

The role of phosphorus in aluminium-induced citrate and malate exudation from rape (*Brassica napus*)

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Received 3 July 2003; revised 22 September 2003

Exudation of organic anions is believed to be a common tolerance mechanism for both aluminium toxicity and phosphorus deficiency. Nevertheless, which of these stresses that actually elicit the exudation of organic anions from rape (*Brassica napus* L) remains unknown, and the combined effects of Al toxicity and P deficiency on rape have not been reported before. Therefore, in the current study, *Brassica napus* var. Natane nourin plants grown with or without 0.25 mM P were exposed to 0 or 50 μ M AlCl₃ and several parameters related to the exudation of organic anions from the roots were investigated. Eight days of P deficiency resulted in a significant growth reduction, but P deficiency alone did not induce exudation of organic anions. In contrast, Al strongly induced organic acid exudation, while simultaneously inhibiting root growth. Increased in-vitro activity of citrate synthase (CS, EC 4.1.3.7), malate dehydrogenase

(MDH, EC 1.1.1.37) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), together with reduced root respiration, indicated that the Al-induced accumulation and subsequent exudation of citrate and malate were associated with both increased biosynthesis and reduced metabolism of citric and malic acid. Phosphorus-sufficient plants showed more pronounced aluminium-induced accumulation and exudation of organic anions than P-deficient plants. A divided root chamber experiment showed the necessity of direct contact between Al and roots to elicit exudation of organic anions. Prolonged exposure (10 days) to Al resulted in a decrease in the net exudation of citrate and malate, and the rate of decrease was much more rapid in P-deficient plants than in P-sufficient plants. It is concluded that P nutrition affects the level of Al-induced synthesis and exudation of organic anions. However, the mechanism needs further investigation.

Introduction

Aluminium toxicity limits crop production on the acidic soils that comprise over 40% of the world's potentially arable lands (von Uexkuell and Mutert 1995). On such soils, P deficiency is commonly reported together with Al toxicity (Clark 1977). When the soil pH drops below 5, the rhizotoxic Al species are solubilized into the soil solution to a level that inhibits root growth and functions. Aluminium renders its toxic effects through binding with cell wall, DNA and membrane (Matsumoto 2000). Higher plants are usually kept stationary with roots in the soil and cannot escape stress. Therefore, they have developed specific strategies to overcome the problem (Matsumoto 2002).

Two mechanisms of plant Al tolerance have been proposed: those that exclude Al from the root apex (Al exclusion) and those that allow Al accumulation in the

symplasm (symplasmic tolerance) (Delhaize and Ryan 1995, Kochian 1995). Al exclusion may involve secretion of Al-chelating ligands, binding of Al with the cell wall and mucilage, plant-induced pH barrier in the rhizosphere or root apoplasm, selective permeability of the plasma membrane and Al efflux (Taylor 1991). The symplasmic tolerance mechanism may include chelation of Al in the cytosol, compartmentation in the vacuole, binding of Al to proteins, and enhancement of enzyme activity.

The release of Al-chelating organic anions has recently been given much more attention as organic anions are powerful chelators of Al (Matsumoto 2002). Accordingly, enhanced exudation of citrate, malate and oxalate in response to Al toxicity has been reported in snap bean, maize, *Cassia tora*, wheat and buckwheat (Miyasaka

Abbreviations – CS, citrate synthase; MDH, malate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PG, phenylglyoxal.

et al. 1991, Delhaize et al. 1993, Pellet et al. 1995, Ma et al. 1997a, b). Similarly, rape (Hoffland et al. 1989), white lupin (Gardner et al. 1983) and alfalfa (Lipton et al. 1987) have been reported to exude organic anions under P-deficient conditions. On the contrary, neither P deficiency nor Al toxicity induced exudation of organic anions in some other plants. In response to P deficiency, exudation of malate, oxalate and citrate was not induced in wheat, taro, buckwheat and soybean (Delhaize et al. 1993, Ma et al. 1997a, Ma and Miyasaka 1998, Yang et al. 2000). Similarly, Al failed to induce exudation in oat (Zheng et al. 1998b), Al-sensitive cultivars of wheat (Ryan et al. 1995b) and maize (Pellet et al. 1995). This indicates that plant species, even cultivars of the same species respond differently to Al toxicity and P deficiency.

Two mechanisms might be involved in the elevated exudation of organic anions under Al and P stresses. One mechanism is enhanced biosynthesis of organic acids following the elevated activity of enzymes relevant to organic acid metabolism. Over-expression of the bacterial CS gene in transgenic tobacco and papaya resulted in a significant increase in both synthesis and release of citrate and hence the transgenic plants became Al tolerant (de la Fuente et al. 1997). However, this result was not readily reproducible in tobacco in an effort to improve Al tolerance (Delhaize et al. 2001). Expression of a *Pseudomonas aeruginosa* citrate synthase gene in tobacco was not associated with either enhanced citrate accumulation or efflux. An increase in the activity of CS and PEPC has been reported under Al-toxic conditions (Mugai et al. 2000). Similarly, an increase in the activity of PEPCase in rape (Hoffland et al. 1992) and of CS, MDH and PEPCase in white lupin (Johnson et al. 1994) have been reported in response to P deficiency. Another mechanism is reduced degradation of organic acids. In line with this, Johnson et al. (1994) and Neumann et al. (1999b) reported that citric acid accumulation and release from developing proteoid roots of white lupin was associated with reduced root respiration.

In the present study we investigated the response of rape to P deficiency, Al toxicity and the additive effects of both. The role of P nutrition in Al-induced exudation of citrate and malate was studied. Moreover, the effects of Al on organic acid metabolism in terms of enzymatic activities, root respiration, and the mechanisms and patterns of citrate and malate release from P-sufficient and P-deficient rape plants were investigated. To our knowledge we report for the first time that elevated exudation of citrate and malate from rape is a more specific response to Al toxicity than to P deficiency and that P supply has a significant effect on the response.

