The Role of Protein Kinases ROG1 and SRF6 in the WAK Stress Response Pathway

Jaepil E. Yoon
Bowdoin College, ericyoon93@gmail.com

Follow this and additional works at: https://digitalcommons.bowdoin.edu/honorsprojects

Part of the Biochemistry Commons, and the Plant Sciences Commons

Recommended Citation
Yoon, Jaepil E., "The Role of Protein Kinases ROG1 and SRF6 in the WAK Stress Response Pathway" (2015). Honors Projects. 79.
https://digitalcommons.bowdoin.edu/honorsprojects/79

This Open Access Thesis is brought to you for free and open access by the Student Scholarship and Creative Work at Bowdoin Digital Commons. It has been accepted for inclusion in Honors Projects by an authorized administrator of Bowdoin Digital Commons. For more information, please contact mdoyle@bowdoin.edu.
The Role of Protein Kinases ROG1 and SRF6 in the WAK Stress Response Pathway

An Honors Project for the Program of Biochemistry

By Jaepil Eric Yoon

Bowdoin College, 2015

© 2015 Jaepil Eric Yoon
# TABLE OF CONTENTS

Acknowledgements ........................................................................................................... iii  
Abstract ................................................................................................................................. iv  
Introduction .......................................................................................................................... 1  
Materials and Methods ........................................................................................................ 8  
  SRF6-GFP cloning and transformation ............................................................... 8  
  SRF6 mutant in phosphorylation sites transformed in plants ................................. 9  
  Purification of proteins in *E. coli* ................................................................. 10  
  Kinase Assays .................................................................................................................. 12  
Results ................................................................................................................................. 14  
  SRF6-GFP Fusion ........................................................................................................... 14  
  SRF6 Mutants ............................................................................................................... 17  
  Kinase Assays .................................................................................................................. 21  
Discussion ............................................................................................................................ 30  
  SRF6-GFP Fusion ........................................................................................................... 31  
  SRF6 Mutants ............................................................................................................... 32  
  Kinase Assays .................................................................................................................. 33  
Color Images in Black and White ..................................................................................... 38  
Literature Cited .................................................................................................................... 48
ACKNOWLEDGEMENTS

I would like to thank Professor Bruce Kohorn and Sue Kohorn for the research experience. From guidance, teaching, lab equipment, concern for health, and deep life conversations, I owe my progress and growth as both a person and scientist to this lab and the people within. I thank my great friend and colleague Josh Benton for his support, light-hearted jokes, and mutual perseverance through the Honors process with me. I thank the Bowdoin College Biochemistry Department for taking me on as an Honors student, all other corroborating peers, and my two readers, Professor McBride and Professor Dube for their help and assistance.
ABSTRACT

The plant cell wall is a complex environment that contains proteins and carbohydrates such as cellulose, hemicellulose, and pectin. The wall-associated kinases (WAKs) are serine/threonine, transmembrane receptor kinases that bind cell wall pectin to activate both an expansion and a stress response pathway in Arabidopsis thaliana. When bound to long pectin polymers, WAKs initiate an expansion pathway, which induces the expression of a vacuolar invertase that increases turgor pressure in the cell to push out against the cell wall. WAKs can also bind pectin fragments called oligogalacturonic acids (OGs), which are generated when plants are wounded or attacked by pathogens. When bound to OGs, WAKs induce a stress response pathway, which leads to the activation of MAP kinases, MPK3 and MPK6. Phosphoproteomics and mass spectrometry were used to identify proteins that were phosphorylated when plants were exposed to OGs. SRF6, a receptor kinase, ROG1, a cytoplasmic kinase, and REM2, a nuclear DNA binding protein, were phosphorylated upon plant exposure to OGs. In order to characterize SRF6 localization in plant cells, a SRF6-GFP fusion was generated in vitro and transformed in Arabidopsis plants, but expression could not be seen under a confocal microscope. The phosphorylation sites of SRF6 were mutated into either glutamic acids (E) or alanines (A) to mimic phosphorylation or to prevent phosphorylation, respectively. The mutants were transformed into Arabidopsis plants. SRF6T361E S362E S364E (p<0.05) and SRF6T361A S362A S364A (p<0.05) mutant plants had leaf lengths that were shorter than those of SRF6 WT plants, and the SRF6T361E S362E S364E mutants displayed curled leaves, which were indicative of a stress response. Kinase assays detected WAK1 phosphorylation of itself. The assays also detected individual
phosphorylation of ROG1 and small levels of phosphomimic mutant ROG1$^{T361E\ S362E\ S364E}$ phosphorylation, but they were not caused by interactions with WAK1, SRF6 or REM2. The phosphorylation of SRF6, SRF6$^{T361E\ S362E\ S364E}$, and REM2 was not detected. Taken together, these results indicate that SRF6 might be involved in the WAK signaling pathways and that other proteins may be required to observe SRF6 and ROG1 activity in vitro.
INTRODUCTION

The plant cell wall is a mosaic environment that defines the size and shape of cells and acts as a barrier to the environment, preventing pathogen entry into the cell. The cell wall is composed of cellulose fibers, hemicellulose, and structural proteins, which reside in a flexible matrix of pectin polysaccharides. Cellulose is a linear chain polysaccharide made of β-1,4-glucose units. Cellulose fibers are held to each other by hydrogen bonds and Van der Waals forces and synthesized by cellulose synthase (CesA) complexes that travel along cortical microtubules near the plasma membrane. Glycans, like pectin and hemicellulose, and proteins are transported from the Golgi to the cell wall where they can be cross-linked by enzymes that are regulated during plant development and by the environment. Pectins, which are polymers of methyl esterified α-1,4 D-galacturonic acid units, are selectively de-esterified at the cell wall by plant or pathogen pectin methylesterases (PME). The resulting negative charge can bind calcium ions to create a cross-linked network, which maintains both the rigidity and structure of the plant cell wall. The PME enzymes are secreted and spatially regulated in specific areas of the cell wall. Thus, the regulation of this cross-linking ultimately dictates the directionality of cell wall expansion during plant development.

A number of different signaling pathways are involved in the regulation and growth of the cell wall. Some examples include the Brassinosteroid (BR) and CrRLK1L pathways, which assist in plant cell growth in diverse ways. There are also pathways dedicated to the perception of pathogens, which must penetrate the cell wall before infection is possible. One such pathway is the flagellin-dependent pathway that activates plant innate immunity when bacterial flagellin is detected. Some signaling mechanisms
are involved in both plant development and pathogen perception. The Wall Associated Kinase (WAK) signaling pathway can initiate both expansion and stress response mechanisms.\textsuperscript{34} These pathways are interconnected because they share certain downstream components and express many related genes. Although the hormones or signaling molecules are different in each pathway, and each has separate receptor-like kinases (RLKs) that initiate a cascade, there seems to be a degree of coordination between these distinct mechanisms.

