

Expression of Aluminum-Induced Genes in Transgenic Arabidopsis Plants Can Ameliorate Aluminum Stress and/or Oxidative Stress¹

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To examine the biological role of Al-stress-induced genes, nine genes derived from Arabidopsis, tobacco (*Nicotiana tabacum* L.), wheat (*Triticum aestivum* L.), and yeast (*Saccharomyces cerevisiae*) were expressed in Arabidopsis ecotype Landsberg. Lines containing eight of these genes were phenotypically normal and were tested in root elongation assays for their sensitivity to Al, Cd, Cu, Na, Zn, and to oxidative stresses. An Arabidopsis blue-copper-binding protein gene (*AtBCB*), a tobacco glutathione S-transferase gene (*parB*), a tobacco peroxidase gene (*NtPox*), and a tobacco GDP-dissociation inhibitor gene (*NtGDI1*) conferred a degree of resistance to Al. Two of these genes, *AtBCB* and *parB*, and a peroxidase gene from Arabidopsis (*AtPox*) also showed increased resistance to oxidative stress induced by diamide, while *parB* conferred resistance to Cu and Na. Al content of Al-treated root tips was reduced in the four Al-resistant plant lines compared with wild-type Ler-0, as judged by morin staining. All four Al-resistant lines also showed reduced staining of roots with 2',7'-dichloro fluorescein diacetate (H₂DCFDA), an indicator of oxidative stress. We conclude that Al-induced genes can serve to protect against Al toxicity, and also provide genetic evidence for a link between Al stress and oxidative stress in plants.

Al ions have a toxic effect on both plant and animal cells (Kochian, 1995). It has been suggested that Al³⁺ ions enhance the peroxidation of phospholipids and proteins in cell membranes (Cakmak and Horst, 1991; Yamamoto et al., 1997), but a range of alternative toxicity mechanisms have also been proposed (Kochian, 1995). Exudation of Al-chelating organic acids such as malate, oxalate, or citrate into the rhizosphere has been proposed as a tolerance mechanism to avoid Al toxicity in many plants (Ryan et al., 1995). Internal oxalate has also been reported to detoxify

cytosolic Al by chelation in an Al-accumulating plant (Ma et al., 1997). Recently, overexpression of a bacterial citrate synthase gene in transgenic plants was shown to confer Al tolerance (de la Fuente et al., 1997). Chelation strategies are very useful, but combining them with additional Al tolerance mechanisms within the plant would be expected to provide more effective protection.

Over 20 genes induced by Al stress have been isolated from a range of plant species, including wheat (*Triticum aestivum* L.) (Snowden and Gardner, 1993; Cruz-Ortega et al., 1997; Hamel et al., 1998; Delhaize et al., 1999), tobacco (*Nicotiana tabacum* L.) (Ezaki et al., 1995, 1996, 1997), and Arabidopsis (Sugimoto and Sakamoto, 1997; Richards et al., 1998). Most of the Al-induced genes seem to be general stress genes that are induced by a range of different plant stresses, including low phosphate (Ezaki et al., 1995), other metal toxicities (Snowden et al., 1995; Sugimoto and Sakamoto, 1997), wounding (Snowden et al., 1995), pathogen infection (Cruz-Ortega et al., 1997; Hamel et al., 1998), or oxidative stress (Sugimoto and Sakamoto, 1997; Richards et al., 1998). Some of the induced genes are well known as anti-oxidation enzymes (e.g. glutathione S-transferase, peroxidase, and superoxide dismutase). It has therefore been proposed that there are common mechanisms for gene induction between Al toxicity and oxidative stress (Richards et al., 1998). The biological role of Al-induced genes in plants is unclear. By analogy with other stress genes, the genes may play a role in protecting cells against Al stress, but experimental evidence on this point is lacking. Recently, we expressed 11 plant Al-induced genes in yeast (*Saccharomyces cerevisiae*) cells and showed that two of these, the tobacco GDP-dissociation inhibitor (GDI) gene (*NtGDI1*) and the gene encoding the Arabidopsis blue copper-binding (BCB) protein (*AtBCB*), conferred Al resistance in yeast cells (Ezaki et al., 1999). Overexpression of another Al-induced gene encoding the wheat phosphatidylserine synthase enzyme also gave Al resistance in yeast (Delhaize et al., 1999).

We describe the construction of transgenic Arabidopsis lines expressing nine Al-induced genes, as well as results of sensitivity tests for Al stress and for oxidative stresses by monitoring root growth. The results suggest that expression of four of the Al-induced genes can ameliorate Al toxicity and that three confer protection against oxidative stress.

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MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis ecotype Landsberg *erecta* (Ler-0) was used for transformation. All *Arabidopsis* plants were grown under fluorescent illumination (approximately $50 \mu\text{E m}^{-2} \text{s}^{-1}$, 16 h of light and 8 h of darkness) at 22°C. A modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing MS salts, B5 vitamins, and 10 g L^{-1} Suc, which was adjusted to pH 5.7, was used for transformation and kanamycin screening. Another modified MS medium, 1/6 MS solution, in which Suc was 10 g L^{-1} , but MS salts and B5 vitamins were 6× diluted, was used for various sensitivity tests in this study. The pH of the medium was adjusted to 4.0 for all sensitivity tests.

