RESEARCH PAPER

Overexpression of an auxilin-like gene (F9E10.5) can suppress Al uptake in roots of Arabidopsis

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Abstract

Plants resistant to aluminium (Al) stress were isolated from Arabidopsis thaliana enhancer-tagged mutant lines. Compared with the parental Col-7 control line, one of the resistant candidates, #355-2, showed a higher expression of the F9E10.5 gene (At1g75100) on chromosome 1, a lower Al content in whole roots, and a shorter root hair length (~30%). Both Al influx and associated oxidative stress occurred in root hairs, as well as in root tips of Col-7, however, they were seen only in root tips of #355-2. Transgenic plants overexpressing the F9E10.5 gene showed a slightly higher Al resistance than their parental control line (Ler). The F9E10.5 gene encodes an auxilin-like protein related to the clathrin-uncoating process in endocytosis. Microscopic observation indicated that both Al ion influx and endocytosis activity were lower in root hair cells of the #355-2 line than in those of Col-7. These results suggested that overexpression of this auxilin-like protein inhibits endocytosis in root hair cells by a disturbance of the transport system as in animal cells shown previously. It was also suggested that a part of the Al influx occurred via endocytosis in root hair cells in Arabidopsis. The Al resistance in the #355-2 line may therefore be due to a lower Al uptake via endocytosis in the root hair region.

Key words: Activation-tagged mutant line, aluminium (Al) resistance mechanism, Al influx, Arabidopsis thaliana, auxilin-like protein, endocytosis, oxidative stress, root hair.

Introduction

Aluminium (Al) ions, especially Al$^{3+}$, have a toxic effect on both plant and animal cells under low pH conditions (pH < 5.0). Inhibition of root growth is the major symptom of Al toxicity in plants, and is accompanied by an accumulation of Al ions in the cell wall of roots (Matsumoto et al., 2003; Kochian et al., 2004). Al ions have been suggested to enhance peroxidation of phospholipids and proteins in root cell membranes (Cakmak and Horst, 1991; Yamamoto et al., 2001). It is also well known that Al ions bind to DNA and RNA molecules in nuclei and inhibit RNA synthesis (Matsumoto et al., 1977). Moreover, cell death accompanied by a degradation of chromosome DNA occurs in the root tip region during Al stress (Ezaki et al., 2001). These interactions finally inhibit cell division and cell elongation in root growth. Several findings also support the idea that Al stress results in callose production in plasmodesmata, leading to an inhibition of auxin transport and blockage of symplasmic transport of auxin, and that these changes might be involved in the inhibition of root elongation (Kollmeier et al., 2000; Sivaguru et al., 2000).

Many studies of Al resistance mechanisms show that secretion of organic acids, such as malate, succinate, oxalate, and so on, from the plant root tip to the soil appears to be a very effective strategy for many plants (Delhaize et al., 1993; de la Fuente et al., 1997; Ma et al., 2001). Recently, the ALMT1 gene encoding a malate transporter was isolated from wheat (Triticum aestivum L.) and was shown to confer Al tolerance in tobacco (Nicotiana tabacum) cells and in barley (Hordeum vulgare) (Sasaki et al., 2004; Delhaize et al., 2004). There are probably additional Al resistance mechanisms in plants. Phenolic compounds, such as flavonoids, alkaloids, terpenoids, and glycosides, form strong complexes with Al ions and were implicated in internal Al detoxification in tea (Camellia sinensis) and other Al-accumulating species (Ofei-Manu et al., 2001). (Kidd et al., 2001) reported that differential Al tolerance in three maize (Zea mays L.) genotypes
showed a better correlation with the rate of Al-stimulated root exudation of the flavonoids catechin and quercetin, than with Al-activated exudation of organic acids. Al resistance in yeast (Saccharomyces cerevisiae) and in plants can be conferred by overexpression of several plant Al-induced genes, such as the Arabidopsis blue copper-binding protein gene (AtBCB), a tobacco GDI-dissociation inhibitor gene (NtGDI2), a tobacco glutathione S-transferase (EC 2.5.1.18) gene (parB), or a tobacco peroxidase (EC 1.11.1.7) gene (NiPox) (Ezaki et al., 1999, 2000, 2001). These experiments clearly suggested that a variety of Al resistance mechanisms can occur. Characterization of these genes in terms of their contribution to Al resistance may supply new strategies for Al resistance in plants in addition to the release of organic acids.

