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Response of Rice to Al Stress and Identification of Quantitative Trait Loci for Al Tolerance

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Rice (*Oryza sativa* L.) shows the highest tolerance to Al toxicity among small-grain cereal crops, however, the mechanisms and genetics responsible for its high Al tolerance are not yet well understood. We investigated the response of rice to Al stress using the *japonica* variety Koshihikari in comparison to the *indica* variety Kasalath. Koshihikari showed higher tolerance at various Al concentrations than Kasalath. The Al content in root apexes was less in Koshihikari than in Kasalath, suggesting that exclusion mechanisms rather than internal detoxification are acting in Koshihikari. Al-induced secretion of citrate was observed in both Koshihikari and Kasalath, however, it is unlikely to be the mechanism for Al tolerance because there was no significant difference in the amount of citrate secreted between Koshihikari and Kasalath. Quantitative trait loci (QTLs) for Al tolerance were mapped in a population of 183 backcross inbred lines (BILs) derived from a cross of Koshihikari and Kasalath. Three putative QTLs controlling Al tolerance were detected on chromosomes 1, 2 and 6. Kasalath QTL alleles on chromosome 1 and 2 reduced Al tolerance but increased tolerance on chromosome 6. The three QTLs explained about 27% of the phenotypic variation in Al tolerance. The existence of QTLs for Al tolerance was confirmed in substitution lines for corresponding chromosomal segments.

Keywords: Aluminum — Backcross inbred line — Exclusion — QTLs — Rice— Tolerance.

Introduction

Low crop production caused by Al toxicity is a major problem in acid soils. Ionic Al (mainly Al^{3+}) inhibits root elongation and the uptake of water and nutrients (for a review, see Kochian 1995, Ma 2000), leading to reduced plant growth and increased susceptibility to environmental stresses. The mechanisms of Al tolerance are not fully understood despite the presence of large variations in Al tolerance between species and among cultivars within species. The degree of Al tolerance among small-grain cereal crops, usually follows the order rice≥rye>wheat>barley although genotypic variation also exists in each species (Foy 1988). Recent research indicated that Al tolerance in rye (*Secale cereal* L.) is associated with the secretion of malate and citrate from root tips (Li et al. 2000). The secretion of organic acids is characterized by a lag phase between exposure to Al and the secretion of organic acids. It has been suggested that alteration in the metabolism of organic acids is involved in the Al-induced secretion of organic acids in rye (Li et al. 2000). Al tolerance mechanisms in wheat (*Triticum aestivum* L.) have also been intensively investigated since Kitagawa et al. (1986) first found that malate was secreted from wheat roots after exposure to Al. Furthermore, more malate was secreted from the Al-tolerant wheat cultivar Atlas 66 than from the Al-sensitive cultivar Brevor. Delhaize et al. (1993) used a pair of near-isogenic wheat lines differing in Al tolerance at a single dominant locus (*Alt1*) and found that Al-tolerant genotypes excreted 5- to 10-fold more malate than Al-sensitive genotypes. A correlation between the amount of malate secreted and Al tolerance was established using a wide range of wheat genotypes differing in Al tolerance (Ryan et al. 1995), suggesting that Al-induced secretion of malate is a general Al tolerance mechanism in wheat. Activation of anion channels on plasma membranes by Al has been suggested to be involved in the secretion of malate in wheat (Ma et al. 2001, Ryan et al. 2001). Recently, malate-permeable channels in the apical cells of wheat roots were characterized using patch clamp techniques (Zhang et al. 2001). However, information on Al tolerance mechanisms in rice is limited.

Al tolerance is a complex trait that seems to be controlled by multiple genes (Aniol and Gustafson 1984, Gallego and Benito 1997). Attempts to clone Al-tolerance genes have been made using various techniques and more than 20 genes induced by Al stress have been isolated. More than ten Al-induced genes (e.g. *wali*1 to *wali*7, *war*4.2, *war*5.2, *war*7.2 and *war*13.2) have been isolated from the roots of different wheat cultivars (Snowden and Gardner 1993, Richards et al. 1994, Hamel et al. 1998). In *Arabidopsis thaliana*, more than ten genes induced by Al have also been identified (Richards et al.

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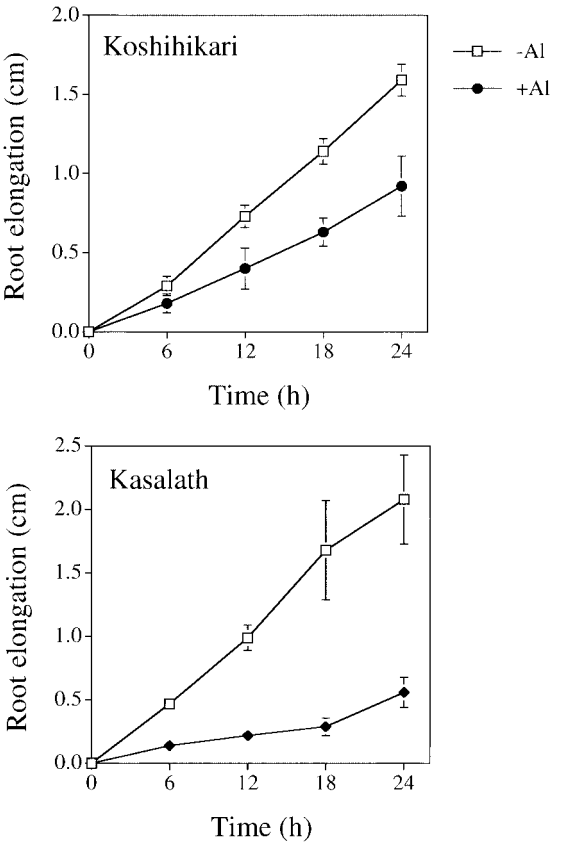

Fig. 1 Effect of Al on root elongation in Koshihikari (*japonica*) and Kasalath (*indica*). Five-day-old seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 or 50 μM $AlCl_3$. The root length was measured periodically. Vertical bars represent ± SD (n = 10).

