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A Cell Wall-associated, Receptor-like Protein Kinase*

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Physical connections between higher plant cell walls and the plasma membrane have been identified visually, but the molecules involved in the contact are unknown. We describe here an *Arabidopsis thaliana* protein kinase, designated Wak1 for wall-associated kinase, whose predicted extracytoplasmic domain contains several epidermal growth factor repeats and identity with a viral movement protein. Wak1 fractionates with insoluble material when plant tissue is ground in a variety of buffers and detergents, suggesting a tight association with the plant extracellular matrix. Immunocytochemistry confirms that Wak1 is associated with the cell wall. Enzymatic digestion of the cell wall allows the release of Wak1 from the insoluble cell wall fraction, and protease experiments indicate that Wak1 likely has a cytoplasmic kinase domain, and the EGF containing domain is extracellular. Wak1 is found in all vegetative tissues of *Arabidopsis*, and has relatives in other angiosperms, but not *Chlamydomonas*. We suggest that Wak1 is a good candidate for a physical continuum between the cell wall and the cytoplasm, and since the kinase is cytoplasmic, it also has the potential to mediate signals to the cytoplasm from the cell wall.

The plant cell wall is composed of an organized matrix of cellulose, hemicellulose, pectin, and protein that is dynamic during the development of a plant and is important in processes such as cell shape and natural defense strategies (1–4). The associations of the cell wall with the plasma membrane and cytoplasm are assumed to be critical for plant cell function, and some have proposed direct connections between the extra- and intracellular environment (5, 6). Despite the importance of these connections, this association remains undefined, although attempts have been made to model the plant cell wall after the extracellular matrix of animal cells (7). Some studies have identified cross-reactive material to human vitronectin antiserum (8, 9). Moreover, plant proteins can be identified that have affinity to the RGD motif that binds fibronectin in mammalian cells, and RGD can have effects on cell wall attachment to plant cells (7). A cDNA for a vitronectin-like protein was recently cloned, but although the antiserum identified a cell wall protein, the cDNA predicted translation elongation factor-1α and the importance was inconclusive (10). Thus, direct evidence for a protein or other physical continuum between the wall and the plasma membrane is still lacking.

We have reported on the isolation of an *Arabidopsis thaliana* cDNA that predicts a receptor-like serine/threonine kinase of 68 kDa and is encoded by a single copy gene (11). This protein is predicted to have several functional domains, one with similarity to the family of serine/threonine kinases, and another domain that contains both epidermal growth factor (EGF)[1]-like repeats and a region similar to a viral movement protein (12). We show here that this protein, called Wak1 for wall-associated kinase (previously called Pro25), is associated with the cell wall and spans the plasma membrane to have its kinase domain within the cytoplasm. Wak1 is found in all vegetative tissues of *Arabidopsis* and in other flowering plants.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions—*A. thaliana* ecotype Columbia was grown in Metro-Mix 220 growing medium (Grace Sierra Horticultural Products Company, Milpitas, CA) under a cycle of 14 h of light and 10 h of darkness at 22 C.

Expression and Purification of Glutathione S-Transferase Fusion Proteins—The coding region for amino acids 78–595 of Wak1 was cloned into the pGEX vector (Pharmacia Biotech Inc.) and transformed into *Escherichia coli* BL21 (13). Cells grown overnight at 37 C in LB medium plus 50 μg/ml ampicillin were diluted 10-fold in LB medium plus 50 μg/ml ampicillin. After shaking at 37 C for 90 min (cell density of A_{600} 0.6–0.8), expression of the fusion protein was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were incubated for an additional 90 min and then harvested at 4 C and resuspended in TSE (50 mM Tris, pH 8.0, 25% sucrose, 1 mM EDTA). Fusion protein was purified according to Smith and Johnson (14) and Hakes and Dixon (15) using glutathione *S*-Sepharose beads (Pharmacia Biotech).

Protein Extraction, Gel Electrophoresis, and Immunoblot Analysis—Plant tissue samples were ground in 1 × protein sample buffer (50 mM Tris-HCl, pH 6.8, 50 mM DTT, 4% SDS, 0.05% bromphenol blue, 10% glycerol) in microcentrifuge tubes using plastic pestles, boiled for 5 min, and the denatured samples were fractionated in 12.5% SDS-polyacrylamide gel electrophoresis (16) and electroblotted to nitrocellulose membranes in 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol (17). Blots were developed using ECL Western blotting detection reagents (Amersham Life Science). Silver staining of gels was performed using a Bio-Rad kit (18). Gel lanes were loaded with samples that represented equal percentages of cell preparations.

