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Rapid Oligo-Galacturonide Induced Changes in Protein Phosphorylation in Arabidopsis*[S]

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The wall-associated kinases (WAKs)[1] are receptor protein kinases that bind to long polymers of cross-linked pectin in the cell wall. These plasma-membrane-associated protein kinases also bind soluble pectin fragments called oligo-galacturonides (OGs) released from the wall after pathogen attack and damage. WAKs are required for cell expansion during development but bind water soluble OGs generated from walls with a higher affinity than the wall-associated polysaccharides. OGs activate a WAK-dependent, distinct stress-like response pathway to help plants resist pathogen attack. In this report, a quantitative mass-spectrometric-based phosphoproteomic analysis was used to identify Arabidopsis cellular events rapidly induced by OGs *in planta*. Using N^{14}/N^{15} isotopic *in vivo* metabolic labeling, we screened 1,000 phosphoproteins for rapid OG-induced changes and found 50 proteins with increased phosphorylation, while there were none that decreased significantly. Seven of the phosphosites within these proteins overlap with those altered by another signaling molecule plants use to indicate the presence of pathogens (the bacterial "elicitor" peptide Flg22), indicating distinct but overlapping pathways activated by these two types of chemicals. Genetic analysis of genes encoding 10 OG-specific and two Flg22/OG-induced phosphoproteins reveals that null mutations in eight proteins compromise the OG response. These phosphorylated proteins with genetic evidence supporting their role in the OG response include two cytoplasmic kinases, two membrane-associated scaffold proteins, a phospholipase C, a CDPK, an unknown cadmium response protein, and a motor protein. Null mutants in two proteins, the putative scaffold protein REM1.3, and a cytoplasmic receptor like kinase ROG2, enhance and suppress, respectively, a dominant *WAK* allele. Altogether, the results of these chemical and genetic experiments reveal the identity of several phosphorylated proteins involved in the kinase/phosphatase-mediated signaling pathway initiated by cell wall changes. ***Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.055368, 1351–1359, 2016.***

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[1] The abbreviations used are: WAKs, wall-associated kinases; OGs, oligo-galacturonides; EGF, epidermal growth factor; PME, pectin methylesterases.

The cell walls of angiosperms are composed of a complex arrangement of cellulose, hemicellulose, and pectin and are assembled through a complex, developmentally regulated coordination of synthesis, turnover, and interactions between protein and carbohydrates (1). The pectins can be selectively and locally cross-linked into a structural network that is subsequently remodeled and degraded by enzymes, and these events have dramatic effects on cell enlargement (2–6). Pathogens and mechanical disruptions also cause fragmentation and, thus, release of the pectin, leading often to a plant stress response (7–9).

A number of receptor kinases such as *THE1*, *FER, HERK*, ANX, and *RLP44* have been termed cell wall sensors (10–18) and typically have extracellular domains containing leucine-rich regions and a malectin carbohydrate-binding domain, although an experimentally demonstrated role for polysaccharide binding to their extracellular domains is unclear. Of the plant putative "wall sensors" only the wall-associated kinases (WAKs) are known to bind to a cell wall component, pectin, and these are distinguished also by their unique extracellular domain that lacks leucine-rich repeats and contains instead epidermal growth factor (EGF) repeats as well as a pectin-binding region (19).

Pectins are synthesized in the Golgi apparatus as methyl esterified 1–4 D-galacturonic acids and are secreted into an extracellular plant cell wall matrix composed of cellulose, hemicellulose, and a variety of proteins (1–5). Pectin methylesterases (PME) in the wall creates negatively charged pectins, leading to calcium-based crosslinking that is hypothesized to provide lateral structure and directionality of growth for a variety of cell types (16, 20–23). Localized digestion by plant-secreted polygalacturonases can also modify the pectin network by loosening the cell wall, thus facilitating directional expansion of cells (24, 25). Abscission zones at the root cap, petiole, and sepal base also express pectin-degrading enzymes that are part of the loosening process. In summary,

current models predict that pectin digestion is a part of numerous regulated developmental processes, and by nature generates local pools of pectin fragments, or oligo-galacturonides (OGs). Indeed, the biological activity of pectin fragments in developmental processes has been suggested for many years, although the molecular mechanisms remain unknown (6, 7, 26–28).

Pectins are also the target of numerous pathogens that digest the wall as they approach the plant cell, thereby generating de-esterified pectin fragments or OGs (7). These OGs can activate a plant stress response, indicating that OGs signal to the plant that a pathogen is present (7, 29). Physical wounding and herbivory can also trigger the accumulation of OGs and a stress response, presumably through a similar signaling pathway.

WAKs Bind to Pectin—WAKs are plasma membrane receptors that bind pectin, thereby activating several different possible responses. During seedling growth, *WAK2* is required for cell expansion and for the pectin activation of MPK3 and vacuolar invertase, presumably to increase turgor-driven expansion (19, 30, 31). Pectin also causes the induction and repression of hundreds of genes involved in cell wall biogenesis and stress responses, and this response is *WAK2* dependent (30, 31). Overall, this work suggested that WAKs serve as pectin receptors, a conclusion that has received additional support from the results of experiments in which the WAK1 extracellular domain was fused to the kinase domain of the EGFR receptor (32). OGs activated a pathway downstream of this hybrid kinase when it was transiently expressed in tobacco leaves.

