Interaction of the *Arabidopsis* Receptor Protein Kinase Wak1 with a Glycine Rich Protein AtGRP-3

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SUMMARY

The *Arabidopsis* wall-associated receptor kinase, Wak1, is a member of Wak family (Wak1-5) that links the plasma membrane to the extracellular matrix. By the yeast two hybrid screen, we found that a glycine rich extracellular protein, AtGRP-3, binds to the extracellular domain of Wak1. Further *in vitro* binding studies indicate that AtGRP-3 is the only isoform among the six tested AtGRPs that specifically interacts with Waks and the cysteine-rich carboxy-terminus of AtGRP-3 is essential for its binding to Wak1. We also show that Wak1 and AtGRP-3 form a complex with a molecular size of approximately 500 kDa *in vivo* in conjunction with the kinase associated protein phosphatase, KAPP, that has been shown to interact with a number of plant receptor-like kinases. Binding of AtGRP-3 to Wak1 is shown to be crucial for the integrity of the complex. *Wak1* and *AtGRP-3* are both induced by salicylic acid treatment. Moreover, exogenously added AtGRP-3 upregulates the expression of *Wak1*, *AtGRP-3*, and *PR-1* (for pathogenesis-related) in protoplasts. Taken together, our data suggest that AtGRP-3 regulates Wak1 function through binding to the cell wall domain of Wak1 and that the interaction of Wak1 with AtGRP-3 occurs in a pathogenesis-related process *in planta*. 
INTRODUCTION

Extracellular matrix (ECM) is a dynamic zone harboring active components that regulate cell-cell interactions of developmental processes and responses to the environment (1). ECM molecules interact with plasma membrane proteins to initiate and modulate diverse signaling pathways that play key roles in essential cellular processes (2). While these events are well understood in animals (3), their study in land plants is in its infancy (4-9). The interactions of cell surface receptors with ligands and extracellular molecules in ECM or cell wall may be involved in these events.

Waks, cell wall associated receptor kinases, physically link the plant ECM to the plasma membrane and can also serve as signaling molecules (10, 11). They contain an extracellular domain that mediates tight association with the cell wall, a transmembrane domain, and a cytoplasmic protein kinase domain (12). While Waks are highly conserved in the kinase domains, they share only 40 to 64% sequence identities in the extracellular domains. Waks have in their extracellular domains epidermal growth factor (EGF)-like repeats and regions similar to the known ECM proteins that include tenascins, collagens, and neurexins (12). Considering the less conserved sequences and the sequence similarity with various ECM proteins in the extracellular domains, it is supposed that the Wak isoforms are functionally distinct and mediate diverse extracellular signals from ECM to the cells.
Wak1 was shown to be implicated in the defense mechanism against pathogen (13). Wak1 is induced by pathogen or exogenously added salicylic acid (SA) or 2,6-dichloroisonicotinic acid (INA), which requires a positive regulator NPR1/NIM1 (14). While Wak1 has thus been regarded as a pathogenesis-related (PR) protein, the physiological functions of Waks are largely unknown. Identification of the upstream and/or downstream molecules of Waks is crucial to elucidate the physiological roles of Waks and the molecular mechanisms underlying the Wak activities.

In this study, we report that a glycine rich secreted protein, AtGRP-3 (15), specifically binds to the extracellular domain of Wak1. The coimmunoprecipitation and size exclusion chromatography experiments combined with Western blotting show that Wak1, AtGRP-3, and kinase associated protein phosphatase (KAPP) are associated into a multimeric complex of 500 kDa in size in vivo and the binding of AtGRP-3 to Wak1 is essential for the structural integrity of the complex. We also demonstrate that AtGRP-3 induces PR-1, a pathogen-related gene, and triggers a positive feedback loop that upregulates the expression of Wak1 and AtGRP-3. We propose that AtGRP-3 is an extracellular molecule that binds to and regulates Wak functions in Arabidopsis.
EXPERIMENTAL PROCEDURES

Yeast Two Hybrid Screen

The MATCHMAKER Two-Hybrid System (Clontech) was used for screening. The Arabidopsis cDNA library was constructed in GAL4 activation domain vector (pGAD424) as described (16). The bait construct was prepared by cloning the region for the extracellular domain of Wak1 (amino acids 178-334) into GAL4 binding domain vector (pGBT9). The HF7c yeast strain was sequentially transformed with the bait construct and then with the cDNA library. Transformants (9x10⁶) were screened for activation of the HIS3 and lacZ reporter genes. The full-length AtGRP-3 and AtGRP-3S genes were isolated from the cDNA library by polymerase chain reaction (PCR). The interaction between the two proteins was quantitated by liquid β-galactosidase assay using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate.