Cell Fractionation—Cell fractionation was performed essentially as described (19, 20). To isolate cell wall, leaf tissues were homogenized in grinding buffer (62.5 mM Tris-HCl, pH 7.5, 5 mM DTT, 1% bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml E-64, 2 μg/ml pepstatin A) using a Polytron (full speed, 3 × 10 s). The homogenate was centrifuged at 1,000 × *g* for 3 min. The pellet was washed with ice-cold grinding buffer (without 1% BSA) 10 times. Finally the (cell wall) pellet was washed by resuspending in 500 mM $CaCl_2$, 20 mM NaCl, 62.5 mM Tris-HCl, pH 7.5, and spinning at 10,000 × *g* for 15 min.

Protoplast Preparation—*Arabidopsis* leaf tissues were incubated in protoplast isolation buffer (1% Cellulase Onozuka R10, 0.25% Macer-

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[1] The abbreviations used are: EGF, epidermal growth factor; DTT, dithiothreitol; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; GST, glutathione *S*-transferase.

ozyme R10, 10 mM $CaCl_2$, 0.4 M mannitol, 10 mM MES, pH 5.7, 50 mM $CaCl_2$, 5 mM β-mercaptoethanol, 0.1% BSA, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml E-64, 2 μg/ml pepstatin A, 2 μg/ml aprotinin) at 22 C for 2–3 h with gentle shaking at 60–80 rpm (21). The isolated protoplasts were filtered through a 70-μm nylon mesh and centrifuged for 10 min at 60–80 × *g*. The pelleted protoplasts were gently resuspended and washed four times with washing buffer (4 mM MES, pH 5.7, 0.5 M mannitol, 2 mM KCl) and visualized by light microscopy. Protoplasts were osmotically ruptured in a hypotonic solution (40 mM Tris-HCl, pH 7.5) on ice for 15 min. Protease treatments were in 0.5 M mannitol, 10 mM Tris-HCl, pH 7.5, and were terminated by the addition of denaturing buffer (16).

Plasma Membrane Preparation—Isolated protoplasts were resuspended in membrane isolation buffer containing 50 mM Tris-HCl, pH 7.5, 8% sucrose, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 95 mM LiCl, 2 mM EGTA, 0.6% polyvinylpolypyrrolidone and homogenized in a Knotes glass homogenizer at 4 C. The homogenate was centrifuged at 2,500 × *g* for 10 min at 4 C, and the generated supernatant was subjected to 100,000 × *g* at 4 C for 20 min. The pellet was resuspended in water and loaded to a two-phase partitioning system to purify plasma membrane (22). The purified plasma membrane was determined to have 95% purity by a vanadate-sensitive ATPase assay using a pyruvate kinase-coupled ATPase assay (23). For NaOH treatment the purified plasma membrane was incubated on ice with or without 0.1 N NaOH for 30 min before ultracentrifugation at 100,000 × *g* for 15 min. The NaOH-treated sample was later neutralized with HCl. The pellets were washed twice with extraction buffer before denaturation and fractionation.

Light and Electron Microscopy—Tissue fixation and Wak1 immunostaining for light microscopy were performed as described (24). For transmission electron microscopy, leaf tissues were fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.05 M potassium phosphate buffer, pH 7.0, washed with the buffer, and dehydrated in an ethanol dilution series. Tissue samples were infiltrated and embedded with London Resin White medium (EM Sciences, Fort Washington, PA) according to the manufacturer's directions. Ultrathin sections collected on gold grids were blocked in 1% BSA in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100) and reacted successively with a 1:100 dilution of anti-Wak1 serum and 1:30 dilution of gold-conjugated rabbit anti-chicken antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Sections were stained in uranyl acetate and poststained in lead citrate. Sections were viewed and photographed with a transmission electron microscope (EM200, Philips Electronic Instruments, Mahwah, NJ).

