A Rice Mutant Sensitive to Al Toxicity is Defective in the Specification of Root Outer Cell Layers

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Outer cell layers of rice roots, which comprise epidermis, exodermis and sclerenchyma, have been proposed to protect the roots from various stresses in soil. Here, we report a mutant which is defective in the specification of outer cell layers, and examined the role of these layers in Al and other metal resistance. Morphological and histochemical observations revealed that the mutant isolated based on Al sensitivity frequently showed a disordered pattern of periclinal cell division in the epidermal layers at a region close to the root apical meristem. The lateral root caps in the mutant became difficult to peel off from the epidermis, and epidermal cells became smaller and irregular with far fewer root hairs. Furthermore, some exodermal cells were transformed into additional sclerenchyma cells. However, there was no difference in the inner cell layers between the wild-type rice and the mutant. The mutant showed similar root growth to the wild-type rice in the absence of Al, but greater inhibition of root elongation by Al was found in the mutant. Morin staining showed that Al penetrated into the inner cortical cells in the mutant. Furthermore, the mutant was also sensitive to other metals including Cd and La. Taken together, our results indicate that root outer cell layers protect the roots against the toxicity of Al and other metals by preventing metal penetration into the inner cells. Genetic analysis showed that the mutant phenotypes were controlled by a single recessive gene, which was located on the short arm of rice chromosome 2.

Keywords: Aluminum • Epidermis • Exodermis • Rice • Root hair • Sclerenchyma.

Abbreviations: PBS, phosphate-buffered saline; RRE, relative root growth; WT, wild type.

Introduction

Uptake of water and nutrients and anchorage are the two major functions of plant roots. However, since the roots are usually exposed to various stresses such as drought, aluminum (Al), heavy metal and salinity in soil, they must exclude harmful or even toxic substances and/or reduce the loss of water, nutrients and phytohormones into the soil solution (Hose et al. 2001, Enstone et al. 2003). Outer cell layers in the roots have been suggested to play an important role in the resistance to these stresses because these layers are the first site in contact with various stresses.

Plants differ in the composition of the root outer cell layers. For example, the outer cell layers of Arabidopsis comprise only epidermis, while that of rice consists of epidermis, exodermis and sclerenchyma (Morita and Abe 1999, Ranathunge et al. 2004). The role of root outer cell layers in stress resistance has been investigated in terms of drought, salinity and flooding. For example, Taleisnik et al. (1999) used a variety of exodermal and non-exodermal species including sorghum and found that the mature exodermis slowed the initial rate of water loss from root segments exposed to air. Reinhardt and Rost (1995) reported that salinity can induce the formation of an exodermis in cotton seedlings to prevent the loss of water and/or solutes from roots.

Al is the most abundant metal in soil. Under acidic conditions, Al is released to soil solution in its ionic form, which is toxic for plant growth. The characteristic symptom of Al toxicity is the inhibition of root elongation. Recent studies showed that the inhibition occurs as early as 30–120 min after the exposure to Al (Barcelo and Poschenrieder 2002, Doncheva et al. 2005). A number of possible mechanisms
responsible for Al-induced inhibition of root elongation have been proposed; however, the exact mechanism by which Al initially causes the inhibition of root elongation has not been understood (Ma 2008). When root elongation is inhibited by Al, most Al is localized on the outer cell layer in the roots (Ma et al. 2004, Jones et al. 2006); however, the role of outer cell layers is unknown. In the present study, we isolated a rice mutant which shows a defect in the formation of outer cell layers. We physiologically characterized this mutant in terms of Al toxicity as well as other metal toxicity. Furthermore, we genetically mapped the gene responsible for the mutation.

**Results**

**Isolation of an Al-sensitive rice mutant**

A rice mutant library, derived from an Al-resistant cultivar (*Oryza sativa* L. cv. Koshihikari) irradiated with γ-rays, was used for screening of Al-sensitive mutants. After an initial screening plus two rounds of confirmatory tests, two mutants showing increased sensitivity to Al were obtained from a total of 1,170 M1 populations. We crossed these two mutants with *als1*, a rice Al-sensitive mutant, which was isolated previously (Ma et al. 2005). Analysis of F1 seedlings showed that one of the mutants was allelic to *als1*, but the other (named *c68*) was not, indicating that *c68* is a novel Al-sensitive rice mutant. This mutant was then used for further physiological and genetic characterization as described below.

