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Examining the role of GRP and LIK1 in Wall Associated Kinase (WAK) perception of pectin in the plant cell wall

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An Honors Project for the Program of Biochemistry

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ABSTRACT

Wall associated kinases (WAKs) are cell membrane bound receptor kinases that bind pectin and pectin fragments (OGs).The binding of WAKs to pectin sends a growth signal required for cell elongation and plant development. WAKs bind OGs with higher affinity than native pectin and instead activate a stress response. Glycine rich proteins (GRPs) are secreted cell wall proteins of unknown function. Seven GRPs with 65% sequence similarity are coded on a 90kb locus of Arabidopsis chromosome 2. GRP3 and WAK1 have been shown to bind *in vitro*, but single null mutations have no discernible phenotype, suggesting that the GRPs are redundant. Low recombination frequency has made multiple mutations difficult to achieve, but in this thesis, CRISPR/Cas9 technology was used to induce deletions of the GRP locus. The promoters pYAO and pICU2 drove Cas9 expression in transformed Arabidopsis plants. The presence of a deletion and Cas9 were detected by PCR. While somatic mutations were induced, there was no inheritance of the GRP deletion, indicating that pYAO and pICU2 do not drive Cas9 to induce deletions in progenitor cells.

LIK1 is a CERK1 interacting kinase implicated in mediating response to various microbe associated molecular patterns (MAMP) such as chitin, flagellin, and peptidoglycans. LIK1 exhibits a drastic increase in phosphorylation in response to OG treatment, making it a candidate for a co-receptor to WAK. T-DNA insertions to the 5'UTR of *LIK1* were used to examine the effect of a *lik1* mutation on the OG induced stress response. *lik1/lik1* mutant seedlings were grown in the presence and absence of OGs, and RNA was isolated. qPCR was used on cDNA to examine *FADLOX* expression, a reporter for the transcriptional response to OGs. The *lik1/lik1* mutant caused a reduction in the OG induced transcriptional response. However, increased *LIK1* expression was associated with the T-DNA insertion indicating that LIK1 inhibits the WAK stress response pathway. Understanding the roles of GRP and LIK1 in moderating WAK mediated pathogenic response in Arabidopsis will enable a better understanding of plant resistance to pathogen invasion in the greater plant kingdom.

Introduction

Cellular interaction with the extracellular environment is crucial to survival in a changing environment. In the multicellular organism, this extracellular environment often involves the extracellular matrix, and interactions between receptors at the cell membrane and ligands in the matrix allow for cellular reaction to signals of adjacent cells and environmental input. In plants, the cell wall is a key extracellular structural component made up of a number of proteins, cellulose, hemicellulose, and pectin polymers, forming a matrix that gives the plant structural integrity (B. Kohorn, 2015). The cell wall also defends the plant against pathogens and environmental stresses such as lack of water and changes in temperature (Hamann, 2015). The cell wall has a vast number of proteins, 715 described already just for the model species *Arabidopsis thaliana* in the *WallProtDB* plant cell wall protein database (Canut, Albenne, & Jamet, 2016). Some of these proteins contribute to cell wall structural integrity, while others are key signal molecules for intercellular communication and for cellular perception of the cell wall (Cosgrove, 2005; Matsubayashi & Sakagami, 2006). The other components of the cell wall also interact with receptors in the cell membrane to modulate cell wall integrity (CWI) and protect the plant from environmental and pathogenic stressors.

This project examines the role of two proteins that possibly interact with wall associated kinase (WAK) perception of the cell wall in order to moderate response to pathogen invasion.

Cell Wall Polysaccharides

Polysaccharides make up the polymer matrix of the cell wall. These carbohydrate polymers are synthesized as the plant grows, and secreted from the cell into the extracellular matrix to be integrated into the wall. Cellulose, perhaps one of the most famous cell wall polymers, has a variety of human uses including paper, textiles, building materials, and biofuels. In the cell wall, cellulose polymers form multiple chain complexes called microfibrils, allowing for a net-like, flexible, yet strong matrix component of the wall (Cosgrove, 2014). It appears that a single cellulose synthase complex is sufficient for cellulose polymer synthesis, and that as cellulose polymers are synthesized at the plasma membrane, microfibrils form with up to 18 chains cross linked, and twist according to the location of the cell wall in the plant and the special biomechanical concerns of each location (Cosgrove, 2014; Zhao et al., 2013). By regulating the properties of cellulose microfibrils, a plant can have adaptable and resilient structure.

Cellulose microfibrils are connected by a matrix of hemicellulose and pectin polymers. Pectin and hemicellulose are synthesized in the Golgi and secreted via vesicles to the extracellular matrix (Cosgrove, 2005; Reiter, 1998; W. Willats et al., 2001). The two most common pectin polysaccharides are rhamnogalacturonan I and homogalacturonan. Pectin and hemicellulose polymers secreted to the cell wall diffuse into the matrix, forming covalent and ionic bonds with other elements of the cell wall, including cross linkages with Ca^{2+} ions. The hemicellulose species xyloglucan, for example, is thought to cross link cellulose microfibrils, binding them together to increase rigidity, and also to bind pectin polymers (Cosgrove, 2005;

Willats, McCartney, Mackie, & Knox, 2001; Wolf, Hématy, & Höfte, 2012). Plant cells grow by increasing internal turgor pressure, and selectively breaking or loosening crosslinkages between cellulose and hemicellulose or pectin polymers, the cell wall is able to elongate in a controlled manner, before being locked into the elongated position by reformation of crosslinkages and interpolymer bonds (Cosgrove, 1999, 2005; W. Willats et al., 2001; Wolf et al., 2012). The enzyme extensin is likely the largest contributor to crosslink disruption for cell wall elongation (Cosgrove, 1999, 2005; B. D. Kohorn, 2001; Reiter, 1998; Wolf et al., 2012).

Pectin and pathogen-induced disruption

The cell wall plays a role in protecting the plant from pathogens physically and chemically, signaling a pathogenic invasion via identification of a polymer disruption. Invasion of the plant by a pathogen frequently results in disruption or reorganization of the cell wall. When pathogens invade a plant, pectin polymers are fragmented into shorter polymers known as oligogalacturonides (OGs) (Benedetti et al., 2015). When the pathogen begins to infect a plant, the pathogen releases cell wall degrading enzymes (CWDEs) to break down the cell wall for easier access to the cell. Pectic enzymes – enzymes that target pectin in the cell wall – are among the most common and earliest secreted CWDEs from bacterial and fungal pathogens (Lionetti, Cervone, & Bellincampi, 2012). These pectic enzymes include polygalacturonases (PGs), pectate lyases (PELs) and pectin methyl esterases (PMEs). PMEs remodel pectin polymers by hydrolyzing methylesters, reducing the amount of methylesterification on the pectin, affecting Ca^{2+} crosslinkages. Pectin

with disordered methylesterification is targeted by pectic enzymes which degrade the polymer into fragments called oligogalacturonides (OG's) (L'Enfant et al., 2015; Lionetti et al., 2012; W. G. T. Willats et al., 2001). Furthermore, decreasing Ca^{2+} crosslinkage is a mechanism for cell wall loosening. Because a limited amount of pectin breakdown is required for the cell wall to allow cell elongation and plant growth, plant cells produce their own PMEs as well in order to facilitate plant growth (Cosgrove, 1999, 2005; Lionetti et al., 2012). Pectin methyl esterase inhibitors (PMEI's), which are produced endogenously by the plant to regulate methylesterification of pectin polymers, decrease PME activity in both cell elongation processes and pathogenic immunity response (L'Enfant et al., 2015; Lionetti et al., 2012). PMEs that are both endogenous and pathogenic to the plant are important to the plant's ability to grow and survive.

Proteins perceiving Cell Wall Integrity are revealed by genetic analysis

Cellular perception of cell wall molecules is key to the organism's adaptation to environmental and pathogenic conditions. Peptide hormones secreted to the cell wall act as ligands to receptors in the cell membrane, aiding intercellular communication, especially during cell elongation (Canut et al., 2016; Haruta, Sabat, Stecker, Minkoff, & Sussman, 2014; Matsubayashi & Sakagami, 2006; Wolf & Höfte, 2014). Signal molecules are able to travel through the cell wall by diffusion, however cell wall components can act as signal molecules as well. The signal and receptor molecules involved in yeast cell wall integrity (CWI) perception are well documented (Hamann, 2015), however the CWI signal pathways in plants are not as

well known. Phosphoproteomic and genetic analyses have helped to reveal a number of CWI perception proteins in Arabidopsis. Loss of function mutations in the Receptor-Like Kinase (RLK) THESEUS (THE1) have been shown to rescue a growth-inhibited phenotype in cellulose deficient Arabidopsis (Wolf et al., 2012). THE1 is required for upregulation of the expression of a number of genes involved in cell wall crosslinking, including a peroxidase, extensins, and reactive oxygen species (ROS) detoxifying enzymes. These data indicate THE1 involvement in inhibiting cell elongation as a response to cell wall perturbations (Wolf et al., 2012). Family members HERK1 and HERK2 are thought to be redundant to THE1, showing cell elongation defects in double mutants with THE1 (B. Kohorn, 2015; Wolf et al., 2012; Wolf & Höfte, 2014).

