

DOWNSTREAM CHITIN SIGNALING MEDIATED BY CERK1 IN ARABIDOPSIS

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INTRODUCTION

Plant innate immunity

Plants are exposed to a large variety of pathogens but, in most cases, a sophisticated network of innate immunity can prevent pathogens from invading the plant and causing disease. Plant innate immunity is composed of repetitive alterations of immunity triggered by pathogen-associated molecular patterns (PAMPs) and pathogen-derived effectors, respectively (1-3). In PAMP-triggered immunity (PTI), plants sense pathogens via perception of PAMPs through membrane-localized pattern recognition receptors (PRRs) (4, 5). To date, a variety of PAMPs have been identified, including flagellin (6) and elongation factor EF-Tu (7) from bacteria, transglutaminase from oomycetes (8) and chitin from fungi (9). PAMP perception activates various defense responses, including production of reactive oxygen species (ROS) (8), deposition of callose (10), production of phytoalexin (11), activation of the mitogen-activated kinase (MAPK) cascade (12) and changes in gene expression which is regulated upon PAMP treatment (1, 13). Most PTI pathways also involve signaling by hormones, including the salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) pathways (14). Apart from being tolerated, some pathogens successfully suppress PTI by secreting fitness-promoting effector proteins into host cells (13). As a consequence, plants have evolved disease resistance (R) proteins to monitor the presence of such effectors. The disease resistance proteins can detect their cognate effectors in pathogens, triggering the second line of innate immunity, the so-called effector-triggered immunity (ETI). ETI is

similar to PTI and is often accompanied by a hypersensitive response (HR), which is manifested as cell death (13). However, in many cases, pathogens may modify the structure or composition of the targeted effectors so that the plants are unable to recognize them. In turn, the plant can target other effector proteins, or evolve to recognize the structure of a new effector protein, thereby restoring resistance to the pathogen (1, 15). While ETI is often higher in intensity and longer in duration (13), PTI occurs earlier and represents a more broad-spectrum immunity that helps plants respond to almost all pathogens.

PAMP-triggered immunity

Mechanisms of PAMP perception

PAMPs are evolutionarily conserved pathogen-derived epitopes or molecules perceived by plant receptor proteins, which are located in the cell membrane (13). Because these molecules can also be found in microbes other than pathogens, PAMPs are often referred to as microbe-associated molecular patterns (MAMPs) (16). Although they are found in a variety of pathogens as vital structures required for pathogen fitness, they have not been reported in plants (13). Therefore, plants can recognize PAMPs as non-self, so as to trigger appropriate defense responses. There is a specificity in the relationship between pathogen PAMPs and host PRRs; in which some epitopes are recognized only by specific hosts and some hosts only perceive specific epitopes. For example, rice plants recognize full-length flagellin, a bacterial PAMP; whereas tomato can only recognize eighteen residues of the N-terminus from flagellin (9, 17).

Arabidopsis plants can recognize harpin (Hrp), flagellin and lipo-polysaccharide (LPS) from bacteria (18); whereas tomato cells can be stimulated by chitin, ergosterol and glycopeptides from fungi (19).

In bacteria, many PAMPs have been studied, including harpin, peptidoglycan (20, 21), LPS, flagellin, cold shock protein (CSP) and elongation factor (EF-Tu) (13, 22). Two well-studied bacterial PAMPs are EF-Tu and flagellin (7). The former is an intracellular protein, the latter is an extracellular protein (13). Arabidopsis PRRs for both PAMPs are leucine-rich-repeat receptor-like kinases (LRR-RLKs), which are thought to signal through the MAPK cascade and to target WRKY transcription factors (23). The EF-Tu receptor is EFR, which binds elf18, a conserved N-acetylated 18 amino acid peptide of EF-Tu (24). The flagellin receptor is FLS2, which also binds flg22, a synthetic 22-amino-acid peptide from a conserved flagellin domain (23, 25). Different epitopes of flagellin are recognized by different host plants, including tobacco, potato and Arabidopsis (4, 26).

In fungi, several PAMPs have been studied, including ergosterol, xylanase and chitin. Chitin, a primary component of the fungal cell wall, is one of the most important fungal PAMPs. Chitin was shown to trigger various defense responses in a variety of plants, including rice (27), Arabidopsis (9, 28), wheat (29), oat (30), alfalfa (31), soybean (32) and melon (33). Chitin is a long chain polymer of β -1,4-linked N-acetylglucosamine (34, 35). Depending on the number of oligosaccharides, different chitin fragments generate a plant defense response with varying strengths (36). For example, chitooctaose triggers the strongest responses (37); whereas chitotetraose and

chitopentaose trigger almost no response (38, 39). Receptors for chitin include the chitin-elicitor binding protein (CEBiP) in rice (27) and LysM-containing receptor-like kinase LysM RLK1/CERK1 in Arabidopsis (9, 28). Unlike the known bacterial LRR PRRs, both chitin receptors contain extracellular LysM domains, which are ancient, ubiquitous protein modules capable of binding peptidoglycan and structurally-related molecules (40, 41). CEBiP has two extracellular LysM motifs, but lacks an intracellular domain(27); whereas CERK1 contains three extracellular LysM motifs and an intracellular kinase domain (9, 28).

To date, most characterized PRRs appear to be receptor-like kinases (RLK) that possess an extracellular elicitor binding domain, a transmembrane domain and an intracellular signaling kinase domain. Although the kinase domain of RLK is essential for signal transduction, simply binding of a PAMP to its cognate RLK is not always enough for triggering intracellular responses. In many cases, signal transduction requires other co-receptors together with the specific PRR. A good example of such a co-receptor is the Brassinosteroid insensitive1-associated kinase 1/SERK3 (BAK1/SERK3). This protein belongs to the Somatic-Embryogenesis Receptor-like Kinase (SERK) protein family (42, 43) and is involved in both elf18 and flg22 signaling (43-45). In both signal transduction pathways, upon PAMP recognition by its specific PRR, BAK1 interacts with the adapting PRR, triggering PTI (43, 44). Interestingly, both EFR and FLS2 were confirmed to interact with various SERK proteins, including BAK1/SERK3 and BAK1-like1/SERK4 (BKK1/SERK4), upon binding of PAMPs to their specific receptors (43). Furthermore, both FLS2 and

BAK1 interact with the cytosolic kinase Botrytis-induced kinase 1 (BIK1) (46). Although BIK1 does not affect the association of FLS2 and BAK1, BIK1 phosphorylation is induced by flg22, and a *bik1* null mutant exhibits a reduction in flg22 sensitivity (46). This suggests that BIK1 is a significant kinase involved in flg22 signaling, downstream of the flg22 perception step. Interestingly, although BAK1 does not appear to interact with AtCERK1 (47), BIK1 was shown to interact with AtCERK1 in a co-Immunoprecipitation assay (48). Moreover, *bik1* mutant plants also have lower responses to chitin, mainly in reactive oxygen species (ROS) production and callose deposition (48). Therefore, it is likely that *BIK1* is involved in chitin signaling at least at an early step, by interacting with AtCERK1. However, whether the protein is required for chitin-triggered immunity is still a question.

Chitin signaling in rice was shown to require OsCERK1, the ortholog of AtCERK1 (49). Suppression of *OsCERK1* expression in rice also impaired chitin signaling. Using a pairwise yeast two hybrid approach, the extracellular domains of OsCEBiP and OsCERK1 were shown to interact, and the extracellular domain of each protein was shown to form a homodimer (49). Unlike the case of OsCERK1 interacting with OsCEBiP, to date, no direct interaction between AtCERK1 and a different LysM protein has been demonstrated. Although it was found that upon chitooctaose recognition, the LysM domain of AtCERK1 does form homodimer (50). One study showed that AtCERK1, despite not being a receptor of peptidoglycan (PGN), is also vital for PGN-triggered immunity (51). The Arabidopsis receptor complex for PGN are

glycosylphosphatidylinositol-anchored LysM proteins LYM1 and LYM3, both of which lack an intracellular signaling domain (51). Therefore, it was hypothesized that although LYM1 and LYM3 specifically bind to PGN, the PRR complex might rely on the AtCERK1 kinase activity to transduce an intracellular signal (51). Thus, LYM1-LYM3 and AtCERK1 may directly interact but there is no evidence for such an interaction. Similar to the ligand-induced dimerization activity of EFR and FLS2, AtCERK1 interacts with itself in the presence of chitooctaose, but not in the presence of chitotetraose or chitopentaose (36). Very recently, our lab showed that AtLYK4, also a LysM RLK, is also involved in chitin-induced innate immunity (52). A T-DNA insertion mutant defective in *AtLYK4* showed a chitin response (e.g., ROS production, calcium spiking and expressions of chitin-responsive genes such as *WRKY53*, *MPK3* and *ZAT12*) significantly less than the wild-type but not as severely affected as *Atcerk1* mutants (52). *Atlyk4* mutant plants are also more susceptible to both hemibiotrophic *Pseudomonas syringae* and necrotrophic *Alternaria brassicicola* (52). AtLYK4, although possessing an intracellular kinase domain, appears to be an inactive kinase (52). It was postulated that, similar to OsCERK1 and OsCEBiP, where only OsCERK1 has an active kinase domain, AtCERK1 and AtLYK4 could form a receptor complex, which would transduce the intracellular response to chitin solely through the AtCERK1 kinase activity. However, there is no direct biochemical evidence for such an interaction.

Another example of the importance of co-receptor functionality is the complex comprised of NFR1 and NFR5 in *Lotus japonicus* (53). This co-receptor complex

recognizes bacterial chitin-like molecules, lipo-chitooligosaccharides (LCO), in the nitrogen fixation in legume–rhizobium symbiosis (40, 53). Again, similar to the complex of OsCERK1/OsCEBiP and that postulated for AtCERK1/AtLYK4, only NFR1 has an active kinase domain in the NFR1/NFR5 receptor complex.

In tomato, ethylene-induced xylanase (EIX), another fungal PAMP, is recognized by LeEIX1 and LeEIX2 (54). Like the system of PGN signaling in Arabidopsis, these two receptors in tomato also function as receptor-like proteins without an intracellular signaling domain (55).

Signal transduction in PTI

Previous studies showed that a common set of downstream pathway components are activated upon PAMP recognition (9, 18, 56). For example, within 60 minutes of PAMP recognition, ion fluxes occur, ROS production is induced, and the MAPK cascade is activated, leading to expression of PAMP-induced genes (57). Within hours, other late responses, such as callose deposition and hormone action, are triggered (57).

ROS has direct antimicrobial activity on pathogen invasion. This toxicity is due to the formation of phenoxy-radicals during cell wall formation, and peroxidation of lipids, which induces phytoalexin synthesis (58). However, ROS production might act downstream and function independently of the MAPK cascade (59). The MAPK cascade in many plants is involved in the intracellular signaling pathway activated in response to

a variety of PAMPs (12). For example, the flagellin-triggered MAPK cascade in *Arabidopsis* is composed of MEKK1, MKK4/5 and MAPK6/3, MKK1 and MAPK4 (56). Similarly, *Agrobacterium* elf18 and *Botrytis cinerea* PAMP-induced MAPK cascades contain MKK4/5 and MPK3/MPK6 (12). The MAPK cascade leads to the activation of transcription factors, notably WRKY22, -25, -29, and -33 (56, 60). However, in most cases, while MAPK3/6 are positive regulators acting on WRKY22 and WRKY29, MAPK4 acts as a negative regulator that functions in a nuclear complex with MAPK substrate 1 (MKS1) and WRKY33 (57, 61).

Transcription factor action, in turn, results in changes in the expression of various genes, including those encoding antimicrobial compounds, RLKs and other receptors of effector proteins (6). Depending on the PAMP stimulation, similar host responses can occur, but with different timing (13). For example, within 30 minutes, the strongest response to chitin addition occurs; whereas at 24 hours, the response to LPS reaches a maximum (18, 60), but by this time the transcriptional response to flg22 treatment is already finished (62). An overlap exists in the genes responding to different PAMPs; this is a key observation that indicates signal convergence after PAMP recognition (13). Previously, a study in our own lab showed that 441 *Arabidopsis* genes are upregulated by treated with each of the three PAMPs chitin, flg22 and elf18 (9). Studies revealed that in *Arabidopsis* LPS and harpin regulate over 600 and 1200 genes, respectively, with 300 genes similarly regulated by both treatments (18). Interestingly, in *Arabidopsis*, out of 800 PAMP-regulated genes, a common set of 96 genes are up-

regulated upon treatment with each of the four different PAMPs (flg22, elf18, LPS and HarpinZ) (13), indicating a critical role of this gene set in PTI (63).

