Aluminum-Induced Gene Expression and Protein Localization of a Cell Wall-Associated Receptor Kinase in Arabidopsis[1]

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Here, we report the aluminum (Al)-induced organ-specific expression of a *WAK1* (cell wall-associated receptor kinase 1) gene and cell type-specific localization of WAK proteins in Arabidopsis. *WAK1*-specific reverse transcriptase-polymerase chain reaction analysis revealed an Al-induced *WAK1* gene expression in roots. Short- and long-term analysis of gene expression in root fractions showed a typical "on" and "off" pattern with a first peak at 3 h of Al exposure followed by a sharp decline at 6 h and a complete disappearance after 9 h of Al exposure, suggesting the *WAK1* is a further representative of Al-induced early genes. In shoots, upon root Al exposure, an increased but stable *WAK1* expression was observed. Using confocal microscopy, we visualized Al-induced closure of leaf stomata, consistent with previous suggestions that the Al stress primarily experienced in roots associated with the transfer of root-shoot signals. Elevated levels of WAK protein in root cells were observed through western blots after 6 h of Al exposure, indicating a lag time between the Al-induced WAK transcription and translation. WAK proteins are localized abundantly to peripheries of cortex cells within the elongation zone of the root apex. In these root cells, disintegration of cortical microtubules was observed after Al treatment but not after the Al analog lanthanum treatments. Tip-growing control root hairs, stem stomata, and leaf stomatal pores are characterized with high amounts of WAKs, suggesting WAKs are accumulating at plasma membrane domains, which suffer from mechanical stress and lack dense arrays of supporting cortical microtubules. Further, transgenic plants overexpressing *WAK1* showed an enhanced Al tolerance in terms of root growth when compared with the wild-type plants, making the *WAK1* one of the important candidates for plant defense against Al toxicity.

In animal and human cells, extracellular matrix (ECM) proteins such as vitronectin and fibronectin are directly linked to transmembrane receptors known as integrins that are associated with components of the cytoskeleton within the cytosol (Miyamoto et al., 1995; Maniotis et al., 1997; Schoenwaelder and Burridge, 1999; Critchley, 2000). Such structural continuity facilitates transfer of external signals from the cell periphery to the cytoplasm, where growth and defense mechanisms in animal cells are orchestrated (Ingber, 1991; Schwartz et al., 1995; Dedhar and Hannigan, 1996). Obviously, plant cells are also equipped with an analogous structural continuum, but individual players, especially the plasma membrane (PM)-associated connectors, have yet to be identified (Wyatt and Carpita, 1993; Fowler and Quatrano, 1997; Miller et al., 1997; Horst et al., 1999; Kohorn, 2000). Integrins and their ECM-binding partners have been proposed repeatedly, but plant proteins reactive to heterologous integrin antibodies often do not localize to the PM (e.g. Lynch et al., 1998). In addition, sequence identities of some potential candidates are too low to be defined as plant homologs of vitronectins (Zhu et al., 1994) or integrins (Nagpal and Quatrano, 1999). In fact, a plant vitronectin-like protein turned out to be more than 93% identical to elongation factor-1α (Zhu et al., 1994). More importantly, recent completion of sequencing of the Arabidopsis genome revealed that this plant, like the budding yeast (*Saccharomyces cerevisiae*) genome, lacks an integrin homolog. The absence of integrins in both yeast and plant may be explained because their ECMs are built largely from unique polysaccharide polymers, which are not related to the proteinaceous polymers of animal ECMs. Moreover, plant cell wall proteins are typically unrelated to proteins of animal ECMs (Bolwell, 1993; Showalter, 1993; Nothnagel, 1997; Cassab, 1998;

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Majewska-Sawka and Nothnagel, 2000). Alternative to animal integrins, plants may utilize unique adhesive proteins. Wall-associated kinases (WAKs), pectins, and arabinogalactan proteins have been proposed recently as key players in the adhesion of the PM to the cell wall (e.g. Miller et al., 1997; Kohorn, 2000; Majewska-Sawka and Nothnagel, 2000; Park et al., 2000; Šamaj et al., 2000; Anderson et al., 2001; Wagner and Kohorn, 2001).

Although plant homologs of integrins, vitronectins, and fibronectins might still be identified in the future, several plant receptor protein kinases characterized during the last few years (for review, see Harmon et al., 1996; Braun et al., 1997; Becraft, 1998; Lease et al., 1998) may serve as possible connecting molecules between the cell wall and the cytoplasmic cytoskeleton. Nevertheless, the actual physical links of receptor protein kinases with components of the cell wall and cytoskeleton remain largely elusive. It was shown that extracellular portions of WAKs (for review, see Anderson et al., 2001) are covalently bound to pectin moieties of plant cell wall (He et al., 1996; Anderson et al., 2001; Cosgrove, 2001; Wagner and Kohorn, 2001). In addition to pectin, the cell wall domain of WAKs is predicted to bind a class of Gly-rich proteins, which could participate in triggering of the cytoplasmic kinase activity. In fact, recent study identified Gly-rich protein AtGRP-3 as a secreted cell wall ligand for WAK (Park et al., 2001). As with other plant receptor kinases, WAKs span the PM and have a conserved Ser/Thr kinase as a cytosolic domain (He et al., 1996; Anderson et al., 2001). There are five members (*WAK1–5*) in the WAK gene family and an additional 21 members that are characterized as WAKL (*WAK*-like kinase) genes in the Arabidopsis genome (He et al., 1996, 1998, 1999; Verica and He, 2002). The WAK/WAKL genes are differentially expressed in various organs of Arabidopsis (He et al., 1996, 1998, 1999; Wagner and Kohorn, 2001; Verica and He, 2002). Recent studies have suggested that *WAK*s may be involved in cell elongation and morphogenesis (Lally et al., 2001). Wounding or pathogen infection triggers additional *WAK1* expression, strongly suggesting a specific role of *WAK1* in the perception and transfer of external stimuli to the cytoplasm (He et al., 1998). Upregulated *WAK1* expression was shown to protect plants from detrimental effects of the pathogen attack. It was also proposed that the increased *WAK1* expression protects plants from abnormally high and toxic levels of salicylic acid, which usually accompany biotic stress. Although pathogen infection induces protective changes in cells, these are ineffective when WAK or other protective molecules are not present in sufficient amounts at critical subcellular locations.