Inhibition of Al uptake is believed to be one of the most effective strategies for resistance in plant cells, because Al uptake into the root region is the first step of toxicity. Akeson et al. (1989) suggested that Al ions are absorbed into liposomes surrounded by mainly phosphatidylcholine-containing lipid bilayers, the most abundant phospholipids in plasma membranes, and then taken up into the cytoplasm by endocytosis. However, to our knowledge, evidence for a clear Al uptake mechanism and the proteins and/or genes related to this Al uptake process have not yet been isolated. Endocytosis is generally defined as the uptake of extracellular substances via the internalization of plasma membrane into cells. A decrease of Al uptake into the cytoplasm of a plant cell via decreased endocytosis would, in principle, be predicted to ameliorate Al toxicity in root cells.

T-DNA and transposon insertion mutagenesis have become important tools for the isolation of new genes and the study of their functions. Walden et al. (1994) developed a method of tagged mutation that used a T-DNA containing multimerized transcriptional enhancers from the cauliflower mosaic virus (CaMV) 35S gene, where the mutation is a consequence not of gene disruption, but rather of the activation of expression of the tagged gene. This method has come to be known as activation tag and has also been developed and applied to Arabidopsis plants. New vectors for activation tags that confer resistance to the antibiotic kanamycin or the herbicide glufosinate have been used to generate several tens of thousands of transformed plants (Weigel et al., 2000). These authors isolated >30 mutants with various phenotypes from these activation enhancer-tagged mutant lines, due to an overexpression of genes which were located immediately adjacent to the inserted CaMV 35S enhancers. Activation-tagged mutant lines have subsequently been used for characterization of a range of gene functions (Hayashi et al., 1992; Kardailsky et al., 1999; Graff et al., 2000; Haung et al., 2001; Li et al., 2001).

In this study, a pool of Arabidopsis activation enhancer-tagged mutant lines were screened and a possible new mechanism of Al resistance in an individual resistant line was characterized. The results suggest that overexpression of an auxilin-like protein decreased endocytosis and Al uptake in Arabidopsis roots, especially in root hairs.

**Materials and methods**

**Plant material and growth conditions**

A pool of Arabidopsis enhancer-tagged mutant lines (Nottingham Arabidopsis Seed Stock Centre, Nottingham, UK) was used to screen for Al-resistant plants. Each tagged mutant line carries enhancer tag(s) inserted in chromosomal DNA at random sites. The tag cassette consisted of a tandem repeated tetramer of an enhancer sequence derived from the CaMV 35S promoter, a kanamycin resistance gene, and a glufosinate resistance gene (Basta-resistance; BAR) (Weigel et al., 2000).

Modified MS agar plates (1.5%) (Murashige and Skoog, 1962), in which MS salts and B5 vitamins were diluted 6-fold (1/6 MS agar plate), were used for plant growth and Al stress testing (adjusted to pH 4.2). The 1/6 MS agar plates also contained 10 g l−1 sucrose as a carbon source. All plants of Arabidopsis were grown on the 1/6 MS agar plates under fluorescent illumination (∼50 μE m−2 s−1, 16 h of light and 8 h of darkness) at 22 °C.

**AI sensitivity tests and screening for Al-resistant candidate lines**

For all Al treatments in this study, sterilized seeds were plated on 1/6 MS agar plates (pH 4.2) containing 0, 300, or 500 μM AlCl₃ and grown at 22 °C for 15 d. In the screening of Al-resistant candidates, plants having longer roots than the wild-type line under the wild-type line under 300 μM Al treatment (first screening) were transferred from the surface of the agar plates to soil and then grown to seed maturation. The seeds independently harvested from each resistant candidate line were tested under 0, 300, or 500 μM Al stress on agar plates for 15 d as the second screening. Al-resistant candidates obtained in this screening were then tested for their AI sensitivity in a third screening to confirm the reproducibility of the phenotypes. Al sensitivity was estimated by comparing relative root growth between the Al treatments (300 μM or 500 μM treatment) and the control treatment (0 μM treatment), and is shown as a percentage.