Later responses to PAMP treatment include callose deposition and hormone signaling. Callose, a β -1,3 glucan polymer, is synthesized between the cell wall and the plasma membrane and accumulates several hours after PAMP treatment (13). Like ROS, callose deposition has antimicrobial activity that depends on PAMP-induced glucosinolates (64). Hormone pathways that are induced during PTI include SA, JA, and ET, each of which functions as a classical defense hormone (14). While the SA-mediated signaling pathway is involved in defense responses to biotrophic and hemibiotrophic pathogens (65), JA/ET-mediated signaling pathways are required for defense responses to necrotrophs and herbivores (14, 66). However, chitin signaling was shown to be independent of the SA, JA and ET-mediated signaling pathways (9, 39, 67), at least during the early responses. In addition, PTI triggered by flg22 signal was also reported to be independent of hormone signaling (6), although the flg22 signal also triggered the pathways affecting SA and ET (68, 69). Depending on the specific PAMP involved, these hormone pathways might be required for local or systemic acquired resistance (SAR). For example, flg22-induced SA is required for both local resistance and SAR; whereas LPS-induced SA is only important for SAR (68, 70). Disruption of SA signaling components suppresses the expression of a set of bacterial PAMP-regulated genes (71). Acting downstream of PAMP recognition, the hormone signaling pathways also depend on both the nature of the specific pathogen, as well as their pathogenicity (14, 66).

CERK1-mediated chitin signaling in Arabidopsis

Chitin is a critical structural component of fungal cell walls, insect exoskeletons, the hard carapace of crustaceans, and the egg-shell of nematodes. It is a linear oligomer of β -1,4-linked N-acetylglucosamine. Despite being widely found in many organisms, chitin has not been found in plants. Chitin generated from the cell walls of pathogenic fungi can trigger defense responses or related cellular responses in many plants (72, 73). It was reported that a purified chitin, N-acetylchitoheptaose (chitoheptaose), added to rice cell suspension culture induced JA responsive genes, leading to the biosynthesis of the antimicrobial compound momilactone A (74). Moreover, chitooctaose induces transient depolarization of membrane potential, protein phosphorylation, ROS production and expression of defense-related genes, such as phenylalanine ammonia lyase, phospholipase, and WRKY transcription factors (74-77). Similarly, in Arabidopsis, ROS production, calcium influx and defense gene expression also occur upon chitooctaose addition (9, 28, 78, 79). Indeed, chitin is well-known to trigger various defense responses to various pathogens (9, 80) in a variety of plants, including wheat (29), oat (30), alfalfa (31), soybean (32) and melon (33).

In Arabidopsis, the receptor for chitin was identified as a LysM Receptor-like kinase1 (LYK1) designated as *Arabidopsis thaliana* CERK1 (9, 28, 36, 81, 82). Mutations that disrupt CERK1 expression compromise not only physiological responses to chitin treatment, such as ROS production and the MAPK cascade, but also expression of chitin-responsive genes, WRKY22/29/33/53 (28, 83). Likewise, CERK1 mutants also show

enhanced plant susceptibility to both hemibiotrophic *Erysiphe cichoracearum* and necrotrophic *A. brassicicola* fungal pathogens, as well as to the bacterial *P. syringae* pv *tomato* strain DC3000 pathogen (9). In vitro binding of chitin to its Arabidopsis receptor was reported (38, 81), and the crystal structure of the extracellular LysM domains of AtCERK1 (CERK1-ECD) was recently published, revealing the binding of different chitooligosaccharide fragments to CERK1-ECD (36). Although chitin fragments of different length can bind to CERK1-ECD, only chitooctaose generates the strongest responses (37) in plants, while other fragments such as chitotetraose and chitopentaose trigger little or no response (38, 39). Consistent with this finding, while CERK1-ED could bind chitotetramer, only binding of the chitooctaose activated dimerization of CERK1-ECD (36).

Upon chitin perception, a MAPK cascade is activated, consisting of MKK4/5-MPK3/6 as well as an unknown kinase in Arabidopsis (79). Activation of the MAPK cascade in turn results in the phosphorylation of a variety of transcription factors (TFs), including ones specifically induced by chitin (60). DNA microarray analysis showed that about 1000 Arabidopsis genes are differentially regulated upon chitin treatment (9, 28, 78, 84). Consistent with this, induction of 118 transcription factors was confirmed by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) (60). An interacting network was characterized based on 34 transcription factors that are induced by chitin and MPK3/MPK6 (85, 86). Notably, the ethylene response factor ERF5

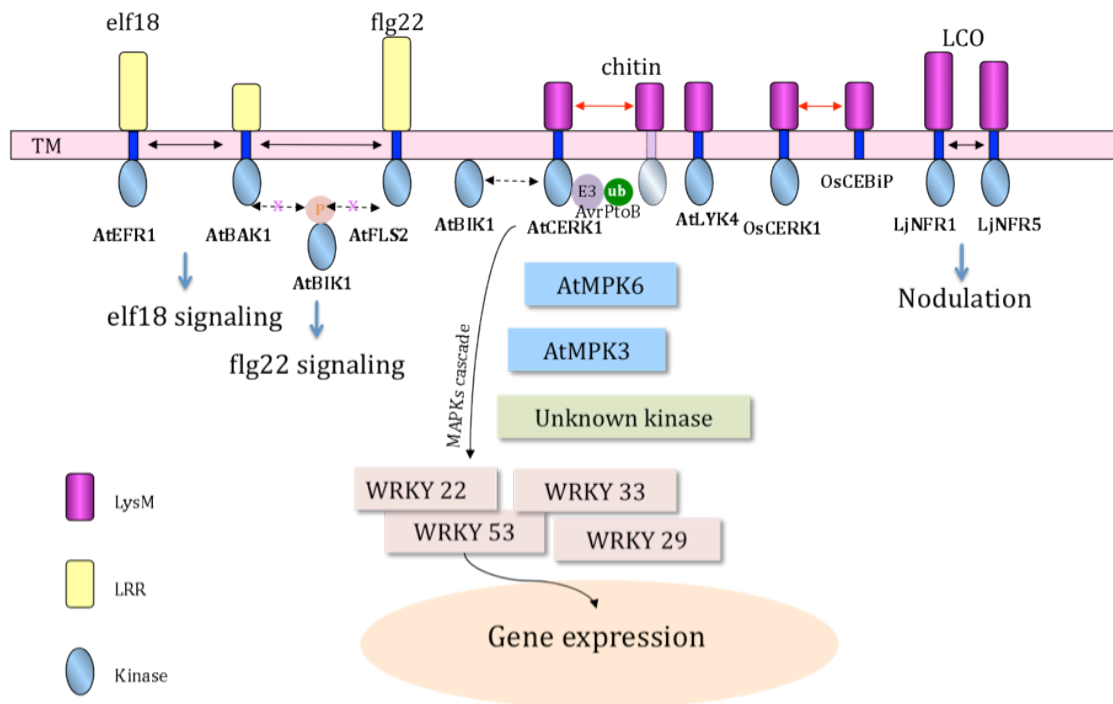
(belonging to the network) was demonstrated to be a regulator of basal defense, likely through its mediation of the ET/JA and/or SA hormone signaling pathway (86).

In addition to its essential role in chitin signaling, AtCERK1 is also involved in defense against bacteria through its recognition of both peptidoglycan and several unknown PAMPs present in bacterial crude extract (51, 83). There is evidence suggesting that virulent bacteria can suppress PTI by targeting AtCERK1 with the effector AvrPtoB (87). Once AvrPtoB is secreted into the host cell, it interacts with the kinase domain of AtCERK1 and targets the domain for ubiquitination with its E3 ligase activity (87). Interestingly, the mechanism to overcome PTI was previously discovered in flagellin signaling, since AvrPtoB also targets the flg22 receptor FLS2 (87, 88). Moreover, both flagellin signaling and chitin signaling likely share a co-receptor, Botrytis induced kinase1 (BIK1) (48). In flg22 signaling, BIK1 was shown to be the first kinase in the pathway and is involved in not only cellular flg22-induced responses but also in flg22-triggered immunity (46, 48). In chitin signaling, *bik1* mutants lose cellular responses to chitin, including ROS production and callose deposition (48). However, to date, the role of BIK1 in chitin-induced immunity remains a mystery.

Although numerous studies of chitin signaling have been published (as described above), it remains unclear how signals downstream of chitin perception are transduced after being recognized by the CERK1 receptor. To this end, I performed an extensive forward genetic screening for chitin insensitive mutants and also high-throughput screening of AtCERK1 interacting proteins using heterologous protein-protein

interaction systems. For the forward genetic screening for chitin insensitive mutants, I sought to discover novel components that are specifically involved in chitin signaling. For the high-throughput screening for AtCERK1 interacting proteins, my goals were to identify potential co-receptors involved in the AtCERK1 receptor complex, as well as other early-functioning components. In this thesis, I shall discuss the current status of chitin signaling based on the function of the novel factors I identified.

Chitin signaling pathway as a model for PTI



In rice, OsCEBiP and the co-receptor OsCERK1 are required for chitin recognition (27, 49). In Arabidopsis, chitin is recognized by LysM RLK1 (LYK1)/CERK1 (9, 28, 36, 81, 82), where dimerization of the LysM domains is induced by chitin binding (e.g., chitoctase) (36). Chitin perception by AtCERK1 activates the MAPK cascade, triggering activation of WRKY transcription factors with subsequent expression of chitin-responsive genes (39, 79, 84). The pathogen *P. syringae* can suppress chitin-triggered immunity by AvrPtoB that targets AtCERK1 for ubiquitination. In an analogous situation, the lipo-chitin Nod factor is recognized by a receptor complex containing two LysM domain proteins, NFR1-NFR5 (53). While flg22 is perceived by FLS2, the signal is transduced only when FLS2

associates with LRR-RLK BAK1 and subsequently dissociates from (after phosphorylating) a cytoplasmic kinase BIK1 (45, 46). AtCERK1 does not interact with BAK1 (47) but does interact with BIK1 (48). However, it is unclear if this interaction is required for chitin-triggered immunity (48). Similar to flg22 signaling, EFR1 interacts with BAK1 upon perception of elf18, triggering elf18 signaling (43). As in nodulation, flg22 signaling, elf18 signaling and chitin signaling in rice, AtCERK1 might function with other proteins in chitin perception.

CHAPTERS

I. SCREENING FOR ARABIDOPSIS MUTANTS DEFECTIVE IN THE CHITIN SIGNALING PATHWAY

1. Introduction

In Arabidopsis, CERK1 was identified as the primary receptor for chitin (9, 28, 36, 81, 82). Mutations that disrupt CERK1 expression compromise not only physiological responses to chitin treatment, such as ROS production and MAPK cascade activation, but also expression of chitin-responsive genes, WRKY22/29/33/53 (28, 83). The AtCERK1 mutant also showed enhanced susceptibility to both hemibiotrophic *E. cichoracearum* and necrotrophic *A. brassicicola* fungal pathogens, as well as to the bacterial *P. syringae* pv tomato DC3000 pathogen (9). In vitro binding of chitin to AtCERK1 was reported (81, 82). In particular, a recent study reported the crystal structure of the AtCERK1 extracellular LysM domain, AtCERK1-ECD, and the binding of different chitin fragments to AtCERK1-ECD (36). Notably, the study showed that while chitotetraose does bind to AtCERK1-ECD, only chitooctaose, which is most active in generating a measurable plant defense response, activated homodimerization of AtCERK1-ECD (36). Chitin perception activates a MAPK cascade consisting of MKK4/5-MPK3/6 and an unknown kinase (79). MAPK cascade activation, in turn phosphorylates a variety of transcription factors (TFs), including ones specifically induced by chitin (60). DNA microarray analyses showed that about 1000 Arabidopsis genes were differentially regulated upon chitin treatment (9,

28, 78, 84). Furthermore, using qRT-PCR, 118 transcription factors from the 1000 chitin-induced genes were identified (60). A recent study from our laboratory identified an interaction network among 34 transcription factors out of the 118 transcription factors and MPK3/ MPK6 (86).

