Metabolism and root exudation of organic acid anions under aluminium stress

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Numerous plant species can release organic acid anions (OA) from their roots in response to toxic aluminium (Al) ions present in the rooting medium. Hypothetically OA complex Al in the root apoplast and/or rhizosphere and thus avoid its interaction with root cellular components and its entry in the root symplast. Two temporal patterns of root OA exudation are observed. In pattern I, OA release is rapidly activated after the contact of the root with Al ions while in pattern II there is a lag phase between the addition of Al and the beginning of OA release. Compounds other than OA have been detected in root exudates and are also correlated with Al resistance in plants. Plant species like buckwheat and tea show mechanisms of Al tolerance, which confer them the capacity to inactivate and store Al internally in the leaves. Disturbances in metabolic pathways induced by Al are still obscure and their relation to the altered OA concentration observed in roots under Al stress is not yet established. High concentrations of OA in roots do not always lead to high rates of OA release even when the spatial distribution of these two characteristics along the root axis is taken into account. Al induces high permeability to OA in young root cells and anion channels located in the cell membrane have been proposed to mediate the transport of OA to outside the cell. Genetically modified plants that overexpress genes involved in the biosynthesis and transport of OA as well as in Al toxicity events at the cell level have been generated. In most cases the transformations resulted in an improved ability of the plant to cope with Al stress. These promising findings reinforce the possibility of engineering plants with superior resistance to Al-toxic acid soils. The environmental impact of the large amounts of root exudates possibly conferred by these genetically modified plants is discussed, with special emphasis on soil microbiota.
Key words: Al resistance, Al toxicity, anion channels, root exudates, transgenic plants.

Metabolismo e exsudação de ânions de ácidos orgânicos sob estresse de alumínio: Várias espécies vegetais liberam ânions de ácidos orgânicos (AO) de suas raízes em resposta a íons tóxicos de alumínio (Al) presentes no ambiente radicular. Hipoteticamente esses AO complexam os íons de Al presentes no apoplasto da raiz e/ou na rizosfera evitando assim sua interação com componentes celulares e ainda sua penetração no simplasto da raiz. Dois padrões temporais de exsudação são reconhecidos. No padrão I, os AO são rapidamente liberados pelas raízes após a exposição das mesmas aos íons de Al. No padrão II de exsudação, ocorre um atraso na liberação de AO após exposição das raízes à solução contendo Al. Outros compostos além dos AO foram detectados em exsudatos radiculares e relacionados com mecanismos de resistência a Al em plantas. Espécies vegetais como trigo sarraceno e chá apresentam mecanismos de tolerância ao Al. Estes mecanismos conferem às plantas capacidade de inativar e de armazenar o Al em formas não tóxicas nas folhas. Os distúrbios induzidos por Al em rotas metabólicas ainda são desconhecidos, assim como a relação desses distúrbios com as mudanças nas concentrações de AO em raízes que estão sob estresse de Al. Altas concentrações internas de AO nas raízes nem sempre levam a altas taxas de exsudação desses compostos, mesmo quando a variabilidade espacial da concentração e da exsudação ao longo do eixo radicular é considerada. Certamente Al induz uma grande permeabilidade de AO em células jovens da raiz. Canais aniônicos localizados na membrana plasmática são os prováveis transportadores desses compostos orgânicos para fora da célula. Plantas que superexpressam genes envolvidos na síntese e na exsudação de AO bem como genes relacionados com a toxidez de Al foram desenvolvidas pela engenharia genética. Na maioria dos casos essas plantas tiveram uma maior capacidade para desenvolver sob estresse de Al. Esses resultados indicam, portanto, novas alternativas para o desenvolvimento de plantas mais adaptadas às condições de solos ácidos e com problemas de toxidez de Al. O impacto ambiental que a grande quantidade de exsudatos radiculares de plantas geneticamente modificadas pode causar, especialmente na microbiota do solo, é discutido.
Palavras-chave: canais aniônicos, exsudatos radiculares, plantas transgênicas, resistência a Al, toxidez de Al.

INTRODUCTION

Organic acids of low molecular weight have often been mentioned as playing an important role in certain mechanisms evolved by plants to cope with environmental stresses like aluminium (Al) toxicity, and phosphorus (P) and iron (Fe) deficiency (Jones, 1998; Ryan et al., 2001; Kochian et al., 2004). These organic acids are carbon compounds with at least one carboxyl group (–COOH), and play a fundamental role in cellular metabolism. Some of them are involved in energy production as intermediates in the tricarboxylic acid (TCA) cycle (e.g. citrate, malate), while others are directly or indirectly involved in many other metabolic processes including the assimilation of carbon and nitrogen, the regulation of cellular pH and osmotic potential, the maintenance of electroneutrality during excess nutrient cation uptake, and the supply of energy to symbiotic bacteria (e.g. malate, malonate, oxalate) (Haynes, 1990; Marschner, 1995; Taiz and Zeiger, 2002). At the near-neutral pH of the cytosol, most of these acids exist there as fully dissociated anions (Taiz and Zeiger, 2002) and are almost certainly released from roots as such (Ryan et al., 2001).

Several plant species are able to release organic acid anions (OA) from their roots in response to toxic Al species present in the rooting medium while others release these compounds when growing under a deficient supply of P. Because OA can carry a varying negative charge, they can form complexes with Al thereby reducing its activity in solution and toxicity to roots (Delhaize and Ryan, 1995; Jones, 1998). Mechanisms that prevent Al from crossing the plasma membrane, entering the symplast, and reaching sensitive intracellular sites (Al exclusion) are mechanisms of Al resistance while those conferring the ability of plants to tolerate Al in the root (and/or shoot) symplast (internal resistance) are mechanisms of Al tolerance (Taylor, 1991; Kochian, 1995).

The processes leading to root OA exudation under Al stress are not yet fully understood. The biochemistry, physiology, and genetic basis of the mechanisms that may confer on root cells a higher capacity to produce and export these metal ligands out to the root apoplast and rhizosphere, are beginning to be understood and will be the main focus of this review.