**Morphological and histochemical observations of *c68***

We compared the morphology of the shoots and roots between *c68* and its wild-type (WT) rice, cv. Koshihikari. There is no visible morphological difference in the shoots under normal conditions (data not shown). However, in roots, there were several differences between the WT rice and the mutant. First, the number of root hairs was far less in the mutant than in the WT (Fig. 1, upper panel). Occasionally, shorter root hairs with different length were observed in the mutant (Fig. 1, lower panel). Secondly, at the root tip region, stratification of epidermal and hypodermal cells became irregular in the mutant (Fig. 2A, B). Thirdly, at the basal root region, some exodermal/hypodermal cells were changed into additional sclerenchyma cells in both seminal and crown roots of the mutant (Fig. 2C–H). Fourthly, the lateral root cap in the mutant was difficult to peel off in the meristem zone (Fig. 3A, B, E, F). In the elongation zone, the epidermal and exodermal/hypodermal cells showed smaller cell size and were disarranged in comparison with those of the WT (Fig. 3C, D). However, in both the root tip and basal zone, there were no morphological changes in the inner layers of root cells between the WT and *c68* (Figs. 2, 3).

![Fig. 1](https://example.com/image1.png) Fewer root hairs in the mutant (*c68*). Root hairs of seminal roots (3 cm from root apexes) of the WT and the mutant (5 d-old) were compared. The lower panel shows staining with the fluorescent dye, propidium iodide. Scale bar: 1 mm.

To confirm the alteration of outer cell layers in the mutant, we performed immunostaining against a marker protein of the exodermis, Lsi1. Lsi1 is a silicon influx transporter which is located at the distal side of the exodermis of rice roots (Fig. 2G, Ma et al. 2006). In contrast to the WT, Lsi1 was localized at two different layers in the mutant (Fig. 2H). In the cells where the exodermal cells were not changed to the sclerenchyma-like cells, Lsi1 protein was localized at the exodermal cells as seen in the WT rice (Fig. 2H), whereas in the cells where two layers of sclerenchyma-like cells are formed, Lsi1 was localized at the outermost cell layer. In both cell layers of the mutant roots, Lsi1 showed polar localization at the distal side (Fig. 2H). These results indicate that with the change of exodermal cells to sclerenchyma-like cells in the mutant, the epidermal cells gain some identity of exodermal cells.

**Root growth and sensitivity to Al and other toxic metals**

Root growth was compared between the WT and the mutant. In the absence of Al, there was no difference in the root elongation between the WT and the mutant (Fig. 4A). A long-term (25 d) hydroponic culture also showed similar root growth between the WT and the mutant (Fig. 4B). These results indicate that although the cell structure and organization of outer layers in the mutant roots were altered, these morphological changes did not influence normal growth of the mutant.
However, when the mutant was exposed to Al, the root growth was inhibited more compared with the WT. At 10, 20, 30 and 50 µM Al, the root elongation of the mutant was inhibited by 29, 59, 69 and 88%, respectively, whereas that of the WT was inhibited by 19, 34, 41 and 57%, respectively (Fig. 5A), confirming the screening result which showed that c68 is a mutant sensitive to Al. We also compared the growth of two lines on an acid soil amended with and without CaCO₃. On soil with pH 5.0 where less Al is present, both the mutant and WT showed similar growth (Fig. 5B, C). However, on soil at pH 4.5 and 4.0 where high Al was present, the root growth of the mutant was more inhibited compared with the WT (Fig. 5B, C). In particular, on soil at pH 4.0, the root growth of c68 was completely inhibited.