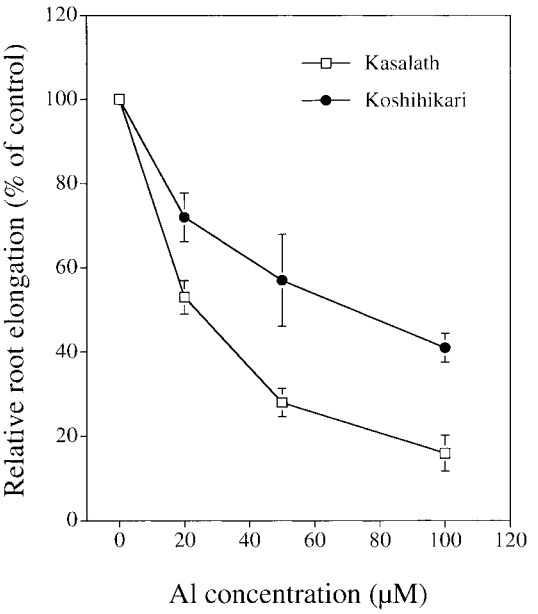

Fig. 2 Effect of increasing Al concentrations on root elongation of Koshihikari (*japonica*) and Kasalath (*indica*). Four-day-old seedlings were exposed for 24 h to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 20, 50 or 100 μM $AlCl_3$. The root length was measured before and after the treatment. Relative root elongation (root elongation with Al/root elongation without Al ×100) is shown. Vertical bars represent ± SD (n = 10).

1998). However, most of these genes are Al-responsive rather than Al-tolerance genes. These genes are also induced by other stresses such as heavy metals, low calcium, wounding (Snowden et al. 1995), pathogens (Hamel et al. 1998) and are expressed equally well in both Al-tolerant and Al-sensitive cultivars (Hamel et al. 1998). Genes conferring Al tolerance therefore remain to be cloned from Al-tolerant plant species or cultivars. Recent progress in DNA marker technology and the availability of detailed linkage maps have provided efficient tools for mapping polygenetic traits, in a process referred to as quantitative trait locus (QTL) analysis (Yano and Sasaki 1997, Yano 2001). The detection of QTLs represents an initial step towards the isolation of target genes (Yano et al. 2000, Takahashi et al. 2001, Yano 2001). Recently, several putative QTLs for Al tolerance in rice have been reported using a population of recombinant inbred (RI) lines (Wu et al. 2000, Nguyen et al. 2001). However, these QTLs have not been confirmed, and the physiological basis responsible for these QTLs in rice has not been examined. In the present study, the response of rice to Al stress was investigated and possible mechanisms involved in high Al tolerance of rice are discussed. The QTLs for Al tolerance in rice were then identified using inbred lines derived from a cross of the Al-tolerant variety Koshihikari (*japonica*) with the sensitive variety Kasalath (*indica*) and further confirmed by the substitution lines.

Results

The effect of Al on the root elongation of rice was compared between the *japonica* variety Koshihikari and the *indica* variety Kasalath. In a time-course experiment, root elongation of Koshihikari was inhibited by 38% after exposure to 50 μM Al for 6 h, while that of Kasalath was inhibited by 70% (Fig. 1). After 24 h, root elongation was inhibited by 42% for Koshihikari and 73% for Kasalath. These results indicate that Koshihikari has a higher degree of Al tolerance than Kasalath. Dose-response experiments further confirmed the higher Al-tolerance of Koshihikari. Root elongation of Koshihikari was inhibited by 27%, 42% and 60% after exposure to 20, 50 and 100 μM Al for 24 h (Fig. 2), while that of Kasalath was inhibited by 47%, 73% and 85%, respectively. The Al content in root apexes (0–1 cm) was higher in Kasalath than in Koshihikari at each Al concentration tested (Fig. 3).

Organic acids secreted from rice roots were examined. Citrate was detected in the presence of Al in both Koshihikari and Kasalath, while no citrate was detected in the absence of Al. Other organic acids such as malic and oxalic acids were not

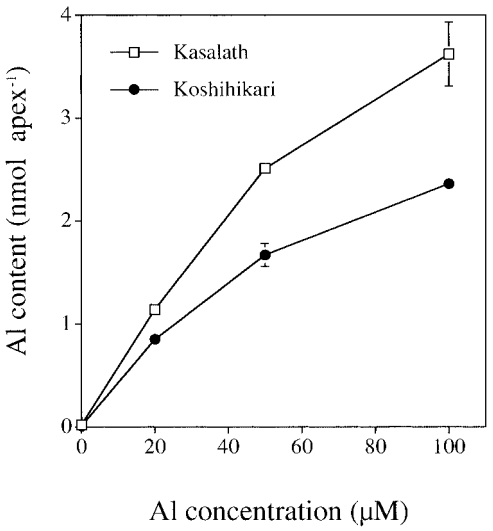

Fig. 3 Al content in the root apex of Koshihikari (*japonica*) and Kasalath (*indica*). Four-day-old seedlings were exposed for 24 h to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 20, 50 or 100 μM $AlCl_3$. Root apexes (1 cm) were excised and the Al concentration was determined by graphite furnace atomic absorption spectrophotometry. Vertical bars represent ± SD (n = 3).