It has been repeatedly shown that aluminum (Al) induces expression of several genes, which share a high degree of similarity to proteins induced by pathogen attack, wounding, or oxidative stresses (e.g. Cruz-Ortega and Ownby, 1993; Cruz-Ortega et al., 1997; Mistrík et al., 1997; Hamel et al., 1998; Ezaki et al., 2000). Although several Al-induced genes belong to a group of general stress-induced genes, we demonstrate here that *WAK1* belongs to the pool of early genes induced by Al and may play a crucial role in plant defense against Al toxicity.

RESULTS

Short-Term Al Impacts on Root Growth

To better understand the early response of root growth to Al, a highly sensitive short-term root growth analysis was carried out. The root growth inhibition by Al became prominent after 6 h of Al treatment. From 9 h onwards, the Al-treated plants showed about 50% of growth inhibition when compared with the control plants (Fig. 1).

Al-Induced WAK1 Gene Expression

To characterize the *WAK1* gene expression in response to Al stress, reverse transcriptase (RT)-PCR was performed using the total RNAs extracted from plants that were exposed to 100 μM Al for various time intervals (0–9 h). The relative mRNA level of the *WAK1* gene was determined by PCR with several different cycles (20, 25, and 30 for the short-term treatments and 25, 30, and 35 cycles for the long-term treatments) to determine optimal number of cycles to estimate the amount of PCR products. In the short-term Al treatment, *WAK1* expression gradually increased over time and reached the maximum level after 1 h of Al treatment (Fig. 2A).

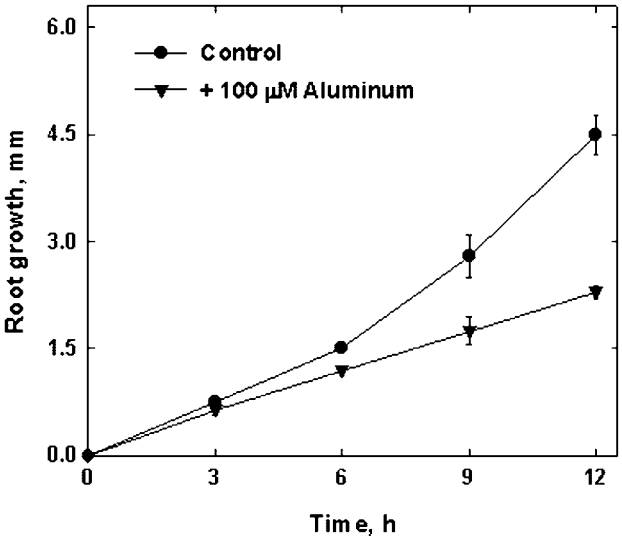

Figure 1. Short-term analysis of root growth in Arabidopsis (ecotype Landsberg-0) seedlings subjected to Al (0 and 100 μM) over a period of 12 h in nutrient solution. Values are mean ± SE of eight to 10 replicates and representative of three independent experiments.

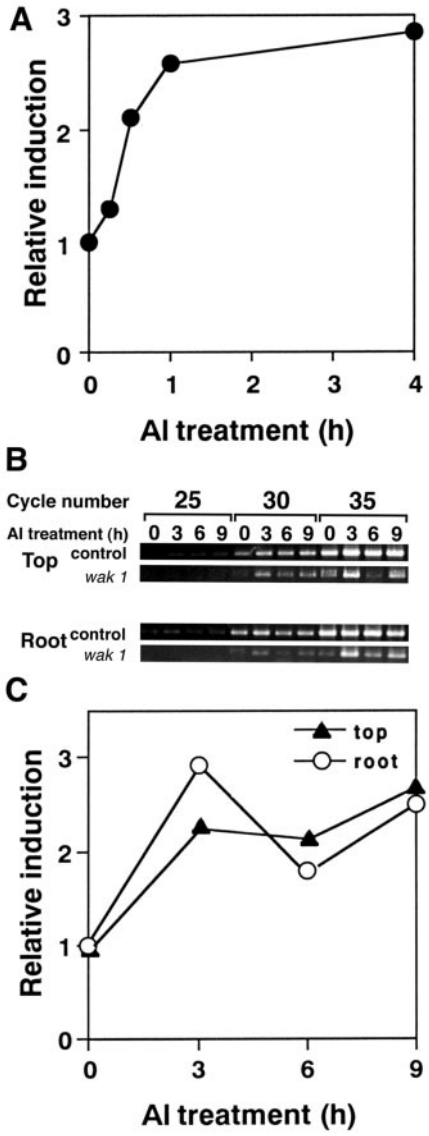

Figure 2. The expression of the *WAK1* gene in Arabidopsis seedlings subjected to Al (100 μM). Short-term (A; 0–4 h) and long-term (C; 0–9 h) Al treatments, where Al was applied only to the root region. B, RT-PCR analysis of *WAK1* expression in response to Al treatment. Loading control (tubulin) was shown at top of each panel. Total RNA was extracted from whole plants (A) at indicated time points and from the root and shoot (top) regions separately (B and C). The relative induction of *WAK1* gene expression computed (30 PCR cycles) at each time point is after setting the level of expression at 0 h as one (A and C). Two independent experiments showed the similar pattern.

The different cycles of PCR for the detection of long-term Al treatment showed similar results. The relative expression induction (Fig. 2C) of *WAK1* gene in the long-term treatment (0, 3, 6, and 9 h) was calculated from the result of 30 cycles in Figure 2B. Maximum accumulation of the *WAK1* transcripts occurs 3 h after Al treatment (Fig. 2C), and a moderate decrease was observed at 6 h of treatment. An apparent gene induction can also be detected in the top regions of the Al-treated Arabidopsis seedlings at 3 h of Al treatment, and this elevated level of *WAK1* transcripts remains unchanged at 6 and 9 h of Al treatment.