Since the discovery of the Brassinosteroid pathway in \textit{Brassica napus} pollen, more than 40 BR-like hormones have been shown to initiate growth and cell elongation in \textit{Arabidopsis}.\textsuperscript{5} The pathway signals through a RLK called brassinosteroid-insensitive 1 (BRI1), which, when mutated, generated plants that were severely dwarfed and sterile despite BR treatment.\textsuperscript{13} BRI1 forms signaling complexes with another LRR(leucine-rich repeat)-RLK called BRI1-associated receptor kinase 1 (BAK1) that activates downstream elements.\textsuperscript{37} The complex induces transcriptional changes controlled by transcription factors BZR1 and BES1/BZR2, which target genes related to cell expansion and cell wall biosynthesis.\textsuperscript{46,56}

The CrRLK1L family of receptors that includes Theseus 1 (THE1) and Feronia (FER) is most notably involved in cell wall integrity and remodeling. Theseus 1 (THE1) is proposed to be a cell wall sensor in plants and was found in a screen for suppressors that inhibited the dark green, short hypocotyl phenotype in a plant lacking CESA6, a cellulose synthase catalytic subunit.\textsuperscript{27} The pathway through which THE1 mediates growth repression under cellulose deficiency is unknown. THE1 represses growth to avoid overloading an already weakened, cellulose-deficient cell wall and to prevent cell death...
The RLK Feronia (FER) was first discovered in pollen tubes in which fer plants were unable to induce pollen tube bursting and sperm cell release. The clustering of FER increases the recruitment of Rho GTPases and NADPH oxidases, which elevates levels of extracellular reactive oxygen species and Ca$^{2+}$ within the cell. The link between 'OH production and calcium influx is still unclear. Researchers have argued that the formation of free radicals in the cell wall can cleave polysaccharides and relax the cell wall. This relaxation allows cells to remodel and to restructure their cell walls. THE1 and FER can be induced by brassinosteroids and positively regulated by BES1 transcription factor, but the mechanism is not understood.

The flagellin-dependent pathway is an innate immune response pathway in Arabidopsis. Flagellin, the main component of bacterial flagella, can be recognized by plants as a signal to activate an immune response. The flagellin-dependent pathway is mediated by the RLK, Flagellin Sensitive 2 (FLS2). Mutants of FLS2 showed a decrease in Arabidopsis immune response, which suggested that FLS2 recognition of flagellin is integral for pathway activation. Upon FLS2 activation, the pathway signals through a cascade of MAPK proteins including AtMEKK1 (MAPKKK), AtMKK4a/5a (MAPKK), and MPK3/MPK6. The cascade activates transcription factors WRKY29 and FRK1, which control the expression of genes related to innate immunity. The MAPK signaling cascade is not limited to the flagellin pathway and is shared by many other signaling mechanisms, including that of BRI1.
The WAK-mediated signaling pathway has a role in both plant cell expansion and in a stress response against wounding, pathogens, and environmental factors.$^{49,44}$ WAKs are RLKs that bind to pectin polymers in the cell wall. They are encoded by five genes clustered in a 30 kb locus of chromosome 1 in *Arabidopsis thaliana* and are characterized by a cytoplasmic serine, threonine kinase domain, a transmembrane domain, and a less conserved extracellular region with EGF repeats.$^{34}$ WAKs are different from WAK-like proteins (WAKLs), which have similar cytoplasmic domains as WAKs, but diverse extracellular EGF domains.$^{48}$ The functions of WAKLs are still unknown in the cell wall.

Cell expansion stems from a synergistic relationship between turgor pressure and the loosening of the cell wall and extracellular matrix.$^{15}$ WAKs are bound to native, wall pectin polymers, and they have a high affinity for negatively charged, de-esterified pectin partly due to ionic bonding.$^{16,7}$ Mutations of the positively charged residues in WAK1 cause loss of binding to negatively charged, de-esterified pectins.$^{16}$ When bound to these de-esterified pectins, WAKs mediate cell wall expansion by regulating the transcription of a vacuolar invertase. This invertase controls polysaccharide concentrations and turgor pressure.$^{41,32}$ When turgor pressure increases within a plant cell, the vacuole pushes out onto the cell wall. The cell wall is selectively loosened in specific areas so that the cell may expand.$^{41}$

While cell expansion is initiated when WAKs are bound to de-esterified pectin polymers, WAKs are also capable of enacting a stress response when they interact with small pectin fragments.$^9$ These fragments with a degree of polymerization (dp) of 9-15 are called oligogalacturonic acids (OGs), which are generated when a plant is wounded or attacked by pathogens.$^{23,44,34}$ *In vitro*, WAK1 and WAK2 have been shown to bind OGs
preferentially over de-esterified pectin polymers.\textsuperscript{16,17} The ability of WAKs to bind pectin of different lengths suggests a degree of competition in ligand interactions.\textsuperscript{36} WAK binding to OGs induces in \textit{A. thaliana} a stress response, which is phenotypically expressed by ectopic lesions, Reactive Oxygen Species (ROS) accumulation, leaf curling, and stunted growth. Such phenotypes were observed in dominant alleles of WAK2 and WAK2\textit{cTAP}, which is a hyperactive, constitutive allele, in the absence of pathogens.\textsuperscript{33,35} Although the activating ligands are different between the WAK stress response pathway and the bacterial flagellin pathway, there is evidence that the two pathways share a MAPK signaling cascade.\textsuperscript{24} It is unclear whether the two pathways can coordinate a stress response pathway when activated. The focus of this study was to identify the components of the WAK-induced stress response pathway.

OGs have been shown to activate protein kinases MPK3 and MPK6, which regulate downstream target genes.\textsuperscript{14,35} The stress response was suppressed in WAK2\textit{cTAP mpk6} plants, which indicated that MPK6 is involved in the stress response.\textsuperscript{35} Hundreds of genes have been found in cell wall biogenesis and immunity that are induced or repressed with pectin treatment in protoplasts.\textsuperscript{32} Many of these genes depend on WAK2 activation and signal through MPK3 and MPK6.\textsuperscript{35} Despite our knowledge of MPK involvement, there are still elements of the pathway that have yet to be identified.

In order to study the components of the WAK-mediated stress response pathway, mass spectrometry and phosphoproteomic assays have been performed to identify proteins that were phosphorylated when Arabidopsis plants were treated with OGs. Initially, fifty proteins were phosphorylated. Of the fifty, proteins that were
phosphorylated upon exposure to flg22, a peptide mimic of an epitope on flagellin, were removed from the list to avoid components already activated by the flagellin pathway. In addition, attention was focused on proteins that showed a two-fold or greater increase in phosphorylation upon OG activation. After applying these two criteria, nineteen candidates were identified. T-DNA (sequence between left and right borders of plasmid that is transferred by Agrobacterium tumefaciens) knockout Arabidopsis lines were ordered for eighteen of the nineteen candidates. None of the lines revealed visible phenotypes. However, six lines displayed diminished OG responses, which were demonstrated by a reduction in FADlox expression, measured with QPCR. FADlox is a highly expressed downstream target gene of the stress response pathway. The six proteins whose knockout lines showed reduced FADlox expression included DNA binding protein REM2, cytoplasmic kinase ROG1, a phospholipase C, and other unknown proteins. Although the protein SRF6 showed only a 1.8 fold change in phosphorylation upon OG induction and its knockout mutant did not show reduced FADlox expression, SRF6 was included in this study because it was the only RLK identified from the phosphoproteomic database. This thesis focused on characterizing cytoplasmic kinase ROG1 and receptor-like kinase SRF6.

SRF6 is a RLK from the Strubbelig-receptor family. Besides the crippling effect of srf4 Arabidopsis plants and possible SRF activity in ovule, anther development, and pectinesterases, the full functions of the family are still unknown. SRF6 has extracellular, transmembrane, and kinase domains (Fig. 1). Therefore, the SRF6 protein was hypothesized to act as a co-receptor to WAK1 and would reside in the plasma membrane near WAK1. WAK1 was not identified in phosphoproteomic analysis because
the RLK was present in the insoluble cell wall fraction, which was not analyzed for technical reasons. Another protein called ROG1 (Responsive to OGs) is predicted to be a cytoplasmic protein kinase and is a part of another family of proteins that includes a close relative called ROG2, which was also identified in phosphoproteomic analysis. ROG2 was not included in this study because it could not be cloned into vectors for expression in *E. coli*.