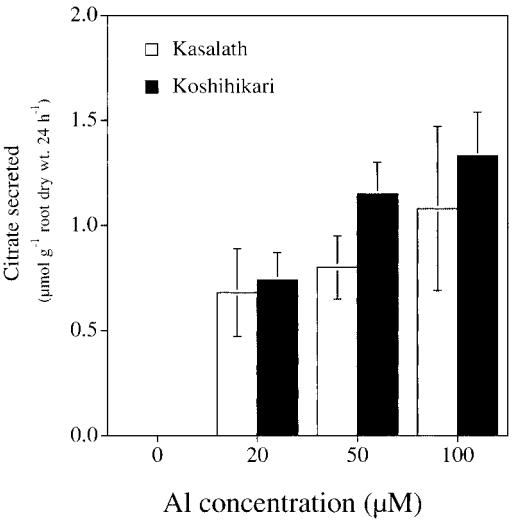

Fig. 4 Effect of increasing Al concentrations on the release of citrate from Koshihikari (*japonica*) and Kasalath (*indica*). Seedlings were exposed for 24 h to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 20, 50, or 100 μM $AlCl_3$. Root exudates were collected after 24 h of exposure to Al and organic acids were analyzed by HPLC. Vertical bars represent ± SD (n = 3).

detected in the exudates. The amount of citrate secreted was small, ranging from 0.74 to 1.33 μmol (g root dry wt.)$^{-1}$ (24 h)$^{-1}$ for Koshihikari and from 0.68 to 1.08 μmol (g root dry wt.)$^{-1}$ (24 h)$^{-1}$ for Kasalath at various Al concentrations (Fig. 4). There was no significant difference between Koshihikari and Kasalath in the amount of citrate secreted and a dose-response effect was not evident (Fig. 4).

A concentration of 50 μM Al was used to evaluate Al tolerance in the QTL mapping experiment. Relative root elongation (RRE) of 183 backcross inbred lines (BILs) and both parents (Koshihikari and Kasalath) was calculated based on the root elongation after 24 h exposure to Al relative to the root elongation in absence of Al. The RRE of Koshihikari and Kasalath was 55±5% and 28±3%, respectively. BILs showed continuous variation in the range of 30–85% (Fig. 5). Transgressive segregants with higher Al tolerance than Koshihikari were observed.

Three putative QTLs for Al tolerance were detected on chromosomes 1, 2 and 6 (Fig. 6). The QTL on chromosome 1 was linked to marker C86 and the QTL on chromosome 2 was linked to R2460. They explained 11.1% and 7.3% of the variation for RRE observed among the BILs, respectively (Table 1). The Koshihikari allele for both QTLs increased tolerance to Al. The QTL on chromosome 6 was linked to marker G200 and explained 8.7% of the variation for RRE, however, the Kasalath allele conferred Al-tolerance for this QTL. The total phenotypic variation explained by all three putative QTLs was 27.1% (Table 1). The effect of all QTLs appeared to be additive. Dominance and epistatic effects were not significant.

Substitution lines (SLs) carrying Kasalath chromosomal segments in a Koshihikari background were used to confirm the detected QTLs. Three lines, SL-C86, SL-R2460 and SL-G200, were selected based on marker information for the putative QTL region (Table 2). SL-C86 carried a Kasalath segment

Table 1 Position and effect of putative QTLs for Al tolerance in rice

Chromosome	Marker interval [a]	Position in cM on chromosome	Distance in cM from nearest marker	LOD score	r^2	Additive effect [b]	Positive allele [c]
1	C86-R2625	122	1	4.39	11.1	–5.74	Koshihikari
2	R2510-R2460	6	2	2.81	7.3	–4.40	Koshihikari
6	S1520-G200	22	3	3.37	8.7	+3.93	Kasalath

Based on relative root elongation (RRE) of 183 BILs after exposure to 50 μM Al for 24 h.
[a] Marker nearest to QTL is underlined.
[b] Effect of substituting one Koshihikari allele by a Kasalath allele (effect in % RRE).
[c] Allele increasing Al tolerance from Koshihikari or from Kasalath.