Chitin signaling is a complex pathway with both unique features, as well as some overlap with other PAMP signaling components. It is apparent that, at least with regard to the early measured response, the chitin-mediated signal transduction pathway is unique and distinct from SA, JA, ET and flagellin-mediated signaling pathways (9, 39). On the other hand, there is some functional overlap with the signaling pathways of other PAMPs, such as flagellin and EF-Tu. Indeed, 441 Arabidopsis genes were shown to be commonly induced upon treatment with any of these three PAMPs (9).

Fungal pathogens are among the most devastating biotic stresses that plants face. Therefore, given the critical importance of chitin signaling in fungal defense, the chitin signaling pathways warrant more intensive scrutiny. Therefore, in my work, an ethyl-methyl sulfonate (EMS) mutagenesis population was generated and screened for mutants specifically defective in chitin signaling. The screening revealed seven mutants defective in chitin signaling responses, very likely representing independent mutagenic events. Interestingly, only one mutant was found to be defective in both chitin and flg22 signaling; whereas the other six mutants are specifically defective in chitin signaling. As would be expected, two out of the six mutants were identified as *AtCERK1* alleles, with mutation sites in the kinase domain.

2. Materials and methods

Plant germination and growth conditions

Arabidopsis thaliana seeds were surface-sterilized with 70% ethanol, incubated in a solution containing 3% sodium hypochlorite and 0.2% Tween-20 (Fisher Scientific) for 5 minutes, washed five-times with sterile distilled water (ddH₂O) and finally re-suspended in 300 µl sterile distilled water. Seeds were then vernalized at 4°C for 3 days in the dark and germinated on solid 1% agar of 0.5X MS medium containing Murashige and Skoog mineral salts (Murashige and Skoog, 1968, Sigma, St. Louis, MO), 0.5 % sucrose (w/v), 0.05 % MES (pH 5.7) and 0.7% agar (Fisher Scientific). For seedling experiments, plants were grown on 0.5X MS agar medium for 3 days and transferred into a liquid 0.5X MS medium. Otherwise, seedlings were grown for up to 2 weeks prior to transfer to soil. Plants were grown in a plant growth chamber (model CU-32L, Percival Scientific Inc., Boone, Iowa) under 8 hour day/ 16 hour night cycle and 60 % humidity. For seed amplification and analysis of mature plants, 14-day-old seedlings were transferred to Pro-mix soil (Premier Horticulture, Red Hill, PA) and grown at 22 °C under continuous fluorescent white light in either a plant growth chamber with 60 % humidity or in a greenhouse.

PAMP treatments

PAMPs were sprayed onto 4-week-old mature leaves or incubated (soaking in liquid) with 4-week-old punched leaf discs and seedlings. The following concentrations

were used for each PAMP: 100ug/mL chitin mixture (csc), 1uM chito-octaose (8mer), 1uM elf18, 1uM flg22 and 4ug/mL purified chitin (spe) (the purification procedure of chitin is described below)

Reactive oxygen species assay

Arabidopsis 14-day-old seedlings or 5-mm diameter 4-week-old leaf discs were incubated in ddH₂O in the growth chamber for one night to recovery from the wound response. One hour prior to each experiment, plant tissues were transferred to the dark (to suppress the light background arising from photon emission for the plastic that could be detected by the camera). Once in darkness, plants were treated with a fresh solution containing the designated PAMP, 0.2% luminol solution (from a stock of 17mg/mL luminol in 200mM KOH) and 0.2% horseradish peroxidase solution (from a stock of 10mg/mL luminol in sterile distilled H₂O) (Sigma, St. Louis, MO, USA). Immediately after treatment, the ROS signal was recorded for 10 minutes; an interval of 20 seconds before and 20 seconds after the maximum signal observed was analyzed using a Photek camera PSU1 (Photek Ltd., San Francisco, CA, USA).

Pathogen assay

P. syringae pv. tomato DC3000 cultures were grown on NYG medium (5 g peptone, 3 g yeast extract, and 20 g glycerol and 1% agar per liter at pH 7.0 (Fisher Scientific) plates for 24 hours, at 30°C prior to inoculation onto plants. Antibiotics were used for plate selection at the following concentrations: 25 µg/mL kanamycin and 100

$\mu\text{g}/\text{mL}$ rifampicin (Fisher Scientific). For PAMP treatment, 4-week-old leaves were sprayed twice with 0.001% silwett and each PAMP prior to pathogen inoculation (24 hours before and 6 hours before). Mature leaves were then infiltrated with bacteria (resuspended in 10 mM MgCl_2 (Fisher Scientific) and inoculated at a concentration of 0.5×10^5 cfu/mL with a needle-less syringe. Alternatively, 10-day old seedlings were treated with PAMPs by soaking seedlings in the PAMP solution for 24 hours. Similarly, seedlings were soaked in the bacterial solution at a concentration of 0.5×10^4 cfu/mL for two hours. The bacterial solution was then removed and seedlings were washed with ddH₂O three times. After 72 hours of inoculation, plants were surface-sterilized for 30 seconds with 70% ethanol, and the 1-cm-diameter punched leaf discs or every three seedlings were ground in 10mM MgCl_2 to extract bacteria. The bacterial extract was plated onto NYG plates with selection antibiotics and grown at 30°C for counting after 48 hours.

Chitin purification

A chitin mixture 100mg/mL (Sigma, St. Louis, MO) was passed through an amine bead column (Invitrogen, Carlsbad, CA) and then washed with acetone nitrile (with the amount of 70% the volume of chitin mixture used) (Fisher Scientific). The concentration of the eluted purified chitin was estimated by measuring OD₂₀₀ of the eluted purified chitin and converting with the linear regression equation:

$$\text{Concentration (ug/mL)} = 85.807 * (\text{OD}200)^{1.5302}$$

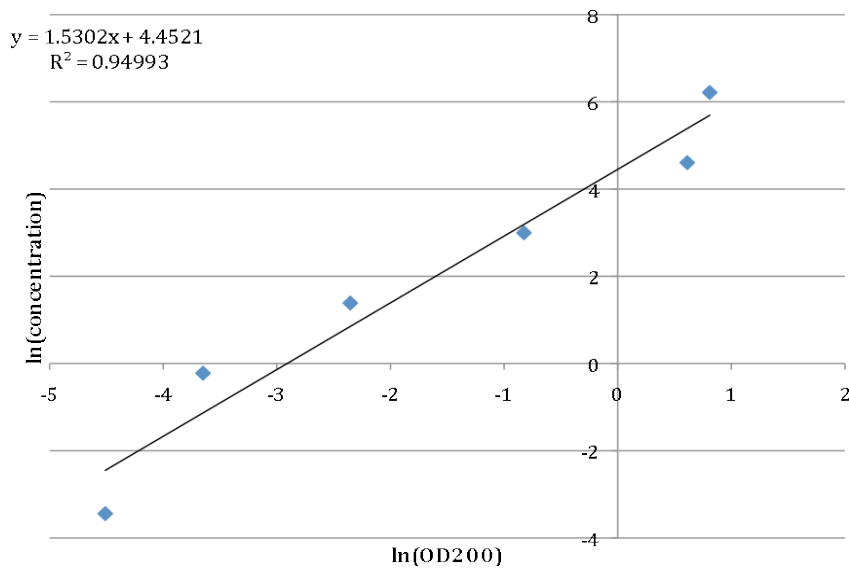
To develop this equation, various known concentrations of chitin were measured to determine the relationship between concentrations and OD200 values. The OD200 was reported as the wavelength best absorbed by oligosaccharides (89). Based on the collected data, the relationship between $\ln(\text{concentration})$ and $\ln(\text{OD200})$ used the simple linear regression described above, with $R^2 = 0.9499$ (Figure I-1)

$$y = 1.5302x + 4.4521$$

In which $y = \ln(\text{concentration})$ and $x = \ln(\text{OD200})$.

Figure I-1 Regression analysis showing the relationship between ln(Concentration) of chitin and ln(OD200 of chitin)

A



B

ln(OD200)	-4.5099	3.649587	-2.353878	-0.825536	0.611937	0.8095993
ln(Concentration)	-3.4421	-0.22314	1.3862944	2.9957323	4.60517	6.2146081

The simple linear regression (A) between ln(OD200) and ln(Concentration) was generated based upon collected data shown in Table B, which was obtained by measuring the OD200 of different concentrations of chitin mixture and then converting the collected data into ln(OD200) and ln(concentration).

EMS mutagenesis of Arabidopsis seeds

Arabidopsis seeds were first hydrated overnight in sterile ddH₂O. The next day, the water was replaced with an EMS (EMS; Sigma, St. Louis, MO, USA) solution at concentrations of 30mM, 40mM or 50mM. This solution was placed on a reciprocal shaker at room temperature for 8 hours and then rinsed at least three times with ddH₂O. After washing, the seeds were shaken in ddH₂O at room temperature overnight. The next day, the mutagenized seeds were sown in a 0.1% Agarose medium (Sigma, St. Louis, MO, USA) and then grown in soil at a density of 500-1000 seeds per 2-ft² flat. The mutagenesis efficiency was calculated based on the frequency of embryonic-lethal mutations, with the target of at least one embryonic-lethal mutation per mutagenized genome, according to the protocol of Weigel & Blazebrook (90).

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 10-day-old seedlings of *Arabidopsis thaliana* using the Trizol[®] reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA concentrations were measured using a Nanodrop-1000 spectrophotometer. RNA quality was assessed by agarose gel electrophoresis. RNA samples were purified using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. To remove genomic DNA contamination, total RNA was treated with TURBO DNase (Ambion Inc., Houston TX) according to the manufacturer's protocol. Two micrograms of RNA were reverse-transcribed to synthesize single-stranded cDNA using Superscript III

reverse transcriptase (Invitrogen, Carlsbad CA), oligo(dT), 10 mM dNTP and RNase Out at 37° C for 1 hour in a 25 µl reaction. The reaction was inactivated by heating at 70° C for 15 minutes.

Quantitative RT-PCR

Quantitative RT-PCR was performed with an ABI 7500 Real Time PCR System with Sequence Detection System (SDS) software version 1.3 (Applied Biosystems, Foster City, CA) utilizing SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). The gene-specific primers utilized are listed in Table 2.2. PCR conditions were as follows: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min, stage 3, 45 cycles of 95°C for 15 sec and 60°C for 1 min; stage 4 (for dissociation), 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Data obtained for three technical replicates of each biological replicate were normalized to AT2G28390 (*SAND*), a gene that is constitutively expressed under the culture conditions used (60). Gene expression levels were calculated by $\text{efficiency}^{-\Delta\text{CT}}$ where ΔCT is calculated by subtracting the cycle threshold (CT) of the reference gene from CT of the each gene of interest, and then converted as relative values to the gene expression level at the starting time of the treatment (initial (0 min) = 1.0). Primers used for *WRKY53* (At4g23810) and *SAND* were 5'-CCTACGAGAGATCTCTTCTTCTG-3' and 5'-AGATCGGAGAACTCTCCACGTG-3'; and 5'-AACTCTATGCAGCATTTGATCCA-3' and 5'-TGATTGCATATCTTTATCGCCATC-3', respectively.

Mapping

Recessive F2 mutants plants arising from a cross with Landsberg *erecta* (*Ler*) plants were mapped using the method described in (91)

Molecular Modeling of proteins

(Protein molecular modeling was done by Cuong The Nguyen, a Ph.D. candidate in the Stacey lab)

Domain localization of the two mutation sites of AtCERK1:

To identify the mutation sites within the AtCERK1 protein, a transmembrane prediction tool (the TMHMM Server version 2.0) (92) was used. The server uses protein sequence as input and provides output with locations of transmembrane domain(s), inside and outside domains, together with their probabilities.