**Characterization of isolated Al-resistant candidates**

The copy number of the enhancer tag in chromosomal DNA was determined by Southern hybridization analysis using the four tandem repeated enhancer tag sequences (1.36 kb) as probe DNA. Chromosomal DNA was extracted from each candidate line, digested with BamHI/XhoI and BamHI/PstI, and then subjected to agarose gel electrophoresis and hybridization (Maniatis et al., 1989). Using the procedure described by Liu et al. (1995), TAIL-PC1R (thermal asymmetric interlaced polymerase chain reaction) was performed to amplify the right border (RB) site of the inserted tag in chromosomal DNA. PCR products carrying the insertion site were sequenced and searched using the homology search software, FASTA (DDBJ; http://www.ddbj.nig.ac.jp), to determine the position of the inserted enhancer tag in chromosomal DNA.

**Semi-quantitative RT–PCR for detection of gene expression**

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Semi-quantitative real-time PCR was carried out using a kit, One Step RNA PCR Kit (TAKARA, Tokyo, Japan), and the Smart Cycler System (Cepheid, Sunnyvale, CA, USA). To identify each PCR product separately, the 12 oligonucleotides
performed with mRNA encoding amounts of RNA were used for amplification, control PCR was also
30 s, and 72 °C for 70 s. To ensure that approximately equal amounts of RNA were used for amplification, control PCR was also performed with mRNA encoding β-tubulin using the two primers, β-tubulin-F and β-tubulin-R.

The primers were used as follows: F9E10.2-F, 5′-AATGAAAGCCGATTTCTCCGGGAG-3′; F9E10.2-R, 5′-GCAGACAAATTGTCGACCTTCTTGTAGTACC-3′; F9E10.3-F, 5′-GTGGTTGGAAGACGAGGAGCTTGCTGA-3′; F9E10.3-R, 5′-CCCTTGTGTGAGACATCGCGGCTGTG-3′; F9E10.4-F, 5′-GATAAACCTGAACGAATGCGCCTTGGTGA-3′; F9E10.4-R, 5′-CTTTGATTTTTCAGCTATAGGGTTCCACCA-3′; F9E10.5-F, 5′-GAGCAGGTTGGGAATGTGTCCCTTGACAAGACGC-3′; F9E10.5-R, 5′-ATTCGGACGTGATAGAAGAGACCC-3′; F9E10.6-F, 5′-CTCGGAAAGCAAGCCAGTGTTGAGGACATG-3′; F9E10.6-R, 5′-GAATTAGACACCCATTAACTCTAAGAGACA-3′; F9E10.7-F, 5′-GAGCAAGGTGGGGAATCTCTTGGACAAAGC-3′; F9E10.7-R, 5′-GTGGTTGGAAGACGAGGAGCTTGCTGA-3′; and β-tubulin-F, 5′-AACATGCGCTGTGAACTGTCCGCA-3′.

**Construction of plasmids and transgenic plants**

A binary vector, pART27 (Gleave, 1992), was used for construction of a plasmid carrying the F9E10.5 gene behind the 35S promoter. Transformation of Arabidopsis by Agrobacterium tumefaciens was performed by the vacuum infiltration method described by Bechtold et al. (1993) using Arabidopsis Landsberg erecta (Ler) as a host plant.

**Determination of Al content in root region**

Ten-day-old plants were exposed to 100 μM Al for 36 h. Al-treated roots were washed completely three times with 1 l of deionized water to remove unabsorbed Al ions. Twenty 15 mm long root tip sections from these washed roots were collected every 3 h or 12 h and disintegrated by treatment with a concentrated acid mixture (HNO₃ and H₂SO₄, 1:1, v/v) at 120 °C for 3 h. The Al content of the solution was determined by a polarized Zeeman atomic absorption spectrophotometer Z-8270 (HITACHI, Tokyo, Japan).

