Journal of Experimental Botany, Vol. 62, No. 1, pp. 9–20, 2011
doi:10.1093/jxb/erq272 Advance Access publication 16 September, 2010

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

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

P. R. Ryan[1,*], S. D. Tyerman[2], T. Sasaki[3], T. Furuichi[3], Y. Yamamoto[3], W. H. Zhang[4] and E. Delhaize[1]

[1] CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia
[2] School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Glen Osmond, SA 5064, Australia
[3] Institute of Plant Science and Resources, Okayama University, Chuo 2-20-1, Kurashiki, Okayama, 710-0046 Japan
[4] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China

* To whom correspondence should be addressed: E-mail: peter.ryan@csiro.au

Received 18 June 2010; Revised 5 August 2010; Accepted 12 August 2010

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 subcellular 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 Al^{3+} resistance genes

Genetic studies over the last 30 years have established that Al^{3+} 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 Al^{3+} 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).

Al^{3+}-resistance genes that contribute to genotypic variation

In the 1990s the genetics and physiology of Al^{3+} 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.*, 1993*a*, *b*; Basu *et al.*, 1994; Ryan *et al.*, 1995). Those studies demonstrated that resistance in a wide range of genotypes relied on the Al^{3+}-activated efflux of malate from root apices, a trait largely controlled by a single genetic locus (Delhaize *et al.*, 1993*b*; Ryan *et al.*, 1995). The model developed suggested that malate anions chelate and reduce the toxicity of the Al^{3+} 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 Al^{3+}-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 Al^{3+} 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 Al^{3+}-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 tabaccum* L.), barley (*Hordeum vulgare* L.) and wheat conferred Al^{3+}-activated malate efflux and enhanced their resistance to Al^{3+} 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 Al^{3+}-activated malate efflux and enhanced resistance to Al^{3+} 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 Al^{3+} in the rhizosphere. Their theoretical models indicated that the concentration of malate at the root surface would be too low to reduce Al^{3+} stress. Instead, they proposed that the epidermal cells near the apex are resilient to Al^{3+} stress and that malate may be more important for reducing Al^{3+} 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 Al^{3+}-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 Al^{3+}-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 Al^{3+} 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 Al^{3+} resistance or tolerance

Genes are organized into different groups: (i) demonstrated Al^{3+}-resistance genes that explain some genotypic variation, (ii) genes demonstrated to be involved in Al^{3+} resistance based on mutational analysis but to date do not explain genotypic variation, and (iii) likely Al^{3+} resistance genes based on segregation analysis, homology with other known genes, and/or functional analysis.

Species	Gene	Protein function	Evidence	Reference
Al^{3+} resistance genes that explains genotypic variation				
Wheat	*TaALMT1*	Malate transport	Segregation, function	Sasaki *et al.*, 2004
Arabidopsis	*AtALMT1*	Malate transport	Homology, function, mutational	Hoekenga *et al.*, 2006
Sorghum	*SbMATE1*	Citrate transport	Segregation, function	Magalhaes *et al.*, 2007
Barley	*HvAACT1*	Citrate transport	Segregation, function	Furukawa *et al.*, 2007
Rye	*ScALMT* gene cluster	Malate transport	Segregation, homology	Collins *et al.*, 2008
Maize	*ZmMATE1*	Citrate transport	Segregation, function	Maron *et al.*, 2010
Al^{3+} resistance genes that do not explain genotypic variation				
Arabidopsis	*AtMATE*	Citrate transport—efflux	Mutational	Liu *et al.*, 2009
Arabidopsis	*AtSTOP1*	C2H2-type Zn finger transcription factor	Mutational	Iuchi *et al.*, 2007
Rice	*OsSTAR1* and *OsSTAR2*	UDP-glucose transport	Mutational	Huang *et al.*, 2009
Rice	*ART1*	C2H2-type Zn finger transcription factor	Mutational	Yamaji *et al.*, 2009
Arabidopsis	*ALS3*	Partial ABC protein—function unclear	Mutational	Larsen *et al.*, 2005
Arabidopsis	*ALS1*	Partial ABC protein—function unclear	Mutational	Larsen *et al.*, 2007
Arabidopsis	*AtSTAR1*	Partial ABC protein—function unclear	Mutational	Huang *et al.*, 2010
Likely Al^{3+} resistance genes				
Wheat	*TaMATE1*	Citrate transport—efflux	Segregation, homology (no mutational or functional data)	Ryan *et al.*, 2009
Brassica napus	*BnALMT1*			
	BnALMT2	Malate transport—efflux	Homology, function (no mutational or segregation data)	Ligaba *et al.*, 2006
Rye	*ScMATE2*	Citrate transport—efflux	Homology, biology (no functional or segregation data)	Yokosho *et al.*, 2010