The HHSearch Server (93) was used to find available homologous templates of the AtCERK1 kinase domain in the PDB (the Protein Data Bank) where their crystallized structures were deposited. The templates were chosen based on their E-values. The secondary structure of AtCERK1 generated by the HHSearch Server was compared with the secondary structure of the template to validate the conserved secondary structure. The best templates were selected based on both low E-values (the lower the E-value, the higher the probability of a non-random hit in the database), and conserved secondary structures sharing between target and template proteins.

Sequence alignment, structure modeling and model selection

An alignment of the AtCERK1 protein sequence to the best template structures was constructed by using the structure sequence alignment module of the Modeller program (94). Two hundred models of the AtCERK1 protein were built from the alignment using the Modeller tool (version 9.10). All 500 models were assessed using the TM-Score program (95) and the best model was selected based on the TM-Score scores.

Model refinement and validation

The best model was checked for clash or contact, and then it was optimized by several cycles of energy minimization using the Chimera program to improve the quality of the model. The absolute quality score of the model (i.e., the expected similarity between the model and the unknown native structure) in terms of the global distance test-total score (96), a standard score between 0 and 1 used to quantify the structural similarity ranging from completely dissimilar to identical, was assessed by the Apollo local program (97). The model was visualized using the Chimera 1.9 software (98). The Rotamer module of the Chimera program was used to modify amino acid side chains with a rotamer library.

Sequencing

The cDNAs of the mutants were used for sequencing with the primers complementary to CERK1 as 5'-ATGAAGCTAAAG-3', 5'-TAGTCTCAAAG-3', 5'-

TACCGCCGGAC-3' and 5'-ACCACCCAAACC-3'. The sequencing process were performed at the DNA core facility, University of Missouri (MO, USA).

3. Results

Screening for EMS mutants identified seven mutants defective in chitin response

To identify mutants involved in chitin signaling, EMS treated populations were screened by measuring chitin-induced ROS production. In the mutation generation step, three different concentrations of EMS were used: 30mM, 40mM and 50mM, resulting in populations with mutagenesis efficiency of 30-35%, 60-65% and a high proportion of more than 80% growth retardation, respectively (see Materials and Methods). The population with 60-65% mutagenesis efficiency was chosen for further screening.

Approximately 135 pools representing 37,260 M2 plants were screened by measuring chitin-induced ROS production by seedlings relative to the wild-type Col-0 and the negative control *cerk1* plants. In the first screening, 1185 M2 mutants were scored as being affected in chitin signaling (both insensitive and hypersensitive). A second screening was then performed on M3 seeds derived from these putative mutants. After challenging with the ROS assay, each candidate seedling was planted into soil with a dome cover. Due to the differences of humidity and nutrient condition between MS medium and the soil environment, and also due to the vitality of the mutagenized plant (e.g., lethality, etc.), survival of the next generation could not be

guaranteed for all mutant candidates. Indeed, only 315 mutants were successfully recovered and produced seeds for the next screening.

These 315 putative mutants were further screened for chitin-induced ROS production using 12 seedlings for each mutant (to provide greater statistical confidence), which resulted in the identification of 79 mutant lines whose ROS response to chitin differed from the wild-type. These mutant lines were again screened using mature leaf discs of 4-week old plants. This third screen confirmed that 45 mutant lines had a chitin response that differed from the wild-type. These lines were sorted into four categories based on the level of ROS produced in response to chitin compared with the wild-type Col-0 (100%). The categories are: ROS production < 20% of the wild-type level (seven mutants), between 20% to 60% (12 mutants), between 60% to 80% (10 mutants), above 80%, but still lower than the wild-type (six mutants) and higher than Col-0 (10 mutants) (Figure I-2). The seven mutants (with designed names as shown in Figure I-2) strongly defective in chitin response were then genetically mapped. Note that each of these seven was identified from different M2 pools and, therefore, likely represent independent mutagenic events.

Involvement of the seven mutants in chitin signaling

Although the seven mutants were chosen based on their deficiency in chitin response based on ROS production in response to chitin (less than 20%), it was still critical to determine if their deficiency in PAMP response was specific to chitin. Therefore, each mutant was also tested for ROS production in response to elicitation

with flg22. Figure I-3 shows the ROS production of the seven mutants and three controls (the positive control Col-0 and two independent negative controls *cerk1* and *fls2*) after independent treatments with each of three PAMPs, chitin mixture, chitooctase, or flg22. In response to treatment with chitin mixture (CSC) and chitooctase (8mer), only the wild-type Col-0 showed ROS production. In all the EMS mutants, ROS production was lower than that generated from the wild-type, ranging from 20%-60% of the Col-0 control. Interestingly, in the flg22 treatment, only the H4-3 mutant showed a deficiency in flg22 signaling, producing no ROS response upon flg22 elicitation. Despite the deficiency in chitin-induced ROS generation, the other six EMS mutants produced the same level of ROS in response to flg22 as the wild-type Col-0 (Figure I-3). Thus, out of seven EMS mutants defective in chitin response, six were specifically defective in chitin signaling, and only one was defective in the response to both chitin and flg22.

EMS mutants showed defects in chitin-induced gene expression

In addition to measuring ROS production in the various mutants, I also examined whether there were defects in chitin-induced gene expression, in particularly WRKY53. Gene expression was measured using mRNA isolated from 10-day-old seedlings. Figure I-4 shows the expression of the chitin-inducible WRKY53 gene (9, 60) in response to chitin addition in the controls and various EMS mutant lines. Upon chitin treatment, WRKY53 was strongly induced (about 55-fold) in the wild-type but showed much lower expression in all of the mutants except D1-11 and H4-3. In particular, WRKY53 expression in mutant J4-7 was as low as in the negative control *cerk1*.

Deficiency in chitin response leads to a loss of innate immunity in some mutants

A defect in the chitin signaling pathway should also compromise the innate immunity response, making the plants more susceptible to infection by pathogens. Therefore, I tested each of the EMS mutants for their resistance to the bacterial pathogen *P. syringae* pv. Tomato strain DC3000 (Figure I-5). Consistent with previous studies (9), chitin treatment triggered PTI in the wild-type, leading to more resistance to DC3000 (demonstrated by less growth on seedlings after chitin treatment compared to the mock treated controls). This response was not seen in *cerk1* mutant seedlings. In contrast to wild-type, four out of seven mutants tested were significantly more susceptible to pathogen infection regardless of chitin treatment. Therefore, it is very likely that the defect in chitin signaling in these mutants resulted in a reduction in PTI upon chitin elicitation, which allowed greater pathogen colonization.

Genotyping of the seven mutants

Although the goal of the EMS mutant screen was to isolate novel mutant alleles defective in PAMP-response, there was also a chance that additional *cerk1* mutant alleles would also be isolated. To test whether any of the seven mutants contained mutations in the *cerk1* gene, each was back-crossed to *cerk1* and the F2 seedlings were tested for their ROS response to chitin addition. This procedure was successful for mutant lines A1-7, D1-11, H4-3, M7-2 and J4-7. For the other two lines, I failed to obtain F2 seeds. The results of back-crossing revealed that three mutants A1-7, D1-11 and H4-3

appeared to be novel mutations, while two mutants, M7-2 and J4-7, were allelic to *cerk1* (Table I-1). The F2 segregation of A1-7, D1-11 and H4-3 crossed with *cerk1* showed a ratio of about 9:7, suggesting that all the lines possess a single recessive mutation, independent from *CERK1* (Table I-1).

Sequencing of the *cerk1*-allelic mutants (Figure I-6) indicated that in M7-2, the codon at position 334 was converted from GGG to GAG, converting a codon for glycine (G), a non-polar, neutral amino acid, to glutamic acid (E), a polar, negatively charged amino acid. Likewise, in J4-7, the codon 454 was converted from CGA encoding arginine (R) to a stop codon, TGA.

Mapping of the mutation in line A1-7

To locate the other mutation sites, the remaining mutants were crossed to *Ler* and resulting F1 progeny were genotyped for mapping. To date, rough mapping has been performed only for A1-7 due to limited availability of F1 seeds, as described in Table I-2. The results indicate that the A1-7 lesion lies in chromosome 1, between the markers T27K12 and NF19K23 (Table I-3)

Figure I-2 ROS production of 45 EMS mutants after 3 rounds of screening

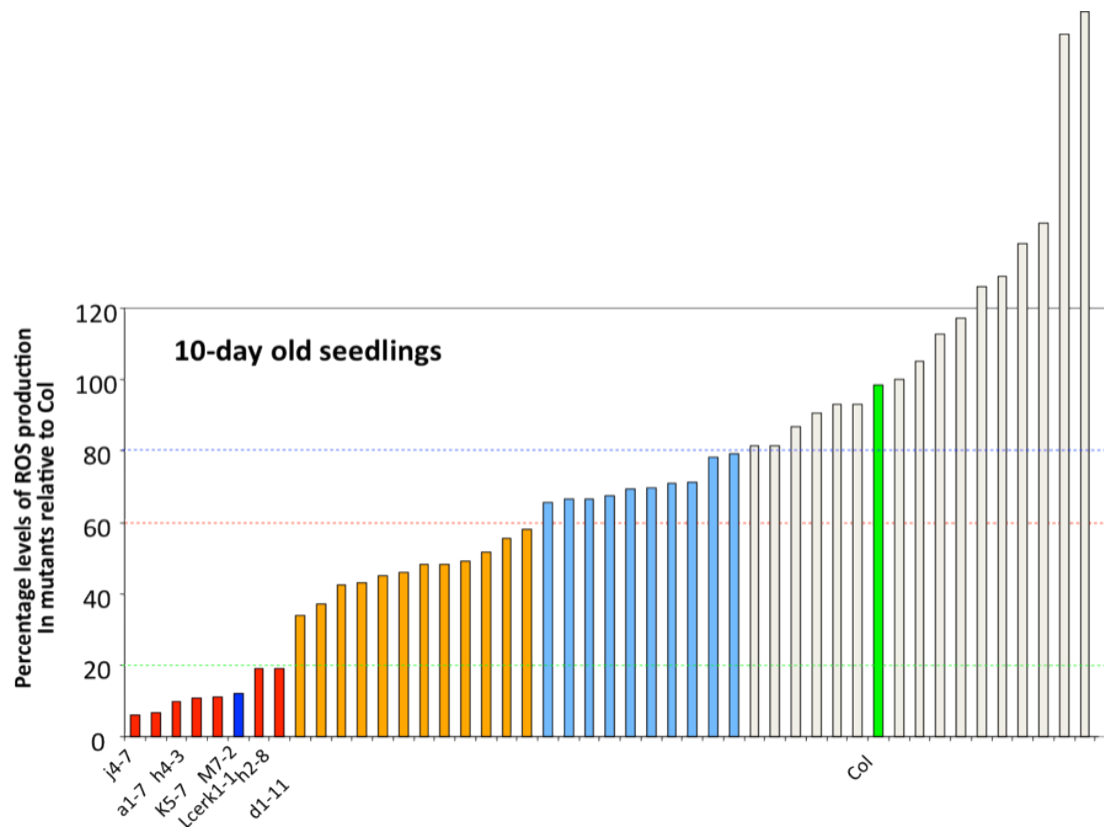


Figure I-2 ROS production of 45 EMS mutants after 3 rounds of screening

The level of ROS production (%) is shown for each mutant relative to the Col-0 wild-type (100%). Original ROS levels represent the average value from 12 10-day-old seedlings, measured 20 seconds before and after the maximum signal was recorded. Based on ROS production compared with Col-O wildtype, mutants are divided into four groups, which are: red bars, 20% or lower ROS compared with Col-O; orange bars, ranging from 30 to 60% ROS production compared with Col-O, blue bars, ranging from 60 to 80% ROS production compared with Col-O; white bar, high ROS production 80% or even higher ROS production compared with Col-O. The first group was chosen for further investigation. Each mutant in the group was labeled with its designated name, in which the first two letters represent the name of the pool. The results were obtained from the first screening.

