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The convergent evolution of aluminium resistance in plants exploits a convenient currency

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Abstract. Suspicions that soluble aluminium (Al) is detrimental to plant growth were reported more than 100 years ago. The rhizotoxicity of Al^{3+} is now accepted as the major limitation to plant production on acidic soils. Plants differ in their susceptibility to Al^{3+} toxicity and significant variation can occur within species, even in some major crops. The physiology of Al^{3+} resistance in some species has been understood for 15 years but the molecular biology has been elucidated only recently. The first gene controlling Al^{3+} resistance was cloned from wheat (*Triticum aestivum* L.) in 2004 but others have now been identified in *Arabidopsis*, barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), sorghum (*Sorghum bicolour* (L.) Moench) and rice (*Oryza sativa* L.) with strong additional candidates in wheat and oilseed rape (*Brassica napus* L.). These genes confer resistance in different ways, but one mechanism occurs in nearly all species examined so far. This mechanism relies on the release of organic anions from roots which bind with the harmful Al^{3+} cations in the apoplast and detoxify them. The genes controlling this response come from at least two distinct families, suggesting that convergent evolution has occurred. We discuss the processes driving this convergence of protein function and offer opinions for why organic anions are central to the mechanisms of resistance in disparate species. We propose that mutations which modify protein expression or their activation by Al^{3+} have played important roles in co-opting different transport proteins from other functions.

Additional keywords: acid soil, aluminum, anion channel, citrate, malate, tolerance, toxicity.

Al^{3+} resistance and tolerance in plants

Most polyvalent anions and cations are harmful to plants at low micromolar concentrations (Kinraide 1991, 1994). They are capable of rapidly inhibiting root growth and damaging cells at the root apex but the mechanisms of their toxicity are not fully understood. Trivalent cations, including Al^{3+}, affect cellular functions through an array of intracellular and extracellular interactions which include blocking ion channels, reducing Ca^{2+} and Mg^{2+} uptake, competing with Ca^{2+} for essential binding sites in the apoplast, altering cytoskeletal structure, binding with DNA, disrupting signal transduction pathways and triggering oxidative stress responses (Taylor 1988*a*; Matsumoto 2000; Yamamoto *et al*. 2003; Kochian *et al*. 2004).

Plants encounter Al^{3+} at harmful concentrations more frequently than any other polyvalent cation. Aluminium is the third most common element in the earth's crust and acidic conditions accelerate its release from soil minerals into the soil solution. Since 30% of arable lands have a pH of less than 5.5, Al^{3+} toxicity is an important limitation to plant production and a prevailing pressure for plant adaptation. Some plant species have evolved mechanisms that allow them to survive acid soils better than others. Indeed, genotypes within species can even differ in their ability to withstand Al^{3+} (Foy 1988). Breeders have exploited this variation to develop cultivars better adapted to acid soils (Garvin and Carver 2003).

The mechanisms that plants have evolved to cope with Al^{3+} stress can be broadly divided into two main strategies: tolerance mechanisms and resistance or exclusion mechanisms although the divisions between these can be blurry (Taylor 1991; Kochian *et al*. 2004; Hiradate *et al*. 2007). Tolerance mechanisms enable plants to safely accommodate Al^{3+} once it enters the symplast either by chelating it in the cytosol to form harmless complexes or by sequestering it to organelles where it cannot disrupt metabolism. Tolerance mechanisms appear to be common in species endemic to regions with acid soils (e.g. the tropics) where the ability to cope with Al^{3+} stress is a prerequisite for survival. Examples include tea (*Camellia sinensis*), buckwheat (*Fagopyrum esculentum*), *Melostoma*, and *Hydrangea* sp., all of which accumulate high concentrations of Al in their leaves (Ma *et al*. 2001).

Resistance or exclusion mechanisms prevent Al^{3+} from accumulating in the symplast and minimise harmful interactions with the plasma membrane, cell wall or other targets in the apoplast. These mechanisms rely on root exudates to bind and detoxify the cations in the apoplast (Delhaize *et al*. 1993), on transport systems to export Al from the symplast or on the capacity to repair damage caused by the Al in the cell wall (Taylor 1991; Huang *et al*. 2009). Our understanding of resistance mechanisms has progressed more rapidly than tolerance mechanisms because they operate in many

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common crops (wheat, sorghum, maize, soybean, barley) as well as the model species *Arabidopsis* and rice and because one or two genes explain most of the phenotypic variation within some of these species. Certainly in wheat, a single locus explains most of the variation in resistance, which led to the conclusion that the trait was not an original condition of this species but appeared more recently in its evolution (Garvin and Carver 2003). Indeed, resistance in wheat appears to be more the exception than the rule. No substantial resistance has been detected in the tetraploid progenitor of hexaploid wheat, *Triticum turgidum* (Slootmaker 1974; Berzonsky and Kimber 1986; Cosic *et al*. 1994), and a moderate level of resistance has only recently been identified in the diploid progenitor of hexaploid wheat, *Aegilops tauschii* (P. R. Ryan and E. Delhaize, unpubl. data).