The aim of this study was to characterize the functions of SRF6 and ROG1 in the WAK-mediated stress response pathway. SRF6 was tagged with GFP in order to find where the protein resides in plant cells. The phosphorylation sites of SRF6 were mutated into glutamic acids (E) and alanines (A) to mimic constitutive phosphorylation and a version of SRF6 that could not be phosphorylated, respectively. These mutants were transformed into Arabidopsis plants to see if they would induce phenotypes different from those of SRF6 WT plants. Lastly, kinase assays were performed *in vitro* with WAK1, SRF6, ROG1, and REM2 to test whether these proteins could phosphorylate each other and reveal specific interactions of the WAK-mediated stress response signaling cascade.
MATERIALS AND METHODS

SRF6-GFP CLONING AND TRANSFORMATION:

SRF6 cDNA was amplified using Phusion Taq PCR with SpeI restriction site forward and reverse primers (Table 1). The product was run on a 1% agarose gel and stained with GelRed, after which the ~2kb band was excised and eluted using QIAquick Gel Extraction Kit (catalog #28704). The construct was ligated into Strataclone pSC-B amp/kan vector and transformed into DH5α E. coli cells, which were grown on LB medium with 50µg/mL ampicillin in addition to 1mM isopropyl D-thiogalactopyranoside (IPTG), and 10 mM Xgal. After a 16 hour incubation at 37°C, white colonies were identified and inoculated into LB+amp liquid broths. After growth overnight, plasmid DNA was extracted from cells by alkaline lysis extraction and digested with SpeI enzyme (New England Biolabs). The digestion reaction was run on 1% agarose gel with GelRed to verify successful cloning, and the smaller ~2kb cloned SRF6 insert was excised. The plant vector pCambia 1302 was cut with SpeI enzyme and subjected to shrimp alkaline phosphatase treatment. The reaction was run on 1% agarose gel with GelRed and screened for a 11kb band. The 11kb pCambia 1302 and ~2kb SRF6 insert were extracted using the QIAquick Gel Extraction kit. The SRF6 insert was ligated with pCambia 1302 and transformed into DH5α E. coli. DNA was isolated from colonies and digested with PstI enzyme (New England Biolabs) to determine insert orientation. The final SRF6-pCambia 1302 plasmid was transformed into Agrobacterium tumefaciens via electroporation, and cells were grown on LB plates with 50µg/mL gentamycin, kanamycin, and rifamycin. A. thaliana WT Columbia and RDR (RNA-dependent RNA Polymerase) mutant plants, whose RNA silencing mechanisms are crippled, were dipped
into a cell suspension of the transformed *A. tumefaciens* cells. Seeds were collected from the F₀ parent (T₀) and subsequently grown on hygromycin B MS plates. Heterozygous seedlings (T₁) and the resulting progeny (T₂) were screened on selection plates and observed under a confocal microscope as seedlings.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpeI Forward</td>
<td>ACTAGTATGAGGAGAAATTGGGCGGTC</td>
</tr>
<tr>
<td>SpeI Reverse</td>
<td>GCTGGTCACTAATGTACGAGCTCTCTGATCA</td>
</tr>
</tbody>
</table>

**Table 1:** Primers with SpeI restriction sites used for SRF6 cDNA amplification. Restriction sites are bolded.

**SRF6 PHOSPHORYLATION SITE MUTANTS WITH GLUTAMIC ACID AND ALANINE RESIDUES (performed by and received from Bruce Kohorn):**

SRF6 cDNA was amplified using Phusion Taq Polymerase with SpeI forward and BstEII reverse restriction sites (Table 2). The product was identified on a gel, and the band was excised and purified using QIAquick Gel Extraction Kit. The construct was ligated into Strataclone pSC-b amp/kan vector and transformed into DH5α *E. coli* cells, which were grown on LB medium with 50µg/mL ampicillin, 1mM IPTG, and 10 mM Xgal. White colonies were inoculated into LB+amp liquid broths and grown overnight. After verifying the correct plasmid through alkaline lysis extraction and digestion with SpeI and BstEII enzymes, a purified plasmid prep was prepared using the Pure Yield™ Plasmid Midiprep System (Promega). PCR reactions were run with mutagenic primers for glutamic acid and alanine mutant versions of SRF6 (below), and the products were transformed into XL-10 Gold Ultracompetent cells after methylated template digestion by DpnI enzyme. Protocols, reagents, and cells came from the QuikChange II XL Site-
Directed Mutagenesis Kit (Agilent Technologies). The cells were grown overnight in LB+amp, and the mutant plasmids were extracted through alkaline lysis extraction. The samples were purified once more using the PCR Extract Mini Kit (5 Prime) prior to being sequenced. With the correct sequences, mutant SRF6 inserts, excised from gels after digestion with SpeI and BstEII, were extracted and ligated into pCambia 1302 vector for transformation into *A. tumefaciens* and eventually into *A. thaliana* WT Columbia and RDR mutant plants.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpeI Forward</td>
<td>ACTAGTATGAGGGAGAATTGGGCGGTC</td>
</tr>
<tr>
<td>BstEII Reverse</td>
<td>GTAGTAGTAGTAGTAGTAATCCCAATGG</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>ACAAAAGAAACTGGACGAGAATTGGAAA</td>
</tr>
<tr>
<td>Alanine mutant</td>
<td>ACAAAAGAAACTGGAGCAGCAGCATTGGCAA</td>
</tr>
</tbody>
</table>

Table 2: Primers used for cloning. Restriction sites and mutagenized nucleotides are bolded.

**PURIFICATION OF WAK1, SRF6, SRF6\(^{T361E\ S362E\ S364E}\)** (phosphomimic):

Coding regions were amplified from cDNA, obtained from the ABRC (Arabidopsis Biological Resource Center). The SRF6\(^{T361E\ S362E\ S364E}\) mutant was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). The WAK1 gene was ligated into pGEX-2TK with a C-terminal glutathione-S-transferase tag, and the SRF6 and SRF6\(^{T361E\ S362E\ S364E}\) genes were ligated into pET28a, containing a C-terminal 6x histidine tag. The plasmids had been generated in advance using similar methods as above. pET28a plasmids were transformed into BL21DE3 cells and grown on LB medium with 50 µg/mL kanamycin, while the pGEX-2TK plasmid was transformed into DH5α *E. coli* cells and grown on LB medium with 50 µg/mL ampicillin. For each
sample, a colony was grown in 250 ml of LB broth with the correct resistant marker until log phase (OD$_{600}$ 0.4-0.6) where 1 mM isopropyl D-thiogalactopyranoside (IPTG) was added to induce protein expression overnight at 30°C. Cells were resuspended in grinding buffer (150mM NaCl, 20mM NaPO$_4$, pH 7.4) with a protease inhibitor cocktail pill (cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack, Roche), and sonicated 3x30 seconds at 70% frequency. Cell debris was separated from supernatant via centrifugation at 10000g at 4°C. WAK1 supernatant was mixed with glutathione beads (Invitrogen, Glutathione Agarose, Linked Through Sulfur), and SRF6 and SRF6$^{T361E}$ S$^{362E}$ S$^{364E}$ supernatants were mixed with Cobalt beads (Clontech, Talon Metal Affinity Resin Beads) on a nutator for an hour at 4°C. After running 100 ml of grinding buffer through mixed samples in a column with a packed volume of 500µL, WAK1 was displaced with elution buffer (50mM HEPES pH 7.6, 20mM glutathione), and SRF6 and SRF6$^{T361E}$ S$^{362E}$ S$^{364E}$ samples were displaced with a different elution buffer (50mM HEPES (pH 7.6), 250 mM imidazole). Fractions were collected in the process, and a Western blot was performed to determine which fractions had the greatest amount of fusion protein.