Resistance genes that do not explain genotypic variation

Subsequently, a different set of Al^{3+} 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. M_2 seedlings are screened and those that grow similarly to wild-type plants under control conditions, but are hypersensitive to Al^{3+} stress or acidity, may carry mutations in genes necessary for Al^{3+} 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, Al^{3+} resistance in rice is not explained by organic anion efflux. Indeed, the Al^{3+}-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 Al^{3+} resistance in rice was achieved when the gene underlying an Al^{3+}-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 Al^{3+} 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 apoplasm by exocytosis and provide protection by modifying the cell walls. It is also plausible that the OsSTAR proteins confer Al^{3+} 'tolerance' rather than 'resistance' by performing other functions in the cytosol. Neither of the *OsSTAR* genes underlies QTLs for Al^{3+} 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 Al^{3+}-induced

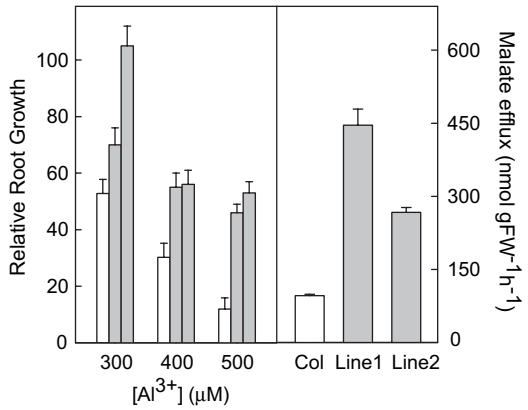

Fig. 1. Expression of the wheat gene *TaALMT1* enhances the Al^{3+} resistance of Arabidopsis. (A) Al^{3+} resistance of wild-type *Arabidopsis* (Col) (white bars) and T2 homozygous transgenic lines (lines 1 and 3) expressing *TaALMT1* (grey bars). Relative root growth was calculated by comparing the length of the longest root on plants grown on control plates (no Al^{3+}) and on plates containing $AlCl_3$. Plants were grown for 14 d on agar plates containing a minimal nutrient solution with different concentrations of $AlCl_3$. Relative root growth was calculated as ((length+Al^{3+})× 100)/(length–Al^{3+}). Data show mean and SE (n=20–25 plants). (B) Malate efflux from wild-type control plants (white bars) and T_2 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 $CaCl_2$ (pH 4.9), containing 100 μM $AlCl_3$. 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 Al^{3+} resistance because they map to previously identified QTLs for Al^{3+} resistance on the rice genome. The observation that the *star1* mutant is as Al^{3+} sensitive as the *art1* mutant suggests that the *OsSTAR* genes control a major mechanism of Al^{3+} resistance in rice.

AtALS1 and *AtALS3* are Al^{3+}-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 Al^{3+} tolerance as well because plants carrying a knockout mutation in *AtSTAR1* were hypersensitive to Al^{3+} 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 M_2 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 Al^{3+}-resistance genes in the strict sense. For instance, a mutation in *AtATR* improved root growth on Al^{3+} 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 Al^{3+} 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-Sarahan 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 Al^{3+}-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 Al^{3+} 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 Al^{3+} 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 Al^{3+} resistance in plants by over-expressing genes whose expression is induced by Al^{3+} 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 Al^{3+} 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 Al^{3+} 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 Δ8 sphingolipid desaturase isolated in this way slightly increased the Al^{3+} resistance of *Arabidopsis* by modifying the composition of cell lipids (Ryan *et al.*, 2007).

