

Identification of Rice Varieties with High Tolerance or Sensitivity to Lead and Characterization of the Mechanism of Tolerance¹

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Pb inhibits plant growth. To study Pb tolerance in rice (*Oryza sativa*), we screened 229 varieties for Pb tolerance or sensitivity. Three-day-old seedlings were treated for 12 d with 20 μM Pb solution. Based on the dry weight of the root, three Pb-tolerant (var CH-55, var KH-2J, var Kumnung) and three Pb-sensitive (var Aixueru, var C-9491, var Milyang23) rice varieties were selected. The root biomasses of the tolerant varieties were approximately 10-fold higher than those of the sensitive ones. The greatest morphological difference between the two groups was in the growth of the adventitious roots, as tolerant lines were able to develop adventitious roots after 6 d of Pb treatment, whereas sensitive ones did not develop any even after 15 d. The growth of adventitious roots in the tolerant varieties was dependent on a mechanism, whereby Pb was altered to a form that cannot be taken up by the tissue, because (a) the solution in which the tolerant varieties of rice had grown still contained Pb but nevertheless did not affect the root growth of new rice seedlings, and (b) the adventitious roots of tolerant seedlings developed in Pb solution contained little Pb. The oxalate content in the root and root exudate increased upon Pb treatment in the tolerant varieties, whereas the opposite was observed for the sensitive ones. Oxalate added to the growth solution ameliorated the inhibition of root growth by Pb. These results suggest that compounds such as oxalate secreted from the root may reduce the bio-availability of Pb, and that this may constitute an important Pb tolerance mechanism in the tolerant rice varieties studied here.

Pb, one of the heavy metal elements, has become the most important metal pollutant of the environment (Salt et al., 1998) due to the development of modern industries. Pb contamination has resulted from mining and smelting activities, Pb-containing paints, gasoline, and explosives, as well as from the disposal of municipal sewage sludges enriched in Pb (Jackson and Watson, 1977; Levine et al., 1989). Because many of the sources of Pb pollution are indispensable for modern human life, soil contamination with Pb is not likely to decrease in the near future.

Pb is not an essential element for the growth of plants and animals, nor does it participate in the process of cell metabolism. The accumulation of a large amount in living organisms results in serious poisoning. For example, Pb accumulation in the human body damages the brain and the nervous system (Body et al., 1991; Ewers and Schlipk er, 1991). Plants are also poisoned by Pb (Buchauer, 1973; Johnson and Eaton, 1980), and Pb-contaminated soil causes sharp decreases in crop productivity. Pb contamination thus poses a serious problem for agriculture. The primary effect of Pb toxicity in plants is a rapid

inhibition of root growth, probably due to the inhibition of cell division in the root tip (Eun et al., 2000). We have shown that Pb-treatment perturbs the alignment of microtubules in a concentration-dependent manner, and that this may, at least in part, contribute to the inhibition of root growth seen in the presence of Pb.

Previous reports have proposed that mechanisms of metal tolerance can be classified into internal tolerance mechanisms in the symplasm and exclusion mechanisms in the apoplasm and at the plasma membrane (Taylor, 1991; Kochian, 1995). The internal tolerance mechanisms immobilize, compartmentalize, or detoxify metals in the symplasm by using metal binding compounds (Hamer, 1986; Rauser, 1990; Hausladen and Alscher, 1993; Grotz et al., 1998). In contrast, the exclusion mechanisms prevent metals from entering or staying in the symplasm and coming in contact with sensitive intracellular sites. For example, Al tolerance is conferred by Al exclusion from the root tip. This has, in several cases, been closely correlated with an increased capacity to release organic acids (Hue et al., 1986) such as citrate (Pellet et al., 1995; Ma et al., 1997), malate (Delhaize et al., 1993; Ryan et al., 1995), and oxalate (Zheng et al., 1998). These acids chelate Al outside the plasma membrane, thereby reducing its uptake.

Despite the significant problem that Pb toxicity poses for agriculture, Pb tolerance mechanisms of plants are to date not well understood. Here we have screened a number of rice (*Oryza sativa*) varieties for

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increased resistance to Pb. We show that the tolerant rice varieties can ameliorate Pb toxicity, and we present evidence that suggests oxalate release may contribute to this tolerance in rice.