Fig. 2  Autofluorescence and immunostaining of roots of the WT and the mutant (c68). (A–F) Autofluorescence observed under UV. (A and B) Cross-section of seminal root tips (2 mm from the apex) of the WT (A) and the mutant (B). The box shows epidermis and hypodermis layers with reduced size and distorted arrangement in the mutant. (C and D) Cross-section of the basal root zone (25 mm from the apex) in seminal roots of the WT (C) and the mutant (D). The arrow shows changes of exodermal cells to sclerenchyma cells. (E and F) Cross-section of the basal root zone (50 mm from the apex) in crown roots of the WT (E) and the mutant (F). The arrow shows changes of exodermal cells to sclerenchyma cells. (G and H) Immunostaining of Lsi1 protein at the cross-section of the basal root zone (50 mm from the apex) in crown roots of the WT (G) and the mutant (H). Arrows indicate the expression of Lsi1 protein in normal expanded exodermal cells. Arrowhead shows the misexpression of Lsi1 in the outermost cells. Scale bar: 100 µm.

Spatial response to Al was also compared between the WT and the mutant by using a multicompartment box. The Al-induced inhibition of root elongation was similar when the whole root or only the root tip part (0–0.5 cm) was exposed to Al in either line (Fig. 6). However, exposure of the basal root part to Al did not inhibit root elongation in either the WT or the mutant (Fig. 6). The difference in the Al-inhibited root elongation between the two lines was only found when the root tips were exposed to Al.
The sensitivity to other metals including Cd and La was also compared between the mutant and WT. In the presence of 30 µM Cd and 5 µM La, root elongation of the WT plant was inhibited by 63 and 37%, respectively; however, that of the mutant was inhibited by 76 and 52%, respectively (Fig. 7). These results indicate that the c68 mutant was sensitive not only to Al but also to Cd and La.

**Al accumulation pattern**

To compare the Al accumulation pattern in roots of the c68 mutant with that of the WT, seedlings (5 d old) were grown in deionized water for 3 d. Root length was measured at 0, 24, 48 and 72 h. Data are means ± SD (n = 15 in the WT and n = 16 in the mutant). (B) Root biomass of the WT and the mutant. Seedlings were grown in a half-strength Kimura nutrient solution in a greenhouse for 25 d. Data are means ± SD (n = 9).

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**Fig. 4** Root growth of the WT and the mutant (c68). (A) Root elongation of the WT and the mutant. Seedlings (5 d old) were grown in deionized water for 3 d. Root length was measured at 0, 24, 48 and 72 h. Data are means ± SD (n = 15 in the WT and n = 16 in the mutant). (B) Root biomass of the WT and the mutant. Seedlings were grown in a half-strength Kimura nutrient solution in a greenhouse for 25 d. Data are means ± SD (n = 9).

**Fig. 5** Effect of Al on root growth. (A) Effect of various Al concentrations on the root elongation of WT rice and the c68 mutant. Seedlings (5 d old) were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) containing 0, 10, 20, 30 or 50 µM Al for 24 h. Relative root elongation (RRE) was used to evaluate their sensitivities to Al and expressed as (root elongation with Al treatment/root elongation without Al) × 100. Data are means ± SD (n = 10). (B and C) Root length of the WT and the mutant grown in an acid soil amended with CaCO₃ at 0.06, 0.13 and 0.25 g kg⁻¹ soil for 8 d. Data are means ± SD (n = 10).
Fig. 6 Spatial response to Al in WT rice and the c68 mutant. Five-day-old seedlings were placed in a multicompartment box and then their root tips (0–0.5 cm) and basal roots were separately exposed to a 0.5 mM CaCl₂ solution (pH 4.5) containing 0 or 50 µM Al for 14 h (for details, see Materials and Methods). Relative root elongation (RRE) was used to evaluate their sensitivities to Al. Data are means ± SD (n = 10).

Fig. 7 Sensitivity to other metals. Five-day-old seedlings of the WT rice and the c68 mutant were exposed to a 0.5 mM CaCl₂ solution containing 0 or 30 µM Cd or 5 µM La at pH 4.5 for 24 h. Root length was measured before and after the treatment, and relative root elongation (RRE) was used to evaluate their sensitivities to metals. An asterisk indicates a significant level, P < 0.001 (Student’s t-test). Values are means ± SD (n = 10).

