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Aluminum Tolerance Genes on the Short Arm of Chromosome 3R Are Linked to Organic Acid Release in Triticale[1]

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Triticale, a hybrid between wheat and rye, shows a high degree of Al tolerance that is inherited from rye, but the mechanisms of high Al tolerance in both rye and triticale are unknown. We found that the short arm of chromosome 3R carries genes necessary for Al tolerance in triticale (× *Triticosecale* Wittmark cv Currency). Detailed comparative studies with a 3DS.3RL translocation line (ST22) and a non-substitution line (ST2) were conducted. Root elongation was similarly inhibited by Al in ST2 and ST22 during the first 12 h of Al treatment, but more strongly in ST22 than in ST2 at 18 h and thereafter. The root inhibition induced by other metals (Cu, Cd, and La) was similar between ST2 and ST22, suggesting that the action of the genes for Al tolerance on the short arm of triticale chromosome 3R is highly specific to Al. A 2-fold larger amount of malate and citrate was released from the roots of ST2 than from ST22 at 12 and 18 h after Al treatment, respectively. The marked lag phase in the inhibition of root elongation and the release of organic acids implies that the expression of genes on the short arm of triticale chromosome 3R is induced by Al, and that these genes are necessary for the release of organic acids.

Genetic variation in response to Al toxicity has been found among plant species and even among cultivars within the same species (Foy and Fleming, 1978; Aniol and Gustafson, 1984). The tribe Triticeae of the grass family, Poaceae (Gramineae), includes some of the most important grain cereal crops, including wheat, barley, rye and triticale. The Al tolerance of Triticeae generally follows the order of rye ≥ triticale > wheat > barley (Aniol and Gustafson, 1984). Triticale is a synthetic wheat/rye hybrid that is largely grown on acid soils in Europe, South America, and Australia (Pfeiffer, 1993). Its Al tolerance is considered to be inherited from rye; however, the mechanisms responsible for the high Al tolerance in both rye and triticale are not understood.

Al toxicity is a major factor limiting crop production in acid soils, which comprise about 40% of the arable land in the world (Foy et al., 1978). Thus, attempts to select and develop crop varieties with greater tolerance to Al toxicity have been made. One technique is cytogenetic manipulation, i.e. the addition, substitution, or translocation of alien chromosomes. This technique has been successfully used to breed new varieties with high stress tolerance. For example, chromosome $5E^b$ of *Thinopyrum bessarabicum* carries genes controlling salt tolerance, and the addition of this chromosome to wheat (cv Chinese Spring) increased the tolerance to salt stress (Forster et al., 1988). Al tolerance is genetically controlled (Chaubey and Senadhira, 1994). Therefore, it is possible to develop new crop varieties with higher Al tolerance using a cytogenetic manipulation approach. For this purpose, it is important to know which chromosomes in plants contain genes for Al tolerance. In hexaploid wheat, major genes influencing tolerance to Al are located on the short arm of chromosome 5A and the long arms of chromosome 2D and 4D (Takagi et al., 1983; Aniol and Gustafson, 1984; Aniol, 1990). Using wheat-rye addition lines, major genes influencing Al tolerance in rye were located on chromosomes 3R, 4R, and the short arm of 6R (Aniol and Gustafson, 1984). However, the physiological processes controlled by these genes are still unknown. In this study, chromosome substitution lines of wheat-triticale were prepared and the chromosomal arm location of genes necessary for Al tolerance in triticale was identified. The mechanisms of Al tolerance were examined in terms of release of organic acids from the roots by comparing an Al-sensitive translocation line with a non-substitution triticale line.

MATERIALS AND METHODS

Breeding Procedures of Cytological Stocks

F_1 hybrids between hexaploid triticale (× *Triticosecale* Wittmack cv Currency) and wheat (*Triticum aestivum* L. cv Chinese Spring) were backcrossed to the triticale parent and the population (BC_1F_1) was raised. The BC_1F_1 population was then self-pollinated for three generations to generate BC_1F_4. In the BC_1F_4 generation, plants with 21 pairs of chromosomes were selected by observing metaphase I chromosome pairing under a microscope. Chromosome constitutions of these plants were determined by C-banding (Taketa et al., 1991), and in some cases genomic in situ hybridization (Taketa et al., 1997) was performed. For genomic in situ hybridization, biotin-labeled total genomic DNA from rye and non-labeled sheared genomic DNA from wheat were used as a probe and a block, respectively,

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and then counterstained with propidium iodide. In the present study, 11 lines in which R- or B-genome triticale chromosomes were substituted by D-genome wheat chromosomes were used (Table I). A line without chromosome substitution (ST2) derived from the above crosses was also used for comparison.

