Involvement of a Cell Wall-Associated Kinase, WAKL4, in Arabidopsis Mineral Responses[1][W]

Xuewen Hou, Hongyun Tong, Jessie Selby[2], Jane DeWitt, Xinxiang Peng[3], and Zheng-Hui He*

Department of Biology (X.H., H.T., J.S., X.P., Z.-H.H.) and Department of Chemistry and Biochemistry (J.D.W.), San Francisco State University, San Francisco, California 94132

The cell wall-associated receptor kinase (WAK) and WAK-like kinase (WAKL) gene family members are good candidates for physical linkers that signal between the cell wall and the cytoplasmic compartment. Previous studies have suggested that while some WAK/WAKL members play a role in bacterial pathogen and heavy-metal aluminum responses, others are involved in cell elongation and plant development. Here, we report a functional role for the WAKL4 gene in Arabidopsis (*Arabidopsis thaliana*) mineral responses. Confocal microscopic studies localized WAKL4-green fluorescent protein fusion proteins on the cell surfaces suggesting that, like other WAK/WAKL proteins, WAKL4 protein is associated with the cell wall. Histochemical analyses of the WAKL4 promoter fused with the β-glucuronidase reporter gene have shown that WAKL4 expression is induced by Na^+, K^+, Cu^{2+}, Ni^{2+}, and Zn^{2+}. A transgenic line with a T-DNA insertion at 40-bp upstream of the WAKL4 start codon was characterized. While the T-DNA insertion had little effect on the WAKL4 transcript levels under normal growth conditions, it significantly altered the expression patterns of WAKL4 under various conditions of mineral nutrients. Semiquantitative and quantitative reverse transcription-PCR analyses showed that the promoter impairment abolished WAKL4-induced expression by Na^+, K^+, Cu^{2+}, and Zn^{2+}, but not by Ni^{2+}. Whereas the WAKL4 promoter impairment resulted in hypersensitivity to K^+, Na^+, Cu^{2+}, and Zn^{2+}, it conferred a better tolerance to toxic levels of the Ni^{2+} heavy metal. WAKL4 was required for the up-regulation of zinc transporter genes during zinc deficiency, and the WAKL4 T-DNA insertion resulted in a reduction of Zn^{2+} accumulation in shoots. A WAKL4-green fluorescent protein fusion gene driven by either the WAKL4 native promoter or the 35S constitutive promoter complemented the phenotypes. Our results suggest versatile roles for WAKL4 in Arabidopsis mineral nutrition responses.

Understanding the cellular and molecular mechanisms of how plant cells monitor and respond to external signals is fundamentally important. Members of the receptor-like kinase (RLK) superfamily are transmembrane proteins that perceive stimuli by their extracellular domains and transmit the signals via their cytoplasmic kinase domains (Shiu and Bleecker, 2001; Morris and Walker, 2003). More than 600 RLKs were identified in Arabidopsis (*Arabidopsis thaliana*), and recent studies have suggested that RLKs are ubiquitous throughout the plant kingdom (Verica and He, 2002; Shiu et al., 2004). While the precise functional roles for most of the RLKs are currently not quite defined, researchers have been able to assign specific signaling functions to several extensively studied members (Torii, 2004). Known functions for these RLKs include phytohormone responses (Chang et al., 1993; Li and Chory, 1997; Clark et al., 1998; Gamble et al., 1998), developmental controls (Becraft et al., 1996; Clark et al., 1997; Yokoyama et al., 1998; Jinn et al., 2000), reproduction regulations (Stein et al., 1991; Mu et al., 1994; Takasaki et al., 2000), nodulations (Endre et al., 2002; Nishimura et al., 2002; Stracke et al., 2002), and pathogen resistance responses (Loh and Martin, 1995; Song et al., 1995). The cell wall-associated kinase (WAK) and the WAK-like kinase (WAKL) represent a unique RLK subfamily whose 26 members are excellent candidates for signaling molecules that directly link and communicate between the cell wall and the cytoplasm (He et al., 1996; Kohorn, 2000; Verica et al., 2003). Prominent unique features for WAK/WAKL members include their epidermal growth factor-like repeats in their extracellular domains and their tight associations with the cell wall (He et al., 1996; Verica et al., 2003). Previous studies have shown that various WAK/WAKL members are involved in biotic and abiotic stress responses and are required for cell elongation and development (He et al., 1996; Lally et al., 2001; Wagner and Kohorn, 2001). Expression of WAK1 can be induced by bacterial pathogens and the induced expression is required for Arabidopsis to survive during pathogenesis (He et al., 1998). WAK1 is an aluminum early responsive gene and its overexpression results in aluminum tolerance (Sivaguru et al., 2003).

[1] This work was supported by the National Institutes of Health (grant no. MBRS SCORE 5 SO6 GM 52533) and by the National Science Foundation (grant no. MCB 9985135).

[2] Present address: Plant Biology Graduate Group, 1002 Life Sciences Addition, University of California, Davis, CA 95616.

[3] Present address: Laboratory of Molecular Plant Physiology, South China Agricultural University, Guangzhou 510642, China.

* Corresponding author; e-mail zhe@sfsu.edu; fax 415–338–2938.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Zheng-Hui He (zhe@sfsu.edu).

[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.066910.

Despite the suggested broad involvement of WAK/WAKL members in plant functions, very little is known about the nature of their ligand binding and the mechanism of their intracellular signal propagation. Biochemical analyses have shown that the extracellular domain of WAK1 binds to cell wall carbohydrate components such as pectins (Wagner and Kohorn, 2001; Decreux and Messiaen, 2005) and cell wall proteins such as the Gly-rich protein, AtGRP-3 (Park et al., 2001). The cytoplasmic domain of WAK1 physically interacts with a type 2C protein phosphatase, KAPP (Anderson et al., 2001). Together with the cytoplasmic KAPP and the extracellular AtGRP3, WAK1 can form a 500-kD protein complex (Park et al., 2001). Recent detailed in vitro studies suggested that the WAK1 extracellular domain (amino acids 67–254), excluding the epidermal growth factor-like repeats, is able to interact with cell wall pectins and the interactions were completely dependent on calcium and ionic conditions that favor the formations of calcium bridges between the pectin oligos and polymers (Decreux and Messiaen, 2005). Cell surface localization and cell wall association for a WAKL member, WAKL6, was also recently confirmed (Verica et al., 2003). Whether and how these associations play roles in signaling between the cell wall and the intracellular compartment are yet to be determined.

Little is known about the functional roles of RLKs in plant root signaling, although various RLKs, including some WAK/WAKL members, are known to be expressed in root tissues (He et al., 1999; Verica et al., 2003, Torii, 2004). Soil abiotic factors, such as availability of mineral nutrients and water, are fundamentally important to plant development and survival (López-Bucio et al., 2003). For example, mineral nutrients, essential elements absorbed by plant root systems in the form of inorganic ions, and their available levels can serve as signals that must be perceived by plants (Clemens, 2001). For most of the mineral nutrients, however, the molecular mechanisms of how exactly the signals are perceived and transduced are currently not well understood. Gene expression studies have suggested that expression of WAKL1-7 is developmentally regulated and tissue specific (Verica et al., 2003). While WAK1-5 genes are largely expressed in green tissues (He et al., 1999), WAKL1-7 genes are highly expressed in roots (Verica et al., 2003). To gain further insight into how WAKL root expression profiles are coupled with their functional roles during plant growth and development, we have further analyzed WAKL4 expression under various mineral nutrient conditions. We report here that WAKL4 expression is highly responsive to various mineral nutrients, including Na^+, K^+, Cu^{2+}, Ni^{2+}, and Zn^{2+}. A WAKL4 T-DNA insertional mutant was isolated and characterized and its phenotypic analysis confirmed an important involvement for WAKL4 in root mineral nutrient responses. This study reports on RLK function in root mineral responses.

RESULTS

WAKL4 Is Localized on the Cell Surface

Previous cellular and biochemical studies have shown that several WAK/WAKL members are localized on the cell surface and tightly associated with the cell wall (He et al., 1996; Verica et al., 2003). The predicted structural features for WAKL4 protein suggest it may also be associated with the cell wall. To test this possibility, we generated transgenic plants that express WAKL4-green fluorescent protein (GFP) fusion proteins. As shown in Figure 1, protein gel-blot

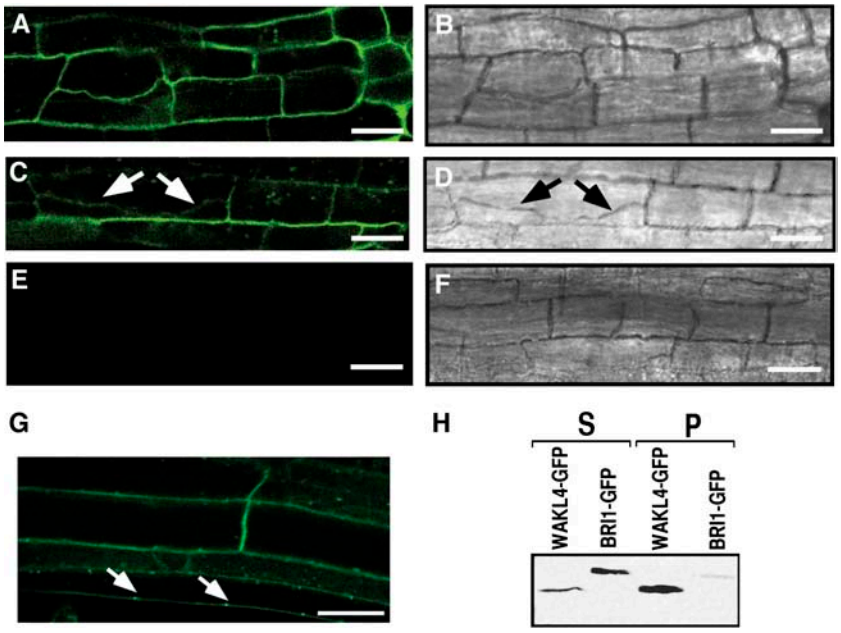

Figure 1. Detection of WAKL4-GFP protein in transgenic plants (in *wakl4-1* background) expressing 35S::WAKL4-GFP. A and C, Confocal microscopic images of GFP fluorescence of the WAKL4-GFP fusion protein in the root epidermal cells of 35S:WALK4-GFP transgenic plants before (A) and after (C) plasmolysis. Arrows indicate plasmolysis occurrence after 0.5 M NaCl treatment. E, Wild-type control. B, D, and F, Bright-field images of the cells shown in A, C, and E, respectively. Bars, 20 μm. G, Closer view of patch-like distribution of WAKL4-GFP fusion proteins on the cell surfaces. Arrows indicate the patch-like patterns. Bar, 20 μm. H, Protein gel-blot analysis of the WAKL4-GFP fusion protein. Total soluble (S) and insoluble (P) proteins were prepared from seedlings of both 35S::WAKL4-GFP (WAKL4-GFP) and BRI1::BRI1-GFP (BRI1-GFP; Nam and Li, 2002) and analyzed with a GFP antibody.