Materials and methods

Plant cultivation

Seeds of rape (*Brassica napus* L. var. Natane nourin # 20) were purchased from Watanabe Seed Company (Miyagi, Japan). The seeds were sown on moist river sand and

germinated in a dark chamber (LNC-131; Tabai, Osaka, Japan) at 25°C. After 3 days, the seedlings were transferred to a naturally illuminated phytotron until they achieved the desired size. Six-day-old, uniformly sized seedlings were transferred to a complete nutrient solution modified from Hoffland et al. (1989) for 7 days. The composition of the nutrient solution was; [in mM]: Ca (NO₃)₂ [5], KNO₃ [1.25], MgSO₄ [2], KCl [0.25], KH₂PO₄ [0.25] and [in μM]: Fe (III)-EDTA [20], H₃BO₃ [25], MnSO₄ [1.5], ZnSO₄ [1.5], CuSO₄ [0.5] (NH₄)₆Mo₇O₂₄ [0.025]. The pH of the nutrient solution was 5.3. The nutrient solution was renewed every 3 days. The seedlings were grown in a cultivation chamber (CFH-405; Tomy Co., Tokyo, Japan) at a cycle of 14 h/25°C day and 10 h/20°C night and a light intensity of 40 μmol m⁻² s⁻¹. The relative humidity of the chamber was adjusted to 65%. After 7 days in a complete nutrient solution, the seedlings were transferred to an aerated nutrient solution with (+P) or without (-P) 0.25 mM P in 3.5-l pots. Eight plants were grown per pot. The plants were grown for an additional 8 days in a naturally illuminated phytotron at a day temperature of 25°C and night temperature of 20°C.

Before Al exposure, the plants were transferred to 0.5 mM CaCl₂ (pH 4.5) for 12 h. After 12 h, all plants grown in the same pot were transferred to 1-l pots filled with fresh Ca-solution (pH 4.5) containing 0, 50 or 100 μM AlCl₃. All treatments were replicated at least three times and each set of experiments was repeated at least twice. Statistical differences were determined using *t*-test when needed.

Determination of root elongation

To determine the inhibitory effect of Al on root elongation, seedlings, which were previously cultured in a complete nutrient solution for 7 days, were transferred to nutrient solution with or without 0.25 mM P for another week. The plants were transferred to 0.5 mM CaCl₂ (pH 4.5) containing 0, 50 or 100 μM AlCl₃. The length of the primary root was determined before and after Al treatment.

Shoots and roots P determination

Phosphorus-concentration in the roots and shoots was determined by the vanado-molybdate yellow method of Gericke and Kurmis (1952). Briefly, 200 mg of milled dry matter was ashed in an oven at a temperature of 480°C for 12 h. Ashed plant material was dissolved in 1:3 diluted HNO₃. The P concentration was determined spectrophotometrically at 450 nm.

The response of rape to short-term and prolonged Al treatment

Twenty-one-day-old plants, previously cultured in +P or -P nutrient solution for 8 days were exposed to 0.5 mM CaCl₂ (pH 4.5) containing 0 or 50 μM AlCl₃ for 6 h. The

solution with root exudates was collected for the analysis of organic anions. The time course effect of Al was studied during 15 h of Al exposure and root exudates were collected every 5 h for analysis of the organic anions. The effect of prolonged Al exposure on the amount and pattern of organic anions exudation was further studied during 10 days of an alternative Al treatment. On each day of Al treatment, plants were exposed to 0.5 mM CaCl₂ (pH 4.5) with or without 50 μM AlCl₃ and root exudates were collected 6 h after exposure. The plants were continuously kept in the same Ca solution with or without 50 μM AlCl₃ until 24 h. The plants were then returned to the +P or -P nutrient solution for 24 h. This treatment was repeated for 10 days.

Divided root chamber experiment/split root experiment

To investigate the involvement of long-distance signal transport in Al-induced citrate and malate exudation, the primary roots of 6-day-old seedlings were carefully removed to achieve uniform lateral roots. The seedlings were first transferred to a complete nutrient solution for 7 days and then to +P or -P nutrient solution for an additional 8 days. The plants were transferred to a root manipulation box as stated in Yang et al. (2001). The root system was divided into two and each half of the root system was separately exposed to 0.5 mM CaCl₂ solution (pH 4.5) with or without 50 μM AlCl₃ for 6 h. The solution with root exudates in each compartment was analysed for organic anions.

The effect of La and phenylglyoxal on Al-induced exudation

To investigate whether the exudation of citrate and malate from rape is a specific response to Al, plants were exposed to 0 or 50 μM LaCl₃ in 0.5 mM CaCl₂ (pH 4.5) containing 0 or 50 μM AlCl₃ for 6 h. Root exudates were collected and analysed for organic anions. To determine the effect of phenylglyoxal (PG) on the release of citrate and malate, plants were treated with 10 μM PG (Katayama, Osaka, Japan) in 0.5 mM CaCl₂ solution containing 0 or 50 μM AlCl₃ for 6 h. Root exudates were collected for the analysis of organic anions.

Analysis of organic anions

Calcium solution containing root exudates was allowed to pass first through a cation exchange column (16 mm × 14 cm) filled with 5 g Dowex 50 W × 8 (50–100, H⁺ form) resin (Muromachi Technos Co. Ltd, Tokyo, Japan) and then through an anion exchange column filled with 2 g Dowex 1 × 8 resin (50–100 mesh, Cl⁻ form) without adjusting the pH. This procedure was carried out in a cold room (4°C) under non-sterile conditions and the organic anions retained in the anion exchange resin were eluted with 8 N formic acid. Organic anion residues were obtained and quantified according to Ma et al. (1997a).