Screening for Al Tolerance

The screening system and procedures for Al tolerance were the same as those described previously (Ma et al., 1997b). Seeds were surface-sterilized by soaking in 0.5% NaClO solution for 20 min and then placed in deionized water for 6 h after rinsing. The seeds were allowed to germinate on moist filter paper in Petri dishes for 2 d at 20°C. Germinated seedlings (root length of approximately 2 cm) of each line were transferred to a net cup (five seedlings for each cup), which was made to just be in contact with the surface of solution (1 mM $CaCl_2$, pH 4.5) in the 50-mL plastic syringe (TerumoR, Tokyo). The solution was renewed daily. After 3 d, the roots were exposed to 1 mM $CaCl_2$ solution containing 5, 10, 20, or 40 μM Al at pH 4.5. The solution of $AlCl_3 \cdot 6H_2O$ was freshly prepared before treatment. After a 24-h treatment with Al, the roots were placed in distilled water for 30 min and then stained with a 0.1% (w/v) aqueous solution of Eriochrome Cyanine R for 10 min. Excess dye was removed by washing with distilled water. Finally, the staining pattern of root tips were recorded and scored to five ranks: 1, highly tolerant; 3, tolerant; 5, intermediate; 7, sensitive; and 9, highly sensitive, according to the staining pattern at various Al concentrations (Ma et al., 1997b). This screening was performed twice independently. Plants were grown in a controlled-environment growth cabinet (TGE-9H-S, TABAI ESPEC, Osaka) with a 14-h/25°C day and 10-h/20°C night regime and a light intensity of 40 W m^{-2}.

Table I. *Al tolerance of chromosome substitution lines of wheat-triticale*

Screening was conducted according to the method of Ma et al. (1997b). In chromosome constitution, only chromosomes substituted are presented. For example, 1D (1B) means that triticale chromosome 1B is substituted by wheat chromosome 1D.

Line	Chromosome Constitution	Al Tolerance[a]
Wheat-triticale substitution line		
ST2	Non-substitution	3
ST5	1D (1B)	3
ST6	6D (6B)	3
ST7	4D (4B), 5RL	3
ST9	1D (1R)	3
ST11	2D (2R)	3
ST14	3D (3R)	5
ST15	5D (5R)	3
ST16	6D (6R)	3
ST18	2D (2R), 5D (5R)	3
ST19	3D (3R), 6D (6R)	5
ST22	T 3DS.3RL[b]	7
Parents		
Triticale	AABBRR	3
Wheat	AABBDD	5

[a]1, Highly tolerant; 3, tolerant; 5, intermediate; 7, sensitive; 9, highly sensitive. [b]T, A translocated chromosome consisting of 3DS and 3RL.

Al Treatment and Root Elongation Measurement

A translocation line (3DS.3RL) (ST22) and a non-substitution line (ST2) were used in the following experiments. In the translocation line (ST22), the short arm of triticale chromosome 3R is substituted with the short arm of wheat chromosome 3D. Seeds were surface-sterilized as described above and then germinated on filter paper saturated with a 0.5 mM $CaCl_2$ (pH 4.5) solution in a Petri dish for 2 d. The seedlings were transferred to a net that was floated on 0.5 mM $CaCl_2$ (pH 4.5) solution in a 2-L plastic container. After 3 d, selected seedlings of similar size were exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution with 20 μM $AlCl_3$ or without Al. Ten replicates were made for each treatment. Root lengths were measured with a ruler at various time points, as shown in Figure 2, and root elongation was calculated based on root length.

In another experiment, seedlings similar to those described above were exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 10, 30, or 50 μM $AlCl_3$ for 24 h. Root lengths were measured at 0 and 24 h. After the treatment, the roots were placed in 0.5 mM $CaCl_2$ (pH 4.5) solution for 15 min, and then the root apex (1 cm) was excised with a razor blade. The method for the determination of Al is described below.

Determination of Al Accumulation in Root Apexes

Excised root apexes (10 for each sample) were placed in a plastic tube (1.5 mL) and 1 mL of 2 N HCl was added. The tubes stood for at least 24 h with occasional shaking. The Al concentration in the solution was determined after appropriate dilution by graphite furnace atomic absorption spectrophotometry (model Z-9000, Hitachi, Tokyo). A predetermination showed that the Al content of root apexes extracted with 2 N HCl was the same as that digested with concentrated HNO_3.

Tolerance to Acid Soil

To confirm the Al sensitivity of the 3DS.3RL translocation line, we planted 10 seeds each of both the 3DS.3RL line (ST22) and the non-substitution line (ST2) directly on an acid soil (pH [H_2O], 4.4) or slightly acid soil (pH [H_2O], 6.5). The acid soil was a non-allophane Andosol (chloritized 2:1 mineral soil), which is distributed in northeastern Japan. A slightly acid soil sample was obtained from the farm of the Research Institute for Bioresources, Okayama University at Kurashiki City. The soil moisture was kept at their field capacity by watering with distilled water daily. After being kept for 6 d in the growth cabinet described above, the roots were carefully taken out of the soil and photographed on color film (ASA 400, Fuji Photo Film, Tokyo). The roots were washed with tap water and stained in 0.1% (w/v) Eriochrome Cyanine R solution as described above.