Construction of Transgenic Arabidopsis Lines

The cDNA fragments containing each Al-induced gene were inserted between the cauliflower mosaic virus 35S promoter and the OCS terminator of pART7 and then cloned into the *NotI* site of pART27 (Gleave, 1992). Constructed plasmids were listed in Table I. All of these constructs were transformed to *Agrobacterium tumefaciens* LBA4404 to get kanamycin-resistant lines. Transformation of *Arabidopsis* by *A. tumefaciens* was performed by the vacuum infiltration method described by Bechtold et al. (1993). T₁ transgenic lines were selected on plates containing 1% (w/v) agar, 200 mg L^{-1} timentin, and 75 mg L^{-1} kanamycin. The kanamycin-resistant seedlings were transferred to soil and grown to maturation. Screening of seeds for kanamycin-resistant progeny was carried out in the same way.

Al Stress and Various Stresses for Plants

Screening for Al resistance was performed according to a vertical-mesh-transfer (VMT) technique developed by Murphy and Taiz (1995). This plate assembly consisted of a 3-mm-thick plastic plate, three squares of 1-mm-thick chromatography paper (3MM CHR, Whatman, Maidstone, UK), and a square of nylon mesh (30- μm mesh). All of these materials were cut to 5- × 5-cm squares. The nylon mesh and chromatography sheets were saturated with the 1/6 MS medium and then set on the plastic plate in this order. Sterilized seeds were incubated at 4°C for 4 d and then

plated in a line on the center of the nylon mesh. The VMT plates were inclined at an angle of more than 80° in a sterilized plant growth rack. Twenty milliliters of 1/6 MS medium was poured into the racks (350 mL volume) to support plant growth. After 5 d of growth, the nylon mesh carrying the young seedlings (now with 1- to 1.5-cm-long roots) was transferred to a second VMT plate on which three new chromatography sheets had been saturated with 1/6 MS medium supplemented with various concentration of metal ions or peroxides. This new VMT plate was rotated 180° with the roots pointing upward, placed in the growth rack, and incubated for a further 2 d. Root growth (the length between the root apex and bending point) of 20 plants selected on the basis of seed availability was measured for each treatment group during the 2-d treatment. Root growth in each treatment was calculated relative to the control treatment.

Microscopic Observations

Localization of Al ions in roots was determined by staining with morin (Sigma, St. Louis), according to the method described by Tice et al. (1992). The formation of peroxides such as H₂O₂ in the root regions was visualized by H₂DCFDA (Molecular Probes, Eugene, OR) as described by Behl et al. (1994). A fluorescent microscope (model MPM800, Carl Zeiss, Oberkochen, Germany) was used for observation.

RESULTS

Construction of Transgenic Arabidopsis Lines Carrying Al-Induced Genes

Larsen et al. (1996) reported differences in Al sensitivity between ecotypes of *Arabidopsis*, with Ler-0 being more sensitive than Columbia (Col-0). We tested these two ecotypes using the gravity bending, root elongation assay (Fig. 1). Ler-0 always showed better root growth in the absence of Al ions (about 1.5 times longer than Col-0), but was more sensitive to Al than Col-0. Root growth of these two ecotypes was inhibited by increasing the Al concentration. A more severe inhibition was observed in Ler-0, especially by 100 to 300 μM Al treatments. We concluded that the more sensitive Ler-0 would be a more useful

Table I. Al-induced genes expressed in *Arabidopsis*

Gene	Identity	Species of Origin	Plasmid and Reference
<i>wali5</i>	Bowman-Birk protease inhibitor	<i>T. aestivum</i>	pAL605 (Snowden and Gardner, 1993)
<i>AtBPI</i>	Bowman-Birk protease inhibitor	<i>Arabidopsis</i>	pAL606 (Richards et al., 1997)
<i>AtBCB</i>	Blue copper binding protein	<i>Arabidopsis</i>	pAL610 (Richards et al., 1997)
<i>AtPOX</i>	Peroxidase	<i>Arabidopsis</i>	pAL611 (Richards et al., 1997)
<i>parA</i>	No homolog	<i>N. tabacum</i>	pAL612 (Ezaki et al., 1995)
<i>parB</i>	GST	<i>N. tabacum</i>	pAL614 (Ezaki et al., 1995)
<i>NtPOX</i>	Anionic peroxidase	<i>N. tabacum</i>	pAL616 (Ezaki et al., 1996)
<i>NtGDI1</i>	GDP-dissociation inhibitor	<i>N. tabacum</i>	pAL618 (Ezaki et al., 1997)
<i>HSP150</i>	Heat shock protein	<i>S. cerevisiae</i>	pAL620 (Ezaki et al., 1998)

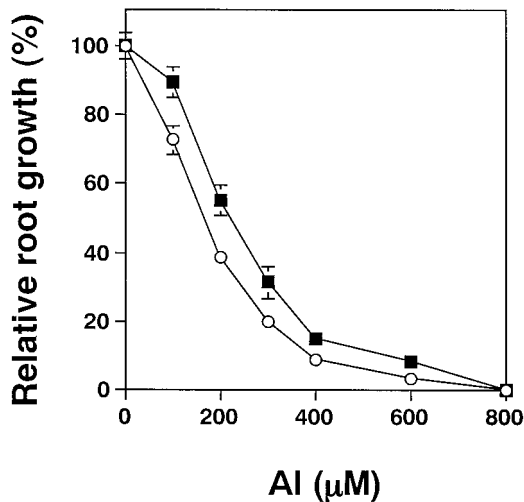


Figure 1. Relative root growth of two ecotypes of *Arabidopsis* in Al stress measured by the gravity bending assay. Relative growth (expressed as a percentage of root growth in the absence of Al) was compared between Ler-0 (○) and Col-0 (■). Error bars represent se values ($n = 20$). The average growth of Ler-0 and Col-0 roots over 2 d were 10.6 and 6.1 mm, respectively.

recipient ecotype for monitoring the effect of potential Al-resistant genes.