Another RLK, FERONIA (FER), is involved in CWI perception. Multiple mutations of FER result in reduced plant stature as well as reduced ROS accumulation in the female gametophyte (Wolf & Höfte, 2014). ROS accumulation in the ovular aperture induces rupture of the pollen tube and release of pollen (Duan et al., 2014). FER is also involved in signaling root hair elongation, fungal pathogen response, and leaf expansion (Wolf et al., 2012). FER is a likely receptor of Rapid Alkalinizing Factor 1 (RALF1), a peptide that induces growth inhibition in plants including Arabidopsis. FER is phosphorylated in 3 locations in the kinase domain as a result of RALF1 exposure, and loss of function mutation *fer-4* causes a loss of RALF1 induced growth inhibition (Haruta et al., 2014).

WAK mediated perception of the cell wall

A family of signal molecules, Wall Associated Kinases (WAKs), found in the plasma membrane of plants, responds to changes in structure and length of pectin polymers in the Arabidopsis cell wall (B. Kohorn, 2015). There have been five forms of WAK identified (WAK 1-5), all containing a cell wall- linked amino- terminus, a trans-membrane domain, and a cytoplasmic protein kinase domain. The variability of the WAK family is primarily in the amino- terminus suggesting that different WAKs bind slightly different pectin components of the cell wall, but since their kinase domains are so similar it has been suggested that they all signal similar cytoplasmic signaling events (Wagner & Kohorn, 2001). Wagner and Kohorn (2001) used β-glucuronidase – WAK promoter fusions and *in situ* hybridization to determine WAK expression patterns during Arabidopsis development, finding that WAK1 and WAK2 were the most active WAK genes and were differentially expressed in seedlings, suggesting slightly different roles in regulating plant development through cell growth.

WAKs are required for plant cell elongation. An antisense knockdown of all five WAKs in Arabidopsis resulted in impaired cell elongation and stunted lateral root development (Lally, Ingmire, Tong, & He, 2001; Wagner & Kohorn, 2001). This effect may be mediated by WAK/pectin interactions. WAKs bind pectin in the cell wall, as evidenced by an experiment where a variety of wall degrading enzymes were used in an attempt to detach WAK from the wall, with only pectinase able to release the WAK (Wagner & Kohorn, 2001). A loss of function WAK2 allele stunted Arabidopsis seedling growth, which was partially rescued by the addition of

metabolized sugars and salts (B. D. Kohorn et al., 2006). It was also observed that vacuolar invertase activity and expression was diminished in the mutant, suggesting that WAK2 signaling results in increased invertase activity as well as expression (B. D. Kohorn et al., 2006). Indeed, pectin activated invertase transcription in a WAK2 dependent fashion (Fig 1). Invertase increases the solute concentration in the vacuole, increasing turgor pressure of the cell and inducing cell elongation when combined with selective loosening of the cell wall.

WAKs respond to fragmented pectin by inducing a pathogenic stress-like response. This phenotype appears as shriveled plant leaves near the infection (B. D. Kohorn, 2001; B. D. Kohorn et al., 2006; Lally et al., 2001). WAKs bind fragmented pectin, oligogalacturonides (OG's), preferentially over full-length pectin, indicating that pectin and OGs compete for the WAK extracellular binding site (Fig 1). OGs infused into Arabidopsis induce rapid phosphorylation of 50 phosphoproteins. Seven of the phosphosites within these proteins are also known to be affected by *Flg22* pathogen induced signal pathway, indicating a distinct OG-dependent signal pathway that overlaps slightly with the *Flg22* induced pathway (B. D. Kohorn, Hoon, Minkoff, Sussman, & Kohorn, 2016). Genetic analysis revealed that 8 of these phosphoproteins are required for OG response and are independent of *Flg22* (B. D. Kohorn et al., 2016). A hyperactive dominant mutant allele of WAK2 induces an always-on pathogenic stress response phenotype. The phenotype is rescued by *mpk6* null mutation (B. D. Kohorn et al., 2012), indicating that *mpk6* is downstream of WAK2, and required for WAK2 pathogenic response signaling (B. D. Kohorn et al., 2016; B. D. Kohorn et al., 2012). The other downstream targets of WAK signaling

require further investigation so that a model of CWI signal transduction similar to the known yeast model can be developed.

Response to OGs is a crucial part of plant innate immunity. Overexpression of WAK1 led to increased pathogen resistance in Arabidopsis, associated with increased OG response (Brutus, Sicilia, Macone, Cervone, & De Lorenzo, 2010). Furthermore, maize resistance to *Sporisorium reilianum* was dependent on WAK expression (Xu et al., 2015; Yang, Balint-Kurti, & Xu, 2017). The WAK mediated response to pathogen invasion confers resistance, making the WAK family and OG induced signal response pathway of special interest in understanding crop disease resistance.

Glycine Rich Protein Family

WAKs may bind and interact with other proteins that help mediate the response to OGs and pectin. One potential protein ligand is Glycine Rich Protein 3 (GRP3), a member of a large family of glycine rich proteins, including a subfamily of seven GRPs that cluster on a 90kb locus of chromosome 2 in Arabidopsis. GRPs are secreted cell wall proteins of unknown function. GRP3 binds WAK1 in a yeast-two-hybrid assay (Fig 1), attaching at the extracellular domain of WAK (B. Kohorn, 2015; B. D. Kohorn, 2001; Park et al., 2001). GRPs are induced under varying circumstances for varying physiological processes not yet well characterized (Sachetto-Martins, Franco, & de Oliveira, 2000), however GRP3 and WAK1 is the only binding pair currently known (B. Kohorn, 2015).

There are seven GRPs that are encoded by tight clusters of genes, making genetic analysis difficult because they do not recombine at high frequency. These

seven GRP proteins are likely redundant, as they share 65% sequence identity, and there is no obvious phenotype in knockouts of single GRP genes (B. Kohorn, 2015; Mousavi & Hotta, 2005). Because of the lack of recombination between these seven GRPs, gaining multiple mutants by T-DNA insertion or other mutagenesis techniques is nearly impossible. By utilizing CRISPR/Cas9 technology, a deletion of the entire 90kb GRP locus can be attained, causing complete loss of function of these highly similar GRPs. The effect of OG/pectin/WAK signaling modulation has yet to be fully explored, so this project examines GRP effects on phenotype via CRISPR/Cas9 deletions of portions of the GRP locus.

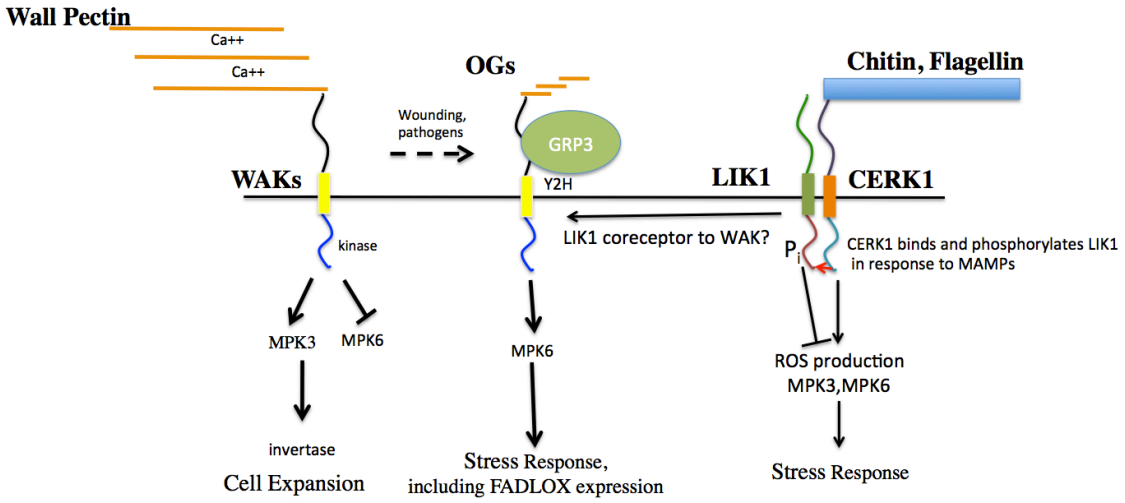

Figure 1. A highly schematic model for WAK mediated pectin perception. GRP3 and WAK1 bind each other in yeast-2-hybrid and co-immunoprecipitation experiments, though the effect on signaling is unknown. CERK1 and LIK1 bind each other in yeast-2-hybrid and co-immunoprecipitation experiments, and LIK1 phosphorylation is reduced in *cerk1-2* mutants. CERK1 is required for response to chitin, flagellin, and peptidoglycans (PGNs), though it does not directly bind PGNs. LIK1 is highly phosphorylated in response to OGs, implicating it in the WAK/OG stress response pathway.