Effect of Al on root exudation of OA by plants

It is well documented that numerous plant species can resist Al through OA exudation, with malate, citrate, and oxalate being the main OA released (Table 1). Two temporal patterns of OA exudation have been observed in Al-resistant plants (Ma, 2000; Ma et al., 2001). In pattern I, observed in wheat (Delhaize et al., 1993), buckwheat (Zheng et al., 1998b) and barley (Zhao et al., 2003), OA release is rapidly activated (15 to 30 min) after exposure of the plants to Al^{3+} solution, and the rate of release remains constant with time. This pattern suggests that Al^{3+} ions activate an existing mechanism of OA transport in the plasma membrane and therefore the activation of genes is not necessary. In pattern II, observed in *Cassia tora* (Ma et al., 1997b), rye (Li et al., 2000) and triticale (Ma et al., 2000), there is an evident delay in the onset of OA release after the addition of the Al^{3+} solution. Furthermore, contrary to pattern I, in pattern II the rates of OA release increase with time. In *C. tora* and rye the

Table 1. Plant species with Al-induced OA release by roots

Plant species	Main OA exuded	Reference[a]
Arabidopsis (*Arabidopsis thaliana* L.)	citrate	(16)
Barley (*Hordeum vulgare* L.)	citrate	(26)
Buckwheat (*Fagopyrum esculentum* Moench)	oxalate	(22,27,28)
Maize (*Zea mays* L.)	citrate	(8,9,11,12,13)
	citrate and malate	(10,15)
	oxalate	(14)
Oat (*Avena sativa* L.)	citrate	(22)
Oilseed rape (*Brassica napus* L.)	citrate and malate	(22)
Radish (*Raphanus sativus* L.)	citrate	(22)
Rice (*Oriza sativa* L.)	citrate	(21)
Rye (*Secale cereale* L.)	citrate and malate	(6)
Sickle senna (*Cassia tora* L.)	citrate	(18)
Snapbean (*Phaseolus vulgaris* L.)	citrate	(29)
Soybean (*Glycine max* L. Merr)	citrate	(19,20)
Sunflower (*Helianthus annuus* L.)	malate	(24)
Taro (*Colocasia esculenta* L.)	oxalate	(25)
Tobacco (*Nicotiana tabacum* L.)	citrate	(17)
Triticale (*Triticale hexaploide* Lart)	citrate and malate	(23)
Wheat (*Triticum aestivum* L.)	malate	(1,2,3,4,5,6,7)

[a] 1. Delhaize et al. (1993); 2. Basu et al. (1994); 3 Pellet et al. (1996); 4 Cocker et al. (1998); 5. Andrade et al. (1997); 6. Li et al. (2000); 7. Zhang et al. (2001); 8. Pellet et al. (1995); 9. Jorge and Arruda (1997); 10. Kollmeier et al. (2001); 11. Piñeros et al. (2002); 12. Mariano and Keltjens (2003); 13. Jorge et al. (2001); 14. Kidd et al. (2001); 15. Wang et al. (2004); 16. Hoekenga et al. (2003); 17. Delhaize et al. (2001); 18. Ma et al. (1997b); 19. Yang et al. (2000); 20. Silva et al. (2001); 21. Ma et al. (2002); 22. Zheng et al. (1998a); 23. Ma et al. (2000); 24. Saber et al. (1999); 25. Ma and Miyasaka (1998); 26. Zhao et al. (2003); 27. Zheng et al. (1998b); 28. Ma et al. (1997a); 29. Miyasaka et al. (1991).

rate of Al-induced OA release was increased after 4 and 10 h of Al exposure, respectively. In triticale, the rate of malate and citrate release was increased after 6 and 12 h of Al treatment, respectively. The lag phase between the moment of addition of Al and the onset of OA release by roots, together with the gradual increase of release with time, indicate that activation of genes related to the metabolism and membrane transport of these compounds might be required. However, to date the product generated by the activation of genes is unknown.

Effect of Al on root exudation of other Al-complexing agents

Organic and inorganic molecules other than OA have been identified in root exudates of plants suffering from Al toxicity. In a study with three maize varieties showing differential resistance to Al, Kidd et al. (2001) observed high exudation rates of the phenolics catechol, catechin, quercetin and curcumin. The amounts released correlated well with the resistance of the varieties to Al. Exudation of OA was also observed with these maize plants, but apart from being released in small amounts compared to the phenolic compounds (only 5% of the total exuded), OA release did not correlate with the general resistance shown by the three varieties to Al. Interestingly, oxalate was the main OA detected in root exudates, with amounts up to 10 times that of citrate or aconitate, the other two acids measured. Further examples are the release of Al-binding polypeptides (Basu et al., 1994) and phosphate (Pellet et al., 1995; Pellet et al., 1996).

Lack of correlation between OA exudation and resistance to Al in plant species where Al resistance is known to exist has not been uncommon (Wenzl et al., 2001; Ma et al., 2002; Piñeros et al., 2005). While investigating the Al resistance of two varieties of rice growing in culture solution containing Al^{3+}, Ma et al. (2002) observed a smaller relative root growth (RRG) in the *indica* variety Kasalath than in the *japonica* variety Koshihikari, together with a higher Al content in root tips of the former compared to the latter. Citrate dominated the OA found in the medium, but it was found at nearly equal amounts for the two rice varieties and furthermore, represented only one tenth of the citrate found in plants of rye used as control. Because rice is the grain cereal showing the highest resistance to Al (Ma et al., 2002), these results suggest that other mechanisms of Al resistance may be operating within this plant species.

Piñeros et al. (2005) made a comparative study of the physiology of Al resistance in maize using three Brazilian and three North American genotypes of significantly different Al resistance. They found an inverse correlation between root tip Al content and RRG. However, a lack of correlation between RRG and root citrate release was observed with the six genotypes. This was mainly due to an Al-sensitive line (Mo17) that had the greatest rate of citrate release among the maize lines. It thus led the authors to investigate other possible mechanisms for conferring resistance to Al such as, alkalinization of the rhizosphere, changes in internal root concentration of citrate, Al translocation to the shoot and exudation of other Al-chelating compounds. Exudation of malate and phosphate was not found to differ among genotypes. Moreover, malate and phosphate release was constitutive, that is, independent of the presence of Al in solution. All the other mechanisms investigated could not explain the differential resistance observed among the maize lines. The authors suggested that, besides citrate, other Al-chelating compounds, including phenolics, occur in maize. They further suggested that Al resistance in maize is a complex trait and will need an interdisciplinary approach (genetic, molecular, and physiological) for its elucidation.

Internal detoxification of Al by OA

About one hundred plant species accumulate Al in their above-ground parts without showing symptoms of Al intoxication (Barceló and Poschenrieder, 2002). Several mechanisms of Al tolerance have been proposed, including chelation of Al in the cytosol, compartmentation in the vacuole, Al-binding proteins, evolution of Al-resistant enzymes, and elevated enzyme activity (Taylor, 1991). Foy (1984) defines Al accumulator plants as those with more than 1000 mg kg^{-1} of Al in the leaves. These localised high concentrations of Al in shoots suggest that Al accumulator plants developed sophisticated mechanisms for the uptake and translocation of Al from roots to leaves. Ma and Hiradate (2000), based on their findings, proposed a conceptual model to explain the high capacity of buckwheat to absorb, transport and accumulate Al in the leaves. In this model, Al^{3+} ions cross the plasma membrane through transport proteins and, once inside the cell, are chelated by oxalate, present in the cytosol at concentrations as high as 8.8 mM (Ma et al., 1998). Before the metal is released to the xylem vessels, ligand exchange occurs and Al is transported to the leaves as Al-citrate complexes. Once in the leaves, ligand exchange occurs again and Al is sequestered in vacuoles as 1:3 Al: oxalate complexes (Shen et al., 2002).

The organic ligand used by the plant to inactivate Al in the symplast varies between species. Al appears mostly complexed with catechins and OA in tea (*Camellia sinensis*

L., Nagata et al., 1992), with citrate in ornamental hydrangea (*Hidrangea macrophylla*, Ma et al., 1997c) and with oxalate in melastoma (*Melastoma malabathricum*, Watanabe et al., 1998). Interestingly, part of Al resistance in buckwheat also correlates to Al-induced oxalate release by the roots (Zheng et al., 1998b).