A dominant *WAK2* allele *WAK2*^{cTAP}, whose encoded protein requires a functional pectin-binding domain and an active kinase, induces a constitutive stress response (33, 34). The response is dependent upon MPK6 and the pathogen response transcription factors EDS1 and PAD4. Importantly, the *WAK2* ^{cTAP} allele is also suppressed by a null allele of a pectin methyl esterase, *pme3* (33). This provides genetic evidence that WAKs are sensing the de-esterified form of pectin, consistent with the higher affinity *in vitro* of WAKs for de-esterified over esterified pectin and for pectin fragments of limited degrees of polymerization, 9–15 sugars (30, 31, 35, 36). The *pme3*/*pme3* mutant plant is more responsive to OGs than WT plants, as measured by the induction of the *FADLox* gene, a robust marker for OG induction of transcription (29, 33, 34). The data support a model in which OGs are competing with native pectin to bind WAKs, thus providing a mechanism for WAKs to distinguish changes in the pectin network and activate alternate pathways.

There are at least 21 other Arabidopsis genes encoding proteins with receptor kinase sequences similar to WAK that also contain EGF repeats, and these have been coined *WAK-like* or *WAKL* genes (37, 38). Unlike the model plant Arabidopsis, in crop plants such as rice and maize, the *WAKLs* have greatly expanded in number and a growing number of reports map disease resistance to specific mutant *wakl* loci (39–43), raising the possibility that WAK and WAKLs may prove to be important targets for future efforts at improving crop yield.

To understand how plant cells respond to pectin fragments that are induced by pathogens, biotic stresses, or during developmental patterns, a phosphoproteomic analysis was carried out to identify proteins phosphorylated as a result of OG treatment. The results reveal a subset of proteins that likely play a role in the pectin-induced signal transduction pathway and provide a new window into how changes to the plant cell wall are perceived.

EXPERIMENTAL PROCEDURES

Arabidopsis Metabolic Labeling and Untargeted MS—Growth conditions were as previously described (44). Treatment was with 50 μg/ml concentration of OG for 5 min. Three A (^{14}N OG-treated, ^{15}N mock-treated) replicates were used, and three B (^{14}N mock-treated, ^{15}N OG-treated) replicates were used. Each A or B experiment consisted of material from two flasks, combined into 85 ml homogenization buffer (44) with 1 mM DTT, 1 mM PMSF, 21.5 mM leupeptin, 1.5 mM pepstatin, 2 mM bestatin, 50 mM 1,10-phenanthroline, 100 mM vanadate, and 2.8 mM E64 added immediately prior to use. After combination, samples were immediately homogenized using a tissue tearer for 10 s at 10,000 rpm. Following filtration through one layer of miracloth, samples were spun at 6000 $\times$ *g* at 4°C for 7 min to pellet debris. Microsomal fractionation was performed by further spinning the supernatant from the 6,000 $\times$ *g* spin for 1 h at 4C and 100,000 $\times$ *g*. Supernatant from this spin yielded soluble protein-enriched fractions, and the pellet was resuspended into 1 ml of resuspension buffer using a Teflon homogenizer to yield an insoluble protein-enriched fraction. Methanol/chloroform precipitation was performed on fractions as previously described. Following protein precipitation, tryptic digestion, and desalting, phosphopeptide enrichment was performed using 200 μl spin-enrichment TiO_2-packed tips (GL Sciences, Inc. Torrance Ca) using supplier protocol.

Dried phosphopeptides were solubilized into Optima LC/MS-grade 0.1% formic acid in water (Fisher Scientific Pittsburg PA) for injection onto an LTQ Orbitrap XL using an Agilent 1100 LC system. Solvent A was 0.1% formic acid, and solvent B was 95% acetonitrile, 0.1% formic acid. Sample was loaded directly onto an analytical column of inner diameter 75 μm and outer diameter 360 μm house-packed with ~13 cm C18 resin (Magic-C18, 200Å, 3 μm, Michrom Biosources, Inc. Auburn Ca) at 0% B and a flow rate of 0.5 μl/min from 0–45 min, then eluted from 45–235 min at a flow rate of 0.2 μl/min and a gradient to 40% B, then from 235–255 min to 60% B, then from 255–260 min to 100% B. The column was then re-equilibrated by flowing 100% A from 260–278 min, stepping flow rate to 0.5 μl/min at minute 265.

MS1 spectra were collected using the Orbitrap at resolution 100,000 with preview mode enabled. The top five MS2 per MS1 were collected in the LTQ, rejecting +1 and unassigned charge states, using CID with an isolation window of 2.5 *m/z*, normalized CE of 35, activation Q of 0.25, activation at 30ms, and a minimum MS1 signal threshold of 500. Raw data were converted to .mgf files using default settings in the Trans-Proteomic Pipeline and searched using Mascot v2.2.2 and the Arabidopsis Information Resource (TAIR) protein database (version 9, June 19, 2009) with reverse sequences and common contaminants manually appended using BioEdit (Ibis Biosciences, 62,522 sequences in total). The enzyme trypsin was specified as the protease and one missed cleavage was allowed. A precursor and fragment ion tolerance of 20 ppm and 0.6 Da, respectively, were used. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation, serine/threonine/tyrosine phosphorylation,

TABLE I
List of primers used for PCR analysis. Gene names are shown on left, with forward (F) and reverse (R) primers, and combinations of T-DNA primers shown to the right of Gene name