analysis detected high levels of the GFP fusion proteins in the transgenic plants (Fig. 1H). Confocal microscopic analyses of the root cells of the transgenic plants confirmed that GFP fluorescence is localized in the cell surface region overlapping the cell wall and the plasma membrane (Fig. 1A). Closer examination of the root cell expressing WAKL4-GFP revealed that the fusion proteins are forming patch-like structures on the cell surface (Fig. 1G). To further verify the nature of the cell wall association of the WAKL4-GFP proteins, roots cells were plasmolyzed in a 0.5 M NaCl solution (Fig. 1D) and the GFP fluorescence was examined. While the majority of the green fluorescence remained in the cell wall region, a small amount migrated together to the plasma membrane. To biochemically confirm the nature of WAKL4-GFP cell wall association, soluble and insoluble proteins were fractionated from the WAKL4-GFP transgenic plants and analyzed by protein gel blotting using a GFP antibody. WAKL4-GFP, shown as a band of 102 kD, was highly enriched in the insoluble pellet (Fig. 1H). Extraction of WAKL4-GFP from the insoluble fraction, like that of WAK1 (He et al., 1996), required boiling in 50 mM dithiothreitol (DTT) and 4% (w/v) SDS. BRI1-GFP, a known plasma membrane protein, was used as a control (Nam and Li, 2002). As expected, BRI1-GFP, shown as a band of 158 kD, was highly enriched in the soluble protein fraction (Fig. 1H). These results suggest WAKL4, like other studied WAK/WAKL members, is a plasma membrane RLK tightly associated with the cell wall.

WAKL4 Expression in Responding to Mineral Nutrients

Prior analyses showed WAKL4 was slightly expressed in the leaf hydathode, stipule, hypocotyl-root junction, and lateral root initiation site (Verica et al., 2003). To gain further insight into the expression profile of the WAKL4 gene in responding to environmental stresses, transgenic plants carrying a construct of the WAKL4 promoter fused to the β-glucuronidase (GUS) reporter gene were analyzed. Seedlings were grown in either 0.1× Murashige and Skoog (MS) medium, as a control, or 0.1× MS medium supplemented with various additional mineral nutrients. As shown in Figure 2, WAKL4::GUS activities strongly responded to elevated levels of Na^+, K^+, Cu^{2+}, Ni^{2+}, and Zn^{2+}. Under the 0.1× MS growth condition, the WAKL4 promoter is slightly active in the vasculature of the leaf petiole and hypocotyl (Fig. 2A, a–d) and little GUS activity was observed in roots (Fig. 2A, e–g). WAKL4::GUS activity in the vasculature was greatly enhanced by all five added mineral nutrients. High levels of GUS activity were observed in vascular tissues of seedlings grown in media with 100 mM NaCl (Fig. 2B, a and c), 40 μM $NiSO_4$ (Fig. 2E, a, c, and d), and 150 μM $ZnSO_4$ (Fig. 2F, a, c, and d). In particular, heavy GUS staining was observed in the vascular tissues of hypocotyls in seedlings grown in media with either nickel or zinc (Fig. 2E, d, and F, d). WAKL4 promoter activity was also greatly induced in hypocotyl-root junctions by all five added mineral nutrients (Fig. 2, B–F, e). In addition, all nutrients, but

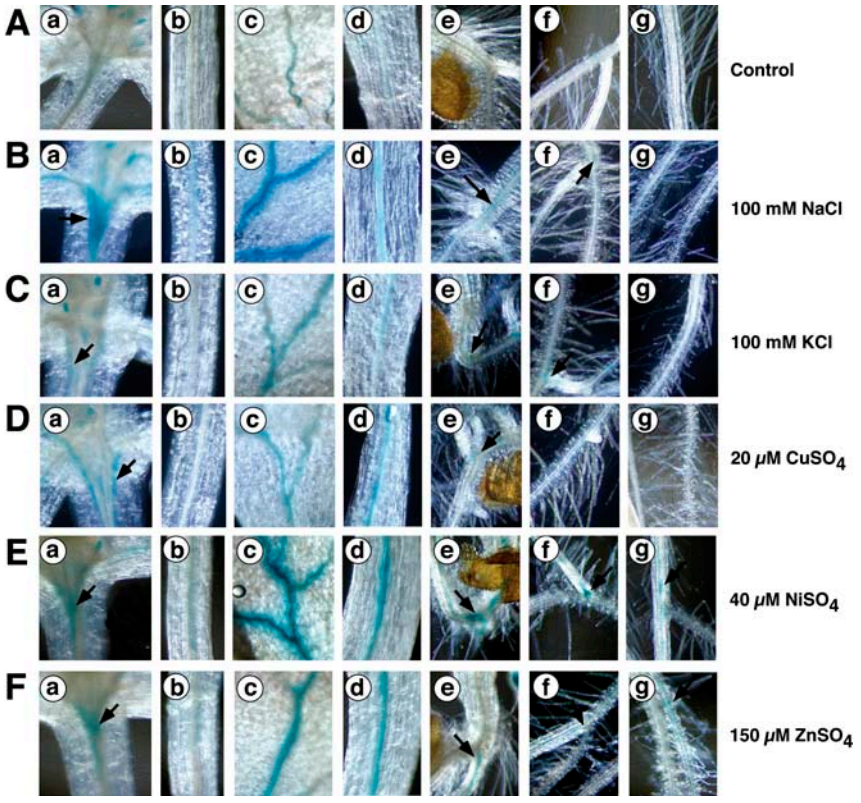

Figure 2. WAKL4 expression in responding to various inorganic minerals and heavy metals. Histochemical staining for transgenic WAKL4::GUS seedlings treated without (A) or with (B–F) various inorganic elements is shown. Eight-day-old seedlings were transferred to MS medium alone (A) or MS medium containing 100 mM NaCl (B), 100 mM KCl (C), 20 μM $CuSO_4$ (D), 40 μM $NiSO_4$ (E), and 150 μM $ZnSO_4$ (F), respectively. After the transfer, seedlings were allowed to grow for an additional 3 d on the designated medium before being harvested for assays. 5-Bromo-4-chloro-3-indolyl β-D-glucuronide staining for the apical meristem/cotyledon (a), leaf petiole (b), leaf vasculature (c), hypocotyl (d), shoot-root junction (e), lateral root initiation (f), and primary roots (g) is shown.

copper, significantly enhanced WAKL4::GUS activity at the lateral root initiation sites (Fig. 2, B–F, f). Similar to that in the hypocotyls (Fig. 2E, d, and F, d), nickel or zinc treatment resulted in a higher GUS expression in the vasculature of the roots (Fig. 2E, f, and F, f).

A comprehensive analysis of publicly available microarray data was carried out. WAKL4 (At1g16150) expression profiles were obtained through GENEVESTIGATOR (Zimmermann et al., 2004) by analyzing data from experiments of more than 1,800 microarray chips and were compared to our GUS expression results. WAKL4 expression patterns in different organs and under various biotic and abiotic stresses revealed by the microarray data were consistent with our GUS results (Fig. 1; Supplemental Fig. 3). Microarray results confirmed that the highest expression of WAKL4 was found in roots and that salt (NaCl) stress significantly induced WAKL4 expression. As much as a 5-fold increase of the WAKL4 transcript was found in salt-treated seedlings (Supplemental Fig. 3). Consistent with our observations, the salt-induced expression seems to be related to elevated minerals and not to general water deprivation as various drought and osmotic stress conditions failed to induce WAKL4 expression (Supplemental Fig. 3).

Impairment of the WAKL4 Promoter by a T-DNA Insertion

To genetically analyze the functions of WAKL4, we attempted to isolate WAKL4 knockout mutants from a number of available collections of T-DNA transgenic plants. Using a primer 1,490-bp upstream of WAKL4 and a T-DNA left-border primer, a PCR screening in a DNA superpool (Arabidopsis Biological Resource Center [ABRC] stock no. CS6503) identified a line with a T-DNA inserted in the promoter region proximal to the beginning of the WAKL4 gene. Sequencing analysis confirmed a T-DNA insertion at 40-bp upstream of the ATG start codon, as illustrated in Figure 3A. This insertional line is hereafter designated as *wakl4-1*. Genomic DNA gel-blot and genetic segregation analyses suggested that the obtained line carried a homozygous mutation for WAKL4. DNA isolated from either the wild type (Wassilewskija [Ws]) or the *wakl4-1* were digested with *Eco*RI and fractionated. A WAKL4-specific fragment used to probe the genomic DNA blot identified one fragment (5.6 kb) in the wild-type lane and two fragments (4.7 and 5.7 kb) in the *wakl4-1* lane (Fig. 3B), suggesting that the T-DNA insertion resulted in homozygous WAKL4 interruption. WAKL4 is part of the WAKL1-7 gene cluster (Verica et al., 2003). Specific reverse transcription (RT)-PCR analyses of WAKL1-7 showed that the T-DNA insertion had little effect on the expression of other clustered WAKL genes (data not shown).