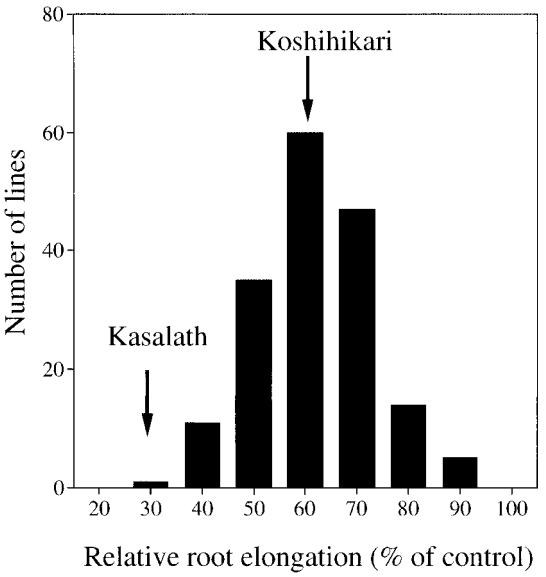

Fig. 5 Frequency distributions for relative root elongation (RRE) of 183 backcross inbred lines. Root elongation during the 24-h period was measured in a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 or 50 μM $AlCl_3$. Relative root elongation refers to root elongation in +Al solution/root elongation in –Al solution ×100. The average of 10 replicates per line is shown.

between markers S13849 and C742 on chromosome 1, SL-R2460 had a Kasalath segment from marker R2510 to G132 on chromosome 2 and SL-G200 from marker R2869 to G200 on chromosome 6. Al-induced inhibition of root elongation was compared between the parents and substitution lines. Exposure to 20 μM Al for 24 h inhibited the root elongation of Koshihikari by 20% and that of Kasalath by 55% (Table 2). Carrying the Al-sensitive Kasalath alleles at QTLs C86 and R2460 lowered the RRE of lines SL-C86 and SL-R2460 to 65% and 67%, respectively. These results confirmed the existence of QTLs detected on chromosomes 1 and 2. Furthermore, they showed that the expected effect of QTLs was closely matched by observed values (Table 2). SL-G200 on the other hand, was slightly more tolerant than Koshihikari but this difference was not significant. The effect of QTL G200 on chromosome 6 could thus not be validated with certainty.

The Al content in the root apexes was higher in SL-C86 and SL-R2460 than that in SL-G200 although a difference between Koshihikari and SL-C86 and SL-R2460 was not detected at 20 μM Al (Table 2). There were no significant differences in the amount of citrate secreted among three substitution lines and two parents.

Discussion

Al tolerance mechanisms in rice

Although rice is generally considered to be the most Al-tolerant species among small-grain cereal crops, the mechanisms responsible for the high Al tolerance of rice are not yet understood. Genotypic variations in Al tolerance have been reported in rice (e.g. Howeler and Cadavid 1976, Khatiwada et al. 1996). In the present study, we found that the *japonica* variety Koshihikari showed higher Al tolerance compared to the *indica* variety Kasalath (Fig. 1, 2). That Koshihikari accumulated less Al in the root apexes than Kasalath (Fig. 3) indicated that Al-exclusion mechanisms rather than internal detoxification would be of importance in rice.

Several mechanisms for Al exclusion have been proposed, including exudation of chelate ligands, formation of a plant-induced pH barrier in the rhizosphere or root apoplasm, immobilization of Al at the cell wall, selective permeability of the plasma membrane and Al efflux (Taylor 1991, Kochian 1995). Recently, a significant amount of evidence has shown that secretion of organic acids with Al-chelating capacity from root apexes is involved in the Al tolerance of plant species such

Table 2 Effect of 24 h exposure to 20 μM Al on root elongation of substitution lines carrying Kasalath chromosomal segments containing putative QTLs for Al tolerance

Genotype	Substituted segment	Chromosome	QTL	Estimated substitution effect [a]	RRE [b] (%)	Observed substitution effect [c]	Al content in root apexes (nmol $apex^{-1}$) [d]	Citrate secreted (μmol (g root dry $wt.)^{-1}$ 24 h^{-1}) [e]
Koshihikari					79.9±5.3		1.42±0.09	0.71±0.04
SL-C86	S13849-C742	1	C86	–11.4%	64.5±4.1	–15.4%	1.35±0.07	0.66±0.02
SL-R2460	R2510-G132	2	R2460	–8.8%	66.9±4.0	–13.0%	1.39±0.08	0.97±0.09
SL-G200	R2869-G200	6	G200	+7.8%	84.2±5.3	+4.3%	1.01±0.05	0.79±0.02
Kasalath					44.6±3.2		1.51±0.18	0.68±0.02

The chromosomal segments were transferred into a Koshihikari background by a series of backcrosses.
[a] Effect of substituting both Koshihikari alleles by Kasalath alleles, based on estimates of the QTL analysis (twice the additive effect).
[b] Relative root elongation (root elongation at 20 μM Al relative to elongation in absence of Al). Data are means ± SD (n = 10).
[c] Relative to the RRE of Koshihikari.
[d] Data are means ± SD (n = 3).
[e] Root exudates were collected in 0.5 mM $CaCl_2$ solution containing 100 μM $AlCl_3$ (pH 4.5) for 24 h. Data are means ± SD (n = 3).