Altered cell OA metabolism induced by Al

Aluminium-induced disturbances in biochemical pathways of OA metabolism as well as their relation with OA accumulation in roots have not yet been comprehensively studied, in contrast to the much better characterised process of Al-induced exudation of OA by plant roots. Cellular OA metabolism involves an array of enzymes that are interrelated and function in coordination with each other but the set of metabolic changes induced by Al that lead to altered concentrations of carboxylic acids in roots remains obscure. A diagrammatic representation of carbon (C) pathways relevant to the status of particularly malate and citrate in root cells is presented in figure 1. Since malate and citrate are both intermediate metabolites of the TCA cycle, their accumulation in root cells may result from an increase in their synthesis and/or from a reduction in their conversion into their succeeding metabolites in the TCA cycle. Therefore, any effect of Al on the activity of citrate synthase (CS), aconitase (Aco), and malate dehydrogenase (MDH), would interfere directly in citrate and malate metabolism. Exclusively in plant cells, malate can be alternatively converted to pyruvate by the action of malic enzyme, and thereby increase the supply of substrate for the synthesis of citrate (Taiz and Zeiger, 2002). Moreover, changes in the activity of phosphoenolpyruvate carboxylase (PEPC) could have a marked effect on cell carbon supply and thus on OA metabolism (Naik and Nicholas, 1986; Ryan et al., 2001).

Studies of the effects of Al on these key enzymes and on OA root accumulation have been undertaken with a number of economically important plant species, with results summarised in table 2. Excess Al leads to higher concentrations of OA in the roots, but they do not result specifically from the metabolic activity of the tissue where these changes are found. These findings raise the question of how root cells can sustain high rates of exudation, since the amounts of OA exuded represent a significant fraction of the OA content found in the exuding root tissue (table 3; Ryan et al., 1995). The activity of the tested root enzymes has, in most cases, not shown any alteration when plants were exposed to Al whereas changes in the concentration of OA in

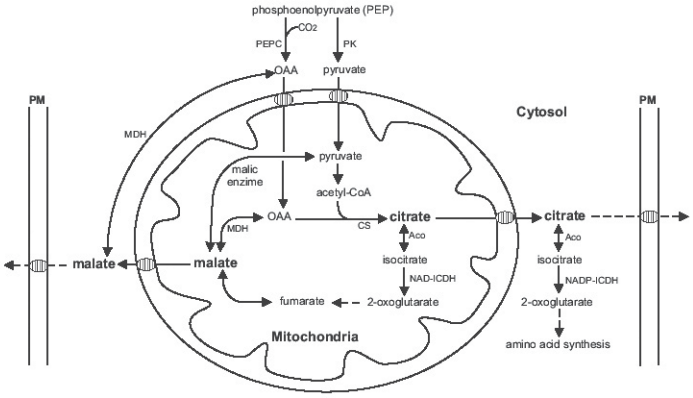

Figure 1. Diagrammatic representation of carbon pathways in plant cells related to malate and citrate metabolism. Aco, aconitase; CS, citrate synthase; MDH, malate dehydrogenase; NAD-ICDH, NAD specific isocitrate dehydrogenase; NADP-ICDH, NADP specific isocitrate dehydrogenase; OAA, oxaloacetate; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PM, plasma membrane. Hatched ellipses on the plasma membrane and mitochondria denote membrane transporters.

root tissues have been found in the majority of the cases, with a notorious inter and intraspecific variation. Examination of the results of citrate and malate accumulation within each plant species suggest a positive relationship between Al resistance and the capacity to increase or at least to maintain the tissue concentration of citrate and malate, and this holds not only for root tips but also for the whole root. Although a similar relationship was observed previously with a group of five wheat genotypes (Foy et al., 1990), the hypothesis that increases in OA accumulation are associated with Al resistance needs to be further investigated, especially because internal root OA concentration itself cannot be used to explain Al resistance (Mariano and Keltjens, 2004). Control of internal OA concentration seems to also involve a feedback mechanism. Blockage of citrate exudation by niflumic acid, a potent anion channel inhibitor, did not result in increased internal concentration of citrate in roots of barley (Zhao et al., 2003).

On the other hand, a recent report presented circumstantial evidence in favour of a regulatory association between metabolic activity, increased production of root OA, and even enhanced root efflux in *Cassia tora* (Yang et al., 2004). A time course study with seedlings of this tropical herb revealed that increases in CS activity and in citrate accumulation in root tips already occurred within the initial 3 h of Al treatment, while a substantial exudation of citrate began 6 h after the Al exposure. Concomitantly, aconitase activity in the tips of Al-treated roots was always suppressed compared with controls. Temporal increases in CS and falls in aconitase activities

Table 2. Relative activity of enzymes and organic acid anion (OA) concentration in roots of plant species where Al-induced root OA exudation is correlated with Al resistance. Values are expressed as no change (=), enhanced activity / increased accumulation (+ %), or reduced activity / decreased accumulation (– %) relative to the respective controls without Al. Symbols: Al, response to Al where R = resistant and S = sensitive; CS, citrate synthase; Aco, aconitase; MDH, malate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; NADP-ICDH, NADP specific isocitrate dehydrogenase; Cit, citrate; Mal, malate; Oxa, oxalate; wr, whole root

Plant species	Root part	Al	CS	Aco	MDH	PEPC	NADP-ICDH	Cit	Mal	Oxa	Ref.[a]
	mm										
Barley	wr	R						– 12	+ 211		(1)
		S						– 51	+ 25		
	0–10	R	=					+ 42			(14)
		S	=					– 7			
S. senna	0–5	R	+ 67	– 58	=	=	=	+ 96			(2)
Maize	wr	R						+ 38	+ 790		(3)
		S						+ 65	+ 620		
	0–20	R						+ 260			(4)
		S						+ 320			
	wr	R						+ 95	+ 81		(5)
		S						+ 165	+ 9		
	0–15	R				+ 124		+ 134	+ 269		(15)
		S				+ 38		+ 120	+ 153		
Rye	0–10	R	+ 30		=	=	=				(6)
	0–5	R	+ 21								(7)
	5–20	R	=								
Soybean	wr	R						– 12	– 84	– 28	(8)
		S						– 37	– 86	+ 67	
	0–10	R	+ 15			=	=	=	=		(9)
	0–5	R						+ 118	+ 13		(10)
		S						– 39	+ 28		
	5–base	R						– 6	+ 175		
		S						– 24	+ 90		
Triticale	0–5	R	=		=	=	+ 30	+ 100	=		(11)
		S	=		=	=	+ 40	+ 100	=		
	10–20	R	=		=	=	=	+ 200	+ 100		
		S						+ 100	+ 100		
Wheat	0–4	R							+ 6		(12)
		S							– 21		
	0–3	R			=	=					(13)
		S			+ 24	+ 51					
	0–10	R	=		=	=	=				(6)

[a] 1. Foy et al. (1987); 2. Yang et al. (2004); 3. Pellet et al. (1995); 4. Piñeros et al. (2005); 5. Mariano and Keltjens (2004); 6. Li et al. (2000); 7. Li et al. (2002); 8. Menosso et al. (2001); 9. Yang et al. (2001); 10. Silva et al. (2001); 11. Hayes and Ma (2003); 12. Delhaize et al. (1993); 13. Ryan et al. (1995); 14. Zhao et al. (2003); 15. Gaume et al. (2001)

Table 3. Root citrate content and exudation of roots of an Al-resistant maize genotype (CMS36) grown at two concentrations of Al (0 or 40 μM) for 28 h. Tissue content was determined at the end of the exudation period (last 4 h of the 28-h Al treatment period).