CRISPR/Cas9

Recent developments in gene editing technology have made specific targeted gene editing and deletion possible and efficient. Bacteria and archaea have the ability to degrade invading nucleotide sequences by using a special and simple short-RNA guided endonuclease called Cas. Cas endonuclease systems are

associated with clustered regularly interspaced short palindromic repeats (CRISPR), whose transcript, CRISPR RNA (crRNA) is sufficient to direct Cas systems to cleave sequence specific target loci on the genome (Mali et al., 2013). Cas and crRNA form a complex with another RNA called trans-activating crRNA (tracrRNA), base pairing with sequences complimentary to the crRNA, separating the dsDNA, and cleaving the DNA at that locus. Because the crRNA directs Cas endonuclease to make a double stranded DNA cleavage at the locus complimentary to the crRNA strand, Cas can be programmed to cleave DNA at almost any locus imaginable with great specificity. By combining the crRNA and tracrRNA loci into one single guide RNA (sgRNA), the construct is simplified (Doench et al., 2014). CRISPR/Cas9 constructs include a promoter for Cas9 ligated to the Cas9 coding region, and a separate promoter, often U6, for the sgRNA sequence (Doench et al., 2014; Hyun et al., 2015; Yan et al., 2015). The CRISPR/Cas9 system is placed on an Agrobacterium plasmid and transformed into the plant so that CRISPR/Cas9 is integrated at a random locus. Two sgRNA then guide the Cas9 endonuclease to cut at either end of a predetermined locus, which is likely to be far enough away from the Cas9 locus to segregate independently.

CRISPR/Cas technology has been utilized successfully in human, mouse, and zebrafish cell lines to edit genes for functional genomic research, and recently has been applied to research on Arabidopsis (Yan et al., 2015). The search for a high-quality promoter for Cas9 so that deletions are found not only in somatic cells but also in germ-line cells has proven difficult. *Ubiquitin* and *35S* promoters have been used in the past for Arabidopsis transgenics, however editing efficiency was low to undetectable (Yan et al., 2015). By using a pYAO promoter rather than 35S promoter

in a pYAO:hSPCas9-BRI1-sgRNA fusion transgenic plant, BRI1 deletions were attained at 90.5% success rate, compared to the 4.3% of plants with BRI1 deletion from 35S:hSPCas9-BRI1-sgRNA (Yan et al., 2015), yet the frequency of inheritance is below 1%.

CRISPR/Cas9 was used to make a deletion of the large 90 kb GRP gene locus in Arabidopsis. It is expected that this deletion will lead to a loss of GRP function so that its role in WAK/Pectin/OG signaling can be further examined.

LIK1, CERK1and the response to OGs.

LIK1 is a candidate for a WAK co-receptor for pectin, and also is required for the response to chitin by CERK1. Lysin motif-containing receptor-like kinase 1 (CERK1) is a membrane associated receptor kinase allowing plant perception of fungal invasion and insect predation; these organisms both contain chitin in their cell walls and exoskeletons, respectively. Arabidopsis CERK1 knockouts completely lose chitin induced ROS production (Miya et al., 2007). Furthermore, Miya et al. (2007) showed that chitin elicitor induced MPK3 and MPK6 activation and transcriptional response to chitin elicitors was completely lost in *cerk1* mutants, indicating that CERK1 is the primary chitin receptor in Arabidopsis (Fig 1). CERK1 may also play a role in Arabidopsis response to bacterial invasion through peptidoglycan (PGN) perception. Willman, et al. (2011) showed that, unlike other LysM receptors LYM1 and LYM3, CERK1 does not directly bind PGNs; however they also showed that PGN induced transcriptional response, including *FRK1* expression, was greatly reduced in *cerk1-2* mutants (2011). This loss of transcriptional response

was associated with decreased immunity to bacterial invasion. These data indicate that CERK1 plays a vital role in the pathogenic response to a wide range of pathogens, fungal and bacterial alike.

LysM RLK1 interacting kinase 1 (LIK1) is implicated in CERK1 mediated pathogenic response. Chitin elicitor induced LIK1 phosphorylation was greatly reduced in *cerk1-2* mutants, and LIK1 and CERK1 interact in yeast-2-hybrid assays and can be co-immunoprecipitated from plant tissue (Le, Cao, Zhang, & Stacey, 2014). These data suggest that LIK1 is directly phosphorylated by CERK1, and moderates the pathogenic response (Fig 1). The effect of LIK1 on pathogen signaling is varied, however. Le, et al. found that *lik1* mutants demonstrated increased ROS production in response to chitin and flagellin elicitors, suggesting LIK1 works as a negative regulator to these pathogenic responses (Le et al., 2014). Furthermore, *lik1* mutants were more resistant to invasion by hemibiotrophic bacterial pathogen *P. syringae*, but less resistant to necrotrophic fungus *S. sclerotiorum*. Thus, LIK1 may help Arabidopsis distinguish between pathogen types. LIK1 was highly phosphorylated in response to OG treatment (B. D. Kohorn et al., 2016), however its role in WAK/OG signaling has not been further examined. This thesis utilizes a T-DNA insertion in the *LIK1* (At3G14840) gene to examine the effect of loss of LIK1 function on transcriptional response to OGs.

Methods

Arabidopsis GRP mutants

Arabidopsis transformed with pYAO:Cas9/pU6:sgRNA(GRP380-580) (contructed by B Kohorn) were self fertilized, and their offspring (T2) provided by

Susan Kohorn. A third construct, pICU2:Cas9/pOLE:RFP/pU6:sgRNA(GRP380-580) tightly linking seed specific RFP expression to the Cas9 locus was utilized to increase throughput by allowing for preliminary Cas9 screening without PCR. T2 plants lacking RFP expression in the seed were provided by Susan Kohorn.

Arabidopsis DNA Extraction

Two to three Arabidopsis leaves were harvested, frozen in liquid nitrogen, and ground in extraction buffer (0.2M Tris pH7.5, 0.25M NaCl, 25mM EDTA, 0.5% SDS) until homogenized. This solution was centrifuged at 15,000 xg at 25°C for 3 minutes. The supernatant was collected and mixed with the same volume of 100% Isopropanol before incubation at 25°C for 15 minutes. The Isopropanol/supernatant solution was then centrifuged at 15,000 xg for 5 minutes, and the resulting pellet washed with 70% ethanol, centrifuged at 15,000 xg for 1 minute, then the supernatant removed and the pellet air dried for 30 min. The dry DNA pellet was resuspended in 100 µL dH_2O.

Detecting presence of Cas9 by polymerase chain reaction (PCR)

The Cas9 gene was amplified from extracted DNA by polymerase chain reaction (PCR) using Titanium Taq Polymerase and Cas9F2 and Cas9R2 primers, using PCR parameters outlined in Table 1. The product was 800bp. PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Detecting deletion success by polymerase chain reaction (PCR)

The success of GRP deletion was determined by PCR. The DNA sequence surrounding the excised GRP380-580 locus was amplified from extracted DNA by polymerase chain reaction (PCR) using Titanium Taq Polymerase and GRP5380F and GRP5580R primers, using PCR parameters outlined in Fig 2 and Table 1. The product was 800bp. If there were no deletion, the GRP380-580 locus would be roughly 90kbp, far too large to amplify (see Figure 2). PCR products were visualized on a 1% agarose gel stained with ethidium bromide. A single band of 800bp indicated GRP deletion success. A plant containing Cas9 endonuclease targeting the GRP380-580 locus was used as a positive control, and Columbia wild-type plant used as negative control.

Table 1. PCR primers for amplifying *Cas9* and *GRP380-580* deletion sites.

Primers	*Primer Sequences*	*PCR Reaction Cycling Parameters*
Cas9F2 Cas9R2	5'-GAT CTG CTA TCT GCA AGA GAT CTT CAG CAA CGA G -3' 5'- CTT GCT CTG GTC GAA GAA AAT CTC TTT GTA CTT C – 3'	Step 1: 95°C for 60 seconds Step 2: 95°C for 30 seconds Step 3: 60°C for 20 seconds Step 4: 68°C for 60 seconds Repeat Steps 2-4 30x Step 5: 68°C for 120 seconds Step 6: 4.0°C storage
GRP5380F GRP5580R	5'- CAT CAC AAG TTT TAA GCA GTA TTT GTA AGA AAA TGG C 3' 5'- CTT TCA TGA TCC TAT AAA CAT TCG GAA AAG TGG TG 3'	Step 1: 95°C for 60 seconds Step 2: 95°C for 30 seconds Step 3: 59°C for 20 seconds Step 4: 68°C for 60 seconds Repeat Steps 2-4 30x Step 5: 68°C for 120 seconds Step 6: 4.0°C storage

Screening for TDNA insertions in LIK1

Arabidopsis Salk_094512C plant line containing a T-DNA insertion in the 5' UTR of At3g14840 (*LIK1*) were obtained from the Arabidopsis stock center (wwww.arabidopsis.org) and grown by Sue Kohorn. The *LIK1* gene and T-DNA location are shown in Figure 5A. Plants were screened for the T-DNA by PCR of

extracted DNA. Wild type *LIK1* was detected with primers LIK1F2 and LIK1R2 and parameters outlined in Table 2. T-DNA insertions were detected using primers LBb1.3 and LIK1R2 and parameters outlined in Table 2. Seeds from plants missing the Wild Type PCR product but containing the T-DNA insertion mutation PCR product were collected.