Figure I-3 Specificity of PAMP-induced ROS generation in the various EMS mutants

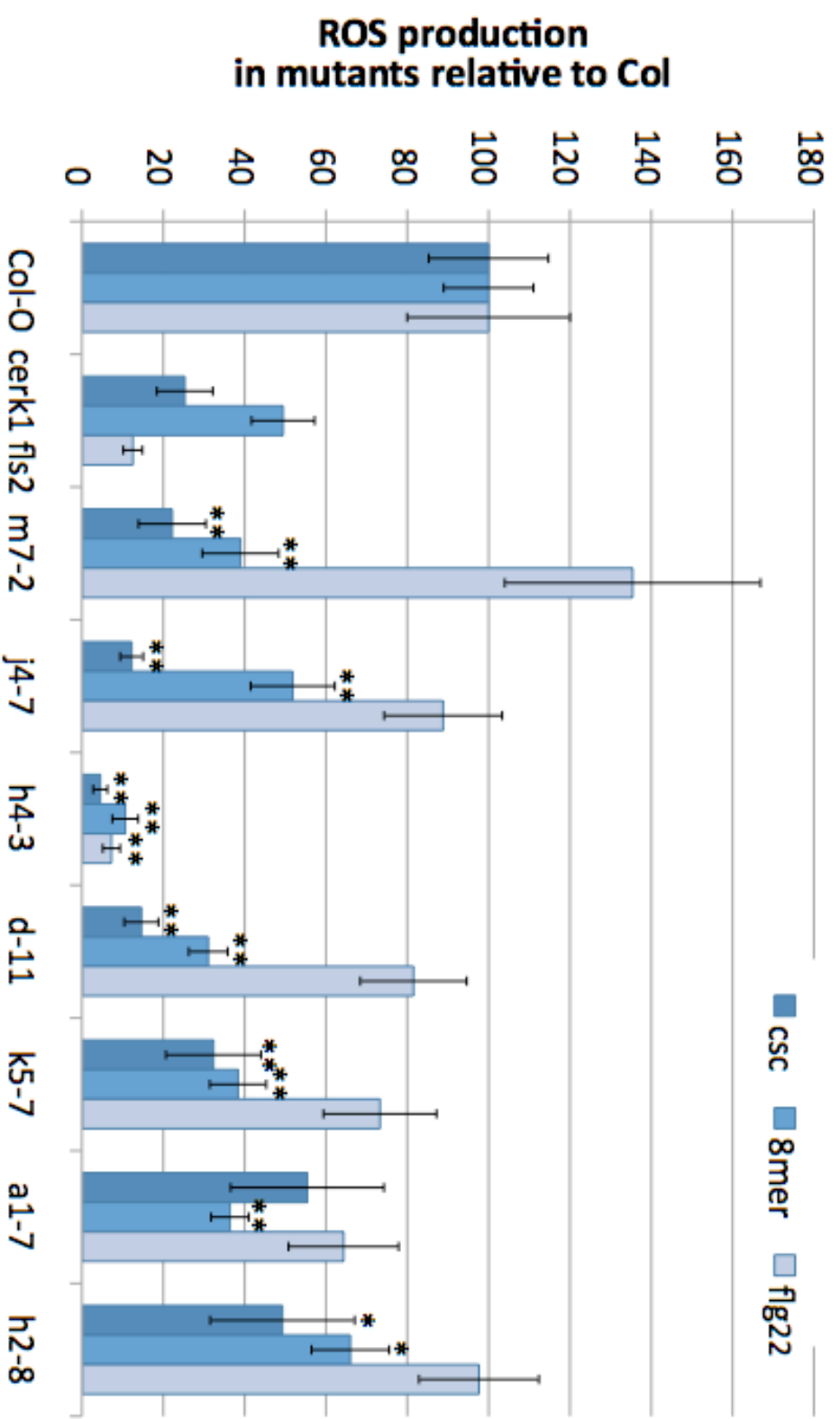


Figure I-3 Specificity of PAMP-induced ROS generation in the various EMS mutants

ROS production by selected EMS mutants is shown in response to treatment with different PAMPs. The data represent an average of ROS production from 12 10-day-old seedlings after application of chitin mixture (csc), chitooctaose (8mer), and fig22. Signal was recorded 20 seconds before and after the maximum signal observed upon treatment with chitin (csc or 8mer) or 2 mutant. These experiments were performed three times, and each replicate gave similar results. Bars represent standard errors.

Student T-test compared between mutants and the control Col-O wild-type in same treatment (*) P<0.05, (**) P<0.01.

Figure 1-4 WRKY53 expression in the EMS mutants

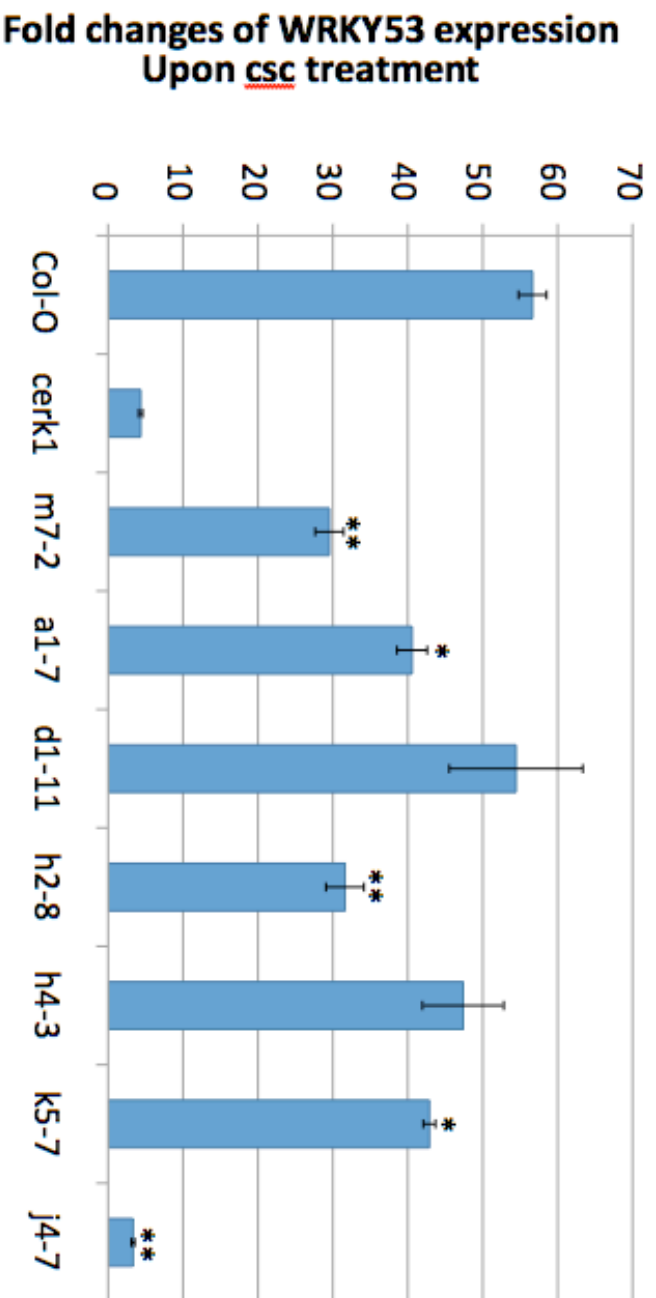


Figure I-4 WRKY53 expression in the EMS mutants

The expression of the *WRKY53* gene in selected EMS mutants is shown, as measured by qRT-PCR. 10-day-old seedlings were treated with the chitin mixture for 30 minutes. The data represent the average of three biological replicates. Bars represent standard errors. Student T-test compared between mutants and the control Col-O wild-type in same treatment (*) P<0.05, (**) P<0.01.

Figure I-5 Some EMS mutants were more susceptible to *P. syringae* pv. *tomato* DC3000

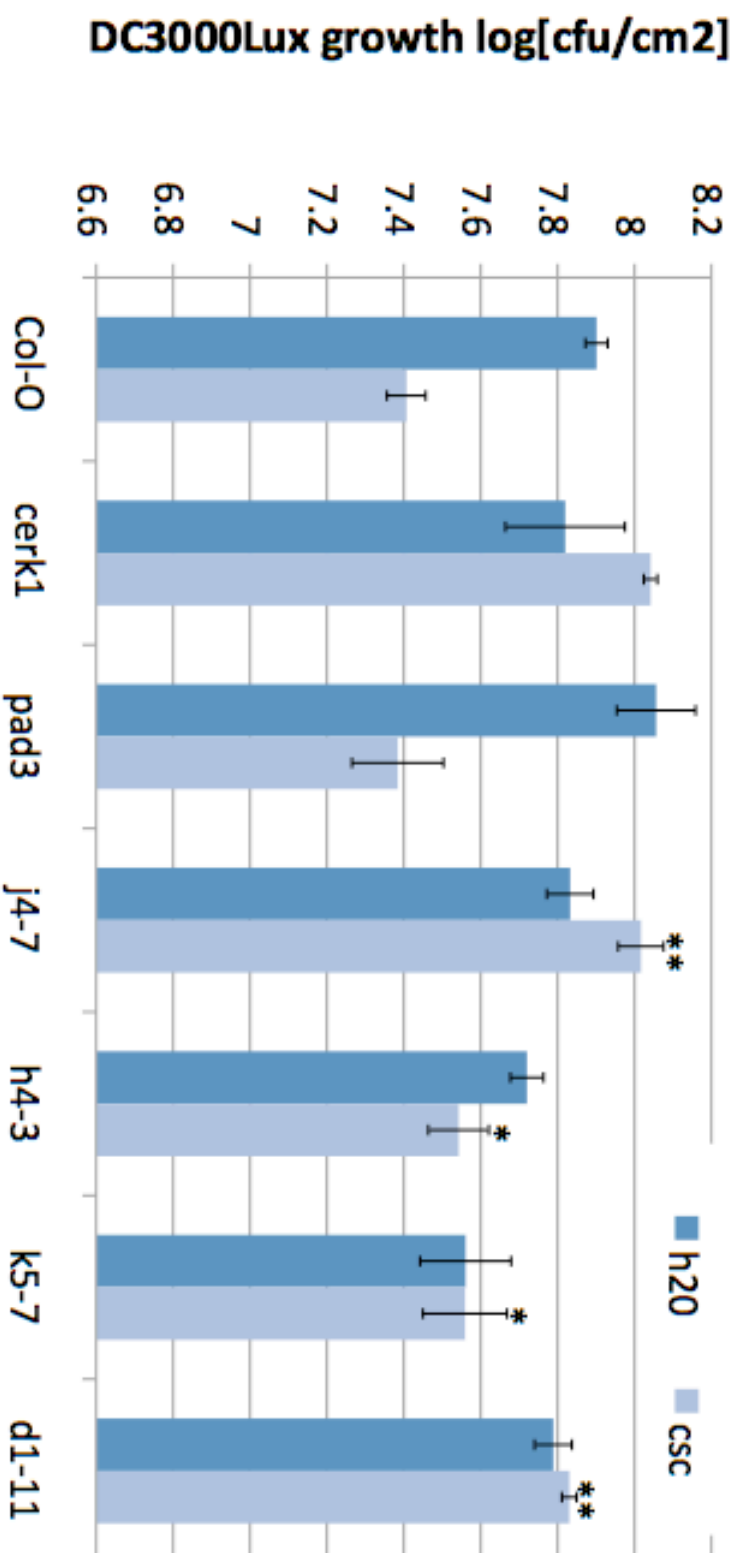


Figure I-5 Some EMS mutants were more susceptible to *P. syringae* pv. tomato DC3000

Growth of *P. syringae* pv. tomato DC3000 on leaves from 4-week-old seedlings. Plants were pre-treated with chitin mixture (csc) or water as control, twice (24 hours and 6 hours prior to pathogen inoculation). The data represent the average bacterial counts from punched leaf discs from each of four plants. These experiments were performed three times, and each replicate gave similar results. Bars represent standard errors. Student T-test compared between mutants and the control Col-O wild-type in same treatment (*) $P < 0.05$, (**) $P < 0.01$.