Construction and Purification of Recombinant Proteins

For Waks, the coding region for the extracellular domain was cloned into pGEX vector (Pharmacia). glutathione S-transferase (GST) fusions include amino acids 25-334 of Wak1, 23-328 of Wak2, 23-327 of Wak3, 23-331 of Wak4, and 20-280 of Wak5. AtGRP-3 (amino acids 25-145) was constructed in pET15b vector (Novagen) that produced a fusion protein
with C-terminal hexahistidine tag. *E. coli* BL21 cells transformed with the constructs were grown in LB containing 50 µg/ml ampicillin to an OD$_{600}$ 0.4-0.5 and the expression of Wak and AtGRP-3 proteins were then induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 30°C or for 2 h at 37°C, respectively. Cells were harvested, resuspended in lysis buffer (20 mM HEPES, pH 7.8, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin), and sonicated. Lysates were centrifuged to remove insoluble material and incubated with Glutathione Sepharose 4B (Pharmacia) for Waks or with Ni$^{2+}$ ProBond (Invitrogen) for AtGRP-3. Fusion proteins were purified according to the manufacturer's instructions.

*In Vitro Binding Assay*

GST-Wak fusion proteins (3-5 µg) and [³⁵S-Met] or [³⁵S-Cys]-labeled *in vitro* translated AtGRPs were incubated in binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 µg/ml leupeptin). Glutathione Sepharose 4B resins were added and incubated for 2 h at 4°C with constant rotation. The resins were washed three times with the binding buffer and resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. Proteins were separated on 15% SDS-polyacrylamide gels and visualized by autoradiography.
Antibody Generation and Protein Analysis

Polyclonal antibodies were raised in mouse to recombinant AtGRP-3 protein and in rabbit to Wak1-specific peptide (amino acids 98-112). The specificities of antibodies were confirmed with both recombinant proteins and plant extracts (Fig. 3 A and B). In Western analysis, the proteins were separated on 12.5% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with these antibodies. The antibody-bound proteins were detected by incubation with secondary antibodies conjugated to horseradish peroxidase using the ECL system (Amersham).

Protoplast Preparation

*Arabidopsis* leaf tissues were incubated in protoplast isolation buffer (1% Cellulase Onozuka R-10, 0.25% Macerozyme R-10, 400 mM mannitol, 8 mM CaCl₂, 5 mM MES, pH 5.6, 1 mM PMSF, 10 µg/ml leupeptin) at 25°C for 2.5-3 h with gentle shaking at 50-75 rpm, as described (17). The isolated protoplasts were filtered through a 70 µm nylon mesh and centrifuged for 10 min at 60 X g. The pelleted protoplasts were gently resuspended and washed two times with 500 mM mannitol. The protoplasts were resuspended to 1 x 10⁶ cells per ml in K₃ medium (400 mM sucrose, 4.3 g/l Murashige and Skoog salts, 100 mg/l *myo*-inositol, 250 mg/l xylose, 460 mg/l CaCl₂, 10 mg/l thiamine, 1 mg/l pyridoxine, 1 mg/l nicotinic acid, pH 5.8, 1 mM PMSF, 10 µg/ml leupeptin). The isolated protoplasts were
incubated with phosphate-buffered saline (PBS) or purified AtGRP-3 proteins at room temperature for the indicated time. The protoplasts were then harvested by centrifugation. In this step, most of unbound AtGRP-3 proteins are removed from protoplasts.

**Protein Preparation**

Protein extracts from *Arabidopsis* seedlings and protoplasts were prepared in the extraction buffer A (50 mM Tris, pH 7.8, 150 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 10 µg/ml leupeptin, protein phosphatase inhibitor cocktail I (Sigma), 1 % Triton X-100). For gel filtration, proteins were extracted in the extraction buffer B (50 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 10 µg/ml leupeptin, protein phosphatase inhibitor cocktail I, 10 % glycerol, 0.1 % Triton X-100). Protein extracts were further centrifuged to remove insoluble materials. Protein concentration was determined with Bio-Rad assay system.

**Immunoprecipitation**

The aliquot (1 mg of protein) in the buffer A was incubated with preimmune or immune serum in the same buffer overnight at 4°C. Protein A/G PLUS-Agarose (Santa Cruz) was added and further incubated for 2 h at 4°C. The immunoprecipitate was washed and subjected to SDS-PAGE and immunoblotting.
**Size Exclusion Chromatography**

Protein extracts in the buffer B were applied to size exclusion fast protein liquid chromatography (FPLC). Proteins were fractionated over Superose 6 HR 10/30 column (Pharmacia) equilibrated with the running buffer (50 mM Tris, pH 7.6, 50 mM NaCl). Fractions (0.5 ml) were collected at a flow rate of 0.3 ml/min.