NP	Oligo
AT1G18150 F and MLB1	F TTCTTGGTACTCCACCTCCTG
	R ATCTTTCGGATCAAAGGCAAG
AT1G53730 R and LBb1.3	F CAA CAT CTC GTG GTC TAT GAA TTC CAC AAA AATGG
	R GAC TGC TCA GAT CTT GAC CTT GTG CTG
AT1G59610 R and LBb1.3	F GGTGCGACACGGGCTCCTATTATC
	R CAGTTGTTTTTGGAGGTCCTTGATTTGAAAGAAG
AT2G01980 F and LBb1.3	F GATTTCTGGAGGAAGCGACCGATTCG
	R CTTAATTTGGTGAACTTCCATTGAAAACGAACTCTC
AT2G32240 F and LBb1.3	F GAGCTTGAAGAAGATTTGAGAATCGCTTTGC
	R CATTACTTCACTTTCCTGTAAGCCAGC
AT2G45820 R and LBb1.3	F CCACCGGTATAAGCTAACGGAGGATATAAAAACTC
	R CAT GCA CAT CAG AGA TCT TCT TTT GTG CC
AT3G08510 F and LBb1.3	F GGCAGCAGAAAAATATGCGAAGCAG
	R GGTTTTCAATTGTTGAGGACCAAGCAGA
AT3G28690 F and LBa1	F ACAGCAGAACCTAAGAAAGTTATTGAGAAGCTTG
	R CTT TGG TGA TTG ACG GGC ATA ACG ATA AG
AT3G61260 R and LBb1.3	F TGGCGGAGGAACAGAAGATAGCGTTAG
	R GGT GCA GGC TCT TCT ACA GG
AT5G19450 R and LBb1.3	F GGAAATTGTTGTGCGAGCCCG
	R CTG AAC AAC TTC AAG AAT AGT CTT CAT CAC TGC AG
AT5G20490 F and LBb1.3	F GATAAATGAGGGCAAGAGCAACTCAATTCTGG
	R AA ACT GAA GCT CGA CAA ACT TTC CAA ATC G
AT5G65950 F and LBb1.3	F GCCTCTTTTCACAATCTGTTCAAGAGCC
	R GGG TAT TGG CTT CAC TGC CAT GTG
LBb1.3	ATTTTGCCGATTTCGGAAC
LBa1	TGGTTCACGTAGTGGGCCATCG
MLB1	GTGGACTCTTGTTCCAAACTG

and asparagine/glutamine deamidation were set as variable modifications. Mascot output was filtered to 1.0% false discovery rate using an in-house written script, and Census (45, 46) was used to extract ion chromatograms and quantify $^{14/15}$N area ratios. Census output was reformatted using an in-house written script, and data were median-normalized per injection. ^{15}N Mascot searches were performed for all B experiment .raw files. Identical filtering and processing was applied, and data were added to the list of phosphochanges. Phosphopeptides of interest with Mascot score $\leq$ 30 or potentially ambiguous phosphorylation localization (*i.e.* multiple phosphoisoforms were identified) were manually validated by raw MS2 spectrum analysis when possible. Ambiguity, even after manual examination, is noted in the supplemental table. All data have been provided as processed Excel files and raw data, which have been uploaded to and is publicly available via Chorus (https://chorusproject.org), and individual spectra are viewable using Scaffold (Proteome Software) and Supplemental File 2. All scripts are available on the Sussman laboratory website (http://www.biotech.wisc.edu/sussmanlab).

Experimental Design and Statistical Rational—To obtain the values highlighted in this manuscript, OG-responsive phosphorylation events had their ratios collapsed into a single data point by averaging all the values for the ^{14}Ntreat/^{15}Ncontrol experiment (A) and the inverse values of the ^{14}Ncontrol/^{15}Ntreat experiment (B). The requirement set for this manual calculation was that the peptides had at least one data point for both the A and the B experiment, and an averaged fold change of $\geq$1.20. *t* tests were performed using the spread of ratios from A experiments and from B experiments prior to inverting the B values, the results of which are the *p* values displayed. In the event that, for example, a peptide was identified in only one of the three A or B experiments, a *p* value could not be calculated; thus, some collapsed ratios have a value of "N/A."

Plant Growth—*Arabidopsis thaliana* columbia was grown on soil or agar plates as described (34) at 22 °C, 16 h light, 8 h dark. For treatment with OGs, seedlings were plated in a microtiter plate with 5 ml of 0.5X MS media plus vitamins, vernalized for 3 days, and incubated at 22 °C with gentle shaking under 24 h light. After 7 days at 22 °C, OGs were added to 50 μg/ml unless otherwise noted, shaken for an additional 3 h, and then seedlings were frozen in liquid nitrogen.

Preparation of OG—400 ml of 1% polygalacturonic acid (PGA Sigma P3850, 85% de-esterified), pH 4.4 (NaOH), was autoclaved for 45 min, and then HCL was added dropwise to pH 2 while stirring. The preparation was centrifuged at 12,000 $\times$ *g* for 20 min and the supernatant was adjusted to 50 mM NaOAc, 22.5% EtOH (should be pH 6 final). The sample was incubated at 4 °C for 12 h and centrifuged at 16,000 $\times$ *g* for 30 min. The pellet was resuspended in 50 ml water, and dialyzed *versus* five changes of water for 2 days at 4 °C using a 1,000 kDa membrane. The solution was then lyophilized to powder. OGs were resuspended in water as needed and analyzed using Dionex chromatography to determine that the preparation had a dp of predominately 9–15. From 4 g of material, 800 mg of OGs were recovered. Esterification was accomplished by adding 800 μl of MeOH and 40 μl H_2SO_4 to 5 mg OGs and incubation for 24 h. The OGs were pelleted in a microfuge and resuspended in 1 ml MeOH, 37.5 μl H_2SO_4 for a further 24 h. The OGs were then washed three times in 1 ml 80% ETOH, dried, and resuspended in water.