To understand the regulatory mechanisms of WAKL4 gene expression and to verify the functional roles for WAKL4 protein, two transformation constructs, WAKL4::WAKL4-GFP and 35S::WAKL4-GFP,

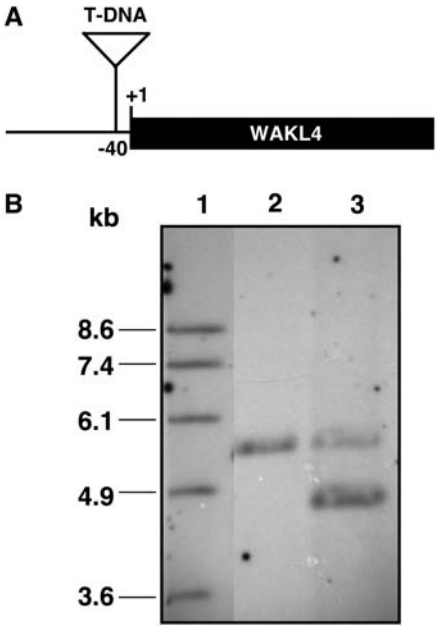

Figure 3. Characterization of a WAKL4 T-DNA insertional line. A, Diagram showing the position of the T-DNA insertion. Sequence analysis confirmed a T-DNA insertion at 40-bp (−40) upstream of the ATG start codon (+1) of the WAKL4 gene. B, Genomic Southern analysis of the WAKL4 T-DNA line. Fifty micrograms of genomic DNA from either the wild-type (lane 2) or the T-DNA line (lane 3) were digested with *Eco*RI and probed with a WAKL4 probe. The DNA M_r ladder is shown in lane 1.

were generated and utilized to transform *wakl4-1* plants. More than 30 independent transgenic lines were obtained for each construct and representative lines were used for detailed molecular characterizations. Seeds for wild-type, *wakl4-1*, WAKL4::WAKL4-GFP, and 35S::WAKL4-GFP transgenic plants were plated in either 0.1× MS medium or 0.1× MS medium supplemented with 50 mM NaCl, 50 mM KCl, 150 μM $ZnSO_4$, 18 μM $CuSO_4$, and 26 μM $NiSO_4$, respectively. Fourteen-day-old seedlings were harvested for total RNA extraction and, subsequently, for RT-PCR analyses. A pair of gene-specific primers was designed in a unique region spanning the two introns of the WAKL4 gene and their WAKL4 specificity was confirmed by sequencing their RT-PCR products (Verica et al., 2003). As shown in Figure 4, consistent with our previously established GUS expression profile (Fig. 2), WAKL4 expression was significantly up-regulated by 50 mM NaCl, 50 mM KCl, and 150 μM $ZnSO_4$. A constitutive actin gene was used to normalize the WAKL4 transcript levels in each sample and the WAKL4 transcript changes were calculated based on that of the wild type grown on MS medium (designated as 1; Fig. 4, bottom). Compared to the wild-type control, both the 35S::WAKL4-GFP and the WAKL4::WAKL4-GFP transgenic plants gave more than 9-fold higher WAKL4 transcripts. While the WAKL4 transcript level in *wakl4-1* slightly dropped in MS medium, it was significantly reduced in medium containing either 50 mM NaCl or 50 mM KCl.

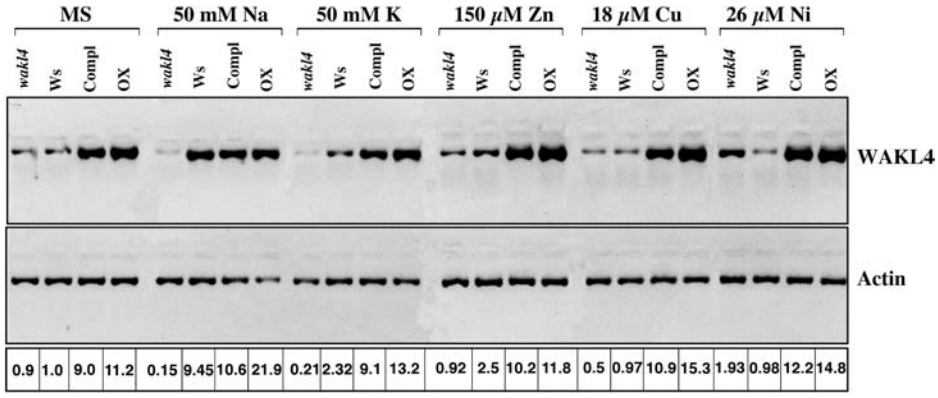

Figure 4. RT-PCR analyses of WAKL4 expression in responding to various mineral nutrients. A gene-specific primer pair was used to analyze WAKL4 transcript levels in seedlings treated without (MS) or with (50 mM NaCl, 50 mM KCl, 150 μM $ZnSO_4$, 18 μM $CuSO_4$, and 26 μM $NiSO_4$) various inorganic ions as indicated on the top. Analyzed plant samples include a T-DNA line (*wakl4-1*), wild type (Ws), WAKL4::WAKL4-GFP (Compl), and a 35S::WAKL4-GFP (OX). The Actin gene was used as a control. Relative WAKL4 transcript levels under various treatments were first normalized against their corresponding actin levels and the calculated changes of WAKL4 transcripts as compared to that of the wild type grown on MS medium (set as 1) are shown at the bottom.

In contrast to other nutrients, nickel has an opposite effect on WAKL4 expression in *wakl4-1*. RT-PCR analysis detected a noticeable increase of the WAKL4 transcript in *wakl4-1* grown in MS medium with nickel (Fig. 4). This observation was confirmed by several independently repeated RT-PCR experiments. To further verify this unexpected result, quantitative real-time RT-PCR analyses were used to determine WAKL4 transcript abundance in samples grown in MS medium with nickel. A constitutive actin gene was included in the same tube real-time RT-PCR assay for normalization purposes and changes of the WAKL4 transcript abundance were calculated based on the level of that in the wild type (Ws) as 1. As shown in Figure 5, the WAKL4 transcript level in the *wakl4-1* mutant grown in the presence of nickel showed a 100% increase when compared to that in the wild type. It also confirmed high levels of WAKL4 transcripts of as much as a 20-fold increase in the 35S overexpression lines (OX; Fig. 5), consistent with the results from the previous semiquantitative RT-PCR experiment (Fig. 4). As a positive control, Na^+ was shown to notably enhance WAKL4 transcript abundance in the wild type (Fig. 5).

wakl4-1 Is Hypersensitive to Various Mineral Nutrients

The results of WAKL4 expression studies strongly suggest a functional role for WAKL4 in mineral nutrient responses. We initially observed that *wakl4-1* seedlings consistently showed retarded root growth when grown in full- or one-half-strength MS nutrients. As shown in Figure 6, while little root length differences between the wild type and *wakl4-1* were observed on 0.1× MS medium, *wakl4-1* root lengths on 0.5× MS were reduced by more than 30% as compared to the wild type.The root length reduction phenotype was complemented when a wild-type copy of the WAKL4 gene in the form of either WAKL4::WAKL4-GFP (complementation line [Compl]) or 35S::WAKL4-GFP (OX) was introduced back into the *wakl4-1* plants (Fig. 6).

To further determine which mineral nutrient in MS medium caused *wakl4-1* root sensitivity, various nutrients were added into 0.1 × MS medium and tested for *wakl4-1* growth. As shown in Figure 7, both 50 mM KCl and 50 mM NaCl had a dramatic effect on *wakl4-1* root growth. Root lengths of *wakl4-1* seedlings were reduced by more than 50% by both salts. Both Compl and OX showed restoration of root growth, although the latter was less effective (Fig. 7).

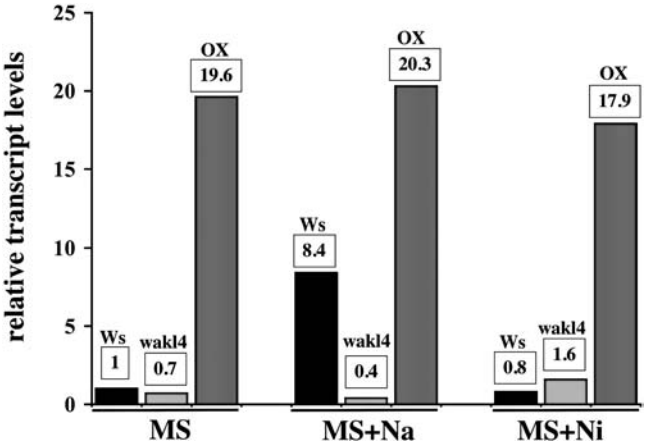

Figure 5. Quantitative real-time RT-PCR analyses of WAKL4 expression. Wild type (Ws), *wakl4-1* (wakl4), and WAKL4 (OX) were grown on regular MS medium (MS), MS supplemented with 50 mM NaCl (MS + Na), or MS supplemented with 26 μM $NiSO_4$ (MS + Ni), respectively. Shown on the top of each vertical bar is the relative WAKL4 transcript level of the indicated sample. All samples were normalized against an actin internal control and calculated based on the value of the wild type (on MS medium) as 1. Values are the average of three independent measurements.

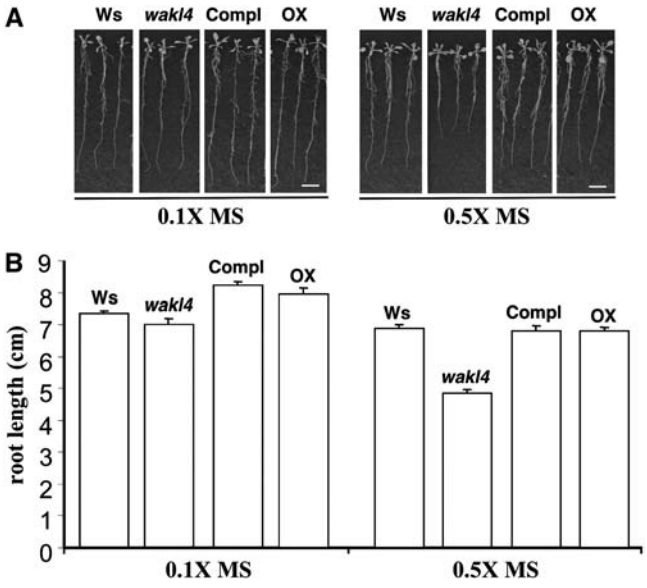

Figure 6. Effect of mineral nutrients on *wakl4-1* root elongation. Seeds were plated on media containing either one-tenth (0.1 × MS) or one-half (0.5 × MS) strength of the MS salt. A, Images of 20-d-old seedlings of wild type (Ws), *wakl4-1* (*wakl4*), WAKL4::WAKL4-GFP (Compl), and 35S::WAKL4-GFP (OX). Bars, 1 cm. B, Root length comparison of seedlings growing in either 0.1 × or 0.5 × MS medium as shown in A. Each value plotted represents the mean of three independent experiments, with each experiment involving 25 seedlings. Error bars = SD.