Metal Specificity Study

Five-day-old seedlings prepared as described in "Al Treatment and Root Elongation Measurement" were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 (−Al), 20 μM Al, 100 μM Cd, 5 μM Cu, or 20 μM La, all of which were the chloride salts of these metals. Root lengths were measured with a ruler before and after the treatments.

Collection of Root Exudates and Determination of Organic Acids

Seedlings prepared as described in "Al Treatment and Root Elongation Measurement" were transplanted to a 1-L plastic pot (12 seedlings per pot) containing aerated nutrient solution. We used one-fifth-strength Hoagland solution containing the following macronutrients: 1.0 mM KNO_3; 1.0 mM $Ca(NO_3)$; 0.4 mM $MgSO_4$; and 0.2 mM $NH_4H_2PO_4$; and the following micronutrients: 20 μM NaFeEDTA; 3 μM H_3BO_3; 0.5 μM $MnCl_2$; 0.2 μM $CuSO_4$; 0.4 μM $ZnSO_4$; and 1 μM $(NH_4)_6Mo_7O_{24}$. The solution was adjusted to pH 4.5 with 1 M HCl and renewed every other day. After a 2-week culture in the above nutrient solution, the roots were placed in 0.5 mM $CaCl_2$ solution at pH 4.5 overnight for cleaning the roots and then exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 50 μM $AlCl_3$. Root exudates were collected and the treatment solution renewed every 6 h. In a dose-response experiment, the seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 10, 30, or 50 μM $AlCl_3$. Root exudates were collected over 24 h.

The root exudates were passed through a cation exchange resin column (16 × 14 mm) filled with 5 g of Amberlite IR-120B resin (H^+ form), followed by an anion-exchange resin column (16 X 14 mm) filled with 2 g of AG 1X8 resin (100–200 mesh, format form) in a cold room. The organic acids retained on an anion-exchange resin were eluted with 1 M HCl, and the eluate was concentrated to dryness with a rotary evaporator (40°C). After the residue was redissolved in dilute $HClO_4$ solution (pH 2.1), the concentration of organic acids was analyzed by HPLC (Ma et al., 1997c).

RESULTS

The screening system for Al tolerance developed by Ma et al. (1997b) classifies Al tolerance into five ranks, from highly tolerant to highly sensitive. Using this system, the Al tolerance of 11 chromosome substitution lines of wheat-triticale, a non-substitution line, and their parents, triticale (cv Currency) and wheat (cv Chinese Spring), were examined. Root tips of the triticale parent were stained with Eriochrome Cyanine R only at 40 μM Al and ranked as tolerant (score 3), while the wheat root tips were stained from 20 μM Al and ranked as intermediate (score 5) (Table I). Substitution of triticale chromosomes 1B, 4B, 6B by homoeologous D-genome wheat chromosomes did not affect Al tolerance. Substitution of triticale chromosomes 1R, 2R, 5R, 6R by homoeologous D-genome wheat chromosomes also did not cause any change in Al tolerance (Table I). However, the substitution of triticale chromosome 3R by wheat chromosome 3D decreased Al tolerance from tolerant (score 3) to intermediate (score 5). In particular, line (3DS. 3RL), in which the short arm of triticale chromosome 3R was substituted by the short arm of wheat chromosome 3D, Al tolerance was dramatically decreased to a sensitive rank (score 7). These results clearly indicated that genes necessary for the complete expression of Al tolerance in triticale are located on the short arm of chromosome 3R.

The mitotic metaphase chromosomes of a non-substitution line (ST2) and a 3DS.3RL translocation line (ST22) are shown in Figure 1. In ST22, a pair of translocated chromosomes 3DS.3RL were clearly indentified by genomic in situ hybridization.

To characterize the physiological process expressed by the genes on the short arm of chromosome 3R that influence Al tolerance, we compared the root response to Al of the 3DS.3RL translocation line (ST22) with that of a non-substitution line (ST2). Root elongation of both lines was similar in the absence of Al (Fig. 2). In the presence of Al