By far the largest increases in Al^{3+} resistance have been achieved by over-expressing organic anion transport proteins (Table 2). These studies targeted the known Al^{3+} 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 Al^{3+}-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, Al^{3+} 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 Al^{3+} 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 Al^{3+} 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 Al^{3+}-sensitive species.

Focus topic: structure–function of TaALMT

It is now clear that many Al^{3+}-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 Al^{3+} resistance genes contribute to other stress pathways, nutrition acquisition, or even pathogen responses (Pineros *et al.*, 2008*b*; 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 Al^{3+}-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.*, 2008*a*) and tobacco suspension cells (Zhang *et al.*, 2008) found that *TaALMT1* facilitated Al^{3+}-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 Al^{3+} resistance by genetic engineering

Shown are studies that have enhanced the Al^{3+} 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 Al^{3+} divided by net root growth in the absence of Al^{3+}.

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

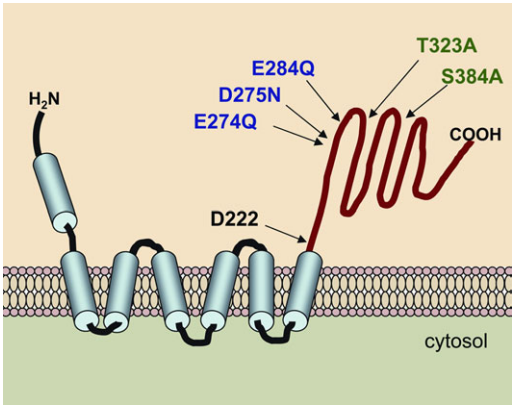

Fig. 2. Diagram depicting the secondary structure of TaALMT1. TaALMT1 (459 amino acids) is predicted to possess six transmembrane regions with the N and C-terminal ends orientated extracellularly (Motoda *et al.*, 2007). Residue D222 is located near the start of the hydrophilic C-terminal region (coloured 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 Al^{3+} 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 F_2 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 F_1 plants from this cross should be sensitive of Al^{3+} but this requires confirmation.

Constraints to further progress

Much of our understanding of Al^{3+} 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.

Acknowledgements

We are grateful to Dr Bei Dong for technical assistance in the transforming Arabidopsis with *TaALMT1*.

References

Aniol A, Gustafson JP. 1984. Chromosome location of genes controlling aluminum tolerance in wheat, rye and triticale. *Canadian Journal of Genetics and Cytology* **26,** 701–705.

Anoop VM, Basu U, McCammon MT, McAlister-Henn L, Taylor GJ. 2003. Modulation of citrate metabolism alters aluminum tolerance in yeast and transgenic canola overexpressing a mitochondrial citrate synthase. *Plant Physiology* **132,** 2205–2217.

Barone P, Rosellini D, LaFayette P, Bouton J, Veronesi F, Parrott W. 2008. Bacterial citrate synthase expression and soil aluminum tolerance in transgenic alfalfa. *Plant Cell Reports* **27,** 893–901.

Basu U, Godbold D, Taylor GJ. 1994. Aluminum resistance in *Triticum aestivum* associated with enhanced exudation of malate. *Journal of Plant Physiology* **144,** 747–753.

Basu U, Good AG, Taylor GJ. 2001. Transgenic *Brassica napus* plants overexpressing aluminium-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminium. *Plant, Cell and Environment* **24,** 1269–1278.

Bhalla PL. 2006. Genetic engineering of wheat: current challenges and opportunities. *Trends in Biotechnology* **24,** 305–311.

Collins NC, Shirley NJ, Saeed M, Pallotta M, Gustafson JP. 2008. An *ALMT1* gene cluster controlling aluminum tolerance at the Alt4 locus of rye (*Secale cereale* L.). *Genetics* **179,** 669–692.