RESULTS

Identification of Pb-Tolerant and -Sensitive Rice Varieties

We screened 229 rice varieties and cultivars for Pb tolerance and sensitivity at the seedling stage. Pb tolerance or sensitivity was assessed from the dry weight of the root since the most conspicuous effect of Pb was inhibition of root growth (Fig. 1A) and development. Measurements of chlorophyll content per gram dry weight (Fig. 1B) correlated with the dry masses of the root. We were able to select three varieties each of Pb-tolerant (var Ch-55, var KH-2J, var Kumnung) and -sensitive (var Aixueru, var Mi-

lyang23, var C9491) rice. var Ch-55, var KH-2J, var Kumnung (KN), and var C9491 are weedy rice, whereas var Aixueru (AXR), and var Milyang23 are agricultural cultivars. At the end of the 12-d experimental period in a solution with 20 μM Pb, the roots of the tolerant varieties were approximately 10-fold heavier than those of the sensitive varieties, whereas the shoot biomass did not differ significantly (Fig. 1A). However, despite the lack of effect of Pb on shoot biomass, shoot elongation was inhibited and lateral growth of the internode increased (swelling) in the Pb-sensitive varieties during the 12 d of Pb treatment (Fig. 2).

Pattern of Root Growth of Pb-Tolerant and -Sensitive Rice Varieties

For the detailed investigation of the growth response of the root to Pb, we used the tolerant var KN and sensitive var AXR because these seedlings did not differ in their patterns of root growth under control conditions (Figs. 2 and 3). In both varieties, growth of the primary root was inhibited by Pb treatment (Fig. 3A). However, the development of the adventitious roots in the presence of Pb differed strikingly (Fig. 3B), as var KN were able to develop adventitious roots after 6 d of Pb treatment, whereas var AXR failed to do so even after 15 d (Figs. 2 and 3B). Thus, the difference in the root weight of these two varieties after Pb treatment could be largely attributed to the difference in their ability to develop adventitious roots. The other tolerant rice varieties, var CH-55 and var KH-2J, were also able to grow adventitious roots after 6 to 8 d of Pb treatment, whereas the other sensitive rice varieties could not (data not shown), suggesting the general importance of this growth pattern in the mechanism of Pb tolerance.

To test whether adventitious roots and primary roots differed in their sensitivity to Pb, seedlings of the two tolerant varieties, var KN (Fig. 3C) or var KH-2J (data not shown), were transferred to a fresh 20 μM Pb solution for 9 d of Pb treatment after the onset of some adventitious roots. The growth of the adventitious roots stopped immediately after the transfer, but within another 5 to 6 d (from 15 d after the onset of the experiment shown in Fig. 3C) new adventitious roots began developing again (Fig. 3C). This response of adventitious roots of var KN to a fresh Pb solution was strikingly similar to that of the primary roots of the same variety of rice shown in Figure 3B, and thus indicated that adventitious roots did not differ from primary roots in their sensitivity to Pb. Furthermore, it suggested that the Pb solution changed during growth of the resistant varieties and that this change permitted the eventual development of new roots.

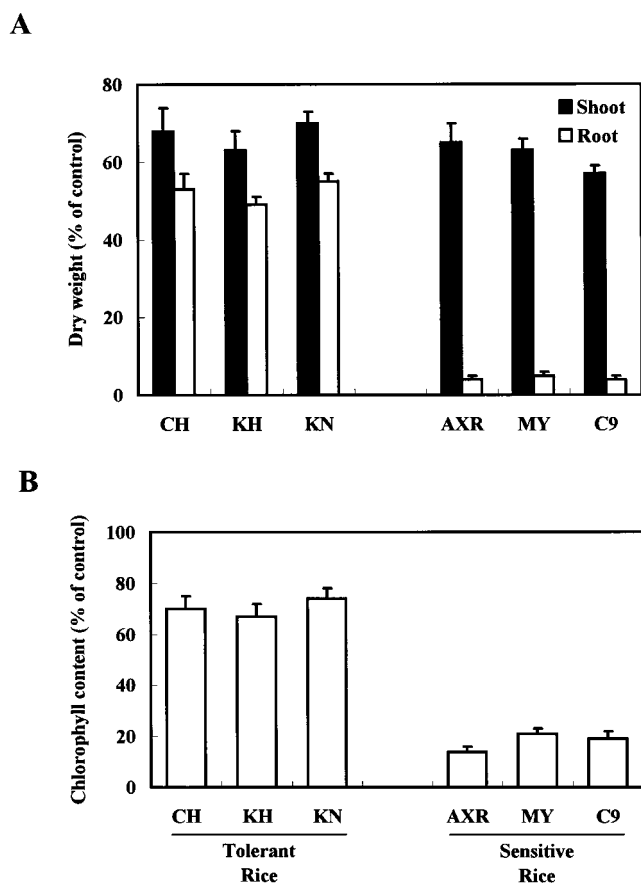


Figure 1. Screening of rice varieties for Pb tolerance or sensitivity. Seedlings were grown for 12 d in 20 μM Pb-containing solution, and the dry weights of shoots and roots (A) and chlorophyll content per gram dry weight (B) were measured. Data are presented as the percent of the controls (seedlings of the same variety grown in distilled water). Error bars represent SE from 10 seedlings. Representative data from three independent experiments are presented.