Nine Al-induced genes, listed in Table I (genes are identified by previously used designations or by a five-letter code), were chosen for expression in plants. Transformation of *Arabidopsis* produced 22 to 80 independent kanamycin-resistant transgenic lines for each gene. To determine the number of T-DNA insertions into the plant genome, 20 kanamycin-resistant lines for each gene were tested for their segregation ratios in the T_2 generation. Approximately 45% to 85% of the tested lines showed ratios of 3:1 (kanamycin-resistant:kanamycin-sensitive seedlings) indicative of single locus insertions in the genome. Five T_2 lines showing single insertions were selected for each gene and northern-blot hybridization analysis was carried out. There was considerable variation in transcript levels between lines and between genes (Fig. 2). Lines containing the *wali5*, *AtBPI*, *AtBCB*, and tobacco peroxidase (*NtPox*) genes generally expressed transcript at high levels, and high-expressing lines could be identified for the *parA*, *parB*, and *HSP150* genes. At the other end of the scale, the *AtPox* and *NtGDI1* genes were poorly expressed in all the lines. Homozygous T_2 plants were identified by screening kanamycin segregation ratios in their T_3 generations; for each transgene we obtained two to four independent lines that showed high gene expression. Most lines were phenotypically normal and showed no significant differences in root growth compared with Ler-0. However, lines carrying the *AtBPI* gene showed poor root growth and were not included in subsequent characterizations.

Sensitivity to Al Stress

As a preliminary screen for Al resistance, one transgenic line containing each gene was used in a root-elongation

assay at three Al concentrations (Fig. 3A). The clearest differences between lines were seen at 200 μM Al. Statistical analyses (Welch's t test and Student's t test) revealed that four transgenic lines, those expressing *AtBCB*, *parB*, *NtPox*, or *NtGDI1*, showed significantly higher relative root growth ($P < 0.01$) than the Ler-0 plants or plants containing the binary vector pART27 only (Fig. 3A). The other three transgenic lines carrying the *wali5*, *AtPox*, or *parA* genes showed growth that was not significantly different from controls. The transgenic line of the *HSP150* gene showed an

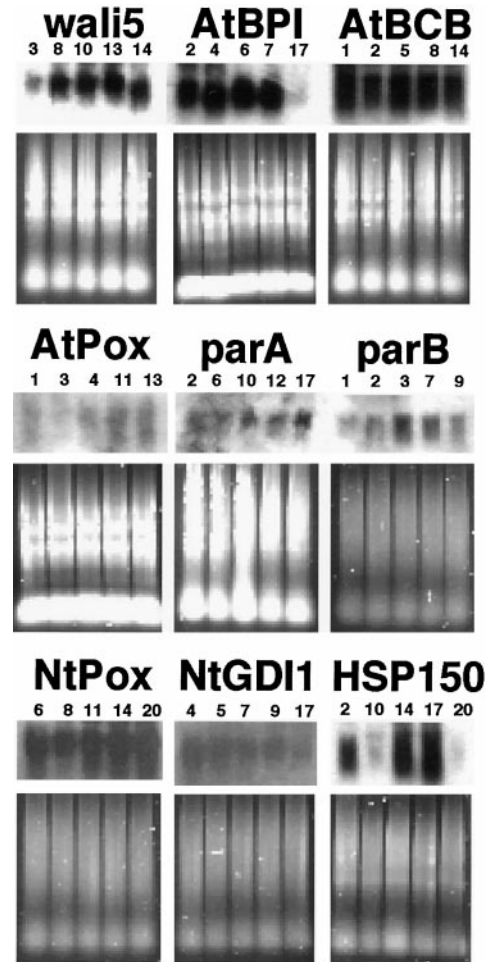


Figure 2. Expression of the Al-induced genes in transgenic *Arabidopsis* lines. Total RNA was extracted from seedlings of each transgenic line, applied to an agarose gel (20 μg for *wali5*, *AtBPI*, *AtBCB*, *AtPox*, and *parA* lines, and 10 μg for the *parB*, *NtPox*, *NtGDI1*, and *HSP150*), and stained with ethidium bromide (bottom part of each figure). DIG-labeled DNA probes were prepared by PCR using each cloned coding region as a template and hybridized to the filters (top part). The numbers above each lane correspond to individual transgenic lines with single-locus inserts of each construct. Thus, for *wali5* we analyzed expression of lines 3, 8, 10, 13, and 14. The highest expressing lines for each construct were chosen and homozygous seeds were obtained for phenotypic analysis (e.g. *wali5*, lines 8, 10, and 14; *parB*, lines 3, 7, and 9). Northern-blot analyses using the total RNA extracted from Ler-0 as control were also performed, and low or undetectable levels of transcript were seen for all the genes (data not shown).