Fig. 8 Al accumulation in WT rice and the c68 mutant. (A) Total Al concentration in the root tip. Twenty seedlings (5 d old) each were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) containing 0, 10, 30 or 50 µM Al for 24 h. Root tips (0–1 cm) were excised and then immersed in 1.5 ml of 2 N HCl. The Al concentration in the solution was determined by graphite furnace atomic absorption spectrophotometry. An asterisk indicates a significant level, P < 0.05 (Student’s t-test). Error bars represent the SD (n = 3). (B and C) Morin staining. Seedlings (5 d old) of WT rice (B) and the mutant (C) were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) containing 50 µM Al for 8 h. Root tips were transversely sectioned at 2 mm from the apexes and stained in 0.01% morin for 15 min. The observation was conducted with a fluorescence microscope.
A fluorescent histochemical indicator for Al, can form a complex with Al to emit fluorescence (Browne 1990). A strong Al-dependent fluorescent signal was observed in outer cell layers and cortical cells of root tips in the mutant (Fig. 8C). In contrast, only faint fluorescence was found in the epidermis and exodermis of WT roots (Fig. 8B). These results indicate that Al was stopped at the exodermis in the WT, but it penetrated into the inner layer cells in the mutant.

Genetic analysis and molecular mapping of c68

To perform genetic analysis of c68, an F₂ population from a backcross between the mutant and WT was used for both morphological observations and evaluation of Al tolerance. Of 109 seedlings tested, 26 plants with fewer root hairs were sensitive to Al [relative root elongation (RRE) ≤ 35%] and 83 with normal root hairs showed tolerance to Al (RRE ≥ 40%) (Fig. 9), which agreed with 1:3 segregation patterns ($\chi^2 = 0.0051$, $P > 0.9$). These results indicate that morphological alterations and sensitivity to Al in the c68 mutant were controlled by the same recessive gene.

To map the responsible gene, we constructed an F₂ population derived from a cross between the mutant and an indica variety Kasalath. The phenotype was evaluated based on occurrence of root hairs. Bulked segregant analysis with 62 polymorphic markers covering the whole rice genome showed that an InDel marker MaOs0204 (boxed) was found to be linked to this gene. Forty-three F₂ plants with fewer root hairs were used to map this gene further and finally the C68 gene was defined between two InDel markers MaOs0212 and MaOs0215. The left bar represents chromosome 2 downloaded from the RGP website (http://rgp.dna.affrc.go.jp). Numbers on the right show recombinants between each marker and C68. Recombinants at marker MaOs0215 are included in those at marker MaOs0204, but not at marker MaOs0212.

Discussion

We isolated a novel mutant (c68) sensitive to Al toxicity based on Al-induced inhibition of root elongation. However, this mutant also showed other phenotypes different from...
the WT rice. First, this mutant was also sensitive to other metals including Cd and La (Fig. 7). Secondly, the mutant showed root morphological alterations including reduction of numbers of root hairs (Fig. 1) and developmental defects of the outer cell layers, which include smaller cell size, irregular arrangement of cells and change from exodermal/sclerenchyma cells to sclerenchyma (Figs. 2, 3). However, genetic analysis showed that these phenotypes are controlled by a single gene (Fig. 9). This means that the phenotypes observed are linked to each other. Therefore, our mutant also provides a good tool for studying the mechanisms underlying the specification of outer cell layers.

**Morphological alterations of the outer cell layers**

Outer cell layers of rice roots consist of epidermis, exodermis and sclerenchyma cells. However, the mechanisms underlying the specification of these cells are still not well understood. Rice roots have multilayers of cortical cells. The formation of multiple cortical layers and endodermis in rice is the result of the sequence of centripetally periclinal divisions in the initials and their derivatives (Kawata and Lai 1965, Lux et al. 2004). The first cells that terminate their periclinal divisions develop into exodermal cells, while the final periclinally dividing cells produce the innermost endodermal cells. Our c68 mutant did not show abnormal periclinal divisions in the cortical cells (Fig. 2). Rather, the specification of the exodermal cells was defective and some exodermal cells were changed into sclerenchyma-like cells with smaller cell size and thicker cell walls (Fig. 2). This alteration occurred in the seminal root as well as in crown roots (Fig. 2D, F, H), indicating that the C68 gene is required for the correct development of exodermis of all primary roots in rice.