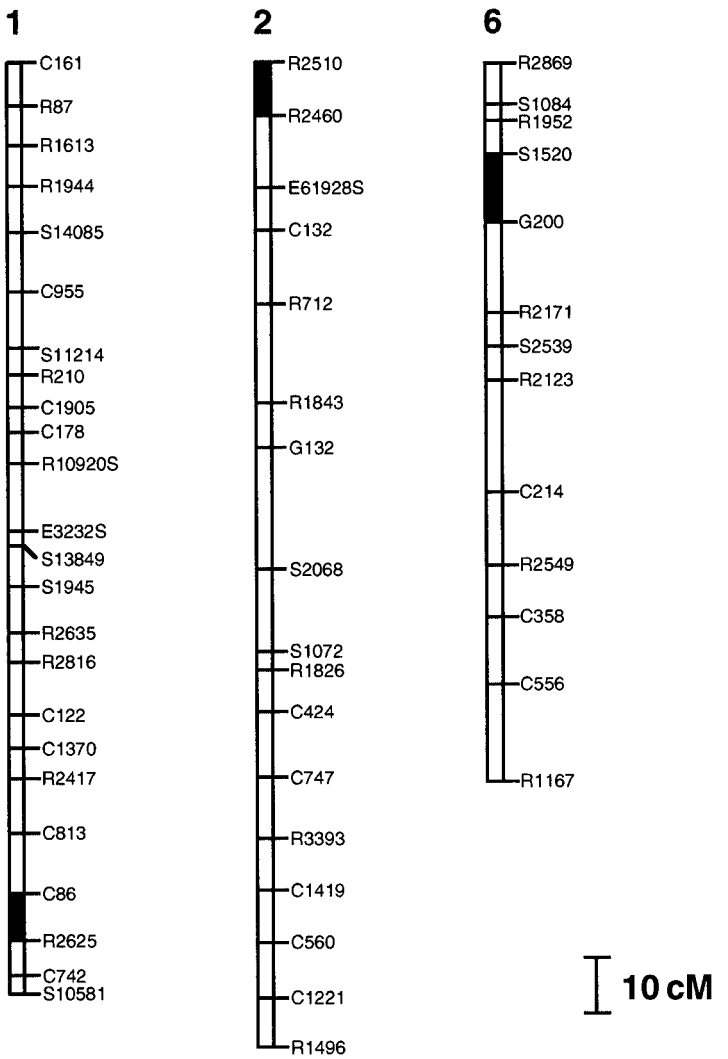

Fig. 6 Positions of QTLs for Al tolerance in rice. RFLP linkage map of chromosomes 1, 2 and 6, where QTLs are contained, is shown. Black bars represent putative map positions of QTLs for Al tolerance.

as snapbean, wheat, maize, *Cassia tora*, soybean, buckwheat, rye, *Arabidopsis thaliana* and triticale (for recent reviews, see Ma 2000, Ma et al. 2001, Ryan et al. 2001). The kind of organic acid secreted depends on plant species and secretion of malate, citrate and oxalate have been reported. In the present study, we examined Al-induced secretion of organic acids in rice. Citrate was found to be secreted with exposure to Al in both Koshihikari and Kasalath (Fig. 4). However, it is unlikely that the secretion of citrate is the major mechanism for Al tolerance in rice for the following reasons: (1) although Koshihikari was more tolerant to Al than Kasalath (Fig. 1, 3), the cultivars did not differ significantly in the amount of citrate secreted (Fig. 4). In addition, there was also no significant difference in the citrate secretion between substitution lines (Table 2); (2) there was no evident increase in the amount of citrate secreted in response to increasing Al concentrations; (3) the amount of citrate secreted was small, being one tenth of rye, which has similar Al tolerance to rice (Li et al. 2000).

It was proposed that a pH shift in the rhizosphere may be responsible for Al tolerance in rice. Sivaguru and Paliwal (1993) and Ganesan et al. (1993) found that an increase in pH of a nutrient solution by Al-tolerant rice cultivars is higher than by Al-sensitive cultivars. However, the pH in their experiments was monitored after a long period of exposure to Al. It is therefore difficult to distinguish whether the pH change is the result or cause of Al tolerance. We also checked Al-induced pH changes in treatment solution during short-term experiments. Changes in solution pH were not significant between Koshihikari and Kasalath and between –Al and +Al treatments after 6 h exposure to 50 μM Al (data not shown), although Al-induced inhibition of root elongation was evident at this time. However, it still needs to be clarified whether Al-induced pH

changes in the rhizosphere are involved in Al tolerance of rice by measuring the pH at the root apex surface as it has been done in *A. thaliana* (Degenhardt et al. 1998).

The Al content of root apexes in rice was much lower compared to other small-grain crops such as wheat and barley (Fig. 3). Binding of Al to the apoplast (cell wall) has been suggested to be involved in Al toxicity (Horst 1995). Different from upland plants, rice roots may have some specific substances coated on the surface or modified components of the cell wall for preventing Al binding. However, these possibilities need to be examined in the future.