Quantification of organic acids and enzyme activities in root apices

To determine organic anions concentration in the root tissue, 40 root apices 1 cm each, which had been exposed to 0 or 50 μM AlCl₃ for 6 h, were excised and thoroughly washed in distilled water. The concentration of organic anions was determined according to Osawa and Matsumoto (2001).

The activity of CS, MDH and PEPC was determined from roots of 21-day-old P-sufficient and P-deficient plants previously exposed to 0 or 50 μM AlCl₃ for 6 h. The roots were thoroughly washed in double-distilled water and 20 root apices (1 cm) were used to extract the enzymes. All procedures were carried out on ice. MDH was assayed spectrophotometrically by monitoring the disappearance of NADH at 340 nm for 1 min according to Johnson et al. (1994). The activity of PEPC was assayed spectrophotometrically in a coupled reaction at 340 nm. This was done by following the reduction of oxalacetate by NADH in the presence of excess MDH for 1 min according to Ashton et al. (1990). The activity of CS was assayed spectrophotometrically according to Srere (1969) by monitoring the reduction of acetyl CoA to CoA with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm for 3 min. Enzyme protein was quantified colorimetrically after Bradford (1976) using bovine serum albumin as a standard.

Determination of root respiration

Plants were treated with 0, 50 or 100 μM AlCl₃ in 0.5 mM CaCl₂ solution for 6 h. Root respiration was measured as the rate of O₂ consumption with excised root apices. Ten root apices (1 cm) were incubated at 25°C in a reaction chamber containing 2 ml air-saturated 0.5 mM CaCl₂ solution. The solution was continuously mixed, and O₂ consumption was recorded during 8 min using a Clark-type oxygen electrode apparatus (Hansatech, King's Lynn, UK).

Results

The effect of P treatment on plant growth

After 8 days of P deficiency, plants showed obvious deficiency symptoms (general growth reduction, purple coloration of leaves). P deficiency resulted in about 50% reduction in shoot dry matter in comparison with P sufficient plants (Table 1). However, root dry matter did not change significantly. The concentration of P in both roots and shoots was about four times higher in P-sufficient plants indicating that P-deficient plants were experiencing severe deficiency.

The effect of Al on root elongation

The effect of Al on root elongation was studied in a simple Ca-solution for 24 h. Al significantly inhibited root elongation in a dose-dependent manner (Fig. 1).

Table 1. The effect of P on biomass and shoot P concentration. Seedlings were raised on moist sand for 6 days. They were cultured in a complete nutrient solution for 7 days. After 7 days, the seedlings were divided into nutrient solution with or without 0.25 mM P for 8 days. Shoot and root dry matter was determined after drying at 70°C. Shoot P concentration was determined as described in materials and methods. Values represent means ± SE of three replicates and at least two independent experiments.

P-status	Dry weight (g)		P concentration (mg P g ⁻¹)	
	Root	Shoot	Root	Shoot
-P	0.13 ± 0.00	1.38 ± 0.05	0.86 ± 0.00	0.55 ± 0.07
+P	0.14 ± 0.01	2.82 ± 0.15	2.96 ± 0.05	2.14 ± 0.04

In the presence of 50 μM AlCl₃, root elongation was about 25% of the control for P-deficient plants and P-sufficient plants. In the presence of 100 μM Al Cl₃, root elongation was further inhibited to about 13% of the control without Al for both P-sufficient plants and P-deficient plants.

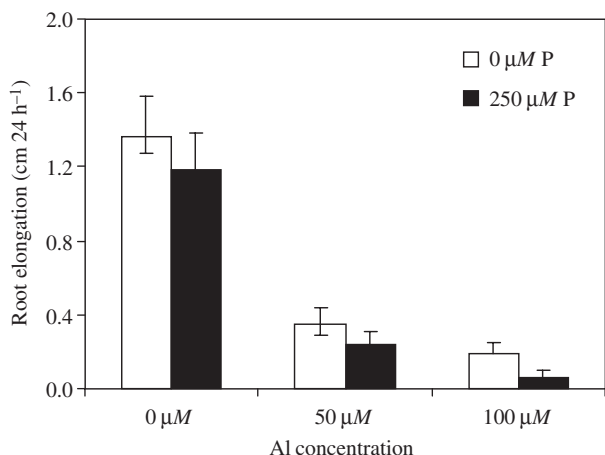


Fig. 1. The effect of aluminium on root elongation. Seedlings were raised on moist sand for 6 days. They were cultured in a complete nutrient solution for 7 days. The seedlings were then transferred to nutrient solution with or without 0.25 mM P for 8 days. The plants were then subjected to 0, 50 or 100 μM AlCl₃ in 0.5 mM CaCl₂ (pH 4.5) for 24 h. The length of primary roots was measured before and after Al treatment. Bars indicate means ± SE of three replicates and two independent experiments.

Aluminium-induced accumulation and exudation of organic anions under P-deficient and P-sufficient conditions

In response to Al exposure, citrate and malate were the only organic anions detected in the root exudates of P-sufficient plants and P-deficient plants. In the presence of 50 μM AlCl₃, both P-sufficient and P-deficient plants exuded significantly more citrate and malate than the control without Al (Fig. 2A and B). In the absence of 50 μM AlCl₃, a negligible amount of citrate was exuded from both P-sufficient and P-deficient plants. On the other hand, the exudation of malate was induced only in the presence of 50 μM AlCl₃. The P-sufficient plants exuded twice as much as the P-deficient plants. In general,

