

Natural variation of *Arabidopsis thaliana* reveals that aluminum resistance and proton resistance are controlled by different genetic factors

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Abstract Root growth of *Arabidopsis thaliana* is inhibited by proton rhizotoxicity in low ionic strength media when the pH of the medium is lower than 5.0. QTL analysis at pH 4.7 revealed that two major QTLs on chromosome 2 and 5 and an additional six epistatic interacting loci pairs control proton resistance in the *Ler/Col* recombinant inbred population. These genetic factors are independently associated with proton resistance in comparison to the known Al resistant QTL and epistases detected in the same RI population at 4 μ M Al at pH 5.0. This indicates that different genetic factors regulate mechanisms of resistance to each stress in this plant species. No correlation was observed between proton resistance and Al resistance among 260 accessions indicating that there is no simple relationship between the genetic factors controlling each trait. Several accessions with different combinations of

proton (pH 4.7) and Al (4 μ M Al at pH 5.0) resistances were identified by phenotypic cluster analysis. Although this grouping was performed using root growth data, the degree of resistance was correlated with their sensitivity to short-term damage in the root tip, indicating that the same resistance mechanism controls proton resistance at different time scales. Resistant accessions grew better than sensitive ones in acid soil culture. This suggests that proton resistance in hydroponic conditions could be an important index in breeding programs to improve productivity in acid soil, at least in acid sensitive plant species.

Introduction

Growth of crop plants is limited in acid soils due to complex stress factors which consist of a series of toxicities and nutrient deficiencies (Kochian 1995; Salazar et al. 1997). Heavy applications of fertilizer, including calcium carbonate (lime stone) and phosphate fertilizer, are a common means of overcoming stress factors in acid soil (Alva et al. 1986; Speher et al. 1993). However, this approach may not be the ideal solution in either developing or developed countries because of the high cost of fertilizers (Ishitani et al. 2004) and greater energy costs. Hence, improvement of growth capability through enhancement of resistance to stress factors would be a more useful solution to reduce inputs of agricultural fertilizers in acid soil areas. To facilitate effective breeding programs, which include marker assisted selection and genetic modification, it is important to understand the mechanisms of resistance to stress factors at the molecular level.

Rhizotoxicity of aluminum (Al) is among the most important of the complex stress factors that cause severe

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inhibition of root growth and thus enhancement of drought sensitivity of crop plants (Foy 1988). However, Al in acid soil also induces phosphorous deficiency due to the formation of Al phosphate which shows very limited solubility (López-Bucio et al. 2000). Other stress factors, such as an excess of manganese and a deficiency of calcium, cause growth reduction in most crop plant species (Rengel 1992; Gonzalez et al. 1998; Horst et al. 1999; Rengel and Zhang 2003). In addition, proton toxicity (low pH of soil solution) may also cause damage to plants in some soil types (Knoepp and Swank 1994; Linkes et al. 1997). For example, plant growth is limited by proton toxicity and free Fe^{2+} toxicity in sulfate soil, but not by Al toxicity. In fact, a screening study of soybean in Brazil indicated that resistance to low Ca, which is connected with low pH resistance (e.g., Kinraide 1998; Koyama et al. 2001), was correlated with growth performance on Cerrado soil containing a large amount of exchangeable Al (Spehar and Souza 1999). These situations indicate that protons must be a stress factor in acid soils and may exert selection pressure in agricultural fields in some areas. These multiple stress factors drastically reduce the yield of crop plants.

This complex series of stress factors can be simulated under laboratory conditions, and this can be useful for physiological and molecular biological studies aimed at identifying genes coding for resistance (Kobayashi et al. 2005). For example, Al in growth media induces phosphate deficiency due to the formation of a precipitate of Al-phosphate. This precipitation becomes significant when growth medium pH is higher than pH 4.5. High concentrations of phosphate drastically decrease Al toxicity, but exacerbate phosphate deficiency stress (Koyama et al. 1988). On the other hand, low pH growth media (pH < 4.5) enhance Al toxicity because Al in the medium can exist in its phytotoxic form, Al^{3+} (Kinraide and Parker 1989, 1990); this induces proton rhizotoxicity in some plant species (Kinraide and Parker 1990; Yokota and Ojima 1995; Koyama et al. 1995). This complex interplay between toxic mechanisms makes identification of resistant mechanisms at the molecular level difficult.

Genetic approaches, for instance mutant studies, could be useful for distinguishing among the complex stress factors acting under experimental conditions and could help to clarify the molecular mechanisms of resistance to each stress factor. For example, two types of mutant carrot cell lines have been selected in Al-containing media, namely Al tolerance (Arihara et al. 1991) and efficient P uptake from insoluble Al-phosphate (Koyama et al. 1988). The latter cell line shares characteristics with a capable of efficient P utilization from Al phosphate, namely white lupin (Neumann et al. 1999; Kihara et al. 2003). These studies contributed to separate Al resistance and resistance to Al-phosphate induced P deficiency, which are involved in complex Al

stress. Similar genetic approaches could be useful for separating other stress factors in acid soils under experimental conditions, such as Al resistance and proton resistance.