QTLs for Al tolerance in rice

Advances in molecular marker technology over the past few years have led to the identification of chromosomal regions associated with many complex traits in rice (Yano and Sasaki 1997). Several rice populations have been developed for analysis of traits such as seed dormancy and heading date (Lin et al. 1998) or P-deficiency tolerance (Wissuwa et al. 1998). In the present study we mapped QTLs controlling Al tolerance using a population of 183 BILs derived from a backcross of the intolerant parent Kasalath to the tolerant recurrent parent Koshihikari. To detect QTLs with minimum error, it is very important to establish a reliable assay system for Al tolerance. As the primary event in Al toxicity is rapid inhibition (within 1 h) of root elongation (e.g. Ownby and Popham 1989, Ryan et al. 1992), we evaluated the Al tolerance by measuring root elongation during a 24-h period. Furthermore, relative root elongation (root elongation in Al solution/root elongation in a solution without Al ×100) was used in QTL analysis to overcome the potentially confounding effect of differences in root growth rates among different lines. As a result, three putative QTLs controlling Al tolerance were detected on chromosomes 1, 2 and 6 (Fig. 6). The direction of gene action for the QTL on chromosome 6 was opposite to that on chromosomes 1 and 2 (Table 1). This result is consistent with the observation of transgressive variation for Al tolerance among BILs (Fig. 5). Subsequently two QTLs were confirmed using substitution lines that carried chromosomal segments containing the Kasalath allele at each QTL (Table 2). This result proved that QTLs continued to show their effect in isolation from much of the donor genome and that both QTLs were not affected by the segregation of other QTLs.

A putative QTL for Al tolerance was also detected on chromosome 1 by Wu et al. (2000) and Nguyen et al. (2001). In their study a population of RI lines had been used that was derived from a cross between the Al-sensitive *indica* variety IR1552 with the Al-tolerant *japonica* variety Azucena (Wu et al. 2000) and between the Al-tolerant *indica* variety Chiembau and the Al-sensitive *indica* variety Omon269–65 (Nguyen et al. 2001). However, it is unclear whether this QTL is at the same position as the QTL reported here because different sets of DNA markers were used. Wu et al. (2000) also detected QTLs for Al tolerance on chromosomes 3, 9 and 12 that could not be detected here. Nguyen et al. (2001) identified four other QTLs for Al tolerance on chromosome 2, 3, 5 and 11. These differences could be ascribed to the different mapping population or differences in evaluation methods. In their experiment, root length after a period of 2 and 4 weeks were used as parameters in QTL analysis. Root growth during such a long period may not only be affected by Al, but also by other indirect factors such as uptake of nutrients.

The genetics of Al tolerance have also been studied in other plant species. 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). 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). Recently, genes controlling Al tolerance in triticale were found to be localized on the short arm of 3R (Ma et al. 2000). Furthermore, these genes are linked to the secretion of organic acids. In barley, the Al tolerance gene (*Alp*) was mapped to the long arm of chromosome 4H, 2.1 cM proximal to the marker *Xbcd*1117 and 2.1 cM distal to *Xwg*464 and *Xcdo*1395 (Tang et al. 2000). In soybean, three QTLs controlling Al tolerance were detected (Bianchi-Hall et al. 2000). These studies that link molecular markers to Al tolerance provide the necessary information to increase the Al tolerance of crops through marker-assisted selection. The complete genome of rice is expected to be sequenced in the near future. This information together with the identification of QTLs for Al tolerance might also lead to the isolation of genes for Al tolerance and will help us to fully understand Al tolerance mechanisms.

Materials and Methods

Plant materials

The *japonica* variety Koshihikari and the *indica* variety Kasalath were used in physiological studies. The population of 183 BILs used for QTL analysis was developed by the single-seed descent method from a backcross of Koshihikari/Kasalath/Koshihikari (Y. Takeuchi and M. Yano unpublished data). The genotypes of each BC_1F_7 line had been determined using 162 RFLP markers equally distributed among the 12 rice chromosomes (Y. Takeuchi and M. Yano unpublished data). Substitution lines (SL-C86, SL-R2460 and SL-G200) containing Kasalath chromosomal segments at each putative QTL region were selected based on graphical genotypes (T. Ebitani, Y. Takeuchi and M. Yano unpublished data).

Evaluation of Al tolerance

For physiological studies, seeds were soaked in deionized water overnight and then germinated on nets that were floated on 0.5 mM $CaCl_2$ (pH 4.5) solution. The solution was renewed daily. After growth at 25°C for 4 d, similar size seedlings were selected and used for the

following experiments. In a time-course experiment, 10 seedlings per genotype were exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0 or 50 μM $AlCl_3$ in a 1.5-liter plastic container. Root lengths were measured every 6 h with a ruler. In a dose-response experiments, 10 seedlings per genotype were exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 20, 50 or 100 μM $AlCl_3$ in 1.5-liter plastic containers for 24 h. Root length was measured before and after the Al treatment. Al tolerance of three substitution lines was evaluated with a similar method except that 20 μM Al was used.

For the QTL analysis, a net (39×24.5 cm) was divided into 40 compartments by plastic bars and floated on a 0.5 mM $CaCl_2$ solution (pH 4.5) in a 20-liter plastic container. The solution was renewed every 2 d. Seeds of BILs (10 seeds per line) were placed on each compartment on the net. On day 5 the seedlings were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) with 50 μM $AlCl_3$ or without Al. Forty lines (38 BILs + two parents) were evaluated each time. The root length was recorded with a ruler before and after the 24 h treatment. RRE was calculated for each line as follows: RRE (%) = root elongation (average of 10 replicates) in Al solution/root elongation in –Al solution ×100. The experiments were conducted at 25°C under natural light.