**Microscopic observation and staining**

Morphological observation was performed using an Olympus CX41 microscope (with U-RFL50, Olympus, Tokyo, Japan). Staining of roots with a tracer dye, SynaptoRed C2 [N-(3-triethylammoniumpropyl)-4-(8-(diethylamino)phenyl)hexatrienyl] pyridinium dibromide (Cosmo Bio, Tokyo, Japan) and monitored microscopically according to Ovecka et al. (2005). SynaptoRed C2 is also known as FM4-64. Quantitative analyses of Al content and endocytosis activity in root hair cells were performed using the software, ImageJ, supplied by NIH.

**Results**

**Screening and molecular genetic characterization of Al-resistant candidate lines**

Screening of Al-resistant plants was performed using a pool of Arabidopsis enhancer-tagged mutant lines (~16 000 lines) in the presence of 300 μM Al. A total of 150 lines were isolated as Al-resistant candidates in the first screening. Finally, 11 lines were selected as Al-resistant candidates after the third screening. Relative root growth of these lines after 300 μM and/or 500 μM Al treatments for 15 d was higher than those of a parental control line (Col-7) (Fig. 1).

Southern hybridization analysis was performed to determine the copy number of the inserter en tag into chromosomal DNA in each Al-resistant candidate. Most of the candidate lines unfortunately showed several bands in their blots, indicating multicopy insertions in their genome DNA (data not shown). One exception was the #355-2 line, in which a single hybridizing band was detected, indicating a single copy insertion of the enhancer tag. This line was therefore subjected to further analyses in this study.

TAIL-PCR was performed to clarify where the tag was inserted in the chromosomes of #355-2. Approximately 450 bp of PCR product derived from the chromosomal DNA was obtained by PCR and sequenced (data not shown). The sequence data indicated that the tag was inserted 28 bp upstream from the ATG start codon of the F9E10.5 gene in chromosome 1 (Fig. 2A). To clarify which genes had their expression enhanced in the inserted

![Fig. 1. Result of the final Al sensitivity test (third screening) for Al-resistant candidate lines isolated in this study. Relative root growth under 300 μM or 500 μM Al treatment for 15 d (shown as percentages of root growth under 0 μM Al treatment) was compared between the Al-resistant candidate lines and the parental control line, Col-7. Error bars represent SE values (n=20). All candidate lines showed similar root growth in 0 μM Al to the control line (within ±10% difference). A significant difference was seen between the control line and #355-2 line under both 300 μM and 500 μM Al treatments (P<0.01).](http://jxb.oxfordjournals.org/DownloadedFrom/jxb.oxfordjournals.org)
#355-2 line, a semi-quantitative real-time PCR experiment was performed. Of six genes near the insertion site (F9E10.2, F9E10.3, F9E10.4, F9E10.5, F9E10.6, and F9E10.7, see Fig. 2A) in the control line Col-7, the highest expression of the F9E10.5 gene (~8-fold) was detected in the #355-2 line (Fig. 2B). DNA database analysis suggested that the F9E10.5 gene (also known as At1g75100) encodes an auxilin-like protein. The deduced amino acid sequence of the F9E10.5 protein shows a high homology to other reported Arabidopsis auxilin-like proteins [e.g. the AtAUL1 gene (AJ007450; Quesada et al., 1999); the Arabidopsis C7A10.840 gene; and the T20K18.120 gene] in the J domain, which is a unique conserved domain in whole organisms.

**Physiological characterization of the #355-2 line**

What is the Al resistance mechanism in the #355-2 line? To address this question, accumulation of Al ions in the root region (15 mm length from root tip) of the #355-2 line during a 36 h period of Al uptake was compared with that of the Col-7 line. The result indicated that the Al content in #355-2 was always lower than in Col-7 (Fig. 3).

Morphological observation showed that the length of root hairs in the #355-2 line was ~30% of that of Col-7 without any Al treatment (Fig. 4, 0 μM Al treatment). An actual measurement of length was performed for 100 root hairs of each line in the without Al condition and the lengths were 120±28 μm for #355-2 and 395±108 μm for Col-7, respectively. At higher Al concentrations (200 μM and 300 μM, 2 d), these two lines showed severe damage to their root hairs, and the number of longer root hairs was decreased, especially in the Col-7 line. All root hairs of Col-7 line showed an aberrant shape with 500 μM treatment for 2 d, while no root hairs could be observed in the #355-2 line at this concentration. In spite of a short root hair phenotype of the #355-2 line, there was no significant difference between the #355-2 line and the Col-7 line (within ±10% difference) in either the root length or the height of the plant under not only a solidified agar medium culture condition, but also a soil culture condition (data not shown).