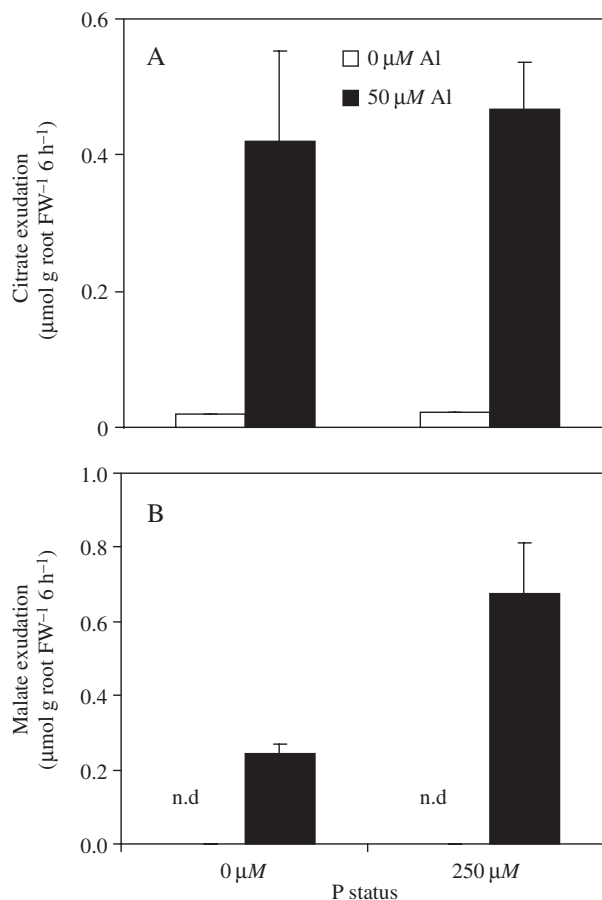


Fig. 2. The effect of aluminium on citrate (A) and malate (B) exudation from +P or -P rape plants. Plants were cultivated in the same way as described in Fig. 1. Then, the plants were subjected to 0 or 50 μM AlCl₃ in 0.5 mM CaCl₂ (pH 4.5) for 6 h and root exudates were analysed for organic anions. Bars indicate means ± SE of three replicates and two independent experiments. (n.d. not detected by HPLC).

P-sufficient plants exuded more organic anions (citrate + malate) in response to Al than P-deficient plants.

As shown in Fig. 3, 50 μM AlCl₃ significantly increased root tissue citrate and malate concentration in both P-sufficient plants and P-deficient plants. This increase was more evident in P-sufficient plants compared with P-deficient plants. Moreover, root tip citrate and malate concentration was about two-fold more for P-sufficient plants. In the absence of 50 μM AlCl₃, both P-sufficient plants and P-deficient plants had very low concentrations of citrate and malate.

Time course effect of Al on organic anions exudation

During the 15 h of Al treatment, P-sufficient plants showed a linear increase in citrate exudation (Fig. 4A), whereas, P-deficient plants showed an increase only during the first 5 h after which it remained more or less the same. Exudation of citrate remained very low in both P-deficient plants and P-sufficient plants in the absence of

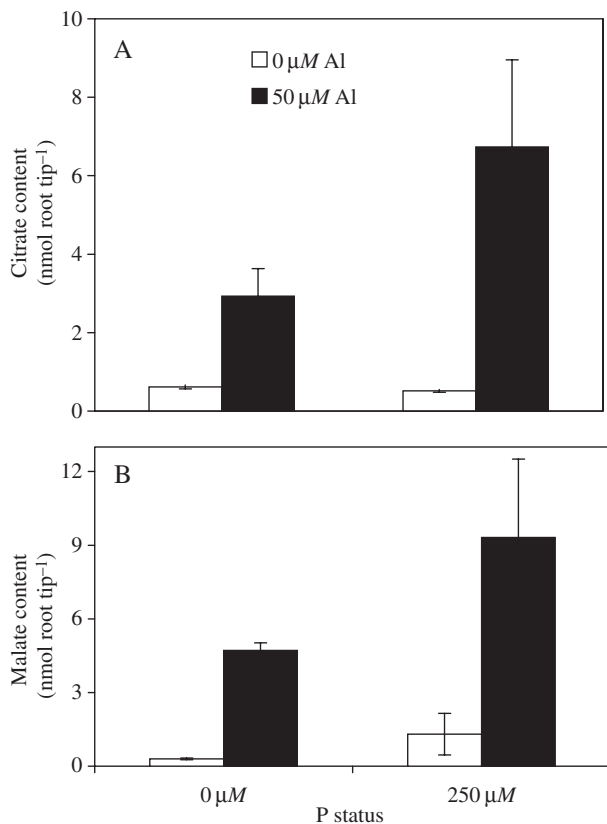


Fig. 3. The effect of aluminium on root tip citrate (A) and malate (B) content in +P or -P rape plants. Plants were treated in the same way as described in Fig. 1. Root tips were collected for the analysis of organic acid. Bars indicate means \pm SE of three replicates and two independent experiments (n.d. not detected by capillary electrophoresis).

50 μ M AlCl₃. Similarly, Al exposure induced exudation of malate from both P-sufficient plants and P-deficient plants (Fig. 4B). The P-sufficient plants showed an increase in the rate of malate exudation until 10 h after Al treatment and gradual decrease during the last 5 h. On the other hand, P-deficient plants did not show much change in the exudation of malate after the first 5 h, similar to citrate exudation. However, unlike citrate, malate was not detected in the absence of 50 μ M AlCl₃.

Divided root chamber experiment/split root experiment

The amount of citrate exudation from one-half of the root system directly exposed to 50 μ M AlCl₃ was significantly higher than that from the other half in Al-free Ca solution (Table 2). There was no significant difference in citrate exudation between P-deficient plants and P-sufficient plants. On the other hand, from the part of the root system exposed to Al, P-sufficient plants exuded a significantly higher amount of malate than P-deficient plants. In agreement with the previous results, malate was not detected in the other part in either P-sufficient or P-deficient plants.