Seedlings of *wakl4-1* were also tested on 0.1 × MS medium supplemented with either 150 μM $ZnSO_4$ or 18 μM $CuSO_4$. As shown in Figure 8, both Zn^{2+} and Cu^{2+} significantly affected root lengths of *wakl4-1* seedlings. Zn^{2+} and Cu^{2+} reduced *wakl4-1* root length by more than 50% and 30%, respectively (Fig. 8). The root length reductions were recovered in both the Compl and the OX lines (Fig. 8).

The Arabidopsis zinc transporter (ZIP) genes were known to be regulated by zinc availability (Grotz et al., 1998). To gain further insight into a possible role for WAKL4 in zinc response, we analyzed ZIP expressions in both root and shoot tissues of wild type, *wakl4-1*, Compl, and OX lines. Seedlings were grown in either 0.1 × MS or 0.1 × MS medium supplemented with 150 μM $ZnSO_4$ and used for total RNA extraction. Pairs of ZIP gene-specific primers were used for RT-PCR analyses. Figure 9A shows that all four ZIP genes were expressed in roots of the wild-type plant, and their expression responded to Zn^{2+} deficiency. Little expression of all ZIP genes but ZIP2 was found in roots when the growth medium was supplemented with 150 μM Zn^{2+} (Fig. 9A). In wild-type shoots, while ZIP1 and ZIP4 were suppressed in the 150 μM Zn^{2+} medium, ZIP2 was slightly induced under the same condition (Fig. 9). The Zn^{2+} deficiency-induced expression for all four ZIP genes appeared to be abolished in *wakl4-1*. When *wakl4-1* seedlings were grown on MS medium, transcripts for ZIP1, ZIP3, and ZIP4 became undetectable and the transcript of ZIP2 was dramatically reduced. Transcript levels for all four ZIP genes were completely recovered in both the WAKL4 Compl and the WAKL4 OX line in both roots and shoots (Fig. 9A).

Zinc content was measured in wild type, *wakl4-1*, Compl, and OX lines. Seedlings were grown on 0.1× MS medium supplemented with 150 μM $ZnSO_4$, and their shoots and roots were separated for Zn^{2+} determination. There was no significant difference in Zn^{2+} levels in roots of the four samples (Fig. 9B). In contrast, Zn^{2+} levels were dramatically reduced in *wakl4-1* shoots (Fig. 9B). This reduction was fully

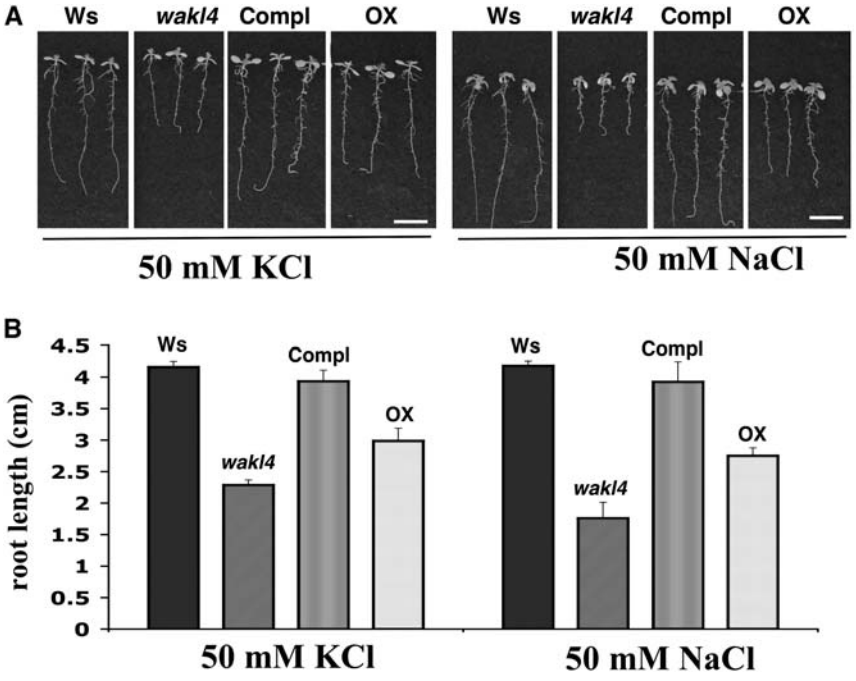

Figure 7. Hypersensitivity of *wakl4-1* to Na^+ and K^+. Seeds were plated on 0.1 × MS medium containing either 50 mM KCl or 50 mM NaCl. A, Images of 20-d-old seedlings of wild type (Ws), *wakl4-1* (*wakl4*), WAKL4::WAKL4-GFP (Compl), and 35S::WAKL4-GFP (OX). Bars, 1 cm. B, Root length measurements of seedlings growing in either 50 mM KCl or 50 mM NaCl, as shown in A. Each value represents the mean of three independent experiments, with each experiment involving 25 seedlings. Error bars = SD.

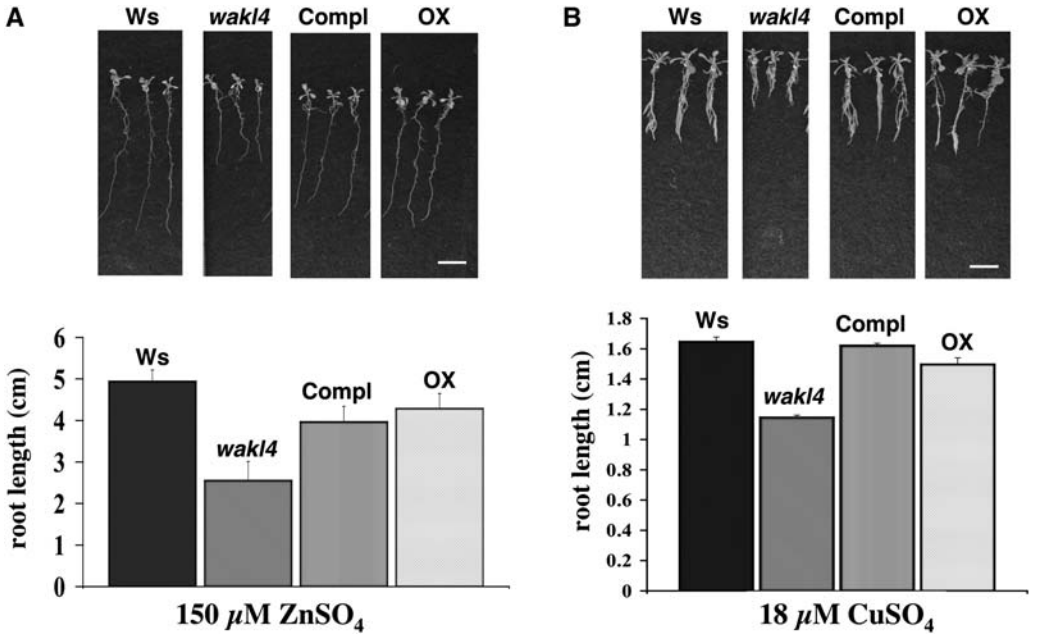

Figure 8. Hypersensitivity of the *wakl4-1* mutant to Zn^{2+} and Cu^{2+}. A and B, Seeds were plated on 0.1 × MS medium containing either 150 μM $ZnSO_4$ (A) or 18 μM $CuSO_4$ (B). Top, Images of 20-d-old seedlings of wild type (Ws), *wakl4-1* (*wakl4*), WAKL4::WAKL4-GFP (Compl), and 35S::WAKL4-GFP (OX) are shown. Bars, 1 cm. Bottom, Root length measurements of seedlings growing on either 150 μM $ZnSO_4$ or 18 μM $CuSO_4$, as indicated. Each value plotted represents the mean of three independent experiments, with each experiment involving 25 seedlings. Error bars = SD.

recovered in both the Compl and OX lines (Fig. 9B). In addition, the OX line contained a higher level of Zn^{2+} in both shoots and roots (Fig. 9B). On 0.1× MS medium supplemented with 150 μM $ZnSO_4$, wild-type roots accumulated twice the amount of Zn^{2+} as wild-type shoots (Fig. 9C). The calculated ratios of Zn^{2+} content in shoots versus in roots of the wild type indicated an average value of 0.5, whereas that of *wakl4-1* decreased to about 0.3 (Fig. 9C). These results suggest a likely role for WAKL4 in Zn^{2+} accumulation in shoots.

WAKL4 and Nickel Tolerance

A slightly increased WAKL4 expression level was detected in *wakl4-1* when treated with Ni^{2+}, and a constitutively enhanced WAKL4 expression was observed in the Compl plants (Figs. 4 and 5). To test

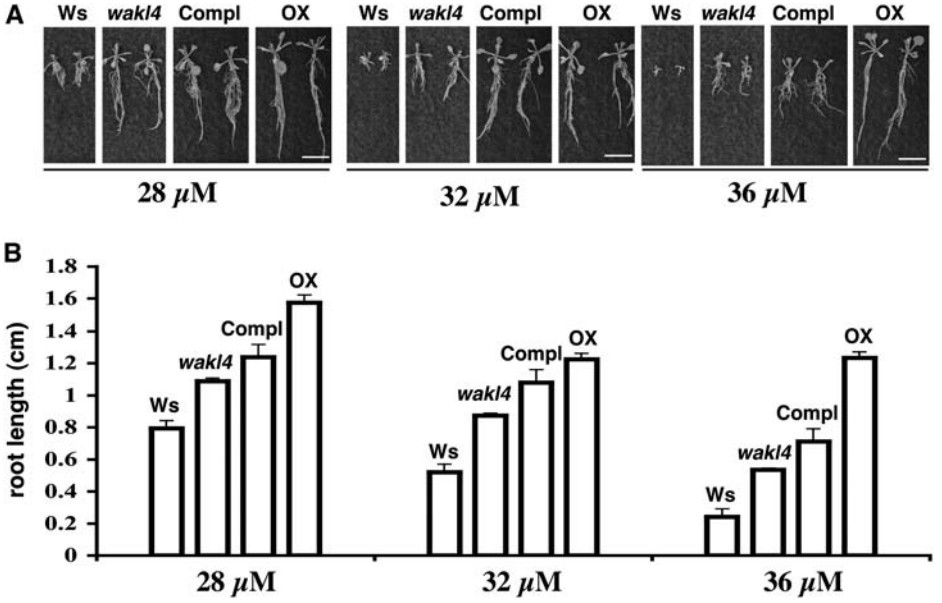

Figure 9. Nickel tolerance of *wakl4-1*. Seeds were plated on 0.1 × MS media containing various concentrations of $NiSO_4$ (28, 32, and 36 μM) as indicated. A, Images of 20-d-old seedlings of wild type (Ws), *wakl4-1* (*wakl4*), WAKL4::WAKL4-GFP (Compl), and 35S::WAKL4-GFP (OX). Bars, 1 cm. B, Root length measurements of seedlings growing under three different $NiSO_4$ concentrations, as shown in A. Each value plotted represents the mean of three independent experiments, with each experiment involving 25 seedlings. Error bars = SD.