In summary, Al^{3+} toxicity is a major selection pressure for plant evolution and many species have evolved tolerance and resistance mechanisms to improve their survival. Some plants appear to display one type of mechanism only (wheat and barley) but others display resistance and tolerance mechanisms which may be additive. *Arabidopsis* appears to have at least two distinct mechanisms relying, first, on the release of different organic anions from roots that bind with Al^{3+} (resistance; Hoekenga *et al*. 2006; Liu *et al*. 2009) and, second, on the redistribution of Al^{3+} in the plant (tolerance; Larsen *et al*. 2005, 2007). Similarly, *Fagopyrum esculentum* (buckwheat) releases organic anions from its roots (resistance; Zheng *et al*. 1998) and safely accumulates high concentrations in its leaves (tolerance; Ma *et al*. 1998).

Al^{3+} resistance in diverse species relies on the efflux of organic anions

This review will focus on a mechanism of Al^{3+} resistance widely spread in the plant kingdom which is associated with the efflux of organic anions from roots. The species using this mechanism represent a range of families including the Poaceae (e.g. wheat, barley, sorghum, maize, rye), Araceae (e.g. taro), Polygonaceae (e.g. buckwheat), Brassicaceae (e.g. *Arabidopsis*) and the Fabaceae (e.g. soybean). Anion efflux is generally restricted to the root apices, the regions most susceptible to Al^{3+} toxicity (Ryan *et al*. 1993; Sivaguru and Horst 1998) and the anions released vary from species to species. Malate and citrate are most common, but oxalate efflux has also been detected in a few species (Ma *et al*. 2001; Ryan *et al*. 2001; Kochian *et al*. 2004). All three anions form complexes with Al^{3+} that are less harmful than the free Al^{3+} cations and not readily adsorbed by roots. Importantly, the organic anions are not released continuously from the roots in most cases but require Al^{3+} to trigger the response.

A case of convergent evolution

The first gene controlling Al^{3+} resistance in plants was isolated from wheat six years ago (Sasaki *et al*. 2004). The *TaALMT1* (*Triticum aestivum* aluminium-activated malate transporter) gene encodes a member of the ALMT family that consists of membrane-bound proteins (Delhaize *et al*. 2007). TaALMT1 is located in the plasma membrane and functions as an Al^{3+}-activated anion channel, releasing malate from root cells (Yamaguchi *et al*. 2005; Zhang *et al*. 2008). This loss of malate will not necessarily deplete the concentration in the root cells because malate released can be replaced by the continual synthesis of new acids. This can even occur in excised tissue. For example, in one study the cumulative loss of malate over a 4-h period from excised root apices was 3-fold greater than the initial malate content of the tissue (Ryan *et al*. 1995).

Soon after this gene was described several other members of the ALMT family were shown to contribute to the Al^{3+} resistance of cereal and non-cereal species in a similar manner (Table 1). These discoveries were exciting at the time because it appeared as though a single gene family controlled Al^{3+} resistance in a diverse range of species (Magalhaes 2006). However, the model soon required revision after the major resistance genes in sorghum and barley were mapped and sequenced. Aluminium resistance in these species relies on citrate efflux and the proteins involved were not ALMTs but members of a completely different family of proteins named the multi-drug and toxic compound extrusion (MATE) family

Table 1. Al^{3+} resistance genes that control organic anion efflux

The table indicates whether gene expression is induced by Al^{3+} treatment (induction) and whether, once expressed, the protein is also activated by Al^{3+} treatment (activation)

Species	Gene name	Induction by Al^{3+}	Activation by Al^{3+}	Organic anion released	Reference
Arabidopsis thaliana	*AtALMT1*	Yes	Yes	Malate	Hoekenga *et al*. (2006)
Arabidopsis thaliana	*AtMATE*	Yes	Yes	Citrate	Liu *et al.* (2009)
Oilseed rape (*Brassica napus*)	*BnALMT1-1* and *1-2*	Yes	Yes	Malate, malate	Ligaba *et al.* (2006)
Barley (*Hordeum vulgare*)	*HvAACT1*[A]	No	Yes	Citrate	Furukawa *et al.* (2007)
Rye (*Secale cereale*)	*ScALMT1-M39.1* and *M39.2*	Yes	Yes[B]	Malate, citrate[B]	Collins *et al.* (2008)
Sorghum (*Sorghum bicolor*)	*SbMATE*[C]	Yes[C]	Yes	Citrate	Magalhaes *et al.* (2007)
Wheat (*Triticum aestivum*)	*TaALMT1*	No	Yes	Malate	Sasaki *et al.* (2004)
Wheat (*Triticum aestivum*)	*TaMATE1*[D]	No	No	Citrate	Ryan *et al.* (2009)

[A]Also referred to as *HvMATE1* (Wang *et al*. 2007).

[B]The contribution of these two *ScALMT1*s to Al^{3+} resistance has not been confirmed directly. The proposal that they transport malate and citrate is based on the observation that resistance alleles of these genes genetically co-segregate with higher levels of Al^{3+}-activated malate and citrate efflux from root apices (N. C. Collins, S. D. Tyerman and S. Ramesh, pers. comm.; Collins *et al*. 2008).

[C]Also referred to as Alt_{SB}. Constitutive expression of *SbMATE* shows a two- to three-fold induction with Al^{3+} treatment over several days.

[D]*TaMATE1* expression correlates with Al^{3+} resistance in segregating populations but its contribution to Al^{3+} resistance has not been confirmed directly.