**PURIFICATION OF REM2, ROG1, ROG1$^{T361E}$ S$^{362E}$ S$^{364E}$ (phosphomimic):**

Coding regions were amplified from cDNA, obtained from ABRC. The ROG1$^{T361E}$ S$^{362E}$ S$^{364E}$ mutant was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) (performed by and received from Josh Benton). Coding regions of all proteins were ligated into pET28a with a C-terminal histidine tag. The plasmids had been generated in advance using similar methods as above. pET28a plasmids were transformed into BL21DE3 cells and grown on LB medium with 50 µg/mL kanamycin.
Colonies were grown in 250 ml of LB broth with the correct resistant marker until log phase (OD<sub>600</sub> 0.4-0.6) where 1 mM isopropyl D-thiogalactopyranoside (IPTG) was added to induce protein expression overnight at 30°C. Cells were lysed in guanidinium hydrochloride buffer (500mM NaCl, 20mM NaPO₄ pH 7.8, 6M gHCl) with a protease inhibitor cocktail pill, and sonicated 3x30 seconds at 70% frequency. After sonication, the total sample was agitated on nutator at 4°C for thirty minutes with frequent pipetting to disperse the aggregates before centrifugation at 5000g at 4°C. Supernatant was mixed with 500µL packed volume cobalt metal affinity resin beads (Clontech, Talon Metal Affinity Resin Beads) for two to four hours on nutator at 4°C. The slurry was poured into a column and washed with 75ml of gHCl buffer before being eluted with 250mM imidazole in gHCl buffer. Fractions were collected and stored at 4°C. After a Western blot to find fractions that contained the greatest concentrations of desired fusion protein, those fractions were dialyzed in three successive 500ml buffers to refold the protein. Fractions were loaded into a 10000 MW cutoff dialysis membrane and placed into the following washes: A. 1M urea, 1M NaCl, 20mM Tris pH 7.5 for three hours, B. 1M NaCl, 20mM Tris (pH 7.5) for three hours, and C. 20mM Tris (pH 7.5), 0.1M NaCl, 10% glycerol, overnight. The fractions were removed from the membrane, and centrifuged in a 10000 MW cutoff centricon unit to concentrate the sample to 500µL. Glycerol was further added to the fractions to make a 20% solution.

**KINASE REACTION:**

The buffer contained a final concentration of 0.4mM DTT, 0.8mM MgCl₂, 1mM CaCl₂, and 4mM ATP in a 50 microliter reaction. Other cofactors used in the kinase buffer were 1mM CoCl₂ and 1mM MnCl₂. Appropriate amounts (2-20µl) of SRF6, SRF<sup>T361E S362E</sup>
S364E, ROG1, ROG1T361E S362E S364E, REM2, WAK1, and kinase buffer, as determined by the Coomassie stain were added to eppendorfs in different combinations. The reactions were brought up to 50 microliters with appropriate amounts of 50mM HEPES. Reactions were incubated at 30°C for 30 minutes.

**WESTERN BLOT DETECTION:**

Samples were denatured at 95°C in 1x Laemmli dye (100mM Tris-HCl (pH 6.8), 100mM DTT, 0.05% bromophenol blue, 20% glycerol) and run on 10% acrylamide gels at 50 mAmps/gel for an hour and subsequently transferred to a nitrocellulose membrane at 300 mAmps over five hours in transfer buffer (50mM Tris, 380mM glycine, and 0.1% SDS). Membranes were blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) with 3% Tween 20. α-phosphothreonine rabbit (Zymed), α-phosphoserine rabbit (Zymed), α-HIS mouse (Santa Cruz), and α-GST mouse (GenScript) primary antibodies were used in 1:5000 dilutions for two hours at room temperature. After three rounds of washing in TBST, 1:5000 incubations with α-rabbit and α-mouse horseradish peroxidase (Pierce) were performed for two hours. SuperSignal® West Pico Stable Peroxide Solution (Thermoscientific, Product# 1859674) was used for developing the blots, and 3603 EZ-RUN Pre-stained Rec Protein Ladder (Fisher Scientific) was run alongside experimental samples. Blots were visualized with the GENESys program for the G:Box Chemi-XRQ series from SYNGENE.
RESULTS

SRF6-GFP:
SRF6 was predicted from its sequence to be a receptor kinase in the plasma membrane (Fig. 1). In order to determine where SRF6 was located in plant cells and what proteins would bind SRF6 from GFP co-immunoprecipitation assays, a SRF6-GFP fusion was generated and expressed in Arabidopsis plants. The coding region of SRF6 was ligated into the pCambia 1302 plant vector such that a GFP coding region was fused to the carboxy-terminus of SRF6 (Fig. 2). The vector was transformed into A. tumefaciens, which subsequently transferred part of the plasmid into the Arabidopsis genome including a 35s promoter and hygromycin selectable marker (Fig. 3). F1 transformed seedlings (T1) were selected on hygromycin plates (Fig. 4), and both F1 and F2 seedlings were eventually screened for GFP expression under a confocal microscope. The expression of SRF6-GFP fusion protein in WT Colombia plants could not be detected using confocal microscopy. The proposed explanation was that the SRF6 gene had been silenced. Therefore, plants that are mutant in gene silencing (RDR Arabidopsis) were transformed with the expectation that SRF6-GFP would be successfully expressed. Imaging of T2 seedlings showed no GFP signal that was higher than background (data not shown) despite the detection of other GFP fusion proteins by the same methods. SRF6-GFP expression was not detected by Western blot, and QPCR had not been performed to determine if SRF6-GFP was transcribed.
Figure 1: The SRF6 coding region includes extracellular, transmembrane, and cytoplasmic kinase domains, suggesting that SRF6 is a receptor kinase.

Figure 2: SRF6 was cloned into pCambia 1302 so that the fusion protein would include a C-terminal GFP tag. pCambia also contains a Cauliflower Mosaic Virus 35s constitutive promoter and hygromycin selectable marker. Agrobacterium transforms sequences between the left border (LB) and the right border (RB) in Arabidopsis.
Figure 3: Arabidopsis plants were dipped into a solution of *A. tumefaciens* that was transformed with the SRF6-GFP construct via electroporation.

Figure 4: SRF6-GFP Arabidopsis T_1_ seedlings were screened for growth on MS/hygromycin plates. Hygromycin resistant plants (larger green seedlings) were further grown to maturity in soil, and they and their T_2_ offspring were screened for GFP expression.
**SRF6 MUTANTS:**

