the whole-cell configuration, especially during voltage ramps and as currents deactivated. Zhang *et al.* (2008) calculated that TaALMT1 was ~18-fold more permeable to malate than Cl^- which was similar to estimates by Pineros *et al.* (2008a) working with *Xenopus* oocytes at the same time. Perhaps more importantly, Zhang *et al.* (2008) revised our understanding of TaALMT1 function by establishing that it is not an inwardly-rectifying channel as first thought. They established that the Al^{3+} -activated currents were fully active at depolarized membrane potentials and partially deactivated at more negative potentials. This deactivation was 'tuned' so that the channel acts like a constant current device. As a result TaALMT1 facilitates an Al^{3+} -activated inward current that is relatively insensitive to membrane potential. This has the effect of providing a relatively constant efflux of malate over a range of physiological membrane potentials. The previous reports of 'inward rectification' (Ryan *et al.*, 1997; Zhang *et al.*, 2001) were artefacts of the concentration gradients of the permeating anions across the plasma membrane and did not result from voltage-dependent changes in gating behaviour. This is an important observation because it helps explain the question raised above of how TaALMT1 sustains malate efflux from depolarized cells. Sustained efflux of malate and K^+ was now feasible because both occurred via separate channels active in depolarized membranes.

Another issue that arises from the sustained release of malate via TaALMT1 is why complexation of Al^{3+} in the immediate vicinity of the channel does not turn off the malate release. The reversibility of the Al^{3+} activation and the effect of external malate on channel activity after activation warrants further investigation. Some anion channels are trans-stimulated by permeating anions (Dietrich and Hedrich, 1998; Diatloff *et al.*, 2004) which means efflux is stimulated by increasing the concentration of permeating anions on the external side. This response has been observed in TaALMT1 in response to external Cl^- (Pineros *et al.*, 2008a) and in HvALMT1 in response to various organic anions (Gruber *et al.*, 2010). This question raises the interesting possibility that malate itself and perhaps other anions that chelate Al^{3+} , may also activate TaALMT1 in order to sustain the malate efflux.

Protein phosphorylation and activation by Al^{3+}

One intriguing aspect of ALMT and MATE involvement in Al^{3+} resistance is the role of post-translational regulation and particularly the requirement for Al^{3+} to activate their function. ALMT1 proteins from wheat, rye, *Arabidopsis*, and *Brassica napus*, and the MATE proteins from sorghum and barley all require external Al^{3+} to trigger transport activity. This is clear from studies that have constitutively expressed these genes in a range of different cell types. In the absence of Al^{3+} the basal level of inward current (organic anion efflux) is consistently greater in transgenic cells compared with controls. However, the addition of Al^{3+} significantly increases transport activity in transgenic cells but not controls. These basal currents are similar to the Al^{3+} -activated currents with regard to selectivity and sensitivity to antagonists, which suggests they reflect the normal function of TaALMT1 (Pineros *et al.*, 2008a; Zhang *et al.*, 2008). How members of the ALMT and MATE protein families acquired such specific interactions with Al^{3+} is an interesting question which is discussed elsewhere (Ryan and Delhaize, 2010). The details of this activation by Al^{3+} remain a mystery, but recent studies investigating this issue are discussed below.

The secondary structure of TaALMT1 (459 amino acids) is predicted to consist of six transmembrane domains in the N-terminal half of the protein and a long C-terminal domain (~240 residues) located extracellularly as shown in Fig. 2 (Motoda *et al.*, 2007). It is tempting to propose that this extracellular domain interacts directly with external Al^{3+} to influence channel function and some support for this is presented later. However, well before *TaALMT1* had even been identified, phosphorylation was predicted to be important because malate efflux from wheat roots was inhibited by the protein kinase inhibitor K252a (Osawa and Matsumoto, 2001). A similar inhibition was also reported for citrate release from soybean roots (Shen *et al.*, 2004). It is now known that *TaALMT1* is constitutively expressed in wheat (Sasaki *et al.*, 2004) which suggests K252a may have been acting on the TaALMT1 protein rather than the pathways regulating expression. Ligaba *et al.* (2009)