whether the elevated expression in Ni^{2+} had any physiological effects on Arabidopsis growth, we plated wild type, *wakl4-1*, Compl, and OX on media containing various levels of Ni^{2+}. In general, Arabidopsis is considered to be nontolerant to Ni^{2+} (Clemens, 2001; Freeman et al., 2005). As shown in Figure 10, in the wild type, as little as 28 μM of Ni^{2+} caused a dramatic inhibition of root growth and 36 μM Ni^{2+} resulted in lethality. In contrast, seedlings of *wakl4-1*, Compl, and OX gave much better root growth than the wild type (Fig. 10A). Both the *wakl4-1* and the Compl plants survived well at 36 μM Ni^{2+}. OX plants consistently showed robust growth at the three Ni^{2+} concentrations. At 28 μM Ni^{2+}, root lengths of wild-type seedlings were about 50% of that of OX. Increased Ni^{2+} concentration had a dramatic effect on wild-type root length but not on OX. At 36 μM Ni^{2+}, root lengths of the wild type dropped to less than 20% of that of OX. These results established a good correlation between WAKL4 expression and the Ni^{2+} tolerance. Measurements of plant Ni^{2+} content showed no significant differences of Ni^{2+} levels in either shoot or root among wild-type, *wakl4-1*, Compl, and OX plants (data not shown), suggesting a possible role of WAKL4 in Ni^{2+} cellular detoxification rather than Ni^{2+} transportation or accumulation.

DISCUSSION

Understanding how plants acquire and use minerals has been fundamentally important to agricultural production and environmental protection. Plants have to constantly monitor and properly respond to the availability of mineral nutrients in soil. While too few minerals in soil hinder plant growth and productivity, excess levels of mineral nutrients can cause toxicity and even lethality to plants. The availability of the mineral nutrients can fundamentally change root architecture (López-Bucio et al., 2003). Minerals and their levels can serve as signals that can be perceived by plants. Due to extensive studies in plant nutrition in the last century, the physiological and biochemical roles for most of the mineral nutrients have been very well defined. For most of the mineral nutrients, however, the molecular mechanisms of how these signals are perceived and transduced are currently not well understood. This study provided evidence suggesting an important involvement of WAKL4 in plant mineral response. Expression of WAKL4 was induced by a number of mineral nutrients, including Na^+, K^+, Cu^{2+}, Ni^{2+}, and Zn^{2+}. While reduced WAKL4 expression resulted in hypersensitivity to Na^+, K^+, Cu^{2+}, and Zn^{2+}, WAKL4 overexpression provided Ni^{2+} tolerance.

WAKL4 Cell Wall Association

The WAKL4 gene is a member of the WAKL1-7 gene cluster (Verica et al., 2003). Previously, another member of the WAKL1-7 cluster, WAKL6, was shown to be localized to the cell wall (Verica et al., 2003). In this study, we have used a WAKL4-GFP fusion gene driven by either the WAKL4 native promoter or the 35S constitutive promoter to complement the *wakl4-1* mutant. Both constructs successfully complemented the

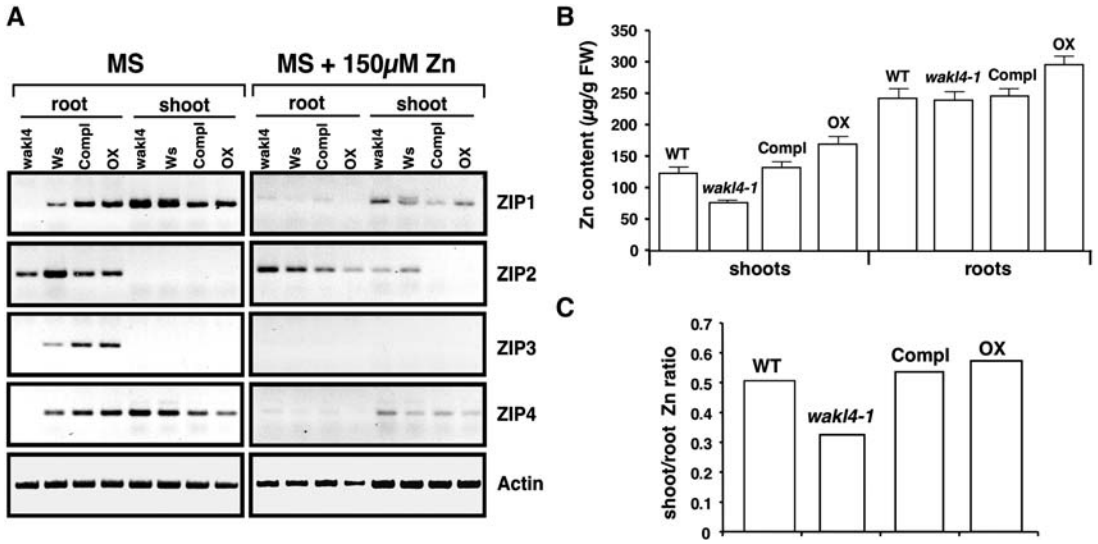

Figure 10. Differences in zinc transporter gene expression and zinc levels in *wakl4-1*. A, RT-PCR analyses of expression comparisons of four zinc transporter genes (ZIP1, ZIP2, ZIP3, and ZIP4) under low or high Zn^{2+} concentrations. Seedlings of *wakl4-1* (*wakl4*), wild type (Ws), WAKL4::WAKL4-GFP (Compl), and 35S::WAKL4-GFP (OX) were grown on either 0.1× MS or 0.1× MS supplemented with 150 μM $ZnSO_4$ (MS + 150 μM Zn^{2+}) and their roots and shoots were separated for RT-PCR analyses. Actin was used as a constitutive control. B, Measurements of Zn^{2+} levels in shoots and roots of wild type, *wakl4-1*, Compl, and OX, as indicated. Error bars = SD. C, Calculated Zn^{2+} content ratios of shoot versus root in the four indicated plant samples.

wakl4-1 mutant, suggesting that the WAKL4-GFP fusion protein is functionally compatible to WAKL4. Confocal microscopic analysis revealed that WAKL4 was localized to the cell surface areas. Under higher magnifications, WAKL4-GFP fluorescence can be observed as patch-like structures in the cell surface area. The mechanism and significance of this patch-like pattern formation on the cell surface is yet to be determined. Further plasmolysis observations confirmed localizations for WAKL4-GFP to both the plasma membrane and the cell wall. After plasmolysis treatment, while the majority of the GFP fluorescence was retained in the cell wall region, a visible amount of fluorescence was detected in the plasma membrane area. Predicted structural features of WAKL4 protein suggested WAKL4, like WAK1 and WAKL6, is likely localized on the cell surface (Verica et al., 2003). Like other typical WAK/WAKL members, WAKL4 has an N-terminal hydrophobic segment of about 24 amino acids predicted to be signal sequences that direct the protein to the endoplasmic reticulum and a transmembrane region characteristic of plasma membrane proteins. GFP fluorescence results in this study are consistent with the localization of WAKL4 in the cell wall.

Protein gel-blot experiments further confirmed the tight association of WAKL4 with the cell wall. Like WAK1 (He et al., 1996), WAKL4 could be efficiently extracted from the insoluble pellet by boiling for 10 min in a buffer containing 50 mM DTT and 4% SDS. The majority of WAKL4-GFP was found in the insoluble fraction (Fig. 1A). A small amount of WAKL4-GFP still remained in the soluble fraction, consistent with the confocal observation in plasmolysis. BRI1-GFP, a known plasma membrane receptor kinase, was largely fractionated with the soluble fraction. The dramatic differences between the fractionations of the two RLKs strongly support that WAKL4 is associated with the cell wall. While the majority of WAKL4 is tightly associated with the cell wall, a fraction of it could become relatively loosely associated, suggesting the association of WAKL4 with the cell wall could be dynamic. Other WAK/WAKL members, such as WAK1, are less tightly associated with the cell wall in young and developing organs when compared to that in older tissues (He et al., 1996; Anderson et al., 2001). The significance of the dynamic nature of cell wall association for the WAK/WAKL members is yet to be determined.

Tissue- and Mineral-Specific WAKL4 Expression

Our analyses suggested WAKL4 expression was mostly in roots and was responding to elevated levels of multiple mineral elements. Previous promoter-GUS analyses suggested WAKL1-7 genes were highly expressed in roots, whereas WAK1-5 genes were mainly expressed in green tissues (Verica et al., 2003). In analyzing WAKL4 promoter-GUS activities under various abiotic conditions, we further determined that the WAKL4 expression in roots strongly responds to mineral strength in the growth media. In our previous report, we used full-strength MS medium that gave among others about 100 μM Na^+, 20 mM K^+, 30 μM Zn^{2+}, and 160 nM Cu^{2+}. Under the full-strength MS, the WAKL4 promoter was active in root-shoot junctions and lateral root initiation sites (Verica et al., 2003). When 0.1× (one-tenth) strength of MS medium was used, the GUS staining in those areas was barely detectable (Fig. 1A, e–g). The 0.1× MS medium became a convenient condition to test WAKL4 responses to various minerals. Various minerals were added to 0.1× MS medium and tested for their effect on the WAKL4 promoter-GUS activity. The WAKL4 promoter can be strongly induced by five minerals, Na^+, K^+, Cu^{2+}, Ni^{2+}, and Zn^{2+} (Fig. 2, B–F). These inductions were mineral specific as a number of other minerals and heavy metals failed to induce WAKL4-GUS expression. For example, while both 100 mM NaCl and 100 mM KCl greatly enhanced WAKL4 promoter activities (Fig. 2, B and C), 100 mM LiCl had little effect (data not shown). Likewise, both 20 μM $CuSO_4$ and 40 μM $NiSO_4$ resulted in strong GUS staining while 40 μM $CdSO_4$ showed no effect. Furthermore, little GUS activity was detected when seedlings were grown on 0.1× MS medium supplemented with 100 μM mannitol. The mineral-specific expression profile strongly suggests WAKL4 may play a role in Arabidopsis mineral nutrient responses.