Delhaize E, Craig S, Beaton CD, Bennet RJ, Jagadish VC, Randall PJ. 1993*a*. Aluminum tolerance in wheat (*Triticum aestivum* L.). 1. Uptake and distribution of aluminum in root apices. *Plant Physiology* **103,** 685–693.

Delhaize E, Gruber BD, Ryan PR. 2007. The roles of organic anion permeases in aluminium resistance and mineral nutrition. *FEBS Letters* **581,** 2255–2262.

Delhaize E, Hebb DM, Richards KD, Lin JM, Ryan PR, Gardner RC. 1999. Cloning and expression of a wheat (*Triticum aestivum* L.) phosphatidylserine synthase cDNA: overexpression in plants alters the composition of phospholipids. *Journal of Biological Chemistry* **274,** 7082–7088.

Delhaize E, Hebb DM, Ryan PR. 2001. Expression of a *Pseudomonas aeruginosa* citrate synthase gene in tobacco is not

associated with either enhanced citrate accumulation or efflux. *Plant Physiology* **125,** 2059–2067.

Delhaize E, Ryan PR, Hebb DM, Yamamoto Y, Sasaki T, Matsumoto H. 2004. Engineering high-level aluminum tolerance in barley with the *ALMT1* gene. *Proceedings of the National Academy of Sciences, USA* **101,** 15249–15254.

Delhaize E, Ryan PR, Randall PJ. 1993 *b*. Aluminum tolerance in wheat (*Triticum aestivum* L.). 2. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiology* **103,** 695–702.

Delhaize E, Taylor P, Hocking PJ, Simpson RJ, Ryan PR, Richardson AE. 2009. Transgenic barley (*Hordeum vulgare* L.) expressing the wheat aluminium resistance gene (*TaALMT1*) shows enhanced phosphorus nutrition and grain production when grown on an acid soil. *Plant Biotechnology Journal* **7,** 391–400.

Diatloff E, Roberts M, Sanders D, Roberts SK. 2004. Characterization of anion channels in the plasma membrane of Arabidopsis epidermal root cells and the identification of a citrate-permeable channel induced by phosphate starvation. *Plant Physiology* **136,** 4136–4149.

Dietrich P, Hedrich R. 1998. Anions permeate and gate GCAC1, a voltage-dependent guard cell anion channel. *The Plant Journal* **15,** 479–487.

Durrett TP, Gassmann W, Rogers EE. 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology* **144,** 197–205.

Eticha D, Zahn M, Bremer M, Yang ZB, Rangel AF, Rao IM, Horst WJ. 2010. Transcriptomic analysis reveals differential gene expression in response to aluminium in common bean (*Phaseolus vulgaris*) genotypes. *Annals of Botany* **105,** 1119–1128.

Ezaki B, Gardner RC, Ezaki Y, Matsumoto H. 2000. Expression of aluminum-induced genes in transgenic Arabidopsis plants can ameliorate aluminum stress and/or oxidative stress. *Plant Physiology* **122,** 657–665.

Ezaki B, Kiyohara H, Matsumoto H, Nakashima S. 2007. Overexpression of an auxilin-like gene (*F9E10.5*) can suppress Al uptake in roots of Arabidopsis. *Journal of Experimental Botany* **58,** 497–506.

Ezaki B, Sasaki K, Matsumoto H, Nakashima S. 2005. Functions of two genes in aluminium (Al) stress resistance: repression of oxidative damage by the *AtBCB* gene and promotion of efflux of Al ions by the *NtGDI1* gene. *Journal of Experimental Botany* **56,** 2661–2671.

Famoso AN, Clark RT, Shaff JE, Craft E, McCouch SR, Kochian LV. 2010. Development of a novel aluminum tolerance phenotyping platform used for comparisons of cereal Al tolerance and investigations into rice Al tolerance mechanisms. *Plant Physiology* **153,** 1678–1691.

Fuente JMdl, Ramirez-Rodriguez V, Cabrera-Ponce JL, Herrera-Estrella L. 1997. Aluminum tolerance in transgenic plants by alteration of citrate synthesis. *Science (Washington)* **276,** 5466–1568.

