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Aluminum Tolerance in Wheat (*Triticum aestivum* L.)

II. Aluminum-Stimulated Excretion of Malic Acid from Root Apices

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We investigated the role of organic acids in conferring Al tolerance in near-isogenic wheat (*Triticum aestivum* L.) lines differing in Al tolerance at the Al tolerance locus (*Alt1*). Addition of Al to nutrient solutions stimulated excretion of malic and succinic acids from roots of wheat seedlings, and Al-tolerant genotypes excreted 5- to 10-fold more malic acid than Al-sensitive genotypes. Malic acid excretion was detectable after 15 min of exposure to 200 μM Al, and the amount excreted increased linearly over 24 h. The amount of malic acid excreted was dependent on the external Al concentration, and excretion was stimulated by as little as 10 μM Al. Malic acid added to nutrient solutions was able to protect Al-sensitive seedlings from normally phytotoxic Al concentrations. Root apices (terminal 3–5 mm of root) were the primary source of the malic acid excreted. Root apices of Al-tolerant and Al-sensitive seedlings contained similar amounts of malic acid before and after a 2-h exposure to 200 μM Al. During this treatment, Al-tolerant seedlings excreted about four times the total amount of malic acid initially present within root apices, indicating that continual synthesis of malic acid was occurring. Malic acid excretion was specifically stimulated by Al, and neither La, Fe, nor the absence of Pi was able to elicit this response. There was a consistent correlation of Al tolerance with high rates of malic acid excretion stimulated by Al in a population of seedlings segregating for Al tolerance. These data are consistent with the hypothesis that the *Alt1* locus in wheat encodes an Al tolerance mechanism based on Al-stimulated excretion of malic acid.

The *Alt1* locus in wheat (*Triticum aestivum* L.) encodes an Al tolerance mechanism that is consistent with exclusion of Al from root apices (Delhaize et al., 1993). Exclusion could be achieved by several mechanisms such as immobilization in the cell wall, selective permeability at the plasmalemma, exudation of chelating ligands, and active efflux of Al^{3+} (Taylor, 1991). Excretion of organic acids that chelate and detoxify Al in the rhizosphere has been implicated in the Al tolerance mechanism of some species (Miyasaka et al., 1991), but there is no direct evidence suggesting that this mechanism operates in Al-tolerant genotypes of wheat.

Release of citric acid from the roots of snapbeans (*Phaseolus vulgaris* L.) is stimulated by Al, and an Al-tolerant genotype was shown to excrete 10 times more citric acid than an Al-sensitive genotype (Miyasaka et al., 1991). Cell cultures of carrot (*Daucus carota* L.) and tobacco (*Nicotiana tabacum* L.) selected for Al tolerance were also shown to possess enhanced ability to excrete citric acid in response to Al treatment (Ojima et al., 1984, 1989; Ojima and Ohira, 1988; Koyama et al., 1990). However, these studies were inconclusive in determining whether organic acid release was triggered by Al or by the onset of P deficiency resulting from the Al treatments. It is well documented that several species excrete organic acids from their roots in response to P deficiency (Gardner et al., 1983; Lipton et al., 1987; Dinkelaker et al., 1989). In their work with snapbeans, Miyasaka et al. (1991) acknowledged that formation of Al-phosphate precipitates could have caused a P deficiency that in turn may have triggered citric acid excretion. Similarly, in subsequent studies on the Al-tolerant tobacco cultures, Ojima et al. (1989) concluded that in addition to the effects of detoxification of Al by organic acids, the improved growth of the cell cultures during Al treatment was due to their ability to access Pi from Al-phosphate precipitates. Therefore, in cases where organic acid release is apparently triggered by Al, the possible involvement of P deficiency in the mechanism must also be considered.

Organic acids have been detected in root exudates of wheat grown under aseptic conditions (Christiansen-Weniger et al., 1992). Carazinho, an Al-tolerant cultivar, was found to excrete about 5-fold more malic and succinic acids than Bolivar, an Al-sensitive cultivar. Because Al was not added to the nutrient solution in these experiments, it appears that excretion was independent of Al stimulation. Christiansen-Weniger et al. (1992) suggested that the Al tolerance of Carazinho was due to its ability to excrete organic acids at a greater rate than Al-sensitive genotypes. It is possible, however, that the differences found in amounts of organic acid excreted reflect cultivar differences that are unrelated to Al tolerance. Carazinho is the source of Al tolerance in the Al-tolerant lines of the near-isogenic wheat lines used in our previous experiments (Delhaize et al., 1991, 1993). In these lines Al tolerance segregates at a single locus called *Alt1*, and in this paper we investigate the possibility that differences in amounts of organic acids excreted are responsible for differences in Al tolerance observed in these lines.

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Abbreviation: *Alt1*, aluminum tolerance locus.