Collection of root exudates

To analyze organic acids secreted from rice roots, root exudates from both Koshihikari and Kasalath were collected. Seedlings (5-day-old) were transplanted to a 1-liter plastic pot (12 seedlings per pot) containing half strength Kimura B nutrient solution. The nutrient solution contained the macronutrients (mM): $(NH_4)_2SO_4$ (0.18), $MgSO_4 \cdot 7H_2O$ (0.27), KNO_3 (0.09), $Ca(NO_3)_2 \cdot 4H_2O$ (0.18) and KH_2PO_4 (0.09), and the micronutrients (μM): NaEDTA-Fe·$3H_2O$ (20), $MnCl_2 \cdot 4H_2O$ (0.5), H_3BO_3 (3), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1), $ZnSO_4 \cdot 7H_2O$ (0.4) and $CuSO_4 \cdot 5H_2O$ (0.2). The pH of this solution without adjustment is 5.5 and the solution was renewed every 3 d. The plants were grown in a temperature-controlled greenhouse (25°C) under natural light. After 10 d of pre-culture, roots were placed in a 0.5 mM $CaCl_2$ (pH 4.5) solution overnight and then exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0, 20, 50 or 100 μM $AlCl_3$. Root exudates were collected after 24 h and 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 (16×14 mm) filled with 2 g of AG 1×8 resin (100–200 mesh, formate form). Organic acids retained on an anion-exchange resin were eluted with 2 M HCl and the eluate was concentrated to dryness with a rotary evaporator (40°C). After the residue was dissolved in 1 ml of milli-Q water, the concentration of organic acids was analyzed by HPLC as described below. Root exudates from three substitution lines during a period of 24 h were also collected similarly as described above in the presence and absence of 100 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution (pH 4.5).

Determination of Al and organic acids

Roots (0–1 cm) exposed to various Al concentrations for 24 h were excised and placed in a plastic tube (1.5 ml) containing 1 ml of 2 M HCl. 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-5000, Hitachi, Tokyo).

Organic acids were determined by HPLC (LC-10A, Shimadzu, Kyoto, Japan) equipped with an ODS column (150×4.6 mm, Shinwa, Japan). The mobile phase was dilute perchloric acid solution (pH 2.1) run at 40°C and peaks were detected at 425 nm after reaction with 0.2 mM bromothymol blue, 15 mM NaH_2PO_4 and 2 mM NaOH in 5% methanol. The flow rate of the mobile phase was 0.6 ml min^{-1} and that of the reactive phase was 0.65 ml min^{-1}.

RFLP mapping and QTL analysis

A linkage map of the 162 RFLP markers used for QTL analysis was obtained from the Rice Genome Project, Japan (Y. Takeuchi and M. Yano unpublished data). QTL analysis was performed using phenotypic data from 183 BILs and both parents (means of 10 replications). Computations were done with the software package PLABQTL (Utz and Melchinger 1996), which uses a multiple regression approach. In a first step, simple interval mapping was performed and cofactors selected. For cofactor selection, F-to-enter and F-to-drop thresholds were set at 6.0 to avoid selecting multiple markers linked to one QTL as cofactors. Using these cofactors to reduce the residual variation, QTLs were detected by a composite interval mapping method. A LOD score >2.5 was considered significant for QTL detection.

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References

Aniol, A. (1990) Genetics of tolerance to aluminum in wheat (*Triticum aestivum* L. Thell). *Plant Soil* 123: 223–227.

Aniol, A. and Gustafson, J.P. (1984) Chromosome location of genes controlling aluminum tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.* 26: 701–705.

Bianchi-Hall, C.M., Carter, T.E., Jr., Bailey, M.A., Mian, M.A.R., Rufty, T.W., Ashley, D.A., Boerma, H.R., Arellano, C., Hussey, R.S. and Parrott, W.A. (2000) Aluminum tolerance associated with quantitative trait loci derived from soybean PI 416937 in hydroponics. *Crop Sci.* 40: 538–545.

Degenhardt, J., Larsen, P.S., Howell, S.H. and Kochian, L.V. (1998) Aluminum resistance in the *Arabidopsis* mutant *alr*-104 is caused by an aluminum-induced increase in rhizosphere pH. *Plant Physiol.* 117: 19–27.

Delhaize, E., Ryan, P.R. and Randall, P.J. (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.) II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiol.* 103: 695–702.

Foy, C.D. (1988) Plant adaptation to acid, aluminum-toxic soils. *Commun. Soil Sci. Plant Anal.* 19: 959–987.

Gallego, F.J. and Benito, C. (1997) Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 95: 393–399.

Gallego, F.J., Lopez-Solanilla, Figueiras, A.M. and Benito, C. (1998) Chromosomal location of PCR fragments as a source of DNA markers linked to aluminium tolerance genes in rye. *Theor. Appl. Genet.* 96: 426–434.

Ganesan, K., Sankaranarayanan, C. and Balakumar, T. (1993) Physiological basis of differential aluminum tolerance in rice genotypes. *Commun. Soil Sci. Plant Anal.* 24: 2179–2191.

Hamel, F., Breton, C. and Houde, M. (1998) Isolation and characterization of wheat aluminum-regulated genes: possible involvement of aluminum as a pathogenesis response elicitor. *Planta* 205: 531–538.

Horst, W.J. (1995) The role of the apoplast in aluminium toxicity and resistance of higher plants: a review. *Z. Pflanzenernah. Bodenk.* 158: 419–428.

Howeler, R.H. and Cadavid, L.F. (1976) Screening of rice cultivars for tolerance to Al-toxicity in nutrient solutions as compared with a field screening method. *Agron. J.* 68: 551–555.