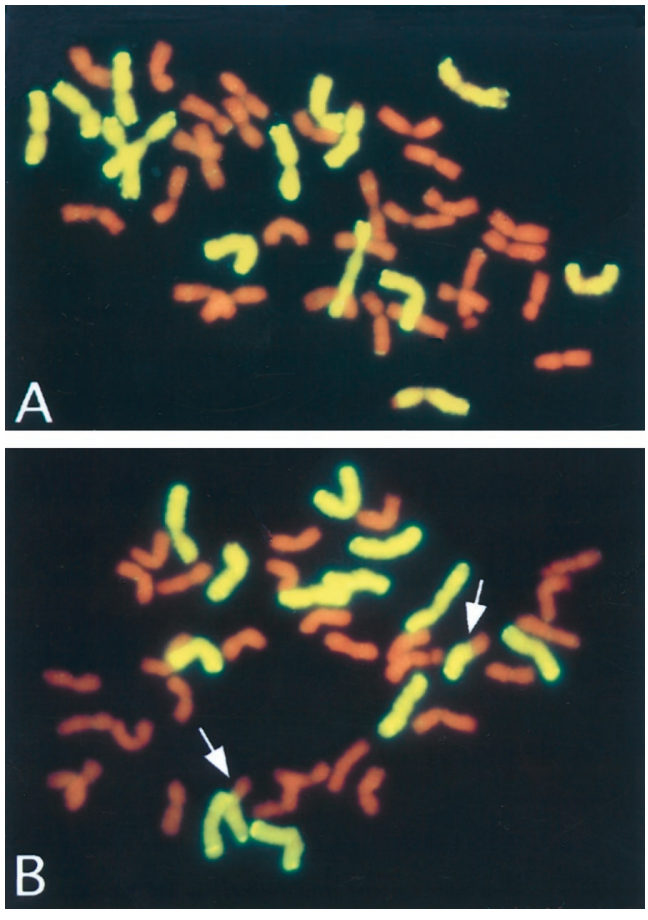

Figure 1. Genomic in situ hybridization to mitotic chromosomes of ST2 (A) and ST22 (B) using biotin-labeled total genomic DNA from rye as a probe (fluorescing yellow) and non-labeled sheared genomic DNA from wheat as a block, counterstained with propidium iodide (red). ST2 is a non-substitution line with 14 rye chromosomes, while ST22 is a translocation line (wheat-triticale ditelosomic substitution line) in which the short arm of triticale chromosome 3R is replaced by the short arm of wheat chromosome 3D. Arrows in B indicate 3DS.3RL translocated chromosomes.

(20 μM), root elongation of both lines was inhibited (Fig. 2). The Al-induced inhibition was similar in both ST2 and ST22 lines during the first 12 h, but was stronger in ST22 than in ST2 at 18 h and thereafter. At 24 h, the root elongation of ST22 was inhibited by 56.1%, and that of ST2 by 26.1%. At 72 h, the root elongation of ST22 and ST2 was inhibited by 79.4% and 38%, respectively (Fig. 2).

Root elongation of ST22 during the 24-h period was inhibited by 43.9%, 66.5%, and 68.9% at 10, 30, and 50 μM Al, respectively (Fig. 3), while that of ST2 was inhibited by 19.4%, 41.6%, and 50%, respectively. This means that root elongation in ST22 and ST2 was inhibited at a similar level by 10 and 50 μM Al, respectively. The Al content in root apexes (1 cm) of ST22 was two to three times higher than that of ST2 at all Al concentrations tested (Fig. 4).

To confirm the Al sensitivity of ST22 due to substitution of the short arm of chromosome 3R, we grew both ST2 and ST22 lines in an acid soil. No difference in root growth was found between ST2 and ST22 lines grown in soil at pH 6.5 (Fig. 5). However, when grown in an acid soil with pH 4.4, root growth of ST22 was significantly inhibited and that of ST2 was not. The root tips of ST22 grown in the acid soil were heavily stained with Eriochrome Cyanine R, but those of ST2 were hardly stained (data not shown). Acid soil may limit plant growth not only by releasing Al, but also by other changes such as phosphorus deficiency, Mn toxicity, and H^+ toxicity, depending on soils. However, the major limiting factor of crop production on the soil used in this study is Al toxicity (Saigusa et al., 1980) and, furthermore, since the growth period was very short (6 d after sowing), the nutritional requirement for root growth could be entirely met by seed reserves. Therefore, the difference in the root growth seen in Figure 5 can be attributable to the different tolerance to Al toxicity of ST2 and ST22 lines, confirming the results obtained in simple salt solution (Table I; Figs. 2 and 3).

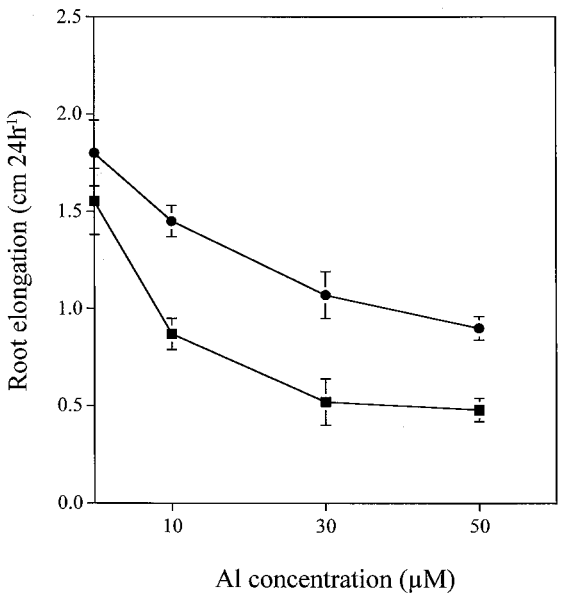

Figure 3. Effect of various Al concentrations on root elongation in a 3DS.3RL translocation line (ST22; ■) and a non-substitution line (ST2; ●). Five-day-old seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 10, 30, or 50 μM $AlCl_3$ for 24 h. The root length was measured before and after the treatment. Vertical bars represent ±SD (n = 10).