One screening study of proton resistance indicated that these factors are genetically different in soybean (Lazof and Holland 1999), but such genetic analysis is very limited in other plant species. In addition, interference of proton toxicity during the selection and evaluation of Al resistant cultivars was suggested by common bean (Rangel et al. 2005) and spinach (Yang et al. 2005). Hence, it would be difficult to conclude that resistance to proton rhizotoxicity is genetically independent from Al resistance. Genetic studies in *Arabidopsis* could be a useful means of answering this question, because each stress factor can be clearly distinguished in this plant species. Al and proton rhizotoxicities cause different patterns of cell damage in growing root tips of *Arabidopsis thaliana* (Koyama et al. 1995). Exposure to low pH growth media damages the plasma membrane of root tip cells irreversibly within a short time (Koyama et al. 2001), whereas exposure to Al solution causes swelling of cells with no visible damage to the plasma membrane (Kobayashi et al. 2005).

Extensive seed collections of natural accessions and mapping populations for QTL analysis are available from public biological resource centers (e.g., NASC, TAIR and RIKEN BRC). These biological resources could be useful for comparing the genetic architecture of these stress factors. In the present study, we used two genetic approaches to compare Al resistance and proton resistance in *Arabidopsis*. Firstly, using the same recombinant inbred (RI) population that was used in a QTL study of Al resistance (Kobayashi and Koyama 2002), we performed QTL analysis of proton resistance in *Arabidopsis*. Secondly we conducted a cluster analysis to identify specific groups with differential Al and proton resistance profiles. Together with physiological characterization, we found that Al resistance and proton resistance in *Arabidopsis* appear to be genetically independent and driven by different functional genes.

Materials and methods

Arabidopsis accessions

Original seed of 260 *A. thaliana* accessions were obtained from Sendai *Arabidopsis* seed stock center, and the seed progenies were derived by single seed descent methods (see supplementary Table 1). These accessions are currently available at RIKEN BRC (SASSC; <http://www.brc.riken.go.jp/lab/epd/SASSC>). The recombinant inbred (RI) population was made by a cross between Col-4 and *Ler-0* (Lister and Dean 1993) at the Nottingham *Arabidopsis* stock center (NASC; <http://www.arabidopsis.info/>).

A subset of the RI population (99 lines) was used for QTL analysis of proton resistance.

Growth conditions

Arabidopsis hydroponic culture was conducted as described previously (Koyama et al. 2001; Kobayashi et al. 2005). Briefly, control culture solutions were prepared by adding 1/50 strength Ca-free and P-free MGRL nutrients (Fujiwara et al. 1992) to 200 μM CaCl_2 solution. Al-toxic solutions were prepared by adding an appropriate amount of Al stock (1 mM AlCl_3) to the control culture solution at pH 5.0, whereas low pH treatment solutions were adjusted to appropriate pH by adding 0.1 N HCl to the control culture solution. Screening of accessions was carried out in Al solution containing 4 μM total Al at pH 5.0, whereas that for proton resistance was done at pH 4.7 with no Al. In this solution, Al is monomer and $\{\text{Al}^{3+}\}$ at the plasma membrane surface is 14.3 μM (personal communication with Dr. Thomas Kinraide at ARS, USDA). Seedlings were synchronously germinated by pre-incubation in distilled water at 4°C for 3 days and then grown on plastic mesh supported by photo slide mounts as described previously (Toda et al. 1999). Seedlings (10 per accession) were grown in plastic containers containing 6 l of culture solution (maximum 130 lines per container) with 12 h of light per day (PPFD 30 $\mu\text{mol E m}^{-1} \text{s}^{-1}$) at 25°C. All culture solutions were renewed every 2 days, in order to maintain rhizotoxicities in the test solutions during growth experiments.

Estimation of root growth

Root length was measured on day 7 using a video microscope (Pico Scopeman, Kenis, Tokyo) equipped with a device for measuring length on a TV monitor (MC-300, Kenis). Ten seedlings of each accession for each treatment were grown and scored for root length. The three highest values were used to omit growth-delayed seedlings. This process was repeated three times, and data were pooled and used for calculating mean relative root length (RRL) and other values. As described previously, this could minimize the effect of growth-delayed individuals and is suitable for estimating the potential capacity of accessions under given conditions (Kobayashi et al. 2005). The RRL was defined as RRL (the mean of the root length in toxic solution/that in the control).

QTL analyses

QTL analyses were carried out following the same methods used in previous QTL studies (Kobayashi and Koyama

2002; Kobayashi et al. 2005). Genetic linkage maps of the *Ler/Col* populations were processed using public data for the genotypes of RI lines obtained from NASC (<http://www.arabidopsis.info/>) using Mapmaker/EXP version 3.0b (Lander et al. 1987). About 195 genetic makers were used for *Ler/Col* RI populations. Using RRL as a resistance index, composite interval mapping was carried out using QTL Cartographer, Model 6 (ver. 1.13; Basten et al. 1994), while epistatic interacting loci pairs were analyzed by complete pair-wise search (i.e., automated genome wide two-way ANOVA) using EPISTAT (Chase et al. 1997). The complete pair-wise search allows detection of epistatic interactions between two loci, even if the marker has not been detected as a significant QTL by the CIM method. To reject false positives, a threshold of the logarithm of odd (LOD) score for CIM analysis was calculated by a permutation test as described by Churchill and Doerge (1994) (1,000 times permutation at the significant level of $\alpha = 0.05$), while the threshold for detection of LLR of EPISTAT was fixed to $P < 0.005$. The R^2 values were calculated by each program.