Genotyping—Plants were genotyped by PCR according to (21) and using primers listed in Table I.

RNA Analysis—RNA was isolated from plant material using the RNeasy Plant Mini Kit (Qiagen Hilden Germany, http://www.qiagen.com). Q-PCR was as described (29, 34); 1 μg of RNA was used for a reverse transcription assay using oligo dT for first-strand synthesis in a Invitrogen Superscript III RTPCR Kit (Invitrogen 18080–051 Carls-

bad Ca). cDNA was then used for QPCR using Power SYBR Green Master Mix (Applied Biosystems Foster City Ca) and an Applied Biosystems StepOne system, version 2.1, which calculated the comparative CT (DDCT) with the following cycles: 95 °C for 15 s, 56 °C for 1 m, repeated 38 times. Primers used for each gene are listed in Table I. Actin expression served as an internal standard and wild-type untreated samples were set as the standard to which other samples were compared, in triplicate. Bar graphs in figures show relative quantitation max and min. Statistical analysis used DCT and DCTSE values in a two-tailed *t* test.

RESULTS AND DISCUSSION

Phosphoproteomics—To identify cellular events affected by the presence of OGs, proteins were identified whose level of phosphorylation was changed rapidly after treatment of plants with OGs. For this quantitative mass-spectrometric-based analysis, seedlings were grown in liquid media containing N^{15} and then treated with OGs for 5 min, a brief period of time that is known to activate a WAK-dependent response (30, 32–34). Mock treated seedlings were grown in N^{14} media. The samples were frozen, combined, fractionated into a membrane and soluble fraction, and analyzed by mass spectrometry, and the N isotope identified the source. The experiment was performed in triplicate. To control for any possible effects of the isotope, the N^{14} and N^{15} were switched between the treatment type and the experiment was performed in triplicate again, for a total of six biological replicates (18, 47–49). From the peptides identified (Supplemental Table 1), phosphopeptides were selected that appeared in at least one of either experimental set's replicates and whose fold change had an average value between experimental sets of $\geq$1.2. The goal was to use the proteomic analysis as a discovery experiment, and this fold level was chosen over one more stringent as it includes a greater number of candidates whose validity could be tested by alternate assays, such as genetic interactions, as described below. Statistical significance of fold change (*p* values) was calculated, and the vast majority were found to be $p \leq 0.05$. This list, comprised of 50 different phosphopeptides, is shown in Table II where "fold change" is relative to mock treatment set to 1.

This list of 50 phosphopeptides was then compared with those identified previously from experiments with the bacterial elicitor Flg22 (50–52), and seven were found to be in common (gray gene number in Table II). Of these seven, only one, the ABC transporter PEN3, had the same phosphorylation site induced by both Flg22 and OGs. Both OG and Flg22 induce phosphorylation but at different sites of: CDPK19, a calcium-dependent kinase; SYP122, a syntaxin-like vesicle transport protein, AHA1 a proton ATPase, MSL6 a mechano-sensitive channel, and REM1.3 a proposed signaling scaffold protein. Beyond these seven phosphorylation events, OGs and Flg22 induce quite different responses, and an overlapping but distinct profile is also observed at the transcriptional level (29, 34) and in the induced plant's phenotypes. Notable too is the absence from OG but not Flg22 treatment of the receptor kinase BIK1 phosphorylation, a common substrate for LRR-type kinase pathways (52, 53). This could be because BIK1 phosphorylation is either not induced by OGs or induced at a level too low to identify using untargeted mass spectrometry.

In order to evaluate the physiological relevance of these phosphorylation events in relation to OG signaling, the list was reduced in size by concentrating on non-Flg22-induced events (*i.e.* OG specific) and on those with a twofold or higher phosphorylation change (Table III). CDPK19 and REM1.3 were retained on the list as examples of overlap with the Flg22 database. Two protein kinases, ROG1 and SRF6, were also included in the shorter list as they have a high potential to describe a signaling cascade, although the significant fold change was less than 2. Most relevant to a potential OG signaling pathway are two cytoplasmic receptor-like kinases we have termed ROG1 and 2 (response to OGs). ROG1 and 2 are members of a large family whose members can associate directly with membrane receptors and/or mediate signal transduction (54, 55). Thus, WAKs may interact with the ROGs, but this remains to be tested. The receptor kinase SRF6 is a member of the STRUBBELIG family whose function is unknown, although one member is important in development (56). Two remorin family members REM1.2 and 1.3 were also identified. The remorins were first observed as being phosphorylated upon OG treatment of seedlings in 1996 and represent a diverse family that may act as scaffolding proteins for signaling at the plasma membrane (57–59). Peptides for MPK3 nor MPK6 were not identified in this untargeted analysis, and thus, although they may play a role in WAK/OG signaling (30, 34), our experiments cannot confirm or refute this. MPK8 was the only phosphorylated MPK induced by OG identified in this analysis. OGs also induced phosphorylation of a motor protein, and we speculate that this may be related to receptor turnover by vesicle traffic, but this remains to be experimental tested. A phospholipase C was also phosphorylated in response to OG treatment, suggesting that OGs may induce the generation of IP_3. The cadmium response protein is phosphorylated but is of unknown function. None of the five WAKs were identified in this analysis, likely because they fractionate with the cell wall rather than the membranes or the cytoplasm. Alternatively, the WAK receptor may have been too rapidly internalized or degraded, as is the case for the FLS2 receptor, which was also not identified in the Flg22 phosphoproteomic analysis (52).