Analysis of publicly available microarray data provided further support for our discovered WAKL4 gene expression patterns. Experimental data from more than 1,800 microarray chips were carefully examined through GENEVESTIGATOR (Zimmermann et al., 2004). Consistent with our GUS data, WAKL4 was mostly expressed in root tissue (Supplemental Fig. 1). During development, the highest WAKL4 expression was found in 14- to 18-d-old seedlings (Supplemental Fig. 2). In addition, WAKL4 responded to a number of abiotic factors, including, most notably, salt stress. Salt stress-treated seedlings gave more than 5-fold of WAKL4 transcripts when compared to control (Supplemental Fig. 3). Interestingly, the microarray data from the osmotic and drought experiments confirmed the results derived from our WALK4 promoter-GUS studies (Fig. 2). Neither the osmotic treatments nor drought stresses had any noticeable effect on WAKL4 expression (Supplemental Fig. 3). The drought and salt stress share some common physiological responses, yet their cellular processes can be distinguished into specific pathways (Hasegawa et al., 2000). Our WAKL4 data suggest two processes may be uncoupled from each other.

WAKL4 Promoter Impairment

The isolated T-DNA line carried a T-DNA insertion in the WAKL4 promoter 40-bp upstream (−40) of the ATG start codon. Within the WAKL1-7 gene cluster, there are 1,159 bp between the TGA stop codon of

WAKL3 and the beginning of WAKL4 ATG start codon (Verica et al., 2003). The WAKL4 promoter region is predicted to be about 1 kb after excluding the 3′ untranslated region of WAKL3 and the 5′ untranslated region of WAKL4. Surprisingly the T-DNA insertion in the proximal region of the WAKL4 promoter failed to completely abolish WAKL4 transcription. In fact, under normal growth conditions, such as 0.1× MS medium, *wakl4-1* could still yield as much as 90% of the WAKL4 transcripts in the wild type (Figs. 4 and 5). As previously noted, WAKL4 expression can be strongly induced by minerals such as Na^+, K^+, and Zn^{2+} (Figs. 4 and 5). For example, our quantitative real-time RT-PCR assays determined an 8-fold increase for WAKL4 transcripts when seedlings were treated by NaCl (Fig. 5). This induced expression was completely suppressed in *wakl4-1*, indicating the T-DNA interruption eliminated the responsiveness of the WAKL4 promoter to these minerals (Figs. 4 and 5). In contrast, the same T-DNA insertion enabled *wakl4-1* to positively respond to elevated levels of Ni^{2+} (Fig. 4). RT-PCR experiments showed there was twice as much WAKL4 transcript in *wakl4-1* seedlings grown in medium containing 26 μM $NiSO_4$ when compared to that in regular medium (Fig. 4). The opposite effects of T-DNA insertion at −40 on WAKL4 expression were not expected. We thus further confirmed the surprisingly elevated level of the WAKL4 transcript by real-time quantitative PCR analyses (Fig. 5). Consistent with the semiquantitative RT-PCR results, almost twice as many WAKL4 transcripts were detected in *wakl4-1* mutants when compared to that of the wild type (Fig. 5). Although multiple copies of the W-box (TTGAC), an element required for pathogenesis-related gene inductions, were discovered in the WAKL4 promoter (Verica et al., 2003), close examination of the −40 region of the WAKL4 promoter in *wakl4-1* did not find any known cis-elements involved in abiotic stress responses. It is possible that there are mineral-responsive cis-acting elements located around the −40 WAKL4 promoter region, and these elements may have overlapping or opposite roles in responding to various mineral nutrients and in maintaining cellular mineral homeostasis. Further detailed promoter structure-function studies will help define such elements.

WAKL4 and Mineral Responses

Hypersensitivity of *wakl4-1* to Na^+, K^+, Cu^{2+}, and Zn^{2+} strongly suggests a functional role for WAKL4 in mineral signaling. The lengths of primary roots in *wakl4-1* were significantly reduced when grown in medium containing elevated Na^+, K^+, Cu^{2+}, and Zn^{2+} (Figs. 7 and 8). As much as more than 50% root length reduction was observed when treated with these minerals. The roles of WAKL4 in response to these minerals were established when a WAKL4-GFP fusion gene was introduced back into *wakl4-1* and proved to be sufficient to restore the primary root lengths (Figs. 7 and 8). WAKL4-GFP expression driven by either the WAKL4 native promoter or the 35S constitutive promoter complemented the phenotypes (Figs. 7 and 8). Semiquantitative and quantitative RT-PCR analyses revealed that the complemented plants produced higher levels of WAKL4 transcripts than the wild type. Protein gel-blot analysis with a GFP antibody verified a high accumulation of the WAKL4-GFP fusion protein. It is not unusual that the complementation by the WAKL4 native promoter-driven WAKL4-GFP resulted in WAKL4-GFP overexpression, which could be due to the specific chromosomal landed positions and possibly multiple copies of the inserted transgene (Nam and Li, 2002). Under standard growth conditions, there appear to be little detectable growth and developmental differences between the OX lines and the wild type.

The five minerals shown to cause phenotypes in *wakl4-1* belong to two biochemically functional groups. Both K^+ and Na^+ are placed in the group of nutrients that remain in ionic forms. They may function in establishing cell turgor and maintaining cell electroneutrality. Cu^{2+}, Ni^{2+}, and Zn^{2+} are micronutrients that belong to the group involved in redox reactions. The mechanism of how WAKL4 is involved in these mineral responses is not known. Plant roots have mechanisms to respond to mineral alternations in the soil environment. All plants have a basic metal tolerance mechanism (Clemens, 2001). Within a tolerable range of mineral concentrations in soil, plants regulate the uptake to maintain mineral homeostasis. Our data suggested that wild-type seedlings could still achieve as much as 70% of the root length when K^+ concentration was increased by 25-fold. Wild-type tolerance on Na^+ is even more dramatic. The Na^+ concentration in regular 0.1× MS medium is about 10 μM (Murashige and Skoog, 1962; Figs. 6 and 7). Wild-type seedlings grown on medium with 50 mM NaCl (a 5,000-fold increase) could reach 60% of the root length when compared to a 0.1× MS medium control (Figs. 6 and 7). Likewise, when Zn^{2+} was increased by 50-fold, wild-type root length was reduced only by about 30% (Figs. 6 and 8). Cu^{2+} has a more dramatic effect on wild-type root length. A 6-fold Cu^{2+} increase resulted in as much as 80% root length reduction for wild type (Figs. 6 and 8). These findings are consistent with the notion that plants have ubiquitous mechanisms for mineral tolerance (Clemens, 2001). WAKL4 expression is mineral regulated and *wakl4-1* is hypersensitive to elevated minerals, suggesting that WAKL4 is required for tolerance responses. However, it is interesting to note that, among all the essential minerals, only the macronutrient K^+ and the subset of the micronutrients (Na^+, Cu^{2+}, Zn^{2+}, and Ni^{2+}) had an impact on WAKL4 expression and caused hypersensitive phenotypes on *wakl4-1*, suggesting that WAKL4 may play specific roles in tolerance responses for these minerals.

The main targets of regulation in mineral uptake processes are usually the mineral transporters. The

measurement of mineral content in wild-type, *wakl4-1*, Compl, and OX plants indicated there were no significant differences among these plants for levels of Na^+, K^+, Cu^{2+}, and Ni^{2+} in both root and shoot tissues (data not shown). The constant steady-state levels of these minerals in the four different plants highly suggest neither suppression nor overexpression of WAKL4 might influence the activity changes of the transporters for these minerals. However, a different scenario for Zn^{2+} content was observed in the four different plants. While Zn^{2+} levels remained relatively the same in the roots, they significantly dropped in *wakl4-1* shoots when compared to that of other plants (Fig. 9B). In general, the OX line showed a consistently higher Zn^{2+} level. Zn^{2+} transport from root to shoot is significantly affected as shown by the calculated Zn^{2+} content ratio of shoot versus root (Fig. 9C). The shoot-to-root Zn^{2+} ratio for the wild type was about 50%, whereas that of *wakl4-1* was about 30%. Consistent with this observation, our analyses indicate that WAKL4 was required for the expression of the Zn^{2+} transporter genes in responding to Zn^{2+} deficiency (Fig. 9A). The Zn^{2+} deficiency-induced expression for the four Arabidopsis zinc transporter genes was abolished in *wakl4-1*. While it has been proposed that specific Zn^{2+} transporters are involved in Zn^{2+} transport between tissues (root to shoot) and between cells, such specific transporters are yet to be assigned (Grotz et al., 1998). The observation that WAKL4 expression is induced by Zn^{2+} at the shoot-root junction is very intriguing (Fig. 2F). Further studies will be needed to dissect the mechanisms of why WAKL4 is required for zinc transporter gene expression and how WAKL4 plays a role in Zn^{2+} uptake, especially in transport between roots and shoots.