MATERIALS AND METHODS

Plant Material and Seedling Growth

The near-isogenic wheat (*Triticum aestivum* L.) lines differing in Al tolerance at the *Alt1* locus are described in a companion paper (Delhaize et al., 1993). Unless specified, the ES3 (Al-sensitive) and ET3 (Al-tolerant) lines were used in the experiments. The lines are derived from a cross between the Al-tolerant cultivar Carazinho and the Al-sensitive cultivar Egret with the resulting progeny backcrossed three times to Egret or derivatives of Egret (Fisher and Scott, 1987). These lines show a 5- to 10-fold difference in Al tolerance, and Al tolerance segregates as a single locus (Delhaize et al., 1991, 1993).

Seedlings were grown in sterile culture to prevent microbial degradation of compounds excreted by the seedlings. The basal nutrient solution was the same composition as previously described (Delhaize et al., 1993) except that the pH was first adjusted to 4.3. The basal nutrient solution was autoclaved prior to addition of filter-sterilized Al as 0.1 M $AlK(SO_4)_2$. After the addition of Al, the pH of the nutrient solution was readjusted under sterile conditions to 4.1. Seed was surface sterilized using NaOCl.

In a typical experiment about 40 seeds were soaked for 20 min in 20 mL of 5 g/L NaOCl that contained about 100 μL of 10% (w/v) SDS. After sterilization the seed was washed eight times with 20-mL portions of sterile water, then seed was added to 125-mL flasks that contained 40 mL of nutrient solution. Typically five seeds were added per flask, but in cases where the amount of organic acid excreted needed to be increased to be above-detection limits, such as in short-term experiments, 10 or 20 seeds were added per flask. The flasks were incubated at 23°C on a rotary shaker (125 rpm) for 4 or 6 d before treatments were imposed. The solution was sufficiently aerated by the shaking and the seedlings immersed in the solution grew vigorously. Prior to treatment, the nutrient solution was decanted from the flasks, the seedlings were rinsed once with 50 mL of basal nutrient solution and rinsed once with 50 mL of the treatment solution, and then the appropriate amount of the treatment solution was added. This procedure removed substances that may have accumulated in the flasks over the 4- or 6-d initial growth of seedlings.

In some experiments organic acids were added to phytotoxic Al solutions to assess their ability to protect against Al stress. Stock solutions of organic acids adjusted to pH 4.1 and filter sterilized were added to nutrient solution that contained 50 μM Al. Seeds were germinated and grown in the solutions as described above, and root elongation was measured after 5 d of growth.

Organic Acid Assays

Malic and citric acids in nutrient solutions and roots were assayed using modifications of previously described enzymic methods (Dagley, 1974; Gutmann and Wahlefeld, 1974). For malic acid, 1.35 mL of sample was incubated with 1.5 mL of buffer (0.5 M Gly, 0.4 M hydrazine, pH 9.0) and 0.1 mL of 40 mM NAD. The reaction mixture was preincubated for 30 to 60 min to obtain a stable A_{340} reading before the addition of 5 μL of malate dehydrogenase (5 mg/mL, Boehringer-Mannheim). The increase in A_{340} due to production of NADH was monitored on a chart recorder and is directly proportional to the amount of malic acid in the sample. Al at 200 μM preincubated with 2 to 80 μM malic acid (the range of malic acid concentrations assayed) did not interfere with the assay. For citric acid, 2.52 mL of sample was incubated with 0.24 mL of buffer (1 M Tris-Cl, pH 7.8), 30 μL of 10 mM NADH, and 10 μL of a lactate dehydrogenase/malate dehydrogenase mixture (0.5 mg/mL for each). After a stable reading was obtained, 10 μL of citrate lyase (Boehringer-Mannheim, dissolved in water to 190 mg/mL) was added and the decline in A_{340} due to oxidation of NADH was monitored on a chart recorder. The decrease in NADH concentration is directly proportional to the amount of citric acid in the sample. Oxalic, succinic, formic, and D-isocitric acids were assayed using enzyme kits following the procedures provided by the supplier (Boehringer-Mannheim). Fumaric acid was assayed by addition of 10 μL of fumarase (2 mg/mL, Boehringer-Mannheim) to the malic acid incubation mixture at the completion of the malic acid assays. Fumarase converts fumaric acid to malic acid, which is in turn estimated by measuring NADH production as described above. Al in the samples did not interfere with the assay for citric or succinic acids because the amount of organic acid detected after addition of known amounts of organic acid to the mixture was similar regardless of the presence of Al.

To analyze malic acid in root apices, 30 root apices (4 mm long) were collected and extracted immediately with a mortar and pestle in 1 mL of ice-cold 0.6 N perchloric acid. The extract was centrifuged at 15,000*g* for 5 min, and 0.9 mL of supernatant solution was collected and neutralized with 80 μL of K_2CO_3 (69 g/100 mL). The neutralized solution was centrifuged at 15,000*g* for 5 min, and 0.5 mL of the supernatant solution was assayed for malic acid as described above after adding 0.85 mL of water to the assay mixture to make up the volume.