Table 2. PCR primers for *LIK1* T-DNA insertion and knockout confirmation.

Primers	*Primer Sequences*	*PCR Reaction Cycling Parameters*
LIK1F2	5'-GAA ATG TCT TCA TGA GTC TCT CTT CTT CTT CCC C -3'	Step 1: 95°C for 60 seconds Step 2: 95°C for 30 seconds Step 3: 56°C for 20 seconds Step 4: 68°C for 60 seconds Repeat Steps 2-4 30x Step 5: 68°C for 120 seconds Step 6: 4.0°C storage
LIK1R2	5'- GCG TTC TTC TCT CTT TTT CAA CAT CCT ATC TTT TCT TTG – 3'	
LBb1.3	5' – ATT TTG CCG ATT TCG GAA C – 3'	
LIK1F	5'- GCT GTT GTT AAG AAA TTT CCT GTC ATG GTT ACA AAT G - 3'	Step 1: 95°C for 60 seconds Step 2: 95°C for 30 seconds Step 3: 58°C for 20 seconds Step 4: 68°C for 60 seconds Repeat Steps 2-4 30x Step 5: 68°C for 120 seconds Step 6: 4.0°C storage
LIK1R	5'- CTT AGA TTT CCA GAT CAG TTC GTT CTC CTT GAG G - 3'	

Comparing OG induced transcriptional response

Wild type and *lik1* mutant seeds were incubated in ½ MS (2.2 mg/L MS) growth media and 0.5% Sucrose medium with 1X MS vitamins and 50µg/ml ampicillin for 2 days at 4°C, then for 7 days at 21°C on orbital shaker set to 80rpm before being treated with OGs at 50 ug/ml, dp 9-15 or dH_2O for 3 hours at 21°C. After OG treatment, seedlings were frozen in liquid nitrogen and homogenized. RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN Sciences, Maryland 20874, USA, catalog #74903). RT-PCR was performed to produce cDNA from 1µg of RNA from the previous step with the SuperScript III First-Strand Synthesis System for

RT-PCR (Invitrogen, Carlsbad, CA 2008, USA, catalog #18080-051). Quantitative PCR (qPCR) was performed with Applied BioSystem StepOne Version 2.1 machine for β-*actin* as a reference and *FADLOX* to measure transcriptional response to OGs. Parameters and primers are shown in Table 3.

Table 3. Primers utilized for qPCR. Beta-actin was used as a reference gene while *FADLOX* reports response to OG exposure.

Gene	*Forward Primer*	*Reverse Primer*	*Parameters*
Beta-actin	5'-AGTGGTCGTAGAACCGGTATTGT-3'	5'-GATGGCATGAGGAAGAGAGAAAC-3'	Step 1: 95°C for 10 min Step 2: 95°C for 15seconds Step 3: 58°C for 60 seconds Repeat Steps 2-3 40x
FADLOX	5'-GACGACACGTAAGAAAGTCC-3'	5'-CGAACCCTAACAACAAAAA-3'	

Results

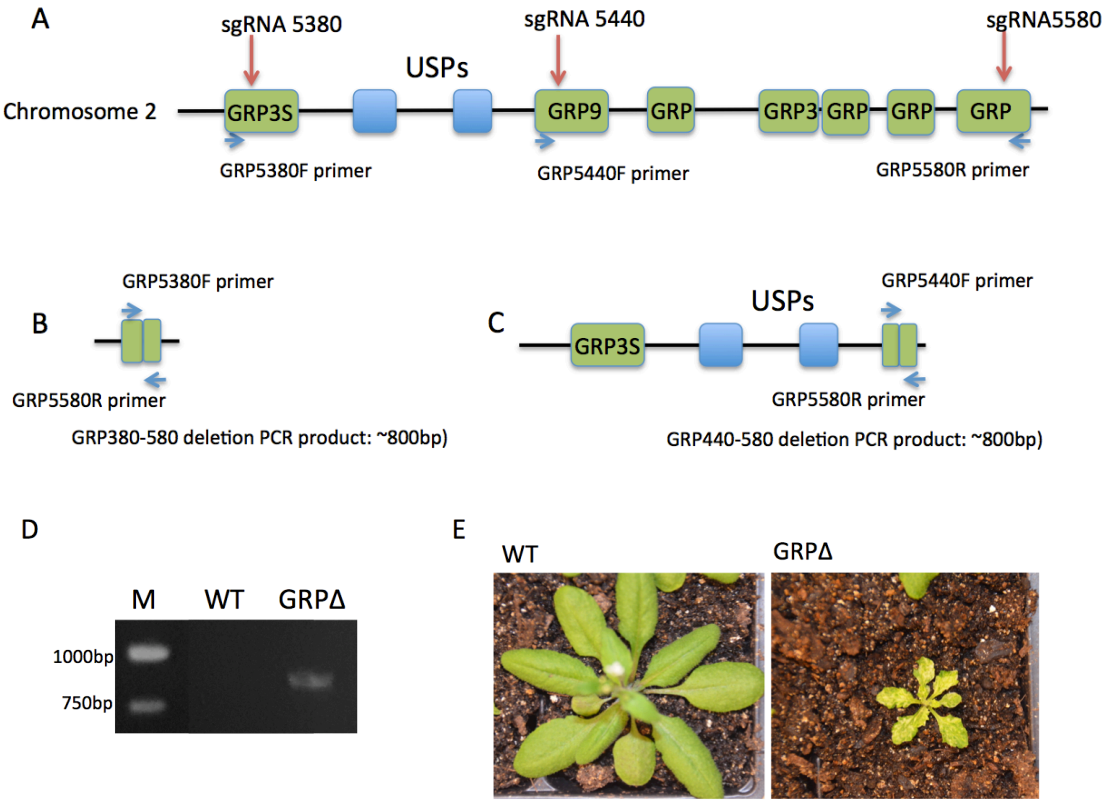

Figure 2. Map of the GRP5380-5580 locus on chromosome 2 before and after Cas9 expression. A; Seven GRP proteins are coded on a 90kbp locus. Red arrows indicate sgRNA binding sites. GRP380-580 deletions are induced using sgRNA 5380 and 5580, while GRP440-580 deletions are induced using sgRNA 5440 and 5580. PCR primers are indicated in blue, selected GRP coding genes shown in green, and ubiquitin specific protease (USP) coding genes shown in blue. The sgRNA target sites for GRP380, GRP 440, and GRP580 are shown in red. GRP380-580 deletions utilize sgRNA 380 and 580, whereas GRP440-580 deletions utilize sgRNA 440 and 580. B; Induction of Cas9 with sgRNA5380 and sgRNA5580 result in deletion of all GRP genes, and an 800bp PCR product with GRP5380F and GRP5580R primers. C; Induction of Cas9 with sgRNA5440 and sgRNA5580 result in deletion of all GRP genes except GRP3S, and an 800bp PCR product with GRP5440F and GRP5580R primers. D; PCR to detect the GRP 380-580 deletion using WT or somatic T1 tissue expressing Cas9 (GRPΔ). The band is 800bp. M: molecular weight marker. E; Columbia WT and a plant with GRP380-580 (GRPΔ) somatic deletion.

GRP deletion induced using pYAO:Cas9/U6:sgRNA

In order to obtain an inherited allele of the GRP380-580 or GRP440-580 deletions, Arabidopsis were first transformed with pYAO:Cas9/U6:sgRNA by floral dip in Agrobacterium, and their seeds collected (T1). Arabidopsis in the T1 generation were

screened for GRP380-580 deletion by using primers GRP5380F and GRP5580R (Fig 2B), or GRP5440F and GRP5580R (Fig 2C). Figure 2D, (GRPΔ) shows the product of the GRP380-580 PCR from DNA extracted from a T1 plant. The fragment was sequenced and shown to have the expected deletion. Some T1 plants with somatic GRP380-580 deletions exhibited dwarf and variegated leaves (Figure 2E, GRPΔ) compared to wild type, indicating that the GRP locus is important for normal growth and development.

The offspring of plants containing the GRP deletion (T2) were grown and screened for Cas9 to find inheritance of GRP deletions. 138 T2 generation Arabidopsis were screened for the absence of Cas9 and absence of GRP in the genome to ensure that the GRP deletion was hereditary, rather than effected by Cas9 expression still present in somatic cells. If the deletion was inherited, every cell in the plant has the GRP deletion, whereas if Cas9 is still present, it's possible that the deletion was newly generated in that generation and not necessarily in all cells. T2 progeny from four self-fertilized T1 individuals were screened: T1#1, T1#2, and T1#4, which all were transformed to have a GRP380-580 deletion. An example of the PCR products for Cas9 and GRP380-580 deletion run in an agarose gel is shown are in Figure 3. Of 66 T2s from T1#1, 13 (19.7%) were Cas9-null as determined by PCR and gel electrophoresis. Of 68 T2s from T1#2, 15 (22.1%) were Cas9-null (Fig 3A). If Cas9 segregates under Mendelian inheritance, 25% are expected to be Cas9-null, and this is close to that observed. However, none of these 28 Cas9-null individuals had inherited the GRP380-580 deletion (Fig 3B). If the deletion were segregating, 3/16 of the individuals would be expected to be Cas9-null and deletion-positive. Of 134 individuals, none were Cas9-null, deletion-positive.