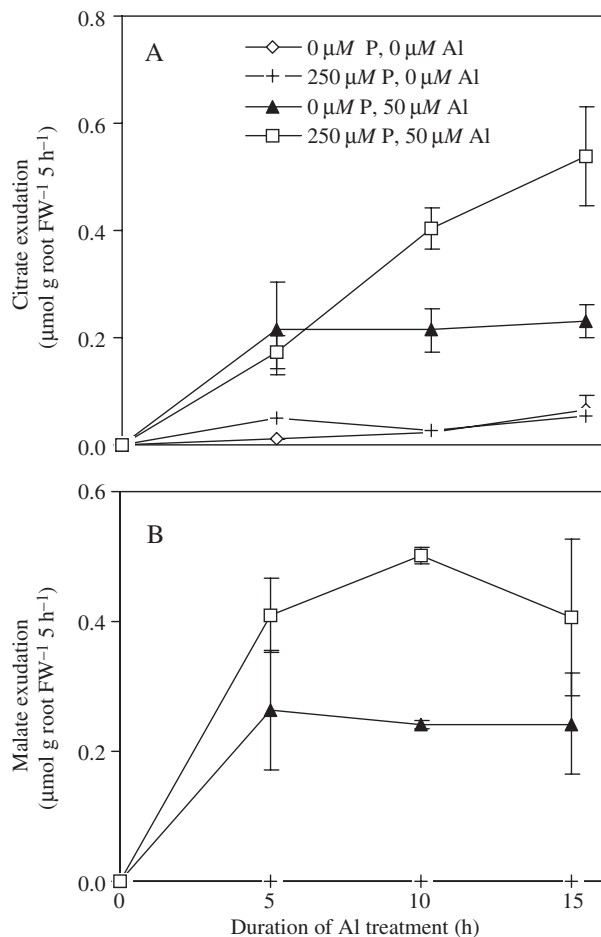


Fig. 4. Time course effect of Al on citrate (A) and malate (B) exudation from +P or -P rape plants. Plants were treated in the same way as described in Fig. 1. Root exudates were collected every 5 h during 15 h of Al treatment and analysed for organic anions. Values represent means \pm SE of three replicates and two independent experiments.

The effect of an alternate Al treatment on the pattern of organic anions exudation

During 10 days of an alternate Al treatment, exudation of citrate remained slightly higher from P-sufficient plants whereas it gradually decreased for P-deficient plants (Fig. 5A). As shown in Fig. 5B, malate exudation tended to decrease in both P-deficient and P-sufficient plants, although rapidly in the former and gradually in the latter. Moreover, from the seventh day of such an alternate Al exposure the exudation of malate was below the detectable range in P-deficient plants. Overall, the total amount of organic anions exuded decreased with prolonged Al exposure and the rate of decrease was pronounced in P-deficient plants.

The effect of Al on enzyme activity

As presented in Table 3, significant differences among treatments were observed in the activity of both CS and

Table 2. The effect of exposing half of the root system to Al on citrate and malate exudation. Plants were cultivated in the same way as described in Table 1. The root system was divided into two parts. Half of the root system was exposed to 50 μM AlCl_3 in 0.5 mM CaCl_2 (pH 4.5). The other half was continuously kept in Al-free Ca solution. After 6 h, root exudates were collected and analysed for organic anions. Values represent means \pm SE of three replicates and two independent experiments. (n.d. not detected by HPLC).

μmol (g root FW) ⁻¹ 6 h ⁻¹	0 μM Al		50 μM Al	
	-P	+P	-P	+P
Citrate	0.05 \pm 0.0	0.10 \pm 0.0	0.23 \pm 0.003	0.38 \pm 0.07
Malate	n. d.	n. d.	0.22 \pm 0.04	0.69 \pm 0.10

PEPCase. However, the activity of MDH did not change significantly. Despite the tremendous increase in both

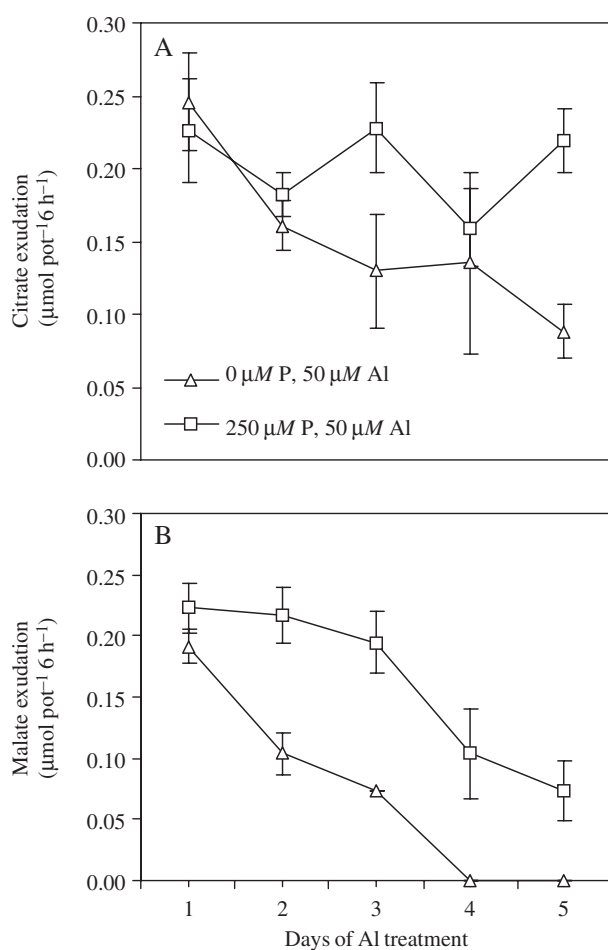


Fig. 5. Changes in the rate of citrate (A) and malate (B) exudation during 10 days of intermittent Al treatment in +P or -P rape plants. Plants were cultivated in the same way as described in Fig. 1. Then, the plants were subjected to 0 or 50 μM AlCl_3 in 0.5 mM CaCl_2 (pH 4.5) every other day for 24 h. After 24 h of Al treatment, plants were transferred back to nutrient solution with or without 0.25 mM P for 1 day. Root exudates were collected 6 h after Al treatment and analysed for organic anions. Values represent means \pm SE of three replicates and two independent experiments.