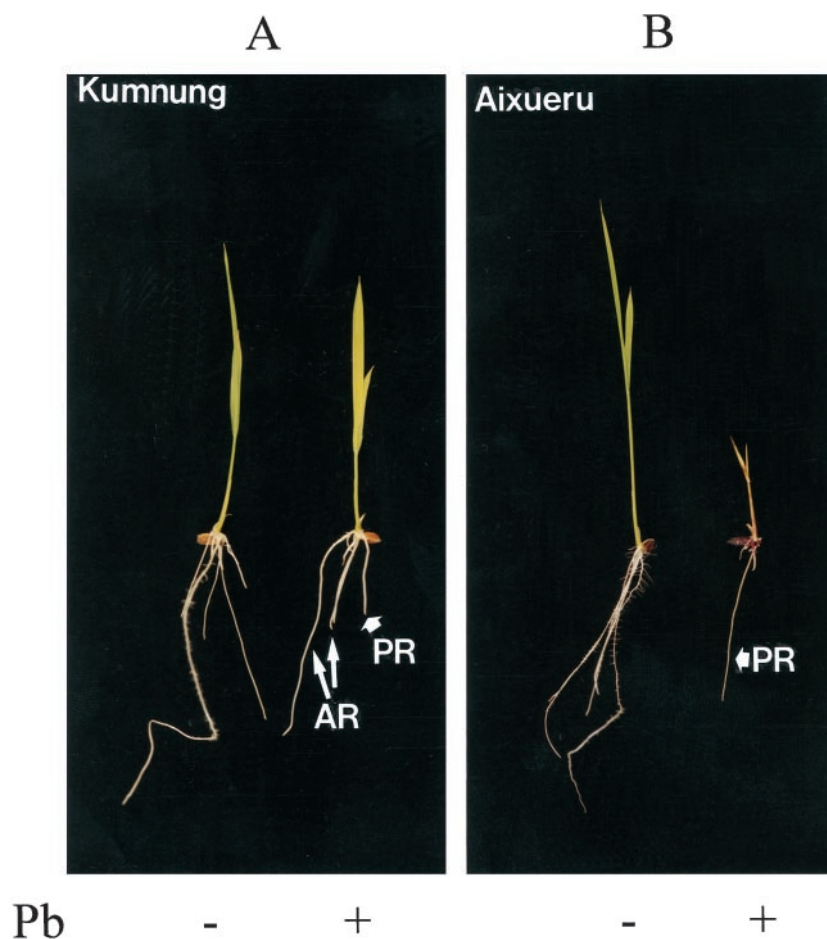


Figure 2. Pb-tolerant (A; var KN) and -sensitive (B; var AXR) rice varieties grown for 15 d in 20- μM Pb-containing solution. PR, Primary root; AR, adventitious root.

Evidence for a Detoxification Mechanism in Pb-Tolerant Rice Varieties

To understand the mechanisms underlying the difference in adventitious root growth between the resistant and sensitive varieties, we first examined whether the growth of the tolerant var KN and sensitive var AXR altered the Pb content in each solution. Approximately 83% Pb added initially was recovered from the growth solution, precipitant in the growth solution, and plant body, in both var KN and var AXR (Fig. 4). Total Pb (solution + precipitant) remaining in the container in which the rice seedlings had grown for 12 d did not differ markedly between the two varieties when measured by Atomic Absorption Spectrometer (which detects both free and bound Pb): 35% and 41% initial Pb levels remained in the var KN and var AXR solutions, respectively (Fig. 4). These levels of Pb, if dissolved, are equivalent to 7 to 8 μM , which should be as effective as 20 μM Pb in inhibiting the primary root growth of var KN, as can be postulated from the effects of fresh 5 and 20 μM Pb solutions (Fig. 5). Pb found in the plant body were also similar in the sensitive and tolerant varieties; the total Pb content of the roots and shoots of var KN were similar to those of var AXR (Fig. 4). However, the dry weight of var KN roots

was 10 times higher than that of var AXR (Fig. 1), and thus the Pb level per unit of root mass in var KN is one-tenth of that in var AXR.