supported this conclusion by showing that protein kinase inhibitors (K252a and staurosporine) inhibit the Al^{3+} -activated currents in *Xenopus* oocytes expressing TaALMT1. Interestingly, pretreatment with a protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) slightly enhanced the TaALMT1-mediated currents. Ligaba *et al.* (2009) generated a series of mutations targeting possible phosphorylation sites on TaALMT1. Two mutations on the C-terminal domain altered protein function. One of these which substituted a threonine for an alanine at residue 323 (T323A) more than doubled the basal and Al^{3+} -activated currents (Fig. 2). No clear explanation is available for this shift in conductance, but suggestions that the mutation altered protein structure are possible. Another mutation located on the C-terminal domain, S384A, significantly reduced both basal and Al^{3+} -activated currents, with the modified proteins being insensitive to staurosporine and PMA treatments (Ligaba *et al.*, 2009). While these results are consistent with phosphorylation being involved in TaALMT1 function, future work should confirm that the S384 residue is actually phosphorylated in wild-type alleles and that the S384A and T323A mutations do not affect the level of protein expression in oocytes. Furthermore, the S384A mutation may have resulted in a dysfunctional protein since both the basal and activated currents were abolished. Nevertheless, the model emerging from these studies indicates that phosphorylation is a prerequisite for TaALMT1 function and perhaps also for its activation by Al^{3+} . It is easy to envision how the addition of a negatively-charged phosphate group on the protein might alter tertiary structure or form part of a binding site for Al^{3+} which then increases transport activity.

Since the C-terminal domain is predicted to be extracellular (Motoda *et al.*, 2007), a surprising corollary from this work is that TaALMT1 may be phosphorylated by a kinase acting in the apoplasm. No extracellular kinases that phosphorylate membrane proteins have been reported to date in plants although ecto-protein kinases that phosphorylate proteins on the cell surface have been characterized in animal cells. Indeed, in a recent review on carboxylate transport in plants, Meyer *et al.* (2010) considered it so unlikely that kinases act extracellularly that they reversed the orientation proposed by Motoda *et al.* (2007). However, the direct experimental evidence supporting the topology predictions is likely to be more reliable than the physiology which could be influenced by other factors. Therefore, we conclude that either ecto-kinase activity does affect TaALMT1 function by processes that are currently unclear or the phosphorylation data require reconsideration.

Another recent study generated different types of mutations to examine TaALMT1 function. A series of truncations were made to the C-terminal domain starting with the terminal 70 residues and working up to the entire C-terminal tail (Furuichi *et al.*, 2010). All truncations abolished the basal currents (occurring in the absence of Al^{3+}) as well as the Al^{3+} -activated transport activity without affecting protein expression. Interestingly, full function was recovered by fusing the N-terminal region of TaALMT1

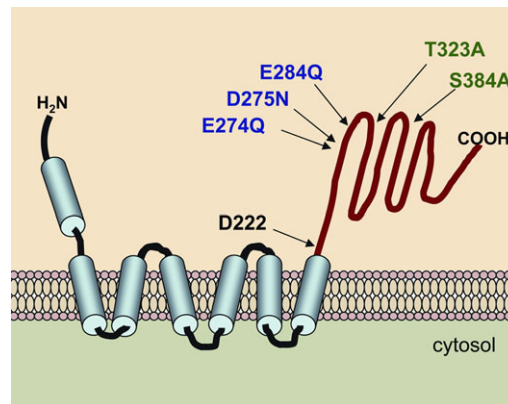


Fig. 2. Diagram depicting the secondary structure of TaALMT1. TaALMT1 (459 amino acids) is predicted to possess six transmembrane regions with the N and C-terminal ends orientated extracellularly (Motoda *et al.*, 2007). Residue D222 is located near the start of the hydrophilic C-terminal region (coloured red). Approximate positions of mutations to the C-terminal domain found to alter protein function are indicated by arrows. Two mutations affecting putative phosphorylation sites are coloured green (Ligaba *et al.*, 2009) and three mutations targeting acidic residues are coloured blue (Furuichi *et al.*, 2010).

with the C-terminal region of AtALMT1 from *Arabidopsis*. These findings demonstrate that the C-terminal domain is required for all functions of TaALMT1 and highlight the close functional similarity between these homologues from wheat and *Arabidopsis*.