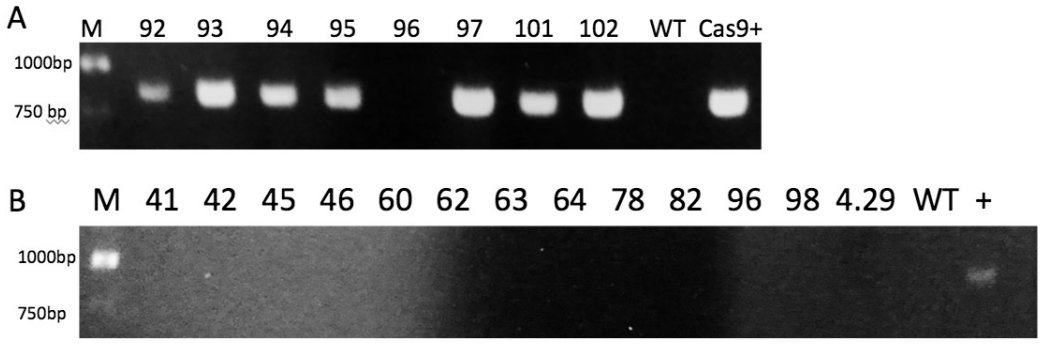

Figure 3. Example PCR products for detecting presence of Cas9 and GRP380-580 deletions. A; The Cas9 gene was amplified by PCR from DNA extracts from second generation (T2) offspring from T1#2, a wild type (WT) negative control and Cas9 positive control (Cas9+), run on 1% agarose gel. One (#96) of 8 (12.5%) individuals were Cas9 null. B; Cas9-null T2 offspring from the T1#1 line (41-98) and one offspring of T1#4 (4.29) were screened for GRP380-580 deletion success. Columbia wild type negative control (WT) and deletion-positive control(+). Lack of deletion indicated by no band, as the region between the primer sequences is too large (90kbp) to amplify. None of the Cas9-null plants had the GRP380-580 deletion. M; mw marker

GRP Deletion induced using pICU2:Cas9 linked pOLE:GFP construct

Since inheritance of GRP380-580 deletion was not accomplished with pYAO:Cas9/pU6:sgRNA transformed plants, a different promoter was used to drive Cas9 expression such that there was an increased chance of expression in cells giving rise to the gametes. Arabidopsis plants were transformed with Agrobacterium containing the CRISPR/Cas9 construct pICU2:Cas9/U6:GRP5380-5580sgRNA with a closely linked pOLE:RFP that expresses only in the seed, indicating presence of Cas9 without requiring PCR. Seeds from deletion positive plants were screened for Cas9 by fluorescent microscopy of the linked pOLE-RFP. Seeds not exhibiting RFP expression were grown and their tissue collected and screened for a GRP deletion. Of 233 individuals from four T1 lines, 15 had the GRP380-580 deletion confirmed by PCR (Fig 4A). These individuals were further

screened for Cas9 using PCR, and surprisingly all were found to be Cas9 positive (Fig 4B).

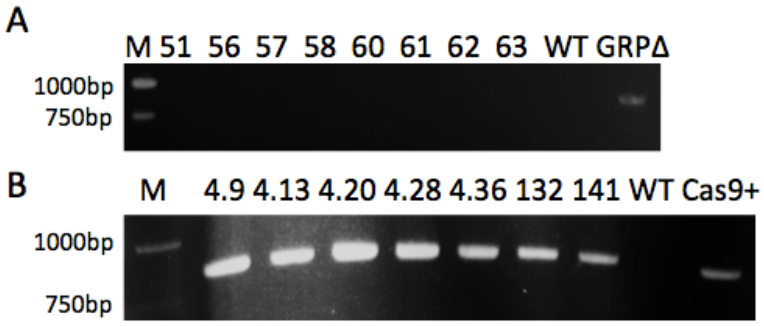

Figure 4. Representative PCR gels for detecting GRP deletion inheritance with pICU2:Cas9pOLE:GFP; U6:sgRNA constructed plants. A; GRP deletion results in an 800bp product, and was absent in 94% of individuals. B; All individuals with GRP deletion also contained Cas9. M indicates molecular weight (mw) marker of the indicated size

LIK1 T-DNA Insertion leads to loss of transcriptional response to OGs

LIK1 is thought to act as a coreceptor for CERK1, the chitin receptor that senses insects and some fungi. LIK1 was phosphorylated upon plant exposure to OGs (Kohorn, et al. 2016), and so LIK1 became a candidate for a WAK coreceptor as well.

To examine the role of LIK1 in OG-induced pathogenic stress response, Arabidopsis seeds from the mutant line Salk_094512C (Arabidopsis.org) containing a T-DNA insertion in the 5'UTR of *LIK1* (Atg14840) were used for a transcriptional response assay. Seeds were provided by the Arabidopsis Biological Resource Center and were selected for the insertion site in the 5' UTR, which is expected to affect expression of *LIK1* mRNA (Fig 5). Figure 5A shows a cartoon of the *LIK1* gene and the location of the T DNA insertion (black triangle). Primers on either side of the insertion and a primer within the T-DNA insertion were used to PCR DNA isolated

from the presumptive *LIK1* mutation. Wild type DNA is expected to produce a 600bp band, and this is shown in Fig 5B, WT. PCR using a primer in the T DNA and in the gene itself would produce a 700bp band, as shown in Fig5C, *LIK1Δ*. Plants homozygous for the insertion should produce no WT band and only one using the T-DNA primers, shown in Fig5B-C, *LIK1Δ*. DNA extracted from plants were found to have no wild type PCR product, and only a T-DNA product, and thus be homozygous for the mutation (Figure 5B-C, *LIK1Δ*), and their seeds were collected.

FADLOX is highly induced by OG treatment in Arabidopsis, and thus is used as a reporter for an activated WAK dependent signal transduction pathway. If LIK1 is involved in this signaling pathway, it was expected that the *lik1/lik1* mutant would reduce the OG induced expression of *FADLOX*, relative to wild type plant. Wild type and mutant plants were grown for 7 days and then treated with OG for 3 hrs (B. D. Kohorn et al., 2012). Total RNA was extracted, and used as template for cDNA. These cDNAs were then used to determine the relative expression of *FADLOX*. In an OG induction of *FADLOX* RT-qPCR assay, it was found that *lik1/lik1* mutants had a 49 ±14% reduction in *FADLOX* expression (mean ±SD) compared to wild-type when incubated in OGs (Figure 5D, t-test, $p<0.0001$).

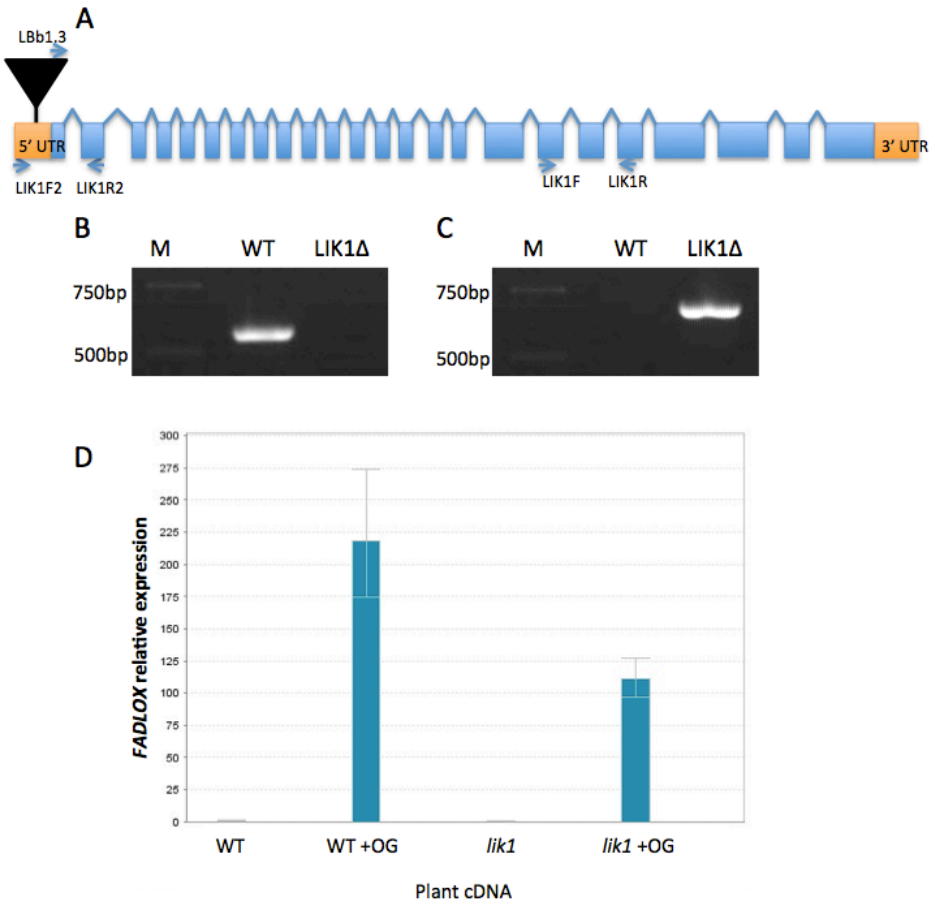

Figure 5. *Lik1* TDNA mutants. A; *LIK1* gene with t-DNA insertion (black triangle) shown in the 5' UTR, and PCR primers shown below (blue arrows). B; *lik1* mutant plants did not contain the 600bp WT *LIK1* product from primers LIK1F2 and LIK1R2. C; *lik1* mutants did contain the 700bp *lik1* mutant allele product with primers LBb1.3 and LIK1R2, indicating homozygosity for the mutant allele. M; mw marker. D; *FADLOX* expression relative to WT, standardized by β-actin. *lik1* mutants exhibited a 49 ± 14% decrease in OG induced *FADLOX* expression.