Phosphoproteomics has revealed that upon OG stimulation of plants, SRF6 is phosphorylated at amino acids $T^{361} S^{362} S^{364}$ (letter indicates amino acid, and the number indicates position relative to the first residue) (Fig. 5). Genes encoding mutant versions of SRF6 at these sites were generated *in vitro* and transformed into plants to see if they would induce phenotypes different than those induced by WT SRF6 plants. Threonine and Serine were changed to alanine in order to create a form of SRF6 that could not be phosphorylated and to glutamic acids in order to mimic a state of phosphorylation. Thus, $\text{SRF6}^{T^{361}A \ S^{362}A \ S^{364}A}$ has neutrally charged alanines and $\text{SRF6}^{T^{361}E \ S^{362}E \ S^{364}E}$ has negatively charged glutamic acids instead of the original residues (Fig. 5). The hypothesis was that $\text{SRF6}^{T^{361}E \ S^{362}E \ S^{364}E}$ would activate the stress response signaling cascade constitutively and that $\text{SRF6}^{T^{361}A \ S^{362}A \ S^{364}A}$ would prevent SRF6 phosphorylation and dampen the cascade (Fig. 5). After the $T_1$ plants had grown for two weeks, a Western blot of individual leaves was performed and probed with anti-histidine antibody to check for the expression of SRF6 in all plants (Fig. 6). In twenty-eight $T_1$ plants, no SRF6-HIS was detected relative to the negative control WT Colombia leaf (Fig. 6, lane 28). A REM2 positive control was run alongside the leaf extracts (Fig. 6, lane 29). A roughly 50 kDa band was expected for SRF6. Despite not detecting the expression of SRF6, there were observable phenotypic differences in the plants. SRF6 WT plants appeared larger than SRF6 $^{T^{361}E \ S^{362}E \ S^{364}E}$ and $\text{SRF6}^{T^{361}A \ S^{362}A \ S^{364}A}$ plants, and the leaves of SRF6 $^{T^{361}E \ S^{362}E \ S^{364}E}$ plants were curled to a greater degree than those of SRF6 WT and SRF6 $^{T^{361}A \ S^{362}A \ S^{364}A}$ (Fig. 7). There was no difference in size between SRF6 $^{T^{361}E \ S^{362}E \ S^{364}E}$ plants and SRF6 $^{T^{361}A \ S^{362}A \ S^{364}A}$ plants (Fig. 7). In order to confirm if the phenotypes were significantly different, leaf lengths were measured for three randomly chosen plants of
SRF6 WT, SRF6^{T361E S362E S364E}, and SRF6^{T361A S362A S364A}. Leaves of Arabidopsis grow successively in a whorl from the meristem. By the second week, all plants had roughly six leaves, ordered one to six, with one being the newest leaf and six being the oldest leaf. Since the sixth leaf had grown to its full size, leaves three, four, and five were chosen because they were still expanding and growing. A plot of average leaf length versus leaf number was generated for the three samples with standard error bars (Fig. 8). Leaves of SRF6 WT plants were significantly longer than those of SRF6^{T361E S362E S364E} plants (paired t-test, p<0.05) and SRF6^{T361A S362A S364A} plants (paired t-test, p<0.05) (Fig. 8). There was no significant difference between the leaf lengths of SRF6^{T361E S362E S364E} and SRF6^{T361A S362A S364A} plants (paired t-test, p>0.05) (Fig. 8).

![Phosphorylation Sites Diagram](image)

**Figure 5:** The phosphorylation sites of SRF6 were mutated into glutamic acid (E) or alanine residues (A). TM; transmembrane domain in pink.
Figure 6: Western blot of leaves picked from individual T₁ plants transformed with wild type and mutant SRF6. All blots were probed with anti-6x histidine antibody. Lanes 1 and 30; marker, lanes 2-5; SRF6 T₃₆₁A S₃₆₂A S₃₆₄A plants, lanes 6-20; SRF6 T₃₆₁E S₃₆₂E S₃₆₄E plants, lanes 21-27; SRF6 WT plants, lane 28; WT non-transformed plant, and lane 29; REM2-HIS as a positive control.
Figure 7: Two week old T₁ seedlings of wild type or mutant SRF6 Arabidopsis plants.
**Figure 8: SRF6$^{T361E \ S362E \ S364E}$ and SRF6$^{T361A \ S362A \ S364A}$ plants had smaller leaves than WT SRF6 plants.** A plot of average leaf length for leaves three, four, and five in three randomly selected samples of SRF6 WT (blue), SRF6$^{T361A \ S362A \ S364A}$ (red), and SRF6$^{T361E \ S362E \ S364E}$ (green) plants, respectively.

**KINASE ASSAYS:**
Null mutants of six proteins, identified in the phosphoproteomic analysis, (DNA binding protein REM2, cytoplasmic kinase ROG1, a phospholipase C, and others) displayed a compromised stress response in Arabidopsis. T-DNA knockout Arabidopsis lines of these six proteins showed decreased OG induced $FAD\text{lo}x$ expression, a gene transcribed in high amounts when a plant is induced by OGs. Null SRF6 alleles had no effect on OG induced gene expression. However, SRF6 was the only RLK found and deemed important enough to include in the kinase assays as other members of the SRF protein family might be redundant and mask the SRF6 mutant allele phenotype. The hypothesis was that WAK1, ROG1, SRF6, and REM2 would be part of a signaling cascade that leads to stress response related gene expression. Kinase Assays were performed to see if these proteins would phosphorylate each other when mixed in various combinations. ROG1 and REM2 were small proteins whose entire coding regions could be ligated into pet28a, but only the catalytic regions of WAK1 and SRF6 were cloned, expressed, and purified for experimentation (Fig. 9). The transmembrane and extracellular domains were left out due to solubility issues that would be complicate protein purification from *E. coli*. In addition, the kinase domains of many proteins have shown ligand-independent activity and interaction. WAK1 was tagged with a C-terminal glutathione-S-transferase tag (cloned previously by B. Kohorn), while the other three proteins had C-terminal 6x histidine tags (Fig. 9). SRF6$^{T361E \ S362E \ S364E}$ and ROG1$^{T361E}$...
mutant proteins with 6x C-terminal histidine tags were also generated because phosphomimic proteins might not require activation (Fig. 9). The SRF6 mutant plasmid was generated by B. Kohorn, and the ROG1 mutant plasmid by Josh Benton. A Western blot was performed to confirm that each protein was purified, could be detected, and was of the expected molecular weight (Fig. 10). WAK1-GST was roughly 75 kDa, SRF6 and SRF6<sup>T361E S362E S364E</sup> were roughly 50 kDa, ROG1 and ROG1<sup>T361E S362E S364E</sup> were roughly 43 kDa, and REM2 showed two bands, one at about 70 kDa and another at 35 kDa. The proteins displayed the desired and expected molecular weights except for REM2. The 70 kDa band of REM2 may be a dimer, but this has not been tested, and the smaller form was larger than the expected 25 kDa molecular weight of REM2 (Fig. 10). A Coomassie stain of a separate, but identical gel was produced to ascertain the amount of protein being loaded and showed that the proteins were enriched with some contaminations (Fig. 10). Even though WAK1 was loaded in relatively small amounts, the affinity of the α-GST was strong enough to produce thick bands on the Western blot, while the α-HIS serum demonstrated a weak signal for abundant levels of HIS-tagged proteins (Fig. 10).

**Figure 9:** Proteins were purified from *E. coli* with 6x HIS or GST tags.
Figure 10: Coomassie Stain and Western blot of protein samples showed amount of protein loaded, size of protein, and strength of antibody detection. WAK1 was probed with anti-GST antibody. All other proteins were probed with anti-6x HIS antibody.