Root zone	Citrate content (nmol)		Citrate exudation (nmol $4h^{-1}$)	
	Al 0	Al 40	Al 0	Al 40
Whole root	21.2	31.9	0.27	1.96
Apical 10 mm segment:				
absolute	4.04	5.16	0.12	1.21
relative (% of whole root)	19	16	44	62

with Al treatments were associated with a gradual increase in root accumulation and exudation of citrate, which clearly characterised and confirmed the pattern II type of exudation in this species (Ma et al., 1997b).

The lack of alteration in the activity of PEPC after Al treatment shown by these studies is particularly interesting, since reactions where phosphoenolpyruvate (PEP) is carboxylated by PEPC to produce oxaloacetate (OAA) are known to function as a source of C for the anaplerotic operation of the TCA cycle (Naik and Nicholas, 1986; Taiz and Zeiger, 2002). Conditions in which OA are being released from roots (e.g. OA exudation by Al-stressed plants) require that C be replaced either by photosynthesis or nonphotosynthetic CO_2 fixation via PEPC (Johnson et al., 1994). Al-induced disturbance of NADP-ICDH function could also affect citrate accumulation in the cell, since cytosolic NADP-ICDH is probably the major pathway for the catabolism of citrate after its transport out of mitochondria and conversion to isocitrate by cytosolic Aco (Delhaize et al., 2003).

Comparatively, studies on OA metabolism and C partitioning within P-starved plants have revealed a multitude of metabolic changes induced by P deficiency that ultimately resulted in accumulation of OA in the root, and eventually in their exudation to the external medium. For instance, OA accumulation in roots and shoots of P-deficient oilseed rape plants coincided with enhanced activities of PEPC in the shoot and in the root tip (Hoffland et al., 1992). With white lupin (*Lupinus albus* L.), a species well-known for its excellent ability to solubilise and absorb P from rock phosphate, Johnson et al. (1994, 1996) observed that under P-stress conditions, C fixation in roots (nonphotosynthetic C fixation) plays an important role in C exudation. Carbon fixed in roots of P-starved plants, via an enhanced activity of PEPC and accompanied by higher MDH and CS activities, contributed about 25 and 35% of the C exuded as citrate and malate respectively.

Aluminium can also have a significant, yet indirect, effect on cell OA metabolism by disturbing the pattern of mineral nutrient uptake and hence altering the cation-anion uptake balance of the cell. The maintenance of electroneutrality within plant cells and organelles is achieved by pH-dependent changes in the size of the OA pool through carboxylation and decarboxylation reactions, often referred to as the biochemical pH-stat (Haynes, 1990). The adverse effects of Al on mineral nutrient uptake are well documented (Foy, 1984), and among its negative effects on absorption of macronutrients, Al has been shown to interfere strongly with the nitrogen nutrition of plants. With maize, Al drastically inhibited the absorption of nitrate (NO_3^{-1}), but not that of ammonium (NH_4^+), which was actually slightly stimulated under Al stress (Keltjens and van Ulden, 1987; Mariano and Keltjens, unpublished data). The resulting alkaline nutrient uptake pattern would result mainly in decarboxylation of malate, but also in H^+ extrusion from roots to compensate for the excess of positive charges, resulting from NH_4^+ uptake and assimilation, over negative ones. In addition, an inherent complicating fact is that the inhibitory effect of Al on the uptake of NO_3^- is stronger in Al-sensitive genotypes than in Al-resistant ones (Keltjens, 1987; Keltjens and van Ulden, 1987). With genotypes showing differential resistance to Al, this toxic metal would almost certainly lead to differences in OA concentration in roots that might be unrelated to its specific action on OA metabolism and/or to the capacity of the genotypes to release these C compounds as a protective mechanism against Al.

We will certainly need a holistic approach that considers enzymes and metabolites both upstream and downstream of citrate and malate as well as their subcellular pools in order to obtain some insight into their dynamics within and transport out of the cell.

Linking internal OA concentration with OA exudation

Attempts to link internal root concentration and root exudation of OA have been made with Al-stressed plants, with an implicit assumption that higher internal concentrations

would lead to higher root exudation rates. However, no correlation is apparent between internal concentration and rate of exudation in plant species where root OA release is stimulated by Al (Delhaize et al., 1993; Pellet et al., 1995; Silva et al., 2001). Aluminium activates a five to tenfold increase in malate exudation in wheat roots (Delhaize et al., 1993; Ryan et al., 1995). Al-stimulated efflux of malate from the terminal 3-mm root segment was tenfold greater than either of the next two 3-mm segments. This pattern was not correlated with the internal distribution of malate, which was actually lower in the apical segment. The high rate of efflux was maintained for several hours and the cumulative release in 4 h represented more than three times the malate present in the tissue before addition of Al, demonstrating that malate synthesis occurred in the excised tissue during the period of efflux. Furthermore, the much greater stimulation of malate efflux from root apices of the Al-resistant compared to those of the Al-sensitive genotype could not be explained by differences in the activities of PEPC or MDH (Ryan et al., 1995).

Given the uneven distribution of organic solutes, including OA, and of OA exudation within a single plant root (Marschner, 1995; Pellet et al., 1995; Jones, 1998; Mariano and Keltjens, 2003), much caution is needed when studying their interrelation in the root system. A remarkable accumulation of the tricarboxylic acid citrate was observed in the apical region (0–10 mm) of maize roots growing under optimal conditions, contrasting with much lower concentrations in the more mature zones, especially in that between the root tip and the basal region with lateral roots (figure 2) (Mariano 2003; Mariano and Keltjens, unpublished data). This great localised concentration reflects the high demand of photosynthates in the growing parts of the root for the formation of new root biomass, followed by high rates of metabolic activity and a concomitant consumption of O_2 in these apical regions (Jones and Darrah, 1996; Li et al., 2002). Conversely, maize roots exposed to Al showed a rather homogeneous spatial internal distribution of citrate along the longitudinal axis (figure 2).