Divided Root Chamber

To determine whether the release of malic acid from roots was localized to a specific region of the root, a divided chamber technique was used. The procedure was a modification of that described by Ryan et al. (1993a). Seeds from the Al-tolerant genotype were germinated under sterile conditions, and sterile apparatus and procedures were used for all subsequent operations. Six-day-old seedlings were placed in large Petri dishes (14 cm diameter) holding approximately 80 mL of nutrient solution. Perspex rings (16 mm diameter × 10 mm high) were smeared with nontoxic vacuum grease (Dow Corning) to enable them to form a water-tight seal with the bottom of the Petri dish and around the root. Each ring had two semicircular notches filed into opposite sides of an edge so that they could harmlessly straddle and isolate a section of root from the solution bathing the remainder of the seedling. A single ring was used to isolate either a 16-mm section of mature root or two root apices each 3 to 5 mm long from separate seedlings. The solution in the Petri dish, both inside and outside the ring, was then removed by suction and replaced with the treatment solution. In one experiment,

control nutrient solution was added to the Perspex ring surrounding root apices while solution containing Al was added to the remainder of the Petri dish. Malic acid released from those sections of root enclosed by the Perspex rings was determined after a 6-h incubation by collecting the solutions and assaying them for malic acid.

Hematoxylin Competition Assay

The ability of various organic acids to compete with hematoxylin for Al in solution was assessed. Hematoxylin solution was prepared the day before the experiment by adding 0.2 g of hematoxylin powder and 0.02 mg of KIO_3 to 100 mL of water and mixing the solution overnight. Basal nutrient solution (3 mL, pH 4.2) that contained 50 μM Al was incubated with 120 μL of hematoxylin solution (final concentration about 250 μM) and 10 μL of Na-acetate buffer (1 M, pH 4.2). Stock solutions of organic acids at pH 4.2 were added to the mixture to yield final concentrations ranging from 25 to 400 μM and a total final volume for the mixture of 3.25 mL. The mixture was incubated for 1 h and the formation of hematoxylin:Al complex was assessed by measuring the A_{540}. The hematoxylin:Al complex at pH 4.2 has an absorption maximum at 540 nm, and the reaction is near completion after 1 h.

RESULTS

Excretion of Organic Acids by Wheat Seedlings

Al in nutrient solution stimulated the excretion of malic and succinic acids from intact seedlings grown under sterile conditions (Table I). Al-tolerant genotypes excreted about 10-fold more malic acid and about 3- to 5-fold more succinic acid than Al-sensitive seedlings over 24 h. Citric acid was detected in the nutrient solutions, but its excretion was not stimulated by Al in either genotype. Fumaric, oxalic, formic, and D-isocitric acids were below the limits of detection for both Al-treated and untreated seedlings. Excised roots excreted copious amounts of malic acid, even in seedlings not exposed to Al, indicating that malic acid was probably diffusing out from the cut surface of the roots (data not shown). For this reason we used intact seedlings for all experiments.

Kinetics of Malic Acid Excretion

The cumulative amount of malic acid excreted by wheat seedlings in response to 50 μM Al was linear over 24 h for both the Al-tolerant and Al-sensitive genotypes (Fig. 1). The Al-tolerant genotype excreted 5- to 10-fold more malic acid than the Al-sensitive genotype at each time point. Amounts of malic acid excreted over 24 h increased with the Al concentration in the nutrient solution up to 100 μM Al for both Al-tolerant and Al-sensitive seedlings (Fig. 2). Malic acid excretion was stimulated by addition of as little as 10 μM Al, and the relative difference in excretion by the two genotypes was maintained at each Al concentration. Because maximal excretion rates were observed with high Al concentrations, 200 μM Al was used in short-term experiments to enhance

Table I. *Excretion of organic acids by Al-tolerant and Al-sensitive wheat seedlings exposed to 50 μM Al for 24 h*

Organic Acid	Organic Acid[a]			
	Sensitive −Al	Sensitive +Al	Tolerant −Al	Tolerant +Al
	nmol seedling^{-1} h^{-1}			
Malic	0.08 ± 0.08	0.33 ± 0.00	<0.08	3.57 ± 0.08
Succinic	0.08 ± 0.08	0.08 ± 0.08	0.08 ± 0.08	0.58 ± 0.08
Citric	0.17 ± 0.08	0.08 ± 0.08	0.08 ± 0.00	0.17 ± 0.00

[a] Mean ± SE from triplicate flasks. Each flask contained five seedlings in 20 mL of nutrient solution.

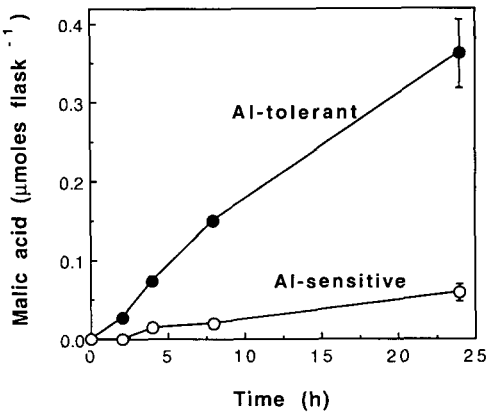

Figure 1. Excretion of malic acid over time by Al-tolerant (●) and Al-sensitive (○) seedlings incubated in nutrient solution that contained 50 μM Al. Five 6-d-old seedlings were incubated in 20 mL of nutrient solution and samples were withdrawn at various times for malic acid assay. The error bars denote the range of the mean from duplicate flasks and are not shown where the error did not exceed the size of the symbol. Malic acid was not detectable (less than 1 nmol seedling^{-1}) over 24 h for either genotype in nutrient solutions that did not contain Al.