(Furukawa *et al*. 2007; Magalhaes *et al*. 2007; Wang *et al*. 2007). Other *MATE* genes were subsequently shown to contribute to the Al^{3+} resistance of *Arabidopsis* (Liu *et al*. 2009) and, probably, wheat (Ryan *et al*. 2009).

Therefore, at least two gene families separately control the release of malate and citrate, sometimes in the same species. The *Arabidopsis* genome alone contains ~14 *ALMT* genes (Delhaize *et al*. 2007) and 58 *MATE* genes (Hvorup *et al*. 2003). The efflux of oxalate from other species may be controlled by a third, as yet, unidentified family. The finding that different gene families confer Al^{3+} resistance via the same general mechanism is indicative of convergent evolution as suggested previously (Delhaize *et al*. 2007; Liu *et al*. 2009). In the remainder of this review we discuss why the efflux of organic anions has emerged as a major mechanism for Al^{3+} resistance in different gene families.

Why are malate and citrate the common currencies of Al^{3+} resistance?

Malate and citrate are cheap to synthesise

The stability of the [Al^{3+}:malate] and [Al^{3+}:citrate] complexes is central to their role in protecting plants from Al^{3+} toxicity. However, inorganic compounds like phosphate and other organic compounds such as cyclic hydroxamates can also form stable complexes with Al^{3+} (Taylor 1988*b*; Matsumoto 2000; Kochian *et al*. 2004; Poschenrieder *et al*. 2005). Indeed the acidic peptides poly-*L*-glutamate and poly-*L*-aspartate are able to protect pollen tube growth and enzyme reactions from Al^{3+} even more effectively than citrate in some conditions (Konishi *et al*. 1988; Putterill and Gardner 1988). The same is likely to be true for other proteins and secondary metabolites that possess carboxyl residues or multiple aldehyde and ketone groups capable of chelating Al^{3+}.

Why then have organic anions emerged as the favoured currency for independently evolved mechanisms of Al^{3+} resistance? The answer may lie in the economy of these small organic compounds. Malate and citrate are ubiquitous in living cells and metabolically cheap to synthesise. These organic anions can almost be considered the 'small change' of cellular metabolism. The synthesis of other types of compounds such as some secondary metabolites, polypeptides or even single amino acids requires minerals from the soil and 4–6 carbon compounds as precursors to initiate much longer pathways. Those compounds are necessarily more metabolically 'expensive' and demanding of cellular resources. The synthesis of glutamate, for example, requires the uptake of nitrogen from the soil, its reduction to ammonium, a pool of α-ketoglutarate precursors from the tricarboxylic acid cycle and NADPH to drive synthesis.

Malate and citrate are ubiquitous in living cells

Malate is among the most prevalent anions in higher plants with vacuolar concentrations exceeding 200 mM in species that undergo crassulacean acid metabolism (Luttge 1987). The sometimes large and dynamic pools of malate and citrate in most cells reflect their central role in the metabolism of all living organisms (Lance and Rustin 1984). They participate in key anabolic and catabolic pathways including the tricarboxylic acid cycle, the glyoxylate cycle, C_4 photosynthetic carbon reduction and crassulacean acid metabolism. Furthermore they contribute directly to nutrient acquisition (phosphorus and iron, Ryan *et al*. 2001) and osmotic adjustment (guard cells, Fernie and Martinoia 2009) and help to maintain electroneutrality during periods of cation absorption (Osmond 1976; Ryan *et al*. 2001). Malate is also involved in regulating cytosolic pH by shuttling across the tonoplast (Kovermann *et al*. 2007) and by participating in the biochemical 'pH stat' reactions (Sakano 1998). The pH-stat model proposes that a series of enzymes finely regulate pH through reactions which produce or consume protons. The combined activities of phosphoenolpyruvate carboxylase (pH optimum ~7.8), malate dehydrogenase and malic enzyme (pH optimum <7.0) buffer cytosolic pH by balancing the synthesis and degradation of malate as well as influencing the rate of glycolysis. These reactions bypass the standard pathway supplying pyruvate for the TCA cycle by allowing oxidation of four-carbon acids in the absence of pyruvate. This shortcut is also useful during periods of phosphate deficiency because the phosphoenolpyruvate carboxylase reaction releases inorganic phosphate (Theodorou and Plaxton 1993).

Malate and citrate need to be shuffled between subcellular compartments

To satisfy their diverse roles, efficient transport systems are required to shuttle malate and citrate in and out of cells and between the subcellular compartments. The movement of these substrates across membranes needs to be mediated by specific membrane-bound proteins. Some of these proteins catalyse energetically passive reactions, some expend energy directly or indirectly and move ions against electrochemical gradients, and others facilitate the coordinated symport or antiport with other substrates. The genes encoding some of these transport proteins have been cloned (Reumann and Weber 2006; Martinoia *et al*. 2007) but many more are yet to be identified. Indeed the 20 or so metabolite transporters now identified and characterised in plastids represent less than 20% of the total predicted to exist from physiological and bioinformatic analyses (Weber and Fischer 2007). The finding that the AtALMT9 protein in *Arabidopsis* resides on the tonoplast demonstrates that some members of the ALMT family function on internal membranes as well as the plasma membrane (Kovermann *et al*. 2007).