Mutations Reduce OG Response—Homozygous null mutations were identified for genes on the reduced list in order to determine which were necessary for the response to OGs. T-DNA knock-out lines all having insertions in exons were available for 12 genes on the short list and homozygous lines for each were isolated. Reverse transcriptase PCR analysis of RNA isolated from plants homozygous for nine of these lines showed that, while mRNA was detected from wild-type loci, no mRNA could be detected from plants homozygous for the mutant gene (ko, Fig. 1). For three lines not shown, no RNA

TABLE II

Phosphopeptides and their corresponding genes induced by OG treatment of seedlings. Bold star indicates phosphorylated residues, with fold induction relative to wild type to the right. Ambiguity in phosphosite assignment is noted in Supplemental Table S1. Fold induction was calculated from as many of six replicates as possible, and a two-tailed t-test p value is shown. Gray gene numbers indicate peptides also induced by Flg22

NP	Name	Sequence	Fold change	*p value*	st. error
AT1G59870.1	PEN3 (penetration 3); ATPase	R.NIEDIF**S***SGS*R.R	10.47	0.022557	3.04
AT2G32240.1	Function unknown;response to cadmium ion	R.DIDLSFSS*PTK.R	4.61	0.000185	0.31
AT4G35060.1	Heavy-metal-associated domain/copper chaperone (CCH)-related	M.GVLDHVSEYFDCSHG**S***S*K.R	4.56	0.002473	0.62
AT2G01980.1	SOS1 (salt overly sensitive 1); sodium:hydrogen antiporter	R.SVS*FGGIYNNK.L	4.35	0.013092	2.70
AT5G65950.1	Unknown protein	K.INLVDVGGGGLFS*PR.E	3.81	0.001071	0.31
AT3G52400.1	SYP122 (syntaxin of plants 122); SNAP receptor	K.V**S***GG**S***CHGGNNLD**T***FFLDVEVVNEDLK.E	2.63	0.002003	0.90
AT3G58730.1	Vacuolar ATP synthase subunit D (VATD)	R.GIS*INAAR.N	2.60	0.006239	0.37
AT2G45820.1	Remorin family protein REM1,3	K.ALAVVEKPIEEHT*PKK.A	2.47	0.000439	0.13
AT4G39680.1	SAP domain-containing protein	K.VPEAQITNSATPTTT*PR.S	2.44	0.000114	0.06
AT3G61260.1	Remorin family protein REM1.2	K.RLS*FVR.A	2.43	0.000231	0.12
AT5G60660.1	PIP2;4 (plasma membrane intrinsic protein 2;4); water channel	K.ALGSFGSFGS*FR.S	2.27	0.003992	0.33
AT3G08510.1	ATPLC2 (phospholipase C 2); phospholipase C	R.EVPS*FIQR.N	2.26	0.000806	0.10
AT2G36380.1	PDR6; ATPase, coupled to transmembrane movement of substances	K.GSHSGTGGSVVELTSTS*SHGPK.K	2.24	N/A	0.16
AT5G19450.1	CDPK19 (calcium-dependent protein kinase 19)	K.SNPFYSEAYTTNG**S***G**T***GFK.L	2.24		0.05
AT4G24520.1	ATR1 (*Arabidopsis* P450 reductase 1)	K.S*MESNVANGNTTIDIHHPCR.V	2.18	0.000003	0.05
AT5G20490.1	XIK; motor/protein binding	R.ENS*GFGFLLTR.K	2.14	0.000119	0.19
AT3G43300.1	ATMIN7 guanyl-nucleotide exchange factor	K.SSVAEVTVPSS*PYK.H	2.08	0.000094	0.25
AT1G59610.1	ADL3 (*Arabidopsis* dynamin-like 3); GTPase	R.AAAASSWSDNSGTESS*PR.T	2.02		0.12
AT1G18150.2	ATMPK8; MAP kinase	R.VSFNDAPTAIFWTDY*VATR.W	2.00	0.000725	0.16
AT4G19490.1	Protein binding	K.SIS*DASSQSLSSILNNPHGGK.S	1.97	N/A	0.15
AT1G04280.1	Unknown protein	R.VIS*TLVEEMR.V	1.88	0.025929	0.15
AT4G39680.1	SAP domain-containing protein	K.VPEAQITN**S***A**T***PTTT*PR.S	1.86	0.000047	0.09
AT3G05200.1	ATL6; protein binding/zinc ion binding	R.TNS*LLVLPR.G	1.85	0.000173	0.11
AT5G46750.1	AGD9 (ARF-GAP domain 9);	K.DNLYEQKPEEPVPVIPAAS*PTNDTSAAGSSFASR.F	1.83	0.000002	0.05
AT2G39970.1	Peroxisomal membrane protein (PMP36)	K.DQTAAPES*PSSNAEALVAVEPRPYGTFNTIR.E	1.81	0.000336	0.11
AT3G03570.1	Molecular_function unknown	R.YSLVLDPNLDAGT*PR.A	1.79	0.000593	0.05
AT5G65950.1	Unknown protein	R.TS**S***FRDPL**S***VSDSASPIPSR.C	1.78	0.000018	0.04
AT2G36380.1	PDR6; ATPase, coupled to transmembrane movement of substances	R.LPT*YDRLR.K	1.76	N/A	0.30
AT4G20780.1	Calcium-binding protein, putative	R.SPS*LNALR.L	1.75	0.006863	0.10
AT2G18960.1	AHA1 (*Arabidopsis* H+ ATPase 1); hydrogen-exporting ATPase	K.GS*YRELSEIAEQAK.R	1.69	0.024305	0.12
AT1G21170.1	SEC5B	K.VVLTSLQS*FPR.G	1.60	0.010393	0.08
AT1G64780.1	ATAMT1;2 (ammonium transporter 1;2)	R.HGGFAYAYNDEDDVS*TKPWGHFAGR.V	1.56	N/A	0.08
AT2G15860.1	Unknown protein	K.LENSVQQGSS*PR.E	1.56	N/A	0.14
AT3G28690.1	Protein kinase, putative ROG1	R.**S***L**S***SLNLPQASPYR.Y	1.54	0.005774	0.16
AT4G29900.1	ACA10 (calcium-transport ATPase/calmodulin binding	K.DVEAGT**S***S***T***EYEDSPFDIASTK.N	1.51	0.000610	0.05
AT5G40390.1	SIP1 (seed imbibition 1-like);	K.SDS*GINGVDFTEK.F	1.48	0.000432	0.06
AT1G76850.1	SEC5A (exocyst complex component SEC5)	K.VALT**S***LQ**S***LPR.G	1.46	0.000023	0.03
AT1G62330.1	Unknown protein	R.VQDLIHGGGGAS*PVQ**S***P**T***R.L	1.45	N/A	0.07
AT2G20960.1	pEARLI4	K.NEESVLLFPELILS*PQERPPSR.L	1.38	0.051386	0.18
AT3G10660.1	CPK2 (calmodulin domain protein kinase)	R.VSS*AGLR.T	1.37	N/A	0.05
AT1G53590.1	NTMC2T6.1	K.KEEFLIGSIEEESQSQS*PR.I	1.36	0.000185	0.03
AT1G78610.1	MSL6 (mechanosensitive channel of small conductance like)	K.SPFLSHVLS*NGGGGGGENK.G	1.35	N/A	0.09
AT5G08080.1	SYP132 (syntaxin of plants 132); SNAP receptor	R.GQ**S***S***T***REGDVELGEQQGGDQGLEDFFK.K	1.35	0.000122	0.03
AT2G36380.1	PDR6; ATPase, coupled to transmembrane movement of substances	K.S*FRDVFAPPTDDVFGR.S	1.34	0.188130	0.16
AT1G05960.1	Binding	K.KVS*FKPNPVTDYK.L	1.33	0.012570	0.07
AT3G11820.2	SYP121 (syntaxin of plants 121); SNAP receptor/protein anchor	R.ASS*FIRGGTDQLQTAR.V	1.31	N/A	0.05
AT1G51690.1	ATB ALPHA; nucleotide binding/protein phosphatase type 2A regulator	R.VV**S***RG**S***ESPGVDGNTNALDYTTK.L	1.30	0.039172	0.09
AT4G32650.2	ATKC1 (*Arabidopsis thaliana* K^+ rectifying channel 1	R.S*PLSILQFR.R	1.30	N/A	0.01
AT1G53730.1	SRF6 (STRUBBELIG-receptor family 6)	K.KLD**T***S***L***S***INLRPPPIDR.N	1.26	0.054814	0.10
AT2G45820.1	DNA-binding protein, putative	K.AS**S***G**S***ADRDVILADLEK.E	1.25	0.009060	0.03