Analysis of WAK Proteins after Al Treatments

The *WAK1* expression induction by Al treatment was investigated with western-blot analysis. The polyclonal WAK antibody recognized a single band (at approximately 69 kD) in whole-plant protein fractions in Arabidopsis seedlings (ecotypes Landsberg and Columbia; Fig. 3A). However, it must be presumed that the WAK1 antibody recognizes all five WAK isoforms because this antibody is directed against epitopes within the cytoplasmic kinase domain, which is 90% identical among five WAKs of Arabidopsis (He et al., 1998). Among the WAK isoforms, because *WAK1* is induced by other abiotic stresses (Verica and He, 2002), we have continued with the analysis of *WAK1* further; however, we cannot rule out the fact that the other WAK isoforms may also have a role in Al response. The direct analysis of proteins in root and shoot regions in parallel with the gene expression (Fig. 2, B and C) experiment to a range of Al treatment durations are shown in Figure 3, B and C. The WAK proteins could be detected both in root and shoot extracts. In shoots, the differences among the protein levels between control and Al-treated samples were not discernibly different. In roots, however, the gene expression pattern showed a noticeable increase after 3 h and a significant peak after 6 h, suggesting there might be a lag between transcription and translation. In general, the protein product in controls at 0 h was clearly different between shoots and root compartments. Consistent with the previous report, very little WAK mRNA could be detected by northern blots in roots under normal growth conditions (He et al., 1998). Nevertheless, WAK1 mRNA could be easily detected in roots that were Al treated.

Impacts of Al on WAK Protein Expression and Distribution

The effect of Al treatment of WAK protein expression and distribution was analyzed by indirect immunofluorescence technique coupled with confocal microscopy. We have processed intact whole mounts of seedlings for immunofluorescence labeling of WAKs in the leaf stomatal guard cells (Fig. 4). WAK proteins are localized heavily in guard cells (Fig. 4A). Almost all leaf stomata were closed after 9 h of Al treatments (Fig. 4B) compared with their respective controls (Fig. 4A). To determine whether this response is Al-specific, parallel experiments performed with lanthanum, an Al analog failed to induce this phenomenon (Fig. 4C). There were no clear differ-

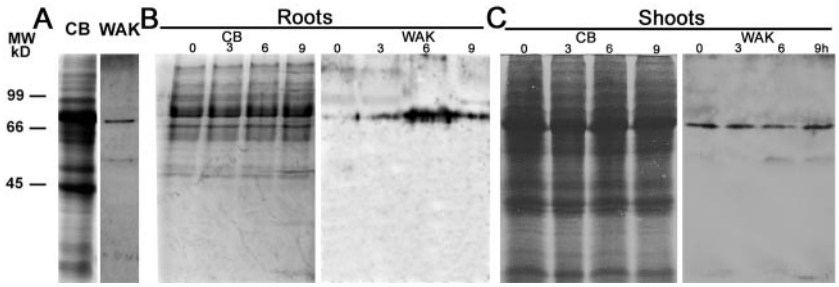

Figure 3. Western-blot analysis showing the induction of WAK protein in Arabidopsis (ecotype Landsberg-0) in response to Al (100 μM) over short-term treatments. A, Recognition of an approximately 69-kD single band in the western blot from the whole-plant protein extracts. Coomassie Blue (CB)-stained gel (left lane) and the WAK western blot of the same lane are shown (right lane). CB staining of root (B) and shoot (C) proteins extracted from plants that were subjected to various periods of Al treatment (CB) are shown. The same replicate gel was probed with the WAK antibody (WAK). Note that there is a detectable amount of WAK protein in roots at 0 h, but the protein level is clearly increased at 6 h of Al treatment (B). There were no differences in the levels of WAK protein in shoots between the Al-treated and untreated plants (C).

ences in stomatal apertures observed from Al treatments in less than the 9-h period (data not shown).

Because cortical microtubules (MTs) play a crucial role in cell elongation and because Al has been shown to disrupt these processes, we have analyzed this in the context of WAK protein expression/localization. Double labeling of WAKs and MTs enabled us simultaneous acquisition and analysis of both WAKs localization and MT arrangement in cells of intact Al-treated root apices. In the root apices of control plants, WAKs are localized in the form of fine cytoplasmic "spots" starting from the basal part of the meristematic zone (Fig. 5A). We imaged cells in individual root growth zones separately. In control plants, there was low WAK expression at the very tip of the root apex (Fig. 5A). The WAK expression in cells of the elongation zone of the same root apex was noticeable (Fig. 5A′). This feature does not alter dramatically after 3 h of Al treatment (data not shown), but after 6 h of Al treatment, the WAK expression increased dramatically in cells of both the meristematic and elongation zones (Fig. 5, B and B′). After 9 h of Al treatment, WAK signal dropped and became diffused as comparable with the 3-h level (data not shown). There was no such tendency found in the root apices treated with lanthanum, particularly 6 h after treatments (Fig. 5, C and C′).

After 9 h of Al treatment, MTs were completely depolymerized (Fig. 6C) compared with their respective controls (Fig. 6A) and the WAK signals emanating as continuous lining along the PM in the basal parts of elongation region (Fig. 6E), whereas WAK-positive cytoplasmic particles also were clearly evident in the mature root zones (Fig. 6G). Neither the complete depolymerization of cortical MTs (Fig. 6B) nor the lining of the PM with WAKs at the basal parts of elongation zone (Fig. 6D) or mature root zone (Fig. 6F) were observed in roots subjected to lanthanum, suggesting the induction of WAK specifically to Al toxicity.