RESULTS

Wak1 Is Found In a Variety of Higher Plant Tissues—Wak1 is 595 amino acids in length and has a putative transmembrane domain that separates the carboxyl-proximal kinase from the EGF-containing region. The coding sequence for a region containing the kinase domain and 2 EGF repeats (amino acids 78–595) was inserted into the pGEX expression plasmid for production of a fusion protein in *E. coli*. The GST-EGF/kinase fusion protein was then purified by glutathione affinity chromatography and used as antigen in rabbits (14, 15). The resulting serum reacted specifically with the GST-EGF/kinase fusion protein and with GST, but not other bacterial proteins. Reaction to the GST-EGF/kinase on Western blots could be eliminated by preincubation of the antiserum with purified GST-EGF/kinase, but not purified GST (data not shown). The serum was then used to probe whole cell extracts from *Arabidopsis* leaves, stems, roots, and flowers that had been run in a denaturing acrylamide gel and Western-blotted, and the results are shown in Fig. 1*A*. A 68-kDa protein is detected in each tissue, and this is the predicted molecular mass of Wak1. Preimmune sera do not detect a 68-kDa protein. The *in vitro* translation products from a full-length Wak1 cDNA were also included in the gel, and the protein detected by the antiserum migrates to the same position as the Wak1 found in plant tissue (Fig. 1*B*, *Trl*.). The Wak1 antiserum was used to probe Western blots of extracts from pea, tobacco, maize, and *Chlamydomonas* (Fig. 1*B*). In all species of higher plants tested but not the unicellular green alga, a cross-reactive 68-kDa protein is detected.

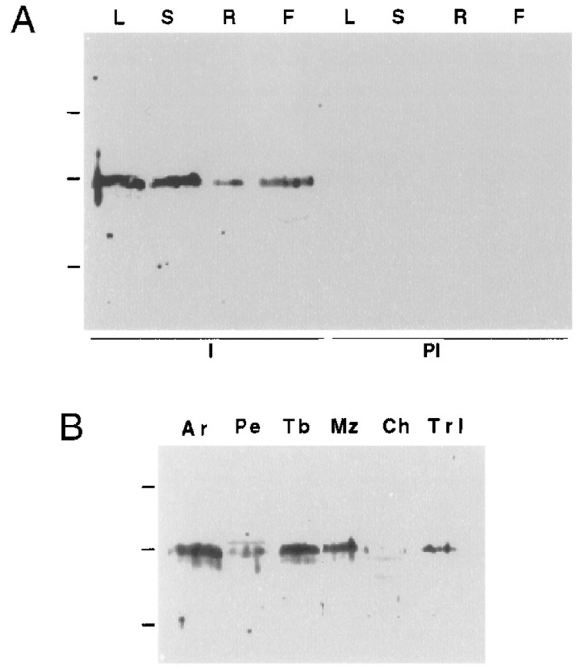

FIG. 1. **Wak1 is detected in different parts of *Arabidopsis* seedlings and in other higher plants.** *A*, immunoblotting of whole cell extracts (60 μg total protein) from various *Arabidopsis* tissues with anti-Wak1 serum. *L*, leaf; *S*, stem; *R*, root; *F*, flower; *I*, immune; *PI*, preimmune serum. *B*, immunoblotting analysis of extracts (60 μg of total protein) from leaves of *Arabidopsis* (*Ar*), pea (*Pe*), tobacco (*Tb*), maize (*Mz*), and from *Chlamydomonas* (*Ch*). *Trl*, *in vitro* unlabeled translation products of full-length Wak1 cDNA. *Lines* on the *left* indicate mass markers 93, 69, and 50 kDa.

Cell Wall Association—To determine the subcellular location of Wak1, we used the Wak1 antiserum to probe plasma membrane, chloroplast, mitochondrial, and cytoplasmic leaf fractions, but Wak1 reactivity was lost during the fractionation, even in the presence of multiple protease inhibitors during the isolation. Previous whole plant extractions (shown in Fig. 1) involved grinding and boiling in a buffer that included 4% SDS and 50 mM DTT, and it was realized that Wak1 was remaining with the insoluble low speed pellet during cell fractionation. Leaves were then ground in a number of diverse buffers and detergents in an attempt to release Wak1 into a low speed (5,000 × *g*) supernatant, and the Western blot of some these supernatants is shown in Fig. 2. Only a combination of 4% SDS and 50 mM DTT, followed by boiling, could release Wak1 into the low speed supernatant. Alteration individually of the pH, ionic, or reducing conditions also could not extract Wak1 (data not shown). These results suggested that the 68-kDa Wak1 fractionated with the cell wall, as much of this material is also insoluble in a variety of detergents and buffers.