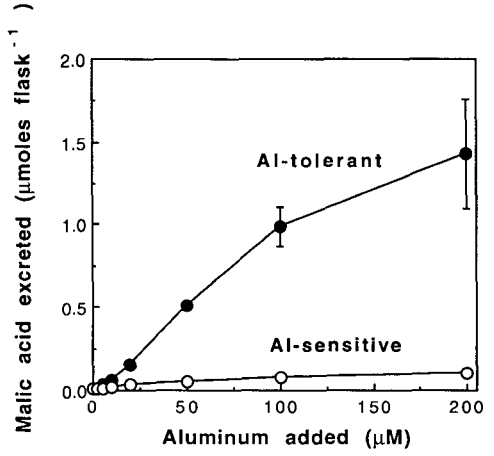

Figure 2. Effect of Al concentration on excretion of malic acid by Al-tolerant (●) and Al-sensitive (○) seedlings. Five 6-d-old seedlings were incubated for 24 h in 20 mL of nutrient solution that contained various concentrations of Al. The error bars denote the range of the mean from duplicate flasks and are not shown where the error did not exceed the size of the symbol.

the sensitivity of the assay. In addition, to increase the sensitivity of the experiment, 10 seedlings were incubated in 10 mL of solution. Malic acid excretion was detected above the untreated controls after 15 min of incubation of tolerant seedlings with 200 μM Al (Fig. 3). The excretion rate was constant over 1 h, and tolerant seedlings excreted more than sensitive seedlings at each time point observed over the 24-h time course.

Location of Malic Acid Excretion from Roots

The relative release of malic acid from the root apex compared with basal root tissues was determined in Al-tolerant seedlings by isolating intact sections of roots with Perspex rings and exposing them to solutions that contained Al. Root apices excreted large amounts of malic acid during Al treatment, whereas negligible amounts were excreted by more mature sections of the root (Table II). Approximately 35-fold more malic acid was released from the root apex than from the mature root per unit area of root. This is an underestimate if the length of root apex releasing the malic acid is significantly smaller than the 3 to 5 mm isolated by the Perspex ring. Application of Al to the mature section of roots only did not stimulate malic acid excretion from root apices.

Because the root apices were the primary source of malic acid excreted, we analyzed their malic acid content. Four-day-old seedlings were used because at that age there were three primary roots per seedling and no laterals. Al-sensitive seedlings had marginally more malic acid in their apices compared with Al-tolerant seedlings when grown in nutrient solution without Al (Table III). After 2 h of incubation in 200 μM Al, malic acid concentrations declined slightly in Al-sensitive root apices and remained constant in Al-tolerant root apices. The amount of malic acid excreted by Al-tolerant seedlings over the 2 h was about four times the total amount present in the root apices before addition of Al. This indicates that new synthesis of malic acid must have occurred in Al-tolerant apices during exposure to Al and that the malic acid pools within these apices were continually replenished.

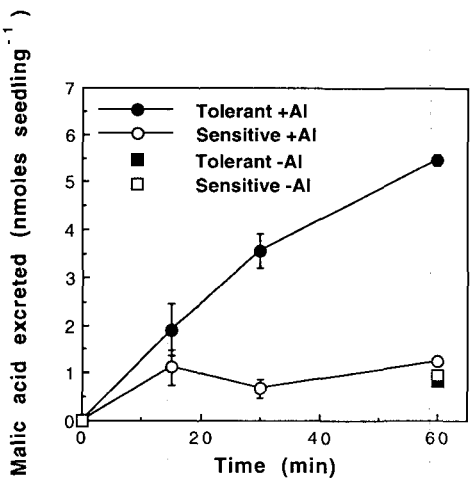

Figure 3. Excretion of malic acid over time by Al-tolerant (●) and Al-sensitive (○) seedlings exposed to nutrient solution that contained 200 μM Al. Excretion of malic acid from seedlings exposed to nutrient solutions without Al was assayed at 60 min (Al-tolerant, ■; Al-sensitive, □). Ten 6-d-old seedlings were incubated in 10 mL of nutrient solution and the solution was collected from the flasks for malic acid assay. The error bars denote the SE values of the mean from triplicate flasks and are not shown where the error did not exceed the size of the symbols.

Table II. *Release of malic acid from different sections of Al-tolerant wheat roots exposed to Al*

Perspex rings were used to form chambers around root sections and to isolate either a 16-mm length of mature root tissue or two root apices.

