

RESEARCH PAPER

# Effect of K-252a and abscisic acid on the efflux of citrate from soybean roots

Hong Shen<sup>1,2</sup>, Ayalew Ligaba<sup>1</sup>, Mineo Yamaguchi<sup>1</sup>, Hiroki Osawa<sup>1</sup>, Koichi Shibata<sup>1</sup>, Xiaolong Yan<sup>2</sup> and Hideaki Matsumoto<sup>1,\*</sup>

<sup>1</sup> Research Institute for Bioresources, Okayama University, 2-20-1 Chuo, Kurashiki 710-0046, Japan

<sup>2</sup> Laboratory of Plant Nutritional Genetics and Root Biology Center, South China Agricultural University, Guangzhou, 510642, PR China

Received 14 August 2003; Accepted 4 November 2003

## Abstract

The Al-induced release of organic acid has been suggested as an important mechanism for Al resistance in plants. In this study, the effect of K-252a and abscisic acid (ABA) on the efflux of citrate was investigated in soybean (*Glycine max* L.) roots. Al initiated citrate efflux from the root apices 30 min after the addition of Al. The Al-triggered efflux of citrate was sensitive to metabolic inhibitors and anion channel inhibitors. Pretreatment or treatment with K-252a, an inhibitor of protein kinase, severely inhibited the Al-induced efflux of citrate accompanying an increase in Al accumulation and intensified Al-induced root growth inhibition. Al-treatment increased the endogenous level of abscisic acid (ABA) in soybean roots in a dose- and time-dependent manner, while K-252a failed to inhibit the Al-induced increase in endogenous ABA. Exogenous application of ABA increased the activity of citrate synthase (EC 4.1.3.7) by 26.2%, and decreased Al accumulation by 32.3%, respectively. ABA-induced increases in citrate efflux and root elongation were suppressed by K-252a, while ABA could not reverse the K-252a effects. Taken together, these results suggest that ABA is probably involved in the early response, after which K-252a-sensitive protein kinases play a key step in regulating the activity of an anion channel, through which citrate is released from the apical cells of soybean roots.

Key words: Abscisic acid, aluminium, citrate release, K-252a, soybean root.

## Introduction

Exclusion of organic-acid anions from the seedling roots is believed to be an important Al-exclusion mechanism. Many plant species have been identified that release organic acids in response to Al stress, such as malate in wheat (Ryan *et al.*, 1995), oxalate in buckwheat and taro (Ma *et al.*, 1997; Ma and Miyasaka, 1998), and citrate in snapbean, soybean (*Glycine max* L.), Cassia tora, and Triticale (Ma *et al.*, 1997; Li *et al.*, 2000; Yang *et al.*, 2001). Bioassay of the toxicity of different Al-citrate complexes indicated that the Al-citrate 1:1 complex is not phytotoxic and its transport through the plasmalemma seems to be very slow (Kochian, 1995). Therefore, the Al-responsible citrate efflux could ameliorate Al toxicity effectively.

In soybean, Al could induce the efflux of a large amount of citrate from Al-resistant roots. The activity of enzymes related to the synthesis and metabolism of citrate did not change greatly in response to Al stress suggesting that the synthesis of citrate in root tissue is not the key step in the Al-induced efflux of citrate (Yang *et al.*, 2001). Recently, the activation of anion channels on the plasma membrane by Al has been found to function as a rate-limiting step, and the activity or open probability of anion channels on the plasma membrane in root apical cells mediates the Al-responsive efflux of organic acid anions (Ryan *et al.*, 1997; Piñeros and Kochian, 2001; Zhang *et al.*, 2001). In wheat, protein phosphorylation is involved in the Al-responsive efflux of malate (Osawa and Matsumoto, 2001). These observations imply that some protein kinases that are responsible for the anion channel activity mediate citrate efflux in response to Al stress. However, whether such kinases exist in soybean remains unknown.

\* To whom correspondence should be addressed. Fax: +81 86 434 1210. E-mail: hmatsumo@rib.okayama-u.ac.jp

The plant hormone ABA plays important roles in responding and adapting to environmental stresses by altering plant cellular metabolism and invoking various defence mechanisms (Schroeder *et al.*, 2001). Matsumoto *et al.* (1996) speculated that the transduction of the Al signal in barley roots is related to an increase in ABA since Al treatment increases ABA levels in barley roots. The exogenous application of ABA increased both ATP-dependent and PPI-dependent H<sup>+</sup>-pumping activities, and these increases, caused by Al stress, could result from increased levels of ABA (Kasai *et al.*, 1993). In Arabidopsis, ABA could activate an open stomatal protein kinase, which mediates the interaction between ABA perception and reactive oxygen species production (Mustilli *et al.*, 2002). In guard cells, cADP-Rib, phospholipase C, phospholipase D, and changes in cytosolic Ca<sup>2+</sup> concentration have been identified as signalling molecules in the ABA signal transduction pathway leading to the stomatal aperture (Wu *et al.*, 1997; Staxen *et al.*, 1999). If ABA is involved in the Al signal transmitting the Al-induced efflux of citrate from soybean roots, treatment with exogenous ABA would influence the citrate efflux in response to Al stress. Moreover, an ABA signal-transmitting pathway should exist.

In the present study the Al-induced efflux of citrate from the root apices of soybean seedlings was investigated using various ion channel modulators and phytohormones. The results in this study indicated that the Al-induced citrate efflux from soybean roots is associated with K-252a-sensitive protein phosphorylation, and that ABA is involved in the early responses of Al signal transduction in the Al-induced efflux of citrate from soybean seedlings.

## Materials and methods

### Reagents

ABA, TEA (tetraethylammonium chloride), and verapamil (soluble in ethanol) were purchased from Wako Chemical (Osaka). K-252a, niflumic acid (NIF), staurosporine (soluble in dimethylsulphoxide), and anthracene-9-carboxylic acid (A9C) (soluble in ethanol) were obtained from Sigma-Aldrich. Phenylglyoxal (PG) (soluble in distilled water) was purchased from Katayama Chemical, Japan. KCN, 2,4-dichlorophenoxy acetate (DCPA) (soluble in distilled water), and 2,4-dinitrophenol (DNP) (soluble in ethanol) were obtained from Wako Chemical (Osaka). All these reagents were prepared as a 1 mM stock solution before use. All other reagents used were of the highest purity obtainable.