![Fig. 2.](image-url)

**Fig. 2.** Insertion site of the enhancer tag in the #355-2 line and comparison of the relative expression level of six genes (F9E10.2–F9E10.7) between Col-7 and #355-2. (A) The insertion site of the tag in whole chromosome 1 (upper) and the precise insertion site of the tag (bottom). Arrows indicate the insertion site of the activation tag in the #355-2 line. The CaMV 35S enhancers (1360 bp) in T-DNA (total length; 6730 bp) represent four repeated enhancer sequences. LB and RB indicate the left border and right border of T-DNA, respectively. F9E10.1–F9E10.7 shown by bold arrows indicate seven genes existing around the insertion sites, and all of them are transcribed from left to right in this figure. The scale bar at the bottom represents 1 kb. (B) The expression level of each gene (F9E10.2–F9E10.7) in the #355-2 line is shown as relative gene expression, when that in the Col-7 line was defined as 1.0. Error bars represent the SE (n=3). A significant difference was seen between the F9E10.5 gene and the five other tested genes in terms of their relative expression level (P <0.01).

![Fig. 3.](image-url)

**Fig. 3.** Al content in the root tip region of #355-2 and Col-7. Following exposure to 100 μM Al for 36 h, 20 root tips (15 mm in length) were collected at the appropriate time points and their Al contents were determined by atomic absorption spectrophotometry. Error bars represent the SE (n=3). A significant difference in Al content was seen between the two lines at 9, 12, and 24 h (P <0.05), but not at the other time points (3, 6, and 36 h).
Al sensitivity test for the transgenic lines overexpressing the F9E10.5 gene

To clarify whether the F9E10.5 gene is related to Al resistance in line #355-2, the Al sensitivity test was performed using overexpressing transgenic plants. A full-length cDNA fragment (pda05281; 2068 bp DNA fragment including 1956 bp of the open reading frame) encoding the F9E10.5 gene was obtained from RIKEN, and three independent transgenic lines that overexpressed the F9E10.5 gene from the 35S promoter were constructed (F9E10.5 #1-4, #4-3, and #5-8). The expression level of the F9E10.5 gene in these lines was determined by semi-quantitative real-time PCR and showed ~2.7–3.7 times higher expression than a parental non-transformant (Ler) (data not shown). These constructed lines showed a slightly higher rate of relative root growth than Ler under 300 μM or 500 μM Al treatments (Fig. 5A). Many (but not all) plants of each F9E10.5-expressing transgenic line (F9E10.5 #1-4, #4-3, and #5-8) also showed shorter root hairs than Ler with a high frequency under the without Al condition (Fig. 5B).

Fig. 4. Microscopic observation of morphological damage in the root hair region of the #355-2 and Col-7 lines after Al treatments (0, 200, 300, or 500 μM for 2 d). Ten-day-old plants grown on 1/6 MS agar plates without Al (pH 4.2) were used for this observation. Higher magnification pictures were only taken under 500 μM Al treatment. White bars indicate 200 μm length.

Fig. 5. Al sensitivity test and microscopic observation of the root hair region of transgenic plants overexpressing the F9E10.5 gene. (A) Relative root growth under 300 μM or 500 μM Al treatment for 15 d (shown as a percentage of root growth in the absence of Al) was compared between the constructed transgenic lines overexpressing the F9E10.5 gene (F9E10.5 #1-4, #4-3, and #5-8) and their parental control line (Ler; non-transformant line). Error bars represent the SE (n=20). * and ** represent a significant difference in Al sensitivity between Ler and each transgenic line at P<0.01 and P<0.05, respectively. (B) Ten-day-old plants of Ler and the three transgenic lines were grown on 1/6 MS agar plates without Al (pH 4.2) and used for microscopic observation. The white bar indicates 200 μm length.
An insertional gene-disrupted mutant line for the F9E10.5 gene (N824458) was also obtained from NASC (Nottingham Arabidopsis Stock Centre). This line showed similar Al sensitivity and root hair length to its parental wild-type line (data not shown). These results suggest that higher expression of the F9E10.5 gene alone can ameliorate Al toxicity, but that depletion of this protein in cells does not affect Al sensitivity.