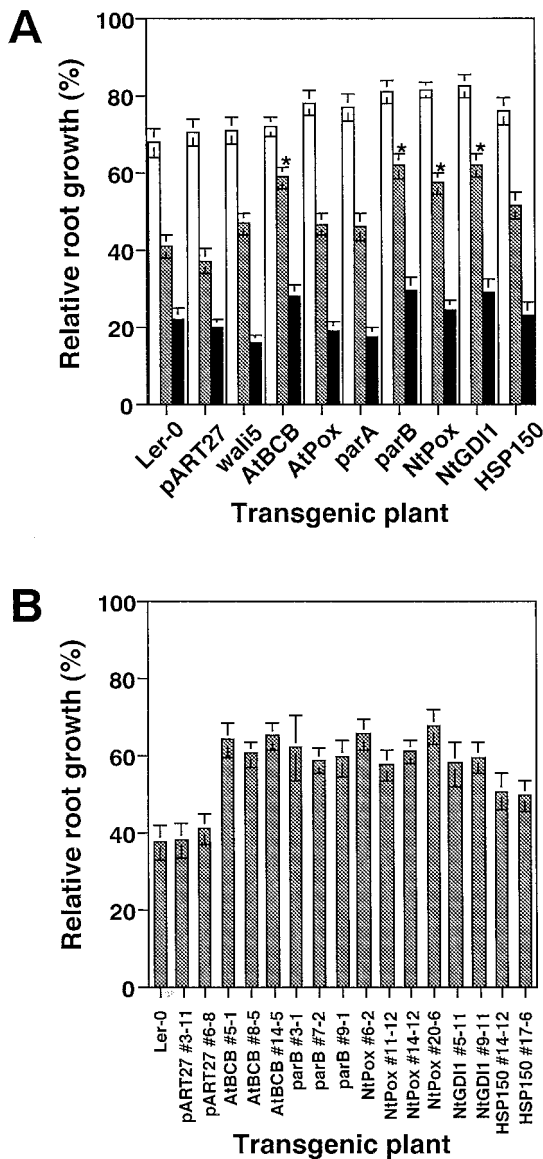


Figure 3. Al resistance of transgenic lines by the gravity bending assay. **A**, One line was selected from each group of transgenic lines and tested in four concentrations of Al (0, 100, 200, and 300 μM) at pH 4.0 for 2 d. Results (white bar, 100 μM ; gray bar, 200 μM ; black bar, 300 μM) are shown as percent root growth compared with 0 μM Al for each line, as in Figure 1. The transgenic lines tested were: pART27 (3–11), *wali5* (8–12), *AtBCB* (5–1), *AtPox* (4–1), *parA* (10–1), *parB* (3–1), *NtPox* (6–2), *NtGDI1* (5–11), and *HSP150* (14–12). Transgenic lines were labeled with nomenclature pART27 (3–11), where the first number refers to the transgenic line (see Fig. 2) and the second number refers to the particular homozygous seed from each line. Values that are significantly different from the controls ($P < 0.01$) are indicated by asterisks. **B**, Variation of Al sensitivity among transgenic lines. Two to four independent transgenic lines containing selected genes were exposed to 0 or 200 μM Al and relative root growth was estimated. Error bars represent SE values ($n = 20$). The average growth rates of Ler-0 roots over 2 d were 9.7 and 10.2 mm in Fig. 3, A and B, respectively; control values for the transgenic lines ranged from 84% to 112% and 78% to 108% of these values, respectively.

intermediate growth between the two groups. At 100 and 300 μM Al, there was no difference in relative root growth for any of the tested lines.

In a second Al sensitivity test, all independent transgenic lines containing the genes *AtBCB*, *parB*, *NtPox*, *NtGDI1*, and *HSP150* were exposed to 200 μM Al (Fig. 3B). Control lines carrying the vector pART27 showed similar sensitivity as Ler-0 for Al treatment. Statistical analyses revealed significant differences in relative root growth at 200 μM Al between the Ler-0 control and all of the individual lines containing the four genes *AtBCB*, *parB*, *NtPox*, and *NtGDI1* ($P < 0.01$). There was no significant difference between individual lines carrying each transgene in the level of Al resistance they conferred, suggesting that the resistance phenotypes of these lines are derived from expression of the transgenes. The relative resistance conferred by the four transgenes has been confirmed by more than three independent experiments. The two lines expressing the *HSP150* gene did not show a significant difference from Ler-0 in relative growth at $P < 0.05$, suggesting that over-expression of the yeast *HSP150* gene in *Arabidopsis* is not sufficient to cause resistance to Al stress.

Sensitivity to Other Metal Stresses and to Oxidative Stresses

Dose-dependent growth inhibition curves for Ler-0 were determined for a range of metal ion stresses using the root-elongation assay. Levels that caused approximately 50% inhibition of root growth were: 50 μM for Cd^{2+} , 20 μM for Cu^{2+} , 100 mM for Na^{+} , and 200 μM for Zn^{2+} (data not shown). None of the transgenic lines showed altered resistance to Cd or Zn stresses. However, the lines carrying the *parB* gene showed higher root growth than Ler-0 or pART27 transgenic lines in both 100 mM Na and 20 μM Cu stress (Fig. 4, A and B).