In addition to defective exodermis, the mutant also showed deficient root epidermis. Smaller (mainly in radial axis) and irregular epidermal cells were formed in the mutant, leading to the disarrangement of epidermal cells (Figs. 2B, 3). Rice root epidermis and root cap are derived from different initials (Clowes 2000, Lux et al. 2004), which is in contrast to Arabidopsis where epidermis and lateral root cap share the same initials (Dolan et al. 1993). In the mutant, the lateral root cap was difficult to peel off from the epidermis (Fig. 3), which might be caused by the defective epidermal cells, but the underlying mechanisms are still unknown. Abnormal periclinal divisions were also found in the epidermal cells of mutant roots (Fig. 3D).

The c68 mutant showed far fewer hair cells than the WT (Fig. 1). The development of hair cells in rice roots is determined by the cell structure of the epidermis, not by position cues. Epidermal cells of rice roots are divided transversely and produce two different sizes of cells: long and short cells, and the initially short cells differentiate into hair cells (Clowes 2000, Kim et al. 2006). Smaller and irregular epidermal cells in the mutant might result in the defect in root hair development. The gene responsible for morphological changes in the mutant has been mapped to the short arm of chromosome 2 (Fig. 10). Identification of this gene will help in understanding the mechanism underlying the specification of root outer cell layers in rice, which is being undertaken in our laboratory.

**Role of outer cell layers in metal resistance**

The mutant isolated in this study provides a good tool for studying the role of outer cell layers in metal resistance. The mutant showed similar root growth to the WT in the absence of metals (Fig. 4), but became more sensitive to Al, La and Cd compared with the WT (Figs. 5, 7). A detailed study with Al showed that the mutant accumulated more Al in the root tips and that Al penetrated into the inner cell layer (Fig. 8). All these results indicate that the outer cell layers function as a physiological barrier, which prevents metals from entering into the inner cortical cells.

The mutant had far fewer root hairs than the WT (Fig. 1). Recently, enhanced Al tolerance was reported in Arabidopsis mutants with shorter root hairs, and this has been attributed to less Al influx in root hair cells of the mutants (Ezaki et al. 2007). However, in the present study, we found that WT root with long root hairs showed higher tolerance to Al than the mutant defective in root hairs. When the basal root zones were exposed to Al, the Al-induced inhibition of root elongation was not observed in both the WT with root hair and the mutant with far fewer root hairs (Figs. 1, 6). These results indicate that root hairs are not involved in the Al toxicity of rice. Al-induced inhibition of root elongation only occurred when the root tip is exposed to Al (Fig. 6), while root hair formation usually occurs in the basal root zone.

The exodermis cells possess Casparian bands and may also develop suberin lamellae and thickened, tertiary walls (Enstone et al. 2003). This cell layer has been suggested to present a barrier for restricting the radial flow of water, solutes and air via the apoplast (Hose et al. 2001, Enstone et al. 2003, Ma and Peterson 2003). On the other hand, the characteristic structure of rice roots is sclerenchyma cells, which are heavily lignified and line the exodermis (Fig. 2C–F, Morita and Abe 1999). This layer has a role in mechanical reinforcement to maintain the root structure with developing aerenchyma. Based on the difference of these two layers, alteration of part of the exodermis to sclerenchyma-like cells and a change in part of the epidermal cells to gain exodermal properties (Fig. 2) imply the discontinuity of physical barrier in the exodermis of the mutant. However, the development of Casparian bands and suberin lamellae of the exodermis and formation of lignin in the sclerenchyma usually occur in the mature zones of the roots (Fig. 2C–F), not in the root tip (Fig. 2A, B). On the other hand, the site of metal toxicity is at the elongation zone of the roots (e.g. Ryan et al. 1993). In this study, we also found that Al-induced inhibition of the
root elongation occurred only when the root tips were exposed to Al in either the WT or the mutant (Fig. 6). These results indicate that a physical barrier different from that of root mature zones is present in the root tips. In the mutant, although sclerenchyma-like cells are formed in the mature zone, an incomplete physical barrier in the root tips might be unable to prevent Al and other metals from entering into the inner cortical cells efficiently, resulting in the increased sensitivity to Al and other metals. These results also suggest that the existence of a layer of exodermis in some plant species such as rice can increase their resistance to Al toxicity compared with those plant species such as Arabidopsis without an exodermal layer. Further characterization of this mutant and cloning of the responsible gene will help us better understand the important role of this gene in the development of epidermis and exodermis, and also in metal resistance, particularly in Al resistance.