Nickel is a micronutrient that is known to be required as a cofactor for urease (Eskew et al., 1983; Zonia et al., 1995). As an essential heavy metal, it can be toxic at excess levels. At 28 μM, Ni^{2+} had a dramatic effect on wild-type growth. The wild type had more than 90% root length reduction (Fig. 10A). A slight increase of Ni^{2+} to 36 μM resulted in extremely stunted wild-type growth. Both the Compl and the WAKL4 OX seedlings can well survive Ni^{2+} at various toxic levels (Fig. 10A). For example, when Ni^{2+} was increased from 28 to 36 μM, the root length of the WAKL4 OX line was just slightly reduced. Similarly, the WAKL4:: WAKL4-GFP Compl lines were also growing much better than the wild type. At toxic levels of Ni^{2+}, like both the WAKL4 Compl line and the WAKL4 OX line, *wakl4-1* seedlings also survived better than the wild type. We further tested more than 10 transgenic lines that gave higher levels of the WAKL4 transcript for Ni^{2+} tolerance. Ni^{2+} tolerance phenotypes were in a good correlation with WAKL4 transcript levels. The molecular mechanism of Ni^{2+} tolerance that resulted from WAKL4 overexpression is currently not known. Studies have suggested Ni^{2+} hypertolerance is correlated with His responses in at least some species (Kerkeb and Krämer, 2003; Wycisk et al., 2004). In addition, Ni^{2+} sequestration in the vacuole appears to play a role in the detoxification process (Krämer et al., 2000). Recently, analyses of Ni^{2+} hyperaccumulation in six diverse Thlaspi hyperaccumulators suggested a role of salicylic acid in a glutathione-mediated Ni^{2+} tolerance (Freeman et al., 2005). Whether and how WAKL4 expression affects cellular levels of His, salicylic acid, and glutathione, or regulates Ni^{2+} vacuole sequestration, are to be determined. Further biochemical and genetic studies on the relationship between Ni^{2+} hypertolerance and WAKL4 overexpression will provide valuable insights into how plants use RLKs to respond to mineral and heavy-metal toxicity.

MATERIALS AND METHODS

Plant Material and Mineral Nutrients

Arabidopsis (*Arabidopsis thaliana*) ecotype Ws and transgenic plants were grown as described before (Verica et al., 2003). Surface-sterilized seeds were cold treated at 4°C for at least 48 h and plated on petri dishes with various media. Both the full-strength 1× MS medium (Murashige and Skoog, 1962) and different diluted solutions were used for various tests. For mineral tests, different mineral solutions were added to 0.1× MS to generate the required medium. Seedlings were grown vertically on square plates (100 × 100 × 15 mm; Fisher Scientific) containing either 0.1× MS medium alone (control) or 0.1× MS medium supplemented with additional mineral nutrients as indicated.

Identification of the WAKL4 T-DNA Insertion Line

Screening for the WAKL4 T-DNA insertion line was carried out by using publicly available T-DNA pools from the ABRC (Ohio State University). DNA pools of various T-DNA collections were used for PCR-based screening (Krysan et al., 1996). T-DNA insertion in WAKL4 was identified in one subpool (pool no. 58, CS2691) of the superpool (ABRC stock no. CS6502; K. Feldmann T-DNA collection containing 6,300 lines) by using a left-border primer (KF-LB, 5′-GCGCAATATTTACACATAGACACACACAT-3′) and a WAKL4 upstream primer (WAKL3-1300R, 5′-ACAGTGCCTTGACCGCCTTGCCCAAG-3′). A positive WAKL4 T-DNA line was identified by PCR screening individual seedlings in the subpool (CS2691; pool of 100 T-DNA lines). T-DNA localization in the WAKL4 T-DNA line was confirmed by sequencing the PCR products (ABI377; Applied Biosystems) using a sequencing primer (KF-LB102, 5′-GATGCAATCGATATCAGCCAATTTAGAC-3′).

Genomic DNA was isolated from the wild type (Ws) and *wakl4-1* as described before (He et al., 1996). Isolated DNA was digested by *Eco*RI before fractionation. A DNA Southern blot was performed as described before (He et al., 1996). A WAKL4-specific probe was generated from the WAKL4 promoter region by using a digoxigenin (DIG) PCR DNA-labeling kit according to the manufacturer's suggested protocol (Roche Diagnostics). Two primers (WAKL4-promotor-500F, 5′-GTGTTGCTGCTACAAAAGTAATAC-3′ and WAKL4-promotor-*Bgl*2-R 5′-AGATCTTTGTTTCTCAACTCGTCAAGTCGGTCTT-3′) were used to amplify the DIG-labeled WAKL4 probe. DIG signals were detected by using a DIG-labeling detection kit (Roche Diagnostics).

WAKL4 Promoter-GUS Assay

The intergenic sequence between the start site of the WAKL4 gene sequence and the end site of the preceding gene was used as the WAKL4. A primer pair was used to clone the WAKL4 promoter (1,159 bp) from genomic DNA using PCR (Verica et al., 2003): 5′-GTCGACCGTCGTAAATCAGACTTTGCTTTAATA-3′ (*Sal*1) and 5′-AGATCTTGTTTCTCAACTCGTCAAGTCGGTCTT-3′ (*Bgl*2). The *Sal*1 and *Bgl*2 sites indicated at the end of the primer sequences were the introduced restriction sites used for cloning. The PCR products were inserted into the promoterless GUS transformation vector pBI101.2 (Verica et al., 2003) and the resulting construct was sequenced for confirmation and introduced into Arabidopsis Columbia (Col-0) wild type via Agrobacterium GV-3101-mediated transformation as described before (Lally et al., 2001). More than 30 independent homozygous lines were generated from the construct. To

assay for GUS activity, eight independent T3-T4 homozygous lines were first germinated and grown on 0.1× MS medium for 10 d. Seedlings were then transferred to a new 0.1× MS medium with or without 100 mM NaCl, 100 mM KCl, 20 μM $CuSO_4$, 150 μM $ZnSO_4$, and 40 μM $NiSO_4$, respectively. Seedlings were grown for 3 d on this new medium until assay. GUS assays were performed as described before (Verica et al., 2003). Photomicrographs were taken by a Nikon 600 fluorescence microscope (Nikon) and arranged by using Adobe Photoshop version 8.0 (Adobe Systems).

Complementation of *wakl4-1*

The WAKL4-GFP fusion gene was created to complement *wakl4-1*. Two complementation constructs were generated. The expression of the WAKL4-GFP fusion gene was driven either by the WAKL4 native promoter or by the constitutive 35S promoter. For generating the WAKL4-promoter::WAKL4-GFP construct, the entire WAKL4 genomic fragment, including the WAKL4 promoter, was PCR amplified using two pair of primers (pair one: WAKL4 promotor-*Kpn*1-F, 5′-TCTCTCGGTACCGATCTGTTCAAGCGGCTTCATGATGAAT-3′ and WAKL4 promotor-*Bgl*2-R, 5′-AGATCTTTGTTTCTCAACTCGTCAAGTCGGTCTT-3′; and pair two: WAKL4 promotor-*Sal*1-F, 5′-GTCGACCGTCGTAAATCAGACTTTGCTTTAATA-3′ and WAKL4-*Bgl*2-R, 5′-TCTCTCAGATCTGCCATGTTCGTAGAGGAACCAGAGGCTC-3′). The introduced restriction sites used for cloning are indicated in the primer names. The PCR products were purified by a PCR purification kit (Qiagen) and ligated to a pGEM T-easy vector (Promega). Positive clones were selected and sequenced to verify sequence accuracy. Fragments of WAKL4-promotor-*Kpn*1-F/WAKL4-promotor-*Bgl*2-R (1.8 kb) and WAKL4-promotor-*Sal*1-F/WAKL4-*Bgl*2-R (2.9 kb) were dropped off from the pGEM vector by cutting with corresponding restriction enzymes and subsequently ligated into the binary pPZP222-GFP vector (opened by *Kpn*1 and *Bgl*2; Nam and Li, 2002). To generate the 35S::WAKL4-GFP construct, a primer pair (WAKL4 coding-*Kpn*1-F, 5′-TTGAGAAGGTACCATGAAGAAAGAAACTC-3′ and WAKL4-2904R, 5′-TAATCATCACATTCATCTCGAAGA-3′) was used for PCR to generate the first part (215 bp) of the WAKL4 gene from the pPZP222-WAKL4 promoter-*WAKL4*-GFP construct. pPZP222-WAKL4 promoter-*WAKL4*-GFP was cut by *Xho*1 and *Bgl*2 to get the rest of the WAKL4 gene. The two parts were ligated into the pCHF1-35S-GFP vector cut by *Kpn*1 and *Bam*H1. Positive clones were identified in *Escherichia coli* XL-1 blue cells and sequenced for sequence confirmation. The two confirmed transformation constructs were used to transform both wild type (Ws) and *wakl4-1* via Agrobacterium-mediated transformation as described before (Lally et al., 2001). More than 30 independent T1 transformants for each construct were identified on MS plates containing 100 mg/L gentamycin. Homozygous T3/T4 lines were used for various assays.

GFP Detection by Protein Gel Blotting and Confocal Laser Scanning Microscopy

Whole protein extraction and protein gel-blot analysis were essentially performed as described before (He et al., 1996; Verica et al., 2003). Soluble proteins were prepared using an extraction buffer (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, and 5 mM DTT) supplemented with a mixture of protease inhibitor (protease inhibitor cocktail, P 9599; Sigma). After extensive washing and extraction, the pellet was used for insoluble protein preparation. Insoluble proteins were extracted by boiling the pellet for 10 min in the extraction buffer (50 mM Tris-HCl, pH 6.8, 50 mM DTT, 4% SDS, 10% glycerol, and 0.05% bromphenol blue). The total GFP was detected by using a horseradish peroxidase-conjugated GFP polyclonal antibody (Vector Laboratories; 1:200 dilution) and developed with enhanced chemiluminescence detection reagents (Amersham-Pharmacia Biotech).

Ten-day-old seedlings were used for GFP detection. GFP fluorescence was excited by a blue argon laser (10 mW, 488-nm blue excitation) and detected at 515- to 530-nm wavelengths in a Nikon C1 confocal E600FN microscope (Nikon). Whole roots were directly mounted in water and observed with water objectives (20× and 60×). Wild-type (Ws) seedlings were used as negative controls. For plasmolysis, roots were treated in a 500 mM NaCl solution for 5 min before observation. Images were processed and arranged by Adobe Photoshop version 8.0.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from various tissues using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. RT-PCR analysis was performed on 0.2 μg total RNA by using the One-Step RT-PCR kit (Qiagen). All RNA samples were treated with a ribonuclease-free deoxyribonuclease to eliminate DNA contamination. In addition, all RT-PCR primers were designed to spin introns to assure gene specificity and to differentiate any potential genomic DNA contamination.