Treatments	Malic Acid Released[a]	Malic Acid Flux[b]
	nmol chamber^{-1} h^{-1}	*nmol m^{-2} s^{-1}*
Mature root −Al	0.05 ± 0.01	0.4 ± 0.1
Mature root +Al	0.13 ± 0.02	1.1 ± 0.2
Root apices −Al	0.10 ± 0.02	1.5 ± 0.2
Root apices +Al	2.44 ± 0.30	36.6 ± 4.4
Root apices −Al while root base +Al	0.08 ± 0.03 (4)	1.3 ± 0.4 (4)

[a] The release of malic acid from either the root apex or basal roots were determined by analyzing the contents of the chamber after a 6-h incubation in the presence or absence of 200 μM Al. Data show the mean and SE (n = 6, unless indicated otherwise in parentheses). [b] A flux of malic acid release was calculated on the basis of the area of root enclosed within the chamber.

Al Specificity of Response

The specificity of Al-stimulated release of malic acid was tested. It was possible that the onset of P deficiency caused by addition of Al to nutrient solution was stimulating malic acid excretion. However, it was unlikely that the seedlings were P deficient after 6 d of growth, and, in addition, Pi-free nutrient solution failed to stimulate malic acid excretion, whereas nutrient solution that contained 100 μM Pi with 50 μM Al did stimulate excretion of malic acid (Table IV). This indicates that Al-induced P deficiency was unlikely to be responsible for triggering malic acid release. Two trivalent ions, La^{3+} and Fe^{3+}, added to 50 μM in nutrient solutions also

Table III. *Effect of Al on amounts of malic acid excreted and the malic acid content of root apices for Al-tolerant and Al-sensitive wheat seedlings*

Treatment[a]	Malic Acid Content	Malic Acid Excreted
	nmol apex^{-1}	*nmol apex^{-1}*
Sensitive −Al	1.52 ± 0.16	0.48 ± 0.10
Sensitive +Al	1.20 ± 0.08	0.70 ± 0.06
Tolerant −Al	1.24 ± 0.07	0.20 ± 0.08
Tolerant +Al	1.32 ± 0.11	5.24 ± 0.48

[a] Ten 4-d-old seedlings per flask were exposed to control nutrient solution (−Al) or nutrient solutions that contained 200 μM Al (+Al) for 2 h. The mean ± SE of five replicate flasks is shown. Each sample consisted of 10 seedlings combined for the analysis of root apices while the solution was analyzed for malic acid excreted.

Table IV. *Specific stimulation of malic acid excretion from wheat roots by Al*

Five 6-d-old seedlings of the Al-tolerant genotype were incubated in the nutrient solutions for 24 h, then malic acid in the nutrient solution was assayed.

Treatment[a]	Malic Acid Excreted[b]
	nmol seedling^{-1} h^{-1}
0 Al; 2 μM Pi	<0.08
50 μM Al; 0 Pi	3.57 ± 0.08
0 Al; 0 Pi	<0.08
50 μM Al; 100 μM Pi	2.57 ± 0.25
50 μM $LaCl_3$	<0.08
50 μM $FeCl_3$	<0.08

[a] The basal nutrient solution described in the "Materials and Methods" is denoted by the 0 Al; 2 μM Pi treatment. Other treatments indicate the changes made to the basal nutrient solution that is described in "Materials and Methods." The pH of all solutions was adjusted to 4.1. [b] The means ± SE of triplicate flasks are shown.

failed to stimulate malic acid excretion above control levels over 24 h (Table IV), indicating a degree of specificity of the response to Al. La shows some similarities to Al in being a potent inhibitor of root growth (Fig. 4). However, La inhibited root elongation of the wheat seedlings regardless of their genotype with respect to Al tolerance, consistent with its inability to elicit malic acid excretion in the Al-tolerant seedlings.

Inactivation and Chelation of Al by Malic Acid

If malic acid has a role in Al tolerance, it should be able to chelate Al in solution and render it nonphytotoxic. In addition, it should be able to explain the difference in staining pattern observed at root apices of wheat genotypes of different Al tolerance after exposure of roots to Al and treatment with hematoxylin. In wheat, the intensity of hematoxylin staining of root apices is inversely related to the Al tolerance of the genotypes (Polle et al., 1978). Malic acid ameliorated Al toxicity; root growth of Al-sensitive seedlings was almost restored to control levels by adding 400 μM malic acid to a nutrient solution containing 50 μM Al (Fig. 5)

In vitro competition experiments were used to determine the affinity of Al binding by malic, succinic, and citric acids relative to hematoxylin. The degree of Al chelation was determined by the ability of the organic acids to "out-compete" hematoxylin for Al binding. Citric acid clearly showed the most avid binding to Al, and malic acid, although not as strong a chelator as citric acid, was able to out-compete hematoxylin at higher concentrations (Fig. 6). Succinic acid, by contrast, was a poor chelator of Al and even at 400 μM did not affect Al binding by hematoxylin.