Wak1 Is on the Cell Surface—The biochemical fractionation of Wak1 suggested a cell wall association, but due to the difficulty of solubilizing Wak1, associated cell wall components are hard to identify. The cell wall location can be confirmed with immunocytochemistry. The Wak1 antiserum was used to probe thin sections of *Arabidopsis* leaves, and these were visualized both under the light and electron microscopes. Gold conjugated secondary antibodies (Fig. 3*A*, *arrows*) to the Wak1 antiserum localize to the surface of cells, specifically in the region of the cell wall found between two adjacent cells (Fig. 3*A*, *W*). The plasma membranes in these sections are separated from the cell wall during the fixation, but these conditions were necessary for efficient antibody detection. Significantly, the gold particles are mostly found in the cell wall, but a few are occasionally seen on the plasma membrane (Fig. 3*A*, *top right*).

These results are consistent with the biochemical observations that most Wak1 fractionates with the insoluble material. Preimmune sera shows no staining (Fig. 3*B*). (Note the ribosomes are slightly larger and less defined than the gold particles.)

Localization by light microscopy reveals that Wak1 is present on the surface of all cells of the leaf. Fluorescent secondary antibodies (Cy3-conjugated) indicate Wak1 antibody is bound to the surface of all cells, as seen in 1-μm cross-sections of a 20-day-old leaf in Fig. 3*C*. Preimmune serum does not detect the cell surface. The chloroplast produces some chlorophyll fluorescence, but preimmune sera does not react with other organelles (Fig. 3*C*). Experiments with peroxidase-conjugated secondary antibody give similar results for all ages of leaves from their tips to bases (data not shown). The Western analysis (Fig. 1) indicated that Wak1 was present in all vegetative tissue, and this has also been confirmed by light microscopy.[2]

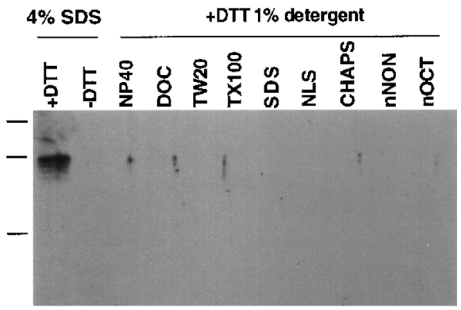

FIG. 2. **Wak1 can be extracted only by 4% SDS 50 mM DTT from mature *Arabidopsis* leaves.** Shown are Western blots of low speed supernatants of *Arabidopsis* leaves (30-day-old) ground in the indicated solution with 10 mM Tris-HCl, pH 7.5. *+DTT*, 50 mM dithiothreitol; *NP-40*, Nonidet P-40; *DOC*, deoxycholic acid; *TW20*, Tween 20; *TX100*, Triton X-100; *SDS*, sodium dodecyl sulfate; *NLS*, lauroylsarcosine, sodium salt; *CHAPS*, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; *nNON*, nonyl-β-D-glucopyranoside; *nOCT*, *n*-octylglucoside. *Lines* on the *left* indicate mass markers 93, 69, and 50 kDa.

Wak1 Is Transmembrane—The Wak1 cDNA predicts a sequence (from amino acid 197–217) that is hydrophobic and could span a membrane and thus separate the EGF and kinase domains. The EGF repeats likely would be extracytoplasmic, as EGF domains are rich in disulfide bonds and are unable to survive the reducing nature of the cytoplasm (25). The kinase domain was therefore predicted to be cytoplasmic, although the possibility of an extracellular kinase could not be ruled out. To probe the topology of Wak1, protoplasts were prepared from leaf cells by digesting the tissue with cellulase and macerozyme to remove the cell wall. Wak1 can be detected in intact protoplasts (Fig. 4, *lane Pp*) and also in a 5,000 $\times$ *g* supernatant of osomotically ruptured protoplasts (*lane So*). The digestion of the cell wall is usually not complete, and thus the protoplast preparation should include a population of cells that have some cell wall and associated Wak1. As expected, some Wak1 does still pellet at 5,000 $\times$ *g* in the osmotically ruptured protoplasts (*lane Po*), even though cell lysis, as assayed by light microscopy, was complete. Nevertheless, Wak1 can be separated from the insoluble material by the digestion of the cell wall.