Thus, despite the presence of toxic levels of Pb in their growth solution, var KN can develop adventitious roots. There are three possible reasons for this. First, var KN is not affected by Pb uptake due to constitutive symplastic tolerance mechanisms. This does not seem likely since growth of primary roots of the var KN seedlings was severely inhibited in fresh Pb solution compared with their controls (Fig. 3). Second, the roots of var KN seedlings may secrete compounds into the solution which transform Pb into a form that is unavailable to the newly growing tissue. This possibility has already been suggested by the experiment described previously where transfer of var KN seedlings after 9 d in the Pb solution to a new Pb solution stopped adventitious root growth until 5 to 6 d later, at which point new adventitious roots began growing again (Fig. 3C). Third, during the 6 d in Pb-containing solution, var KN is able to induce a symplastic tolerance mechanism or a mechanism that allows the newly developing tissue to actively exclude Pb.

If the mechanism of Pb tolerance requires an induction step, new seedlings that have never been ex-

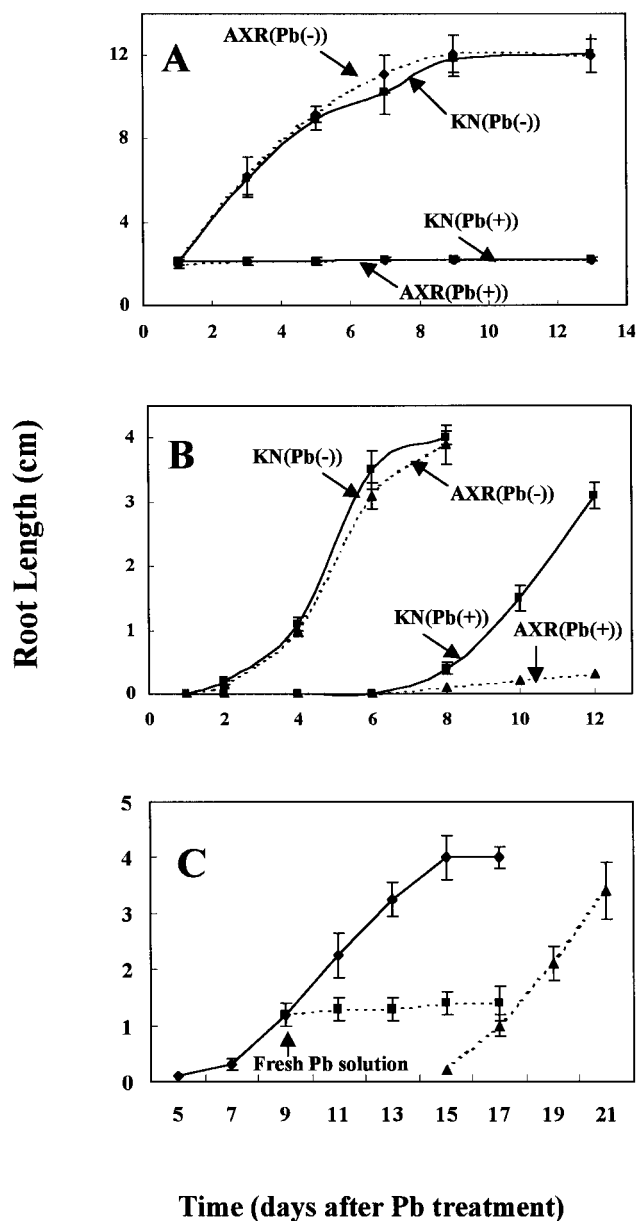


Figure 3. The primary (A) and adventitious (B and C) roots of Pb-tolerant (var KN) and -sensitive (var AXR) rice varieties grown in the presence or absence of 20 μM of Pb. In B and C, the longest, and usually the oldest, adventitious roots are presented. In C, var KN seedlings were either continuously exposed to the same 20- μM Pb solution for 17 d (solid line) or exposed to 20 μM Pb for 9 d and then transferred to a fresh 20- μM Pb solution (dashed line). Note in C that the adventitious roots stopped growth immediately after the transfer, and 6 d thereafter new adventitious roots started developing. Error bars represent SE from six seedlings. Representative data from three independent experiments are presented.

posed to Pb should not be able to grow in the solution containing 7 to 8 μM Pb that remains after growth of a rice strain. In contrast, if Pb is altered to a nonavailable form, then new seedlings should be able to grow in the "aged" solution as well as seedlings adapted over 12 d in the solution. To test these

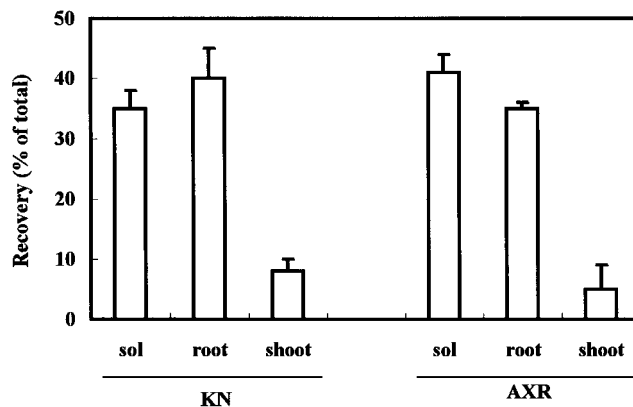


Figure 4. Pb content in shoots, roots, and the growth solution after 12 d treatment with 20 μM Pb. Seedlings were separated into roots and shoots. Pb content was analyzed by an Atomic Absorption Spectrometer. Error bars represent SE from 10 seedlings.