Al Tolerance in Transgenic Plants Overexpressing WAK1

To test whether *WAK1* actually plays a physiological role in Arabidopsis Al response, transgenic plants overexpressing *WAK1* were generated. In the transgenic plants, an ectopic copy of the *WAK1* gene was fused to the constitutive 35S promoter. The construct was transformed into Arabidopsis wild-type (WT) plants (ecotype Columbia), and 30 transgenic lines were produced. Western-blot analysis confirmed that there was a severalfold increased level of WAK1 protein in one-third of the generated *WAK1* overexpression lines (data not shown). Figure 7B shows severalfold higher level of WAK1 protein in one of the overexpression lines (WAK10X) when compared with WT. For the Al response test, 5-d-old seedlings of both the WT and WAK10X were transferred to Murashige and Skoog or Murashige and Skoog with Al media, and their root growth was monitored by measuring the positions of the root tips every 24 h. Seedlings of both the WT and the WAK10X had similar root lengths (10 mm) before the transfer of seedlings to a new set of plates with or without Al. One hundred micromolar Al had a noticeable effect on the root growth of both the WT and WAK1-overexpressing plants. The WAK1-overexpressing plants showed a 3-fold increase in growth in the presence of Al (Fig. 7, A and C) compared with the growth of WT plants in the presence of Al.

DISCUSSION

Over 20 Al-induced genes have been isolated from wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*), and yeast cells so far. Some of them, such as pEARLI1 and pEARLI8 genes, show an early response to Al stress manifested within the first few hours (Richards et al., 1998). In this paper, we demonstrate that *WAK1* is a further representative of

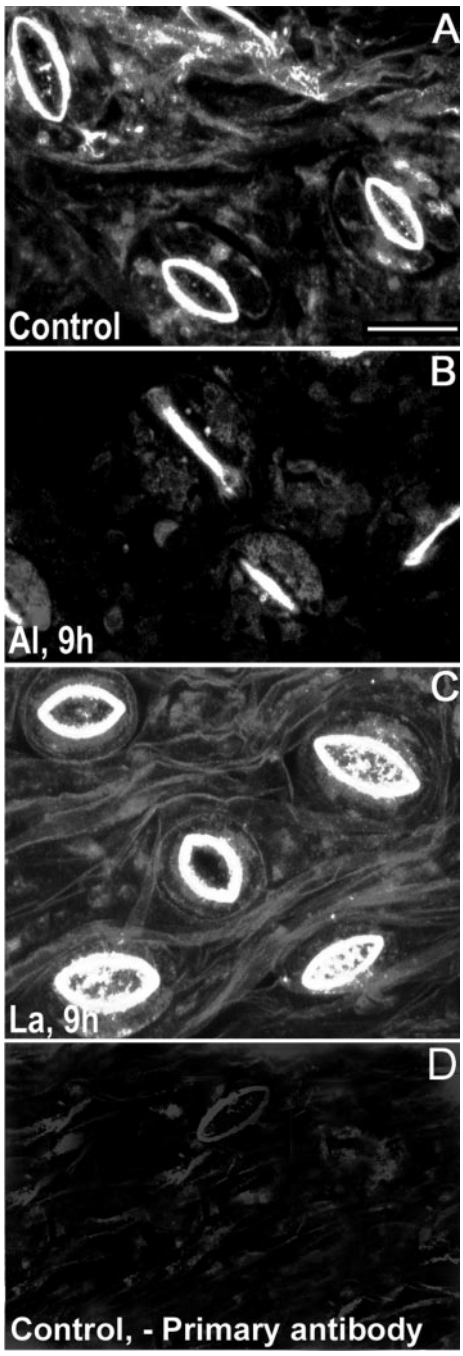

Figure 4. Confocal images showing WAK localization in the leaf stomatal guard cell pore complexes in control plants (A) and plants treated with either 100 μM Al (B) or lanthanum (C) for 9 h that were supplied to roots. D, Negative controls of leaf preparations in which the primary antibody was removed. Note the preferential localization of WAK only at the inner ridges of the guard cell stomatal pore complexes (A). The Al-induced closure of stomata was evidenced by the closed configuration (B) compared with widely opened control (A) as well as after lanthanum treatment (C). Representative images from at least 10 replicate plants for each treatment. Bar in A = 25 μm for all images.

early genes induced by Al in Arabidopsis. Our results with the *WAK1* expression pattern and the site of WAK1 protein localization (elongation zone), together with *WAK1* overexpression studies, strongly suggest a functional role of *WAK1* in the Al tolerance. Also, our RT-PCR results suggest that the mRNA level of the *WAK1* gene is extremely low in roots. The rapid Al-mediated up-regulation identifies *WAK1* gene as a good candidate for genes rendering tolerance toward the Al toxicity in Arabidopsis. WAK1 has been shown to play a role in abiotic stress; therefore, we have focused on this expression to Al toxicity because there are several isoforms of WAKs present in plants, and we cannot rule out the fact that other isoforms may also play a role in Al response.

The *WAK1* gene has been reported to be a member of the pathogenesis-related (PR) genes in Arabidopsis (He et al., 1998). Many of the Al-induced genes seem to be general stress genes that are induced by oxidative stress, hormone treatment, other metal toxicity, phosphate starvation, wounding, and pathogen infection (e.g. Cruz-Ortega and Ownby, 1993; Cruz-Ortega et al., 1997; Mistrík et al., 1997; Hamel et al., 1998). For instance, the PR-2 gene is up regulated during pathogen infection, and it is also induced by Al treatment in wheat (Cruz-Ortega and Ownby, 1993). Intriguingly, most of the genes up-regulated by Al show high homologies with PR genes induced during the pathogen attack (Hamel et al., 1998). All this strongly suggests that Al might be considered for a pathogenesis response elicitor and that it might trigger PR signal transduction pathways. Our present data strongly suggest that WAKs could mediate these cross talks between the pathogen- and Al-mediated signaling pathways.