TABLE III

*Partial list of peptides (and their identifying gene) phosphorylated upon OG treatment of seedlings. Bold star indicates phosphorylated residue, with fold induction relative to wild type to the right. Percentage on far right column indicates FADlox mRNA induction relative to wild type (RT QPCR in triplicate,) by OG of plants homozygous for a null allele of the indicated gene (NP). * % indicates significantly different from wild-type induction (t-test $p < 0.01$) taken from Fig 2. ND, not determined due to lack of mutant. Gray gene numbers refer to those identified in Flg22 analysis*

NP	Name	Sequence	Fold change	OG induction
AT2G32240.1	Cadmium response	R.DIDLSFSS*PTK.R	4.61	*55%
AT4G35060.1	copper chaperone	M.GVLDHVSEYFDCSHG**S*S***K.R	4.56	ND
AT2G01980.1	SOS1	R.SVS*FGGIYNNK.L	4.35	99%
AT5G65950.1	ROG2	K.INLVDVGGGGLFS*PR.E	3.81	*55%
AT3G58730.1	V-ATPase	R.GIS*INAAR.N	2.60	ND
AT2G45820.1	Rem1.3	K.ALAVVEKPIEEHT*PKK.A	2.47	*34%
AT4G39680.1	SAP domain	K.VPEAQITNSATPTTT*PR.S	2.44	ND
AT3G61260.1	Rem1.2	K.RLS*FVR.A	2.43	*56%
AT5G60660.1	PIP2;4	K.ALGSFGSFGS*FR.S	2.27	ND
AT3G08510.1	PLC2	R.EVPS*FIQR.N	2.26	*80%
AT2G36380.1	PDR6; ATPase	K.GSHSGTGGSVVELTSTS*SHGPK.K	2.24	ND
AT5G19450.1	CDPK19	K.SNPFYSEAYTTNG**S***G**T***GFK.L	2.24	*61%
AT4G24520.1	ATR1 P450 Reductase	K.S*MESNVANGNTTIDIHHPCR.V	2.18	ND
AT5G20490.1	motor	R.ENS*GFGFLLTR.K	2.14	*78%
AT3G43300.1	ATMIN7 GEF	K.SSVAEVTVPSS*PYK.H	2.08	ND
AT1G59610.1	ADL3 Dynamin-like	R.AAAASSWSDNSGTESS*PR.T	2.02	106%
AT1G18150.2	MPK8	R.VSFNDAPTAIFWTDY*VATR.W	2.00	94%
AT3G28690.1	ROG1	R.**S***L**S***SLNLPQASPYR.Y	1.54	*67%
AT1G53730.1	SRF6	K.KLD**T*S***L**S***INLRPPPIDR.N	1.26	103%