possibilities, we exposed new seedlings to the solution in which different seedlings had previously grown for 12 d. Thus, four varieties of rice seedlings were grown in a 20 μM Pb solution for 12 d, after which they were discarded and 3-d-old seedlings of tolerant var KH-2J were grown in the aged solution for 2 d. It is remarkable that the primary roots of the new var KH-2J seedlings were able to grow in the solutions in which tolerant seedlings had been grown to an extent comparable with growth in the control solution, whereas solutions in which sensitive seedlings had been grown inhibited var KH-2J root growth (Fig. 5). These results support the possibility that the Pb in the solutions in which tolerant varieties of rice had grown become altered into a form not available to the newly developing root.

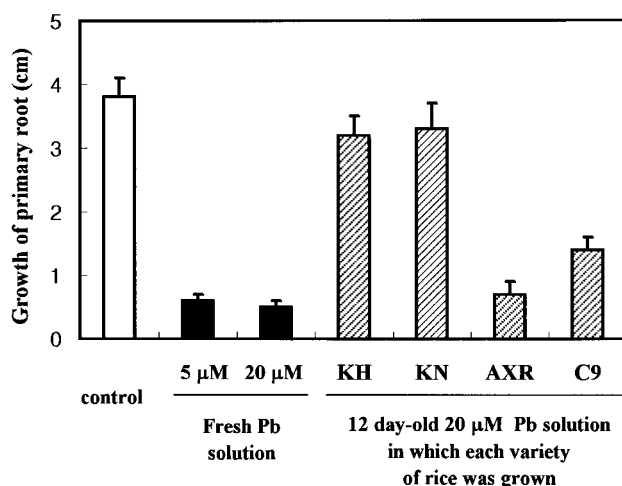


Figure 5. Evidence for a mechanism that ameliorates Pb in tolerant rice varieties. Seedlings of four rice varieties ($n = 10$ each) were grown in 20 μM Pb solution for 12 d and then discarded. Three-day-old var KH-2J seedlings were then grown for 2 d in either the remaining solutions, distilled water (control), or fresh 5- and 20- μM Pb solutions. Error bars represent SE from 10 seedlings. Representative data from two independent experiments are presented.

If resistant var KN is able to grow adventitious roots, as shown in Figure 3, because Pb is converted to an unavailable form after 6 d of growth, then it follows that the adventitious roots of var KN should not absorb as much Pb from the aged solution as the primary root. It has already been shown in a previous experiment that adventitious roots do not differ intrinsically from the primary root in their sensitivity to Pb (Fig. 3C). Pb contents of the Pb-exposed primary root and the adventitious roots that developed 6 d after Pb treatment were both measured after 12 d of Pb treatment using the x-ray fluorescence microprobe in the Pohang synchrotron facility. The Pb content per unit of area in adventitious root was less than 3% of that of the primary root (Fig. 6). This result further supports the notion that by the time adventitious roots develop, the Pb in the solution is no longer in a bio-available form.

Oxalate in Relation to Pb Tolerance

The release of organic acids for mitigation of Al toxicity has been previously reported (Hue et al., 1986; Delhaize et al., 1993; Pellet et al., 1995; Ryan et al., 1995; Ma et al., 1997; Zheng et al., 1998), and thus we tested the correlation between Pb tolerance in rice and increased levels of synthesis and excretion of organic acids in the roots. To do this, we measured free oxalate contents in root and root exudates of both the tolerant and sensitive rice varieties using HPLC. The free oxalate content in the exudates and roots was increased by 20 μM Pb treatment in tolerant var KH-2J and var KN but decreased in sensitive var AXR and var C-9491 (Table I). The time course of exuded oxalate (Fig. 7) showed that the free oxalate levels in growth solutions of tolerant varieties were higher than those of the sensitive ones throughout the duration of the experiment, supporting the role of