In order to determine how the T-DNA insertion affects the expression of the *LIK1* mRNA, a PCR was performed on the cDNA derived from the *LIK1* mRNA using LIK1F and LIK1R primers (Table 2). The PCR from cDNA was expected to be 100 bp shorter than one from genomic DNA due to the presence of an intron in the *LIK1* gene. The results are shown in Fig. 6A. The PCR of the cDNA product (Fig 6A, wt, wt +OG, *lik1, lik1* +OG) was indeed 100bp shorter than wild type genomic DNA product (Fig 6A, WT). However, the mutant and wild type plant both showed mRNA

indicating that the T-DNA did not reduce expression, and in fact the bands for mutant plants looked stronger than the wild type bands (Fig 6A, *lik1*, *lik1* +OG). In order to confirm that there was an increase in *LIK1* mRNA in mutant seedlings, an RT-qPCR was performed on wt and *lik1/lik1* seedlings with and without OG treatment (Fig 6B). Indeed, the *lik1* mutants exhibited a 4-fold increase in *LIK1* mRNA (ANOVA, $p = 0.0012$). Furthermore, *LIK1* expression is slightly increased in response to OGs in both wild type and *lik1* mutant seedlings (ANOVA, $p < 0.0001$).

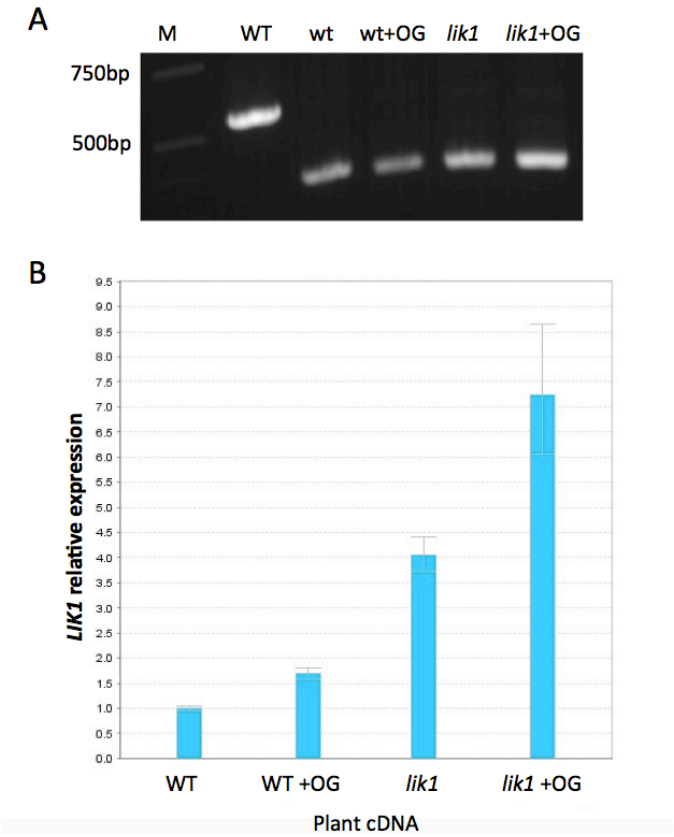

Figure 6. *LIK1* expression in *lik1/lik1* mutant plants. A; PCR of wild-type genomic DNA (WT), and cDNA for wild type (wt) and *lik1* seedlings untreated and treated with OGs (+OG). B; RT-qPCR of cDNA from wild type and *lik1* seedlings for *LIK1* mRNA using LIK1F and LIK1R primers and normalized to β-actin as reference mRNA. M; mw marker.

Discussion

pYAO and pICU2 promoters driving Cas9 induce T1 GRP deletions that are not inherited

CRISPR/Cas9 gene editing technology was used to induce a deletion in the GRP locus of Arabidopsis. Two promoters were used to drive Cas9 expression: pYAO and pICU2. Cas9 was inherited in a mendelian fashion, however there was no inheritance of GRP380-580 deletions with either promoter, though there was a high rate of somatic deletion when Cas9 was present. The somatic T1 cells still had a wild type PCR product and thus were mosaic for the deletion (Kohorn, unpublished). Nevertheless there were sufficient cells affected to provide a strong phenotype. Not all plants exhibited this dwarf and necrotic phenotype and this correlated with the amount of deletion present in the mosaic tissue. It remains to be determined why the loss of the GRP locus leads to this strong phenotype, but it does resemble the effect of a strong dominant active allele of WAK that induces a pathogen response and loss of cell expansion (Kohorn et al., 2012). This phenotype is consistent with previous hypotheses that GRP acts to inhibit WAK stress response (Gramegna et al., 2016), but inherited deletion alleles and rescue experiments are necessary to confirm this effect.

Plants containing the pYAO:Cas9/U6:sgRNA construct did not inherit GRP380-580 deletions. While it was reported that the YAO gene was required and highly active in developing Arabidopsis embryo, even as early as the four-cell stage (Li, Liu, Shi, Liu, & Yang, 2010; Yan et al., 2015), there is the possibility that YAO was an insufficiently active promoter to express Cas9 in progenitor cells. Yan, et al

(2015) reported that among Cas9-null plants in the T2 generation, 6.7% had inherited Cas9 induced BRI1 mutation. Our results show a lower transmittance rate of 0%, suggesting the need for a different promoter be utilized.

The promoter p*ICU2* has been shown previously to express in the progenitor cells of the pollen and egg that give rise to the subsequent generation, allowing for high gene editing efficiency, making it an excellent candidate for finding a heritable GRP deletion (Hyun et al., 2015). Using the pICU2:Cas9/pOLE:RFP/pU6:sgRNA allowed for screening of Cas9 positive and Cas9 minus individuals without PCR. The pOLE:RFP reporter was tightly linked to pICU2:Cas9, allowing for screening of Cas9 by observing seeds under fluorescent microscopy. This prescreen for Cas9 was highly effective at increasing screening throughput, and only 6% were found to be Cas9 positive. It is not clear why these RFP negative plants did have Cas9, and the frequency is higher that one might expect due to recombination, and thus segregation. It is possible that the RFP was subjected to gene silencing, although if Cas9 is still linked, this too would have been expected to be silenced. It is also possible that natural variation in seed coating thickness made RFP fluorescence harder to distinguish in some seeds. While Hyun, et al. found very low frequency (1%) stable heritability of mutations induced using the pICU2:Cas9/pU6:sgRNA system (2015), we observed no inheritance of GRP380-580 deletion whatsoever.

It is possible that the promoters are not expressing in progenitor cells, or are only active in a small number of progenitor cells. This possibility seems unlikely, however, because both ICU2 and U6 are crucial genes for cell function, so their promoters have high activity. It is possible that these promoters are silenced in the

embryo progenitor cells. ICU2 encodes part of the DNA Polymerase α subunit in Arabidopsis, and *icu2-2* and *icu2-3* strong mutants exhibited zygotic lethality, indicating that the gene is expressed in the earliest stages of development (Barrero, González-Bayón, del Pozo, Ponce, & Micol, 2007). The U6 short nuclear RNA promoter is widely used to drive sgRNA expression in CRISPR systems and small interfering RNA (siRNA) for RNA interference experiments (Hyun et al., 2015; Makinen et al., 2005; Yan et al., 2015). Thus pICU2:Cas9/pU6:sgRNA should drive high, or at least adequate Cas9 and sgRNA expression in progenitor cells to see heritable mutations in the GRP locus.