**Materials and Methods**

**Screening of Al-sensitive mutants**

The procedure for generating the M1 mutant library from an Al-tolerant cultivar (O. sativa L. cv. Koshihikari) irradiated with γ-rays has been described previously (Ma et al. 2005). Three rounds of evaluation of Al sensitivity were conducted to screen mutants sensitive to Al. In the first screening, 10 seeds of each M1 line were placed on a net floating on a 0.5 mM CaCl2 solution in a 20 liter container at 25°C. After growth for 5 d, seedlings were exposed to a 0.5 mM CaCl2 solution containing 0 or 30 µM CdCl2, or 5 µM LaCl3 for 24 h. Root lengths were measured with a ruler before and after treatment and then root elongation was calculated. Seedlings with poor root elongation compared with the WT plant were selected as candidate mutants for further screening. In the second screening, roots of candidate mutants were cut and grown in a 0.5 mM CaCl2 solution. After a week, the root length of the longest newly generated root of each mutant was measured and then exposed to a 0.5 mM CaCl2 solution at pH 4.5. After 24 h, the root length was measured again and subsequently exposed to a 0.5 mM CaCl2 solution (pH 4.5) containing 30 µM AlCl3 for a further 24 h. The root length was measured at the end of the treatment. RRE was expressed as (root elongation with Al treatment/root elongation without Al) × 100. Seedlings with a lower RRE than the WT were selected as candidate lines and grown in a field to obtain M2 seeds. For the third screening, the M2 progeny were re-evaluated for their sensitivities to Al based on their RRE as described above.

**Microscopic observation**

Seedlings (5 d old) of both WT rice and the mutant were used for preparation of root sections. Both cross-sections and longitudinal sections (100 µm thickness) of root tip (2 mm from the root apex), the basal part of the seminal root (25 mm from the apex) and the crown root (50 mm from the apex) were prepared with a micro-slicer (ZERO1, Dosaka EM, Kyoto, Japan). Cell wall autofluorescence or staining by propidium iodide were observed with a microscope (Axio Imager with Apotome, Carl Zeiss).

**Immunohistological fluorescence staining**

Rice roots were fixed in 4% (w/v) paraformaldehyde and 60 mM sucrose and then embedded in 5% agar. Sections sliced to 50 µm thickness were incubated in phosphate-buffered saline (PBS) containing 0.1% (w/v) pectolyase Y-23 and then in PBS containing 0.3% (v/v) Triton X-100. The non-specific reaction was blocked with 5% (w/v) bovine serum albumin (BSA) in PBS. Then we incubated the slides with purified rabbit anti-Lsi1 polyclonal antibodies and subsequently with secondary antibodies (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes, Eugene, OR, USA). We observed the sections with a microscope (Axio Imager with Apotome, Carl Zeiss).

**Root growth and sensitivity to Al, Cd and La**

Seeds of the WT and the mutant (c68) were soaked in deionized water in the dark for 1 d. After 5 d, the seedlings were used for root growth experiments. The root length was measured with 15 replicates at 0, 24, 48 and 72 h in deionized water, and the root elongation was then calculated. To compare the root growth for a relatively long-term period, seedlings of both lines were grown in half-strength Kimura nutrient solution in a greenhouse. After 25 d growth, roots and shoots were harvested and the dry weight was recorded after drying in an oven at 70°C for 3 d. Nine replicates were made for each line.

For a dose–response experiment, seedlings (5 d old) were exposed to a 0.5 mM CaCl2 solution containing 0, 10, 20, 30 or 50 µM Al at pH 4.5 for 24 h. Root length was measured before and after treatment. RRE described above was used to evaluate the sensitivity to Al. For evaluation of sensitivity to other metals, seedlings (5 d old) were exposed to a 0.5 mM CaCl2 solution containing 0 or 30 µM CdCl2, or 5 µM LaCl3 for 24 h. Ten replicates per treatment were conducted.