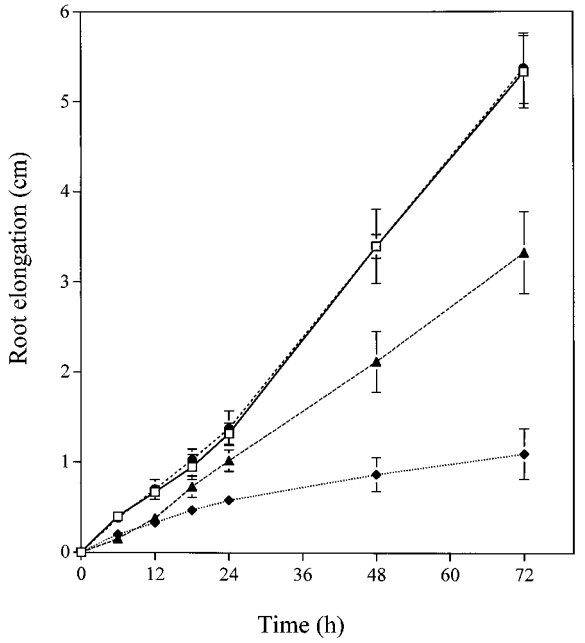

Figure 2. Effect of Al on root elongation in a 3DS.3RL translocation line (ST22) and a non-substitution line (ST2). Five-day-old seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing no Al or 20 μM $AlCl_3$. The root length was measured periodically. Vertical bars represent ±SD (n = 10). □, ST22 (−Al); ◆, ST22 (+Al); ●, ST2 (−Al); ▲, ST2 (+Al).

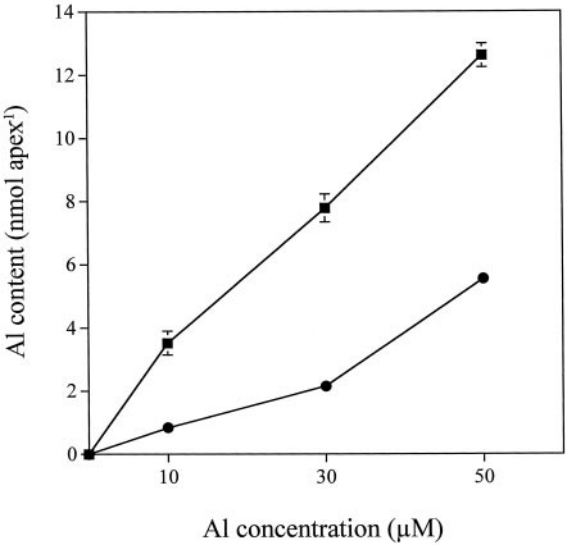

Figure 4. Al content in root apex of a 3DS.3RL translocation line (ST22; ■) and a non-substitution line (ST2; ●). Five-day-old seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 10, 30, or 50 μM $AlCl_3$ for 24 h, and the root apexes (1 cm) were excised. The Al concentration was determined by graphite furnace atomic absorption spectrophotometry. Vertical bars represent ±SD (n = 3).

Figure 5. Tolerance of a 3DS.3RL translocation line (ST22) and a non-substitution line (ST2) to acid soil. Both lines were grown in non-allophane Andosol (pH 4.4) or slightly acid soil (pH 6.5) for 6 d.

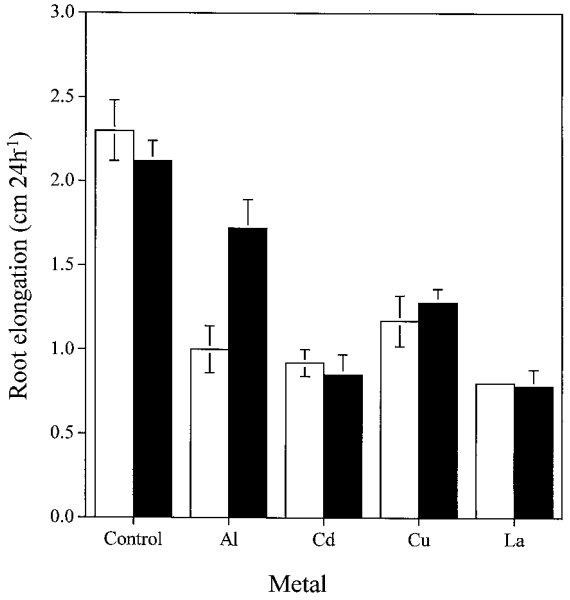

Figure 6. Effect of metals on root elongation in a 3DS.3RL translocation line (ST22; white bars) and a non-substitution line (ST2; black bars). Five-day-old seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 20 μM Al, 100 μM Cd, 5 μM Cu, or 20 μM La in their chloride form for 24 h. The root length was measured before and after the treatment. Vertical bars represent ±SD (n = 10).