Deletion of the GRP locus may be lethal to germinating seedlings. However, all T2 seeds germinated. Alternatively, deletion of the GRP locus may be gametophytic lethal. If it were only lethal in either male or female gametophytes, then the male or the female gamete would survive and heterozygous individuals would be observed. If a GRP deletion were lethal in male and female gametophytes, it would be obvious if GRP deletions were fatal because roughly half of all pollen and eggs would be non-viable. This could be easily observed with an Alexander stain for pollen abortion (Alexander, 1969; H. Wang et al., 2015), or by observation of seed and ovule viability as seen in the seed pod or silique. (2004). We observed no signs of gametophytic or zygotic lethality in T2 plants. However if inheritance of GRP mutation were very low – roughly 1- 5% then lethality would be difficult to detect, as such rates are not much more than expected in wild type Arabidopsis. The GRPs in the 380-580 locus are not known to be expressed in gametophytes, so it is not expected that a deletion would be lethal (Anderson et al., 2001; B. Kohorn, 2015;

Mousavi & Hotta, 2005). If, however, the GRP380-580 deletion were lethal, a number of experiments could be done to allow for inheritance. First, it is possible that, given the high similarity of the GRPs on the 380-580 locus, one GRP is sufficient for rescue of lethality. The GRP440-580 deletion maintains GRP3S, the short isoform of GRP3 (Figure 2C). While it is possible that GRP3S is sufficient for plant function, GRP3S lacks all the binding function of GRP3, able to bind WAK1 but not WAK2 *in vitro* (Kohorn et al., 2016, Anderson and Kohorn, unpublished). Another option is to transform a GRP3 gene into Arabidopsis at the same time as the CRISPR machinery, in a way that GRP3 is not linked to or available as target to the CRISPR machinery. GRP3 is expected to rescue any lethality, and could be crossed out to lose GRP function.

Towards understanding the role of GRPs in WAK signaling

Once GRP null, Cas9 null individuals are identified, GRP function can be further examined. Heterozygotes will be self-fertilized to yield plants homozygous for the GRP deletion. It has been previously hypothesized that GRP3 acts to inhibit the WAK mediated pathogenic response (Gramegna et al., 2016). If this is so, GRP-null individuals are expected to exhibit increased transcriptional response to OGs, and an always-on pathogenic stress-like response similar to WAK2cTAP hyperactive mutant (B. D. Kohorn et al., 2012). The phenotype, if detected, will be rescued by reintroducing GRP3 by plant transformation. Because there are some non-GRP genes included in the 380-580 deletion, including two ubiquitin specific proteases (Fig 2A), it is possible, yet unlikely, that phenotypes found in the GRP380-580 deleted plants are caused by deletion of these genes, a possibility that could be ruled

out by selectively reintroducing those genes to GRP null T3s. The GRP440-580 deletion also avoids this problem by not deleting these genes. Work was done primarily on the GRP380-580 deletion because it would be difficult to cross a loss of function mutation in GRP3S into a GRP440-580 deletion after the fact, and the pICU2:Cas9/U6:sgRNA380-580 construct is more readily available.

LIK1 is involved in signaling transcriptional response to OGs

LIK1 plays a role in signaling oxidative burst and transcriptional pathogenic responses through a CERK1 mediated pathway (Le et al., 2014), and that LIK1 and CERK1 displayed high increases in phosphorylation in response to OG treatment (Kohorn, 2016). It was determined whether *lik1* mutants lost the transcriptional OG response. Mutants exhibited increased *LIK1* transcription, the opposite of what was expected from a T-DNA insertion in the 5'UTR of the *LIK1* gene (Figure 6). This is not an entirely extraordinary event: an analysis of 173 T-DNA lines where the T-DNA was 5' to the start codon by 1000bp or less revealed that insertion increased transcription 5% of the time (Y. H. Wang, 2008). Furthermore, a T-DNA insertion in the 5' UTR of the Arabidopsis telomerase (TERT) gene resulted in marked increase in TERT transcription (Fojtová et al., 2011). It is possible that promoter elements within the T-DNA act to increase the amount of transcription of the gene downstream. These data emphasize the importance of confirming the effect of T-DNA on transcription of the gene of interest.

Homozygous *lik1/lik1* plants were attained and grown with and without OGs, and *FADLOX* expression was measured by qPCR (Figure 5D). The loss of OG induced increase in *FADLOX* expression compared to Columbia wild type shows that LIK1

inhibits the transcriptional response to OGs. LIK1 can inhibit responses to certain microbe associated molecular patterns and enhance others (Le et al., 2014). LIK1 is a necessary part of the Arabidopsis pathogenic response mechanism across many elicitor types, including OGs. The response to chitin and flagellin elicitors were affected by *lik1* loss of function, and that LIK1 may be crucial to distinguishing between varied fungal and bacterial pathogens (Le et al., 2014; Miya et al., 2007). The fine-tuning of stress response level is likely crucial to prevent a plant from overreacting to a pathogenic or environmental stress.

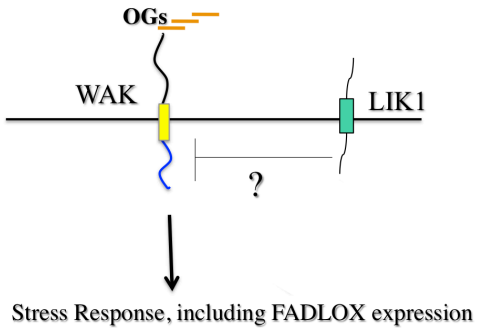

Figure 7. LIK1 inhibits WAK mediated response to OGs. LIK1 is phosphorylated in response to plant OG treatment, and inhibits the transcriptional response to OGs, as measured by the reporter *FADLOX.* LIK1 may bind directly to WAK and inhibit the kinase domain, or it may inhibit the signal transduction pathway at downstream components.

Future research should aim to place LIK1 in the OG signaling pathway (Fig 7). *cerk1* mutants do not lose *FADLOX* OG induction (Feroe and Kohorn, unpublished), suggesting that LIK1 acts as a coreceptor to OGs independently of CERK1 to inhibit WAK mediated response to OGs. LIK1 and WAK could have independent, parallel OG signal transduction pathways, and there could be a downstream signal that induces LIK1 phosphorylation in response to OGs. These interactions might subsequently inhibit further OG transcriptional responses, or LIK1 may bind OGs in tandem with

WAKs and inhibit WAK signaling (Fig 7). However, there is no evidence that LIK1 binds cell wall components. LIK1 and WAK interaction should be characterized using *in vitro* and *in vivo* binding assays. Further, it must be determined whether LIK1 binds OGs directly or is phosphorylated by another pathway. By utilizing loss of function experiments in putative WAK signal components discovered by Kohorn et al (2016), and observing changes in OG induced phosphorylation state in CERK1 and LIK1, the sequence of OG signaling will be further elucidated. Furthermore, a true loss of function experiment in LIK1 must be performed to confirm its inhibitory role in response to OGs.

Color Figures in Black and White

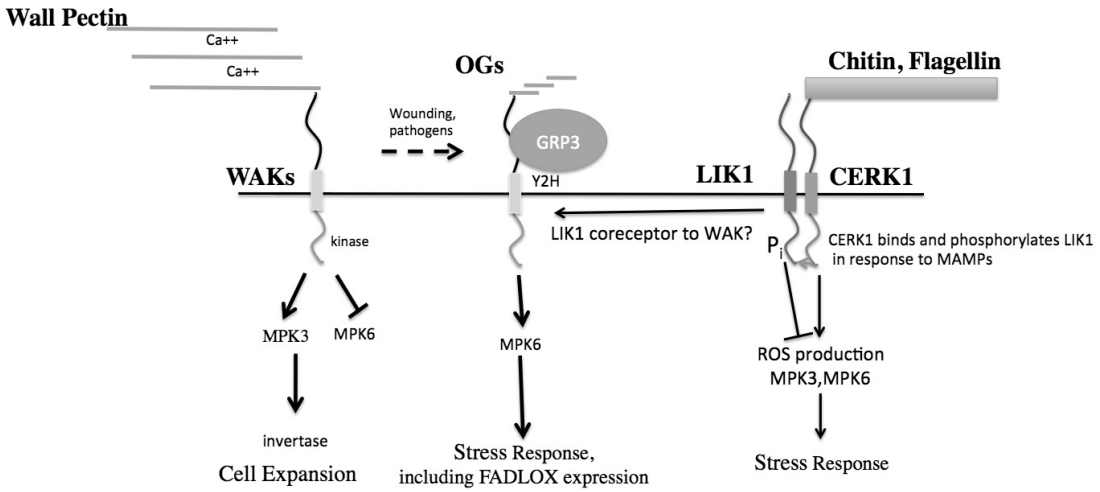

Figure 1. A highly schematic model for WAK mediated pectin perception. GRP3 and WAK1 bind each other in yeast-2-hybrid and co-immunoprecipitation experiments, though the effect on signaling is unknown. CERK1 and LIK1 bind each other in yeast-2-hybrid and co-immunoprecipitation experiments, and LIK1 phosphorylation is reduced in *cerk1-2* mutants. CERK1 is required for response to chitin, flagellin, and peptidoglycans (PGNs), though it has not been shown to directly bind PGNs. LIK1 is highly phosphorylated in response to OGs, implicating it in the WAK/OG stress response pathway.