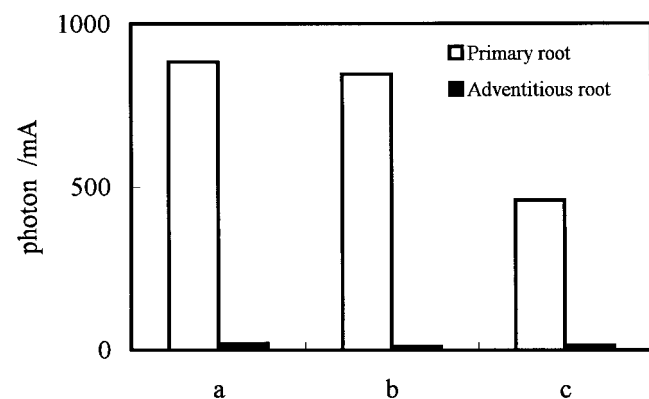


Figure 6. X-ray fluorescence (XRF) analysis of Pb levels in resistant seedling roots. Specific areas of the primary and adventitious roots from var KN were line scanned every 100 μm across the long axis of the seedlings. The scanned areas were 0.5 mm (a), 1 mm (b), and 6 mm (c) from the root tip. Counts at 10.5 keV (specific for Pb) were analyzed from XRF spectra. Mean values of the highest counts from each scan area of roots from three seedlings are presented.

Table I. Changes in root oxalate content in response to Pb treatment

Oxalate contents were measured in Pb-tolerant (var KH-2J and var Kummung) and -sensitive (var Aixueru and var C-9491) rice seedlings ($n = 10$ each) grown in the presence or absence of 20 μM Pb for 6 d. Numbers in parenthesis indicate the results of a second experiment.

Rice Varieties	Oxalate (μg)/10 Seedling			
	Root		Exudates	
	Pb(-)	Pb(+)	Pb(-)	Pb(+)
var KN	50 (46)	68 (70)	7 (10)	18 (16)
var KH-2J	48 (40)	78 (64)	5 (6)	12 (14)
var AXR	32 (35)	11 (9)	8 (9)	4 (6)
var C9	20 (22)	3 (7)	6 (8)	3 (5)

oxalate in Pb tolerance. It is noteworthy that the free oxalate levels in growth solutions of sensitive lines also increased with time of exposure to Pb, which suggests that the sensitive lines were not completely poisoned by 20 μM Pb, and in the long term they may also be able to overcome the toxicity of the Pb solution by secreting oxalate. Citrate and malate levels were below the limits of detection for both Pb-treated and -untreated seedlings. We then tested the effect of exogenously added oxalate on Pb toxicity. Ten micromolar Pb inhibited root elongation of the Pb-sensitive rice var AXR by approximately 80% during 30 h. However, when oxalate was added to the Pb solution, the inhibition of root elongation was ameliorated in an oxalate concentration-dependent manner (Fig. 8).

DISCUSSION

In this study, we screened 229 rice varieties for plants that are tolerant or sensitive to Pb and inves-

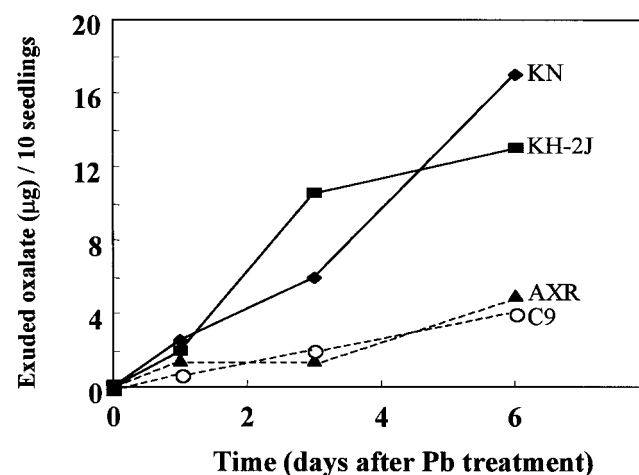


Figure 7. Time course of the changes in free oxalate contents of the Pb solutions in which rice seedlings grew. Using HPLC, free oxalate contents were measured in solutions that Pb-tolerant (var KH-2J and var KN) and -sensitive (var AXR and var C-9491) rice seedlings ($n = 10$ each) had been grown in the presence of 20 μM Pb for 1, 3, and 6 d. Averages from two separate experiments are presented.