Hypothesis: the evolution of Al^{3+} resistance via organic anion efflux arose from mutations that co-opted malate and citrate transport proteins from other functions

As discussed above, Al^{3+} resistance is unlikely to have been an early trait for plants that did not evolve on acidic environments. Instead, it would have evolved after their distribution extended into regions with acid soils (Garvin and Carver 2003). We propose that Al^{3+} resistance arose from mutations that co-opted some of the many malate or citrate transport proteins that originally performed different functions (Fig. 1). For example, if a protein facilitating malate release from a stomatal guard cell in leaves incurs a mutation that redirects or extends its expression to roots, the efflux of malate from the root cells could afford some

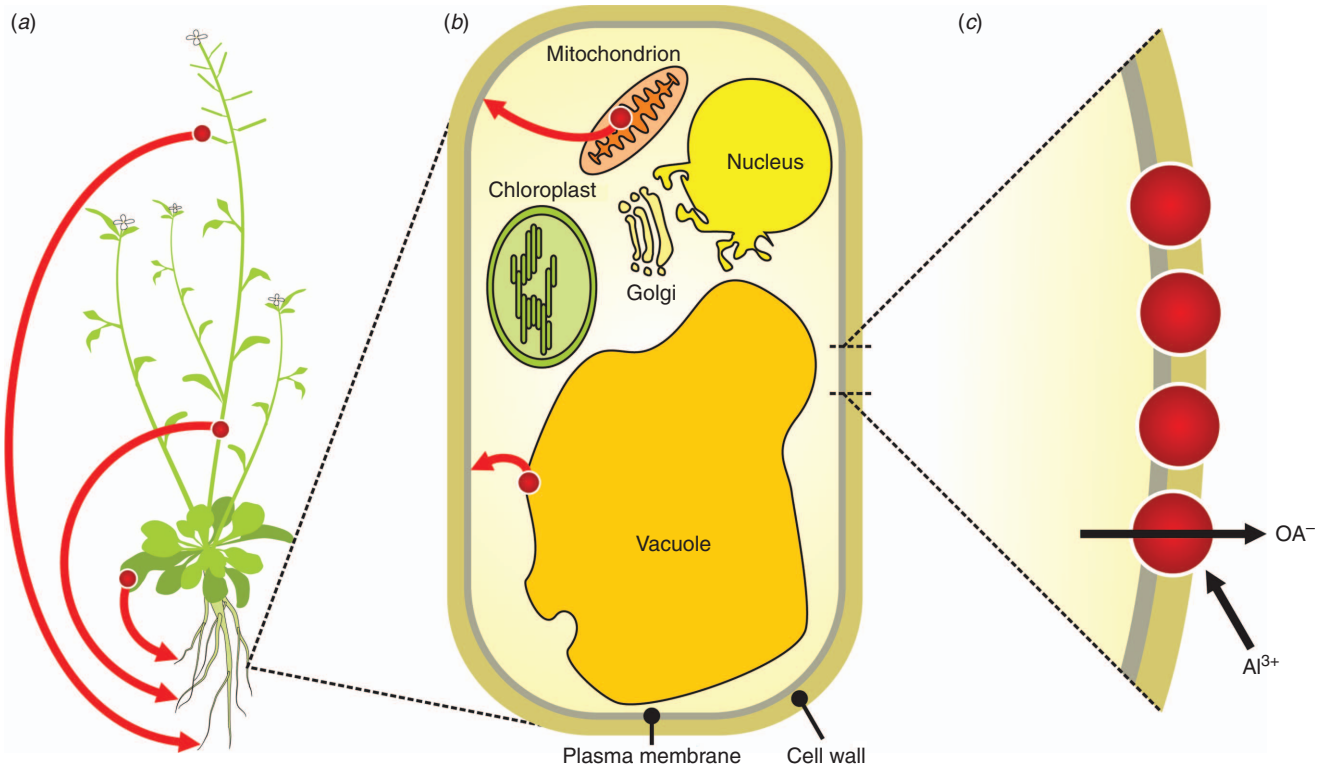

Fig. 1. Pictorial summary explaining how mutations in genes encoding organic anion transport proteins could confer Al^{3+} resistance by altering their pattern of expression or membrane location. The cartoon illustrates the two types of mutations that could increase Al^{3+} resistance by changing the expression or function of genes encoding organic anion transport proteins. Mutations occurring in *cis*-regulatory elements (promoter sequence and other non-coding regions) could alter (*a*) the tissue specificity of protein expression as well as (*c*) the level of expression. For instance, if the expression of proteins normally facilitating organic anion efflux from cells in the seeds, stem or leaves is extended to the roots then they could increase Al^{3+} resistance by releasing organic anions from root cells. Mutations occurring in the coding region of the transport proteins could modify the targeting domains which alter (*b*) their intracellular targeting or otherwise (*c*) enable their activity to be increased when interacting with Al^{3+}. For example, mutations to the targeting domains of an organic anion transport protein might alter its location from an internal membrane of a root cell (e.g. mitochondrial membrane or tonoplast) to the plasma membrane where they would then release organic anions into the apoplast and provide Al^{3+} resistance.