The initial hypothesis was that SRF6 would be able to act as a co-receptor to WAK1 and that ROG1 would be an immediate downstream target. The three proteins were mixed in different combinations to test for phosphorylation. In lanes one, three, and seven, WAK1, SRF6, and ROG1 were added individually (Fig. 11). The combinations
tested whether WAK1 would be able to phosphorylate ROG1 (Lane 2) or SRF6 (Lane 5) separately, whether SRF6 could phosphorylate ROG1 (Lane 4), and whether WAK1, ROG1, and SRF6 would act in a complex or would require each other’s presence for phosphorylation (Lane 6) (Fig. 11). An individual ROG1 sample was loaded without kinase buffer or incubation (Lane 8) to see whether ROG1 was already phosphorylated in *E. coli* (Fig. 11). WAK1 threonine autophosphorylation, and serine, threonine phosphorylation of ROG1 were observed (Fig. 11). Faint bands with the shape of SRF6 appeared in the α-Phosphoserine blot, but it was not certain whether the bands were non-specific antibody binding to abundant protein or small amounts of phosphorylation. However, the band in lane 8 of the α-Phosphoserine and α-Phosphothreonine blots suggests that the ROG1 sample was already phosphorylated prior to kinase buffer incubation (Fig. 11). The ROG1 sample without kinase buffer in lane 8 was included after ROG1 phosphorylation had been seen in prior blots. An explanation was that ROG1 had phosphorylated itself or had been phosphorylated by other proteins in *E. coli* as it was being expressed. In addition, an unidentified smearing was present when SRF6 and WAK1 were mixed (Fig. 11, lanes 5+6). There were also three unidentified bands with MW 100 kDa that were seen in the α-HIS blot (Fig. 11). Non-specific aggregation or specific binding interactions between proteins may have caused the smearing or the three unidentified bands.
Figure 11: Western blot of WAK1 (circle), SRF6 (pentagon), and ROG1 (triangle) kinase assay. Autophosphorylation of WAK1 (circle) and phosphorylation of ROG1 (triangle) were detected. The phosphorylation of SRF6 was uncertain (square). SRF6 and WAK1 created an unidentified smearing in the α-Phosphothreonine blot (lanes 5+6). Three unidentified bands with MW 100 kDa were seen in the α-HIS blot (diamond). ROG1 was incubated with kinase buffer (Lane 7) or was not incubated (Lane 8).
Replicates of the first kinase assay repeatedly showed that WAK1, SRF6, and ROG1 did not phosphorylate each other. Therefore, a second assay was performed with the addition of REM2 to examine whether the three proteins would phosphorylate the transcription factor. Lanes 1-4 contained WAK1, SRF6, ROG1, and REM2, respectively, as controls (Fig. 12). The combinations tested were whether ROG1 would phosphorylate REM2 (lane 5), whether SRF6 would phosphorylate REM2 (lane 6), whether SRF6 and ROG1 were both required for the phosphorylation of REM2 (lane 7), whether WAK1 would directly phosphorylate REM2 (lane 8), and whether WAK1, SRF6, and ROG1 would be required altogether for REM2 phosphorylation (lane 9). The same WAK1 and SRF6 autophosphorylation and ROG1 phosphorylation were found in blots of α-Phosphothreonine and α-Phosphoserine (Fig. 12). The smearing observed in lanes 5 and 6 of the previous assay was not observed in the ninth lane of the α-Phosphothreonine where WAK1, SRF6, ROG1, and REM2 were added altogether (Fig. 12). It was unclear whether the addition of REM2 had prevented the formation of this aggregation or interaction. The phosphorylation of REM2 was not detected in any lane (Fig. 12).
Figure 12: Western blot of WAK1 (circle), SRF6 (pentagon), ROG1 (triangle), and REM2 (hexagon) kinase assay. Autophosphorylation of WAK1 (circle) was present in addition to the phosphorylation of ROG1 (triangle). SRF6 bands appeared in the α-Phosphoserine blot (square). Smearing was not found as in the previous kinase assay. No REM2 (hexagon) phosphorylation was detected.
Since there had not been any evidence of a signaling cascade between the four proteins, a hypothesis was that the proteins might require additional cofactors to undergo phosphorylation \textit{in vitro}. Two cofactors, Co\(^{2+}\) and Mn\(^{2+}\), were added to the kinase buffer in order to test whether new phosphorylation events could be detected and whether levels of phosphorylation would be increased when compared to the regular buffer that contained Mg\(^{2+}\) and Ca\(^{2+}\). In addition, phosphomimic SRF6\(^{T361E\; S362E\; S364E}\) and ROG1\(^{T361E\; S362E\; S364E}\) might phosphorylate each other or REM2 at more robust levels due to their constitutively active, phosphorylated states. To test these hypotheses, WAK1, SRF6, ROG1, and REM2 (lanes 1 + 3 + 5) and WAK1, SRF6\(^{T361E\; S362E\; S364E}\), ROG1\(^{T361E\; S362E\; S364E}\), and REM2 (lanes 2 + 4 + 6) were mixed separately, incubated together for twenty minutes, and run on gels for Western blots. In addition, REM2 was added to lanes 7, 8, and 9 individually to test whether different cofactors would bring about its phosphorylation. The lanes were subjected to three different kinase buffers (regular in lanes 1 + 2 + 7, regular + Co\(^{2+}\) in lanes 3 + 4 + 8, and regular + Mn\(^{2+}\) in lanes 5 + 6 + 9). No new phosphorylation events were detected (Fig. 13). The expectation was that ROG1\(^{T361E\; S362E\; S364E}\) would not be phosphorylated because the glutamic acids were mimicking phosphorylated residues. However, slight phosphorylation of ROG1\(^{T361E\; S362E\; S364E}\) was observed (Fig. 13, Lanes 2 + 4 + 6), perhaps indicating that the protein was being phosphorylated on other serine residues in the kinase buffer or in \textit{E. coli} (Fig. 13). No significant difference in phosphorylation levels was observed when Co\(^{2+}\) and Mn\(^{2+}\) were used as additional cofactors (Fig. 13).
Figure 13: Western blot of kinase assay mixing WAK1 (circle), ROG1 (triangle), SRF6 (pentagon), and REM2 (hexagon) and WAK1, ROG1\textsuperscript{T361E S362E S364E} (triangle), SRF6\textsuperscript{T361E S362E S364E} (pentagon), and REM2 separately with three different buffers (regular, regular + Co\textsuperscript{2+}, and regular + Mn\textsuperscript{2+}). There was no difference in levels of phosphorylation between regular kinase buffer and adding 1mM Co\textsuperscript{2+} or 1mM Mn\textsuperscript{2+}. REM2 phosphorylation was not detected when phosphomimic ROG1\textsuperscript{T361E S362E S364E} and SRF6\textsuperscript{T361E S362E S364E} were added together with WAK1 and REM2. Autophosphorylation of WAK1 (circle) and the phosphorylation of ROG1 (triangle) were detected. Low levels of ROG1\textsuperscript{T361E S362E S364E} phosphorylation (triangle) were also detected.

DISCUSSION

SRF6 was predicted to be a receptor plasma membrane protein that would interact with WAK1 to effect OG signal transduction (Fig. 1). Therefore, a SRF6-GFP fusion was generated and transformed into WT and RDR Arabidopsis to detect where the protein resides in plant cells. The SRF6-GFP fusion protein could not be detected in Arabidopsis seedlings by confocal microscopy. SRF6 was the only RLK found from phosphoproteomic data. Thus, the protein was hypothesized to be involved in the stress response signaling cascade. SRF6 mutants that had its phosphorylation sites replaced with negatively charged glutamic acids (SRF6\textsuperscript{T361E S362E S364E}) and neutrally charged alanines (SRF6\textsuperscript{T361A S362A S364A}) were created to mimic phosphorylation and to prevent phosphorylation, respectively. They were transformed into Arabidopsis to see if they would induce phenotypes different from those of SRF6 WT plants. Leaves of SRF6\textsuperscript{T361E S362E S364E} plants (p<0.05) and SRF6\textsuperscript{T361A S362A S364A} plants (p<0.05) were smaller than those of SRF6 WT plants, and SRF6\textsuperscript{T361E S362E S364E} plants showed more leaf curling, a phenotype associated with a stress response (Fig. 7+8). However, SRF6 expression was not detected by Western blot and did not provide further evidence that the mutants had induced these phenotypic differences (Fig. 6). Phosphoproteomic analysis suggested that WAK1, ROG1, SRF6, and REM2 would create a signaling cascade when plants were
induced with OGs. Therefore, WAK1, ROG1, ROG1$^{T361E \ S362E \ S364E}$, SRF6, SRF6$^{T361E \ S362E \ S364E}$, and REM2 were expressed and purified from *E. coli* as glutathione-S-transferase or histidine tagged fusion proteins. They were then mixed in various combinations to test for kinase activity. The phosphorylation of WAK1 and the phosphorylation of ROG1 and ROG1$^{T361E \ S362E \ S364E}$ were detected (Fig. 11-13). The phosphorylation of ROG1, however, was also seen in the non-incubated lane without kinase buffer (Fig. 11, Lane 8), suggesting that ROG1 was phosphorylated in *E. coli*. The phosphorylation of SRF6 was very weak, but could have been either nonspecific binding or low levels of phosphorylation (Fig. 11). The phosphorylation of SRF6$^{T361E \ S362E \ S364E}$ and REM2 was not detected (Fig. 12+13), and cofactors, Co$^{2+}$ and Mn$^{2+}$, did not increase levels of phosphorylation.