**RNA Analysis**

RNA was extracted using 0.5 ml of Trizol reagent (GIBCO-BRL) per $10^6$ cells of protoplasts. Contaminated genomic DNA from total RNA was removed as described (18). Total RNA (1 µg) was treated with 6 units of RNase-free DNase I (GIBCO-BRL) for 5 min at 37°C. The activity of DNase I was inactivated by boiling. The RNA was then cleaned up by RNeasy mini kit (Qiagen). Reverse transcriptase-PCR (RT-PCR) was performed with 0.1 µg of total RNA using Acess RT-PCR system (Promega).
RESULTS

Interaction of Wak1 with Glycine Rich Proteins AtGRP-3 and AtGRP-3S

To identify proteins that bind to the extracellular domain of Wak1, we have performed the yeast two hybrid screen (19). A library of Arabidopsis seedling cDNAs fused to the GAL4 activation domain was screened against the construct encoding the extracellular domain of Wak1 fused to the GAL4 DNA binding domain. Among the positive clones, of interest was AtGRP-3 that is a member of a previously described family of glycine-rich secreted cell wall proteins (20-22). While we were trying to isolate full length AtGRP-3 cDNA by PCR, we identified a closely related sequence that is 81% identical to AtGRP-3 and lacks ca. 25 amino acids near the amino-terminus (Fig. 1A). This short form of AtGRP-3 is hereafter referred to as AtGRP-3S.

To confirm the interaction between Wak1 and AtGRP-3, the extracellular domain of Wak1 (ED/pGBT9) and full length AtGRP-3 (AtGRP-3/pGAD424) or AtGRP-3S (AtGRP-3S/pGAD424) fusion constructs were cotransformed into a yeast reporter strain HF7c. The transformants were then tested for their ability to grow on the selective plates lacking histidine and the expression of the β-galactosidase activity (Fig. 1B). The results indicated that both AtGRP-3 and AtGRP-3S bind to the extracellular domain of Wak1.

To further confirm the interaction of Wak1 with AtGRP-3 and AtGRP-3S, an in vitro
Interaction of Wak1 with AtGRP-3

binding assay was performed (Fig. 1C). Purified GST (lane 1) or GST-extracellular domain of the Wak1 fusion protein (GST-ED) (lane 2) was incubated with the \textit{in vitro} translated $[^{35}\text{S}-\text{Cys}]-labeled$ AtGRP-3S (lane 3) or AtGRP-3 (lane 4), and the resulting complexes were isolated by glutathione Sepharose beads. Both AtGRP-3S and AtGRP-3 bound to GST-ED (lanes 6 and 8) but not to GST alone (lanes 5 and 7), indicating that Wak1 specifically interacts with both AtGRP-3 and AtGRP-3S.

\textit{Isoform-Specific Interaction between Wak1 and AtGRP-3}

Five \textit{Wak} genes (\textit{Wak1-}Wak5) (12) and multiple \textit{AtGRP} genes (15, 23-25) have previously been identified from \textit{Arabidopsis}. The complete \textit{Arabidopsis} genome sequence also reveals that there exist additional Wak and GRP family members. Therefore, we tested whether the interaction between Wak1 and AtGRP-3 we have shown above is isoform-specific or it merely represents one of the general interactions between Waks and AtGRPs. The purified GST-ED was incubated with the \textit{in vitro} translated $[^{35}\text{S}-\text{Cys}]-$ or $[^{35}\text{S}-\text{Met}]-labeled$ AtGRPs, and the resulting complexes were collected by glutathione Sepharose beads and separated on a SDS-gel (Fig. 2A). The results demonstrated that Wak1 binds only to AtGRP-3 but not other tested AtGRPs (AtGRP-2, 4, 6, 7, 8). The binding specificity of AtGRP-3 was then tested against the five Wak isoforms. The \textit{in vitro} translated $[^{35}\text{S}-\text{Cys}]-labeled$ AtGRP-3 was incubated with the purified GST-ED of the Wak fusion proteins, and
the complexes were analyzed (Fig. 2B). The results indicated that AtGRP-3 binds to Wak1, Wak3, and Wak5. We obtained the same results with AtGRP-3S (data not shown), implying that AtGRP-3 and AtGRP-3S may play a similar physiological role. Notably, AtGRP-2, 4, 6, 7, and 8 did not bind to any of the Wak isoforms (data not shown), indicating that AtGRP-3 and AtGRP-3S are unique among AtGRPs in their ability to interact with Waks.