protection from Al^{3+} toxicity and provide the plant with a selective advantage on acid soils. This trait could undergo further selection in subsequent generations and become more widely represented in the population if the selection pressure is sustained. A recent study on the *Arabidopsis* gene *AtFRD3* provides 'in principal' support for the idea that extending the expression of a gene can confer a new Al^{3+}-resistance phenotype. *AtFRD3* encodes a MATE protein expressed in cells surrounding the root vasculature where it facilitates the efflux of citrate into the xylem to accompany iron movement to the shoots (Rogers and Guerinot 2002; Durrett *et al*. 2007). Ectopic expression of *AtFRD3* with the CaMV35S promoter causes no deleterious effects to the *Arabidopsis* plants but the resulting constitutive efflux of citrate from the roots enhances Al^{3+} resistance (Durrett *et al*. 2007). The original location of AtFRD3 on the plasma membrane of vascular cells makes this protein predisposed to mutations that extend its function to include citrate efflux from roots. Overexpression of HvALMT1 in barley also confers Al^{3+} resistance through constitutive malate efflux from roots but the transgenic plants show severe stunting and leaf necrosis (Gruber 2009). This deleterious phenotype might be related to the disruption of organic anion homeostasis internally, since the HvALMT1 proteins localise to internal membranes as well as the plasma membrane.

Similar outcomes are possible for proteins redirected from other tissues to the roots or from internal membranes to the plasma membrane as long as the protein still functions in its new location. Perhaps the simplest scenario would involve proteins already located on the plasma membrane of root cells which incur mutations that increase their expression or enable their function to be activated by Al^{3+} (see later). These proteins could be facilitating organic anion release from root cells for other reasons such as osmotic adjustment or nutrient acquisition.

The question of why some species release malate from their roots and others release citrate might not depend on the concentration of organic anions in the tissue or on their biochemistry. Instead, it might simply relate to whether the original function of the transport protein co-opted to perform this new role involved malate transport or citrate transport. This is supported by transgenic experiments that overexpressed the wheat *TaALMT1* gene in barley (Delhaize *et al*. 2004). The relatively small variation in the Al^{3+} resistance among genotypes of barley relies on Al^{3+}-activated citrate efflux, not malate efflux, yet barley plants expressing *TaALMT1* show the

customary Al^{3+}-activated malate efflux. This demonstrates that barley is capable of releasing malate from its roots providing a suitable transport mechanism is present.

Presumably it would be necessary for the original function of the protein to be maintained or replaced so that these mutations are not detrimental. If the mutation simply extends the expression of the gene to new tissues or membranes then function could continue in the original location. If the protein is completely relocated then other proteins with similar function (redundancy) need to be able to compensate for their loss. A common source of redundancy in plants is local gene duplication where individual genes within chromosomes are copied. Redundancy can also arise from the hybridisation of entire sets of chromosomes as has occurred in wheat (polyploidisation). Redundancy provides insurance against lethal mutations and enables genes to be changed or re-deployed without adverse effects. Therefore, redundancy can benefit the fitness of organisms by facilitating evolutionary experimentation in changing environments (Otto and Whitton 2000).

Of course, not all transport proteins will be suitable for facilitating the efflux of useful anions from root cells. For instance, the energetics and mechanism of the transport protein, their pH sensitivity or their phosphorylation state are some factors that can affect function. The efflux of malate and citrate from root cells involves the movement of anions from the cytosol to the apoplast. This is an energetically 'down-hill' or passive process due to the large negative electrical potential of the cytosol relative to the outside of –100 to –200 mV. Ion channels are one type of transport protein that would be suitable because they facilitate passive ion movement and physiological studies indicate that TaALMT1 functions as an anion channel (Zhang *et al*. 2008). By contrast, the malate/oxaloacetate antiporter found on the inner mitochondrial membrane is an example of a malate transporter that is unlikely to work on the plasma membrane of root cells unless there is sufficient oxaloacetate in the apoplasm to satisfy the exchange reaction.

What type of mutations could generate these changes in tissue expression or membrane localisation?

Where a protein is finally located in a cell relies on short amino acid motifs called targeting domains. The sequence of these targeting domains and their position on the protein contain information that ensures soluble proteins reach their appropriate subcellular compartment (e.g. plastid, mitochondria, vacuole) and that transport proteins and other membrane-bound enzymes reach their target membranes (e.g. tonoplast, plasma membrane, mitochondrial membrane). Targeting domains occur at the *N*- and *C*-terminal ends of the protein and in some cases they are removed once the protein has reached its final destination. Intracellular localisation of a protein can therefore be affected by mutations to the DNA sequences encoding these targeting domains.

By contrast, the level of expression and tissue specificity are controlled by *cis* regulatory elements generally found in the promoter region of the gene. *Cis*-regulatory elements are untranscribed DNA sequences that influence when, where, and to what level genes are expressed. Relatively simple mutations in these elements could influence phenotype by redirecting proteins to different tissues or by altering their level of expression. Nucleotide substitutions, deletions or tandem repeats in *cis*-regulatory elements are common mutations that have the potential to alter gene expression and, consequently, plant phenotype.

Modifications to the target sequences and *cis*-regulatory elements can influence evolutionary change just as mutations in the coding regions do by changing protein function (Wray *et al*. 2003). The role of *cis* mutations and transcriptional regulation in species diversification is receiving increased attention. Its importance has been strengthened by reports directly linking phenotype to gene expression (Wray *et al*. 2003). We already have evidence that *cis* mutations have contributed to the evolution of Al^{3+} resistance in wheat by altering the expression of *TaALMT1* and this is discussed in more detail later.