Phenotypic cluster analysis

The data sets of phenotypes (root length in control solution and RRLs of low pH treatment and Al treatment) were imported into the CLUSTER program (Eisen et al. 1998; <http://www.rana.lbl.gov/EisenSoftware.htm>). The data were first mean-centered for each condition, and then hierarchical cluster analysis was performed using an uncentered matrix and average linkage method. Resulting tree figures were displayed using the software package, Treeview (<http://www.rana.lbl.gov/EisenSoftware.htm>).

Statistical analysis

The coefficient of variation (CV; %) for each condition was calculated by dividing the standard deviation by the mean. Broad sense heritability (H_2^b) was calculated from RRL values ($n = 9$) of accessions using the following formula

$$H_2^b = \sigma_g^2 / [(\sigma_e^2 / r) + \sigma_g^2],$$

where σ_g^2 is genetic variance, σ_e^2 environment variance, and r number of data points employed. Significant differences among the means of all accessions were estimated using one-way ANOVA followed by a least significant difference (LSD) test ($P < 0.01$). The coefficient of determination (r^2) between Al and low pH treatments was calculated using the RRLs of accessions in each treatment.

Root staining and microscopic observations

Histochemical analyses of the root tips derived from the proton and Al treatments were performed as described previously (proton stress, Koyama et al. 2001; Al stress, Kobayashi et al. 2005). Briefly, growing roots (day 5–7 in the control MGRL medium that contained 1/50 MGRL nutrients and additional Ca to give a final concentration of 200 μM) were transferred to stress conditions (proton stress: 200 μM CaCl_2 at pH 4.7 for 1 or 24 h; Al stress: 4 μM Al at pH 5.0 for 24 h). Damage caused by proton stress was visualized using propidium iodide (PI; 4.5 μM of PI for 1 m), while callose and H_2O_2 accumulation caused by Al stress were visualized using aniline blue [AB; 0.1% (w/v) aniline blue in 0.15 M K_2HPO_4 at pH 9.5 for 15 min] and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; 10 μM for 10 min), respectively. Root apices were stained directly using PI and H_2DCFDA , while the roots were pre-fixed in 10% (w/v) formaldehyde, 5% (w/v) glacial acetic acid and 45% (w/v) ethanol in vacuo for 3 h and then used for the AB staining. Fluorescence in the root tip was observed using a fluorescent microscope (IMT-2-21-RFL, Olympus, Tokyo) equipped with appropriate dichroic mirror units (IMT-2-DMG for PI; IMT-2-DMIB for H_2DCFDA ; and IMT-2-DMV for AB-callose, respectively). Images were photographed using a digital camera (PMDC $\alpha/\text{OL-1}$, Olympus).

Soil culture of specific responses in accessions extracted from cluster analysis

Soil culture was performed with an acid soil, namely Kawatabi soil, which had been employed in several previous studies for estimating the resistance of varieties and transformants in acid soil environments (e.g., Kobayashi et al. 2005; Ma et al. 2005). Basal soil was prepared by adding 250 mg NaH_2PO_4 , 48 mg KCl, 36 mg MgSO_4 , and 132 mg $(\text{NH}_4)_2\text{SO}_4$ to 100 g of air-dried soil. Either 0.06 or 0.25 g of CaCO_3 was added to 100 g aliquots of basal soil which was then designated as partially neutralized soil or neutralized soil, respectively. Seedlings were grown for 3 weeks under a 12 h light and dark photoperiod (PPFD; 100 $\mu\text{mol E m}^{-2} \text{s}^{-1}$) at 20–25°C.

Results

QTL controlling proton resistance in two RI populations

The QTLs controlling Al resistance have been identified in the *Ler/Col* RI population (Kobayashi and Koyama 2002;

Hoekenga et al. 2003). The QTLs controlling proton resistance in this RI population have not been identified. We therefore performed QTL analysis of proton resistance using relative root length (RRL) at pH 4.7 as the resistance index. There was a small difference in RRL between the parental accessions, *Ler* and *Col*. The RRLs of *Ler* and *Col* were 49.0 and 44.1%, respectively (Table 1). The RI population showed a normal distribution of RRL values ($0.025 < P < 0.050$) when assessed using the χ^2 test ($\chi^2 = 12.51$) with a coefficient of variation of 20.7% (*Ler/Col*). The broad sense heritability (H_2^b) of RRL in the RI population was 0.99. Using these data sets, we identified two major QTLs ($P < 0.05$) by the CIM method and six significant epistatic interacting loci pairs ($P < 0.005$) using a complete pairwise search (Tables 2, 3). Major QTLs were detected in the middle of chromosome 2 (73.9 cM from the top and linked to genetic marker nga1126) and in the middle of chromosome 5 (109.49 cM from the top and linked to genetic marker mi219), which together explained about 35% of the proton resistance phenotypic variation within the *Ler/Col* RI population. Although the *Ler* allele showed a positive effect on the RRL at both QTL positions, three of the heterologous epistatic loci pairs (e.g., CL and LC of $\text{nga139} \times \text{mi2}$) had more positive effects on the RRL than the *Ler* homologous loci pairs. This variation in the genetic influences on RRL could explain transgressive segregation among the *Ler/Col* RI population. No major QTL detected by the proton resistance test overlapped that associated with Al resistance previously identified in the same mapping population (Kobayashi and Koyama 2002) (Table 2).