We next localized the region in AtGRP-3 that is responsible for the interaction with Wak1. Constructs for the amino- and carboxy-terminus deleted forms (AtGRP-dN and AtGRP-dC, respectively) and carboxy-terminal region (AtGRP-CT) of AtGRP-3 were generated (Fig. 2C). The proteins were in vitro-translated and subjected to the in vitro binding assay with GST alone or GST-ED (Fig. 2D). Interactions were observed with full length AtGRP-3, AtGRP-dN and AtGRP-CT but not with AtGRP-dC, indicating that the cysteine-rich carboxy-terminus is essential for the association of Wak1 and AtGRP-3.

Association of Wak1, AtGRP-3, and KAPP

We have generated anti-Wak1 and anti-AtGRP-3 antibodies as described in the “Experimental Procedures”. The anti-Wak1 antibody specifically recognized only the ED of Wak1 but not those of other tested Wak proteins (Fig. 3A). Western blotting of Arabidopsis protein extracts with anti-Wak1 antibody detected two bands that migrate as approximately 78 kDa and 100 kDa proteins on a SDS-gel. Since the expected size of Wak1 is 78 kDa, the
slowly migrating band is likely to be a posttranslationally modified form (e.g. glycosylated) of Wak1 (Fig. 3B, lane 1). Anti-AtGRP-3 antibody recognized an 18 kDa band that is larger than the expected 12 kDa of AtGRP-3 (lane 2). This discrepancy is unlikely to be due to a posttranslational modification, because the in vitro translated (Fig. 1C) or recombinant AtGRP-3 protein purified from E. coli (Fig. 3A) also migrated as an 18 kDa protein. Protein extracts from Arabidopsis seedlings were subjected to immunoprecipitation with preimmune or anti-AtGRP-3 antibody (Fig. 3C). The precipitated proteins were separated on a SDS-gel and probed with anti-Wak1 antibody. The results showed that both 78 and 100 kDa Wak1 proteins were co-precipitated with AtGRP-3, indicating that Wak1 and AtGRP-3 form a complex in vivo.

As KAPP has been shown to interact with a number of plant receptor-like kinases (RLKs) (26), we tested whether KAPP is also associated with the Wak1-AtGRP-3 complex. The proteins immunoprecipitated by anti-AtGRP-3 antibody were probed with anti-KAPP antibody that recognized a 65 kDa KAPP protein on a Western blot (Fig. 3B, lane 3). The presence of KAPP in the immunoprecipitates (Fig. 3C) suggests that KAPP is a component of the complex that includes Wak1 and AtGRP-3.

**Association of Wak1 and AtGRP-3 in the Multimeric Complex in Vivo**

To further analyze the protein complex of Wak1, AtGRP-3, and KAPP, protein extracts
of Arabidopsis seedlings were subjected to size exclusion chromatography. The fractions were then probed with anti-Wak1, anti-AtGRP-3, and anti-KAPP antibodies (Fig. 4A). The molecular sizes were extrapolated from a standard curve prepared with a set of marker proteins. Wak1 proteins were present in two complexes with the apparent molecular sizes of 500 and 200 kDa, respectively. It is noticeable that 100 kDa and 78 kDa Wak1 proteins were separated into the 500 and 200 kDa complexes. AtGRP-3 was fractionated along with the 500 kDa complex, but not with the 200 kDa complex. This suggests that AtGRP-3 is stably associated with the 100 kDa Wak1 in the 500 kDa complex. AtGRP-3 that runs as a 48 kDa is likely the unbound form of AtGRP-3. KAPP was detected mostly in the range of 500-600 kDa, implying the association of KAPP with a number of RLKs as reported (26).

To elucidate the role of AtGRP-3 in the formation of the 500 kDa complex, we analyzed protein extracts of protoplasts by size exclusion chromatography (Fig. 4B). AtGRP-3 was completely absent in the protoplasts as determined by Western blotting. In the absence of AtGRP-3, Wak1 was detected mostly in the 200 kDa complex (~AtGRP-3). Upon the addition of the recombinant AtGRP-3 protein to protoplasts, Wak1 reappeared associated with the 500 kDa complex and the added AtGRP-3 was also eluted along with the 500 kDa complex (+AtGRP-3). These data suggest that AtGRP-3 is essential for the structural integrity and/or probably the formation of 500 kDa complex that includes Wak1 and AtGRP-3. The recombinant AtGRP-dC that lacks the 35 carboxy-terminal amino acids was
ineffective (+AtGRP-dC), suggesting that the direct interaction between Wak1 and AtGRP-3 is critical for the formation and/or maintenance of the integrity of the 500 kDa complex.