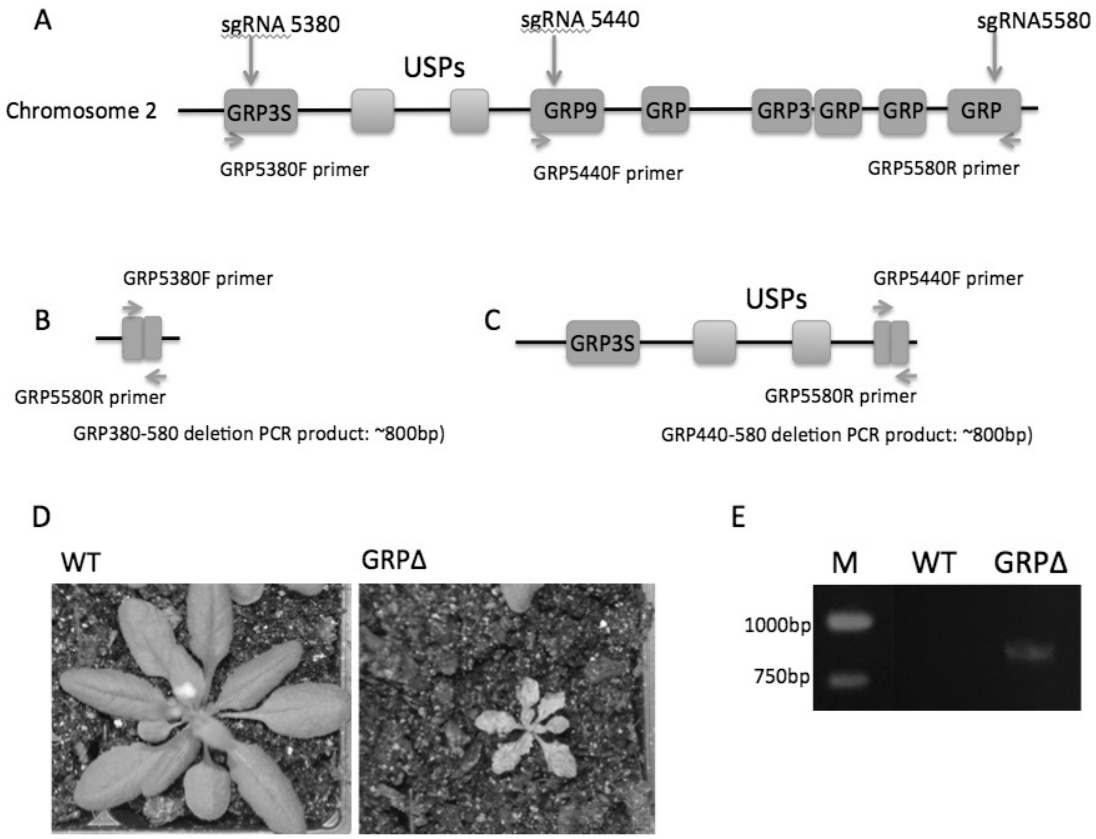

Figure 2. Map of the GRP5380-5580 locus on chromosome 2 before and after Cas9 expression. A; Seven GRP proteins are coded on a 90kbp locus. Vertical arrows indicate sgRNA binding sites. GRP380-580 deletions are induced using sgRNA 5380 and 5580, while GRP440-580 deletions are induced using sgRNA 5440 and 5580. PCR primers (horizontal arrows), selected GRP coding genes, and ubiquitin specific protease (USP) coding genes shown. The sgRNA target sites for GRP380, GRP 440, and GRP580 are shown. GRP380-580 deletions utilize sgRNA 380 and 580, whereas GRP440-580 deletions utilize sgRNA 440 and 580. B; Induction of Cas9 with sgRNA5380 and sgRNA5580 result in deletion of all GRP genes, and an 800bp PCR product with GRP5380F and GRP5580R primers. C; Induction of Cas9 with sgRNA5440 and sgRNA5580 result in deletion of all GRP genes except GRP3S, and an 800bp PCR product with GRP5440F and GRP5580R primers. D; Columbia WT and a plant with GRP380-580 (GRPΔ) somatic deletion. E; PCR to detect the GRP 380-580 deletion using WT or somatic T1 tissue expressing Cas9 (GRPΔ). The band is 800bp. M: molecular weight marker

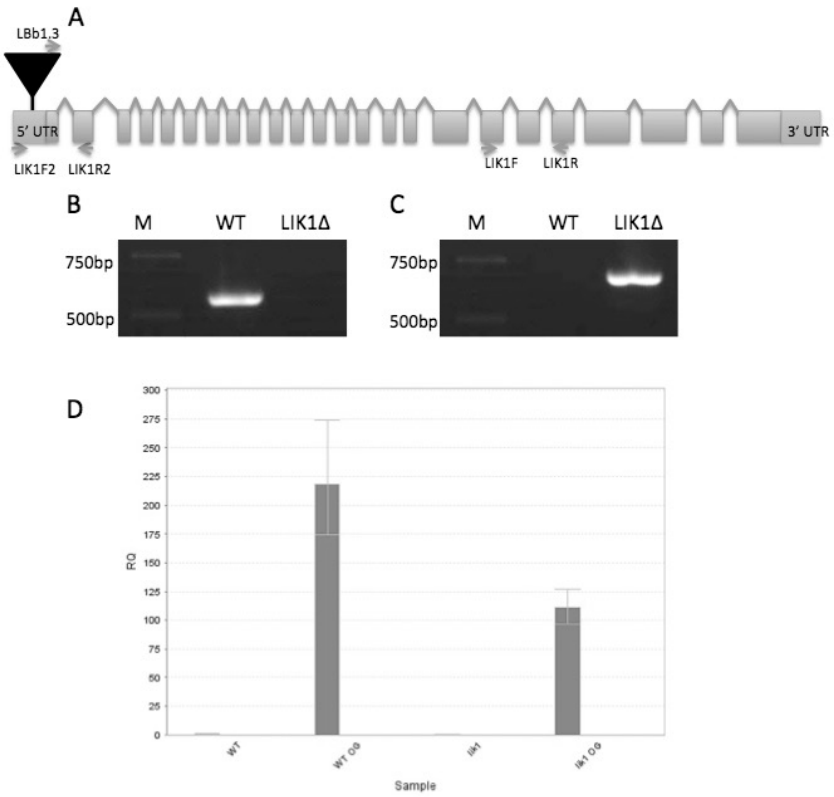

Figure 5. *Lik1* TDNA mutants. A; *LIK1* gene with t-DNA insertion (black triangle) shown in the 5' UTR, and PCR primers shown below (arrows). B; *lik1* mutant plants did not contain the 600bp WT *LIK1* product from primers LIK1F2 and LIK1R2. C; *lik1* mutants did contain the 700bp *lik1* mutant allele product with primers LBb1.3 and LIK1R2, indicating homozygosity for the mutant allele. M; mw marker. D; *FADLOX* expression relative to WT, standardized by *β-actin*. *lik1* mutants exhibited a 49 ± 14% decrease in OG induced *FADLOX* expression.

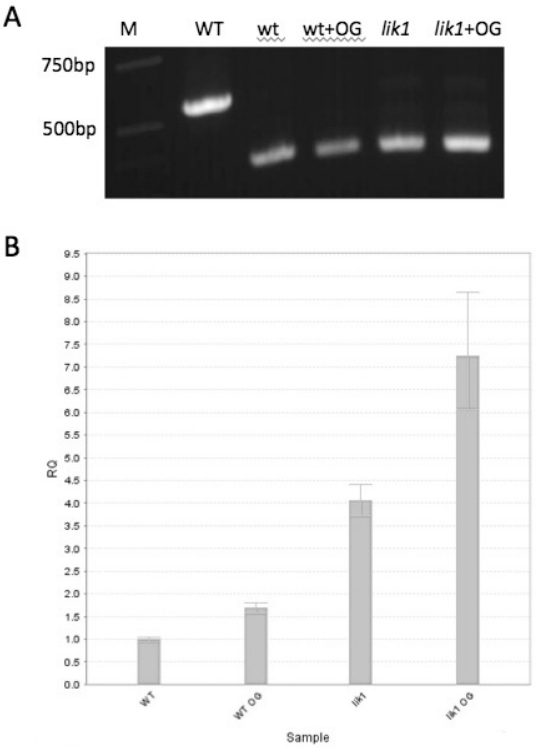

Figure 6. *LIK1* expression in *lik1/lik1* mutant plants. A; PCR of wild-type DNA (WT), and cDNA for wild type (wt) and *lik1* seedlings untreated and treated with OGs (+OG). B; RT-qPCR of cDNA from wild type and *lik1* seedlings for *LIK1* mRNA using LIK1F and LIK1R primers and β-actin as reference mRNA. M; mw marker.

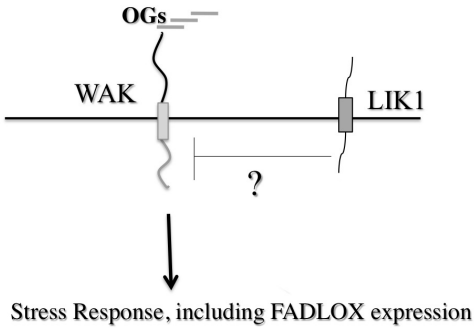

Figure 7. Proposed mechanism for LIK1 inhibition of WAK mediated response to OGs. LIK1 may bind directly to WAK and inhibit the kinase domain, or it may inhibit the signal transduction pathway at downstream components.

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