### Plant material and seedling growth

Seeds of *Glycine max* were gently ground with sea sand (20–30 mesh) for 10 s to facilitate germination. Pretreated seeds were soaked in a solution containing 0.5 mM CaCl<sub>2</sub> for 1 h and then germinated in peat moss mixed with sand quartz for 3 d at 25 °C. After germination, the seedlings were transferred to nutrient solution in 2.0 l plastic pots containing (μM): KNO<sub>3</sub> (750), Ca(NO<sub>3</sub>)<sub>2</sub> (250), MgSO<sub>4</sub> (325), KH<sub>2</sub>PO<sub>4</sub> (20), Fe-EDTA (20), H<sub>3</sub>BO<sub>3</sub> (8), CuSO<sub>4</sub> (0.2), ZnSO<sub>4</sub> (0.2), MnCl<sub>2</sub> (0.2), and (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> (0.2). The

solution was adjusted to pH 6.0 with 1 mM HCl and renewed every 3 d. The seedlings were grown in a growth cabinet at 25/20 °C and 14/10 h day/night cycles, 40 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity, and 70% relative humidity. Each experiment was conducted with three replicates and repeated at least twice.

### Analysis of citrate efflux from root apices and intact roots

Citrate efflux from 5 mm root apices was analysed enzymatically by the method of Delhaize *et al.* (1993) with minor modifications. Briefly, 30 root apices were transferred into a 3.5 cm Petri dish, washed for 2 h with 0.2 mM CaCl<sub>2</sub> solution (pH 4.2). After applying the test solution, the Petri dishes were placed on a reciprocal shaker at 80 rpm. The solution was then collected for citrate analysis. Two ml of the sample solution was incubated in the solution consisting of 95 μl of buffer (1 M TRIS-HCl, pH 7.8), 12 μl of 10 mM NADH, and 4 μl of a lactate dehydrogenase (LDH)/malate dehydrogenase (MDH) mixture. After a stable A<sub>340</sub>-reading was obtained, 4 μl of citrate lyase was added and the decline in A<sub>340</sub> was recorded. The decrease in NADH concentration was directly proportional to the amount of citric acid in the sample. Aluminium in the sample solution did not interfere with the assay for citrate because the exact amount of citrate could be detected when standard citrate was added regardless of the presence of Al. Reagents as indicated were added to the root-apex incubation medium for 30 min. Root apices were then rinsed three times with 0.2 mM CaCl<sub>2</sub> solution to remove excess modulators and exposed to 0.2 mM CaCl<sub>2</sub> solution containing 200 μM Al for 120 min. K<sup>+</sup> efflux from root apices was determined by graphite furnace atomic absorption spectrophotometry (Z-8270; Hitachi, Tokyo, Japan).

Citrate efflux from intact roots was measured according to the method of Yang *et al.* (2001). After incubation in nutrient solution for 8 d, the seedling roots were exposed to 0.5 mM CaCl<sub>2</sub> solution overnight for acclimatization and then transferred to different treatments. After treatment, the solution was collected and passed through a cation exchange column (16×14 mm) filled with 4 g of Amberlite IR-120B resin (H<sup>+</sup> form), followed by an anion-exchange column (16×14 mm) filled with 2 g of Dowex 1×8 resin (100–200 mesh, formate form) in a cold room. Citrate retained on anion exchange resin were eluted by 1 M of HCl, and the eluate was concentrated to dryness by a rotatory evaporator (40 °C). After the residue was redissolved in deionized water, the concentration of citrate was analysed by high-performance liquid chromatography (HPLC, LC-10A, Shimadzu, Kyoto, Japan).

### Analysis of Al content

After treatments, the seedling roots were washed three times with deionized water. Excised 5 mm root apices were transferred to 1.5 ml Eppendorf cups containing 2 N HCl for 48 h. The Al content in the HCl solution was measured by graphite furnace atomic absorption spectrophotometry (Z-8270; Hitachi, Tokyo, Japan).

### Analysis of citrate content and the activity of citrate synthase

Citrate content in root apices was determined by capillary electrophoresis with a PACE 5510 system (Beckman Instruments, Fullerton, CA) equipped with UV detector (254 nm) as follows: After treatment, 50 root apices (5 mm long) were excised and placed in an 80% (v/v) ethanol solution in a microcentrifuge tube, and boiled for 5 min at 80 °C. The root apices were ground using a microhomogenizer (model NS-310E, Tokyo), and centrifuged for 5 min at 10 000 g. After collecting the supernatant, the pellet was re-extracted twice by the same procedure. The supernatants of three replicates were mixed, freeze-dried to remove excess reagent, and reconstituted in 100 μl of ultra pure water. Reconstituted samples were filtered on 0.45 μm sterilized filters (Millipore, Tokyo), and used for analysis of citrate content. For the enzyme assay, 20 root

apices (5 mm) were excised and transferred to 1.5 ml microcentrifuge tube containing 0.5 ml cold 50 mM HEPES-NaOH buffer (pH 7.5) with the following components: 5 mM MgCl<sub>2</sub>, 5 mM EDTA, 10% (v/v) glycerol, and 0.1% (v/v) Triton X-100. After homogenizing, the homogenate was centrifuged at 10 000 g for 5 min, and the supernatant was used for enzyme assay. Citrate synthase was assayed spectrophotometrically according to Johnson *et al.* (1994) by monitoring the reduction of acetyl-S-CoA in the presence of 5,5'-bisthiol(2-nitrobenzoic acid) at 412 nm for 3 min. Protein was quantified colorimetrically according to Bradford (1976).