Sensitivity to three different oxidative stresses, H_2O_2 , diamide, or methyl viologen, was also examined to determine whether the Al-induced genes can confer resistance to these oxidative stresses in plants. In Ler-0, concentrations of 1 mM H_2O_2 , 1 mM diamide, or 15 μM methyl viologen each gave approximately 50% inhibition of root growth (data not shown). No Al-induced genes conferred resistance to H_2O_2 or methyl viologen. However, transgenic lines with the *AtBCB*, *AtPox*, or *parB* genes showed a higher relative growth than Ler-0 and pART27 lines in both 1 and 1.5 mM diamide (Fig. 4C). The sensitivity tests for Cu, Na, and diamide were repeated two or three times for each transgenic line and similar results were obtained. There were also no significant differences between individual lines carrying each transgene in the level of resistance conferred for each stress, suggesting that the resistance phenotypes of these lines are derived from expression of the transgenes.

Estimation of Oxidative Stress Using a Fluorescent Indicator

To characterize the difference in diamide sensitivity between Ler-0 and the three resistant lines in more detail, the

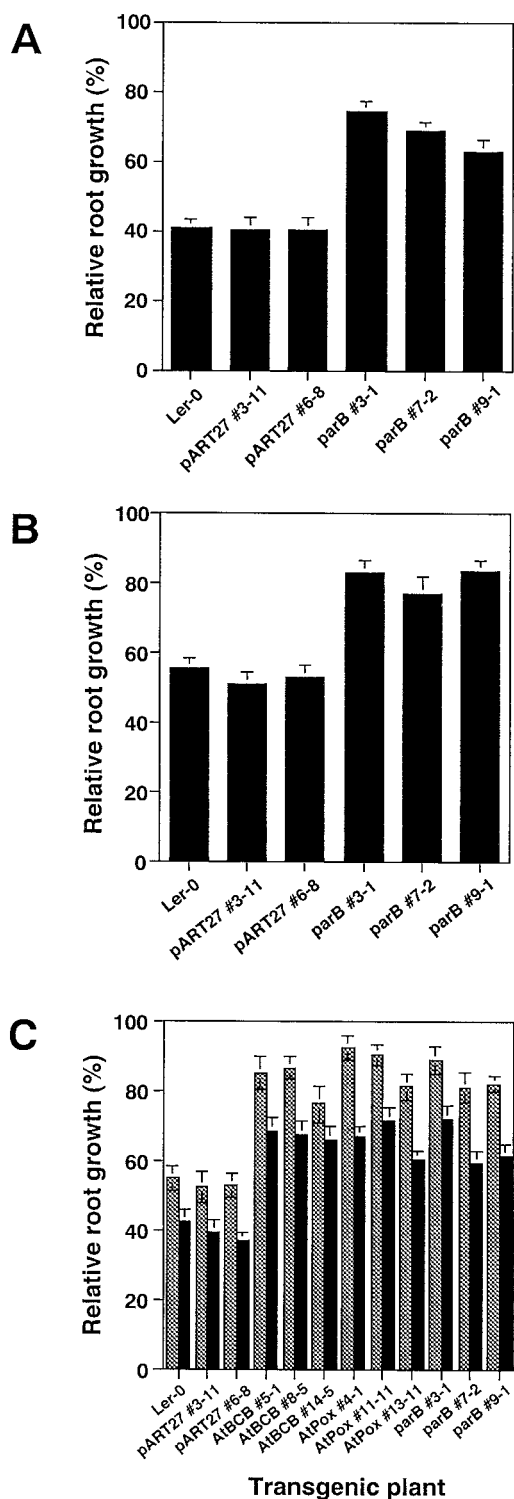


Figure 4. Resistance of transgenic lines to other stresses. A, 100 mM NaCl; B, 20 μ M CuSO_4 ; C, oxidative stress generated by diamide (gray bar, 1 mM; black bar, 1.5 mM). We present only those results for transgenic lines showing a higher relative growth than Ler-0. Results are shown as the percent root growth compared with control treatment for each line, as in Figure 1. Error bars represent SE values ($n = 20$). The average growth of Ler-0 roots was 16.2 mm over 2 d, and the control values for the transgenic lines ranged from 83% to 104% of this value.

roots were examined microscopically after staining with H_2DCFDA , which has been used as a high-sensitivity indicator to the formation of peroxides such as H_2O_2 and lipid hydroperoxides (Behl et al., 1994). Untreated roots of Ler-0, as well as those of all transgenic lines, had very low fluorescent signals in the tip region. With increasing concentrations of diamide, stronger H_2DCFDA -dependent fluorescent signals could be detected in roots of Ler-0 (Fig. 5A, 1–4). Similar staining was also seen in roots treated with 1 mM H_2O_2 (Fig. 5A, 5), indicating that H_2DCFDA is a useful indicator for oxidative stress in plants.

Three diamide-resistant transgenic lines were also stained with H_2DCFDA after 1 mM diamide treatment, and two of them expressing *AtBCB* and *AtPox* showed clearly lower fluorescent signals in their root tip region (compare Fig. 5A, 6–8 with Fig. 5A, 3). On the contrary, a signal strength similar to that of Ler-0 was detected in the *NtPox* transgenic line (Fig. 5A, 9), which showed diamide sensitivity in the root elongation assay. These results confirm that oxidative stress caused by diamide was reduced in the these resistant lines.