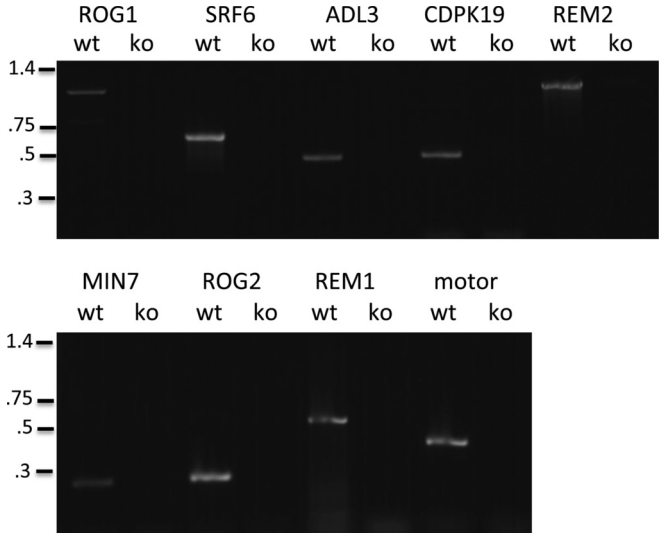

FIG. 1. **T-DNA insertions block mRNA accumulation.** qRT-PCR of RNA isolated from wild type (wt) or the homozygous mutant (ko) for the indicated gene (above gel). Primers used for the analysis are listed in Table I. Numbers to the left indicate kb.

could be detected in wild-type plants (data not shown). None of the plants homozygous for the T-DNA insertion had visible phenotypes on soil or MS agar (data not shown) in the absence of added OGs. To determine if plants that were homozygous mutant for these knockout alleles had an altered response to OGs, the level of OG -*FADlox* transcript relative to mock treated was measured by qRT-PCR of isolated RNA, with actin as a standard relative control. The results are compiled in Table III along with the phospho-peptide information, and the qRT-PCR results relative to wild type are shown in Fig. 2. In wild-type (WT) plants, *FADlox* expression was induced by OGs ~1,500-fold relative to untreated plants (29, 30, 33, 34). Of the 12 mutant lines tested, 8 showed a compromised OG response, the level of which relative to wild type (WT) is indicated in the Table III ("OG induction," differences are significant $p < 0.01$ indicated by an *). These include the potential scaffold proteins REM1.2 and REM1.3, two receptor-like cytoplasmic kinases ROG1 and ROG2, one receptor kinase SRF6, a phospholipase C, a motor, and a protein associated with a cadmium response. Thus, when plants contain knockout mutations in 8 of the 12 proteins with OG-induced phosphorylation, the OG transcriptional response is reduced.

Genetic Interactions—To further test the biological relevance of the phosphorylation events detected in the 12 candidates, the homozygous mutant line of each was independently crossed with a hyperactive dominant *WAK* allele, $WAK2^{cTAP}$ that induces stress-related genes (e.g. *FADLox*), curling of leaves, and stunted growth (30, 34). The F2 of each cross was screened by PCR for plants homozygous for the candidate gene T-DNA and also the $WAK2^{cTAP}$ allele. Of the 12 lines tested, two alter the $WAK2^{cTAP}$ phenotype and 10 have no visible affect. The two mutants that alter the $WAK2^{cTAP}$ phenotype are shown in Fig. 3, and the PCR-based genotyping confirming all mutant alleles are present is shown in Fig. 4. Compared with WT plants, $WAK2^{cTAP}$ exhibit the dwarfed, curly leaf phenotype, and both *rem1.3*−/− and

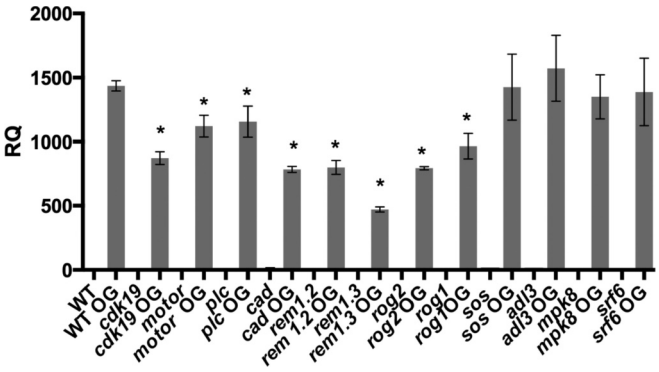

FIG. 2. **qRT-PCR of RNA isolated from plants of the indicated genotype treated or not treated with OG.** Gene name on X-axis indicates homozygous recessive allele. RQ, relative gene expression level with actin as internal control. * indicates significantly different ($p < 0.01$ relative to wild type, WT).