Furuichi T, Sasaki T, Tsuchiya Y, Ryan PR, Delhaize E, Yamamoto Y. 2010. Extracellular hydrophilic carboxy-terminal domain regulates the activity of TaALMT1, the aluminum-activated malate transport protein of wheat. *The Plant Journal* doi: 10.1111/j.1365–313X.2010.04309.x.

Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF. 2007. An aluminum-activated citrate transporter in barley. *Plant and Cell Physiology* **48,** 1081–1091.

Gabrielson KM, Cancel JD, Morua LF, Larsen PB. 2006. Identification of dominant mutations that confer increased aluminium tolerance through mutagenesis of the Al-sensitive Arabidopsis mutant. *als3-1. Journal of Experimental Botany* **57,** 943–951.

Garvin DF, Carver BF. 2003. Role of genotype in tolerance to acidity and aluminum toxicity. In: Rengel Z, ed. *Handbook of soil acidity*. New York: Marcel Dekker Inc, 387–406.

Gruber BD. 2009. Characterisation of the HvALMT1 gene from barley. *PhD thesis*. Canberra, ACT, Australia: The Australian National University.

Gruber BD, Ryan PR, Richardson AE, Tyerman SD, Ramesh S, Hebb DM, Howitt SM, Delhaize E. 2010. HvALMT1 from barley is involved in the transport of organic anions. *Journal of Experimental Botany* **61,** 1455–1467.

Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P. 2006. Harnessing the potential of hairy roots: dawn of a new era. *Trends in Biotechnology* **24,** 403–409.

Hiradate S, Ma JF, Matsumoto H. 2007. Strategies of plants to adapt to mineral stresses in problem soils. *Advances in Agronomy* **96,** 65–132.

Hoekenga OA, Maron LG, Pineros MA, *et al*. 2006. *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **103,** 9738–9743.

Hoisington D. 2002. Opportunities for nutritionally enhancing maize and wheat varieties to combat protein and micronutrient malnutrition. *Food Nutrition Bulletin* **23,** 376–377.

Horst WJ, Wang YX, Eticha D. 2010. The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review. *Annals of Botany* **106,** 185–197.

Huang CF, Yamaji N, Ma JF. 2010. Knockout of a bacterial-type ABC transporter gene, AtSTAR1, results in increased Al sensitivity in *Arabidopsis*. *Plant Physiology* **153,** 1669–1677.

Huang CF, Yamaji N, Mitani N, Yano M, Nagamura Y, Ma JF. 2009. A bacterial-type ABC transporter is involved in aluminum tolerance in rice. *The Plant Cell* **21,** 655–667.

Iuchi S, Koyama H, Iuchi A, Kobayashi Y, Kitabayashi S, Ikka T, Hirayama T, Shinozaki K, Kobayashi M. 2007. Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. *Proceedings of the National Academy of Sciences, USA* **104,** 9900–9905.

Kinraide TB, Parker DR, Zobel RW. 2005. Organic acid secretion as a mechanism of aluminium resistance: a model incorporating the root cortex, epidermis, and the external unstirred layer. *Journal of Experimental Botany* **56,** 1853–1865.

Kobayashi Y, Hoekenga OA, Itoh H, Nakashima M, Saito S, Shaff JE, Maron LG, Pineros MA, Kochian LV, Koyama H. 2007. Characterization of AtALMT1 expression in aluminum-inducible malate

release and its role for rhizotoxic stress tolerance in arabidopsis. *Plant Physiology* **145,** 843–852.

Kochian LV. 1995. Cellular mechanisms of aluminum toxicity and resistance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 237–260.

Kochian LV, Hoekenga OA, Pineros MA. 2004. How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. *Annual Review of Plant Biology* **55,** 459–493.

Koyama H, Kawamura A, Kihara T, Hara T, Takita E, Shibata D. 2000. Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus-limited soil. *Plant and Cell Physiology* **41,** 1030–1037.

Larsen PB. 2009. Unraveling the mechanisms underlying aluminum dependent root growth inhibition. In: Jenks MA, Wood AJ, eds. *Genes for plant abiotic stress*. Ames, Iowa: Wiley-Blackwell, 113–142.