Can this model accommodate other observations on Al^{3+} resistance?

The cases of STOP1 *and* ART1

STOP1 in *Arabidopsis* and *ART1* in rice encode C2H2 zinc finger-type transcription factors that control the expression of several genes providing resistance to Al^{3+} stress (*ART1*; Yamaji *et al*. 2009) or to low pH and Al^{3+} stress (*STOP1*; Sawaki *et al*. 2009). *STOP1* regulates two genes involved in Al^{3+}-activated malate and citrate efflux (*AtALMT1* and *AtMATE1*) both of which contribute to the Al^{3+} resistance of *Arabidopsis* (Table 1; Liu *et al*. 2009; Sawaki *et al*. 2009). The involvement of transcription factors such as STOP1 and ART1 can be accommodated by our model. The proposed mutations that divert malate and citrate transport proteins from their original sites of expression or membrane location to the plasma membrane of root cells would not necessarily interfere with any pre-existing regulation by transcription factors. The sequences recognised by transcription factors tend to be short modular regions of the promoter and these will not be affected by changes occurring in the coding region. Those sequences can also be physically separated from mutations to neighbouring regions of the promoter that control expression level or tissue specificity.

Activation and induction by Al^{3+}

The *ALMT* and *MATE* genes conferring Al^{3+} resistance are constitutively expressed in some plants and induced by Al^{3+} treatment in others. However, regardless of whether or not the genes are induced by Al^{3+}, nearly all the proteins they encode require external Al^{3+} to activate their function and release organic anions (Table 1). It is unclear how this activation occurs or even which form of Al is responsible for activation because soluble Al exists in a pH-dependent equilibrium among several ionic species (Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$ and $Al(OH)_4^{-}$; Kinraide 1991). Nevertheless Al^{3+}, the most likely candidate (Ryan *et al*. 1995), could directly trigger efflux, by interacting with the proteins, or indirectly, by first initiating a signal cascade or stress response, which then activates transport activity (Ryan *et al*. 2001). The direct-trigger explanation is more likely given this same dependence for Al^{3+} is observed in every heterologous expression system used to examine the function of these proteins. For example, *TaALMT1* has now been expressed in a range of

plants (e.g. rice, barley, tobacco, *Arabidopsis*), cell cultures (tobacco suspension cells) and animal cells (*Xenopus laevis* oocytes) using constitutive promoters or cRNA injection and, in every case, addition of Al^{3+} to the bathing solution increases malate efflux. It seems simpler for Al^{3+} to directly activate anion efflux than to expect that all those different cell types share pathways capable of first interacting with Al^{3+} and then transducing that signal to activate function. An exception to this dependency on Al^{3+} is in wheat where a constitutive efflux of citrate contributes to the Al^{3+} resistance in a few genotypes (Table 1; Ryan *et al*. 2009).

We view the induction of protein expression and the activation of protein function by Al^{3+} as useful traits for minimising unnecessary carbon loss in the absence of toxicity. Since Al^{3+} poses little problem in soils with pH >5.5 it would be wasteful for plants to continually release organic anions, even at a small metabolic cost, when there is no benefit. This requirement for Al^{3+} to activate efflux would also help stabilise the trait in a population because the resistance gene would not be selected against in non-acid conditions when there is no penalty to fitness.

In some species Al^{3+} both induces expression of the *ALMT* and *MATE* genes and activates protein function (Table 1). Why should both responses occur in a single species when these dual 'safety' mechanisms appear redundant? Part of the explanation might be that the induction of expression is a general response to stress that predates the involvement of the transport protein in Al^{3+} resistance whereas the capacity for the protein to be activated by Al^{3+} is a trait acquired more recently. In other words, the expression of these genes might have already responded to some other stress before they were co-opted from their original function to contribute to Al^{3+} resistance. There is some evidence for this because the major Al^{3+} resistance genes in *Arabidopsis*, *AtALMT1* and *AtMATE1*, are induced by other stimuli. Both genes are partly induced by low pH (Kobayashi *et al*. 2007; Liu *et al*. 2009) and *AtALMT1* is strongly induced by foliar infection with *Pseudomonas syringae* (Rudrappa *et al*. 2008). Similarly, the expression of *BnALMT1-1* and *BnALMT1-2* in oilseed rape is induced by metal ions other than Al^{3+} (Ligaba *et al*. 2006). Therefore, in some cases at least, the Al^{3+}-dependent increase in expression might reflect the induction by general stress and not a specific response to Al^{3+} toxicity. If so, the activation by Al^{3+} becomes an even more important point of control because, regardless of the stress inducing ALMT or MATE expression, anion efflux will only occur when toxic Al^{3+} is present in the soil.