#### ABA analysis

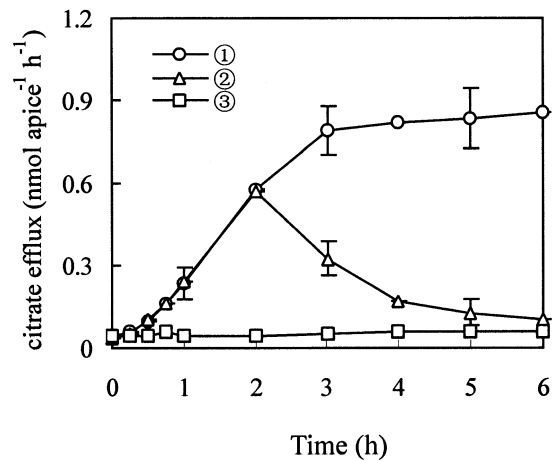
After treatment, root tissues were cut, weighed, and immediately placed in liquid nitrogen then stored at -80 °C until extraction. Freeze-dried tissue samples were weighed, homogenized, and extracted with homogenization buffer [80% (v/v) aqueous methanol+10 mg l<sup>-1</sup> butylated hydroxytoluene]. To avoid non-physiological increases in xanthoxal levels through the breakdown of carotenoids and to minimize isomerization and degradation of xanthoxal and ABA, the extracts were passed through a C<sub>18</sub>-reversed phase prepacked column immediately under dim light conditions at 4 °C. The methanol solution was dried under reduced pressure and the aqueous residue partitioned three times against ethyl acetate at pH 2.5. Ethyl acetate in the combined organic fractions was evaporated to dryness under a N<sub>2</sub> stream and the residue was dissolved in 0.5 ml of TBS-buffer (TRIS-buffered saline: 150 mM NaCl, 1 mM MgCl<sub>2</sub>, and 50 mM TRIS; pH 7.5) for an immunological ABA assay (ELISA) as described by Mertens *et al.* (1983).

## Results

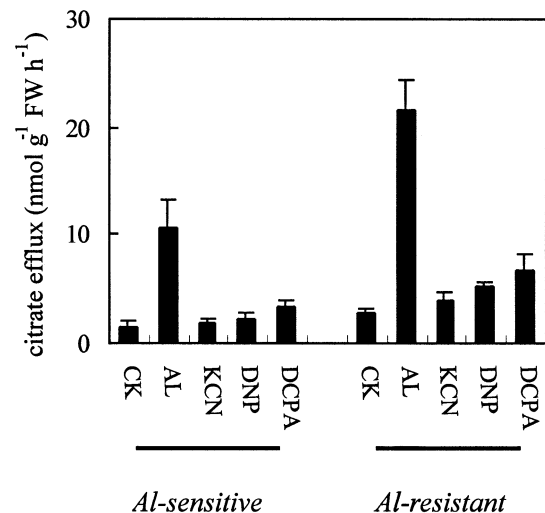
#### Al-induced efflux of citrate

Efflux of citrate from the intact roots of soybean seedlings was induced by Al in a dose-dependent manner (Yang *et al.*, 2001). The preliminary results from this study using root apices showed that citrate efflux increased with increasing Al levels, and this efflux was induced by Al primarily at the terminal root tips (data not shown). To elucidate the regulatory mechanism for Al-induced efflux of citrate, the time for the initiation of citrate efflux was examined after exposure to Al stress (Fig. 1). The citrate efflux was initiated 30 min after the addition of Al, and the efflux rate reached a maximum level at 180 min after Al treatment, then remained stable. To examine whether Al is required for citrate efflux after the initiation of citrate efflux, Al was withdrawn from the medium. The citrate efflux declined quickly (Fig. 1). This suggested that Al contact was required for citrate efflux. Al-induced efflux of citrate from soybean roots ceased at 4 °C (data not shown). Metabolic inhibitors (KCN, DNP, DCPA) severely suppressed the Al-induced citrate efflux in both Al-sensitive and Al-resistant soybean cultivars (Fig. 2).

Liu and Luan (2001) reported that Al could enter plant cells through a Ca<sup>2+</sup> channel-like pathway. The effects of two antagonists of a cation channel-like pathway (verapamil and TEA) on the efflux of citrate remain to be identified. The results from Table 1 indicated that



**Fig. 1.** Time-course of citrate efflux from the root apices of Al-resistant (cv. Suzunari) soybean seedlings. Excised root apices (5 mm in length from root apices) were pretreated with 0.2 mM CaCl<sub>2</sub> solution for 2 h, then with different treatments. During the first hour, root exudate was collected every 15 min, then the sample was collected after 1 h. (1) Exposure to 200 μM Al for 6 h; (2) exposure to 200 μM Al for the first 2 h, then to 0.2 mM CaCl<sub>2</sub> solution for 4 h; (3) exposure to 0.2 mM CaCl<sub>2</sub> solution for 6 h. Citrate efflux was determined enzymatically. Values are means ± SE (*n*=3).



**Fig. 2.** Effects of metabolic inhibitors on Al-induced efflux of citrate from Al-sensitive and Al-resistant soybean roots. Intact roots of Suzunari and Shishio seedlings were pretreated with 0.5 mM CaCl<sub>2</sub> containing 5 mM KCN, or 0.5 mM DNP, or 0.5 mM DCPA for 30 min, washed three times with Ca solution, and then exposed to 0.5 mM CaCl<sub>2</sub> solution containing 40 μM Al for 6 h. Citrate efflux was determined by HPLC. pH in all solutions is 4.2. Values are means ± SE (*n*=3).

verapamil and TEA failed to induce citrate efflux in the presence or absence of Al. Anion-channel inhibitors (NIF, A9C and PG at 20 μM, respectively) decreased the Al-induced citrate efflux by 29–55%. The order of effectiveness of these inhibitors was niflumic acid>PG>A9C (Table 1). These results suggested that Al-induced efflux

**Table 1.** Effect of ion channel modulators on Al-induced efflux of citrate from the root apices of an Al-resistant cultivar

Excised root apices were pretreated with 0.2 mM CaCl<sub>2</sub> solution containing or not containing either type of inhibitor, then exposed to the Ca solution containing 200 μM Al (pH 4.2). Dimethylsulphoxide or ethanol concentration in the Ca solution was less than 0.5 %, which did not inhibit the Al-induced efflux of citrate. After the pretreatment, root apices were rinsed three times with the Ca solution to remove excess modulators. Periods of pretreatment and treatment were 30 and 120 min, respectively. Values are means ±SE (n=3).