Table 1 Root growth of *Ler/Col* RI population and two parental lines after 7 days hydroponic culture in control (pH 5.0) or low pH (pH 4.7) media

	Root length (mm)		Relative root length (%; pH 4.7/control)
	Control	pH 4.7	
<i>Col</i>	17.6 \pm 0.6	6.7 \pm 0.1	44.1 \pm 0.7
<i>Ler</i>	17.3 \pm 0.8	8.5 \pm 0.7	49.0 \pm 4.1
Range of RI lines	3.5–25.4	5.8–10.6	27.2–69.8
Mean of RI lines	19.3 \pm 0.3	8.0 \pm 1.0	42.3 \pm 8.8
CV	15.7	12.6	20.7
Heritability (H_2^b)	0.98	0.97	0.99
LSD	2.2	1.0	5.5

Relative root length of parental lines and RI lines were calculated
CV coefficient of variation, Heritability (H_2^b) broad sense heritability (see text), LSD least significant difference value of each data set at $P < 0.01$

Table 2 Closely linked markers for QTLs associated with low pH stress (pH 4.7) or Al stress (4 μ M, pH 5.0; Kobayashi and Koyama 2002) detected by the composite interval mapping method at a $P < 0.05$ threshold

Chrom.	Marker interval	Linked marker	LOD	Additive effect	R^2 ^a	Allele mean ^b (%; control)	
						Col	Ler
QTL detected by low pH							
2	GPA1–nga1126	nga1126	4.61	5.49	0.22	38.6	43.6
5	mi219–mi291b	mi219	2.97	2.93	0.13	41.1	42.5
QTL detected by Al ^c							
1	apx1A–ATTS0477	ARR4	6.67	–10.2	0.32	54.6	36.9
4	mi51–mi204	mi51	2.52	5.5	0.11	43.5	51.0

Chrom. chromosome numbers, LOD additive logarithm of odds value

^a Proportion of the variance explained by each QTL

^b Genotypic mean of the relative root length of RI lines at QTL position

^c QTL detected with aluminum in our previous study (Kobayashi and Koyama 2002)

Distribution of Al resistance and low pH resistance of *Arabidopsis* accessions

We used 260 of 300 natural accessions (ecotypes) based on their suitability for hydroponic culture, and grew them in control (pH 5.0), Al (4 μ M Al at pH 5.0), and low pH (pH 4.7) solutions. Root growth of the accessions ranged from 8.1 to 20.2 mm in the control solution, while those in the proton (pH 4.7) and the Al (4 μ M) toxic solutions ranged from 4.0 to 13.7 mm and from 1.0 to 16.1 mm, respectively (Table 4). In all cases, broad sense heritability was higher than 0.90. Using these data sets, we calculated RRL as the resistance index of accessions for each stress factor and found each to conform to a typical normal distribution judged by χ^2 test (pH, $\chi^2 = 8.45$; Al, $\chi^2 = 8.77$;

0.250 < $P < 0.500$) (Fig. 1a, b). Coefficients of variation for RRL in low pH and Al solutions were 13.0 and 22.4, respectively. Larger CV values suggested that the genetic factor controlling root growth in the Al solution would be more valuable for breeding purposes than that controlling root growth in the low pH solution. No significant (i.e., $r^2 = 0.003$, $P = 0.393$) correlation of RRL in the Al and proton toxic solutions indicated that there was no simple genetic interaction between these two traits (Fig. 1c).

Cluster analysis of Al resistance and proton resistance among accessions

Cluster analysis of the phenotypes of natural accessions is one approach to analyzing genetic interactions between various traits (Maloof et al. 2001). Using root length in

Table 3 Epistatic interactions between two loci for low pH resistance Ler/Col RILs detected by a complete pair-wise search at $P < 0.005$ to reject false positives

Epistatic marker interaction	Chrom.	LLR	RRL means (%; pH 4.7/control)			
			Allelic combination at epistatic loci pairs			
			C \times C	C \times L	L \times C	L \times L
nga111 \times mi204	1 \times 4	6.8	43.4 ^b	40.7 ^{a,b}	38.5 ^a	44.1 ^b
nga1145 \times mi32	2 \times 4	7.2	42.1 ^b	40.1 ^{a,b}	39.2 ^a	46.5 ^c
O802F \times nga1107	2 \times 4	10.3	44.8 ^b	37.2 ^a	41.2 ^b	43.3 ^b
nga1111 \times fm4	4 \times 5	9.0	36.0 ^a	42.7 ^b	44.5 ^b	41.4 ^b
mi32 \times m331	4 \times 5	7.8	38.7 ^a	41.7 ^a	46.8 ^b	40.9 ^a
nga139 \times mi2	5 \times 5	6.8	39.2 ^a	44.4 ^b	44.8 ^b	40.3 ^a

Different letters indicate significant difference among four combinations of each epistatic interacting loci pair estimated by one-way ANOVA ($P < 0.05$)

Chrom. chromosome numbers of epistatic loci, LLR log likelihood ratio, RRL relative root length, C Col allele, L Ler allele

Table 4 Root growth of *A. thaliana* accessions after 7 days in hydroponic culture with low pH and Al treatments

	Root length (mm)			Relative root length (%; control)	
	Control	pH 4.7	AlCl ₃	pH 4.7	AlCl ₃
	Mean of lines	13.6 ± 2.2	7.5 ± 1.4	9.3 ± 2.4	55.8 ± 7.2
Minimum line mean	8.1	4.0	1.0	34.7	7.5
Maximum line mean	20.2	13.7	16.1	74.6	106.4
CV	16.2	18.3	26.0	13.0	22.4
Heritability (H_2^b)	0.96	0.97	0.99	0.94	0.99
LSD	3.5	1.7	1.5	12.8	11.7