A constitutive basal release of OA into the soil is observed in the apical parts of roots growing without any external stimuli (Jones, 1998, Ryan et al., 2001). This constant and low release is independent of the internal pool of citrate found in this part of the root. Moreover, high concentrations of citrate in spatially-isolated segments of intact maize roots did not confer high rates of exudation, as shown by the lack of correlation between internal concentration and exudation observed with and without Al (figure 3). It indicates that the internal concentration of citrate itself is not the driving force of citrate exudation from roots, but Al can induce a high permeability to citrate in root cells. Furthermore, contrary to the distribution of the internal citrate content of the root, where the apical 10 mm accounted for 15–20% of the total root citrate, this apical part accounted for as much as 44 and 62% of the total citrate exuded by the entire root in 4 h (table 3). Location of sites of high OA release in apices not only coincides with this segment being the most Al-sensitive region of the root but also shows that only cells at a certain stage of development are able to release OA in response to toxic Al ions.

Besides showing an altered distribution of internal root citrate, Al-treated roots contained 50% more citrate than roots not treated with Al (table 3). The cortical as well as the stellar tissue of maize roots show a capacity to increase their citrate concentrations after root exposure to Al (Piñeros et al., 2002). Because citrate and malate are simple intermediate metabolites in the TCA cycle and the enzymatic machinery for their production is already present in the cell, it would appear, in principle, that cells along the whole root can respond to Al through an altered production and storage of these two OA.

The stimulated release of citrate verified with maize roots treated with 40 μM Al resulted in a partial loss of root citrate to the outer medium. This loss represented about 5% of the total citrate content of the root but could represent much more (about 25%) if only the apical root segment were considered (table 3). These data reinforce the hypothesis that a continual synthesis of citrate is required to sustain such a high rate of citrate exudation, which would otherwise completely deplete the citrate reserve of that root segment within a

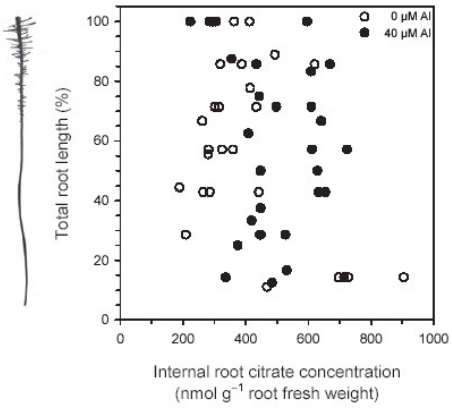

Figure 2. Distribution of internal citrate concentration along the main root axis of an Al-resistant maize genotype (CMS36) exposed to 0 or 40 μM Al for 28 h. A root axis as used in the experiments is schematised on the left-hand side for reference.

few hours. It becomes very likely therefore that apart from the synthesised OA, that transported from other parts of the root system or the plant should also be contributing to the continual release of OA by root apices. Equally possible is the basipetal transport of photosynthetically fixed C compounds, including sugars, that can be locally converted into OA before release to the external medium. Transport of sugars might constitute an important source of energy as well as C itself to the C drain that exudation may represent (up to 25% of the photosynthetically fixed C, Johnson et al., 1996; Jones, 1998). With plants of oilseed rape suffering from P deficiency, Hoffland et al. (1992) demonstrated that part of the citrate accumulated in the roots had its origin in the shoots. Citrate produced from photosynthetically fixed C was transported via the phloem towards the root where it was stored in the exudation region. Deficiency of P also led to an increase in the transport of sugars from shoots to roots. Because no increase in the concentration of malate was observed in the phloem sap, these authors suggested that malate was probably newly synthesised in the cells of the accumulating root segment from sugars imported from the shoots. The pattern of citrate concentration shown by maize roots after 28 h of Al treatment (figure 2) should reflect the net result of a combination of the different processes described, i.e. cell OA metabolism of roots and shoots (biosynthesis and decomposition), reallocation and storage, and root exudation. For such a process, a regulation mechanism operating at whole plant level might be expected.

Assuming that the aerial parts of the plant would also contribute to root exudation (Yang et al., 2001), the role of signal molecules in altering the OA metabolism in the shoot soon after Al exposure would be of primary importance. Due to the high binding affinity of Al for cellular components of the roots, Al is not usually translocated to the upper parts of plants, even when root growth is severely inhibited (Marschner, 1995). Therefore, it is most unlikely that Al itself can trigger increases in OA biosynthesis in the shoots soon after Al exposure, especially with those plant species that show a minimal transport of Al to leaves.

It is noteworthy that current studies with OA metabolism, transport and exudation in response to Al stress have either focused on the whole root system or on the first few millimetres of the root tip, which does not allow the assessment of C partitioning in Al-stressed plants. In future studies we will need a more integrative model to understand the changes in OA metabolism induced by Al that lead to an altered accumulation of OA in shoots and roots and eventually to an enhanced release by roots and resistance to Al.

Al-activated transport of OA across the cell membrane

Monomeric Al ions induce a high permeability to AO in root cell membranes. This induction is highly specific to Al since other di and trivalent metals like lead (Pb^{2+}), cadmium (Cd^{2+}), manganese (Mn^{2+}), lanthanum (La^{3+}), gallium (Ga^{3+}), indium (In^{3+}), and ytterbium (Yb^{3+}) were unable to induce exudation of OA from roots of several plant species (Ryan et al., 1995; Ma et al., 1997b; Li et al., 2000, 2002).

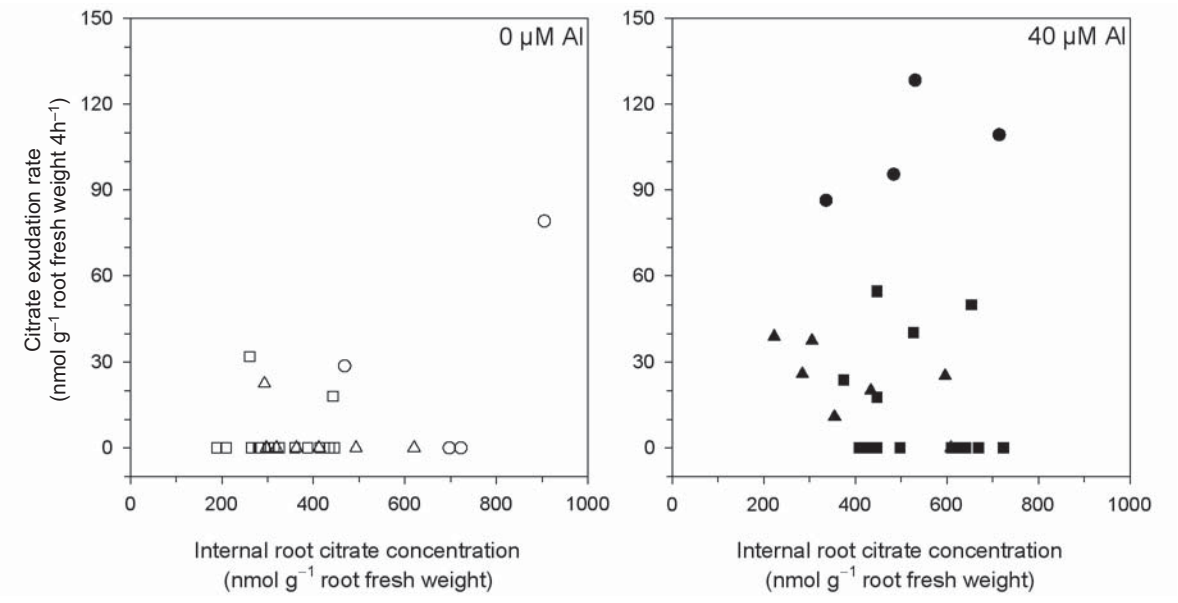

Figure 3. Internal root citrate concentration and root citrate exudation of root segments of the intact main root of an Al-resistant maize genotype (CMS36) exposed to 0 or 40 µM Al for 28 h. ,○●, root tip; □■, mature zone without lateral roots; △ ▲, mature zone with lateral roots.