Localization of Al Ions and Reactive Oxygen Species Induced in Root Tips by Al Treatment

Morin fluorescence has been used to detect the localization of Al in plant tissue (Tice et al., 1992; Larsen et al., 1996). To characterize the phenotype of the transgenic lines in more detail, roots of Ler-0 or transgenic lines were directly exposed to 1/6 MS medium containing Al ions for 5 h and then stained with morin. Root tips (a zone approximately 0–1 mm from the root apex in *Arabidopsis*) were analyzed, since they are the tissues initially targeted by Al in plants. Negligible levels of fluorescence signal were seen in untreated roots of Ler-0, but increasing fluorescent signals could be seen in root tips treated with 50 and 100 μ M Al for 5 h (Fig. 5B, 1–3). All eight transgenic lines of *Arabidopsis* were compared for the strength of their morin signal at 100 μ M Al.

The tested lines were classified into three groups. The first group, expressing *wali5*, *AtPox*, and *parA*, showed almost the same fluorescence as Ler-0 (Fig. 5B, 4–6). A second group, expressing *parB*, *NtGDI1* and *HSP150*, showed an intermediate level of morin staining (Fig. 5B, 7–9). The final group, expressing *AtBCB* and *NtPox*, had significantly lower signals in their roots (Fig. 5B, 10 and 11). In particular, these latter two transgenic lines had considerably less fluorescent signal in their root tip region (approximately 0–0.5 mm from root apex) than in Ler-0. The four Al-resistant lines all had reduced morin staining compared with Ler-0. However, there were differences in their relative morin staining that were not reflected in differences in Al resistance, e.g. the *HSP150* transgenic line, which could not demonstrate an improved Al resistance, showed considerably reduced morin staining.

To characterize the relationship between Al stress and oxidative stress, roots of Ler-0 were treated with Al and then stained with H_2DCFDA . Higher concentrations of Al (50 and 100 μ M) caused stronger fluorescent signals (Fig. 5C, 1 and 2). Staining was more intense in the region back