**SRF6-GFP:**

Since expression of the fusion protein could not be detected, the location of SRF6 in plant cells is still unknown. The level of SRF6 mRNA transcript could be measured by QPCR in order to verify the transcription of the SRF6-GFP construct, but limited time has prevented the analysis. Some explanations for the lack of SRF6-GFP expression include the stability of the protein and its mRNA transcript. Although the constitutive Cauliflower Mosaic Virus 35s promoter was used, the rate of degradation or removal of SRF6-GFP could have outpaced the rate of its formation, preventing the accumulation of GFP signal. Since endogenous SRF6 is expressed in lower quantities, relative to ROG1, as measured previously by QPCR (Kohorn unpublished), only small amounts of SRF6 may be needed to induce a stress response. The lack of GFP signal may suggest that the cells have removed the greater amounts of SRF6 produced by the 35s promoter. The
SRF6-GFP fusion was also generated from SRF6 cDNA. Introns that may assist in mRNA transcript stability or nuclear export may have been required to detect GFP signal. Finally, the SRF6 construct may not have been expressed at high levels due to the site of chromosomal insertion. Although multiple transformants were identified, all had no detectable expression. Using a different plant vector with a GFP coding region and the entire transcribed region of SRF6 may be possible solutions for SRF6-GFP expression.

Although the lack of GFP expression has hindered its characterization, SRF6 may cluster in the plasma membrane under OG treatment conditions to enact changes in the cellular environment. The clustering of ethylene receptor kinases has been previously reported in plants. A similar type of clustering or movement may be possible for SRF6. If a strongly GFP expressing plant were found, the treatment with or without OGs could shed light on the protein’s movements. In addition, an anti-GFP co-immunoprecipitation experiment had been planned for whole cell lysates from plants treated with or without OGs to detect proteins that would bind SRF6. After using mass-spectrometry based methods for identifying these proteins, this result could highlight additional candidates that may be part of the stress response pathway.

**SRF6 MUTANTS:**

Although SRF$^{T361E\ S362E\ S364E}$ and SRF$^{T361A\ S362A\ S364A}$ plants displayed smaller average leaf lengths than SRF6 WT plants, whether the mutant proteins had caused these changes was unclear because SRF6 expression was not detected. A possible solution for SRF6 detection could be using co-immunoprecipitation to concentrate SRF6 levels from leaves prior to loading in gels. The better solution might be to tag SRF6 with different epitopes and to use antibodies that have stronger affinities for those epitopes. Weak, but
not detectable expression of SRF6 mutants may have been sufficient to cause phenotypic differences. Two possibilities might explain the difference in phenotypes of these plants. A constitutively active, phosphomimic SRF6\textsuperscript{T361E S362E S364E} might activate the stress response pathway, resulting in smaller-sized plants and curled leaves. In SRF6\textsuperscript{T361A S362A S364A} mutants, the reduced size was also observed, but the stress response phenotype was not. Therefore, SRF6\textsuperscript{T361A S362A S364A} might not have activated a stress response as robustly as SRF6\textsuperscript{T361E S362E S364E} did. The smaller-sized plants also suggested that SRF6\textsuperscript{T361A S362A S364A} could be affecting the expansion pathway. Therefore, SRF6 may be a component of both the WAK-mediated expansion and stress response pathways. However, a plant with stronger SRF6 expression is needed for further interpretations.

**KINASE ASSAYS:**

Kinase assays were performed with WAK1, SRF6, ROG1, SRF6\textsuperscript{T361E S362E S364E}, ROG1\textsuperscript{T361E S362E S364E}, and REM2 in various combinations and conditions to observe whether the proteins would phosphorylate each other. Results suggest that WAK1, SRF6, ROG1, and REM2 do not phosphorylate each other in vitro (Fig. 11-13). WAK1 and ROG1 appeared to be phosphorylated when incubated alone (Fig. 11). WAK1 required incubation in kinase buffer, but ROG1 did not, suggesting that *E. coli* proteins had phosphorylated ROG1 or ROG1 had phosphorylated itself (Fig. 12, lane 8). ROG1 is less likely to have self-phosphorylated, as cytoplasmic receptor kinases have not been observed to perform this function. As a soluble protein, ROG1 may have interacted with proteins in the cytoplasm of *E. coli* during the purification process. The protein also seems to have been phosphorylated on incorrect residues because the sequence contains serine and threonine residues other than those in its phosphorylation sites. *E. coli* proteins
are unlikely to interact specifically with ROG1 as Arabidopsis proteins would in its native environment. Smear bands above WAK1 were observed when WAK1 was mixed with SRF6. These bands may have been composed of non-specific aggregations or specific protein interactions (Fig. 12). The bands were not seen with the addition of REM2 to WAK1, SRF6, and ROG1 and may suggest that REM2 had inhibited the smearing. REM2 was predicted to be a nuclear DNA binding protein and a component of the signaling pathway that would activate the transcription of relevant genes. However, no REM2 phosphorylation was detected (Fig. 13). Finally, the use of different cofactors, Co^{2+} and Mn^{2+}, and phosphomimic SRF6^{T361E S362E S364E} and ROG1^{T361E S362E S364E} did not induce kinase activity. Levels of phosphorylation were unchanged, and no new bands were seen (Fig. 13).

Taken together, our data suggest at least two possibilities. The first is that other key components of the WAK-mediated stress response pathway have yet to be identified. Our kinase assays utilized the only known kinases found from phosphoproteomics of the stress response pathway. Crucial adaptor proteins that could connect the four proteins may have been absent, preventing specific protein interactions. A SRF6-GFP co-immunoprecipitation assay would be helpful in finding proteins that may bind to SRF6. Differences between in vitro and in vivo conditions may also account for lack of phosphorylation interactions. The kinase buffer may not have been conducive to the interactions of WAK1, SRF6, ROG1, and REM2. The plant cell environment is different than that of our in vitro studies, and changes in pH, intracellular concentrations, and substrates may have contributed to the lack of specific phosphorylation interactions. In addition, the catalytic regions of WAK1 and SRF6 were purified instead of the whole
coding region of the protein. The transmembrane and extracellular regions of the proteins may have been crucial for binding. This possibility, however, seems unlikely because the kinase domains of many proteins have shown ligand-independent activity and interaction.

The second interpretation is that our proteins may not have been active or properly folded into their native forms to be used in the kinase assays. Abnormalities in structure can prevent specific protein interactions. Since ROG1 and REM2 formed inclusion bodies when they were expressed in *E. coli*, they were subjected to 6M gHCl treatment to break up aggregations prior to binding with Cobalt beads and 1M urea to step down the denaturant gradually during the refolding steps. gHCl and urea are denaturing and reducing agents that break cysteine and hydrogen bonds and completely denature proteins. Although fractions were later dialyzed to switch the gHCl buffer for phosphate buffer, the proteins may not have returned to their native structure, which could display catalytic activity. Another issue with our protein samples was that the half-lives of ROG1 and REM2 were very short. ROG1 degrades and REM2 completely dimerizes in a couple weeks. The proteins were re-purified often and used within a day or two for experimentation. Additional reductants were not tried before gel electrophoresis, and this might resolve the issue of the potential dimer. As shown in the Coomassie stain, large amounts of protein were loaded into the gels. Overloading in the kinase reactions could have also prevented specific protein interactions.