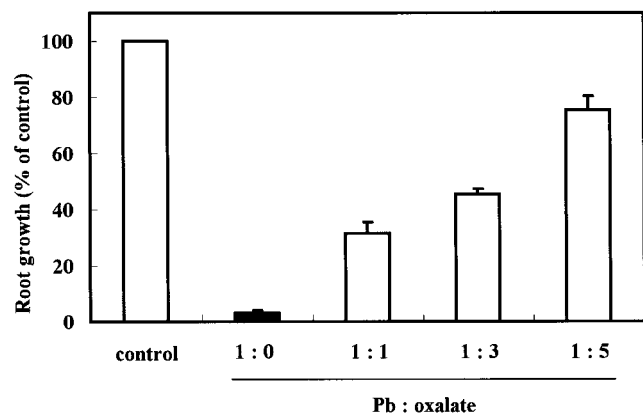


Figure 8. Oxalate added to the Pb solution ameliorates Pb-induced root growth inhibition. Pb concentration was $10 \mu\text{M}$ throughout. Six 3-d-old seedlings of a Pb-sensitive rice var AXR were exposed to different ratios of Pb and oxalate (Pb:oxalate = 1:0, 1:1, 1:3, 1:5) for 30 h. Control seedlings were grown in distilled water. Error bars represent SE. Representative data from three independent experiments are presented.

investigated the mechanisms of Pb tolerance in the selected plants. Three varieties each of Pb-tolerant and -sensitive rice were selected. When exposed to a solution containing Pb, the root biomasses of the tolerant varieties were higher than those of the sensitive rice varieties because of the ability of the tolerant varieties to develop adventitious roots. Root development in the tolerant varieties was associated with a mechanism that altered the Pb in the solution into a form that could no longer be taken up by newly growing tissue. We suggest that oxalate release may contribute toward this mechanism of Pb detoxification in rice.

The initial indication of Pb toxicity was profound inhibition of root growth, which resulted in a smaller and damaged root system. Both of the tolerant and sensitive rice varieties ceased to elongate their primary roots when placed in a Pb-containing solution (Fig. 3A). Thus the rice varieties were not different in their Pb tolerance at the initial stage of exposure to Pb. However, the tolerant varieties were able to develop adventitious roots 6 d after initiation of Pb treatment, whereas the sensitive varieties did not develop them even after 15 d (Fig. 3B). The major difference between tolerant and sensitive lines was not in their Pb contents (Fig. 4) but in their ability to reduce Pb toxicity in the solution; only tolerant seedlings could alter their growth solution to allow the growth of the primary roots of new seedlings (Fig. 5). Furthermore, the Pb content of the adventitious roots developed 6 d after Pb treatment was only 3% of that of the primary roots that were initially exposed to fresh Pb solution (Fig. 6), suggesting that the tolerant seedlings changed Pb in the solution into a form not available to the newly growing tissue. Induction of

mechanisms of symplastic tolerance or active exclusion of Pb in the tolerant rice does not seem strong, because when the tolerant plants that had been grown in Pb to the point of initiation of adventitious root development were placed in a fresh Pb solution, they were not able to continue root growth until 5 to 6 d later (Fig. 3C).

Recent reports have indicated the existence in plants of an Al-resistance mechanism that results in the exclusion of Al from the root apex through the release of Al-binding ligands such as organic acids (Jones, 1998). When these ligands are released into the rhizosphere, they effectively chelate Al^{+3} and reduce its entry into the root. Furthermore, in several species, including snapbean, maize, wheat, and buckwheat, increased Al resistance is correlated with Al-dependent citrate, malate, or oxalate release (Miyasaka et al., 1991; Delhaize et al., 1993; Basu et al., 1994; Pellet et al., 1995; Zheng et al., 1998). To determine whether our Pb-tolerant rice varieties might have increased levels of synthesis and excretion of organic acids in roots, we measured the free organic acid content of the roots and root exudates of the tolerant and sensitive rice varieties by HPLC. Upon 6 d of $20 \mu\text{M}$ Pb treatment, the oxalic acid content in exudates and roots was increased in tolerant varieties but decreased in sensitive varieties, compared with their non-treated controls (Table I). Moreover, free oxalate contents in exudates of both tolerant and sensitive varieties increased with time of Pb treatment throughout the experiment (Fig. 7), supporting its role in detoxification of Pb in the growth solution. Rice roots have been reported to be able to synthesize oxalate via the oxidation of glycolate to glyoxylate and then oxidation of glyoxylate (Kumazawa, 1985). Although element analysis using Atomic Absorption Spectrometer showed that the precipitant formed in the solution in which tolerant variety of rice had been grown was enriched with Pb (data not shown), we were unable to identify the precipitant as the oxalate-Pb complex because it was insoluble in both water and organic solvents. We were, however, able to show a mitigating effect of exogenous oxalate on Pb toxicity. When oxalate was added to the Pb solution at Pb:oxalate ratios of 1:1, 1:3, and 1:5, the inhibition of root elongation of a sensitive variety was ameliorated in an oxalate dose-dependent manner (Fig. 8). Thus, we propose that tolerant rice varieties up-regulated the synthesis and secretion of oxalate, a compound that precipitates Pb, thereby reduced Pb uptake by the roots.