Table I-1 Genetic analysis of EMS mutants defective in the chitin response

Cross	(ROS) ^a	(No ROS) ^a	(χ^2) ^b
M7-2 x <i>cerk1-1</i>	0	24	30.86 (P<0.001) ^b
J4-7 x <i>cerk1-1</i>	0	24	30.86 (P<0.001) ^b
A1-7 x <i>cerk1-1</i>	15	9	0.38 (P>0.5) ^b
D1-11 x <i>cerk1-1</i>	15	9	0.38 (P>0.5) ^b
H4-3 x <i>cerk1-1</i>	13	19	0.126 (P>0.5) ^b

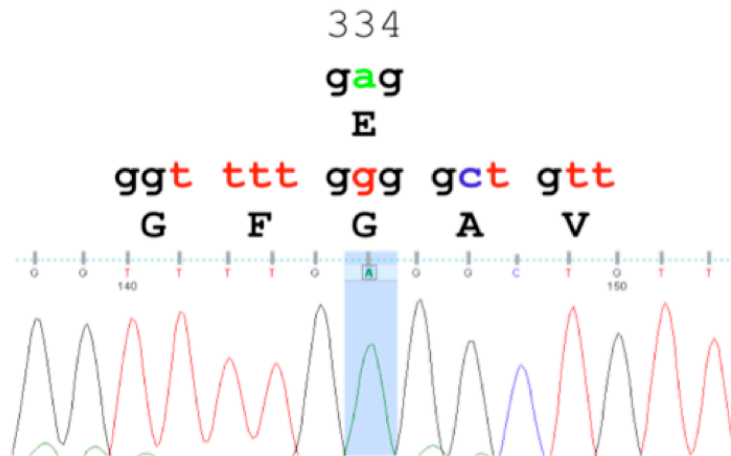
ROS assay on F2 segregating progeny from crosses between the various mutants and *cerk1-1*

^aEMS mutants were crossed to *Atcerk1-1*. Ten-day old seedlings derived from the F2 populations were tested for ROS production in response to chitin. The photon counting was measured 20 seconds before and after the maximum signal observed.

(χ^2)^b value for expected ratio of 9 ROS : 7 No ROS

Figure I-6 Sequencing of the mutation in the *CERK1* gene

(A) M7-2



(B) J4-7

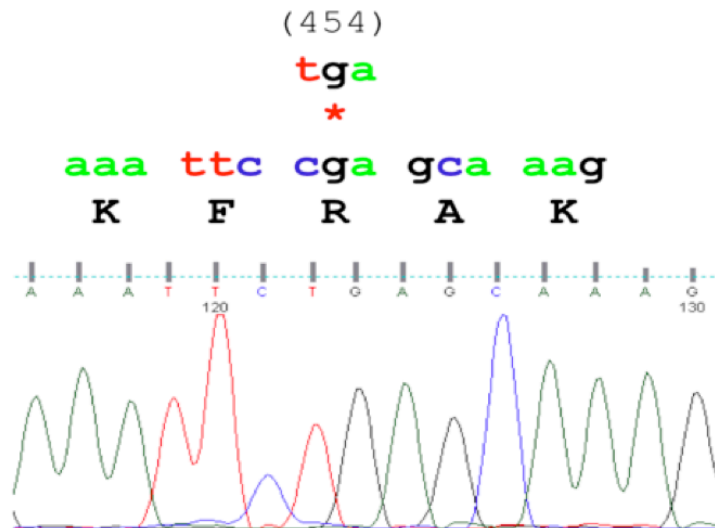
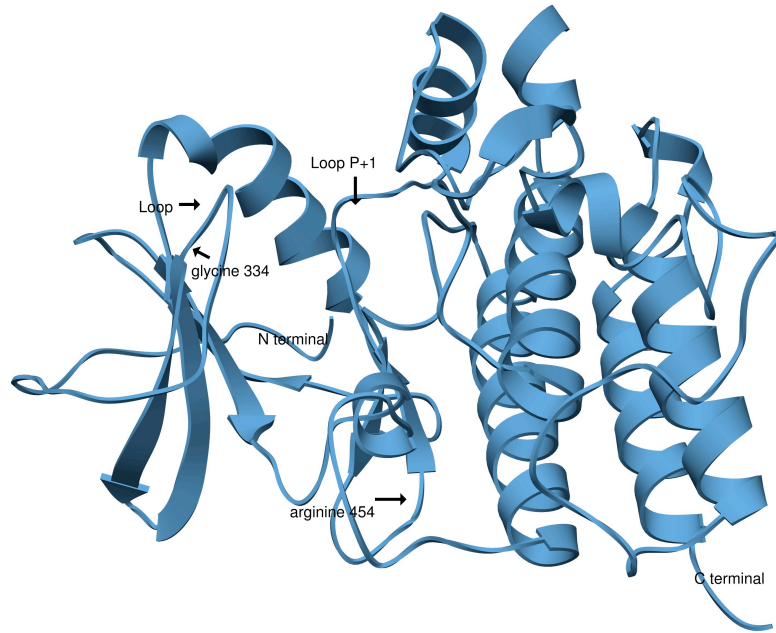


Figure I-6 Sequencing of the mutation in the *CERK1* gene

Sequencing of mutated codons (A: 334rd in M7-2 and B: 454 rd in J4-7) with the corresponding amino acid of the *AtCERK1* gene derived from the M7-2 (leucine) and J4-7 (stop codon) mutants, compared with relevant cDNA and protein sequences derived from the wild-type of M7-2 (glutamic acid) and J4-7 (arginine).

Figure I-7 Prediction of protein structures of *CERK1*-allelic M7-2

A



B

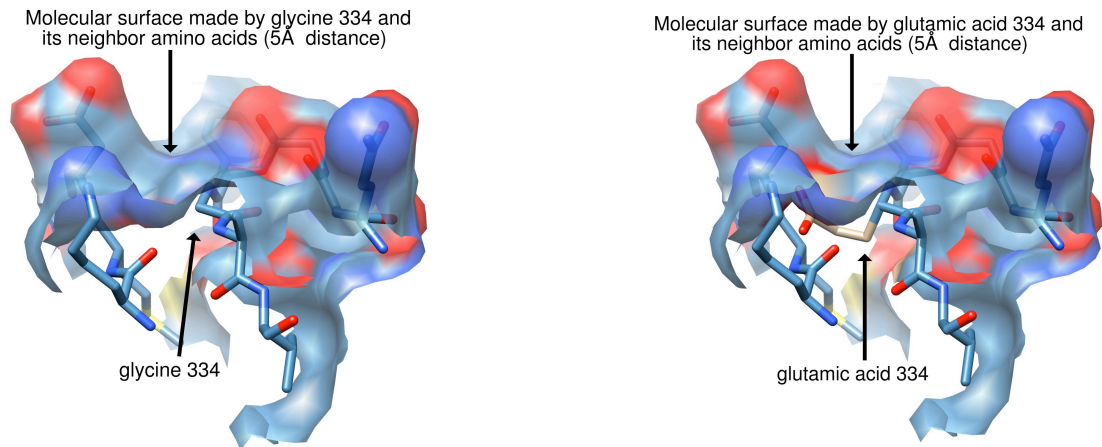


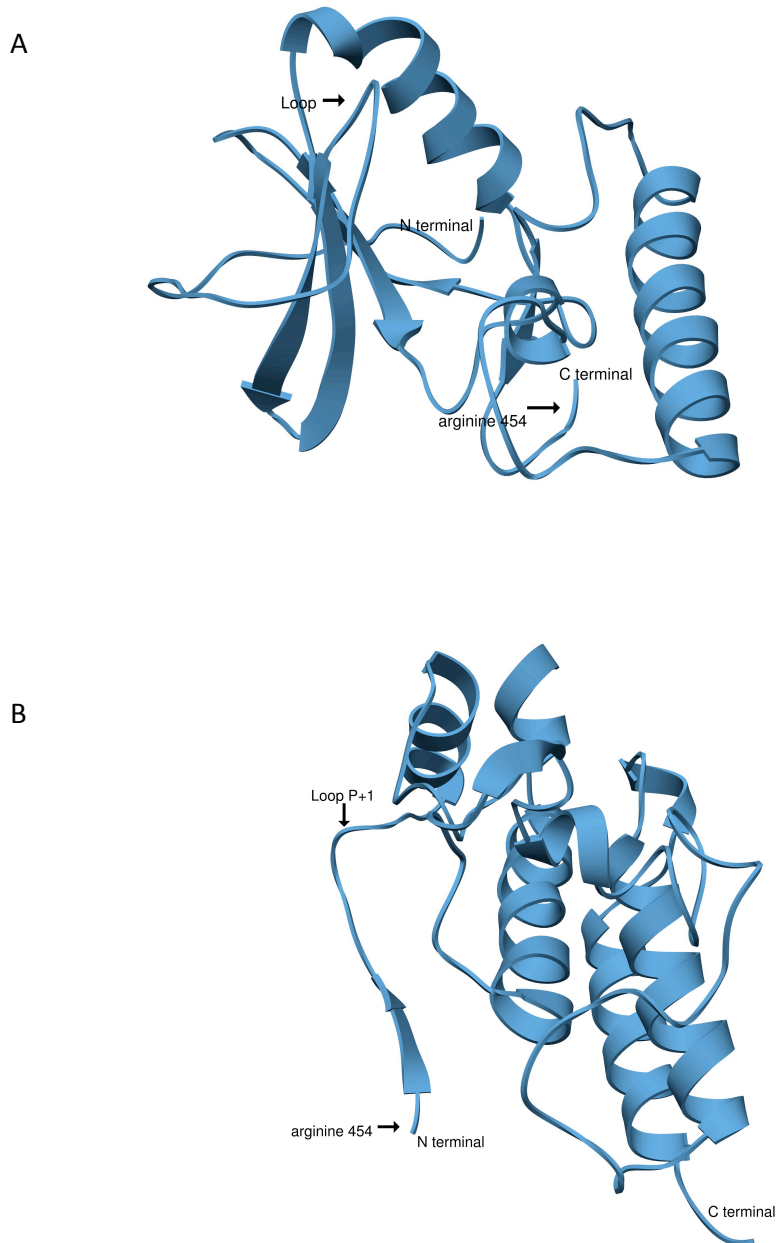
Figure I-7 Prediction of protein structures of CERK1-allelic M7-2

A) The best tertiary structure of CERK1 was selected among 500 predicted models based on quality assessment score (TM-Score) (95). Gly 334 and Agr 454 are the two mutation sites of the two *CERK1* mutant alleles.

B) The molecular surface created by glycine 334 and its adjacent amino acids (5 angstrom distance) (left panel) and the molecular surface created by glutamic acid 334 and its adjacent amino acids (5 angstrom distance) (right panel).

The figure was generated by Cuong The Nguyen, a Ph.D. student in Dr. Gary Stacey's lab.

Figure I-8 Predicted protein structure of CERK1 allelic J4-7



Normal kinase domain of CERK1 protein (A) was modified with a stop codon #454 (B).

The figure was generated by Cuong The Nguyen, a Ph.D. student in Dr. Gary Stacey's lab.

Table I-2 Rough mapping of the A1-7 mutation

Marker^(a)	Length (cM)	Chromosome	Recombinant Chromosome^(b)	Total number of chromosome	Recombinant frequency (%)
nga63	3.55	1	40	90	44.4
ciw12	9.62	1	32	88	36.4
SO392	10.86	1	33	90	36.7
ciw1	18.36	1	29	90	32.2
nga280	20.87	1	30	86	34.9
NF5I14	24.37	1	31	90	34.4
ATPase	28.53	1	34	90	37.8
nga1145	0.68	2	51	90	56.7
ciw3	6.4	2	36	86	41.9
nga1126	11.7	2	35	88	40
nga168	16.29	2	39	88	44.3
nga172	0.92	3	33	90	36.7
nga162	4.6	3	32	90	35.6
ciw11	9.77	3	35	88	39.8
ciw4	18.89	3	48	90	53.3

nga6	23	3	49	90	54.4
ciw5	0.73	4	46	88	52.3
nga8	5.63	4	39	88	44.3
ciw6	7.89	4	49	86	57.09
ciw7	11.5	4	45	90	50
nga1139	16.44	4	44	90	48.9
nga1107	18.09	4	42	90	46.7
nga225	1.51	5	46	90	51.1
nga151	4.67	5	52	84	61.9
nga139	8.42	5	44	90	48.9
nga76	11.1	5	36	88	40.9
PHYC3	14	5	38	84	45.2
ciw9	17	5	44	86	51.2
ciw10	24.5	5	42	80	52.5

Table I-2 Rough mapping of the A1-7 mutant

A1-7 was crossed with *Ler* and genomic DNA of about 40 to 45 F1 plants were extracted for PCR using specific primer pairs of each mapping marker (a). Information about the markers (a), including position, sequence, length and specific primers were obtained from the protocol of Ponce et al. (91). PCR results were analyzed to identify the number of recombinant chromosomes (b), in which for each band representing *Ler* indicated two chromosomes recombined, each pair of bands representing heterozygous indicated one chromosome recombined and bands representing Col-O showed no recombination event. The recombinat frequency was calculated by the percentage ratio between the number of recombinant chromosomes (b) and the total number of chromosomes (two chromosomes are involved in each PCR).