If the co-opted ALMT or MATE proteins were originally located on intracellular membranes or in shoot tissues, then the requirement for Al^{3+} to activate their function is more likely to have appeared after the proteins were relocated to the plasma membrane of root cells. The reason for this is that intracellular proteins and leaf tissues are not usually exposed to soluble Al^{3+}. Even if Al^{3+} manages to enter the symplast, the alkaline pH and plethora of potential binding compounds maintain the concentration of free Al^{3+} cations extremely low. It is doubtful that proteins are specifically activated by a ligand they rarely, if ever, encounter. If we conclude then, that activation by Al^{3+} evolved after the proteins were co-opted to their new role then we are also forced to conclude that this change occurred independently in the ALMT and MATE families. The surprising corollary to this position is that relatively simple changes in the amino acid sequence of very different proteins can alter their function in a similar manner (i.e. allow them to be activated by Al^{3+}). If so, we may get some idea what these changes are by comparing the sequence of proteins that do or do not show activation in order to identify residues associated with this phenotype. Mutational analysis could then confirm the importance of any candidate residues. Nevertheless it remains possible that activation of the proteins by Al^{3+} did not evolve as described above but is a non-specific response. For instance, the highly charged Al^{3+} ions might simply induce conformational changes in these proteins that alter their activity. Previous reports showing that other trivalent cations, such as erbium, can also activate TaALMT1, albeit to lesser extent, support this idea by demonstrating that activation is not absolutely specific for Al^{3+} (Kataoka *et al*. 2002; Delhaize *et al*. 2004). One last example shows that it is even possible to totally circumvent this requirement for Al^{3+}. The foliar infection of *Arabidopsis* plants with a strain of *Pseudomonas syringae* not only induces AtALMT1 expression but triggers malate efflux (Rudrappa *et al*. 2008). This intriguing response, which reportedly encourages the proliferation of beneficial microorganisms to the rhizosphere, suggests that AtALMT1 may have multiple functions. It also begs the question of whether AtALMT1 was originally co-opted from a plant-microbe function to contribute to Al^{3+} resistance or *vise versa*.

Supportive evidence

Hypotheses which attempt to explain evolutionary processes are inherently difficult to prove or disprove. Nevertheless, we can ask what type of data would be consistent with the hypothesis and see if those data are available or whether they can be obtained. For instance, what evidence would support the idea proposed here that mutations in *cis* regulatory elements may have altered the level of protein expression or its tissue specificity? The demonstration that differences in promoter sequence can change the pattern or level of gene expression would support the hypothesis.

One example is illustrated by the perfect tandem repeats that occur in the promoter of *TaALMT1* in most Al^{3+}-resistant wheats (Sasaki *et al*. 2006). These repeats occur as duplications or triplications 31–803 bp long that are associated with increased expression of *TaALMT1* and greater malate efflux (Raman *et al*. 2008). Four of the tandem repeat patterns appear to have evolved independently of one another (Delhaize *et al*. 2007) with at least two conferring enhanced *TaALMT1* expression as demonstrated experimentally by transforming rice with several promoter regions fused to the reporter gene encoding green fluorescent protein (E. Delhaize and P. R. Ryan, unpubl. data). These tandem repeats appear to have arisen after the appearance of *T. aestivum* some 10 000 years ago (Dubcovsky and Dvorak 2007) because they have not been detected in *Ae. tauschii* the D-genome progenitor of hexaploid wheat. Polyploidy may have been helpful in fixing these mutations in subpopulations of *T. aestivum* as discussed above. How these tandem repeats formed is not known but may be due to the activation of mobile elements associated with allopolyploidy in cereals

(Kashkush *et al*. 2003). For instance, transposable elements such as *Helitrons* possess the rolling-circle machinery capable of generating these types of tandem repeats as suggested by Piffanelli *et al*. (2004). Illegitimate recombination is another mechanism that can generate duplications but the signature short direct repeats that usually flank these duplications (Wicker *et al*. 2007) are absent from the tandem repeats in the *TaALMT1* promoter. Once a region of the genome is duplicated by whatever means, additional copies can be generated by unequal crossing over.

Variations in the promoter regions of *MATE* genes are also associated with enhanced Al^{3+} resistance. Major genes for Al^{3+} resistance in sorghum (*SbMATE*) and barley (*HvMATE*, also named *HvAACT1*) encode MATE proteins that are activated by Al^{3+} and efflux citrate (Furukawa *et al*. 2007; Magalhaes *et al*. 2007; Wang *et al*. 2007). The sorghum *SbMATE* promoter possesses multiple insertions of a tourist-like miniature inverted repeat transposable element (MITE) and the number of MITES correlates with the level of expression and Al^{3+} resistance (Magalhaes *et al*. 2007). However, direct evidence, such as those obtained from reporter-gene studies, showing that these insertions enhance expression level is currently lacking. Similarly, the promoter region of *HvMATE* in an Al^{3+}-resistant barley cultivar possesses a 1023-bp insertion 4.6 kb upstream of the open reading frame start site that is absent from sensitive cultivars (Fujii *et al*. 2009). This insertion contains multiple transcription start sites that may be responsible for the high level of HvMATE expression found in Al^{3+} resistant genotypes.

The above examples described one type of *cis* mutation that confers a novel phenotype by changing the level of transcription but others can be investigated. The related idea that mutations to the targeting sequences of a protein can generate new phenotypes can be examined by first establishing a correlation between the coding alleles of a gene and Al^{3+} resistance. We can then test whether allelic variants direct the protein to different cellular membranes when fused to a reporter gene and expressed in a heterologous system. Once again, evidence establishing that certain coding alleles generate new phenotypes via protein targeting would provide additional support to the hypotheses proposed in this review.