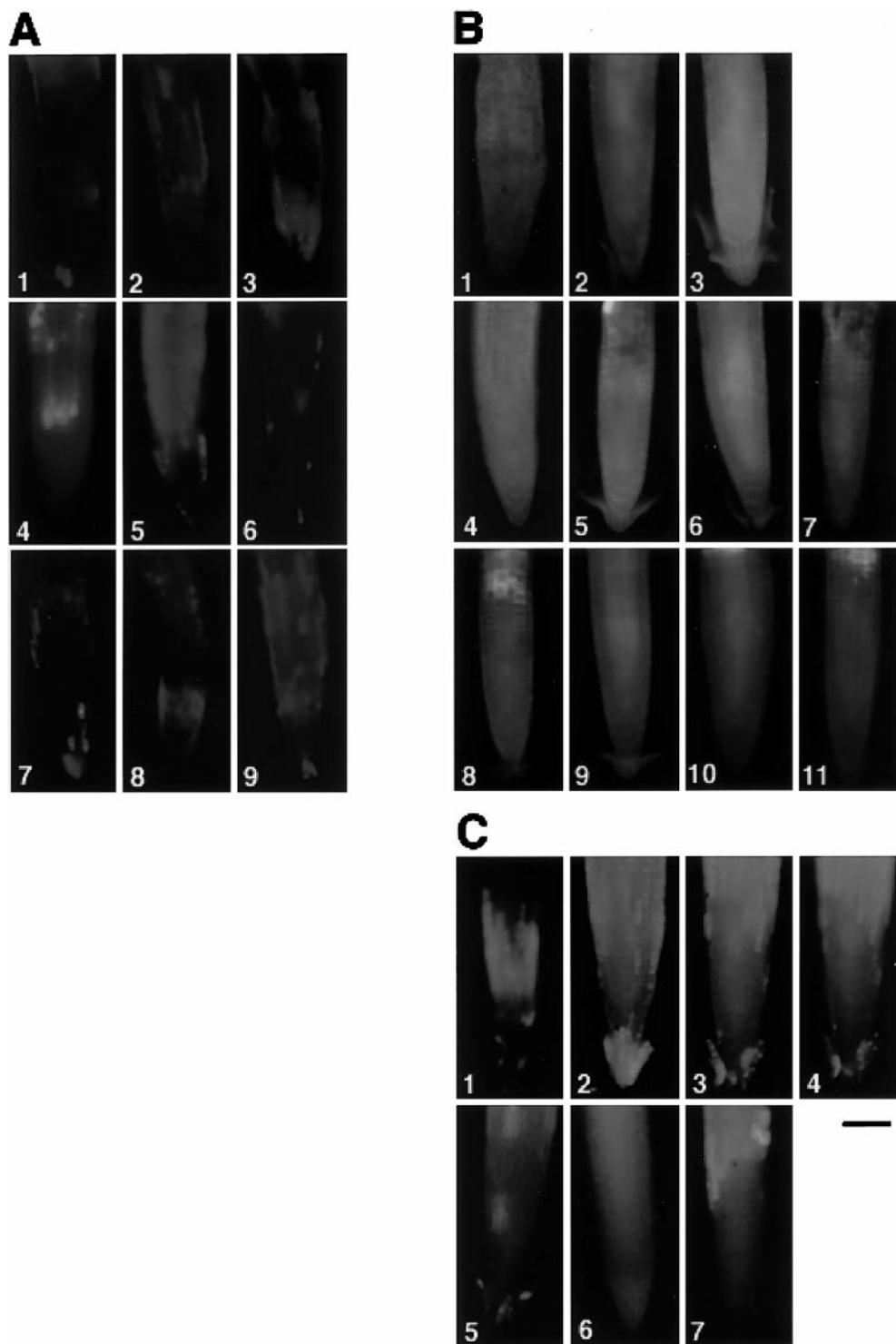


Figure 5. Microscopic observations of roots of Ler-0 and transgenic lines. A, H₂DCFDA-stained roots after diamide treatment. 1 to 5, Ler-0 with various treatments for 5 h; 1, non-treated (control); 2, 0.5 mM diamide; 3, 1 mM diamide; 4, 1.5 mM diamide; 5, 1 mM H₂O₂. 6 to 9, Transgenic lines with 1 mM diamide treatment for 5 h: 6, *AtBCB* (5–1); 7, *AtPox* (4–1); 8, *parB* (3–1); 9, *NtPox* (6–2). B, Morin-stained roots after Al stress. 1, 2, and 3 are Ler-0 plants: 1, 0 μ M Al for 5 h; 2, 50 μ M; and 3, 100 μ M. All the remaining plants were treated with 100 μ M Al for 5 h. 4, *wali5* (8–12); 5, *AtPox* (4–1); 6, *parA* (10–1); 7, *parB* (3–1); 8, *NtGDI1* (5–11); 9, *HSP150* (14–12); 10, *AtBCB* (5–1); 11, *NtPox* (6–2). C, H₂DCFDA-stained roots after Al stress. 1 and 2, Ler-0 plants: 1, 50 μ M Al for 5 h; 2, 100 μ M Al. All of the remaining plants were treated with 100 μ M Al for 5 h. 3, *AtBCB* (5–1); 4, *parB* (3–1); 5, *NtPox* (6–2); 6, *NtGDI1* (5–11); 7, *HSP150* (14–12). Ten-day-old roots directly exposed to 1/6 MS media containing diamide (0, 1, and 1.5 mM), H₂O₂ (1 mM), or Al (0, 50, or 100 μ M) for 5 h were used for observations; the bar represents 200 μ m.

from the root tip, as well as in the root cap cells. These results are consistent with the idea that Al stress induces the formation of reactive oxygen species in Arabidopsis root tips. All eight transgenic lines were also stained with H₂DCFDA after treatment with 100 μ M Al for 5 h. Three transgenic lines, expressing the *wali5*, *AtPox*, and *parA* genes, showed no difference in signal pattern and intensity compared with Ler-0 (data not shown). There was significantly lower signal in the transgenic line carrying the *NtPox* gene (Fig. 5C, 5); this lower signal was seen in both root cap cells and throughout the whole root apex compared with Ler-0. Lower signals were also seen in the root tip region (0–0.5 mm behind the root cap) in the *AtBCB*, *parB*, and *HSP150* transgenic lines (Fig. 5C, 3, 4, and 7); however, in these three lines, the staining intensities farther back in the root were similar to those seen in Ler-0. A somewhat different pattern of staining was seen in the transgenic line expressing *NtGD11*, with a more even distribution of H₂DCFDA staining in whole root tip region. However, the overall intensity of signal was slightly lower than Ler-0 (Fig. 5C, 6).

DISCUSSION

These results demonstrate that overexpression of any one of four Al-induced genes can confer Al resistance. The levels of resistance conferred were not very high, and differences in resistance were only observed over a narrow window of Al concentrations. Nonetheless, the differences in relative root growth were significant and reproducible. Furthermore, lines expressing these four genes showed lower Al content and less oxidative damage than Ler-0 in their root tip regions. The findings provide the first evidence (to our knowledge) that Al-induced genes can protect cells against Al toxicity in plants. We did not analyze levels of protein expression in transformants using specific antibodies. These experiments may show that some of the differences in Al resistance were due to differences in protein levels.

Plant genes induced by a particular stress often serve to protect against that stress. This approach has been successful for oxidative stress (Gupta et al., 1993; Camp et al., 1996; Veena et al., 1999), salt stress (Veena et al., 1999), heat stress (Lee et al., 1995; Prandl et al., 1998), and pathogen attack (Alexander et al., 1993). We have previously shown that inactivation of the *HSP150* gene in yeast increases Al sensitivity, demonstrating that this Al-induced gene normally plays a protective role against Al stress (Ezaki et al., 1998). Therefore, our demonstration that overexpression of Al-induced plant genes can confer Al resistance is perhaps not surprising.