Furuichi *et al.* (2010) then mutated acidic residues on the C-terminal domain since these were considered most likely to interact with extracellular Al^{3+} . Fifteen acidic residues were individually replaced with uncharged residues and the transport behaviour of the modified proteins were compared in *Xenopus* oocytes. Three mutations (E274Q, D275N, and E284Q) abolished the Al^{3+} -activated transport activity without affecting the basal transport activity which remained the same as wild-type TaALMT1 (Fig. 2). The authors proposed that all three anionic residues are necessary for Al^{3+} to activate TaALMT1 function. They further argued that these mutations do not grossly disrupt the tertiary structure of the protein because the basal currents were unaffected.

The *AtALMT1* gene from *Arabidopsis* requires Al^{3+} treatment both to induce gene expression and to activate protein function (Hoekenga *et al.*, 2006; Kobayashi *et al.*, 2007). This differs from *TaALMT1* in wheat which is constitutively expressed and only requires Al^{3+} to activate function. Kobayashi *et al.* (2007) showed the induction of *AtALMT1* expression by Al^{3+} is inhibited by staurosporine (kinase inhibitor) and calyculin A (phosphatase inhibitor) and K252a inhibited Al^{3+} -dependent malate efflux without reducing gene expression. These results suggest that induction of *AtALMT1* expression by Al^{3+} may involve reversible protein phosphorylation. Furthermore, either AtALMT1 itself or an upstream component needs to be phosphorylated for Al^{3+} to activate transport activity, which is similar to the conclusions drawn above for TaALMT1.

Insights into the structure–function relationships of AtALMT1 were also obtained from the *Arabidopsis* ecotype Warschau-1 which is hypersensitive to Al³⁺ stress. A single nucleotide substitution was responsible for the phenotype because it introduced a premature stop codon in the N-terminal half of the *AtALMT1* coding region (Kobayashi *et al.*, 2007). The resulting protein is a naturally occurring example of the truncation experiments described above for TaALMT1 but it is not known whether the truncated protein accumulates in the plant. The mutation in Warschau-1 generated a dominant negative phenotype in an F₂ population developed from Warschau-1 and Col-4 ecotypes and the authors speculated that the presence of truncated monomers disrupt the formation of functioning multimeric complex necessary for a mature transport protein. If correct, all F₁ plants from this cross should be sensitive of Al³⁺ but this requires confirmation.

Constraints to further progress

Much of our understanding of Al³⁺ resistance conferred by organic anions is derived from heterologous expression of ALMTs and MATEs in plant and animal cells. Further advances will rely on the systematic generation and characterization of mutations in these proteins. Mutations can be efficiently made in yeast and bacteria by standard techniques, but a suitable expression system remains an obstacle. ALMTs and MATEs have been successfully expressed in plants (wheat, barley, *Arabidopsis*, and rice) as well as tobacco suspension cells. While increasingly routine, these procedures take months to complete and it is difficult to carry scores of different lines through tissue culture.

Xenopus oocytes have proved themselves to be very useful for characterizing the electrophysiological behaviour of transport proteins and already many studies have used these cells to assess the effect that specific mutations have on ALMT transport activity. The procedure is rapid, only taking a few days and *Xenopus* oocytes will continue to be an important experimental system in the future. Nevertheless, there is always some concern whether the behaviour of proteins expressed in this animal system truly reflects their behaviour *in planta*. The ionic composition of the cytosol in oocytes and root-cells are not identical and so transport behaviour could differ. Furthermore, oocyte experiments require substantial infrastructure to raise the frogs and analyse protein function with electrophysiological techniques. Few laboratories can easily afford the expense to maintain this system.

Are there practical alternatives to *Xenopus* oocytes? Plant cells are preferred but single-celled systems or other animal cells offer some advantages. Insect cell lines (e.g. *Spodoptera frugiperda* and *Trichoplusia ni*) are available but these may present the same difficulties as oocytes. Attempts to express ALMTs in yeast and bacteria were unable to detect function, despite confirming protein expression in yeast (W. Chen and P. Ryan, unpublished; BD Gruber, personal communication; Sasaki T, personal communication). Unfortunately, this precludes the use of a number of powerful genetic and

molecular techniques for analysing these proteins. One alternative to using stably-transformed plants is the hairy root systems which have been successfully established in several dicotyledonous species (Guillon *et al.*, 2006). More rapid than whole-plant transformation, this technique provides a transgenic root system suitable for experiments ranging from whole roots to single cells. The choice of which system to use comes down to a balance between using plant cells to ensure a faithful phenotype and the time, effort and expense in generating the transgenic lines.

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