This Al-specific induction is confined to the regions of root meristems (Pellet et al., 1995; Kollmeier et al., 2001; Mariano and Keltjens, 2003) and Al must be in direct and continuous contact with the root before activated exudation is observed. Soybean plants whose root system was spatially divided and partially treated with Al showed exudation of citrate only from (tips of) roots exposed to the toxic metal (Yang et al., 2001). The specificity of the mechanism controlling exudation under Al stress can be further appreciated by comparing it to the mechanism controlling root exudation induced by P deficiency, where the site of exudation occurs along the root axis as far back from the tip as where the root has contact with the rock phosphate (Hoffland et al., 1992).

Anion channels are found in plants cells and play an important role in cell signal transduction, osmoregulation, nutrition and metabolism (Barbier-Brygoo et al., 2000; Ma et al., 2001; Ryan et al., 2001). Protons (H^+) are continuously pumped from the cytoplasm to the apoplast by H^+-ATPases, thus generating a negative potential (–100 to –200 mV) across the plasma membrane (negative inside). This negative potential and the gradient of concentration between cytoplasm and apoplast allow the passive efflux of anions to the apoplast via anion channels (Barbier-Brygoo et al., 2000).

Anion channels that are specifically activated by extracellular Al^{3+} have been reported in protoplasts isolated from root apices of Al-resistant wheat (Ryan et al., 1997; Zhang et al., 2001) and maize (Kollmeier et al., 2001; Piñeros and Kochian, 2001; Piñeros et al., 2002), and have been proposed as the mediators of OA transport across the cell membrane. Indeed, the species-specific pattern of OA release (Table 1), together with the observation that root segments containing OA in the highest concentration are not the ones showing the highest rates of release (i.e. the gradient of concentration itself does not determine efflux rate), suggest that specific membrane transporters that can be activated by Al might well be involved. Further evidences that support this hypothesis are: 1) these anion channels are activated specifically by Al^{3+}, while La^{3+} is not able to replace it; 2) apart from the anion Cl^- that is mostly used in electrophysiological studies, Al-activated anion channels have shown permeability to the most important OA involved in the Al resistance mechanism (i.e. malate^{2-} and citrate^{3-}); 3) in the presence of anion channel blockers, anion currents as well as exudation rates are strongly inhibited; 4) Al-activated anion channels have been observed in the apical region of roots, more specifically in the distal transition zone (DTZ, 1–2 mm from the tip), which coincides with this region being the most sensitive to Al; 5) trivalent Al ions activate anion channels more often in cells from Al-resistant than from Al-sensitive lines, which correlates with higher rates of OA release observed in roots of the former.

The mechanism by which Al activates these channels is not known precisely. A model proposed in details by Ma et al. (2001) and Ryan et al. (2001), suggests that the Al activation of these anion channels could occur through the following possibilities: exudation pattern I – a) direct interaction between Al^{3+} and the channel in the cellular membrane; b) indirect interaction between Al^{3+} and the channel, via a specific receptor in the cellular membrane and close to the channel; c) Al^{3+} enters the symplast to activate the anion channels; exudation pattern II – Al^{3+} interacts with a receptor in the cellular membrane that in turn is able to activate the channel through a signal cascade that can involve gene activation, protein synthesis, OA metabolism and transport. For example, Piñeros and Kochian (2001) observed that the anion current is activated by Al^{3+} in outside-out patches of Al-resistant maize, suggesting that Al does not need to enter the symplast to activate the anion channels.

Recently, an Al-activated membrane protein that mediates the transport of malate out of the cell was reported by Sasaki et al. (2004). This protein is encoded by a wheat gene named *ALMT1* (aluminum-activated malate transporter), and is constitutively expressed in the root apices, with higher expression in the Al-resistant lines than in the near-isogenic but Al-sensitive ones. Further studies on the factors controlling the permeability of this protein to malate will certainly produce interesting insights into Al resistance.

Genetic manipulation of OA metabolism and exudation in plants

Plant breeders can take advantage of genetic engineering by which useful genes are made available from virtually any species and this can speed up the introgression of new desirable traits in crop species. Researchers have manipulated the biosynthetic capacity of cells with a view to produce and accumulate higher amounts of OA, in the hope that this will ultimately result in an altered root exudation profile and Al resistance of a given plant genotype.

As pointed out by Rengel (2002), increase of synthesis and, eventually, accumulation of OA in root cells are not a guarantee of high rates of root exudation. Rather, effective exudation of OA into the rhizosphere relies on at least three processes: a signalling sequence, effective biosynthetic machinery producing relatively large amounts of OA, and a

membrane transporter that allows transfer of OA out into the root apoplast and rhizosphere.

Table 4 summarises the attempts to obtain transgenic plants with higher Al resistance by enhancing OA exudation. The most well-known example of a successful achievement in this direction is the work of de la Fuente et al. (1997). These authors transferred a citrate synthase gene from the bacterium *Pseudomonas aeruginosa* into tobacco and papaya (*Carica papaya* L.) and recorded increases of 2- to 3-fold in CS activity, up to 10-fold in internal citrate concentration and up to 4-fold in root citrate efflux with plants of both species grown in Al-containing nutrient solution. The transgenic tobacco plants also had lower root growth inhibition and lower hematoxylin staining of the root apex than tobacco plants containing an empty vector, when growing under Al stress. The strategy of overexpressing enzymes involved in OA metabolism has proven to be effective to enhance OA exudation and to increase Al resistance with other transgenic plants. *Arabidopsis thaliana* expressing a CS from carrot (*Dancus carota* L.) (Koyama et al., 2000), alfalfa overexpressing an alfalfa (*Medicago sativa* L.) MDH (Tesfaye et al., 2001), and oilseed rape transformed with a CS from *A. thaliana* (Anoop et al., 2003) all had increased concentrations and/or activity of CS or MDH, that were correlated with higher release of OA by roots.