WAK1 expression induced by Al proceeds in both root and aboveground organs without any considerable lag time, although the Al treatment was applied only to the root region. Therefore, the *WAK1* induction in aboveground organs could not be caused by a direct Al effect but must result from Al-induced signals originating from roots. Larsen et al. (1996) proposed that there is an Al-specific signal transduction between roots and shoots and presented evidence that exposure of roots to Al rapidly induced callose formation in the shoot apex. Such transfer of signals is not characterized and it may involve plant hormones, like auxin and abscisic acid (ABA). The latter stress hormone coordinates several intrinsic functions including stomatal closure and opening (e.g. Li et al., 2000), Al-induced organic acid (citrate in rye [*Secale cereale*]) release (Ma et al., 2001), and other abiotic stress-induced stomatal closures (Cochard et al., 2002) such as drought (Mata and Lamattina, 2001), flooding (Else et al., 1996), and nitric oxide (Mata and Lamattina, 2001). Our original finding that Al induces closure of stomata could be added to this phenomenon. We speculate that Al may interfere with ABA transport and signaling, which lead to the

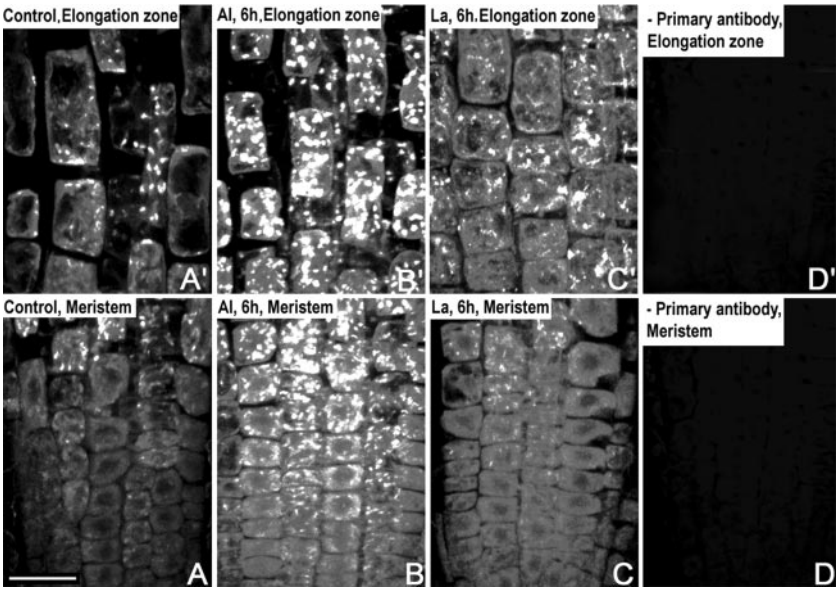

Figure 5. Confocal images showing WAK expression and localization in Arabidopsis roots. Whole-plant mounts of control root meristematic zone (A) and part of the elongation zone (A′), identical regions either after 6 h of 100 μM Al (B and B′) or after 6 h of lanthanum treatments (C and C′). Negative control of the same regions of roots prepared after the omission of the primary antibody (D and D′). There was no WAK expression in cells of the meristem but moderate localization starts from basal (A) and middle part of elongation zone in control root apices (A′). This WAK expression significantly increased upon the 6-h Al treatment (B and B′). In contrast, no such effect on WAK expression was observed in root apices treated with lanthanum (C and C′). Images are representative of at least 10 plants for each treatment. Bar in A = 25 μm for all images.

closure of stomata. In line with this, Al is known to increase ABA levels in plant roots (Kasai et al., 1993). Therefore, the *WAK1* may be included in this root-shoot stress signal transduction system. However, specific mechanisms controlling this signal transduction cascade must be analyzed further to better explain this phenomenon. On the other hand, we cannot rule out the fact that Al could interfere with the stomatal behavior involving alterations to redox system like other abiotic stresses.

Although the mechanism of *WAK1* involvement in Al tolerance is not clear, our northern-blot results suggests that the Al-induced *WAK1* gene expression is part of the early response to the Al stress. Furthermore, the cytoplasmic kinase activity of these PM-associated receptor kinases (He et al., 1996, 1998, 1999) makes WAKs rather strong candidates for putative effectors of the Al signal transduction pathway via utilization of the cell wall-PM-cytoskeleton continuum (Wyatt and Carpita, 1993; Miller et al., 1997; Horst et al., 1999; Kohorn, 2000). Transgenic plants overexpressing *WAK1* showed an enhanced Al tolerance suggesting an important role for *WAK1* in Al response. In the presence of Al, *WAK1* overexpression plants yielded as much as 3 times root growth when compared with WT plants. Because it has been already shown that inhibitors of kinases and phosphatases alter growth and development of plants, particularly roots (Baskin and Wilson, 1997), it would be intriguing to investigate the specific mechanism of sustained growth in the *WAK1*-overexpressing plants. Experiments are underway to address this phenomenon, and we could envision following scenarios on this front.

Al is well known to alter properties of biological membranes, which appear as the major target of Al toxicity (Wagatsuma et al., 1995; Matsumoto et al., 1996; Ahn et al., 2001), and the Al tolerance is associated with the ability to maintain normal ion fluxes across plant membranes (Miyasaka et al., 1989; Huang et al., 1996; Jones et al., 1998). Al alters membrane fluidity, perhaps via changes in the architecture of membrane lipids (Vierstra and Haug, 1978; Zhao et al., 1987). For instance, it is known that Al forms poorly reversible complexes with the minor membrane phospholipid phosphatidylinositol-4,5-bisphosphate (Hilgemann and Ball, 1996). For wheat roots, it was shown that Al inhibits the signal transduction pathway based on cleavage of this critical lipid (Jones and Kochian, 1995). Moreover, Al seems to bind directly to, and cross link, negatively charged sites on the PM surface of beet (*Beta vulgaris*) protoplasts (Lee et al., 2001). This might be expected to affect the function of WAKs. Increased expression of *WAK1* and its accumulation at those cell periphery domains, which are affected by apoplasmic Al and marked via dramatic deposition of callose (M. Sivaguru, W.J. Horst, H. Schneider, T. Schulze, N. Sehmohl, Z. Yang, and H. Matsumoto, unpublished data), might be relevant for maintaining physiological functions of the PM. However, there are also other plausible scenarios for how increased expression of WAKs might contribute to the Al tolerance of plants. The cell wall-embedded domain of WAKs might relieve Al toxicity via interactions with critical cell wall components such as callose, pectins, or arabinogalactan proteoglycans. WAKs are firmly associated with components of cell walls, especially pectins (He et al., 1996; Anderson et al., 2001; Cosgrove, 2001; Wagner and Kohorn, 2001). In this respect, pectin content of cell walls is shown to modulate Al sensitivity in maize (*Zea mays*) cells grown in suspension culture (Schmohl and Horst, 2000). However, Al interaction with other known cell wall cross-linking components such as expansins (proposed by Horst, 1995) should also be taken into consideration.