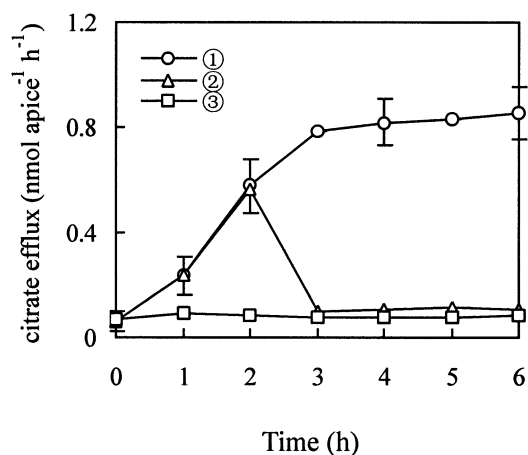
Treatment	Citrate efflux (nmol apex <sup>-1</sup> h <sup>-1</sup> )	Percentages (compared with AlCl <sub>3</sub> ) (%)
Control	0.069±0.029 d <sup>a</sup>	9.37
AlCl <sub>3</sub>	0.736±0.122 a	100
Cation channel inhibitors		
10 mM Verapamil	0.791±0.105 a	107
10 mM TEA	0.851±0.122 a	115
Anion channel inhibitors		
20 μM A9C	0.523±0.050 b	71.1
20 μM PG	0.490±0.036 b	66.6
20 μM NIF	0.334±0.055 c	45.4
Inhibitors of protein kinase		
1 μM K-252a	0.334±0.013 c	45.4
5 μM K-252a	0.118±0.019 d	16.0
10 μM Staurosporine	0.703±0.105 a	95.1

<sup>a</sup> Different letters in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range tests.

of citrate was via an anion channel and independent of the cation channels on the plasma membrane.

In these preliminary experiments ATP-binding cassette inhibitors (diphenylamine-2-carboxylic acid and glibenclamide), protein phosphatase inhibitors (cyclosporin A, okadaic acid, and U73343), and G protein modulators (PD98059 and Mastoparan) were also examined for their effects on the Al-induced efflux of citrate. These modulators had no effects or only slightly influenced the Al-induced efflux of citrate (data not shown). Among the modulators tested here, K-252a, a broad-range inhibitor of protein kinases, was the most effective and strongly suppressed the Al-induced efflux of citrate (Table 1). Pretreatment with K-252a for 30 min or the addition of K-252a after 2 h Al treatment blocked the Al-induced efflux of citrate almost completely. Treatment with Al plus K-252a could not induce any citrate efflux (Fig. 3; Table 1). These results indicated that K252a-sensitive protein kinases probably played an important role in modulating the Al-induced efflux of citrate. Moreover, pretreatment with K-252a increased the Al content in the root apices of cv. Suzunari, and this increase was in a concentration-dependent manner (Fig. 4A).

To investigate the role of citrate efflux in ameliorating Al-inhibition of root elongation, Suzunari intact roots were pretreated with K-252a and then exposed to Al treatment. In the absence of Al, pretreatment with 5 μM K-252a did not affect root elongation. While in the presence of 20 or



**Fig. 3.** Effect of K-252a, an inhibitor of protein kinase, on the Al-induced efflux of citrate from root apices of an Al-tolerant cultivar. Excised root apices were pretreated with 0.2 mM CaCl<sub>2</sub> solution for 1 h, then for different treatments. (1) Exposure to 200 μM Al for 6 h; (2) exposure to 200 μM Al for 2 h, then to 5 μM K-252a for 1 h, followed by exposure to 200 μM Al for 3 h; (3) exposure to 200 μM AlCl<sub>3</sub> plus 5 μM K-252a for 6 h. pH in all solutions is 4.2. Citrate efflux was determined enzymatically. Values are means ±SE (n=3).

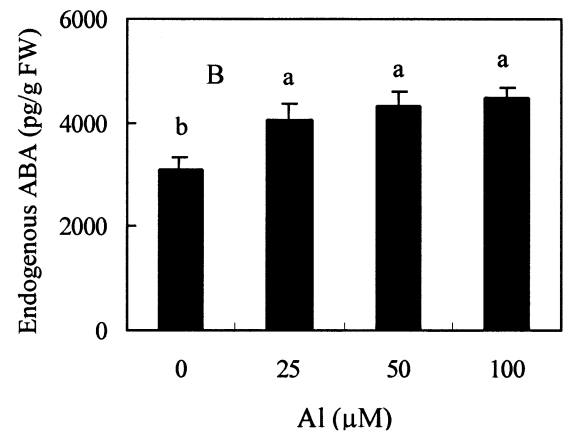
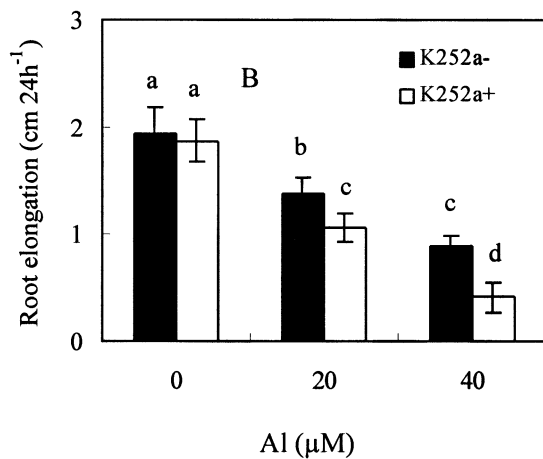
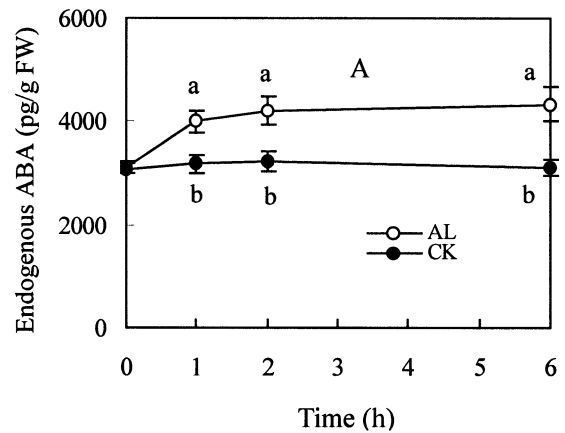
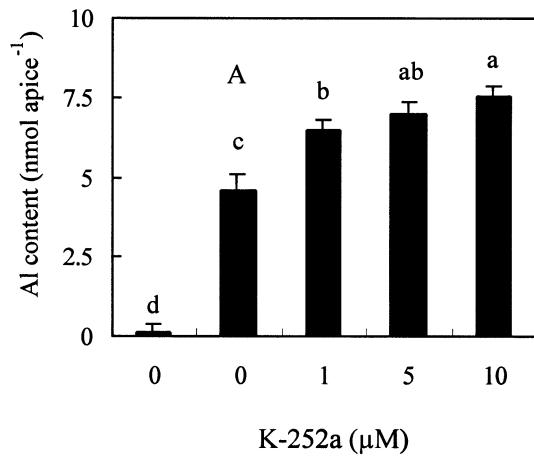
40 μM Al, K-252a intensified the inhibition of root elongation by Al (Fig. 4B). Staurosporine has previously been demonstrated to be an effective inhibitor of protein kinases in disassembling the actin network in soybean cells (Chandra and Low, 1995). However, staurosporine inhibited the Al-induced efflux of citrate only slightly, even at 50 μM, and did not enhance either Al accumulation or Al-induced inhibition of root elongation (data not shown).