Co-expression of Wak1 and AtGRP-3 in Tissues and Co-induction by Salicylic Acid Treatment

In an effort to evaluate the biological relevance of the interaction between Wak1 and AtGRP-3, we examined the expression pattern of *Wak1* and *AtGRP-3* in *Arabidopsis* tissues by northern analysis. *Wak1* and *AtGRP-3* were co-expressed predominantly in leaves and stems (Fig. 5A), which is consistent with previous reports (12, 13, 15). It was also shown that *Wak1* expression is induced by SA and this induction requires the positive regulator NPR1/NIM1 (12). We thus analyzed the expression of *Wak1* and *AtGRP-3* in response to SA. Northern analysis showed that both *AtGRP-3* and *Wak1* were induced upon SA treatment (Fig. 5B). This induction was abolished by mutation in *npr1* and by overexpression of *nahG* which degrades SA (27, 28) (Fig. 5C). These data suggest that the functions of Wak1 and AtGRP-3 are closely related and implicated in the SA signaling.

Upregulation of Gene Expression by Exogenously Added AtGRP-3 in Protoplasts

We tested by RT-PCR whether the treatment of protoplasts with AtGRP-3 resulted in the regulation of gene expression (Fig. 6). The expression of *Wak1* and *AtGRP-3* was upregulated by the addition of AtGRP-3 in a dose-dependent manner (Fig. 6 A and C),
indicating that *Wak1* and *AtGRP-3* are in a positive feedback loop that enhances the *AtGRP-3*-Wak1 signaling. The addition of *AtGRP-3* to protoplasts also induced *PR-1*, a molecular marker of SA-dependent defense response, but not *PDF1.2* (for plant defensin), a molecular marker of JA-dependent defense response (29, 30). It is worth noting that the expression of *Wak1* and *AtGRP-3* was induced by SA (Fig. 5B). In contrast to *AtGRP-3*, the exogenously added *AtGRP-dC* and bovine serum albumin (BSA) barely affected the expression level of the tested genes (Fig. 6B and D). Taken together with the fact that *AtGRP-3* binds to Wak1 in a specific manner, these data suggest that *AtGRP-3* is a physiological ligand of Wak1, and the *AtGRP-3*-Wak1 signaling pathway is involved in the SA-dependent defense response.
DISCUSSION

Plant cells are encompassed by the cell wall or ECM that is complex in structure and function (5, 8, 20, 22). In addition to conferring physical strength, the plant cell wall regulates cell growth, development, and cell-cell and cell-environment interactions. While the plant cell wall harbors carbohydrates and proteins that actively change in structure and composition, little is known about the precise functions of the cell wall components and their interactions.

Waks are cell wall-associated kinases and the different isoforms vary significantly in the extracellular domains (12). This raises the possibility that the extracellular domains of Wak proteins may be involved in the interaction with diverse wall components in cell type- or stage-dependent manner. Waks may perceive differential signals from the changing environment through the interaction of extracellular domain with cell wall molecules and transduce the signals to the cell.

AtGRP-3 is a member of GRP family and likely a cell wall protein with a predicted signal peptide at the amino-terminus (15). Its amino acid sequence has high content of glycine (31%) and its mid-region is marked by a glycine-rich domain of 57 amino acids with the sequence Gly$_4$-Asn/Arg-Tyr-Gln repeated six times. The signal peptide and carboxy-terminal cysteine-rich motif show sequence similarity to several nodule-specific plant-encoded proteins (nodulins) (31). In a recent report, the cysteine-rich carboxy-terminal region of
NtTLRP, a cell wall protein in tobacco that is highly homologous to that of AtGRP-3 was sufficient for cross-linking of proteins to cell walls (32). However, the physiological functions of GRPs are unknown. The sequences of the GRP family members are divergent with little conserved regions, which suggests that GRPs may play diverse biological roles (21).

In this study, we have shown that AtGRP-3 specifically binds to Wak1 both in vitro and in vivo. We also provide several lines of evidence suggesting that Wak1-AtGRP-3 interaction is physiologically relevant and the binding of AtGRP-3 regulates the signaling activity of Wak1. First, Wak1, AtGRP-3 and KAPP are associated into a 500 kDa complex in vivo that may represent the activated signalosome (33-35) and the direct binding of AtGRP-3 to Wak1 is critical for the integrity of the 500 kDa complex. These interactions share many features with the regulation of CLAVATA1 (CLV1) receptor kinase (36, 37). Second, exogenously added recombinant AtGRP-3 to protoplasts upregulated the expression of PR-1 as well as Wak1 and AtGRP-3. In the previous report, the expression of antisense Wak1 allele in transgenic plants inhibits the induction of PR-1 response (13). Involvement of both Wak1 and AtGRP-3 in PR-1 induction suggests that these molecules are closely related in their biological functions.