In the Western blots, problems with antibody affinity were also discovered. The Coomassie stain showed that small amounts of WAK1 were added in our kinase assays, but the α-GST antibody displayed strong affinity for the GST-tag on the C-terminal end of WAK1 (Fig. 10). The HIS-tagged proteins were loaded in greater amounts than
WAK1, but the α-6xHIS antibody showed weaker bands relative to the amount loaded (Fig. 10). Two possible ideas might explain this phenomenon. Either the α-6xHIS antibody had a weaker affinity for its epitope or portions of the HIS tag were removed from the protein. The HIS tag consists of six histidine residues, and if a few of the histidines were removed from the tag, fewer antibodies might bind to the tag and yield a weaker signal. A higher concentration of protease inhibitor can be used to prevent such degradations. However, whether the histidine tag had degraded was uncertain. If the α-HIS antibody had bound to its epitope weakly, this problem could potentially explain why SRF6<sup>T361E S362E S364E</sup>, SRF6<sup>T361A S362A S364A</sup>, and SRF6 WT expression in mutant RDR plants could not be detected (Fig. 6). If the plants were to express SRF6 in small amounts, a more sensitive α-HIS antibody or a different epitope tag such as the myc or hemagglutinin tag could be used to detect lower expression levels.

WAK1, SRF6, ROG1, and REM2 did not phosphorylate each other in vitro. Reasons include the fact that other unidentified components in the signaling cascade may be required or that the purified proteins were improperly folded and inactive. Thus, co-immunoprecipitation assays of these proteins from whole cell lysates could reveal adaptors or proteins that could bind to them. In order to check whether proteins may be properly folded, tryptophan fluorescence or CD (Circular Dichroism) assays could be performed to demonstrate the presence of alpha helices and beta sheets. However, these assays only verify the existence of properly folded alpha helices and beta sheets or tryptophan residues in hydrophobic regions, but not whether the protein had correctly folded. If these structural properties could be shown in our protein samples, the hypothesis that WAK1, ROG1, SRF6, and REM2 require other proteins to phosphorylate
each other would be more likely. Significant phenotypic differences were seen in SRF₆₃₆₁₋₃₆₄T₃₆₁₋₃₆₄E and SRF₆₃₆₁₋₃₆₄T₃₆₁₋₃₆₄A plants when compared to SRF6 WT plants. Yet, SRF6 was not detected in Western blots. Other epitope tags and antibodies will be used to detect the expression of SRF6 in leaves. Overall, results, primarily phosphoproteomics, suggest that SRF6 may be an important receptor in the stress response pathway and warrant further investigation into SRF6’s identity and its ability to bind other proteins. A SRF6 knockout plant did not display reduced expression of the FADlox gene as a ROG1 knockout plant did, but this may be due to redundancy in the SRF gene family. A more comprehensive study of ROG1 and REM2 must be performed, as knockout mutants of both proteins have already demonstrated reduced expression of the FADlox gene.
COLOR IMAGES IN BLACK AND WHITE

Figure 1: The SRF6 coding region includes extracellular, transmembrane, and cytoplasmic kinase domains, suggesting that SRF6 is a receptor kinase.

Figure 2: SRF6 was cloned into pCambia 1302 so that the fusion protein would include a C-terminal GFP tag. pCambia also contains a Cauliflower Mosaic Virus 35s constitutive promoter and hygromycin selectable marker. Agrobacterium transforms sequences between the left border (LB) and the right border (RB) in Arabidopsis.
Figure 3: Arabidopsis plants were dipped into a solution of *A. tumefaciens* that was transformed with the SRF6-GFP construct via electroporation.

Figure 4: SRF6-GFP Arabidopsis T₁ seedlings were screened for growth on MS/hygromycin plates. Hygromycin resistant plants (larger green seedlings) were further grown to maturity in soil, and they and their T₂ offspring were screened for GFP expression.
Figure 5: The phosphorylation sites of SRF6 were mutated into glutamic acid (E) or alanine residues (A). TM; transmembrane domain in pink.

Figure 6: Western blot of leaves picked from individual T₁ plants transformed with wild type and mutant SRF6. All blots were probed with anti-6x histidine antibody. Lanes 1 and 30; marker, lanes 2-5; SRF6 T₃₆¹ΔS₃₆²ΔS₃₆⁴Δ plants, lanes 6-20; SRF6 T₃₆¹E S₃₆²E S₃₆⁴E plants, lanes 21-27; SRF6 WT plants, lane 28; WT non-transformed plant, and lane 29; REM2-HIS as a positive control.
Figure 7: Two week old T₁ seedlings of wild type or mutant SRF6 Arabidopsis plants.
Figure 8: SRF6<sup>T361E S362E S364E</sup> and SRF6<sup>T361A S362A S364A</sup> plants had smaller leaves than WT SRF6 plants. A plot of average leaf length for leaves three, four, and five in three randomly selected samples of SRF6 WT (blue), SRF6<sup>T361A S362A S364A</sup> (red), and SRF6<sup>T361E S362E S364E</sup> (green) plants, respectively.

Figure 9: Proteins were purified from <i>E. coli</i> with 6x HIS or GST tags.
Figure 10: Coomassie Stain and Western blot of protein samples showed amount of protein loaded, size of protein, and strength of antibody detection. WAK1 was probed with anti-GST antibody. All other proteins were probed with anti-6x HIS antibody.
**Figure 11:** Western blot of WAK1 (circle), SRF6 (pentagon), and ROG1 (triangle) kinase assay. Autophosphorylation of WAK1 (circle) and phosphorylation of ROG1 (triangle) were detected. The phosphorylation of SRF6 was uncertain (square). SRF6 and WAK1 created an unidentified smearing in the α-Phosphothreonine blot (lanes 5+6). Three unidentified bands with MW 100 kDa were seen in the α-HIS blot (diamond). ROG1 was incubated with kinase buffer (Lane 7) or not incubated (Lanes 8).
Figure 12: Western blot of WAK1 (circle), SRF6 (pentagon), ROG1 (triangle), and REM2 (hexagon) kinase assay. Autophosphorylation of WAK1 (circle) was present in addition to the phosphorylation of ROG1 (triangle). SRF6 bands appeared in the α-Phosphoserine blot (square). Smearing was not found as in the previous kinase assay. No REM2 (hexagon) phosphorylation was detected.
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAK1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRF6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRF6S→E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROG1S→E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REM2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1mM Mn²⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1mM Co²⁺</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

![Image of protein bands](image)

- **α-Phosphothreonine**
- **α-Phosphoserine**
- **α-HIS**
- **α-GST**

<table>
<thead>
<tr>
<th>kDa</th>
<th>130</th>
<th>95</th>
<th>72</th>
<th>56</th>
<th>43</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 13: Western blot of kinase assay mixing WAK1 (circle), ROG1 (triangle), SRF6 (pentagon), and REM2 (hexagon) and WAK1, ROG1$^{T361E \ S362E \ S364E}$ (triangle), SRF6$^{T361E \ S362E \ S364E}$ (pentagon), and REM2 separately with three different buffers (regular, regular + Co$^{2+}$, and regular + Mn$^{2+}$). There was no difference in levels of phosphorylation between regular kinase buffer and adding 1mM Co$^{2+}$ or 1mM Mn$^{2+}$. REM2 phosphorylation was not detected when phosphomimic ROG1$^{T361E \ S362E \ S364E}$ and SRF6$^{T361E \ S362E \ S364E}$ were added together with WAK1 and REM2. Autophosphorylation of WAK1 (circle) and the phosphorylation of ROG1 (triangle) were detected. Low levels of ROG1$^{T361E \ S362E \ S364E}$ phosphorylation (triangle) were also detected.
LITERATURE CITED