#### Endogenous ABA

Al treatment increased the endogenous level of abscisic acid (ABA) in soybean roots, and this increase was dependent on the time and concentration of Al treatment (Fig. 5A). Interestingly, endogenous ABA increased rapidly in the first 2 h after the addition of Al, then became stable in response to Al. Furthermore, endogenous ABA hardly changed when Al levels exceeded 25 μM (Fig. 5B). A larger Al-responsive increase of endogenous ABA was observed in Al-sensitive cv. Shishio than in Al-resistant cv. Suzunari. Pretreatment with K-252a failed to inhibit an increase in endogenous ABA triggered by Al in both Al-resistant and Al-sensitive cultivars (Fig. 5C).

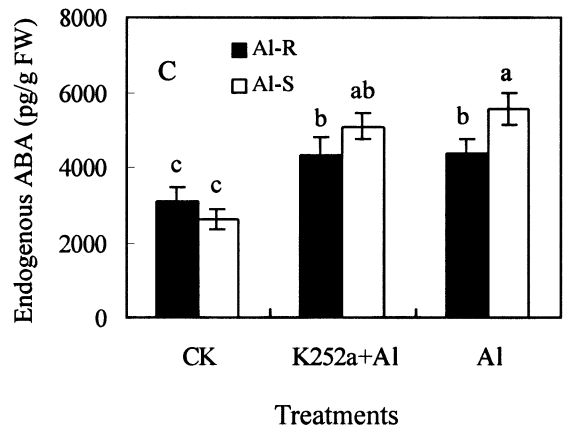
#### ABA and K-252a effects

Since Al treatment could increase the level of endogenous ABA, the possible role of elevated endogenous ABA was examined. In addition, the effects of many other phytohormone molecules on the Al-induced efflux of citrate were also investigated, which included gibberellin, indoleacetic acid, jasmonic acid, kinetin, and salicylic acid on the Al-induced efflux of citrate. However, only ABA



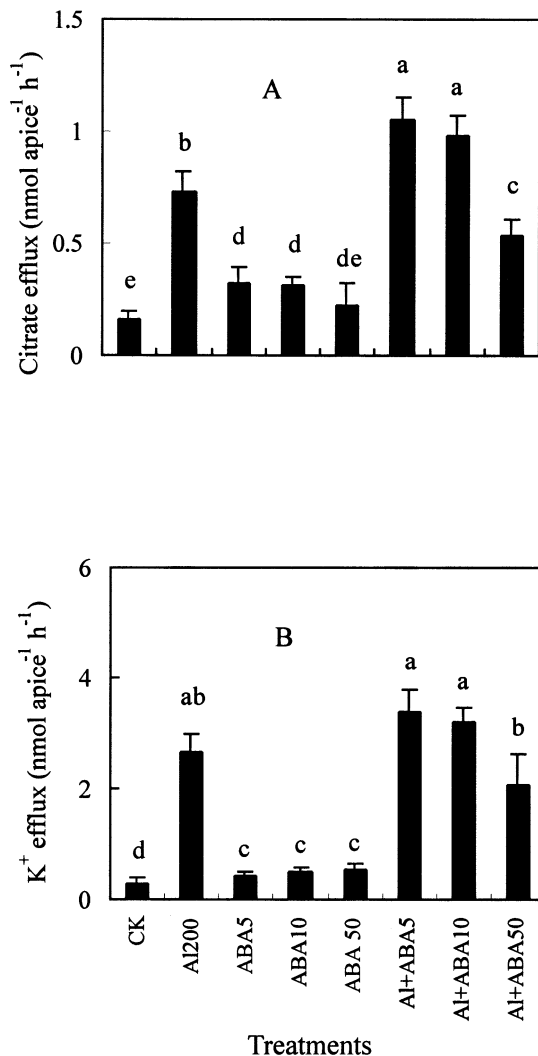
**Fig. 4.** Effect of K-252a on Al content in the root apices (A) and Al-induced inhibition of root elongation (B) in an Al-resistant soybean. (A) Intact roots of soybean were pretreated with 0.5 mM CaCl<sub>2</sub> solution containing 0, 1, 5, or 10 μM K-252a for 30 min and then exposed to 0.5 mM CaCl<sub>2</sub> solution containing 40 μM Al for 6 h. After treatment, root apices of 5 mm length were excised with a blade and used for analysis of Al content. Values are means ± SE ( $n=3$ ). (B) Intact roots were pretreated with 0.5 mM CaCl<sub>2</sub> solution containing 0 or 5 μM K-252a for 30 min, then exposed to 0.5 mM CaCl<sub>2</sub> solution containing 0, 20, or 40 μM Al for 24 h. pH in all solutions is 4.2. Root elongation was measured with a ruler before and after Al treatment. Values are means ± SE ( $n=8$ ). Different letters indicate significant difference at the 0.05 level, according to Duncan's multiple range test.

affected the citrate efflux, and the other hormone molecules just slightly influenced or had no effect on the citrate efflux in the presence or absence of Al (data not shown). Exogenous application of 5 μM ABA enhanced the Al-induced efflux of citrate from the root apices of cv. Suzunari seedlings by 42.7% (Fig. 6A). The corresponding value from the intact roots was 59.4% (Table 2). However, 50 μM ABA decreased the Al-induced efflux of citrate by 36.3% (Fig. 6A). ABA slightly increased K<sup>+</sup> efflux in comparison to Al treatment. Similar effects of ABA on citrate and K<sup>+</sup> efflux were observed in Fig. 6.