CV coefficient of variation, Heritability (H_2^b) broad sense heritability, LSD least significant difference value of each data set at $P < 0.01$

Fig. 1 Distribution of relative root lengths of 260 *Arabidopsis thaliana* accessions in low pH (a) and Al (b) treatments. Root length in control solution (pH 5.0, no Al) was expressed as 100%. Seedlings of accessions were grown hydroponically for 7 days in low pH (pH 4.7) or in Al (4 μ M, pH 5.0) solutions. Correlation of the RRL in low pH and Al is shown in c

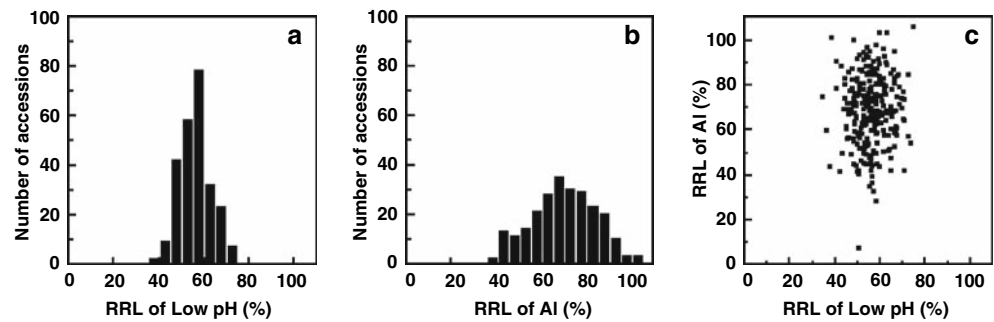
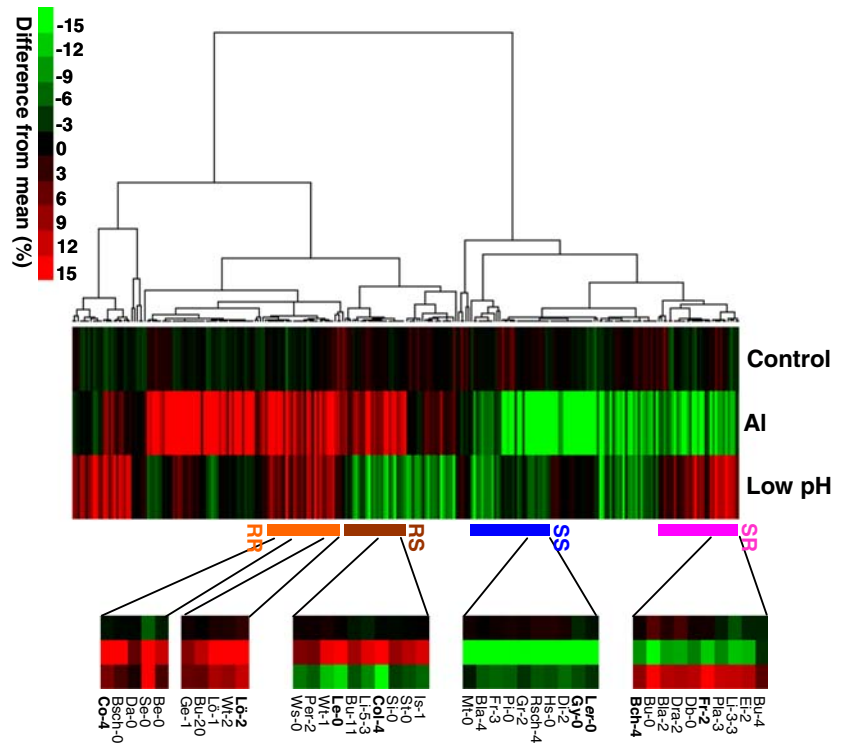


Fig. 2 Hierarchical cluster analysis to group accessions that showed similar responses for proton and Al toxicities. Columns indicate the values for each accession. Root length in control solution is shown in the upper row (control) and followed by the relative root length values of 4 μ M Al (Al) and pH 4.7 (low pH). Differences from the mean of each condition are indicated by colors in rows as follows: intermediate (black), less than average (green), and greater than average (red). Typical accession groups are highlighted by bars at the lower side of rows. RR Al and low pH resistant (orange), RS Al resistant but low pH sensitive (brown), SR Al sensitive but low pH resistant (pink), and SS Al and low pH sensitive (blue)



control solution as a reference, we performed hierarchical cluster analysis for the RRL of Al and low pH solutions. As shown in Fig. 2, accessions can be grouped by different combinations of Al-resistance and proton-resistance. Accessions in the right side cluster are mostly sensitive to Al (green at the second line), while the left side cluster contains both Al-resistant and intermediate accessions (red or black). Five intermediate accessions (i.e., both black for Al resistance and proton resistance) were grouped in the right side cluster. The right side cluster also contained one group of Al-sensitive/proton-resistant accessions (SR, 32 accessions; pink bar) and two groups of Al/proton-sensitive accessions (SS, 31 accessions; blue bar). On the other hand, the Al/proton-resistant group (RR, 28 accessions; orange bar) and the Al resistant/proton-sensitive group (RS, 24 accessions; brown bar) were identified in the left side cluster. To examine the reliability of the grouping by cluster analysis, we compared root growth of selected accessions

(RR: Lö-2 and Co-4; RS: Col-4 and Le-0; SR: Bch-4, Fr-2, and SS: Ler-0 and Gy-0) at the usual screening conditions (i.e., 4 μ M Al at pH 5.0 and zero Al at pH 4.7) and at more severe screening conditions (i.e., 6 μ M Al at pH 5.0 and zero Al at pH 4.6). As shown in Fig. 3, resistant accessions grew better than sensitive accessions under both the more severe and less severe conditions, indicating that the reliability of the grouping under variable degrees of stress.