To our knowledge, this study is the first report identifying rice varieties that are highly tolerant to Pb. Isolation of such tolerant and sensitive rice varieties may permit the characterization of novel Pb tolerance mechanisms. In addition, the identification of Pb-tolerant rice varieties may permit the isolation of those genes responsible for tolerance.

MATERIALS AND METHODS

Plant Culture and Identification of Pb-Tolerant and -Sensitive Rice Varieties

Seeds of a number of rice (*Oryza sativa*, six agricultural cultivars, 205 weedy rice, and 18 wild-type rice) varieties, collected and maintained by Wild Crop Germplasma Bank (College of Natural Resources, Yeungnam University, Kyongsan, Korea), were soaked in distilled water in the dark at 37°C for 2 d and then germinated on a plastic screen floating on distilled water at 26°C for 4 d. After 3 d, 10 seedlings from each strain were transferred to a paper cup (7-cm diameter), containing 140 mL of 20 μM $\text{Pb}(\text{NO}_3)_2$ (or water alone as a control). After 12 d of growth in a greenhouse, the seedlings were separated into roots and shoots and oven dried at 70°C for 2 d. The weight of the dry parts was used to assess the Pb tolerance or sensitivity of the rice varieties. Tolerance was also assessed from the chlorophyll content in the shoots, which was assayed according to the method of Lichtenthaler and Wellburn (1983).

Determination of Pb Content in Plant Material

Plants were rinsed with 1 mM citrate for 30 min at 4°C to remove surface Pb, then separated into shoots and roots, and oven dried at 70°C for 2 d. The samples were digested in glass tubes containing 5 mL of concentrated HNO_3 placed in a heat block at 100°C until the solution became clear. The sample volumes were raised to 10 mL with distilled water. The concentration of total Pb in the tissues was measured by an atomic absorption spectrometer (model SpectrAA-800, Varian, Palo Alto, CA).

For the comparison of Pb content in primary and adventitious roots, we used the 1B2 beam line at the Pohang Light Source (Pohang, Korea), which has XRF facilities with a critical energy of 4.8 keV at 2.4 GeV as described (Eun et al., 2000). Unlike the atomic absorption spectrometer, the XRF makes it possible to depict elements in specific areas of hydrated, unprocessed biological samples, thus reducing experimental steps and providing improved spatial resolution (Iida and Gohshi, 1991). After Pb treatment and the ensuing desorption, whole roots were mounted on the acrylic plate, and Pb content was measured by line scanning every 100 μm across the long axis of the seedlings using a white beam through a pinhole of 30 $\mu\text{m} \times 30 \mu\text{m}$.

Examination of Pb Detoxification by Rice Seedlings

To test if the mechanism of Pb tolerance involves an alteration of the growth solution by the tolerant varieties, tolerant (var KH-2J, var KN) and sensitive (var AXR, var C9) varieties of rice seedlings were grown separately in 140 mL of the 20- μM Pb solution for 12 d, then discarded, after which 3-d-old var KH-2J seedlings were grown for 2 d in the aged solutions in which four different varieties had been grown. Root lengths of var KH-2J seedlings were measured before and after growth in the aged solutions.

Measurement In Vitro of the Effect of Oxalate on Pb Toxicity

Effects of oxalate were assayed using 3-d-old seedlings of Pb-sensitive var AXU in solutions containing different Pb:oxalate ratios, namely 1:0, 1:1, 1:3, and 1:5. A control without any added chemicals was also included for comparison. The pH of each Pb oxalate solution was adjusted to 6.3. Root length was measured before and after 30 h of treatment.

Determination of Organic Acid Levels in Roots and Root Exudates

For analysis of organic acid contents in roots, seedlings were rinsed in distilled water, their roots were harvested, and ground in 5 mL of 60% (v/v) ethanol. Cellular debris was pelleted, the supernatant collected, and the pellet resuspended in 95% (v/v) ethanol. The debris was pelleted again and the supernatant was collected and combined with the first supernatant. The samples were then dried under vacuum and resuspended in distilled water. For analysis of organic acid contents in root exudates, the solutions in which the seedlings grew were concentrated to dryness with *n*-butanol in a rotary evaporator (37°C). The samples were passed through a octadecylsilane cartridge column (Waters, Milford, MA). Organic acids were eluted by 50% (v/v) methanol, and the eluate was concentrated to dryness in a rotary evaporator. The residue was redissolved in distilled water. The concentration of organic acids was analyzed by HPLC (Coppola and Starr, 1986). Identification of organic acids in roots and exudates was achieved by comparison of retention times with those of authentic organic acid standards (Sigma, St. Louis). Quantification was based on standard curves generated from peak integration.

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