The intact protoplasted cells were also treated with protease to determine whether the kinase domain was protected by the

[2] Z.-H. He and B. D. Kohorn, unpublished results.

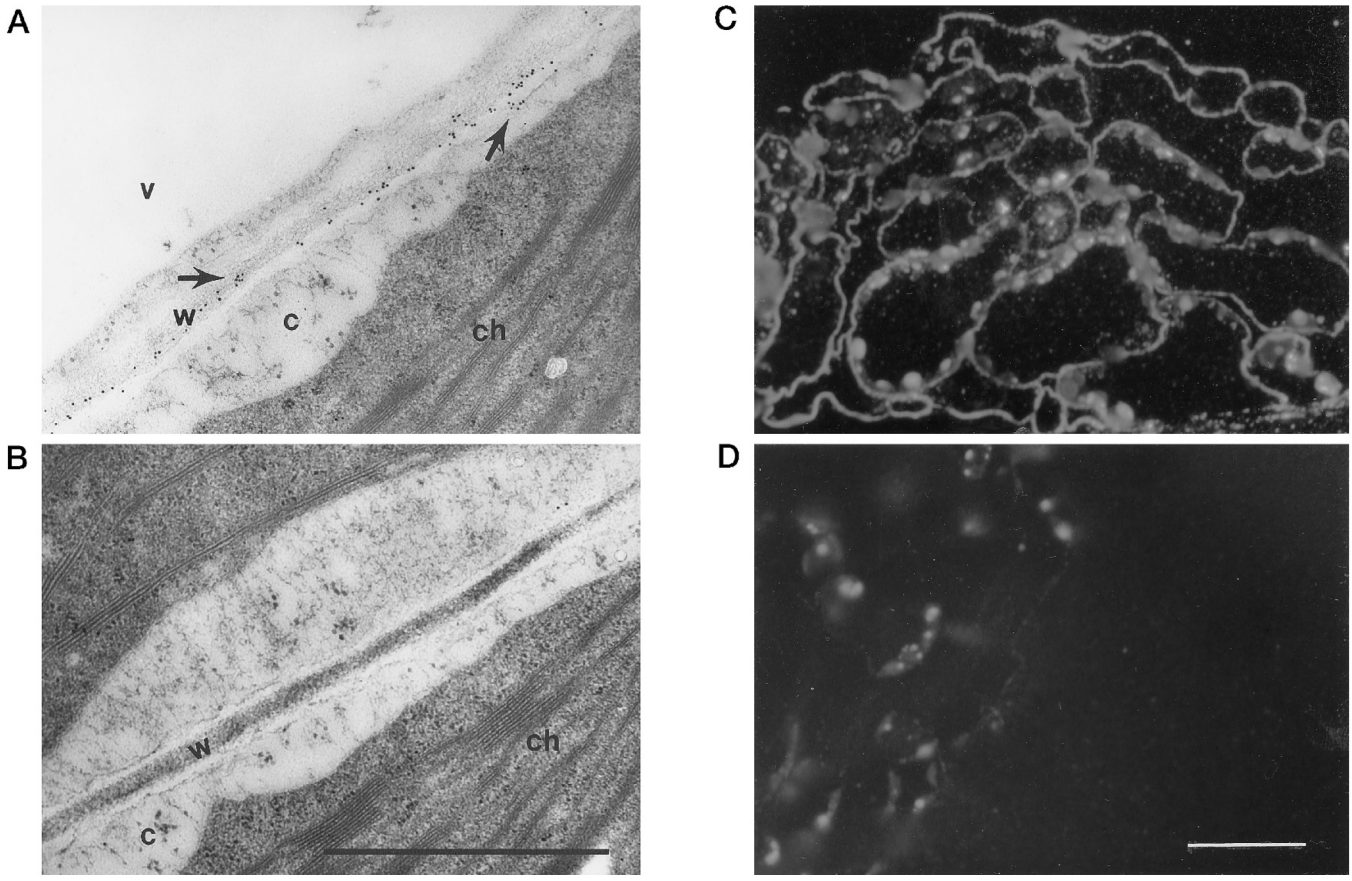

FIG. 3. *A* and *B*, immunocytochemical localization of Wak1 by electron microscopy. *A*, 0.5-μm leaf sections reacted with anti-Wak1 serum, and gold-conjugated secondary antibodies. *B*, preimmune staining. *Bar* = 1 μm. *c*, cytoplasm; *ch*, chloroplast; *w*, cell wall; *arrow* points to representative gold particles to be distinguished from the slightly larger and less defined ribosomes. *C* and *D*, localization by light microscopy reveals that Wak1 is present on the surface of all leaf cells. *C*, 1-μm cross-sections reacted with anti-Wak1 serum and subsequently stained with fluorescent secondary antibodies (Cy3-conjugated). *D*, preimmune serum staining. *Bar* = 10 μm.

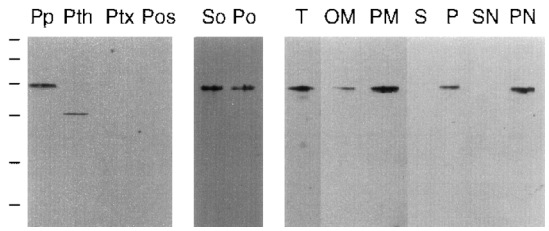

FIG. 4. **Wak1 is in the protoplast plasma membrane.** Western blots with Wak1 antiserum of total protoplasts (*Pp*) were osmotically ruptured and separated into a 6,000 × *g* supernatant (*So*) and pellet (*Po*). Total protoplasts were also treated with thermolysin (*Pth*), Triton X-100 and thermolysin (*Ptx*), or osmoticum and thermolysin (*Pos*). Total celluar membranes prepared from protoplasts (*T*) were fractionated into plasma membrane (*PM*) and other membranes (*OM*). The plasma membrane was subjected to 100,000 × *g* to create a pellet (*P*) and supernatant (*S*). Plasma membrane was also incubated with NaOH and then separated into a pellet (*PN*) and supernatant (*SN*) after 100,000 × *g*. *Lines* on the *left* indicate mass markers 220, 97.4, 69, 46, 30, and 21.5 kDa.