Our model that convergent evolution has resulted from the procurement of different gene families to enhance Al^{3+} resistance can even be extended to different genes within the same family. For instance, although barley *HvMATE*, sorghum *SbMATE* and *Arabidposis AtMATE* all belong to the same gene family, they appear not to have originated from a common ancestral *MATE* gene since they do not cluster as a group distinct from other MATEs that confer constitutive citrate efflux (Fig. 2*a*). The same conclusion can be drawn from the ALMTs because among the proteins characterised so far those involved in Al^{3+} resistance do not cluster together in a phylogenetic tree (Fig. 2*b*). We know that ALMT proteins occur on different membranes and have functional roles beyond Al^{3+} resistance. Both *ZmALMT1* and *HvALMT1* have greater similarity to *TaALMT1* than *AtALMT1* yet neither appears to have a role in Al^{3+} resistance (Piñeros *et al*. 2008; Gruber 2009). These observations suggest that two or more genes from each of these families were independently recruited for a role in Al^{3+} resistance and each independently evolved the capacity for their function to be activated by Al^{3+}. Alternatively, it

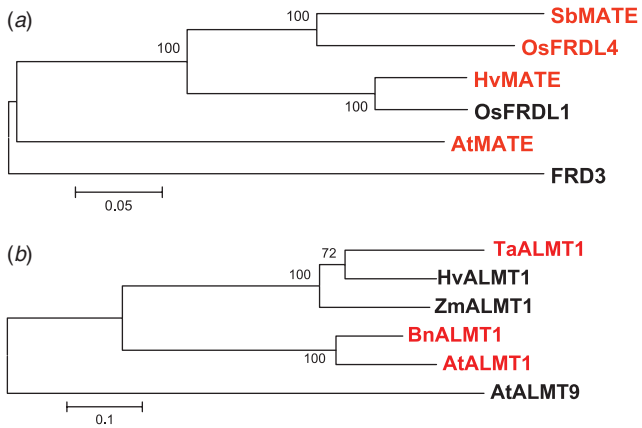

Fig. 2. Unrooted phylogenetic tree of the functionally characterised MATE and ALMT proteins. Direct physiological investigations have demonstrated that the proteins shown are capable of transporting organic anions. (*a*) The MATE proteins in red are those that are activated by Al^{3+} and have roles in resistance. The genes encoding SbMATE (GenBank ABS89149), HvMATE (GenBank BAF75823) and AtMATE (GenBank NP_974000) are described in Table 1 and FRD3 (GenBank NP_187461) is described by Durrett *et al*. (2007), OsFRDL4 (GenBank BAD87624) by Yokosho *et al*. (2009*a*) and OsFRDL1 (GenBank BAF11300) by Yokosho *et al*. (2009*b*). (*b*) The ALMT proteins in red are those that are activated by Al^{3+} and have roles in resistance. The genes encoding TaALMT1 (GenBank BAD10882), BnALMT1 (GenBank BAE97280) and AtALMT1 (GenBank AAF22890) are described in Table 1 and ZmALMT1 (GenBank ABC86748) is described by Piñeros *et al*. (2008), AtALMT9 (GenBank NP_188473) is described by Kovermann *et al*. (2007) and HvALMT1 (GenBank EF424084) is described by Gruber (2009). The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 10 000 replicates is taken to represent the evolutionary history of the taxa analysed and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*. 2007).

is also plausible that an ancestral *MATE* gene did encode a protein that was activated by Al^{3+} and that OsFRDL4 and AtFRD3 subsequently lost this activation domain to assume roles in the long distant transport of iron (Durrett *et al*. 2007; Yokosho *et al*. 2009*b*). A similar argument for the involvement of *ALMT* genes in Al^{3+} resistance has been made previously (Delhaize *et al*. 2007) despite apparent conservation of Al^{3+} resistance genes between monocots and dicots (Magalhaes 2006). The rye genes (*ScALMT1-M39.1* and *M39.2*) which are closely related to *TaALMT1* and appear to confer resistance based on Al^{3+}-activated efflux of organic anions (Table 1) probably share a common origin. However, the more distantly-related *Arabidopsis* gene *AtALMT1*, which is not the most similar member of this family in that genome to *TaALMT1*, is likely to have developed a role in Al^{3+} resistance independently (Delhaize *et al*. 2007).

Summary

The emergence of Al^{3+} resistance in plants appears to be a classic example of convergent evolution. This is based on the

finding that the same mechanism of Al^{3+} resistance operating in a range of plants is encoded by distinct gene families. The criteria that make malate and citrate anions the ideal currency of this mechanism include their ability to form stable complexes with Al^{3+}, their prevalence in plant cells, the economy of their synthesis and their requirement to be moved across most cellular membranes by an array of different transport proteins. We propose that the mechanism of Al^{3+} resistance based on organic anion efflux from root cells evolved relatively recently from mutations that co-opted transport proteins from other locations or other functions. Among the many proteins transporting organic anions across plant-cell membranes, members of the MATE and ALMT families appear most suited and readily recruited to assume roles in Al^{3+} resistance.

Note added in proof

A relevant paper was published after these proofs were prepared. Maron *et al*. (2010) identified and characterised two MATE genes that map to major aluminium-resistance QTLs in maize.

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