The following primers were used for RT-PCR analyses of gene expression: ZIP1 (At3G12750.1): ZIP1-cDNA-381F 5′-TGCTGGTTTTGTAGCGATGCTGTCGG-3′, ZIP1-cDNA-880R 5′-CCTCTTTGCTCTCTTTCCTGTAGCC-3′; ZIP2 (At5G59520.1): ZIP2-cDNA-447F 5′-GTTTGTAGCGGCTGGGAGTAATAAC-3′, ZIP2-cDNA-801R 5′-GGAGTAGACGACAGTGAGGAAGAATG-3′; ZIP3 (At2G32270.1): ZIP3-cDNA-478F 5′-TCTAAGAGGGTCAGTGATGGAGAAACC-3′, ZIP3-cDNA-916R 5′-AATCTGCTGCGAGAAGGTCAACCAAAG-3′; ZIP4 (At1G10970.1): ZIP4-cDNA-518F 5′-AAGCCGCTGCTGGTAGTGAAGAGATTGC-3′, ZIP4-cDNA-820R 5′-GGGATAAACCGATGATGATTGAGTGC-3′; WAKL4 (At1G16150): WAKL4-cDNA-810F 5′-AAGATGGCGGGTATGGTTCCGAGAC-3′, WAKL4-cDNA-1140R 5′-AGAACACTCTCATTCGACTGCTCCT-3′; and Actin (At3G46520): Actin-cDNA-200F 5′-AACCAATCGTGTGTGACAATGGT-3′, Actin-cDNA-620R 5′-TACGACCACTGGCGTACAAGGA-3′.

For quantitative real-time RT-PCR analyses, purified total RNA was transcribed using a Bio-Rad iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's recommended protocol. The generated cDNA was then used for a TaqMan-based quantitative real-time PCR assay. TaqMan primers were designed using Primer Express version 2.0 (Applied Biosystems). For WAKL4 gene expression analysis, the following primers were used: WAKL4-274-F (5′-CAATCCTGAATACGTTGAATGGACTA-3′), WAKL4-354R (5′-CAGCTAATGGCAAGCAGAATGA-3′), and WAKL4-TaqMan-301 (5′-AATTGTTCTTG^GAACCACTATCGGCTTCTTG-3′). The WAKL4-TaqMan-301 probe was designed to spin the second intron (position indicated as ^ in the listed sequence) of the WAKL4 gene. The following primers were designed for the Actin2 (ACT2) gene used as an endogenous control: ACT2-F (5′-ATCGGTGGTTCCATTCTTGCT-3′), ACT2-R (5′-GCTTTTTAAGCTTTTGATCTTGAGAG-3′), and ACT2-TaqMan probe (5′-AGCACATTCCAGCAG^ATGTGGATCTCCAA-3′). Similarly, the ACT2-TaqMan probe was designed to spin an intron (position indicated as ^ in the listed sequence) of the ACT2 gene. The WAKL4-TaqMan-301 probe was carboxytetramethylrhodamine labeled and the ACT2-TaqMan probe was carboxyfluorescein labeled (Applied Biosystems). Fluorescence dye reactive oxygen was added in the master PCR mix to normalize pipetting errors. Multiplex real-time PCR assays (mixing WAKL4-TaqMan probe and ACT2-TaqMan probe) were performed in an ABI 7300 real-time PCR system (Applied Biosystems). Relative quantitations of WAKL4 gene expression were calculated using the comparative threshold cycle method.

Zn^{2+}, Cu^{2+}, Ni^{2+}, K^+, and Na^+ Content in Shoots and Roots

Seedlings of wild-type (Ws), *Wakl4-1*, WAKL4::WAKL4-GFP, and 35S::WAKL4-GFP transgenic lines were grown on 0.1× MS supplemented with 50 mM NaCl, 50 mM KCl, 18 μM $CuSO_4$, 26 μM $NiSO_4$, and 150 μM $ZnSO_4$, respectively. Fourteen-day-old seedlings were carefully washed in distilled water and their shoots and roots were separated. The harvested tissues were blotted dry by Kimwipes and weighed. Shoot tissues were added in an acid solution (10:1 nitric acid:perchloric acid). For every 1 mg of shoot tissue, 10 μL of acid were added. Tissues were digested in a boiling water bath until all solid material dissolved. The same procedures were used for root tissue digestion, except 20 μL of acid solution were added for each 1 mg of root tissue. Mineral levels were assayed using an atomic absorption spectrometer (ATI Unicam Solaar 929) with various standards. The graphite furnace mode was used for Cu^{2+}, Ni^{2+}, and Zn^{2+} assays and the flame mode for K^+ and Na^+ assays.

Received June 9, 2005; revised July 25, 2005; accepted September 22, 2005; published November 11, 2005.

LITERATURE CITED

Anderson CM, Wagner TA, Perret M, He ZH, He D, Kohorn BD (2001) WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. Plant Mol Biol **47:** 197–206

Becraft PW, Stinard PS, McCarty DR (1996) CRINKLY4: a TNFR-like receptor kinase involved in maize epidermal differentiation. Science **273:** 1406–1409

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science **262:** 539–544

Clark KL, Larsen PB, Wang X, Chang C (1998) Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc Natl Acad Sci USA **95:** 5401–5406

Clark SE, Williams RW, Meyerowitz EM (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell **89:** 575–585

Clemens S (2001) Molecular mechanism of plant tolerance and homeostasis. Planta **212:** 475–486

Decreux A, Messiaen J (2005) Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. Plant Cell Physiol **46:** 268–278

Endre G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss G (2002) A receptor kinase gene regulating symbiotic nodule development. Nature **417:** 962–966

Eskew DL, Welch RM, Carey EE (1983) Nickel: an essential micronutrient for legumes and possibly all higher plants. Science **222:** 621–623

Freeman JL, Garcia D, Kim D, Hopf A, Salt DE (2005) Constitutively elevated salicylic acid signals glutathione-mediated nickel tolerance in Thlaspi nickel hyperaccumulators. Plant Physiol **137:** 1082–1091

Gamble RL, Coonfield ML, Schaller GE (1998) Histidine kinase activity of the ETR1 ethylene receptor from Arabidopsis. Proc Natl Acad Sci USA **95:** 7825–7829

Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. Proc Natl Acad Sci USA **95:** 7220–7224

Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51:** 463–499

He ZH, Cheeseman I, He D, Kohorn BD (1999) A cluster of five cell wall-associated receptor kinase genes, WAK1-5, are expressed in specific organs of Arabidopsis. Plant Mol Biol **39:** 1189–1196

He ZH, Fujiki M, Kohorn BD (1996) A cell wall-associated, receptor-like protein kinase. J Biol Chem **271:** 19789–19793

He ZH, He D, Kohorn BD (1998) Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response. Plant J **14:** 55–63

Jinn TL, Stone JM, Walker JC (2000) HAESA, an Arabidopsis leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev **14:** 108–117

Kerkeb L, Krämer U (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. Plant Physiol **131:** 716–724

Kohorn B (2000) Plasma membrane-cell wall contacts. Plant Physiol **124:** 31–38

Krämer U, Pickering IJ, Prince RC, Raskin I, Salt DE (2000) Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator Thlaspi species. Plant Physiol **122:** 1343–1354

Krysan PJ, Young JC, Tax F, Sussman MR (1996) Identification of transferred DNA insertions within Arabidopsis genes involved in signal transduction and ion transport. Proc Natl Acad Sci USA **93:** 8145–8150

Lally D, Ingmire P, Tong HY, He ZH (2001) Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. Plant Cell **13:** 1317–1333

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell **90:** 929–938

Loh YT, Martin GB (1995) The Pto bacterial resistance gene and the Fen insecticide sensitivity gene encode functional protein kinases with serine/threonine specificity. Plant Physiol **108:** 1735–1739

López-Bucio J, Cruz-Ramirez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol **6:** 280–287

Morris ER, Walker JC (2003) Receptor-like protein kinases: the keys to response. Curr Opin Plant Biol **6:** 339–342

Mu JH, Lee HS, Kao TH (1994) Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. Plant Cell **6:** 709–721

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 472–497

Nam KH, Li J (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell **110:** 203–212

Nishimura R, Hayashi M, Wu GJ, Kouchi H, Imaizumi-Anraku H, Murakami Y, Kawasaki S, Akao S, Ohmori M, Nagasawa M, et al (2002) HAR1 mediates systemic regulation of symbiotic organ development. Nature **420:** 426–429

Park AR, Cho SK, Yun UJ, Jin MY, Lee SH, Sachetto-Martins G, Park OK (2001) Interaction of the Arabidopsis receptor protein kinase Wak1 with a glycine-rich protein AtGRP-3. J Biol Chem **276:** 26688–26693

Shiu SH, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA **98:** 10763–10768

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. Plant Cell **16:** 1220–1234

Sivaguru M, Ezaki B, He Z-H, Tong H, Osawa H, Baluska F, Volkmann D, Matsumoto H (2003) Aluminum induced gene expression and protein localization of a cell wall-associated receptor protein kinase in *Arabidopsis thaliana*. Plant Physiol **132:** 2256–2266

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhe LH, et al (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science **270:** 1804–1806

Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor protein kinase encoded at the self incompatibility locus of Brassica oleracea. Proc Natl Acad Sci USA **88:** 8816–8820

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, et al (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature **417:** 959–962

Takasaki T, Katsunori H, Suzuki G, Watanabe M, Isogai A, Hinata K (2000) The S receptor kinase determines self-incompatibility in Brassica stigma. Nature **403:** 913–916

Torii KU (2004) Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways. Int Rev Cytol **234:** 1–46

Verica JA, Chae L, Tong H, Ingmire P, He ZH (2003) Tissue-specific and developmentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in Arabidopsis. Plant Physiol **133:** 1732–1746

Verica JA, He ZH (2002) The cell wall-associated kinase (WAK) and WAK-like kinase gene family. Plant Physiol **129:** 455–459

Wagner T, Kohorn B (2001) Wall-associated kinases are expressed throughout plant development and are required for cell expansion. Plant Cell **13:** 303–318

Wycisk K, Kim EJ, Schroeder JI, Krämer U (2004) Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in Arabidopsis thaliana. FEBS Lett **578:** 128–134

Yokoyama R, Takahashi T, Kato A, Torii KU, Komeda Y (1998) The Arabidopsis ERECTA gene is expressed in the shoot apical meristem and organ primordia. Plant J **15:** 301–310

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR, Arabidopsis microarray database and analysis toolbox. Plant Physiol **136:** 2621–2632

Zonia LE, Stebbins NE, Polacco JC (1995) Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. Plant Physiol **107:** 1097–1103