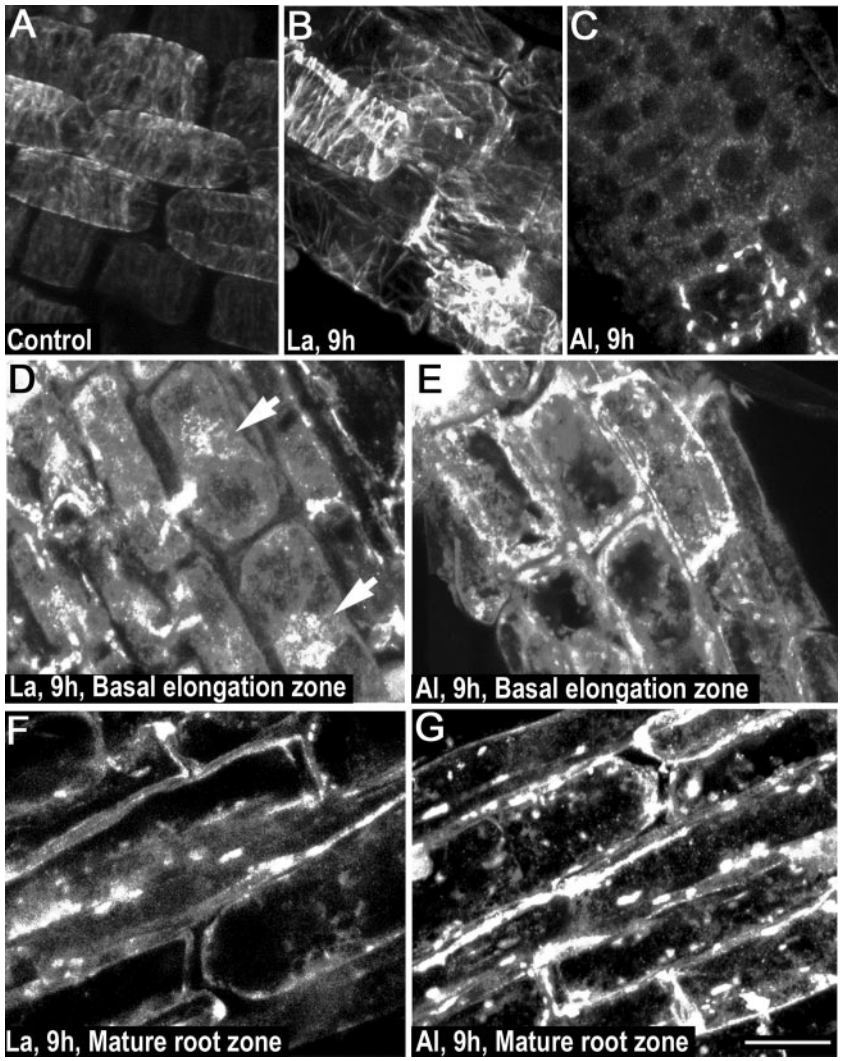

Figure 6. Confocal images of cortical MTs (A–C) and WAK localization (D and E) in cells of the elongation zone, basal part of the elongation zone (D and E), and in mature root zones (F and G) of Arabidopsis. A, Whole-plant mounts of control; B, D, and F, after lanthanum (9 h); and C, E, and G, after Al (100 μM for 9 h) treatments. Note that cortical MT arrays are still present after lanthanum treatments, albeit heavily bundled (B) compared with control (A). Completely depolymerized MTs after Al treatments result in "MT-free" cells (C). Note at this time diffused WAK lining of cellular peripheries in cells of Al-treated root apices (E). This could not be observed after lanthanum treatments (D). Also, note the developing root hair cells showing WAK at the tip regions (arrows in D). The mature part of elongation zone also shows similar but less intense WAK lining, which is relatively higher after Al (G) but less dramatic after lanthanum (F) treatments. Images are representative of at least 10 plants for each treatment. Bar in G = 25 μm for all images.

On the other hand, an intriguing possibility remains that cytoplasmic kinase domains can be cleaved off from WAKs and then participate in the putative cytoplasmic Al response pathways. In fact, a significant portion of the WAK fluorescence is cytoplasmic, indicating that the protein kinase part of WAK, against which the polyclonal antibody used in our study has been raised (He et al., 1996), can be localized independently of the PM within the cytoplasm. For instance, such a situation is known for neurite Notch receptors, which initiate the evolutionary conserved Notch signaling pathway (e.g. Schroeter et al., 1998; Struhl and Adachi, 1998; Šestan et al., 1999). After its proteolytic cleavage, the intracellular domain of Notch receptor is rapidly translocated into the nucleus, where it is presumed to regulate the gene transcription. Similarly, the 55-kD N terminus cleavage form of the PM- and F-actin-associated ezrin is translocated to the nucleus, which has been speculated to be relevant for communication between the PM-associated cytoskeleton and the nucleus (Kaul et al., 1999).