The effect of Cd, Cu, and La on root elongation was compared with that of Al. Exposure to Cd, Cu, and La also caused inhibition of root elongation in both the ST2 and the ST22 lines (Fig. 6). No significant differences in the tolerance to Cd, Cu, or La were found between lines ST2 and ST22, although tolerance to Al differed greatly (Fig. 6).

Both citrate and malate were detected in root exudates of both lines exposed to Al (Fig. 7). In the present study, the rate of organic acid efflux (nmol 6 h^{-1} g^{-1} root dry weight) was shown in Figure 7 rather than the cumulative amount of organic acids, which was used in most previous studies (e.g. Delhaize et al., 1993; Ryan et al., 1995; Ma et al., 1997a, 1997c). There was no significant difference between the two lines in the rate of malate efflux at 6 h (Fig. 7B) and the rate of citrate efflux at 12 h (Fig. 7A) after exposure to Al; the rate of both malate and citrate efflux was low. However, at

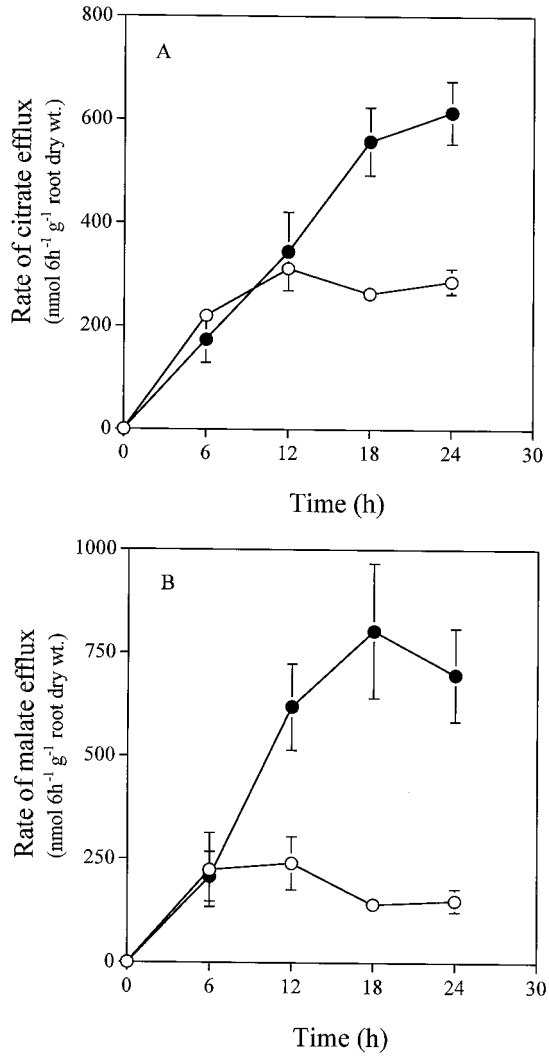

Figure 7. Time course of Al-induced release of citrate (A) and malate (B) in a 3DS.3RL translocation line (ST22; ○) and a non-substitution line (ST2; ●). Seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 50 μM $AlCl_3$. Root exudates were collected every 6 h after initiation of Al treatment. Organic acids were analyzed by HPLC. Vertical bars represent ±SD (n = 3).