Aluminum and proton resistance of accessions judged by root tip damage

To further test the reliability of grouping, we performed histochemical analyses using known methods for evaluating Al resistance and low pH resistance. To test Al resistance, we observed accumulation of ROS (i.e., H_2O_2) and callose in the root tip following 24 h Al treatment; both

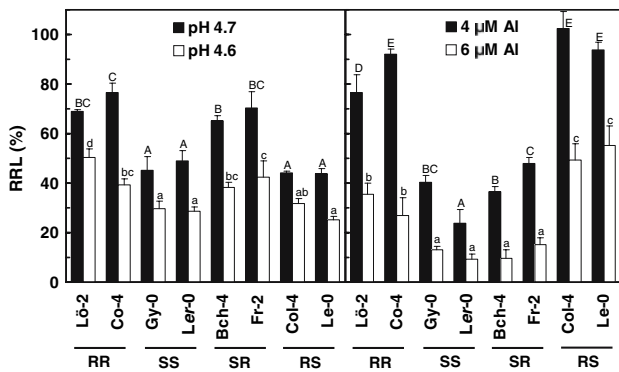


Fig. 3 Relative root length of selected accession in low pH (pH 4.7 and 4.6, *left panel*) and Al (4 and 6 μ M at pH 5.0, *right panel*) solutions. Each two accessions with different combinations of Al resistance and low pH resistance were selected from 260 accessions by the phenotypic cluster analysis in Fig. 2 (RR Al and low pH resistant, RS Al resistant but low pH sensitive, SR Al sensitive but low pH resistant, and SS Al and low pH sensitive). Mean \pm SD ($n = 9$) of the relative root length [(growth in Al or at low pH)/control (no Al at pH 5.0)] from three replications are shown. Different letters indicate significant difference by LSD test ($P < 0.01$)

accumulations are sensitive indicators of Al injury. Both SR and SS groups showed brighter fluorescence than RR and RS, indicating that the Al-sensitive groups accumulate greater amounts of ROS (Fig. 4Aa) and callose (Fig. 4Ab) than Al-resistant groups. On the other hand, the damage in the root tip by proton toxicity can be visualized by propidium iodide staining (Koyama et al. 2001). By this method, proton-sensitive accession groups (RS and SS) showed more damage than resistant groups (RR and SR) after growing in the low pH solution for 1 day (Fig. 4B). This provided confirmation of the reliability of assessments of Al and proton resistance judged by RRL.

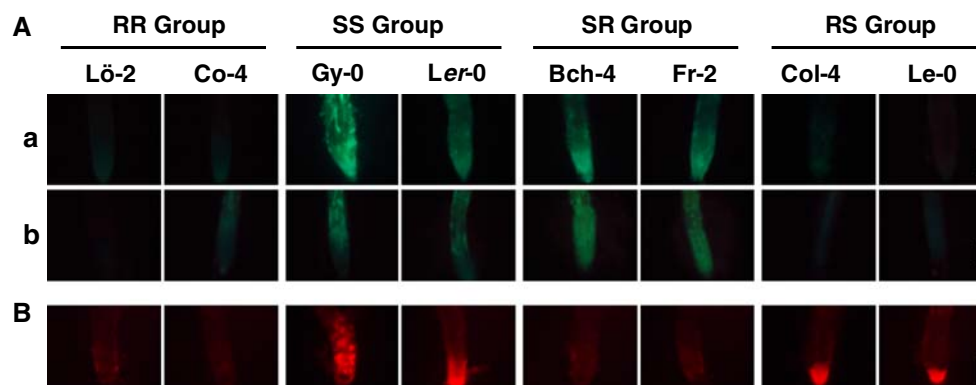


Fig. 4 Histochemical analyses of the root tip damage caused by Al (A) and low pH (B) treatments. Accessions showing different combinations of Al and low pH resistances (RR Al and low pH resistant, RS Al resistant but low pH sensitive, SR Al sensitive but low pH resistant, and SS Al and low pH sensitive) were exposed to Al (4 μ M Al, pH 5.0) for 24 and 12 h, then stained by H_2 DCFDA (Aa;

It would also indicate that long-term resistance judged by RRL at 1 week is correlated with that of short-term resistance at 24 h. Although Al injury cannot be detected in less than 1 day by the current procedures, proton damage can be detected within a shorter term (<1 h) by identifying seedlings that have damaged cells in portions of the root tip. When the same number (15 seedlings) of the growing roots of each accession were exposed to a low pH simple solution for a short-term (pH 4.7, $CaCl_2$ 200 μ M, 30 min), the number of seedlings with damaged cells in the root tip of sensitive accessions (RS and SS) was significantly greater than in the resistant accessions (RR and SR) (Table 5).