Difference in localization of Al ions and lipid peroxides in roots

To identify differences in the localization of Al ions between #355-2 and the control line, Al-treated roots were stained with an Al-specific fluorescent dye, morin. Negligible fluorescence was seen in both lines without Al (data not shown). However, the root tip region of both lines showed a strong Al-dependent fluorescent signal after 200 μM Al treatment for 6 h (Fig. 6). Root hairs and the associated stem region of the wild-type Col-7 line also showed a strong Al-dependent fluorescent signal. In contrast, no significant signals were seen in the corresponding regions of the #355-2 line.

To detect the localization of oxidative damage caused by Al treatment, Al-treated roots of both lines were also stained with DCFDA, which can specifically detect the products of oxidative damage, such as lipid peroxides (Fig. 6). Negligible fluorescence was also seen in both lines in the without Al condition by DCFDA staining (data not shown). Both lines showed a strong fluorescent signal derived from oxidative damage in their root tip region. In addition, the Col-7 line also showed severe oxidative damage in many root hairs and their associated stem regions; this fluorescence was not seen in the #355-2 line.

Al sensitivity tests of root hair mutants

Three Arabidopsis root hair mutants, rhd4 (Schiefelbein and Somerville, 1990), aux1 (Pitts et al., 1998), and ire (Oyama et al., 2002), show shorter root hair phenotypes than their individual parental control lines. Are there any differences in Al sensitivity between these root hair mutants and their parental lines, if the formation of short root hairs is related to Al toxic mechanism? All three mutants showed slightly higher rates of relative root growth under 300 μM and/or 500 μM Al treatments, compared with their parental wild-type lines (Col-0 or WS) (Fig. 7). Accumulation of both Al ions and oxidative damage estimated by morin or DCFDA staining were again observed in root tips, but not in the root hairs of each Al-treated mutant line (200 μM Al, 6 h). The results for the aux1 mutant line only are shown in Fig. 6. The results observed in all three mutants were very similar to those in the #355-2 line.

Fig. 6. Morin- or DCFDA-stained roots after Al treatment. Ten-day-old plants (Col-7, #355-2, and the aux1 mutant line) grown on 1/6 MS agar plates without Al (pH 4.2) were exposed to 200 μM Al treatment for 6 h and then stained with either 10 μM morin or 10 μM DCFDA for 10 min. Fluorescent signals in both the root tip region (left) and the middle position (right) of Al-treated roots of each line are shown here. The white bar indicates 200 μm length.

Fig. 7. Al sensitivity test for the root hair mutant lines. Relative root growth under 300 μM or 500 μM Al treatment for 15 d (expressed as a percentage of root growth in the absence of Al) was compared between the root hair mutant lines (rhd4, aux1, and ire) and their parental control lines (Col-0 or WS). Error bars represent SE values (n=20). The three mutant lines showed almost similar root growth under the 0 μM Al condition to that of their control lines (within ±10% difference). * and ** represent a significant difference in Al sensitivity between control lines and mutant lines at P <0.01 and P <0.05, respectively.
Vesicle trafficking via endocytosis and Al uptake in root hairs

DNA database analysis suggested that the F9E10.5 gene encodes an auxilin-like protein. Many proteins in the auxilin family are known to function in endocytosis (Ahle and Ungewickell, 1990; Ungewickell et al., 1995; Lemmon, 2001). Suetsugu et al. (2005) have suggested that the F9E10.5 protein regulates chloroplast movement in the cells of Arabidopsis. The present microscopic observations showing lower Al concentrations in the root hair region of the #355-2 line (Fig. 6) suggested the possibility that overexpression of the F9E10.5 gene in the #355-2 line may be related to reduced endocytosis and therefore to a lower Al uptake.