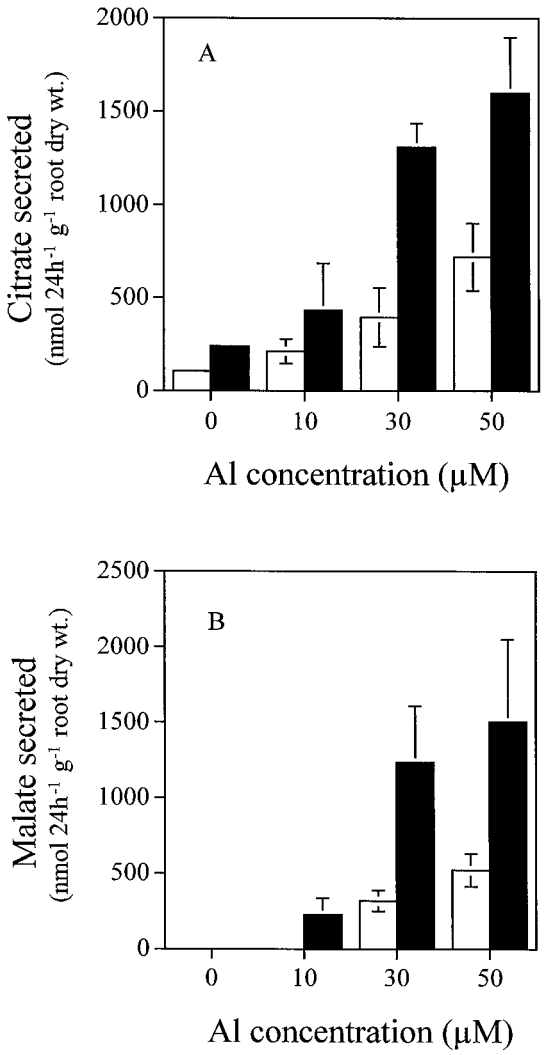

Figure 8. Effect of various Al concentrations on the release of citrate (A) and malate (B) in a 3DS.3RL translocation line (ST22; white bars) and a non-substitution line (ST2; black bars). Seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 10, 30, or 50 μM $AlCl_3$. Root exudates were collected for 24 h, and organic acids were analyzed by HPLC. Vertical bars represent ±SD (n = 3).

18 h, the rate of citrate efflux of ST2 increased 1.62 times compared with that at 12 h (Fig. 7A), while the rate of citrate efflux in the ST22 line did not increase or decrease. The rate of malate efflux of ST2 also increased after 6 h, and was 2.6-fold higher than that of ST22 at 12 h, and a high level was maintained thereafter (Fig. 7B).

A dose-response experiment showed that both malate and citrate secreted during 24-h period increased with increasing in Al concentrations in both the ST2 and the ST22 line (Fig. 8). However, the amount of citrate and malate secreted from ST2 was two times larger than that from ST22 at all Al concentrations tested (Fig. 8).

DISCUSSION

Hexaploid triticale consists of A, B, and R genomes. The A and B genomes are from wheat, and the R genome is from rye, which usually shows a higher tolerance to mineral stress. Rye is the species most tolerant to Al toxicity among the tribe Triticeae (Aniol and Gustafson, 1984), and this tolerance is also expressed in triticale. Gallego and Benito (1997) found that Al tolerance is controlled by at least two major dominant and independent loci in rye (*Alt1* and *Alt3*). DNA markers linked to Al tolerance loci were also selected in rye (Gallego et al., 1998). However, neither the mechanisms of Al tolerance nor the link between Al-tolerant genes and physiological processes have been elucidated in rye and triticale. To understand the role of R-genomes in Al tolerance, we developed a set of chromosome substitution lines by crossing triticale (cv Currency) and wheat (cv Chinese Spring). Such substitution lines are not uniform in the genetic background of A and B genome chromosomes. However, in the present study, the participation of A and B genome chromosomes in Al tolerance appears to be negligible because a non-substitution line with recombined A and B genomes (ST2) and the triticale parent showed a similar Al tolerance (Table I).

Screening results of Al tolerance clearly showed that the genes required for the complete expression of Al tolerance in triticale were located on the short arm of chromosome 3R (Table I). This is in agreement with previous findings with wheat-rye addition lines that one of major genes for Al tolerance in rye is located on chromosome 3R (Aniol and Gustafson, 1984). The genes on chromosome 4R and the short arm of chromosome 6R are also necessary for Al tolerance in rye (Aniol and Gustafson, 1984), but in the present study the substitution of chromosome 6R by homoeologous D-genome chromosomes did not cause any change in Al tolerance (Table I). This discrepancy may be attributable to the different parents used for crossing and to different approaches used (addition or substitution lines).

To link the Al-tolerance genes on the short arm of chromosome 3R with physiological processes, detailed comparative studies were conducted using the 3DS.3RL translocation line (ST22) and a non-substitution line (ST2). A dose-response experiment showed that root elongation inhibition in ST2 at 50 μM is comparable to that of ST22 at 10 μM (Fig. 3). The tolerance of ST2 to acid soil was much higher than that of ST22 (Fig. 5). All of these results confirmed that genes critical to the full expression of Al tolerance in triticale were located on the short arm of chromosome 3R (Table I). Interestingly, there was no significant difference in Al-induced root inhibition between ST2 and ST22 until 12 h after Al treatment, but a significant difference appeared at 18 h and thereafter (Fig. 2). This suggests that some Al-tolerance mechanisms were induced during 0 to 12 h in the ST2 line.

Because the Al content in the root apex (1 cm) of ST2 was less than one-half of that of ST22 at any Al concentration tested, Al-excluding mechanisms seem to be operating in the ST2 line. Several mechanisms for exclusion of Al from the root apex have been proposed (Delhaize and Ryan, 1995; Kochian, 1995). Recently, more and more evidence has accumulated showing that the release of organic acids with Al-chelating capacity is involved in the Al exclusion mechanism (e.g. Miyasaka et al., 1991; Delhaize et al., 1993;