Correlation of Malic Acid Excretion with the *Alt1* Genotype

Malic acid excretion was greater in Carazinho, the Al-tolerant parental cultivar of the near-isogenic lines, than in Egret, the Al-sensitive parental cultivar. When five seedlings were exposed to 50 μM Al for 24 h, Carazinho produced 4.1 nmol malic acid seedling^{-1} h^{-1} while Egret produced 0.7 nmol malic acid seedling^{-1} h^{-1}. A similar difference in malic acid excretion was observed between the near-isogenic lines ET8 and ES8 (five seedlings exposed to 50 μM Al for 24 h: ET8, 3.5 ± 0.1 nmol malic acid seedling^{-1} h^{-1}; ES8, 0.2 ± 0.1 nmol malic acid seedling^{-1} h^{-1} [mean ± SE, n = 3]).

The progeny of a cross between ES8 and ET8 were assayed for malic acid excretion to determine whether Al tolerance was consistently associated with high rates of malic acid excretion. It would have been difficult to determine both the Al tolerance and malic acid excretion of individual F_2 seedlings, so bulked F_3 seed from individual F_2 plants that were self-fertilized was used for these experiments. Progeny from F_2 plants that were either homozygous *Alt1* or homozygous

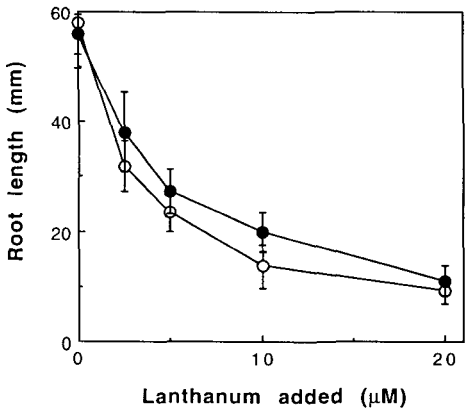

Figure 4. Effect of La on root elongation of Al-tolerant (●) and Al-sensitive (○) seedlings. Seedlings were germinated and grown for 5 d in aerated nutrient solution that contained various concentrations of La. Nutrient solutions were changed daily, and seedlings were grown under nonsterile conditions. The error bars denote the SE values of the mean of five seedlings.

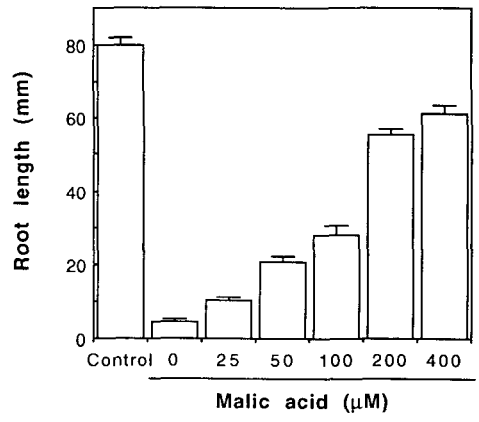

Figure 5. Amelioration of Al toxicity by malic acid. Al-sensitive seedlings were grown in nutrient solution that contained 50 μM Al and various concentrations of added malic acid. The error bars denote the SE values of the mean root length of 15 seedlings after 5 d of growth. The control consisted of seedlings grown without Al or malic acid.

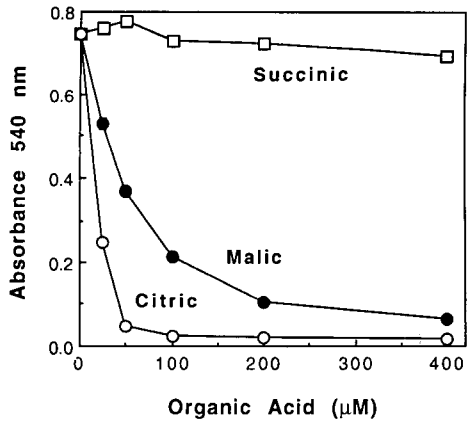

Figure 6. Chelation of Al by organic acids as determined from competition assays with hematoxylin. Citric (○), malic (●), or succinic (□) acid was incubated with nutrient solution that contained 50 μM Al, 3.2 mM Na-acetate buffer (pH 4.2), and 250 μM hematoxylin. The mixture was incubated for 1 h and the formation of Al:hematoxylin complex was determined by the A_{540}. A decrease in A in the presence of organic acid indicates that the organic acid is chelating a proportion of the Al, making it unavailable for chelation by hematoxylin. The values are the means of triplicate assays and the SE values did not exceed the size of the symbols.

alt1 would have all been Al-tolerant or Al-sensitive, reflecting the genotype of the parent. Progeny from F_2 plants heterozygous for *Alt1* were segregating approximately 3 to 1 for *Alt1*, reflecting the heterozygous genotype of the F_2 parent. There was a consistent correlation of high malic acid excretion rates with Al tolerance in all F_3 lines tested (Fig. 7)