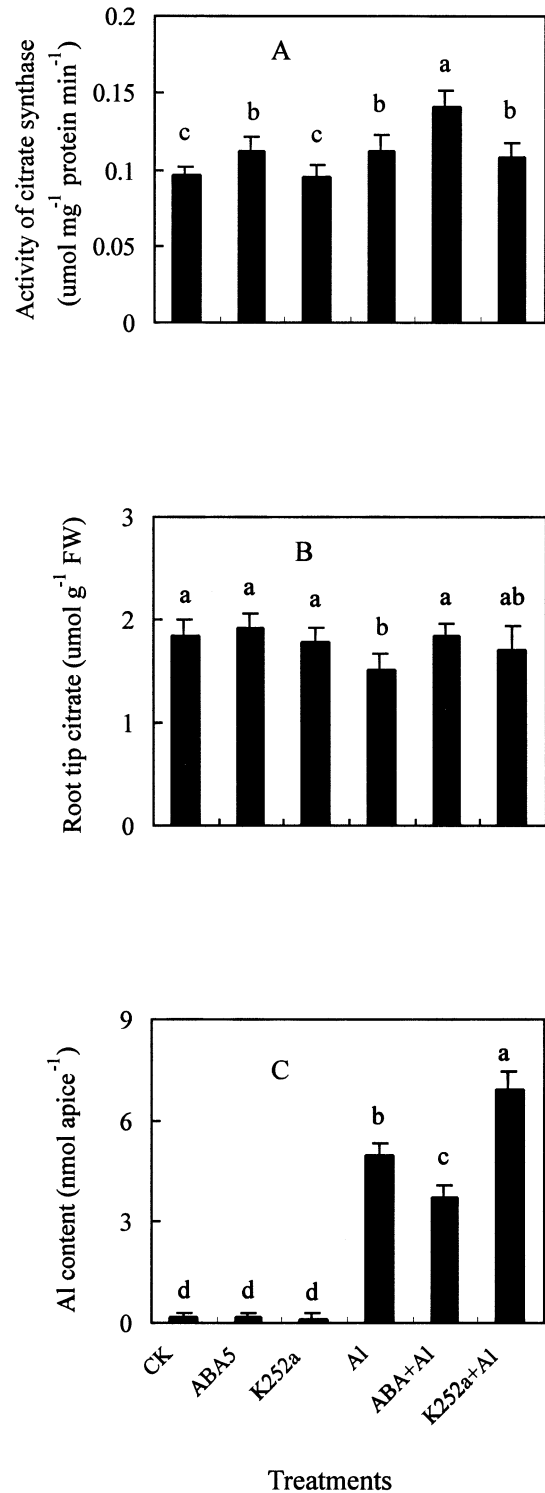


**Fig. 5.** Effects of Al and K-252a treatments on endogenous ABA in intact roots of soybean seedlings. (A) The intact roots of Suzunari seedlings were exposed to 0.5 mM CaCl<sub>2</sub> solution (pH 4.2) containing 0 or 40 μM AlCl<sub>3</sub>. The samples were collected after 1 h treatment. (B) Suzunari roots were exposed to 0.5 mM CaCl<sub>2</sub> solution containing 0, 25, 50, or 100 μM AlCl<sub>3</sub> for 6 h. (C) Intact roots of Suzunari (Al-resistant) and Shishio (Al-sensitive) seedlings were pretreated in the solution containing 5 μM K-252a for 30 min, then transferred to 0.5 mM CaCl<sub>2</sub> solution containing 50 μM AlCl<sub>3</sub> for 6 h. ABA was determined by ELISA method. Values are means ± SE ( $n=3$ ). Different letters indicate a significant difference at 0.05 level, according to Duncan's multiple range test.

Citrate content, activity of citrate synthase, and Al content in the 5 mm root apices of soybean seedlings were further investigated in response to ABA and K-252a treatments. Exogenous application of 5  $\mu\text{M}$  ABA increased Al-induced citrate synthase activity by 26.2% (Fig. 7A). Al treatment decreased the citrate content in the terminal 5 mm apices by 22.5%, while ABA treatments could compensate for Al effects (Fig. 7B). Exogenous application of ABA decreased Al content by 32.3% (Fig. 7C). K-252a had no effects on both citrate synthase activity and citrate content (Fig. 7A, B).



**Fig. 6.** Effects of ABA on citrate (A) and K<sup>+</sup> (B) efflux from the apices of Suzunari roots. Excised root apices of 5 mm length were pretreated with 0, 5, 10, or 50  $\mu\text{M}$  ABA in 0.2 mM CaCl<sub>2</sub> solution for 30 min, and then exposed to 0.2 mM CaCl<sub>2</sub> solution containing 0 or 200  $\mu\text{M}$  Al (pH 4.2) for 120 min. Ethanol concentration in the solution was less than 0.1%, which did not inhibit citrate and K<sup>+</sup> efflux. Values are means  $\pm$ SE ( $n=3$ ). Different letters indicate a significant difference at the 0.05 level, according to Duncan's multiple range test.



**Fig. 7.** Effects of ABA and K-252a on the activity of citrate synthase (A), citrate content (B), and Al content (C) in the root apices of Suzunari seedlings. For ABA treatment, the seedling roots were treated with 5  $\mu\text{M}$  ABA or 5  $\mu\text{M}$  ABA plus 40  $\mu\text{M}$  Al for 6 h. For K-252a treatment, the seedling roots were pretreated with 5  $\mu\text{M}$  K-252a for 30 min, and then exposed to 0 or 40  $\mu\text{M}$  Al for 6 h. Citrate synthase, citrate content, and Al content in 5 mm root apices were determined, respectively. Values are means  $\pm$ SE ( $n=3$ ). Different letters indicate significant difference at 0.05 level, according to Duncan's multiple range test.

**Table 2.** Effect of ABA and k-252a on the Al-induced citrate efflux and inhibition of root elongation in Al-resistant soybean

The seedling roots were pretreated with 0.5 mM CaCl<sub>2</sub> containing 0 or 5 μM ABA, or 5 μM K-252a for 30 min, and then placed in 0.5 mM CaCl<sub>2</sub> solution containing 0 or 40 μM Al, or 40 μM Al plus 5 μM K-252a, or 40 μM Al plus 5 μM ABA for 24 h. pH in all solutions is 4.2. After treatment, the solution was collected for assay of citrate. Values are means ±SE (n=3). Root elongation was measured with a ruler before and after Al treatment. Values are means ±SE (n=7).