To compare the growth on acid soil, 10 geminated seeds each of the WT and c68 mutant were sown on an acid soil (non-allophane Andisol) amended with CaCO3 at 0.06, 0.13 and 0.25 g kg⁻¹ soil, resulting in a soil pH of 4.0, 4.5 and 5.0, respectively. The seedlings were grown for 8 d and then the root length was measured with a ruler.

**Multicompartment box experiment**

The response of different root zones to Al was investigated by using a multicompartment box (height = 1.4 cm, length = 4.7 cm, width = 1.0 cm) (Ma et al. 2001). Five 5-day-old seedlings each of both the WT and the mutant were...
placed in the same box divided into two compartments and two boxes were used for each treatment. Treatments included: (i) root tips (<0.5 cm) in the first compartment were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) containing 50 µM Al while the whole basal roots in the second compartment were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) without Al; (ii) basal roots were exposed to 50 µM Al solution while the root tips were placed in a solution free of Al; (iii) both root tips and basal roots were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) without Al; and (iv) both root tips and basal roots were exposed to 50 µM Al. After 14 h, the root length in the first compartment was measured before and after the treatment. RRE was used to evaluate their sensitivities to Al.

**Determination of Al concentration**

Five-day-old seedlings of the WT and the mutant were exposed to a 0.5 mM CaCl₂ solution containing 0, 10, 30 or 50 µM Al for 24 h. Root tips (0–1 cm) were excised and placed in a plastic tube containing 1.5 ml of 2 N HCl. The tubes were laid on the bench for at least 24 h with vortexing more than five times. The Al concentration in the solution was measured with a graphite furnace atomic absorption spectrophotometer (model Z-2000, Hitachi, Tokyo, Japan).

**Morin staining**

Seedlings (5 d old) were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) containing 50 µM Al for 8 h. Root tips were transversely sectioned at 2 mm from their apexes and stained in 0.01% morin for 15 min. The fluorescence signal was observed with a microscope (Axio Imager with Apotome, Carl Zeiss).

**Genetic analysis and molecular mapping**

To perform genetic analysis, the mutant was crossed with the WT plant and then the resultant F₁ plants were grown to maturity to obtain the F₂ seeds. The F₂ seeds were germinated and grown in a 0.5 mM CaCl₂ solution for 5 d and then exposed to a 0.5 mM CaCl₂ solution at pH 4.5 for 24 h. Afterwards, the seedlings were exposed to a 0.5 mM CaCl₂ solution containing 50 µM Al at pH 4.5 for a further 24 h. RRE described above was used to evaluate their sensitivity to Al. Finally, the number of root hairs of each F₂ seedling was checked with a microscope. χ² tests were used to evaluate their segregating patterns.

For mapping the responsible gene C68, an F₂ mapping population was constructed by crossing the mutant with an indica cultivar Kasalath. Bulked segregant analysis was used roughly to determine the chromosome site of this gene. Briefly, two DNA pools were made by extraction of DNAs from mixed leaves of 10 seedlings with fewer root hairs or 10 seedlings with normal root hairs. Sixty-two polymorphic markers developed in our lab (Ma et al. 2005) were used to examine the linkage to the gene by using four DNA samples consisting of two parent DNAs and the two DNA pools. To map this gene further, 43 F₂ mutants with fewer root hairs were used. The primer sequences of flanking markers were as follows: MaOs0209, 5′-GAAACCCAAGCAGAAA ACCG-3′ (forward) and 5′-GAAAGGAAGAAGCGGAG AA-3′ (reverse); MaOs0210, 5′-TAAGTTGATCATGAAATAA GCTGC-3′ and 5′-AATGATTTTTTGGGGGGAG-3′; MaOs0s212, 5′-CAGTGATTTGCAATCCTTTG-3′ and 5′-GGGGT GAGTATTTCTTCTTCTT-3′; and MaOs0215, 5′-TTGC TTACTTGCACCACCTCTCC-3′ and 5′-CTCATACTGACCC CACACCC-3′.

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**References**


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