Larsen PB, Cancel J, Rounds M, Ochoa V. 2007. Arabidopsis *ALS1* encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. *Planta* **225,** 1447–1458.

Larsen PB, Geisler MJB, Jones CA, Williams KM, Cancel JD. 2005. *ALS3* encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in Arabidopsis. *The Plant Journal* **41,** 353–363.

Li XF, Ma JF, Matsumoto H. 2000. Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiology* **123,** 1537–1543.

Ligaba A, Katsuhara M, Ryan PR, Shibasaka M, Matsumoto H. 2006. The *BnALMT1* and *BnALMT2* genes from rape encode aluminum-activated malate transporters that enhance the aluminum resistance of plant cells. *Plant Physiology* **142,** 1294–1303.

Ligaba A, Kochian L, Pineros M. 2009. Phosphorylation at S384 regulates the activity of the TaALMT1 malate transporter that underlies aluminum resistance in wheat. *The Plant Journal* **60,** 411–423.

Liu JP, Magalhaes JV, Shaff J, Kochian LV. 2009. Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance. *The Plant Journal* **57,** 389–399.

Luo MC, Dvorak J. 1996. Molecular mapping of an aluminum tolerance locus on chromosome 4D of Chinese Spring wheat. *Euphytica* **91,** 31–35.

Ma JF. 2007. Syndrome of aluminum toxicity and diversity of aluminum resistance in higher plants. *Survey of Cell Biology* **264,** 225–252.

Ma JF, Nagao S, Sato K, Ito H, Furukawa J, Takeda K. 2004. Molecular mapping of a gene responsible for Al-activated secretion of citrate in barley. *Journal of Experimental Botany* **55,** 1335–1341.

Magalhaes JV. 2010. How a microbial drug transporter became essential for crop cultivation in acid soils. Aluminum tolerance conferred by the multidrug and toxic compound efflux (MATE) family. *Annals of Botany* **106,** doi:10.1093/aob/mcq1115.

Magalhaes JV, Garvin DF, Wang YH, Sorrells ME, Klein PE, Schaffert RE, Li L, Kochian LV. 2004. Comparative mapping of a major aluminum tolerance gene in sorghum and other species in the Poaceae. *Genetics* **167,** 1905–1914.

Magalhaes JV, Liu J, Guimaraes CT, *et al*. 2007. A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. *Nature Genetics* **39,** 1156–1161.

Maron LG, Pineros MA, Guimaraes CT, Magalhaes JV, Pleiman JK, Mao CZ, Shaff J, Belicuas SNJ, Kochian LV. 2010. Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *The Plant Journal* **61,** 728–740.

Matsumoto H. 2000. Cell biology of aluminum toxicity and tolerance in higher plants. *International Review of Cytology—a Survey of Cell Biology* **200,** 1–46.

Meyer S, De Angeli A, Fernie AR, Martinoia E. 2010. Intra- and extra-cellular excretion of carboxylates. *Trends in Plant Science* **15,** 40–47.

Motoda H, Sasaki T, Kano Y, Ryan PR, Delhaize E, Matsumoto H, Yamamoto Y. 2007. The membrane topology of ALMT1, an aluminum-activated malate transport protein in wheat (*Triticum aestivum*). *Plant Signal Behavior* **2,** 467–472.

Omote H, Hiasa M, Matsumoto T, Otsuka M, Moriyama Y. 2006. The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations. *Trends in Pharmacological Sciences* **27,** 587–593.

Osawa H, Matsumoto H. 2001. Possible involvement of protein phosphorylation in aluminum-responsive malate efflux from wheat root apex. *Plant Physiology* **126,** 411–420.

Papernik LA, Bethea AS, Singleton TE, Magalhaes JV, Garvin DF, Kochian LV. 2001. Physiological basis of reduced Al tolerance in ditelosomic lines of Chinese Spring wheat. *Planta* **212,** 829–834.