Three predictions can be made from these results. First, the diversity of genes acting to promote resistance suggests that the genes operate via different mechanisms. If this is the case, increased levels of resistance could be obtained by combinations of transgenes in the same line. Second, our demonstration that Al resistance can be improved by enhanced expression of Al-induced genes suggests that Al-induced genes contribute to the natural polygenic variation in Al resistance found in many inheritance tests (Carver

and Ownby, 1995). We predict that some Al-induced genes will map to quantitative trait loci affecting Al resistance. Third, it is likely that many of the Al-induced genes, perhaps including some of the four for which overexpression did not confer resistance, will prove to be necessary for Al resistance when knockout mutations in each gene are analyzed in Arabidopsis. For example, disruption of the yeast Al-induced gene *HSP150* reduced both Al and oxidative stress resistance (Ezaki et al., 1998), but did not promote resistance to either Al or oxidative stress when overexpressed in yeast (B. Ezaki, R.C. Gardner, Y. Ezaki, and H. Matsumoto, unpublished data). It is likely that Al-induced genes contribute to the Al-sensitive mutants found in both Arabidopsis (Larsen et al., 1996) and yeast (Schott and Gardner, 1997). Indeed, in the case of yeast, a large proportion of Al-sensitive strains showed altered stress tolerance (Schott and Gardner, 1997).

Morin staining of the Al-treated roots indicated that the four Al-resistant transgenic lines accumulated less Al than Ler-0 in their root tip region, particularly the 0.5-mm apical tip which includes the cell division and cell elongation zones. Interpretation of these results is somewhat complicated. One explanation might be that all four genes acted to confer Al resistance by reducing cellular Al content in root tips (e.g. by decreasing uptake or by increasing efflux). There is some support for this explanation. The *AtBCB* gene is likely located in the cell walls and appears to confer Al resistance in yeast by decreasing uptake, while the *NtGD11* gene product likely plays a role in intracellular vesicle transport and appears to act in yeast by increasing Al efflux (Ezaki et al., 1999). We have no data as to the precise mechanism of action for the other two genes that conferred Al resistance in this study, the tobacco anionic peroxidase (*NtPox*) and glutathione *S*-transferase (*parB*) genes. However, it is likely that they both encode intracellular proteins whose cellular role involves detoxifying reactive oxygen species, since it has been reported that the total activity of peroxidase and other anti-peroxidation enzymes increases during Al treatment (Cakmak and Horst, 1991). It is less likely that these two genes also act directly by reducing Al content in the cell, but there is a possibility that they act to restrict lipid peroxidation in cell membrane regions of these transgenic plants. These cell membranes may be able to keep the influx of Al ions into the cytosol at a reduced level. An alternative explanation of the lower morin staining in the resistant lines may be that Al uptake into the root tip occurs as the result of Al toxicity rather than being a cause of it. Thus, an increase in resistance via any mechanism would be reflected in reduced Al content in root tips. However, it should be noted that the correlation between Al resistance and morin staining was not complete. Similarly, lines expressing *HSP150* showed lower morin staining than Ler-0, but were not significantly different in Al resistance. It is possible that these differences may reflect the non-quantitative nature of our estimate of morin staining or a lack of sensitivity of the root elongation assay used here for measuring Al resistance. However, we consider it more likely that the two assays are in fact measuring different parameters, and that any correlation between them may be coincidental. Larsen et al.

(1996) noted changes in Al content in eight Al-sensitive mutant lines, which correlated with Al sensitivity in some cases but not others.

Our results suggest that the fluorescent stain H₂DCFDA is an indicator of oxidative stress in Arabidopsis root tips. The increase in H₂DCFDA staining seen with increasing Al confirms previous suggestions that Al induces oxidative stress in Arabidopsis roots (Sugimoto and Sakamoto, 1997; Richards et al., 1998). These results are consistent with the idea that oxidative stress (e.g. Al-induced lipid peroxidation) is the primary cause of Al stress. However, they do not rule out the idea that oxidative stress is a result of Al toxicity rather than a cause. H₂DCFDA staining suggested that there is some reduction of oxidative stress in the root tips of the four Al-resistant transgenic lines, especially in the *NtPox* lines. This result provides confirmation of the Al resistance of these lines. However, there were significant spatial differences in the staining patterns observed in the various transgenic lines, suggesting that the situation may be quite complex; additional analysis of the changes in reactive oxygen species under Al stress in these lines is necessary.

Our results also provide genetic evidence supporting previous suggestions (Cakmak and Horst, 1991; Sugimoto and Sakamoto, 1997; Richards et al., 1998) that Al stress and oxidative stress are strongly linked in plants. We have shown that overexpression of three Al-induced genes in plants conferred oxidative stress resistance. In particular, overexpression of the *parB* gene simultaneously conferred resistance to both Al and oxidative stresses. Therefore, at least some of the genes induced during Al and oxidative stresses play protective roles against both stresses. We found similar results with yeast (Ezaki et al., 1998).

Recently we overexpressed a selection of 11 Al-induced genes in yeast, including all nine of the genes expressed here in Arabidopsis. Only two of the genes, *AtBCB* and *NtGDI1*, conferred Al resistance to yeast (Ezaki et al., 1999). Both genes also conferred Al resistance to Arabidopsis, suggesting that there is an overlap in the protection mechanisms that operate in yeast and plants. There are several possible reasons why the genes encoding anti-peroxidation enzymes, *NtPox* and *parB*, conferred resistance in Arabidopsis but not in yeast. One simple explanation is that the plant gene products are not expressed in an active form in yeast, or that the overexpression of the transcript in yeast had no effect on their enzyme activities. Alternatively, yeast and Arabidopsis may differ in their regulation of oxidative stress pathways or in which anti-oxidant enzyme systems are induced in response to Al stress.

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