DISCUSSION

Al stimulated the excretion of malic and succinic acids from root apices of wheat, and malic acid excretion was 5- to 10-fold greater in Al-tolerant seedlings than in Al-sensitive seedlings. We propose that the release of malic acid from roots exposed to Al is the Al tolerance mechanism encoded by the *Alt1* locus for the following reasons: (a) there was a consistent correlation of the *Alt1* locus with malic acid excretion in a population of seedlings segregating for Al tolerance; (b) Al stimulated malic acid excretion within 15 min, consistent with observations that Al tolerance is apparent after short exposures to Al; (c) malic acid excretion was localized at root apices, the primary site of Al toxicity; (d) malic acid excretion may provide an explanation for the different hematoxylin staining observed at root apices of wheat seedlings with different Al tolerance; and (e) malic acid added to nutrient solution was shown to ameliorate Al toxicity. Preliminary experiments with three other Al-tolerant wheat cultivars, Atlas, Warigal, and Toropi, showed that they excreted amounts of malic acid approximately equivalent to the ET lines in response to Al exposure (data not shown). This suggests that excretion of malic acid may be a widespread Al tolerance mechanism in wheat.

Although citric acid is a stronger chelator of Al than malic acid and can better protect wheat seedlings from Al (Ownby and Popham, 1989), only relatively small quantities of citric acid were released by either genotype in response to Al. Succinic acid is a relatively poor chelator of Al and, although its excretion was stimulated by Al, it is unlikely that it contributes to the Al tolerance of seedlings. Malic acid was the most abundant organic acid excreted by root apices, and its binding affinity for Al, as determined by the hematoxylin competition assays (Fig. 6), suggests that it is the main contributor to the Al tolerance of the lines used in our study. Studies have shown that malic acid ameliorates the toxic effects of Al in vitro on membranes (Suhayda and Haug, 1986) and on calmodulin (Haug and Caldwell, 1985). Amelioration of Al toxicity was observed at high malic acid concentrations relative to the Al concentration, and even with an 8-fold greater concentration of malic acid than Al, seedlings were not completely protected (Fig. 5). However, this ratio of malic acid to Al in the bathing solution required for amelioration may be misleading. A concentration gradient of both solutes will result when root apices excrete malic acid into a solution containing Al, and the ratio at the membrane surface will be the more important factor. Because the seedlings were grown in a small volume of solution for 5 d without changes, it is possible that uptake or absorption of the added malic acid by seedlings lowered the concentration during much of that period. Furthermore, malic acid was excreted almost exclusively from the root apex, and the concentration at the surface of the cells in that region will be greater than in the bulk solution.

Given a rate of malic acid release of about 40 nmol m^{-2} s^{-1} (Table II) and using a simple planar diffusion model to approximate the system, the estimated malic acid concentration at the root surface will be 1 to 10 μM above the concen-

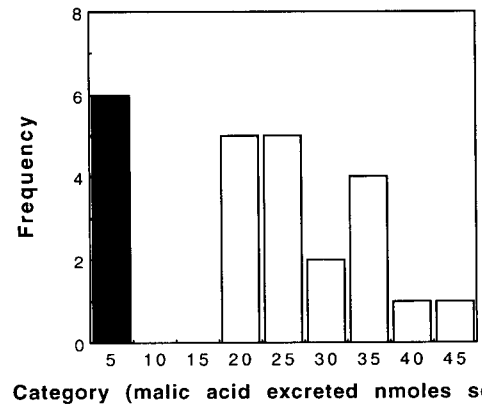

Figure 7. Excretion of malic acid after exposure to 200 μM Al for 6 h of F_3 seed collected from 24 selfed F_2 individuals from a cross between the near-isogenic lines ES8 and ET8. The Al tolerance of the F_3 seedlings (20 seedlings per flask) was determined after measuring malic acid excretion by growing the seedlings for a additional 3 d in 20 μM Al and assessing root growth. The data for flasks that contained seed populations segregating for Al tolerance are expressed as malic acid excreted per Al-tolerant seedling. Malic acid is expressed to the nearest nmol excreted per seedling and is denoted as categories 5 (0–5 nmol seedling^{-1}), 10 (6–10 nmol seedling^{-1}), etc. The homozygous batches of Al-sensitive seedlings are denoted by solid bars, and homozygous or heterozygous Al-tolerant batches are denoted by the empty bars.

tration of the bulk solution (assuming a diffusion coefficient for malic acid of 0.5×10^{-9} m^2 s^{-2} and an unstirred layer of thickness 10–100 μm). Although this concentration would appear from Figure 5 to be too low to provide sufficient protection from Al^{3+}, many uncertainties are involved. These include (a) the actual portion of area within the terminal 5 mm of root responsible for malic release, (b) the diffusion coefficients for malic acid and Al^{3+} in cell walls, (c) the effect of membrane surface potential on Al^{3+} and malic acid activities, and (d) the effect of mucilage on these parameters, particularly its effect on the thickness of the unstirred layer and on diffusion coefficients of the solutes involved. Wheat roots have 0.5 to 1.0 μL of mucilage surrounding each apex (Puthota et al., 1991), which will substantially increase the unstirred layer.