On a SDS-gel, Wak1 migrates as two bands with the molecular sizes of 78 and 100 kDa, respectively. The slowly migrating 100 kDa protein appears to be the functional Wak1
because this protein but not the 78 kDa protein is associated with AtGRP-3 and KAPP to form the 500 kDa complex. In protoplasts, all the Wak1 proteins exist as the seemingly incompetent 78 kDa protein. Upon the addition of recombinant AtGRP-3, the 100 kDa Wak1 reappeared. It is possible that either AtGRP-3 invokes the modification of 78 kDa resulting in the appearance of 100 kDa Wak1 or AtGRP-3 prevents the 100 kDa Wak1 from being cleaved to 78 kDa Wak1 thus resulting in the accumulation of 100 kDa Wak1. The detailed molecular mechanism underlying the communication between Wak1 and ArGRP-3 remains to be seen. However, switching between active and inactive forms of Wak1 by the posttranslational modification triggered by AtGRP-3 provides a unique mechanism for the modulation of receptor function by ligand molecules.

The noticeable feature of the carboxy-terminal region of AtGRP-3 is the presence of six conserved cysteine residues. The sequence alignment of Wak isoforms also exhibits the conserved cysteine residues in twenty-four places (12). It would be interesting to see whether the conserved cysteine residues in AtGRP-3 and Wak1 participate in the interaction between two molecules and with others. Disulfide bonding is reversibly dependent on the redox state in the cell wall that would be changing in different developmental stages. The reactive oxygen species generated under various stress conditions such as wounding, pathogen attack, cold or ozone stress would also affect the redox condition of the cell wall (38-41). There have been reports that the oxidative cross-linking of the cell wall proteins plays an important role
as a defense mechanism in response to pathogen attack (42, 43). It is conceivable that the oxidative modifications of cysteine and other residues may regulate the interaction of AtGRP-3, Wak1, and other proteins and therefore the signaling of Wak1. In view of the finding that both \textit{Wak1} and \textit{AtGRP-3} are induced in response to SA and modulate \textit{PR-1} in a positive way as shown in this study and others (13, 15), it appears likely that Wak1 and AtGRP-3 are involved in pathogen response. AtGRP-3 and its homologues in tobacco and petunia have expression patterns that suggest a role in the defense mechanism (44, 45). It is also conceivable that oxidative change in AtGRP-3 and Wak1 proteins may be the underlying mechanism of pathogenesis-related response of Wak1 and AtGRP-3.

To date only a few plant plasma membrane receptors have been characterized in association with their ligands. A good example is the meristem regulator CLV1, the \textit{Arabidopsis} receptor kinase that regulates cell proliferation and organ formation at the meristems (46). Both genetic and biochemical studies provided evidence that CLV3 acts as the ligand for CLV1 as part of a multimeric complex (34, 35, 47). Another illustrative example would be BRI1 and SRK that are implicated in brassinosteroid signaling and self-incompatibility, respectively (48-50). Here the results add to the observation from other RLKs, that plant receptor kinases can be regulated by soluble secreted ligands.
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The abbreviations used are: KAPP, kinase associated protein phosphatase; ECM, extracellular matrix; RLK, receptor-like kinase; SA, salicylic acid; INA, 2,6-dichloroisonicotinic acid; PR, pathogenesis-related; EGF, epidermal growth factor; GST, glutathione S-transferase; ED, extracellular domain; CLV, CLAVATA; ONPG, o-nitrophenyl β-D-galactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; RT-PCR, reverse transcriptase-polymerase chain reaction.
FIGURE LEGENDS

Fig. 1. **Wak1 interacts with AtGRP-3 and AtGRP-3S.** *A*, amino acid sequence comparison of AtGRP-3 and AtGRP-3S. The accession numbers are S47409 and AF104330 for AtGRP-3 and AtGRP-3S, respectively. Identical residues are shaded. *B*, interaction of Wak1 with AtGRP-3 and AtGRP-3S in yeast. The interaction was monitored by the growth of yeast cells on the selective media lacking histidine (-His) and by assaying the β-galactosidase (β-gal) activity using ONPG. *C*, *in vitro* binding of Wak1 to AtGRP-3 and AtGRP-3S. Coomassie Blue-stained gel of GST control (lane 1) and the GST-ED fusion protein (lane 2). Autoradiogram of *in vitro* translated [35S-Cys]-labeled AtGRP-3S and AtGRP-3 (lanes 3 and 4). Shown is 10% of the amount used in binding reactions. AtGRP-3S and AtGRP-3 bind to GST-ED (lanes 6 and 8) but not to GST (lanes 5 and 7).