Basu et al., 1994; Pellet et al., 1995; Ryan et al., 1995; Ma et al., 1997a, 1997c). We therefore compared the Al-induced release of organic acids from the roots between the ST2 and the ST22 lines. Both malate and citrate were detected in the root exudates (Figs. 7 and 8). Between 0 and 6 h after exposure to Al, the efflux rate of malate was low in both lines, and there was no significant difference between ST2 and ST22 lines (Fig. 7B). However, at 12 h, the efflux rate of malate was 2.6-fold higher in ST2 than in ST22 (Fig. 7B). There was also no significant difference in the rate of citrate efflux during 0 to 12 h between ST22 and ST2 lines (Fig. 7A), but a significant difference (2.1-fold higher in ST2 than in ST22) was observed at 12 h and thereafter. This discernible delay in the increased release of organic acids is consistent with that found in the root elongation inhibition in the ST2 line (Fig. 2). These results suggest that increased organic acid release was induced 0 to 12 h after exposure to Al in ST2, resulting in different expression in the Al-induced inhibition of root elongation after 12 h (Fig. 2).

Based on previous studies on Al-induced release of organic acids, two patterns can be classified. One is that there is no discernible delay between the addition of Al and the onset of release of organic acids. For example, in an Al-tolerant genotype of wheat, ET3, Al-stimulated secretion of malate from both intact roots and excised root apexes was observed within 20 min after exposure to Al (Delhaize et al., 1993; Ryan et al., 1995). In buckwheat, the secretion of oxalic acid occurred within 30 min after the exposure to Al (Ma et al., 1997a). The efflux rate in this pattern is the same at any time after the exposure to Al. The other pattern is that there is a marked lag phase between the addition of Al and the onset of organic acid release. In *Cassia tora*, secretion of citrate in response to Al was increased after 4 h (Ma et al., 1997c). In an Al-resistant cultivar of maize, a considerable lag phase before maximal citrate efflux is observed (Pellet et al., 1995). Therefore, the efflux rate of organic acids in this pattern varies with the time after exposure to Al, being low at the initial time, and high at a later time.

The secretion pattern observed in the triticale (ST2) in this study belongs to the latter pattern (Fig. 7). Different mechanisms seem to be involved in the two secretion patterns. Organic acids have been suggested to be secreted through an anion channel located on the plasma membrane (Ryan et al., 1995). The rapid secretion of organic acids upon Al exposure in the former pattern suggests that gene induction is not involved. Activation of the anion channel by Al is a possible mechanism involved in rapid release (Delhaize and Ryan, 1995). Three possibilities have been proposed by Delhaize and Ryan (1995). The first is that Al interacts directly with a channel protein, causing a change in the conformation and increasing its mean open time or conductance. The second is that Al interacts with a specific receptor on the membrane surface or with the membrane itself, which, through a series of secondary messengers in the cytoplasm, changes channel activity. And the third possibility is that Al enters the cytoplasm and alters channel activity either directly, by binding with the channel, or indirectly, through a signal transduction pathway. In fact, the activities of phospho*enol*pyruvate carboxylase (PEPC) and NAD-malate dehydrogenase did not differ between Al-sensitive and Al-tolerant cultivars of wheat and between the plants treated and not treated with Al (Ryan et al., 1995). The internal malate content was not changed by the exposure to Al during a short time (Delhaize et al., 1993).

All of these facts suggest that the in vivo synthesis of organic acids is not altered by Al in wheat plants containing the Al tolerance gene. In contrast, gene induction may be involved in the latter secretion pattern with the lag phase. The gene(s) may be related to biosynthesis of organic acids, to anion channels on the plasma membrane and/or tonoplast, or to the transport of citrate from mitochondria. Phosphorus deficiency also induces the release of citrate in *Lupinus albus* (Johnson et al., 1996). It has been shown that PEPC mRNA, PEPC enzyme, and PEPC specific activity increase under P deficiency, suggesting that PEPC is in part under transcriptional regulation (Johnson et al., 1996). Although the process leading to the release of organic acids is different between plants under Al stress and those under phosphorus-deficiency stress (Ma et al., 1997b), the effect of Al on the activity of enzymes related to the biosynthesis of citrate and malate and to gene induction need to be examined in this pattern with lag phase.

Although root elongation was also inhibited by exposure to Cd, Cu, and La, no difference in tolerance to these metals was found between ST2 and ST22 (Fig. 6). This suggests that the action of the genes for Al tolerance on the short arm of triticale chromosome 3R is highly specific to Al. An Al-tolerant wheat genotype is not La tolerant (Ryan et al., 1995), nor are Al-tolerant Arabidopsis mutants (Larsen et al., 1998). In Al-tolerant species or cultivars, Al induces the release of organic acids but La does not (e.g. Delhaize et al., 1993; Ma et al., 1997c). This may be why Al-tolerant species or cultivars are not La tolerant.

In conclusion, genes necessary for Al tolerance in triticale are located on the short arm of chromosome 3R. Secretion of malate and citrate is one of the mechanisms involved in Al tolerance in triticale, and the genes on the short arm of chromosome 3R are essential for the release of organic acids in response to Al. To our knowledge, this is the first report that links chromosomal arm location of Al-tolerant genes with organic acids release in triticale.

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