Table 3. The effects of Al on the activity of CS, MDH and PEPC in the root apices of +P and -P rape plants. Plants were cultivated same way as described in Table 1. The plants were pretreated in 0.5 mM CaCl_2 (pH 4.5) for 12 h. Then, they were subjected to 0 or 50 μM AlCl_3 in 0.5 mM CaCl_2 (pH 4.5) for 6 h. Twenty root apices were excised to determine enzymes activity. Values represent means \pm SE of three replicates and two independent experiments. Enzyme activity is expressed in [$\mu\text{mol min}^{-1}(\text{mg protein})^{-1}$].

AlCl_3 (μM)	P-status	CS	MDH	PEPC
0 μM Al	-P	0.90 \pm 0.12	12.19 \pm 1.93	0.18 \pm 0.02
	+P	1.11 \pm 0.06	12.72 \pm 2.01	0.23 \pm 0.01
50 μM Al	-P	1.34 \pm 0.18	14.88 \pm 2.35	0.19 \pm 0.02
	+P	1.37 \pm 0.09	16.11 \pm 2.54	0.27 \pm 0.01

accumulation in the root tissue and exudation of citrate and malate from the roots in the presence of 50 μM AlCl_3 , the increase in the specific activities of CS, MDH and PEPC was less than 30% in both P-sufficient plants and P-deficient plants (Table 3). In comparison with the control without Al, a 20 and 30% increase in the activity of CS was observed in P-sufficient plants and P-deficient plants, respectively. Similarly, a 20% increase in MDH was observed in P-sufficient plants and P-deficient plants. In the presence of 50 μM AlCl_3 , PEPC activity was increased by 16% in P-sufficient plants but only 5% in P-deficient plants. The activity of PEPC remained significantly higher in P-sufficient plants than in P-deficient plants.

The effect of Al on root respiration in P-sufficient plants and P-deficient plants

Root respiration was determined as a rate of oxygen consumption by P-sufficient plants and P-deficient plants. An increase in the concentration of Al resulted in a significant decrease in root respiration (Fig. 6). At 0 and 50 μM AlCl_3 , the rate of root respiration was 16% lower in P-sufficient plants than in P-deficient plants. In P-sufficient plants, 100 μM AlCl_3 did not affect the rate of root respiration compared with 50 μM AlCl_3 .

Al specificity of organic anions exudation

The effect of La^{3+} was studied in the presence or in the absence of 50 μM AlCl_3 . As shown in Fig. 7A and B, in comparison with the control (without both La and Al), La induced exudation of citrate to a lesser extent than 50 μM AlCl_3 . On the other hand, 50 μM LaCl_3 did not induce exudation of malate. Moreover, La suppressed the Al-induced exudation of citrate and malate. Similarly, P deficiency did not induce exudation of organic anions from rape. However, Al exposure induced citrate and malate exudation from both P-sufficient plants and P-deficient plants.

Discussion

In the current study, Al exposure significantly inhibited root elongation in a dose-dependent manner (Fig. 1).

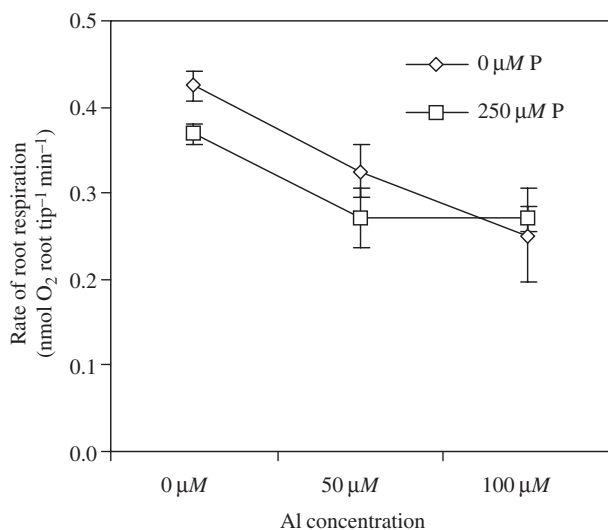


Fig. 6. The effects of Al on the rate of root respiration in +P and -P plants. Plants were cultivated in the same way as described in Fig. 1. Then, they were subjected to 0, 50 or 100 μM AlCl₃ in 0.5 mM CaCl₂ (pH 4.5) for 6 h. Ten root apices were excised and root respiration was determined by measuring the rate of oxygen consumption. Values represent means ± SE of three replicates and two independent experiments.

Nevertheless, the degree of inhibition of Al-induced root elongation followed more or less the same tendency in both P-sufficient and P-deficient plants. A similar effect of Al on root elongation was previously reported in pea (Matsumoto et al. 1996). Although P-sufficient plants exuded more organic anions that are believed to alleviate Al-induced root elongation inhibition, the level of Al tolerance based on root elongation was more or less the same in P-deficient plants. This is mainly because of the fact that the severity of Al toxicity in P-deficient plants was masked by the stimulating effect of P deficiency on root elongation.