Pretreatment	Treatment		Citrate efflux (nmol g <sup>-1</sup> FW 24 h <sup>-1</sup> )	Root elongation (cm 24 h <sup>-1</sup> )
	AlCl <sub>3</sub>	K-252a/ABA		
ABA/K252a				
–	–	–	26.6±5.80 c <sup>a</sup>	1.66±0.19 a
–	+	–	187.7±19.3 b	0.76±0.11 c
ABA	–	–	45.8±8.00 c	1.63±0.15 a
ABA	+	–	298.0±23.4 a	1.12±0.07 b
K-252a	+	–	30.6±4.60 c	0.43±0.05 d
ABA	+	K-252a	35.8±5.35 c	0.40±0.07 d
K-252a	+	ABA	42.2±12.1 c	0.42±0.08 d

<sup>a</sup> Different letters in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

ABA increased the Al-induced efflux of citrate, while K-252a suppressed the citrate efflux dramatically, the relationship between ABA and K-252a in the process of Al-induced efflux of citrate was studied in terms of citrate efflux and root elongation (Table 2). It was observed that pretreatment with ABA increased citrate efflux and root elongation in the presence of Al. While pretreatment with K-252a strongly blocked the Al-induced efflux of citrate and intensified the Al-induced inhibition of root elongation. Pretreatment or treatment with K-252a could abolish the effects of ABA on citrate efflux and root elongation. Moreover, the K-252a-suppressed citrate efflux and K-252a-strengthened inhibition of root elongation by Al were not reversed by ABA treatment (Table 2).

## Discussion

Two patterns of Al-induced efflux of organic acids have been identified (Ma *et al.*, 2001). In Pattern I, no discernible delay is observed between the addition of Al and the onset of organic acid efflux. By contrast, in Pattern II, organic acid efflux is delayed for several hours after exposure to Al. The efflux of citrate from the root apices of soybean seedlings was induced 30 min after the addition of Al suggesting Pattern I in soybean (Fig. 1). While a 4 h lag phase or induction period between Al addition and citrate efflux existed using intact roots of soybean seedlings (Yang *et al.*, 2001). These results suggested that two Patterns probably existed simultaneously in the Al-induced efflux of citrate from soybean roots. Short-term transcriptional and translational regulation of the Al-activated anion channel on the plasma membrane might be involved in the Al-induced efflux of citrate in soybean roots (Ma *et al.*, 2001; Yang *et al.*, 2001). Co-efflux of K<sup>+</sup> plays an important role in a charge-balance transport with citrate anions (Fig. 6A, B). Verapamil, TEA, and lanthanides (La<sup>3+</sup> and Yb<sup>3+</sup>) could not trigger the efflux of citrate, and anion channel inhibitors (niflumic acid, PG, and A9C)

significantly blocked the Al-induced citrate efflux (Table 1), suggesting that citrate is released from soybean via an anion channel and specific to Al stress.

Al probably alters the gating behaviour of anion channels via a series of secondary signals such as transient increases in plant hormones, cytoplasmic Ca<sup>2+</sup> concentrations, or protein phosphorylation (Haug *et al.*, 1994). The Al-induced citrate efflux ceased at low temperatures and was inhibited by metabolic inhibitors (Fig. 2), indicating that energy-dependent activities are required for the Al-induced efflux of citrate from soybean roots. In line with these results, Al-induced efflux of malate from wheat and citrate from barley also showed the involvement of an energy-dependent process (Osawa and Matsumoto, 2001; Zhao *et al.*, 2003).

Several studies indicated that protein phosphorylation was involved in the process of mediating the activity of anion channels and the induction of Al-responsive efflux of malate in wheat (Pei *et al.*, 1997; Osawa and Matsumoto, 2001). K-252a effectively blocked the Al-induced efflux of citrate from root apices of soybean (Table 1; Fig. 3). Furthermore, pretreatment with K-252a could promote Al content and intensify the Al-dependent inhibition of root elongation (Fig. 4), indicating that some K-252a-sensitive protein kinases might be involved in a direct phosphorylation of an anion channel or phosphorylation of related protein(s), which regulates the activity of anion channels and, finally, leads to citrate efflux. K-252a is an inhibitor of a broad range of protein kinases, though it remains speculative whether increased Al accumulation and inhibition of root elongation were due to the decrease of citrate efflux or other Al exclusion mechanisms that are sensitive to K-252a, the results of this study provide one possibility that phosphorylation-dependent citrate efflux is able to decrease the Al accumulation and to restrain Al-induced root growth inhibition.

ABA, known as a stress-inducible phytohormone, has been shown to enhance the adaptability of plants to various

types of stress (Zeevaert and Creelman, 1988; Kasai *et al.*, 1993). The activation of ABA-responsive protein kinase (ABR\* kinase) was dependent on the time and concentration of ABA (Mori *et al.*, 2000). Increased activity of ABR\* kinase could enhance ABA signal transmission (Schmidt *et al.*, 1995), and this transmission could lead to protein phosphorylation (Pei *et al.*, 1997; Osawa and Matsumoto, 2001) and regulate gene expression (Leung and Giraudat, 1998). Al-induced increase in endogenous ABA (Fig. 5) and increased citrate efflux due to exogenous ABA application (Fig. 6A; Table 2) suggested that the Al signal might be mediated by the ABA signal transduction pathway, and the ABA signal transduction pathway was involved in the regulation of Al-induced efflux of citrate in soybean roots. Al as an initial stimulus might switch on some molecular responses and require several endogenous signal components such as ABA, protein kinases or other phytohormones to transmit its signal. The application of exogenous ABA might amplify the Al signal and thus intensify the subsequent physiological responses, which finally results in citrate efflux.

Citrate synthase activity and citrate accumulation in root tips were reported to be associated with Al-induced citrate efflux (Li *et al.*, 2000; Yang *et al.*, 2001). ABA increased both citrate synthase activity and citrate content in the root apices, while K-252a could not (Fig. 7A, B), suggesting that ABA rather than K-252a was involved in regulating the process of citrate production. In guard cells, ABA could activate a 48 kDa protein kinase (Mori and Muto, 1997). In the present study, the mechanism by which ABA promoted the Al-induced efflux of citrate remains unknown. However, it may be possible that ABA is involved in the activation of several other downstream components in the ABA signal transduction pathway. These assumed components should mediate the ABA signal as well as modify the activity of anion channel for citrate efflux. Pei *et al.* (1997) found that the ABA-induced activation of anion channels is under the control of K-252a-sensitive protein kinases. In this study, K-252a strongly inhibited Al-induced efflux of citrate (Fig. 3; Table 1), but failed to block the Al-induced increase in endogenous ABA (Fig. 5C). ABA-induced increases in citrate efflux and root elongation were strongly suppressed by K-252a, while pretreatment or treatment with ABA could not reverse the inhibitory effect of K-252a on citrate efflux and root elongation (Table 2). Taken together, these results suggested that ABA was probably involved in the early response to the Al signal, after which, K-252a-sensitive protein kinases play a key step in regulating the activity of anion channels on the plasma membrane, through which citrate was released from the apical cells of soybean roots. The signal transduction pathway participating in anion channel activation, as well as the elucidation of additional physiological mechanisms conferring Al resistance are subjects for future research.