To clarify this hypothesis, vesicle trafficking via endocytosis and Al uptake in the root hair region were determined and compared between the Col-7 and #355-2 line (Fig. 8A, B). A fluorescent dye, SynaptoRed C2 (also called FM4-64), has been widely used to monitor vesicle trafficking activity via endocytosis (Ovecka et al., 2005). Al-treated root hairs were simultaneously stained with morin and SynaptoRed C2, and observed by a fluorescent microscope with selective filters (green or red fluorescent patterns in Fig. 8A, respectively). Under SynaptoRed C2 staining, transport vesicles were detected as fine red particles or red zones in both lines (Fig. 8A, a–f for Col-7 and g–j' for #355-2). Most of these particles or zones were also detected as green fluorescent signals by morin staining (Fig. 8A, a–f for Col-7 and g–j for #355-2). These results indicate that a part of the Al influx actually occurred via endocytosis in root hair cells. The strength of the red fluorescence (endocytosis activity) and green fluorescence (Al content) in root hair cells was clearly lower in #355-2 than in Col-7 in the Al-treated condition (Fig. 8A, B). These results suggested that a lower Al influx is caused by a lower endocytosis activity in the #355-2 line.

Al-untreated root hair cells of each line showed a basal level of morin-dependent fluorescent signal (Fig. 8B; photographs for Al-untreated root hair cells showing a negligible fluorescence in both lines were not shown in Fig. 8A). Al content was increased in both lines, with an individual level by Al treatment, while there was no clear change in endocytosis activity in either line (Fig. 8B). It was suggested that Al toxicity does not affect endocytosis activity in these lines. Both endocytosis activity and Al uptake were observed in the root hair region of the F9E10.5 null mutant (N824458), at levels similar to those in the control line (data not shown).

Discussion

The #355-2 line showed a much higher gene expression of the F9E10.5 gene (At1g75100), compared with the control line (Col-7), presumably due to the insertion of the...
enhancer tag into the upstream region of this gene. DNA database analysis suggested that this gene encodes an auxilin-like protein previously related to endocytosis in various organisms (Lemmon, 2001). Recently, Suetsugu et al. (2005) designated the F9E10.5 gene as the jac1 gene and reported that the protein regulates phototropin-mediated chloroplast movement in Arabidopsis. As the JAC1 protein (F9E10.5 protein) has a conserved C-terminal J-domain and a Phe-X-Asp-X-Phe motif (an AP-2 α appendage-binding motif) exists in the first 33–37 amino acid residues, they also speculate on the possibility that this protein functions in the endocytosis process. What is the biochemical function of the overexpression of the F9E10.5 gene for Al resistance in the #355-2 line? The #355-2 line shows an ~8-fold higher gene expression of the F9E10.5 gene and a higher Al resistance than the Col-7 line. Transgenic lines expressing the F9E10.5 gene ~2.7–3.7 times more than the wild-type line also could partially ameliorate Al toxicity in the Al sensitivity test. These results suggested that dose-dependent Al resistance can be obtained by expression of the F9E10.5 gene in plants. Moreover, microscopic observation after morin and SynaptoRed C2 staining strongly suggested that a part of the Al influx actually occurred via endocytosis in the root hair region and that the influx was clearly lower in the #355-2 line than in the Col-7 line due to an overexpression of this gene. Why does the overexpressed F9E10.5 gene show a lower endocytosis activity in the #355-2 line? It has already been reported that auxilin proteins function in uncoating of clathrin from the mature vesicles to promote the recycling of clathrin during the endocytosis process and that the J domain of auxilin protein functions in this process (Ahle and Ungewickell, 1990; Ungewickell et al., 1995; Lemmon, 2001). It has also been reported that overexpression of auxilin can inhibit endocytosis in neuronal cells and HeLa cells (Umeda et al., 2000; Zhao et al., 2001; Newmyer et al., 2003). No clear mechanism for the latter inhibition has been established. However, it is speculated that, as in these animal cells, overexpression of the auxilin-like F9E10.5 protein in Arabidopsis inhibits and/or disturbs the endocytosis process in root hair cells (Fig. 8). Endocytosis activity and Al content in the root hair region of the insertion type of the gene-disrupted mutant line, N824458, were also determined in this study, but the results indicated that there is no difference between the mutant and the control line (data not shown). There is as yet no clear explanation for this result, but a simple explanation may be that there is another hypothesized protein(s) having a similar function to the F9E10.5 protein that can complement the disruption phenotype of the N824458 line. Another possibility is that the insertion mutation in the F9E10.5 gene of the N824458 line does not completely disrupt the function of this gene in endocytosis. From DNA database analyses, it has been deduced that the F9E10.5 gene has nine exons, and the insertion has occurred in the sixth exon of the F9E10.4 gene in the mutant line. The mutated exon is far away from the hypothesized active domain, the J domain, which is located in the C-terminal region of F9E10.5 gene (ninth exon). It is therefore suggested that the mutation in the sixth exon cannot effectively produce an inactivation of this protein in the N824458 line. Mutant lines disrupted in the J domain should be constructed and used for these experiments.