Despite various reports on P deficiency-induced exudation of organic anions from white lupin, alfalfa and rape (Gardner et al. 1983, Lipton et al. 1987, Hoffland et al. 1989), the current study showed a negligible amount of citrate and an absence of malate exudation from P-deficient rape plants (Fig. 2A and B). Similarly, P deficiency did not induce exudation of organic anions from wheat (Delhaize et al. 1993), buckwheat and taro (Ma et al. 1997a, 1998) and soybean (Yang et al. 2000). On the other hand, there are overwhelming reports on the release of organic anions as one of the Al tolerance mechanisms. Snapbean, wheat, maize, *C. tora*, buckwheat and soybean have been reported to exude malate, citrate and oxalate in response to Al (Miyasaka et al. 1991, Delhaize et al. 1993, Pellet et al. 1995, Ma et al. 1997a, b and Yang et al. 2000). In agreement with these findings, both P-sufficient and P-deficient plants showed a significant increase in exudation of both citrate and malate in the presence of 50 μM Al (Fig. 2A and B). This significantly lower level of citrate exudation and absence of malate indicates that Al is a prerequisite for the

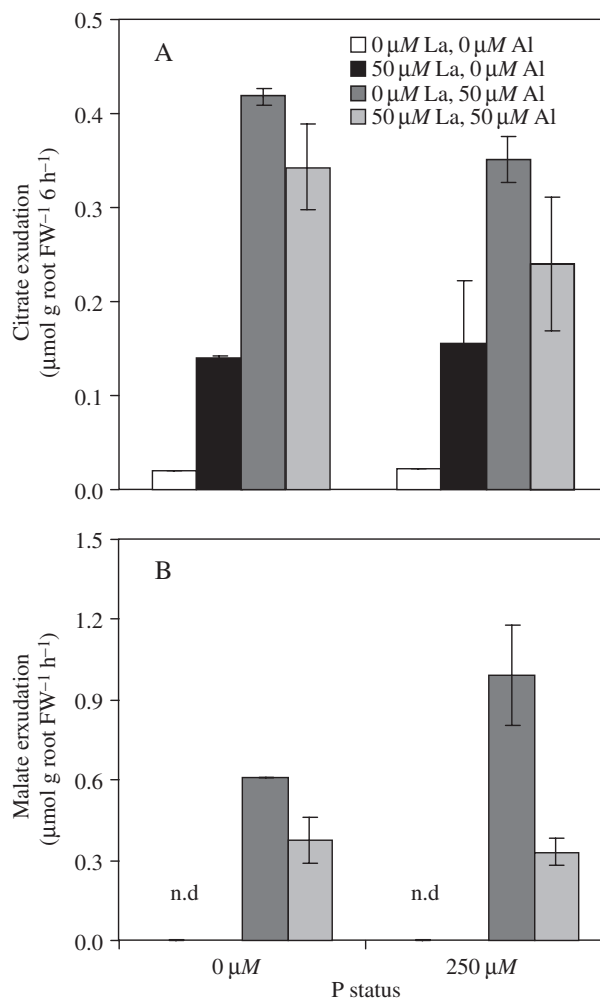


Fig. 7. The effect of lanthanum on aluminium induced citrate (A) and malate (B) exudation from +P or -P rape plants. Plants were cultivated in the same way as described in Fig. 1. Then, the plants were subjected to 0 or 50 μM LaCl₃ in 0.5 mM CaCl₂ (pH 4.5) containing 0 or 50 μM AlCl₃. Root exudates were collected 6 h after treatment and analysed for organic anions. Bars represent means ± SE of three replicates and two independent experiments. (n.d. not detected by HPLC).

response. Furthermore, P-sufficient plants exuded a significantly higher amount of organic anions (citrate + malate) than P-deficient plants. Similarly, Nian et al. 2003), reported that three soybean varieties showed a significantly higher rate of citrate exudation in the presence of 10 μM P than in the control without P. Therefore, P nutrition affects Al-induced synthesis and exudation of organic acids significantly.

Although Hoffland et al. (1989) in their work with rape and Neumann and Römhel (1999a) with white lupin reported a significantly higher accumulation of citrate and malate in the roots in response to P deficiency, the present result showed a significant increase in the root tip accumulation of citrate and malate in response to 50 μM AlCl₃ (Fig. 3A and B). The rate of increase in citrate and malate accumulation was more

evident for P-sufficient plants in the presence of 50 μM AlCl_3 than in its absence. In agreement with the current study, Piñeros et al. (2002) recently reported an increase in root tip citrate content in maize in response to Al. Similarly, Pellet et al. (1995) observed an Al-induced increase in malate content in the root tip of both Al-tolerant and Al-sensitive maize cultivars whereas the citrate content was unaffected. On the other hand, Delhaize et al. (1993) and Ryan et al. 1995b) did not observe any such alterations in the malate content of Al-treated wheat. Nevertheless, Yang et al. (2001) reported a rapid decline in malate content in the root tip after a 3-h Al treatment, although they did not observe any change in the citrate content. These differences might indicate the presence of plant-specific regulation mechanisms.

To understand the mechanism involved in Al-induced accumulation and exudation of citrate and malate, we investigated the activity of enzymes involved in citrate and malate metabolism. Accordingly, the activity of CS, MDH and PEPC showed an increase of about 30, 20 and 16%, respectively, at 50 μM Al compared with the control (Table 3). Similarly, de la Fuente et al. (1997), reported that transgenic tobacco and papaya into which the *Pseudomonas auregnosa* CS gene had been introduced was found to be Al-tolerant due to the increase in the synthesis and exudation of citrate. Moreover, Koyama et al. (2000) reported that over-expression of *Daucus carota* CS gene in *Arabidopsis thaliana* improved the growth on phosphate-limited soil as a result of enhanced citrate exudation from the root. Therefore, the increase in both citrate synthesis and release in rape can be partly explained by the increase in the CS activity. On the other hand, Hoffland et al. (1992) reported that a transfer of newly synthesized citrate from the shoot to the root was the main source of citrate accumulated in and exuded from the roots of P-deficient rape plants. If this were the case, P-deficient plants would show a higher rate of Al-induced citrate exudation. However, in the current study P-sufficient plants showed a two-fold increase in citrate accumulation in root tip and also a higher rate of malate exudation. Moreover, we cannot rule out the transport of more carbon from the shoot to the root. Non-photosynthetic CO_2 fixation mediated by an increased activity of PEPC has been implicated as an anaplerotic carbon supply (>30%) for the biosynthesis of organic acids induced by P deficiency in many plant species (Hoffland et al. 1992, Johnson et al. 1994). Furthermore, as reported for proteoid roots of P-deficient white lupin (Johnson et al. 1994), the activity of MDH, an enzyme that catalyses the conversion of oxalacetate to malate was enhanced in Al-treated plants. Thus, the increased activities of CS, PEPC and MDH can partly explain the increase in both accumulation and release of citrate and malate.