Fig. 2. **Wak1 and AtGRP-3 show specific interaction.** *A*, comparative binding analysis of Wak1 with various AtGRPs. Autoradiograms of *in vitro* translated [35S-Cys] or [35S-Met]-labeled AtGRPs (top). Shown is 10% of the input used in binding reactions. Bound proteins are shown in the bottom panel. GST-ED (3 μg) was incubated with similar amounts of *in vitro*-translated AtGRPs (10-30 μl). *B*, comparative binding analysis of AtGRP-3 with various Waks. Coomassie Blue-stained gel of purified GST-ED of various Waks (top).
Autoradiogram of AtGRP-3 (15 µl) that binds to GST-ED (5 µg) (bottom). C, schematic representation of AtGRP-3 and the various constructs used in these experiments. The putative signal peptide (amino-terminal 23 amino acids) was removed. D, *in vitro* binding of Wak1 and various truncated forms of AtGRP-3. Autoradiogram of *in vitro* translated $[^{35}\text{S}}\text{-Cys]}$ or $[^{35}\text{S}}\text{-Met]}$-labeled AtGRP-3 wild type and truncated forms (left). Shown is 10 % of the amount used in binding reactions. Bound proteins are shown in the right panel. GST-ED (3 µg) was incubated with similar amounts of various *in vitro*-translated AtGRP-3 deletion proteins (10-30 µl). AtGRP-dN, amino-terminus deleted AtGRP-3; AtGRP-dC, carboxy-terminus deleted AtGRP-3; AtGRP-CT, carboxy-terminal region of AtGRP-3.

Fig. 3. **AtGRP-3 is associated with Wak1 and KAPP in vivo.** A, specificity test of anti-Wak1 and anti-AtGRP-3 antibodies. Purified GST and GST-ED of various Waks, and AtGRP-3 separated on SDS-polyacrylamide gels were Coomassie blue-stained (top), or immunoblotted with anti-Wak1 (lanes 1-6) or anti-AtGRP-3 (lane 7) antibody (bottom). B, Western analysis of *Arabidopsis* protein extracts with anti-Wak1 (lane 1), anti-AtGRP-3 (lane 2), and anti-KAPP (lane 3) antibodies. C, Western analysis of AtGRP-3-associated complex immunoprecipitated from *Arabidopsis* tissue. Tissue extracts were immunoprecipitated with preimmune (PI) (lane 1) or anti-AtGRP-3 (lane 2) antibody. Immunoprecipitated proteins were subjected to immunoblotting with anti-Wak1 (top), anti-AtGRP-3 (middle), and anti-
KAPP (bottom) antibodies. IP, immunoprecipitation; IB, immunoblotting.

Fig. 4. **Wak1 and AtGRP-3 are associated in the multimeric complex.** The multimeric status of Wak1 and AtGRP-3 was investigated by size exclusion chromatography. The fractions (0.5 ml) collected from Superose 6 FPLC were subjected to Western analysis with the antibodies indicated on the right side of the panels. The distribution of standard protein markers is indicated on top. FN, fraction number; IB, immunoblotting. **A**, size fractionation of protein extracts from *Arabidopsis* seedlings (Tissue). **B**, size fractionation of protein extracts from protoplasts treated with PBS (−AtGRP-3), AtGRP-3 (+AtGRP-3), or AtGRP-dC (+AtGRP-dC) as indicated on the left side. After treatment with proteins (50 µg per 10⁶ cells) for 30 min, protoplasts were harvested to remove unbound proteins.

Fig. 5. **Wak1 and AtGRP-3 are expressed in the same tissues and induced by SA treatment.** Total RNA from leaves and stems was extracted 1 day after application of SA or water. The RNA gel blots containing 20 µg of total RNA per lane were hybridized with a digoxigenin-dUTP labeled *Wak1* or *AtGRP-3* probe. The ethidium bromide-stained 25S rRNA served as a loading control. **A**, northern analysis of *Wak1* and *AtGRP-3* expression in various tissues. **B**, northern analysis of the induction of *Wak1* and *AtGRP-3* in response to SA treatment. A solution of SA with the indicated concentration or water was applied to the
entire *Arabidopsis* plants. Total RNA was isolated from leaves or stems 1 day after treatment.

*C*, northern analysis of the induction of *Wak1* and *AtGRP-3* upon SA treatment in wild type, *npr1*, and *nahG* plants.