Table I-3 Mapping of A1-7 on Chromosome 1

Marker	Position (cM)	Recombinant Chromosome	Total number of chromosome	Recombinant frequency (%)
SO392	10.86	33	90	36.7
T27K12	15.9	28	88	31.8
ciw1	18.36	33	90	36.7
nga128	20.6	33	90	36.7
nga280	20.87	33	90	36.7
NF19K23	22.9	32	90	35.6
NF5I14	24.37	33	90	36.7
ATPase	28.53	34	90	37.8

Markers (a) information (position, sequence, length and specific primers) was obtained from the protocol by Ponce et al. (91). The number of recombinant chromosomes and recombinant frequency (%) were calculated using the same methods for rough mapping of the A1-7 mutant (Table I-3).

4. Discussion

Potential novel genes specifically involved in chitin signaling

Seven EMS mutants highly defective in chitin signaling were identified, each likely derived from an independent mutagenesis event. Notably, only one mutant was defective in both chitin and flg22 signaling pathway, while six mutants differed from wild-type in their response to chitin. Previous studies showed that chitin and flg22 signaling share many downstream components; including the MAPK cascade and an overlap of 441 genes whose expression is induced by either PAMP treatment (9, 34, 35, 79). Therefore, it would not be surprising to find mutants blocked in both chitin and flg22 response. However, only one of the seven mutants identified showed this phenotype, while the remainder appeared to be specific altered in their response to chitin. These six mutants are highly and specifically impaired in many chitin responses, including ROS production (early response) (Figure I.2, Figure I.3), chitin-responsive gene expression (late response) (Figure I.4) and chitin-induced resistance to bacteria (Figure I.5). Excluding the two *AtCERK1* alleles, the mutant screened identified four novel mutants that warrant further investigation.

The kinase domain of CERK1 is critical for the protein to function in signal transduction

Out of six mutants defective in chitin signaling, two were found to be allelic to *Atcerk1* (i.e., mutants M7-2 and J4-7). Given the methods used for mutant screening it

was not surprising to find alleles of *Atcerk1*, identification of these mutants give us added confidence that the remaining mutants will reveal important genes involved in chitin signaling. The known alleles of *Atcerk1* studied included a Ds-transposon mutant *Atcerk1-1* (28) and two T-DNA insertion mutants *Atcerk1-2/ LysM RLK1* insertion mutant 096409 (9, 28, 87) and *Atcerk1-3* (87). All three mutants have insertions within the 10th intron, leading to a total loss of *CERK1* expression (9, 28, 87). The two *Atcerk1* mutant alleles identified in this study allow for the expression of CERK1 protein, but with loss of function presumably due to a lack of kinase activity, either through missense mutation (M7-2) or premature termination (J4-7). All known *Atcerk1* mutant alleles exhibit the same phenotype with regard to impaired response to chitin elicitation. The predicted structure of CERK1 in the M7-2 mutant (i.e., *Atcerk1-4*), resulting from the replacement of G334 by E334, suggests the formation of a smaller molecular surface, likely because of side chain containing the E334 residue (Figure I-7). This change may directly disrupt kinase activity and/or affect the interaction with other downstream signaling components. The predicted model of the protein produced in the J4-7 (i.e., *Atcerk1-5*) mutant indicates the loss of a significant portion of the C-terminus of the kinase domain, likely leading to a loss of kinase function (Figure I-8). Miya et al. demonstrated that AtCERK1 kinase domain is a functional kinase, as determined by autophosphorylation activity and the ability to phosphorylate myelin basic protein (MBP) in vitro (28). Furthermore, the AtCERK1 kinase domain was shown to be the target of AvrPtoB by ubiquitination, resulting in degradation of the receptor, and suppression of PTI (87). My

data about the phenotype of the two *Atcerk1* alleles also support the critical role of the AtCERK1 kinase domain.

II. IDENTIFICATION OF PROTEINS INTERACTING WITH ARABIDOPSIS LYSM RLK1/CERK1 USING A YEAST TWO HYBRID SCREENING APPROACH

1. Introduction

To combat pathogens, plants rely on an innate immunity system comprised of PTI and pathogen-derived effector-triggered immunity (ETI) (1, 13, 15). In PTI, plants sense pathogens via perception of PAMPs through membrane-localized PRRs. However, sometimes PTI is not sufficient to combat all pathogens. Some pathogens can secrete fitness-promoting effector proteins into host cells that function as virulence factors and can suppress PTI, resulting in effector-triggered susceptibility (ETS) (4, 8, 16, 99). Consequently, plants have evolved disease resistance (R) proteins to monitor the presence of such effectors, resulting in ETI. Some pathogens acquire new effectors during their evolution that cannot be recognized by host R proteins, which again restore ETS. Plants may counter with the development of R proteins that recognize these new effectors, again triggering ETI. It is the back and forth of PTI and ETI, combating the adaptations of the pathogens, that controls the balance of plant disease resistance (3). Generally, ETI is longer in duration, higher in sensitivity and specificity, while PTI provides a broad-spectrum resistance to a variety of pathogens (13).

Chitin is a polymer of β -1,4 linked N-acetyl glucosamine and is an important component of the fungal cell wall. It is one of the best studied PAMPs and is capable of eliciting basal defense responses in plants against fungal pathogens. Recently, two

research groups independently demonstrated that the Arabidopsis LysM-containing receptor-like kinase 1 (LysM RLK1/CERK1) plays a critical role in chitin signaling (9, 28). AtCERK1 is a LysM receptor-like kinase comprised of an intracellular kinase domain, a transmembrane domain and three extracellular LysM domains (9, 28). Much research has focused on both the interaction between AtCERK1 and PAMPs, as well as on AtCERK1 and pathogen effectors. The critical role of AtCERK1 in chitin perception has been studied using a variety of molecular, genetic (9, 28), and biochemical methods (38, 81). Genetic studies showed that *Atcerk1* mutants are impaired in all chitin signaling responses, including ROS production, the activation of a MAPK cascade, and the expression of chitin-induced genes, eventually resulting in the failure of chitin-induced pathogen resistance (9, 28). Biochemical analysis confirmed direct binding between AtCERK1 and chitin (81) (82). Moreover, very recently, the X-ray crystal structure of the extracellular, LysM domain of AtCERK1 (AtCERK1-ECD) was elucidated (36). The structure predicts that a single AtCERK1 monomer can bind chitotetraose, but such binding results in little or no induction of PTI. Binding of chitooctaose resulted in homodimerization of AtCERK1-ECD. Since chitooctaose treatment results in a strong PTI response (37), the authors suggested that AtCERK1 homodimerization may be essential for signal transduction (36).

Other proteins are also known to impact chitin signaling by direct interaction with AtCERK1. For example, AvrPtoB, a type III secretion system effector from *P. syringae* interacts with kinase domain of AtCERK1, targeting the domain for ubiquitination by

AvrPtoB's E3 ligation activity (87). This leads to degradation of CERK1 protein, suppressing PTI and promoting susceptibility to the pathogen. AtCERK1 also appears to function in conjunction with a variety of putative co-receptors or co-adaptors. For example, AtCERK1 kinase was found to interact with BIK1 (46). Interestingly, BIK1 was reported to be the first kinase in the flg22 signaling pathway, immediately downstream of the FLS2/BAK1 receptor complex that perceives flg22 (46, 48). BIK1 is required for the phosphorylation activity of the FLS2/BAK1 receptor complex, as well as for flg22-triggered immunity (46, 48). Although *bik1* mutants show a reduction in some chitin responses, including ROS production and callose deposition (46), it is not clear if the interaction between BIK1 and AtCERK1 is required for chitin signaling or, especially, for chitin-triggered immunity.

In order to identify other protein components that directly interact with At CERK1 and, therefore, may be important for chitin signaling, I performed a yeast two-hybrid screening using the AtCERK1 intracellular kinase domain as the 'bait'. This screen identified fifty-four putative AtCERK1-interactors. Annotations of these putative interactors identified them as both transmembrane and intracellular proteins, including various endoplasmic reticulum proteins, transcription factors, pathogen-related genes, and kinases. Further investigation of 43 (out of the 54) putative interactors was performed based on the phenotypes of the corresponding T-DNA insertion mutants. This additional screening identified 16 mutants that were clearly affected in their production of ROS upon chitin elicitation. Finally, screening of these mutant lines

identified two mutants; one of which showed significantly greater susceptibility to pathogen infection and the other showed greater disease resistance.

2. Materials and methods

Table II-1 Bacterial/ yeast strains and plasmids used in the Y2H screening

Strains or Plasmids	Relevant Genotype or Characteristic	Source or Reference
Eschericia coli		
DH5a	supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Bethesda Research labs
KC8	leuB600, trpC9830, pyr::Tn5, hisB463, lacDeltaX74	OriGene Technologies, Inc.
Yeast		
EGY194	MATa trp1 his3 ura3 leu2::4 LexAop-LEU2	OriGene Technologies, Inc.
Plasmid		
Reporter gene (LacZ) plasmids		
pSH18-34	URA3, 2m, Amp ^r , 8 ops.- LacZ (high sensitivity)	OriGene Technologies, Inc.
pJK103	URA3, 2m, Amp ^r , 2 ops.- LacZ (medium sensitivity)	OriGene Technologies, Inc.
pRB1840	URA3, 2m, Amp ^r , 2 ops.- LacZ (low sensitivity)	OriGene Technologies, Inc.
pJK101	URA3, 2m, Amp ^r , Amp ^r , GAL 1-2 ops.-LacZ (repression assay)	

Bait plasmids		
pEG202	HIS3, 2m, Amp ^r , (constitutive ADH promoter expresses LexA and is followed by a polylinker for making the bait fusion protein)	OriGene Technologies, Inc.
pEG202-NLS	HIS3, 2m, Amp ^r , (similar to p EG202 but with SV40 nuclear localization sequence between LexA and polylinker)	OriGene Technologies, Inc.
pNLexA	HIS3, 2m, Amp ^r , (similar to p EG202 except that the LexA sequence is 3' rather than 5' of the polylinker)	OriGene Technologies, Inc.
Prey plasmid		
pJG4-5	TRP1, 2m, Amp ^r , (inducible GAL1 promoter expresses B42-HA tag and is followed by a polylinker for making target fusion protein expression libraries from cDNA)	OriGene Technologies, Inc.
Control plasmids		
pRHF1	His3, 2m, Amp ^r , (ADH promoter expresses LexA- Bicoid homeodomain)	OriGene Technologies, Inc.

	fusion; used as a positive control in the repression assay and a negative control in the DupLEX-A screen)	
pSH17-4	HIS3, 2m, Amp ^r , (ADH promoter expresses LexA-GAL4 activation domain, used as a positive control in the DupLEX-A screen)	OriGene Technologies, Inc.
pEG202-Max	Expresses LexA-Max fusion constitutively; used as a negative control when testing isolated target proteins or as a positive control in the repression assay	OriGene Technologies, Inc.
pBait	Constitutively expresses a LexA-bait fusion protein that interacts with the fusion protein from pTarget; can also be used as a negative control when testing isolated target proteins or as a positive control in the repression assay)	OriGene Technologies, Inc.
pTarget	Expresses (galactose-	OriGene Technologies, Inc.

dependently) a B42-target
fusion protein that
interacts with the fusion
protein from pBait

Table II-2 Primers used in the Y2H screening

Primers	Sequences	Purpose
5' target fusion primer	CTG AGT GGA GAT GCC TCC	Determine the reading frame and identity of positive clones
3' target fusion primer	GCC GAC AAC CTT GAT TG	Determine the identity of positive clones
K1KaEcoRI	ACGAATTCATGAAGTCGAAGGGTGATTCGTTT	Amplify CERK1 construct as the bait
K1KaBamHI	ACGGATCCCCTACCGGCCGGACATAAGACT	Amplify CERK1 construct as the bait

Molecular cloning of plasmids

The coding region of a construct (K1), containing the transmembrane and kinase domain of CERK1, was amplified from cDNA of Arabidopsis Col-0 and cloned into pGEM-T easy using T-A cloning, as directed by the manufacturer's protocol (Promega corporation, Madison, WI, USA). This construct was then digested with BamHI and EcoRI, and the resulting fragment was cloned into the pEG202 bait vector. The primers used for verification of the cloning are indicated in Table II-2.