Pereira JF, Zhou G, Delhaize E, Richardson T, Ryan PR. 2010. Engineering greater aluminium resistance in wheat by over-expressing TaALMT1. *Annals of Botany* **106,** 205–214.

Pineros MA, Cancado GMA, Kochian LV. 2008 *a*. Novel properties of the wheat aluminum tolerance organic acid transporter (TaALMT1) revealed by electrophysiological characterization in *Xenopus* oocytes: functional and structural implications. *Plant Physiology* **147,** 2131–2146.

Pineros MA, Cancado GMA, Maron LG, Lyi SM, Menossi M, Kochian LV. 2008 *b*. Not all ALMT1-type transporters mediate aluminum-activated organic acid responses: the case of ZmALMT1, an anion-selective transporter. *The Plant Journal* **53,** 352–367.

Poschenrieder C, Gunse B, Corrales I, Barcelo J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* **400,** 356–368.

Raman H, Ryan PR, Raman R, *et al*. 2008. Analysis of *TaALMT1* traces the transmission of aluminum resistance in cultivated common wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* **116,** 343–354.

Raman H, Zhang KR, Cakir M, *et al*. 2005. Molecular characterization and mapping of *ALMT1*, the aluminium-tolerance gene of bread wheat (*Triticum aestivum* L.). *Genome* **48,** 781–791.

Ratledge C. 2000. Look before you clone. *FEMS Microbiology Letters* **189,** 317–319.

Reide CR, Anderson JA. 1996. Linkage of RFLP markers to an aluminum tolerance gene in wheat. *Crop Science* **36,** 905–909.

Rudrappa T, Czymmek KJ, Pare PW, Bais HP. 2008. Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiology* **148,** 1547–1556.

Ryan PR, Delhaize E. 2010. The convergent evolution of aluminium resistance in plants exploits a convenient currency. *Functional Plant Biology* **37,** 275–284.

Ryan PR, Delhaize E, Jones DL. 2001. Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 527–560.

Ryan PR, Delhaize E, Randall PJ. 1995. Characterization of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. *Planta* **196,** 103–110.

Ryan PR, Liu Q, Sperling P, Dong B, Franke S, Delhaize E. 2007. A higher plant Delta 8 sphingolipid desaturase with a preference for (Z)-isomer formation confers aluminum tolerance to yeast and plants. *Plant Physiology* **144,** 1968–1977.

Ryan PR, Raman H, Gupta S, Horst WJ, Delhaize E. 2009. A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. *Plant Physiology* **149,** 340–351.

Ryan PR, Raman H, Gupta S, Sasaki T, Yamamoto Y, Delhaize E. 2010. Multiple origins of aluminium resistance in hexaploid wheat are derived from *Aegilops tauschii* and from more recent *cis* mutations to *TaALMT1*. *The Plant Journal* doi: 10.1111/j.1365-313X.2010.04338.x.

Ryan PR, Skerrett M, Findlay GP, Delhaize E, Tyerman SD. 1997. Aluminum activates an anion channel in the apical cells of wheat roots. *Proceedings of the National Academy of Sciences, USA* **94,** 6547–6552.

Sasaki T, Ryan PR, Delhaize E, *et al*. 2006. Sequence upstream of the wheat (*Triticum aestivum* L.) *ALMT1* gene and its relationship to aluminum resistance. *Plant and Cell Physiology* **47,** 1343–1354.

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H. 2004. A wheat gene encoding an aluminum-activated malate transporter. *The Plant Journal* **37,** 645–653.

Sawaki Y, Iuchi S, Kobayashi Y, Ikka T, Sakurai N, Fujita M, Shinozaki K, Shibata D, Kobayashi M, Koyama H. 2009. STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminum toxicities. *Plant Physiology* **150,** 281–294.

Shen H, Ligaba A, Yamaguchi M, Osawa H, Shibata K, Yan XL, Matsumoto H. 2004. Effect of K-252a and abscisic acid on the efflux of citrate from soybean roots. *Journal of Experimental Botany* **55,** 663–671.