Growth of proton resistance and sensitive accessions on acid soil

To determine whether proton resistance judged by hydroponic culture was correlated with resistance in acid soil, we grew two proton-resistant and two proton-sensitive accessions in acid soil. To minimize the effect of Al toxicity in the soil, accessions were selected to have similar levels of Al-resistance (SR and SS) (Fig. 5a). All four accessions grew similarly in neutralized soil (pH 5.5). However, proton-sensitive accessions, Gy-0 and Ler-0, showed growth inhibition in partially neutralized soil (pH 4.6), while no significant growth reduction was observed in Bch-4 and Fr-2 (Fig. 5b).

Discussion

Physiological and genetic studies to investigate the mechanisms of proton toxicity and resistance are limited when

for H_2O_2) and aniline blue (Ab; for callose). On the other hand, the same accessions were incubated in the low pH solution (pH 4.7) for 24 h and then stained by propidium iodide (B; damage in the plasma membrane) to visualize damages caused by protons on the plasma membrane. The bar indicates 50 μ m

Table 5 Number of plants showed damage in the plasma membrane in the root tip of *A. thaliana* during brief exposure to low pH solution

Accession	Number of seedlings with damaged roots	
	Control	pH 4.7
Lö-2	1.7 ± 2.1 ^a	1.3 ± 0.6 ^a
Co-4	1.3 ± 1.5 ^a	3.7 ± 1.2 ^a
Bch-4	2.3 ± 1.5 ^a	4.0 ± 1.0 ^a
Fr-2	2.3 ± 2.5 ^a	4.0 ± 2.0 ^a
Col-4	3.0 ± 1.7 ^a	8.0 ± 2.0 ^b
Le-0	2.3 ± 2.3 ^a	8.3 ± 2.5 ^b
Gy-0	3.7 ± 2.9 ^a	9.7 ± 1.5 ^b
Ler-0	1.7 ± 2.1 ^a	8.3 ± 1.2 ^b

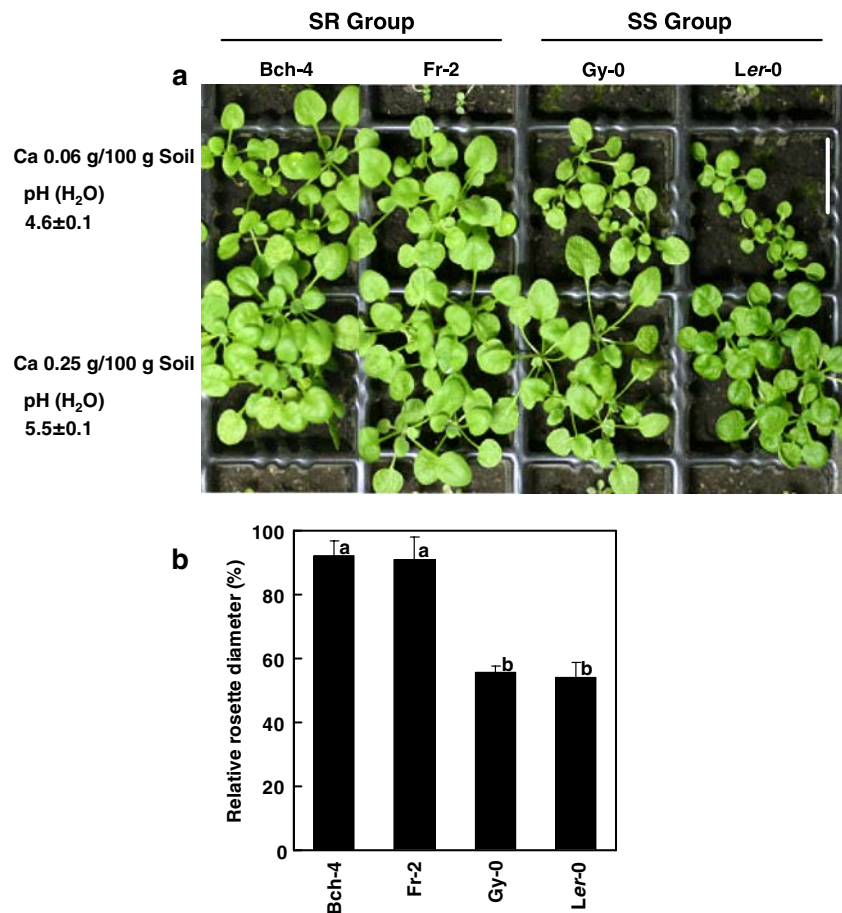
The root of 15 seedlings were immersed in the basal test solution containing 200 µM CaCl₂ added at pH 5.0 (control) or pH 4.7 for 30 min and then stained with PI. Mean ± SD from three replications are shown. Different letters indicate significant difference by LSD test ($P < 0.01$)

compared to those of Al toxicity. One reason for this may be the uncertain nature of rhizotoxicity of protons in hydroponic culture due to the alleviative effects of other cations in culture solutions (Kinraide et al. 1992). For example, high concentrations of coexisting Ca²⁺ ion in low

pH solutions drastically alleviate proton rhizotoxicity by three separate mechanisms (Kinraide 1998). However, the toxicity is clear in low ionic strength solutions (Koyama et al. 2001). In the present study, we employed a low ionic strength solution to enhance proton rhizotoxicity (Koyama et al. 2001). In this culture solution, growth of accessions were inhibited by lowering pH (Figs. 3, 4B, Table 5). A significant difference in RRL was observed between resistant and sensitive accessions (Fig. 3), indicating that genetic variation must exist to control proton resistance of *Arabidopsis*.