In addition to lines overexpressing F9E10.5, it was shown that three unrelated mutants with a phenotype of short root hairs (rhd4, aux1, and ire) were resistant to Al stress. These three mutants are defective in very different pathways leading to root hair formation. However, all of them showed the ability to continue growing longer roots during exposure to Al stress. In addition, all showed reduced Al accumulation and oxidative damage in their root hairs. These results provide arguments for a role for root hairs in Al uptake and toxicity in Arabidopsis. As the total surface area of the shorter root hairs in #355-2 and the three short root hair mutants is smaller than in their wild-type parental lines, Al uptake in the root hair region by endocytosis is probably also less in these lines. This speculation seems to be consistent with the results shown in Figs 6 and 8. Although a short root hair phenotype is clearly not essential for Al resistance, these results suggest that it provides an alternative way of obtaining tolerance, at least in Arabidopsis. It is hypothesized that short root hair formation is a passive strategy rather than an active one against Al toxicity.

Another important conclusion from this study is the suggestion that root hairs, along with the root tip, may be a significant region of the plant for both Al toxicity and Al resistance. Determination of Al content in the root region indicated a lower accumulation of Al in the #355-2 line than in the Col-7 line (Fig. 3). The morin staining experiment for root tips of both lines showed a similar strength of fluorescent signals with 200 μM Al treatment. However, many root hairs and the related stem region of the Col-7 line also showed a strong Al-dependent fluorescent signal. A much lower fluorescent signal was detected in the short root hair region of the #355-2 line. Only a few studies concerning the relationship between Al toxicity and root hairs have been reported, and the mechanistic basis for Al toxicity is still uncertain. Gassmann and Schroeder (1994) described the blockage of the low affinity K+ uptake channel in the root hair region by Al. Jones et al. (1998) reported that Al stress causes an alteration in cellular Ca2+ homeostasis, but that the phytotoxic action of Al in root hairs is not through blockage of Ca2+-permeable channels required for Ca2+ influx into the cytoplasm. The present results clearly showed damage of root hair cells by Al stress in this study. It is proposed that the lower accumulation of Al ions from the root hair region may be one of the Al
resistance mechanisms in the #355-2 line. Most reports have focused on the accumulation of Al ions only in the root tip region, but it is speculated that root hairs are also one of the targets for Al toxicity and that the Al influx probably occurs via root hairs as well as at the root tip. Al-dependent oxidative damage was observed in both the root tip region and in many root hair cells of the Col-7 line, but not in most of the short root hairs of the #355-2 line. This result strongly suggests that accumulation of Al ions causes oxidative damage in the root hair regions of Arabidopsis. The lower oxidative damage in the root hair region of both the #355-2 line and the root hair mutants may be derived from a lower accumulation of Al ions in the root hair region.

Eleven Al-resistant candidates were isolated from ~16,000 Arabidopsis activation enhancer-tagged mutant lines in the present screening, but 10 of 11 lines unfortunately showed multiple insertions of the activation tag in each chromosomal DNA. Since molecular genetic characterization of these lines was complicated and difficult, the #355-2 line was therefore used for further analyses. Multiplicity of inserts might be due to the structure of the plasmid vector used for construction of the tagged mutant lines. However, activation-tagged mutant lines have proven useful for characterization of many genes (see Introduction), and in the present case have provided a novel resistance gene that has contributed a new insight to our understanding of how Al inhibits the growth of plant roots.

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