membrane. Fig. 4 shows that thermolysin treatment of protoplasts removes ~22 kDa from the 68-kDa Wak1 (*lane Pth*). The predicted extracytoplasmic domain is 200 amino acids, and this correlates exactly with the size of the portion that is removed. The kinase domain is predicted to be ~40 kDa. Protease digestion of the protoplasts in the presence of Triton X-100 to disrupt the membrane (Fig. 4, *lane Ptx*), or osmoticum to burst open the protoplasts (*lane Pos*) renders Wak1 not detectable, and thus likely mostly digested. These data are therefore consistent with an extracellular Wak1 EGF containing domain tightly associated with the cell wall and a cytoplasmic kinase.

In a separate method to determine whether Wak1 is integral to the outer membrane of protoplasts, we purified plasma membrane through phase partitioning of total membrane from protoplasted leaf cells. In non-protoplasted cells, less than 0.1% of the total cell Wak1 could be detected in a total cellular membrane fraction (data not shown). Fig. 4 displays the Western analysis of Wak1 in a plasma membrane preparation from leaf protoplasts. Wak1 is enriched in the purified plasma membrane fraction (*lane PM*), and only a small amount is detected in the fraction that represents other cellular membranes (*lane OM*). The purity of the PM fraction was determined to be 95%, as assayed by the vanadate sensitivity of ATPase activity (23). When the PM fraction was subjected to 100,000 × *g*, Wak1 was found with the pellet and not supernatant (Fig. 4, *lanes P versus S*), even when the preparation was treated with the chaotropic agent 0.1 N NaOH prior to 100,000 × *g* (*lanes PN versus SN*). Thus Wak1 is an integral membrane protein of the plasma membrane.