The solutions used in our study combined higher-than-usual pH for Al studies, low ionic strength, and low Al concentration. This combination of conditions allowed the imposition of Al stress in the absence of proton stress and prevented the formation of polynuclear or solid-phase Al. For the solution that was used for screening of Al sensitivity (4 µM Al, Ca 200 µM at pH5.0), no Al precipitation was expected by GEOCHEM analysis (data not shown), but the Al toxicity to judge by {Al³⁺} at the plasma membrane surface as estimated by a Gouy–Chapman–Stern model, which is more reliable index for the assessment of Al toxicity than bulk-phase {Al³⁺} (e.g., Kinraide et al. 1992). The {Al³⁺}_{PM} in the screening solution is 14.3 µM,

Fig. 5 Growth of low pH resistant and sensitive accessions with same level of resistance to Al on a typical acidic andosol of Japan, namely Kawatabi andosol. Plants of SR (Al sensitive but low pH resistance) and SS (Al sensitive and low pH sensitive) were grown for 3 weeks on the neutralized (pH 5.5) and partially neutralized (pH 4.6) andosol which contained different levels of CaCO₃ (a). Relative rosette diameter in soil culture (%; pH 4.6/pH 5.5) are also shown (b). Different letters indicate significant difference by LSD test ($P < 0.01$). The bar indicates 1 cm



which is very similar to that in a common Al test solution (i.e., 100 μM Al in 400 μM Ca at pH 4.4; $\{\text{Al}\}_{\text{PM}} = 14.7 \mu\text{M}$) (personal communication with Dr. Thomas Kinraide at ARS, USDA). In fact, sensitive biochemical indicators of Al toxicity such as callose formation (e.g., Wissemeier et al. 1992) were identified in the same solution (Fig. 4). It was thus we could infer that we could estimate Al sensitivity of accessions by minimizing proton toxicity. In this experimental system, no significant correlations of the RRL in Al and in low pH (Fig. 1c) and differential genetic architecture of QTL in a *Ler/Col* RI population were observed. These results indicated that proton resistance and Al resistance are mostly regulated by different genetic mechanisms which regulate different biological system.

Cluster analysis with phenotypic values allowed us to group typical accessions that showed different combinations of proton and Al resistance. Because the degree of resistance of accessions was similar even when tested under two degrees of severity, we infer that resistance indices used for phenotypic cluster analysis is reliable (Fig. 2). Resistance of accessions to both toxicities judged by root growth was correlated to that of short-term responses. For example, typical Al damage symptoms, namely callose accumulation (e.g., Schireiner et al. 1994; Kaneko et al. 1999; Sivaguru et al. 2000) and H_2O_2 generation (Ezaki et al. 2001; Basu et al. 2001) in Al-resistant accessions were less than those in sensitive accessions (Fig. 4Aa, Ab). In addition, an early symptom of proton toxicity in growing roots, namely the short-term damage in the root tip (Koyama et al. 2001), was marked in the proton-sensitive accessions while it was ambiguous in the resistant accessions (Fig. 4B). These results may suggest that the same mechanisms control both long-term and short-term resistance to each stress factor. For example, *Arabidopsis* Al resistance is mainly regulated by malate release from the roots (Hoekenga et al. 2006), and it can be observed at similar time scales as those employed here to observe callose and H_2O_2 generation (Yamamoto et al. 2002). Similar relationships are common in other plant species that employ organic acid release as an Al resistance mechanism, such as wheat (Delhaize et al. 1993; Ryan et al. 1995; Sasaki et al. 2004), barley (Ma et al. 2004) and snap bean (Miyasaka et al. 1991).

We were able to identify the genetic architecture of proton resistance in a *Ler/Col* RI population including two major QTLs detected by CIM and six epistatic interacting loci pairs (Tables 2, 3). The major QTLs for proton resistance did not overlap with previously identified Al-resistant QTLs (Kobayashi and Koyama 2002; Hoekenga et al. 2003). This indicates that the major genetic factors controlling Al resistance and proton resistance are clearly distinct at least in this RI population. In fact, Al responsive malate excretion controlled by the *Col* allele at the top of

chromosome 1 (QTL1 of Al resistance; Hoekenga et al. 2003) was identified as the Al-resistant mechanism in the RI population. However, a T-DNA insertion mutant (KO in *AtALMT1*) lacks Al-responsive malate excretion and showed no change in low pH sensitivity (pH 4.2; Hoekenga et al. 2006), indicating that malate release is not involved in proton resistance. However, cluster analysis indicates that different mechanisms drive proton resistance, which is distinct from that for Al resistance. At present, there are several possible mechanisms of proton resistance, such as enhanced proton efflux, as a part of integrated reactions (see review Netting 2002) that could contribute phenotypic variations in proton resistance. Comparison of such capacities among accession groups would be a useful approach for answering this question. In this context, recent advances of public resource centers and databases in *A. thaliana* could accelerate functional biological approaches for understanding the mechanisms of proton resistance at the molecular level.

In soil culture using acid soil, growth of proton-sensitive accessions was more inhibited than growth of resistant accessions (Fig. 5). Since, in this experiment, we used four accessions with similar levels of Al-resistance, the observed growth differences may be caused by differences in their sensitivity to protons. Although Al toxicity is believed as most the severe stress factor in acid soil, our results suggest that protons can be stress factor in some plant species sensitive to protons such as spinach (Yang et al. 2005) and turnip (Kinraide and Parker 1990). This factor would be one of the important breeding targets for improving crop productivity in acid soil. Further research is needed to examine this possibility.

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