Nevertheless, the strategy has also failed in some cases. Delhaize et al. (2001) argued that expression of the *P. aeruginosa* citrate synthase gene in plants is unlikely to be a robust strategy for enhancing the Al resistance of plants. Delhaize and co-workers used the same lines from de la Fuente et al. (1997) and generated novel tobacco lines expressing the same *P. aeruginosa* gene. The CS activity in the novel lines was up to 100-fold greater than in the non-transformed plants but no increase in root citrate accumulation, citrate root efflux or Al resistance was observed. Moreover, Delhaize et al. (2001) could not repeat the findings of de la Fuente et al. (1997) using the same tobacco lines. Transference of the same construct to alfalfa resulted in high values of CS expression, but again it did not confer higher resistance to Al compared to non-transformed plants. Delhaize et al. (2001) then suggested that either the *P. aeruginosa* CS gene could be sensitive to the different environmental conditions used by the two research groups or the previous results on Al resistance (de la Fuente et al., 1997) and enhanced P acquisition (López-Bucio et al., 2000) could be due to other variables.

In another study, Delhaize et al. (2003) produced transgenic plants of tobacco that had marked increases in mitochondrial CS activity and large reductions in cytosolic NADP-ICDH, two enzymes with a key role in controlling cell citrate concentration, but with no differential internal citrate concentration or citrate efflux. An increased turnover of citrate where increased biosynthesis was matched by increased degradation was considered as a possible explanation but the authors also argued that the limiting step may not be the production, but rather the transport of citrate across the plasma membrane.

An important step in the use of membrane transporters of OA with an aim to improve Al resistance was recently taken by Delhaize et al. (2004) using the *ALMT1* gene encoding a membrane malate transporter. Sasaki et al. (2004) observed that transgenic rice plants overexpressing the *ALMT1* gene did not show increases in Al resistance and concluded that this was because of the natural high Al resistance observed in rice plants. Based on these findings Delhaize et al. (2004) used barley, a highly Al-sensitive species, and found that

Table 4. Transgenic plants overexpressing genes involved in OA synthesis and release.

Gene	Organism of origin	Transformed species	Al resistance	Ref.[a]
CSb (citrate synthase)	*P. aeruginosa*	Tobacco, papaya	Increased	(1)
DcCS (citrate synthase)	Carrot	Arabidopsis	Increased	(2)
CSb (citrate synthase)	*P. aeruginosa*	Tobacco, alfalfa	Not changed	(3)
neMDH (malate dehydrogenase)	Alfalfa	Alfalfa	Increased	(4)
PEPC (phosphoenolpyruvate carboxylase)	Alfalfa	Alfalfa	Not changed	(4)
cit1 (citrate synthase)	Tobacco	Tobacco	Not changed	(5)
At-mtCS (citrate synthase)	Arabidopsis	Oilseed rape	Increased	(6)
ALMT1 (malate channel)	Wheat	Rice	Not changed	(7)
ALMT1 (malate channel)	Wheat	Barley	Increased	(8)

[a] 1. de la Fuente et al. (1997); 2. Koyama et al. (2000); 3. Delhaize et al. (2001); 4. Tesfaye et al. (2001); 5. Delhaize et al. (2003); 6. Anoop et al. (2003); 7. Sasaki et al. (2004); 8. Delhaize et al. (2004)

Al triggered a perceptible exudation of malate from roots. The rate of malate release accompanied Al concentrations in solution in a dose-dependent manner, confirming the previous findings that the ALMT1 protein is an Al-gated anion channel (Sasaki et al., 2004). Moreover, the fact that malate release can be increased by the expression of an anion channel protein indicates that this mechanism of transport can be easily modulated by root apex cells and is not rate-limiting within the Al concentrations normally used in Al toxicity/resistance studies. Unfortunately, no information was made available on the internal malate concentration with the transformed plants.

Root release of OA can increase soil-P mobilisation and availability to plants (Kochian et al., 2004), and the performance of transgenic plants with higher efflux of OA has also been evaluated in P-limiting conditions. Growing in soils with low available P, transgenic tobacco plants produced more biomass and had higher concentrations of P in their leaves than the control plants (López-Bucio et al., 2000). Overexpression of CS in *A. thaliana* (Koyama et al., 2000) and of MDH in alfalfa (Tesfaye et al., 2001) conferred higher exudation of citrate in the former and of several OA in the latter, which in both cases resulted in increased P uptake from acidic low-P soils.

It is worth noting that performance of a transgenic plant compared to non-transformed plants cannot be taken as the unique variable to assess Al resistance and/or P efficiency. The use of Al resistant/P efficient germplasm and (field) assays using acid soils is highly desirable to get stronger evidence on their potential use in agricultural systems. From all the studies presented in table 4, only the overexpression of the *neMDH* gene in alfalfa (Tesfaye et al., 2001) and the *ALTM1* gene in barley (Delhaize et al., 2004) produced transgenic plants with growth comparable to an Al-resistant standard.

Knowledge of the genetic basis of OA release by roots brings, therefore, new perspectives on the use of genetically modified organisms to cultivate soils often considered Al-toxic/P-deficient problem soils. Plants with superior resistance to Al and higher capacity to acquire P conferred by exudation of high amounts of OA may help to reduce the use of soil amendments (e.g. lime) and P fertilisers, hence reducing financial and environmental costs.

Transgenic plants overexpressing Al-induced genes

There are several genes that have been found to be induced by Al stress and the effect of their overexpression in transgenic plants evaluated (Table 5). Ezaki et al. (2000) found that transgenic *A. thaliana* plants expressing a blue-copper-binding protein, a guanosine 5' diphosphate (GDP) dissociation inhibitor, a peroxidase and a glutathione S-transferase had up to 1.5-fold higher root growth at 100 µM Al compared to wild-type plants. Although modest, the increase in Al resistance did help to better understand the mechanisms of Al toxicity, reinforcing the former view that Al causes oxidative stress, since both peroxidase and glutathione S-transferase are well-known enzymes involved in the detoxification of reactive oxygen species. In another study, the survival of *A. thaliana* seedlings in 200 µM Al solution was strongly enhanced by the overexpression of the soybean Al-induced *IMPDH* gene, which encodes a protein homologous

Table 5. Transgenic plants overexpressing genes induced by Al stress.

Gene	Organism of origin	Transformed species	Al resistance	Ref.[a]
AtBCP (Blue copper binding protein)	Arabidopsis	Arabidopsis	Increased	(1)
AtBPI (protease inhibitor)	Arabidopsis	Arabidopsis	Not changed	(1)
AtPOX (peroxidase)	Arabidopsis	Arabidopsis	Not changed	(1)
HSP150 (heat shock protein)	*S. cerevisiae*	Arabidopsis	Not changed	(1)
NtGDI (GDP dissociation inhibitor)	Tobacco	Arabidopsis	Increased	(1)
NtPOX (peroxidase)	Tobacco	Arabidopsis	Increased	(1)
parA (unknown function)	Tobacco	Arabidopsis	Not changed	(1)
parB (glutathione S-transferase)	Tobacco	Arabidopsis	Increased	(1)
Wali5 (Bowman-Birk protease inhibitor)	Wheat	Arabidopsis	Not changed	(1)
WMnSOD (mitochondrial manganese superoxide dismutase)	Wheat	Oilseed rape	Increased	(2)
Wak1 (cell wall-associated receptor kinase 1)	Arabidopsis	Arabidopsis	Increased	(3)
IMPDH (inosine-5'-monophosphate dehydrogenase)	Soybean	Arabidopsis	Increased	(4)

[a] 1. Ezaki et al. (2000); 2. Basu et al. (2001); 3. Sivaguru et al. (2003); 4. Ermolayev et al. (2003)

to inosine-5'-monophosphate dehydrogenase (Ermolayev et al., 2003). This protein is involved in the synthesis of guanine nucleotides (RNA and DNA precursors) and in cell differentiation (Ermolayev et al., 2003 and references therein).