DISCUSSION

The plant cell wall, or extracellular matrix, is a complex arrangement of carbohydrate and protein whose composition has been difficult to study, due in part to its limited solubility. A number of proteins have been identified as integral components of the cell wall, but suggestions for a cell wall-cytoplasmic continuum have been few. The predicted topology of Wak1 from the cDNA sequence suggests a receptor-like function, such that this transmembrane protein would have a cytoplasmic kinase and an extracellular domain that contains the EGF repeats. The combination of the biochemical and microscopic evidence supports this predicted topology. Moreover, they suggest that Wak1 is a strong candidate for a physical connection between the ECM and the cytoplasm.

Wak1 appears to be tightly associated with the cell wall, as only after cell wall digestion can it be released into a plasma membrane fraction. Wak1 can only be extracted non-enzymatically from tissue by boiling in 4% SDS and DTT, and this property suggests that Wak1 is associated with the cell wall. The electron micrographs demonstrate that this indeed is true, although the exact location within the cell wall is not clear.

The nature of the cell wall-Wak1 association also remains unknown. The requirement for DTT for the release into a 4% SDS low speed supernatant indicates the presence of a covalent attachment, but it is not known if this is entirely proteinaceous. Alternatively, DTT may disrupt the disulfide-dependent EGF domain (25) and thereby disrupt a non-covalent association with a cell wall component. A series of detergent and MeOH washes can be used to purify Wak1 (data not shown), and so it is not likely that Wak1 is covalently attached to the ECM. The cell wall association of Wak1 is not induced by the fractionation procedure, as addition of *in vitro* translated, radiolabeled Wak1 to a leaf and subsequent extraction, allows all of the labeled product to be recovered in the water-soluble fraction (data not shown).

We suggest that the Wak1 in the cell wall is also membrane-associated, but as it is tightly associated with the cell wall, it cannot fractionate with the plasma membrane unless released by digestion of the wall. The electron micrographs showing Wak1 in the cell wall of cells in which the plasma membrane has shrunk away from the cell wall supports this suggestion.

Wak1 in the plasma membrane of protoplasts is partly resistant to digestion by added protease, and the portion that is removed from the outside surface of protoplasts corresponds exactly in size to that expected for the entire predicted amino-terminal extracytoplasmic domain. In addition, plasma membrane Wak1 is not extracted by the chaotropic agent NaOH. These results are consistent with a trans-membrane topology of Wak1, where the kinase is cytoplasmic. Domain-specific antisera, however, will be required to verify this conclusion.

A plant cell wall-associated kinase activity has been reported to phosphorylate a viral movement protein (26). This activity is associated with mature leaves and is enriched in plasmodesmata. However, Wak1 is present in all vegetative tissues, and antiserum do not decorate plasmodesmata in thin sections. Moreover, Wak1 antiserum does not react with any protein in extracts that contain the plasmodesmata kinase activity.[3] We have detected autophosphorylation of Wak1 in bacterial extracts containing recombinant Wak1 protein, and nonspecific phosphorylation of substrates *in vitro*, and thus Wak1 does indeed have kinase activity (12).

The Wak1 cDNA was initially isolated through a selection assay (27, 28) that was designed to isolate molecules that interacted with the amino terminus of a thylakoid membrane protein. The Wak1 cDNA indeed interacted genetically with the target in yeast (11) and can phosphorylate the target sequence *in vitro* (12). However, as the target and Wak1 are present in separate cellular compartments, it is probable that the substrate specificity of Wak1 is relaxed when it is expressed in yeast or when it is provided with an abundant substrate. We have generated an additional antiserum to the Wak1 kinase domain, and this serum specifically recognizes a 50-kDa thylakoid protein called Tak1, that does interact with LHCP.[2] On prolonged exposures of Westerns, Wak1 antiserum can detect Tak1, and this cross-reactivity may explain the few gold particles observed within the chloroplast after the immunostaining shown in Fig. 3*A*.

It remains possible that Wak1 serves a structural role in serving as a membrane anchor for the cell wall, and while signaling and structural roles have been assigned to distinct molecules in metazoans, these functions may not be mutually exclusive in the plant cell wall. Future genetic and develop-

[3] Z.-H. He, B. D. Kohorn, and V. Citovsky, unpublished results.

mental analysis in *Arabidopsis* will help to establish the role of Wak1 in communication between the plant extracellular matrix and the cytoplasm.

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