**Fig. 6.** *AtGRP-3* treatment induces gene expression in protoplasts. RT-PCR analysis of *AtGRP-3*, *Wak1*, *PR-1*, and *PDF1.2* in protoplasts. *Actin1* was used as a control. **A**, protoplasts were treated with different amounts of *AtGRP-3* proteins (µg per 10⁶ cells) for the indicated time. **B**, RNA levels were compared in the protoplasts treated with PBS or proteins (50 µg per 10⁶ cells) such as *AtGRP-3*, *AtGRP-dC*, and BSA for 30 min. **C**, quantitative analysis of the data in **A**. The accumulated RNA levels in the protoplasts treated with *AtGRP-3* for 30 min were assessed by densitometric measurement and calculated as the fold induction, compared to the RNA level in the untreated (0 µg) protoplasts. **D**, quantitative analysis of the data in **B**. The induction of RNA levels were estimated as described in **C**.
A

AtGRP-3 1 MALKAVLLGLFAVLLVSEVAAASSATVNSSEKETVQPDQRYGDNGGN
AtGRP-3S 1 MALKAVLLGLLAPFLVSEMAAAAC--TVKSEETVQPEQH

AtGRP-3 51 YNNCCYQSCCGNYYQGGNYQSGGYQCCGYQCCGYQGCGQRYQ
AtGRP-3S 41 ---------------GGFGDGNGGQGYQGHHGQGQGGQRYQ

AtGRP-3 101 GGGRQGGGQYCRHCCYRNYCSCSCTCYAEGAVQTQPH
AtGRP-3S 75 GGGRQGGGQ---CSCFHCYCCYKGYHCSCTCTCYAEGAVQTQPH

B

|                   | pGBT9 |               | ED/pGBT9 |
|-------------------|-------|----------------|----------|
|                   | +His  | −His | β-gal assay | +His  | −His | β-gal assay |
| AtGRP-3/ pGAD424 | +     | −    | 0           | +     | +    | 0.648±0.088 |
| AtGRP-3S/ pGAD424| +     | −    | 0           | +     | +    | 0.785±0.107 |

C

[Image of protein expression analysis]
Fig. 2

A

| Mr(K) | AtGRP-3 | AtGRP-2 | AtGRP-4 | AtGRP-6 | AtGRP-7 | AtGRP-8 |
|-------|---------|---------|---------|---------|---------|---------|
| 45    |         |         |         |         |         |         |
| 30    |         |         |         |         |         |         |
| 18.4  |         |         |         |         |         |         |
| 45    |         |         |         |         |         |         |
| 30    |         |         |         |         |         |         |
| 18.4  |         |         |         |         |         |         |

B

| Mr(K) | Wak1 | Wak2 | Wak3 | Wak4 | Wak5 |
|-------|------|------|------|------|------|
| 68    |      |      |      |      |      |
| 45    |      |      |      |      |      |
| 18.4  |      |      |      |      |      |
| 14.3  |      |      |      |      |      |

C

AtGRP-3

- ASSATYNSESKEKTVKP
- DQRGYGDNGGNYNN
- G-rich domain (G4R/NYQ)₆

AtGRP-dN

- 29 aa
- 57 aa
- 35 aa

AtGRP-dC

AtGRP-CT

SRGSCYCRHGCYCYRGYN
CSRCYCSYAGEAVQTQPQH

D

| Mr(K) | AtGRP-3 | AtGRP-dN | AtGRP-dC | AtGRP-CT |
|-------|---------|----------|----------|----------|
| 18.4  |         |          |          |          |
| 14.3  |         |          |          |          |
| 6.2   |         |          |          |          |

| Mr(K) | GST | GST-ED | GST | GST-ED | GST | GST-ED | GST | GST-ED |
|-------|-----|--------|-----|--------|-----|--------|-----|--------|
| 18.4  |     |        |     |        |     |        |     |        |
| 14.3  |     |        |     |        |     |        |     |        |
| 6.2   |     |        |     |        |     |        |     |        |
Fig. 3

A

B

C

IB

Wak1

AtGRP-3

KAPP

IB

AtGRP-3

KAPP

IB

Wak1

AtGRP-3

KAPP
**Fig. 5**

A and B show Western blot analysis of different tissues (Leaf, Stem, Flower, Root) for various proteins and RNA. The blot images include Wak1, 25S rRNA, AtGRP-3, and AtGRP-3 in different treatments.

C compares the expression levels of Wak1, AtGRP-3, and rRNA in Wild type, npr1, and nahG mutants across different SA concentrations (0, 1, 2 mM).
Interaction of the Arabidopsis Receptor Protein Kinase Wak1 with a Glycine Rich Protein AtGRP-3
Ae Ran Park, Somi K. Cho, Ui Jeong Yun, Mi Young Jin, Seoung Hyun Lee, Gilberto Sachetto-Martins and Ohkmae K. Park

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