Sivaguru et al. (2003) superexpressed the *WAK1* (cell wall-associated receptor kinase 1) in *A. thaliana* and found that the growth of transgenic plants at 100 μM Al was 3-fold higher than that of wild-type plants. The gene *WMn-SOD* is induced by Al and encodes a manganese superoxide dismutase (SOD) that co-segregated with Al resistance in a wheat population (Basu et al., 1999, 2001). Transgenic oilseed rape plants overexpressing this gene had 1.5- to 2.5-fold higher SOD activity, which was associated with a lower content of malondialdehyde, a marker of lipid peroxidation (Basu et al., 2001). The root growth of the genetically modified plants exposed to Al was 1.5- to 2.3-fold higher than the root growth of wild-type plants.

The findings with transgenic plants along with the wide array of function of Al-induced genes and Al toxicity symptoms indicate that the plant defences against this metal are controlled by multiple genes. This makes it quite difficult to produce Al-resistant transgenic plants using only one of these genes. A good alternative would be to use gene pyramidization, with the expression of several genes simultaneously. In fact, Ezaki et al. (2001) produced hybrids from their previous work with transgenic *A. thaliana* overexpressing the *AtBCB*, *parB*, *NtPox,* and *NtGDI1* genes (see table 4 and Ezaki et al., 2000). While the lines containing only one transgene showed around 40% of root growth reduction, any transgenic hybrid containing two of these genes had only 20% of root growth reduction at 200 μM Al solution (Ezaki et al., 2001). The combination of genes involved in both Al exclusion and Al tolerance will certainly produce transgenic plants with higher total Al resistance.

Another alternative is the identification of transcriptional factors that are at the top of the cascade that triggers the expression of several genes involved in plant defences against Al toxicity. There are several examples of the expression of a single transcriptional factor that triggers plant defences, as the case of the *A. thaliana CBF1* and *CBF4* genes which activate several genes, increasing tolerance to abiotic stress such as low temperature and water deficit (Haake et al., 2002; Lee et al., 2003). To this end it is expected that experiments with DNA microarrays, which allow high throughput gene expression profiling, will have a huge impact on the discovery of new Al-induced genes. It is surprising that so far only one preliminary experiment on Al resistance has been evaluated using this technology (Hoekenga et al., 2003).

Promoters used to overexpress transgenes

The strong 35S promoter has been widely used to direct the expression of the transgenes shown in table 4. The only exception is the work of Delhaize et al. (2004), where the maize Ubi1 promoter from an ubiquitin gene was used. It is well known that the 35S promoter is strongly expressed in most plant tissues. Since the site of Al toxicity is the root apex, promoters that express transgenes only in this region would avoid the waste of metabolic energy and undesirable phenotypes due to ectopic expression. For instance, the low biomass production of transgenic plants grown in soil with neutral pH, where the OA release mechanism was not expected to be activated, might be associated with the use of the 35S promoter to express the *neMDH* gene, as pointed out by Tesfaye et al. (2001). Interestingly, no differences were observed in root growth between wild-type and transgenic barley plants expressing the *ALMT1* gene under the control of the constitutive Ubi1 promoter (Delhaize et al., 2004). Unfortunately, these authors did not present data on the total plant biomass that would permit a comparison with data of Tesfaye et al. (2001).

It is worth mentioning that public concerns towards genetically modified crops must also be taken into account when planning the production of transgenic plants (Doering, 2004). In this sense, the expression of transgenes in plant parts that will be used for human and animal nutrition is not desirable, reinforcing the need of specific promoters. In any case, a desirable promoter for engineering Al resistance would be highly expressed in the root apex and only in the presence of Al. Recently Ezaki et al. (2004) evaluated the expression pattern of two Al-induced genes encoding GST proteins from *Arabidopsis*. Although both *AtGST1* and *AtGST11* promoters are induced by Al, they were mainly active in the leaves and showed very low activity in roots, making them unsuitable candidates to direct the expression of genes aimed at Al resistance. We have isolated a promoter from a maize gene that is expressed only in epidermis of the root tip (Maron et al., unpublished data). Transgenic maize plants with this promoter are being obtained and should help to evaluate the hypothesis that localised expression of transgenes is a good strategy to obtain Al resistance.

Transgenic plants with increased OA efflux and soil microbiota

Root exudates have great impact on soil microbiota, which are key components for plant nutrient supply. For example, the rhizosecretion of glutamate, aspartate and dicar-

boxylic acids attracts *Bradyrhizobium japonicum* to soybean roots (Barbour et al., 1991), while flavonoids released by several legume species induce *nod* genes in rhizobia, with an important role in nodulation (Peters et al., 1986). Transgenic plants exudates have been shown to change root-associated bacterial populations, as observed by Oger et al. (2000), who found that the opine-utilizing bacteria population increased in soil cultivated with transgenic *Lotus corniculatus* that exudes opines.

The impact of the release of OA from transgenic plants has only recently been addressed. It might be expected that bacteria that use organic acids could have a selective advantage in soils where transgenic plants with increased efflux of OA are being grown. Tesfaye et al. (2003) evaluated the diversity of rhizobacteria and soil nutrient availability in soils cultivated with transgenic alfalfa plants expressing the *neMDH* gene, using a non-transformed plant as control. Total DNA was extracted from soil samples and sequences from the 16S ribossomal DNA was used to infer the relative abundance of bacteria. Significant differences between soil planted with transgenic and non-transformed plants were found for a few bacterial groups. Untransformed alfalfa had higher numbers of flexibacter and unknown bacteria, while transgenic alfalfa had higher diversity of gram-positive bacteria with high G+C DNA content, nitrospira, and fibrobacterium/acidobacterium. This finding is in line with the fact that the rhizosphere soil associated with transgenic alfalfa roots had bacteria with a higher array of substrate utilisation and greater functional diversity. Furthermore, Tesfaye et al. (2003) observed that several mineral nutrients like P, K, Mn, Cu, and Zn were more highly available in the rhizosphere soil of transformed plants, showing that these plants have a great potential to mobilise soil nutrients.

These specific effects of transgenic plants on soil microbiota are not surprising if we consider that common agricultural practices such as crop rotation and use of fertilisers are anthropogenic activities that also cause significant changes in microbial biomass and community structure (O'Donnell et al., 2001). Although these changes are well known, knowledge of their relationship to soil processes is scarce (O'Donnell et al., 2001) and further studies on rhizosphere ecology with other transgenic plants should contribute to a better understanding of this subject.

Acknowledgements: The financial support provided by FAPESP, through a postdoctoral fellowship to EDM (04/04821-0), and a grant to MM (04/05131-7), is gratefully acknowledged.

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