Additionally, an increase in the concentration of Al resulted in a significant decrease in the rate of root respiration measured as O_2 consumption for both P-sufficient and P-deficient rape plants (Fig. 6). Similarly, citric acid accumulation in developing proteoid roots of white lupin was also associated with reduced root

respiration (Johnson et al. 1994, Neumann and Römheld 1999a). Therefore, it can be concluded that Al-induced exudation of citrate and malate in rape is attributed to enhanced biosynthesis and decreased degradation of these anions. Moreover, the higher rate of accumulation and exudation in P-sufficient plants in comparison with P-deficient plants can be attributed to the slightly higher PEPC activity (16% in P-sufficient plants, and 5% in P-deficient plants), the lower rate of respiration in the presence of an Al signal and higher biomass production, which might serve a carbon source.

In the current study, the pattern of organic anions exudation was monitored during 10 days of an alternate Al treatment. As Fig. 5A and B show, the total amount of citrate and malate exuded was higher at the onset of Al exposure and declined in a later stage. In agreement with this finding Zheng et al. (1998b) examined Al-induced organic acid secretion in eight plant cultivars belonging to five species and identified three patterns of exudation. In pattern I, the amount of organic acid secreted during the whole treatment period was very low, Scout 66 and two cultivars of oat belong to this group. Pattern II included Atlas 66 and two cultivars of oilseed rape, which showed a quite high rate of exudation during the first exposure and declined during the later stage. Therefore, the cultivar used in the current study can be categorized in this group since the pattern of organic anion exudation showed the same trend. The decline in a later stages might be attributed to: first, the prolonged Al treatment, which might have injured the root system and hence inhibited the biosynthesis and release of organic anions. Second, the amount of organic anions secreted in response to Al might vary with plant age. Therefore, there might be an optimum growth stage for Al-induced exudation after which it may decline or completely cease. In line with this conclusion, Ma (2000) indicated that the amount of organic acid exuded in response to Al varies with plant species, plant age, Al activity and exposure time. Neumann et al. (1999b) also reported that increased root exudation of organic acid in response to P deficiency was detectable 23 days after sowing. Moreover, exudation and internal concentration of malic acid decreased with increasing age of the root clusters. In pattern III (buckwheat and radish), the amount of organic anions exuded maintained a high level.

Although Blackman and Davies (1985) reported a long-distance signal transport from maize root to the shoot under water stress, such signal transport was not observed in the current study. The exudation of citrate remained significantly lower in the part of the root system bathing in Al-free Ca solution (Table 2) compared with the other half treated with 50 μM Al. On the other hand, malate was not detected in the absence of Al. A similar result was previously reported for Al-induced citrate exudation from soybean (Yang et al. 2001). Recently, Nian et al. (2003), also reported that citrate and malate were exuded only from the part of the root system that was directly exposed to either Al or Cu. Hence, these reports suggest the

absence of a long-distance signal transport in the Al-induced exudation of citrate and malate in rape and that direct contact with Al is a prerequisite to elicit the response.

It was previously reported for wheat (Delhaize et al. 1993) and buckwheat (Zheng et al. 1998a) that neither La^{3+} nor P deficiency induced exudation of malate or oxalate. Similarly, the current study showed that 8 days of P deficiency did not induce exudation of citrate and malate in rape. However, $50 \mu\text{M}$ LaCl_3 could slightly elicit exudation of citrate. Although Ryan et al. (1995a) and Kataoka et al. (2002) reported that La^{3+} could elicit the efflux of malate from root apices of wheat at a much lower level than observed for Al, malate was not exuded from rape roots in the presence of $50 \mu\text{M}$ LaCl_3 . Hence, exudation of malate is specific to Al exposure. In the current study, La^{3+} significantly suppressed Al-induced exudation (Fig. 7A and B). Similarly, La^{3+} suppressed Cu-induced citrate exudation in wheat (Nian et al. 2003). The suppression of Al-induced exudation by La^{3+} might be because of the competition of the two trivalent cations for binding sites. As suggested by Ryan and Kochian (1993), Al^{3+} and La^{3+} might act similarly at the level of the cell surface due to their close ionic radius (Al 4.75 Å and La 4.52 Å). Hence, enhanced exudation of citrate and malate is a clear response to Al toxicity in rape. Exogenous application of an anion channel inhibitor, PG resulted in about 50% inhibition of citrate and malate (data not shown) and this may suggest the involvement of an anion channel in the process of exudation.

In conclusion, both P-deficient and P-sufficient plants showed significant increase in synthesis and exudation of both citrate and malate in response to Al exposure. Phosphorus deficiency by itself failed to elicit exudation. The P-sufficient plants showed significantly higher rates of synthesis and exudation of mainly malate. However, the mechanism needs to be further scrutinized.

Acknowledgements – This research was supported by the Program for Promotion of Basic Research Activities in Innovative Biosciences (PROBRAIN) to H.M., Grant-in-Aid for General Research (A) (14206008) from the Ministry of Education, Science, Sports and Culture of Japan to H.M and Ohara Foundation for Agricultural Sciences. We are grateful to Mrs Sanae Rikiishi, Dr Akira Tabuchi and Dr Hiroki Osawa for their technical support.

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