Khatiwada, S.P., Senadhira, Carpena, A.L., Zeigler, R.S. and Fernandez, P.G. (1996) Variability and genetics of tolerance for aluminum toxicity in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 93: 738–744.

Kitagawa, T., Morishita, T., Tachibana, Y., Namai, H. and Ohta, Y. (1986) Differential aluminum tolerance in wheat and secretion of organic acids. *Jpn. J. Soil Sci. Plant Nutr.* 57: 352–358.

Kochian, L.V. (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46: 237–260.

Li, X.F., Ma, J.F. and Matsumoto, H. (2000) Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123: 1537–1543.

Lin, S.Y., Sasaki, T. and Yano, M. (1998) Mapping quantitative trait loci controlling seed dormancy and heading date in rice (*Oryza sativa* L.), using backcross inbred lines. *Theor. Appl. Genet.* 96: 997–1003.

Ma, J.F. (2000) Role of organic acids in detoxification of aluminum in higher plants. *Plant Cell Physiol.* 41: 383–390.

Ma, J.F., Ryan, P.R. and Delhaize, E. (2001) Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* 6: 273–278.

Ma, J.F., Taketa, S. and Yang, Z.M. (2000) Aluminum tolerance genes on the short arm of chromosome 3R are linked to organic acid release in triticale. *Plant Physiol.* 122: 687–694.

Nguyen, V.T., Burow, M.D., Nguyen, H.T., Le, B.T., Le, T.D. and Paterson, A.H. (2001) Molecular mapping of genes conferring aluminum tolerance in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 102: 1002–1010.

Ownby, J.D. and Popham, H.R. (1989) Citrate reverses the inhibition of wheat root growth caused by aluminum. *J. Plant Physiol.* 135: 588–591.

Richards, K.D., Schott, E.J., Sharma, Y.K., Davis, K.R. and Gardner, R.C. (1998) Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. *Plant Physiol.* 116: 409–418.

Richards, K.D., Snowden, K.C. and Gardner, R.C. (1994) *wali*6 and *wali*7. Genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. *Plant Physiol.* 105: 1455–1456.

Ryan, P.R., Delhaize, E. and Jones, D.L. (2001) Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 527–560.

Ryan, P.R., Delhaize, E. and Randall, P.J. (1995) Characterization of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. *Planta* 196: 103–110.

Ryan, P.R., Shaff, J.E. and Kochian, L.V. (1992) Aluminum toxicity in roots: Correlation among ionic currents, ion fluxes, and root elongation in aluminum-sensitive and aluminum-tolerant wheat cultivars. *Plant Physiol.* 99: 1193–1200.

Sivaguru, M. and Paliwal, K. (1993) Differential aluminum tolerance in some tropical rice cultivars. II. Mechanism of aluminum tolerance. *J. Plant Nutr.* 16: 1717–1732.

Snowden, K.C. and Gardner, R.C. (1993) Five genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. *Plant Physiol.* 103: 855–861.

Snowden, K.C., Richards, K.D. and Gardner, R.C. (1995) Aluminum-induced genes. Induction by toxic metals, low calcium, and wounding and pattern of expression in root tips. *Plant Physiol.* 107: 341–348.

Takagi, H., Namai, H. and Murakami, K. (1983) Exploration of aluminum tolerance genes in wheat. *In* Proceedings of the 6th International Wheat Genetics Symposium. pp. 141–146, Kyoto, Japan.

Takahashi, Y., Shomura, A., Sasaki, T. and Yano, M. (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the α subunit of protein kinase CK2. *Proc. Natl. Acad. Sci. USA* 98: 7922–7927.

Tang, Y., Sorrells, M.E., Kochian, L.V. and Garvin, D.F. (2000) Identification of RFLP makers linked to the barley aluminum tolerance gene *Alp*. *Crop Sci.* 40: 778–782.

Taylor, G. (1991) Current views of the aluminum stress response; the physiological basis of tolerance. *Curr. Top. Plant Biochem. Physiol.* 10: 57–93.

Utz, H.F. and Melchinger, A.E. (1996) PLABQTL: A program for composite interval mapping of QTL. *J. Quant. Trait. Loci.* 2(1). http://probe.nalusda.gov:8000/otherdocs/jqtl.

Wissuwa, M., Yano, M. and Ae, N. (1998) Mapping of QTLs for phosphorus-deficiency tolerance in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 97: 777–783.

Wu, P., Liao, C.Y., Hu, B., Yi, K.K., Jin, W.Z., Ni, J.J. and He, C. (2000) QTLs and epistasis for aluminum tolerance in rice (*Oryza sativa* L.) at different seedling stages. *Theor. Appl. Genet.* 100: 1295–1303.

Yano, M. (2001) Genetic and molecular dissection of naturally occurring variation. *Curr. Opin. Plant Biol.* 14: 130–135.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y. and Sasaki, T. (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell* 12: 2473–2483.

Yano, M. and Sasaki, T. (1997) Genetic and molecular dissection of quantitative traits in rice. *Plant Mol. Biol.* 35: 145–153.

Zhang, W.H., Ryan, P.R. and Tyerman, S.D. (2001) Malate-permeable channels and cation channels activated by aluminum in the apical cells of wheat roots. *Plant Physiol.* 125: 1459–1472.

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