We found that PM-associated WAKs accumulate in large amounts specifically at such subcellular domains that suffer from internal mechanical stresses and that lack dense supporting arrays of cortical MTs underlying the PM. Here, we document this feature for extending tips of root hairs and stomatal pores. It may not be just a coincidence that these cell periphery domains also accumulate callose/pectin and are depleted in cellulosic microfibrils (e.g. Kumarasinghe and Nutman, 1977; Waterkeyn and Bienfait, 1979; Palevitz, 1981; Derksen et al., 1999). Because Al toxicity induces exactly the same situation (e.g. Sivaguru and Horst, 1998; Horst et al., 1999; Sivaguru et al., 1999, 2000), we propose that WAKs are involved in the protection of stressed PM portions, irrespective if these suffer from natural mechanical stresses associated with abundant exocytosis/endocytosis events (for tip growth, see Derksen et al., 1995; for stomatal pores, see discussion in Leyman et al., 1999) or from injuries associated with the Al toxicity. In this respect, it is interesting to note that Al induces bursting of tip-growing pollen tubes (Zhang et al., 1999) and

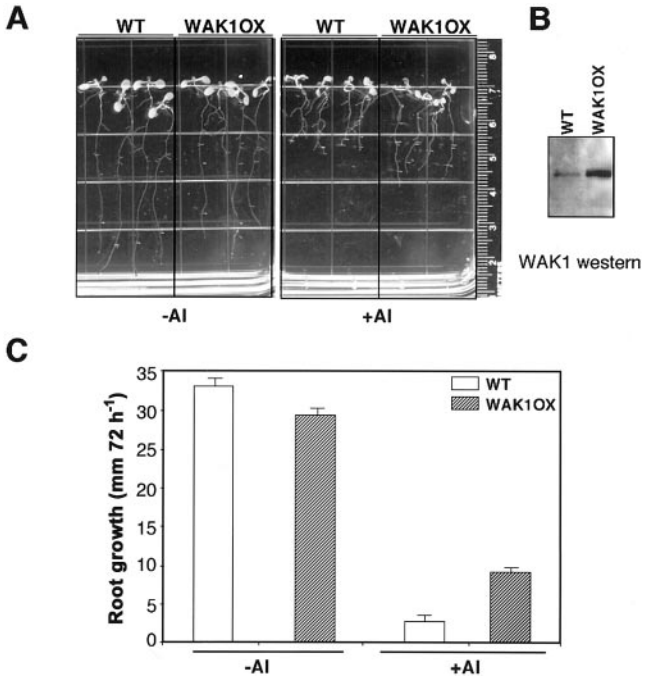

Figure 7. Overexpression of *WAK1* results in an enhanced Al tolerance. A, Responses of WT and *WAK1*-overexpressing seedlings (WAK1OX) to Al. Five-day-old seedlings were transplanted to plates with (+Al) or without (−Al) Al and continued to grow for another 72 h. B, Western-blot analysis of *WAK1* protein in WT and *WAK1*-overexpressing (WAK1OX) plants. Equal amount of total protein was fractionated and probed with the WAK antibody. C, Analysis of root growth of WT and *WAK1*-overexpressing plants (WAK1OX) in plates with (+Al) or without (−Al) Al as shown in A. Root lengths were around 10 mm (average) at the time of transfer to Al plates, and the values in C reflect root growth in the 72 h after the transplantation. Values are mean ± SE of at least 30 seedlings in three independent replicate plates.

beet protoplasts (Lee et al., 2001), suggesting that a delicate balance between cytoplasmic pushing forces and mechanical resistance of the cell periphery complex are disturbed in Al-exposed pollen tube apices.

PM-associated callose synthesis is the alternative to cellulose synthesis (Jacob and Northcote, 1985; Kauss, 1996) and typically is associated with the depletion of dense cortical MTs underlying the PM (Baluška et al., 2000). Callose can be rapidly induced in response to a wide range of physical and environmental factors that affect stability and integrity of the PM, ranging from glutaraldehyde fixation (Hughes and Gunning, 1980) via detergent treatments (Li et al., 1997), plasmolysis (Baluška et al., 1999), mechanical stress (Takahashi and Jaffe, 1984), wounding (Galway and McCully, 1987), to pathogen attack (Hussey et al., 1992; Rodriguez-Galvez and Mendgen, 1995; Škalamera and Heath, 1996). It is imperative for further studies to assess possible roles of WAKs in coping with all these stresses affecting primarily the integrity of the PM and the plasma membrane-cell wall interface.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis ecotype Landsberg *erecta* (Ler-0) seeds, sterilized using commercial bleach, were incubated at 4°C for 4 d and grown for 7 d on the nylon membrane described previously (Ezaki et al., 2000). The plants were grown under fluorescent illumination (approximately 50 μmol m^{-2} s^{-1}, 16 h of light and 8 h of darkness) at 22°C. A diluted (one-sixth) Murashige and Skoog medium (Murashige and Skoog, 1962), adjusted to pH 4.0 consisting of 10 g L^{-1} Suc, was used for plant growth and Al sensitivity tests.

Al Treatments and Growth Measurements

Seedlings with 2- to 3-cm root length were removed from the nylon membrane and subjected to one-sixth Murashige and Skoog containing 100 μM Al or lanthanum (chloride salts) for 9 h (approximately 60 seedlings per 120 mL of medium). Some portions of Al-treated plants were removed from Al medium after designated time points (0, 15, 30, 45, 60, and 240 min for short-term Al treatment and after every 3 h until 12 h for long-term Al treatment), washed profusely in distilled water, and stored at −80°C until extraction of total RNA or proteins. Root growth rate was measured at 3-h intervals during the 12-h Al treatment (100 μM). For root growth measurements (for Fig. 1), six to 10 plants were removed from the treatment solution, and after determining the initial root lengths, the root growth was measured at 3-h intervals using an Olympus stereomicroscope (SZ 60, Olympus Company Ltd., Tokyo) under 20× magnification.

For root growth analyses of the *WAK1* overexpression transgenic lines, seeds were surface sterilized, cold treated for more than 48 h, and germinated in plates containing a growth medium as described above without Al. After 5 d, seedlings were transferred to the same medium that contains Al, and the plates were placed vertically in a growth chamber at 22°C with 14 h of light (200 μmol m^{-2} s^{-1}). Root growth was measured by marking the positions of the root tips every 24 h. Root length measurements were made as described previously (Lally et al., 2001). Root images were recorded using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using the National Institutes of Health ImageJ 1.29 software (free download from http://rsb.info.nih.gov/ij/).