Shi BJ, Gustafson JP, Button J, Miyazaki J, Pallotta M, Gustafson N, Zhou H, Langridge P, Collins NC. 2009. Physical analysis of the complex rye (*Secale cereale* L.) Alt4 aluminium (aluminum) tolerance locus using a whole-genome BAC library of rye cv. *Blanco. Theoretical and Applied Genetics* **119,** 695–704.

Sivaguru M, Ezaki B, He ZH, Tong HY, Osawa H, Baluska F, Volkmann D, Matsumoto H. 2003. Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in Arabidopsis. *Plant Physiology* **132,** 2256–2266.

Sivaguru M, Fujiwara T, Samaj J, Baluska F, Yang ZM, Osawa H, Maeda T, Mori T, Volkmann D, Matsumoto H. 2000. Aluminum-induced 1→3-beta-D-glucan inhibits cell-to-cell trafficking of molecules through plasmodesmata. A new mechanism of aluminum toxicity in plants. *Plant Physiology* **124,** 991–1005.

Taylor GJ. 1991. Current views of the aluminum stress response; the physiological basis of tolerance. *Current Topics in Plant Biochemistry and Physiology* **10,** 57–93.

Tesfaye M, Temple SJ, Allan DL, Vance CP, Samac DA. 2001. Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. *Plant Physiology* **127,** 1836–1844.

Trejo-Tellez LI, Stenzel R, Gomez-Merino FC, Schmitt JM. 2010. Transgenic tobacco plants overexpressing pyruvate phosphate dikinase increase exudation of organic acids and decrease accumulation of aluminum in the roots. *Plant and Soil* **326,** 187–198.

Wang JP, Raman H, Zhou MX, Ryan PR, Delhaize E, Hebb DM, Coombes N, Mendham N. 2007. High-resolution mapping of the Alp locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **115,** 265–276.

Wang Q-F, Zhao Y, Yi Q, Li K-Z, Yu Y-X, Chen L- M. 2010. Overexpression of malate dehydrogenase in transgenic tobacco leaves: enhanced malate synthesis and augmented Al-resistance. *Acta Physiologia Plantarum* doi: 10.1007/s11738-010-0522-x

Wang WZ, Pan JW, Zheng K, Chen H, Shao HH, Guo YJ, Bian HW, Han N, Wang JH, Zhu MY. 2009. Ced-9 inhibits Al-induced programmed cell death and promotes Al tolerance in tobacco. *Biochemical and Biophysical Research Communications* **383,** 141–145.

Wherrett T, Ryan PR, Delhaize E, Shabala S. 2005. Effect of aluminium on membrane potential and ion fluxes at the apices of wheat roots. *Functional Plant Biology* **32,** 199–208.

Yamaji N, Huang CF, Nagao S, Yano M, Sato Y, Nagamura Y, Ma JF. 2009. A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. *The Plant Cell* **21,** 3339–3349.

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H. 2002. Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. *Plant Physiology* **128,** 63–72.

Yang H, Knapp J, Koirala P, Rajagopal D, Peer WA, Silbart LK, Murphy A, Gaxiola RA. 2007. Enhanced phosphorus nutrition in monocots and dicots over-expressing a phosphorus-responsive type I H^+-pyrophosphatase. *Plant Biotechnology Journal* **5,** 735–745.

Yin LN, Wang SW, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, Takeuchi Y, Tanaka K. 2010. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. *Planta* **231,** 609–621.

Yokosho K, Yamaji N, Ma JF. 2010. Isolation and characterisation of two MATE genes in rye. *Functional Plant Biology* **37,** 296–303.

Zhang WH, Ryan PR, Sasaki T, Yamamoto Y, Sullivan W, Tyerman SD. 2008. Characterization of the TaALMT1 protein as an Al^{3+}-activated anion channel in transformed tobacco (*Nicotiana tabacum* L.) cells. *Plant and Cell Physiology* **49,** 1316–1330.

Zhang WH, Ryan PR, Tyerman SD. 2001. Malate-permeable channels and cation channels activated by aluminum in the apical cells of wheat roots. *Plant Physiology* **125,** 1459–1472.