## Acknowledgements

This research was supported by the Program for the Promotion of Basic Research Activities in Innovative Biosciences (PROBRAIN) to HM, Grant-in-Aid for General Research (A) (grant no. 14206008) from the Ministry of Education, Science, Sports and Culture of Japan to HM, the Ohara Foundation for Agricultural Sciences and Postdoctoral Fellowships from Japan Society for the Promotion of Science (JSPS) to HS.

## References

- Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- Chandra S, Low PS.** 1995. Role of phosphorylation in elicitation of the oxidative burst in cultured soybean cells. *Proceedings of the National Academy of Sciences, USA* **92**, 4120–4123.
- Delhaize E, Ryan PR, Randall PJ.** 1993. Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiology* **103**, 695–702.
- Haug A, Shi B, Vitrorello V.** 1994. Aluminum interaction with phosphoinositide—associated signal transduction. *Archives of Toxicology* **68**, 1–7.
- Johnson JF, Vance CP, Allan DL.** 1994. Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. *Plant Physiology* **104**, 657–665.
- Kasai M, Sasaki M, Tanakamaru S, Yamamoto Y, Matsumoto H.** 1993. Possible involvement of abscisic acid in increases in activities of two vacuolar H<sup>+</sup>-pumps in barley roots under aluminum stress. *Plant Cell Physiology* **34**, 1335–1338.
- Kochian LV.** 1995. Cellular mechanisms of aluminum toxicity and resistance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 237–260.
- Leung J, Giraudat J.** 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 199–222.
- Li XF, Ma JF, Matsumoto H.** 2000. Pattern of Al-induced secretion of organic acids differs between rye and wheat. *Plant Physiology* **123**, 1537–1543.
- Liu K, Luan S.** 2001. Internal aluminum block of plant inward K<sup>+</sup> channels. *The Plant Cell* **13**, 1453–1466.
- Ma JF, Ryan PR, Delhaize E.** 2001. Aluminum tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* **6**, 273–278.
- Ma JF, Zheng SJ, Matsumoto H, Hiradate S.** 1997. Detoxifying aluminum with buckwheat. *Nature* **390**, 569–570.
- Ma Z, Miyasaka SC.** 1998. Oxalate exudation by taro in response to Al. *Plant Physiology* **118**, 861–865.
- Matsumoto H, Senoo Y, Kasai M, Maeshima M.** 1996. Response of the plant root to aluminum stress: analysis of the inhibition of the root elongation and changes in membrane function. *Journal of Plant Research* **109**, 99–105.
- Mertens R, Neumann D, Weiler EW.** 1983. Monoclonal antibodies for the detection and quantitation of the endogenous plant growth regulator, abscisic acid. *FEBS Letters* **160**, 269–272.
- Mori IC, Muto S.** 1997. Abscisic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. *Plant Physiology* **113**, 587–594.
- Mori IC, Uozumi N, Muto S.** 2000. Phosphorylation of the inward-rectifying potassium channel KAT1 by ABR kinase in *Vicia* guard cells. *Plant Cell Physiology* **41**, 850–856.
- Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J.** 2002. *Arabidopsis* OST1 protein kinase mediates the regulation of

- stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *The Plant Cell* **14**, 3089–3099.
- Osawa H, Matsumoto H.** 2001. Possible involvement of protein phosphorylation in aluminum-responsive malate efflux from wheat root apex. *Plant Physiology* **126**, 411–420.
- Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI.** 1997. Differential abscisic acid regulation of guard cell slow anion channels in arabidopsis wild-type and *abi1* and *abi2* mutants. *The Plant Cell* **9**, 409–423.
- Piñeros MA, Kochian LV.** 2001. A patch-clamp study on the physiology of aluminum toxicity and aluminum tolerance in maize: identification and characterization of Al<sup>3+</sup>-induced anion channels. *Plant Physiology* **125**, 292–305.
- Ryan PR, Delhaize E, Randall PJ.** 1995. Characterization of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. *Planta* **196**, 103–110.
- Ryan PR, Skerrett M, Findlay GP, Delhaize E, Tyerman SD.** 1997. Aluminum activates an anion channel in the apical cells of wheat roots. *Proceedings of the National Academy of Sciences, USA* **94**, 6547–6552.
- Schmidt C, Schelle I, Liao YJ, Schroeder JI.** 1995. Strong regulation of slow anion channels and abscisic acid signalling in guard cells by phosphorylation and dephosphorylation events. *Proceedings of the National Academy of Sciences, USA* **92**, 9535–9539.
- Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D.** 2001. Guard cell signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 627–658.
- Staxen I, Pical C, Montgomery L, Gray J, Hetherington AM, McAinsh MR.** 1999. Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proceedings of the National Academy of Sciences, USA* **96**, 1779–1784.
- Wu Y, Kuzma J, Marechal E, Graeff R, Lee HC, Foster R, Chua NH.** 1997. Abscisic acid signalling through cyclic ADP-Ribose in plants. *Science* **278**, 2054–2055.
- Yang ZM, Nian H, Sivaguru M, Tanakamaru S, Matsumoto H.** 2001. Characterization of aluminum-induced citrate secretion in aluminum-tolerant soybean (*Glycine max* L.) plants. *Physiologia Plantarum* **113**, 64–71.
- Zeevaart JAD, Creelman RA.** 1988. Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* **39**, 439–473.
- Zhang WH, Ryan PR, Tyerman SD.** 2001. Malate-permeable channels and cation channels activated by aluminum in the apical cells of wheat roots. *Plant Physiology* **125**, 1459–1472.
- Zhao ZQ, Ma JF, Sato K, Takeda K.** 2003. Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). *Planta* **217**, 794–800.