Determination of *WAK1* Gene Expression by RT-PCR

Total RNA was extracted from root and shoot (stem and leaves) regions separately or from whole plants after Al treatments using an extraction reagent (TRIZOL Reagent, GIBCO-BRL, Rockville, MD). First strand cDNA was synthesized in a 25-μL reaction containing approximately 2 μg of total RNA using Superscript Pre-amplification System for First Strand cDNA Synthesis (Life Technologies, Inc., Rockville, MD) and oligo(dT) 12-18 as a primer. Using the synthesized first strand cDNA as a template and two primers derived from the *WAK1* gene (5′-CTACATCAGGTCGCATCAAGC-3′ and 5′-TCATAGT-GGTGGTATCTAAGCGG-3′) as primers, PCR with different cycles (25, 30, and 35 cycles) was performed. The PCR products were applied to 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The strength of fluorescent signal derived from ethidium bromide in each lane was determined using the National Institutes of Health Image analysis program (version 1.57). By using the two primers derived from the β-tubulin gene (Knight et al., 1999), tubulin forward (5′-CCTGATAACTTCGTCTTTGG-3′) and tubulin reverse (5′-GTGAACTCCATCTCG-TCCAT-3′) as primers, PCR with different cycles (20, 25, and 30) was also performed to normalize the amount of first strand cDNA.

Localization of MTs and WAK Proteins Using Indirect Immunofluorescence

After experimental treatments, the whole Arabidopsis plants were handled as described elsewhere (Harper et al., 1996; Sivaguru et al., 1999). In brief, the plants were transferred into 5 mL of a buffer (50 mM PIPES, 5 mM EGTA, and 5 mM $MgSO_4$ [pH 6.9]) containing 5% (v/v) dimethyl sulfoxide for 15 min at room temperature. Afterward, they were fixed with 4% (w/v) paraformaldehyde in the above buffer containing 10% (v/v) dimethyl sulfoxide for 60 min at 20°C with initial 10 min under vacuum. After three rinses (10 min each) in phosphate-buffered saline (PBS, pH 7.4) to facilitate

tubulin antibody penetration, the fixed intact plants were digested with an enzymatic cocktail (1% [w/v] Hemicellulase [from *Aspergillus niger*, Sigma-Aldrich, Tokyo], 2% [w/v] Pectolyase [Seishin Corporation, Tokyo], 0.5 M EGTA, 0.4 M mannitol, 1% [v/v] Triton X-100, and 0.3 mM phenylmethylsulfonyl fluoride, all dissolved in the above buffer) for 60 min in an rotary shaker. The digestion reaction was terminated by transferring the plants to the above buffer for 15 min followed by 1% (v/v) Triton X-100 in the same buffer for 10 min. After a brief rinse in the buffer, plants were incubated with mouse monoclonal antibody raised against chick brain α-tubulin (Amersham, Buckinghamshire, UK) and diluted 1:200 (v/v) in PBS for 12 h at room temperature in darkness. After a further rinse in the buffer, they were incubated with rabbit polyclonal antibody raised against Arabidopsis WAK1 (He et al., 1996) and diluted 1:100 (v/v) in PBS for 12 h at room temperature. After double labeling with two primary antibodies, the plants were further incubated in fluorescein isothiocyanate-conjugated anti-mouse IgG raised in goat (Sigma, St. Louis) and tetraethylrhodamine isothiocyanate-conjugated anti rabbit IgG raised in goat, both diluted 1:200 (v/v) in PBS, for an additional 12 h each in complete darkness.

A set of plants incubated only with secondary antibodies served as negative controls. The double-labeled whole seedlings including leaves were treated with 0.01% (w/v) Toluidine Blue to diminish the natural autofluorescence of plant tissues. The labeled plants were mounted in 0.2 M Tris (pH 8.5) and 10% (v/v) glycerol containing Mowiol (Calbiochem, La Jolla, CA) to avoid fluorescence fading.

Localization of MTs and WAKs Using Confocal Laser Scanning Microscopy

The images of MTs from roots and leaf stomatal guard cells were obtained using the 488-nm excitation line (long pass 560) of an argon laser, and the tetramethylshortamine-isothiocyanate fluorescence of WAKs was imaged using the 543-nm excitation line of the He-Ne laser in a Zeiss microscope (Axioplan II) coupled with the confocal module (LSM 510, Carl Zeiss, Oberkochen, Germany). The images obtained from different distances from tip positions of the root surface and shoots/leaves from at least 10 replicate plants in a treatment, using the Ph3-Plan-Neofluar 100× oil immersion (1.3 numerital aperture) objective were documented systematically. The root surface confocal images were the overlay of seven to 11 optical sections (1.0 μm thick) of roots from the surface. In the case of leaves, stem, and root hairs, the top 5 to 10 μm was optically sectioned at 0.55-μm intervals, and the composite images were displayed. By using the reuse mode of the LSM software, uniform scanning parameters such as pinhole, amplifier offset, and gain were used for both MTs and WAK images and between treatments to facilitate intensity comparisons. All images stored permanently in the computer hard disc in RGB format were organized using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Protein Extraction and Western-Blot Analysis

Total proteins for tissue-specific western blots were extracted according to the method of He et al. (1996). Tissue samples were ground in extraction buffer (50 mM Tris-HCl [pH 6.8], 50 mM dithiothreitol, 4% [w/v] SDS, 10% [w/v] glycerol, and 1% [w/v] polyvinylpolypyrrolidone) in microcentrifuge tubes using plastic pestles. The homogenates were boiled for 5 min to release cell wall-bound proteins, followed by centrifugation at 3,000*g* to recover the proteins. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Total protein (60 μg) was fractionated by 12.5% (w/v) SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane using a semidry blotting apparatus at 2 mA constant current cm^{-1} of membrane for 2 h at 4°C, blocked with 2% (v/v) blocking reagent (Amersham) in PBS (10 mM phosphate buffer [pH 7.4] containing 138 mM NaCl and 2.7 mM KCl) for 1 h, and then incubated with WAK polyclonal antibody at a dilution of 1:500 (v/v) in PBS for 1 h at room temperature. After three washes in PBS (15 min each), the membrane was incubated with a goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) at a dilution of 1:3,000 (v/v) in PBS for 1 h at room temperature. After further washing (3 × 15 min) in PBS, the antigen-antibody complex was visualized by a chemiluminescence system according to the manufacturer's protocol (Amersham).

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