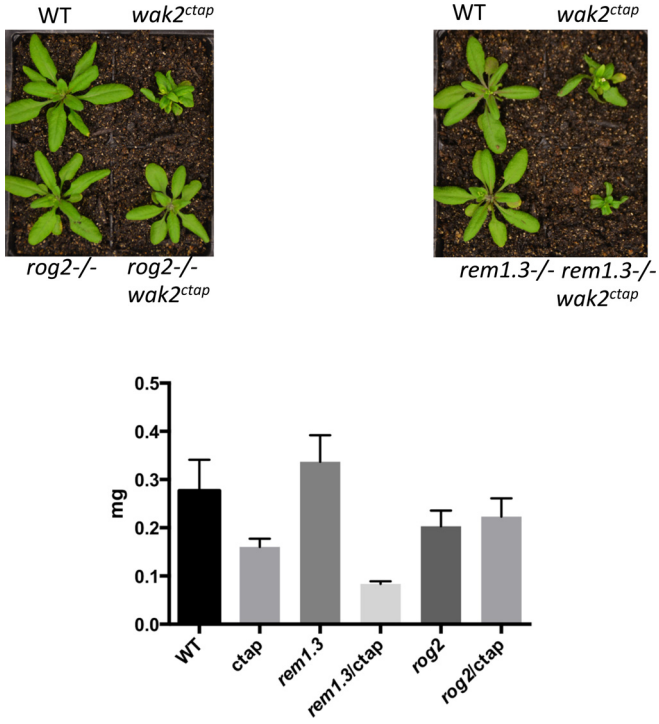

FIG. 3. **Suppression and enhancement of WAK2^{cTAP} dominant allele by *rog2* and *rem1.3*.** Plants are homozygous for all indicated alleles (see Fig. 4). Plants of the indicated genotype grown on soil (*top panel*). +/+; homozygous wild-type allele, −/− homozygous mutant. The mass of five plants of each genotype was determined and compared.

rog2−/− plants appear normal. However, compared with the WAK2cTAP single mutant background, the double mutant *rog2*−/− *WAK2^{cTAP}* plant is larger and has no curled leaves, indicating that *rog 2* suppresses the *WAK* dominant allele. By contrast, *rem1.3*−/− *WAK2^{cTAP}* double mutants have a more severe phenotype than *WAK2^{cTAP}*. The wet weight of five isolates of whole plants of each genotype was measured, and the results are shown in Fig. 3, indicating that the *rog2*−/−

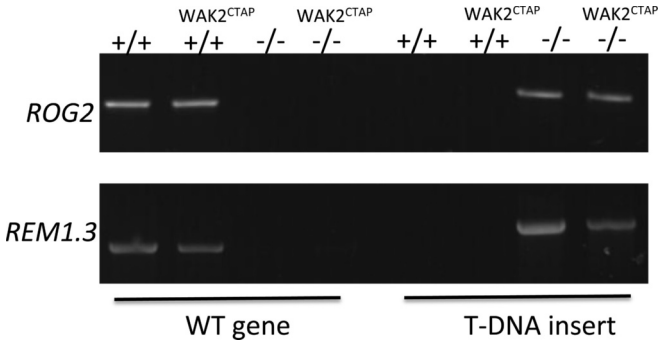

FIG. 4. **Genotypes of *rog2* and *rem1.3*** WAK2^{cTAP} double mutants. PCR using DNA isolated from plants having the indicated genotype (above each lane) for either *ROG2* or *REM1.3* genes (left of gels). +/+; homozygous wild-type allele, −/− homozygous mutant. Primers detected the WT alleles or T-DNA alleles (indicated at bottom of gels). WAK2^{cTAP} was detected using segregation of linked Basta resistance and Western blotting for TAP tag.

WAK2^{cTAP} plant is significantly larger than *WAK2^{cTAP}* (*t* test, $p < 0.01$) and therefore suppresses the dwarfed phenotype, and the *rem1*−/− *WAK2^{cTAP}* is significantly smaller than *WAK2^{cTAP}* (*t* test, $p < 0.01$) and therefore enhances the dwarfed phenotype. The mass of *rem1.3*−/−, *rog2*−/− and *rog2*−/− *WAK2^{cTAP}* plants are not significantly different from wild type (*t* test, $p < 0.01$). All plants with a *WAK2^{cTAP}* allele express wild-type levels of WAK2^{cTAP}, indicating suppression and enhancement is not due to altered gene expression of the mutant WAK allele (data not shown).

In conclusion, our results indicate that the cytoplasmic kinase ROG2 is required for the effect of the hyperactivated *WAK2^{cTAP}* allele and, hence, by inference, for WAK action. This is supported by the observation that WAK2^{cTAP} has been shown to be pectin responsive and dependent upon the pectin binding and kinase domains in the receptor (34). These results also corroborate the finding that the OG transcriptional response in *rog2* homozygotes is compromised (Table III, Fig. 2). REM1.3 is predicted to be a scaffold protein that might bind signaling proteins near the plasma membrane (58). The observation that *rem1.3* homozygotes compromise OG induced *FADlox* gene expression but enhance the dominant WAK phenotype suggests that its absence has an effect on a mechanism that induces the WAK2^{cTAP} phenotype independent of *FADlox* gene expression reduction. The reduction of *FADLox* induction may also be insufficient to abate a phenotype that is still enhanced by *rem1.3* mutants. Nevertheless, the results demonstrate that both REM1.3 and ROG2 affect both OG activation and interact genetically with *WAK2*, providing further support of their role in OG signal transduction. However, the results here have yet to indicate if the binding of OGs to WAKs are inducing the observed phosphorylation events.

By using a robust phosphoproteomic analysis of Arabidopsis seedlings and genetic validation through testing of mutants in the genes encoding these peptides, results we have

obtained have implicated a receptor-like kinase SRF6, two cytoplasmic kinases ROG1 and ROG2, and putative scaffolding proteins REM1.2 and REM1.3 in OG signaling. Other possible candidates specific for an OG signaling cascade include a phospholipase C, a motor protein, and an unknown Cd response protein. Further genetic and biochemical analysis will be required to determine how these combine to elicit the perception of pectin fragments in the plant cell wall.

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