Although organic acids excreted by plants grown in soil are likely to be rapidly degraded by microorganisms, continual excretion from apices could provide sufficient protection from Al toxicity. It is not necessary that the Al in bulk soil be chelated for a plant to be Al tolerant, and the energetic cost of continual excretion of malic acid is reduced by protecting only those regions of the root susceptible to Al toxicity (Taylor, 1991; Ryan et al., 1993a). Other benefits to the plant of high rates of organic acid excretion have been suggested and include (a) enhanced colonization of the rhizosphere by *Azospirillium* species with resulting greater nitrogen fixation around roots (Christiansen-Weniger et al., 1992) and (b) solubilization of soil phosphate compounds, resulting in mobilization of phosphate to roots of plants excreting the organic acid and to plants growing nearby (Gardner and Boundy, 1983; Gardner et al., 1983).

Al stimulated the excretion of malic acid from root apices within 15 min of exposure, and the rate of excretion remained constant over 24 h. The rapidity of the malic acid response to Al is consistent with observations that Ca fluxes around root apices (Huang et al., 1992a) and hematoxylin staining of root apices (Delhaize et al., 1993) can both differentiate wheat genotypes of different Al tolerance within 10 min of Al exposure. Al is known to be a potent inhibitor of Ca uptake by roots (Johnson and Jackson, 1964; Clarkson and Sanderson, 1971; Huang et al., 1992b) and recently a link was established between Al tolerance in certain cultivars of wheat and their ability to maintain Ca influx at the root apex during Al stress (Huang et al., 1992a, 1992b). However, in three separate pairs of near-isogenic wheat lines this association was found to be less convincing, suggestive of an indirect relationship only (Ryan and Kochian, 1993; Ryan et al., 1993b). An explanation for those results may lie in the release of malic acid from root apices, by maintaining a lower concentration of Al in the rhizosphere of Al-tolerant plants. This is consistent with the conclusions of Kinraide et al. (1992), who showed a correlation between relative root growth and the predicted Al^{3+} activity at the membrane surface. This is also likely to result in an indirect correlation between Al tolerance and the maintenance of Ca uptake during Al stress, as discussed above.

Excretion of malic acid was specifically stimulated by Al, and two other trivalent cations, La^{3+} and Fe^{3+}, were unable to trigger the response. Several plant species respond to P deficiency by excreting organic acids from their roots, and it has been difficult to dissociate the effect of Al stress from the effect of Al-induced P deficiency on organic acid excretion (Ojima et al., 1984, 1989; Ojima and Ohira, 1988; Miyasaka et al., 1991). In our experiments we have shown that low external Pi conditions did not stimulate malic acid excretion over 24 h, and high external Pi concentrations did not prevent Al from stimulating malic acid excretion (Table IV). If low external Pi concentrations were controlling the excretion of malic acid, we would have expected malic acid to be excreted by seedlings in the absence of Pi and excretion to be suppressed with a high external Pi concentration. It is possible that Al affects P metabolism in root cells, resulting in a localized region of P deficiency and subsequent release of malic acid. Pfeffer et al. (1986) have shown by NMR that Al affects P metabolism in roots after 20 h of exposure to Al. However, for this mechanism to be responsible for malic acid excretion it would need to be rapid (within 15 min) and affect Al-tolerant seedlings only, even though it is these seedlings that maintain greater growth and vigor during Al treatments.

Christiansen-Weniger et al. (1992) reported that Carazinho excreted more malic and succinic acids than a sensitive wheat cultivar when grown without Al exposure. By contrast, we did not observe differential excretion of malic acid in the tolerant and sensitive lines when Al was absent from the nutrient solutions. The experiments of Christiansen-Weniger et al. (1992) were done over several weeks with mature seedlings, and it is possible that a difference in organic acid excretion was undetectable over the relatively short times (24 h or less) used in our experiments. Alternatively, small amounts of Al contaminating the nutrient solution may have been sufficient to trigger a response in their experiments.

The ability to release large amounts of malic acid appears to be independent of the endogenous malic acid content of root apices, since similar amounts were present in both Al-tolerant and Al-sensitive seedlings (Table III). These results are consistent with the results of Foy et al. (1990), who found that internal concentrations of malic acid in roots and shoots of a range of wheat cultivars were not correlated with Al tolerance. Exposure of seedlings to Al caused little change in the concentration of malic acid in root apices of either genotype, even though Al-tolerant seedlings excreted sufficient malic acid to turn over the resting malic acid pool at least four times in 2 h. These results suggest that transport of malic acid out of root apices, and not the ability to accumulate malic acid within apices, is the limiting factor. If synthesis were limiting the amount of malic acid excreted by Al-sensitive apices, we would have expected malic acid concentrations to be depleted rapidly in Al-sensitive apices in response to Al and not be replenished unless there is a threshold level of malic acid required to activate its efflux. Alternatively, it is possible that only a small proportion of the cells in root apices are responsible for malic acid excretion, and a decline in malic acid concentrations within these cells could be masked by the remainder of the cells, resulting in no measurable difference between genotypes. Further work will be aimed at determining the biochemical basis for the difference in malic acid excretion between the different genotypes and at determining how Al triggers malic acid excretion from root apices.

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