Yeast two-hybrid screening

The bait vector was transformed into yeast EGY194 strains harboring each of four different reporter plasmids (as shown in Table II-1) to test for bait self-activation and nuclear localization following the DupLEX-A yeast transformation protocol (OriGene Technologies, Inc., MD, USA). The prey library was created from 10-day-old seedlings pretreated with 100ng/ml chitin for 30 minutes under the construction of the DupLEX-A yeast-two hybrid system pJG4-5 vector (OriGene Technologies, Inc., MD, USA) (Shuqun Zhang lab, University of Missouri, MO, USA). The prey library was transformed into the yeast strain EGY194 harboring the bait vector and the reporter plasmid pJK103 following the DupLEX-A yeast two-hybrid system protocol (OriGene Technologies, Inc., MD, USA). The transformation mixture was plated on YNB medium with galactose, but lacking uracil (the reporter gene plasmid marker), histidine (the bait plasmid marker) and threonine (the library plasmid marker). The plates were incubated at 30°C and examined

after 4 to 5 days. Colonies growing on the medium were purified by streaking onto YNB medium lacking selection nutrients (as indicated as selection vector in Table II-1).

Plasmids from positive colonies were isolated from yeast using a Zymoprep II-yeast plasmid miniprep kit (Zymo Research, CA, USA). The purified plasmids were subsequently heat-shock transformed into *E. coli* strain KC8, which was grown on M9 glucose medium lacking tryptophan to eliminate the bait vector and to selectively recover each cDNA-containing plasmid. Each positive clone was then re-transformed into a yeast strain EGY194 harboring the bait and reporter gene plasmid (pJK103) and again screened on selective medium to confirm interactions. Confirmed prey plasmids were then sequenced to characterize the positive clones.

Plant germination and growth conditions

As described in Materials and Methods of chapter I.

PAMP treatments

As described in Materials and Methods of chapter I.

Reactive oxygen species assay

As described in Materials and Methods of chapter I.

Pathogen assay

As described in Materials and Methods of chapter I. Ten day-old seedlings were treated with purified chitin (spe) that was produced using the procedure described in the Materials and Methods of Chapter I. *P. syringae* DC3000 lux DCABE (100) was used in the pathogen assay. Bacterial growth was quantified using a Photech PSU1 camera to detect luminescence 24 hours after inoculation.

LacZ filter assay

Yeast colonies were transferred onto No # 30 Whatman paper filters (Fisher Scientific) and fixed by freezing in liquid nitrogen. Filters were then soaked in Z buffer containing 2% (v/v) X gal solution (Sigma, St. Louis, MO), 0.8% 2-mercaptoethanol (Fisher Scientific) and incubated at 30°C and checked for signal every 20 minutes.

3. Results

The bait with K1 is compatible with the LexA-based yeast two-hybrid screening system

To search for candidate AtCERK1 interactors, a LexA-based yeast two-hybrid (Y2H) screen was performed. Because LexA-based Y2H screening is most appropriate for cytosolic proteins, the kinase domain of AtCERK1 (K1) was used as bait to screen an Arabidopsis chitin-treated 10-day old seedling cDNA library.

The bait was first tested in two assays: An autoactivation assay and a nuclear localization assay. K1 plasmids (HIS3, AmpR) and reporter gene plasmids (URA3, AmpR) were co-transformed into the EGY48 yeast strain. The yeast did not grow on leucine minus medium and did not turn blue in the X gal filter assay (Figure II-1 A). Thus, the K1 bait alone did not activate the reporter genes LEU2 (encoding β -isopropylmalate dehydrogenase involved in leucine synthesis) or LacZ. The ability of the K1 bait to enter the nucleus and bind LexA proteins was then tested. To this end, the K1 plasmids were co-transformed with pJK101 plasmid (URA, AmpR), a construct that contains a LacZ reporter gene driven by the yeast GAL1 promoter. The colonies did not turn blue on galactose SD-(Ura/His)-(Figure II-1 B), indicating that K1 does indeed enter the nucleus and bind to the LexA operators located between the GAL1 promoter and the LacZ gene, thereby repressing LacZ expression. Therefore, the bait is suitable for Y2H screening.

Lex A-based Y2H screening revealing 54 interactors with AtCERK1

A yeast EGY48 strain containing the K1 bait plasmid and reporter gene plasmid was transformed with 100µg cDNA target plasmid (galactose-dependent expressed, TRP1, AmpR). About 600 out of 4.5×10^6 transformants grew on the galactose-SD-(Ura/Trp/His/Leu)-medium. Fifty-four colonies turned blue on X-gal galactose-SD-(Ura/Trp/His)-but not on X-gal galactose-SD-(Ura/Trp/His)-and did not grow on glucose-SD-(Ura/Trp/His/Leu)-medium (Figure II-2). These results indicate that K1 interacts with the targeting proteins due to the prediction that K1 and the target proteins together bound and activated the LEU2 and LacZ reporter genes. Furthermore, the interactions were specific because they required galactose, on which expression of the target protein depends.

The yeast plasmids isolated from these 54 positive clones were transformed into *E. coli* strain KC8 grown on M9 glucose medium lacking tryptophan to eliminate the bait vector and to recover the cDNA target plasmids. To confirm the interactions, the cDNA target plasmids were individually co-transformed with the bait, and the resultant transformants were grown and turned blue as in the previous screening procedures. In addition, the cDNA target plasmids were also individually transformed into EGY48 to determine if they were false positive interactors (i.e., if they interacted with proteins other than the bait). The EGY48 yeast strains containing the cDNA target plasmid did not grow on galactose SD-(Ura/Trp/Leu)-medium, indicating that the cDNA clones alone

could not activate the reporter genes. Thus, these cDNA clones are positive interactors with AtCERK1.

Table II-3 shows the 54 interactors with the number of times each protein was recovered in the screening. Four of the interactors were recovered twice and, one was recovered three times. Notably, one interacting class, represented by six different clones pulled out from the screening, was found to interact with AtCERK1. Based on the annotations of the putative interactor genes, they were classified into nuclear, transmembrane, cytoplasmic, endoplasmic reticulum and organellar proteins, representing 4%, 4%, 9%, 13% and 23%, respectively, of the total.

Further screening for proteins involved in chitin signaling from the 54 putative interactors

To determine if the interactors are required for chitin signaling, the phenotypes of the corresponding mutants of each gene were assessed for their response to chitin.

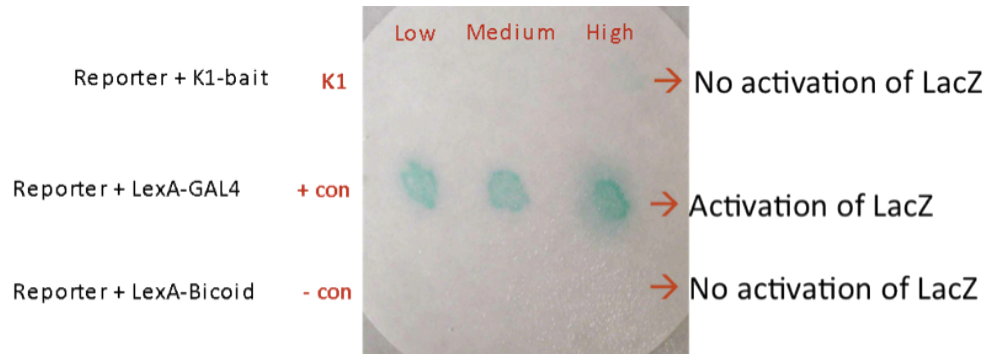
One hundred twenty-three T-DNA insertion lines of these 54 genes were obtained. Of the 110 lines genotyped, 81 homozygous lines were generated for 43 genes. After repeated attempts, homozygous lines could not be recovered for the remaining 11 genes. It is likely that these mutations are lethal when in a homozygous state. The 81 homozygous lines recovered were screened for the ability to produce ROS upon chitin addition.

The results of this screen showed that mutations in 16 of the 43 genes analyzed showed a significant variation in ROS production upon chitin elicitation when compared to wild-type plants (Figure II-3). Among these mutants, seven showed a reduced chitin response; whereas the other nine mutants showed stronger ROS production compared to wild-type. One mutant gene (At5G47210) produced very large amounts of ROS when challenged by chitin.

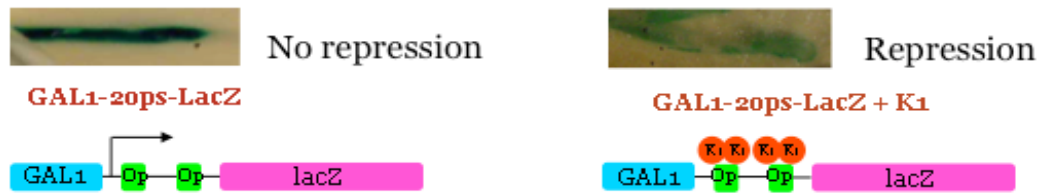
The purpose of my study was to identify AtCERK1 interactors that play a critical role in PTI. Therefore, I tested each of the mutants for their susceptibility to the pathogen *P. syringae* DC3000 lux DCABE (100), as shown in Figure II-4. This bacterial strain constitutively expresses luciferase and, therefore, population levels can be indirectly estimated by the level of light produced. As shown in Figure II-4, only two of the 16 mutants tested had a statistically significant difference in pathogen response when compared to the wild-type. One mutant line, in gene AT3G10190, was significantly more susceptible to the pathogen, while the other mutant (in gene AT3G14840) was significantly more resistant to the pathogen.

Figure II-1 Standardization of the bait to prior to Y2H screening

(A) The bait construct showed no self-activation with different reporter genes



(B) The bait can enter the nucleus and bind to operons upstream lacZ

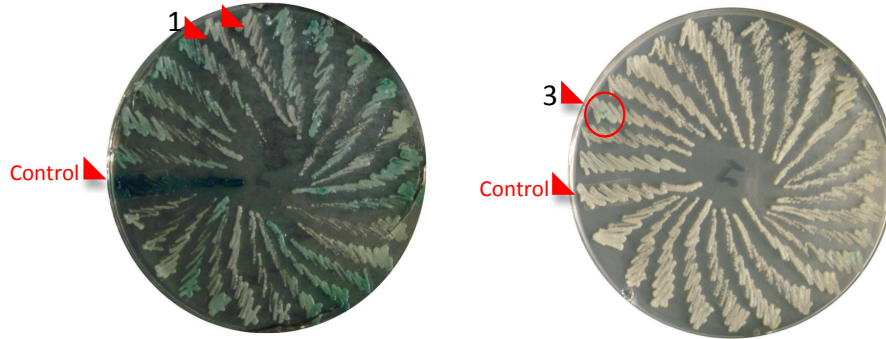


(A) The bait vector and various reporter genes (low, medium and high sensitivity as indicated in Materials and methods) were co-transformed into EGY198 and dropped onto selection medium (UH)⁻. After one week of growth, the lacZ filter assay was performed. This image was collected 30 minutes after the assay was initiated.

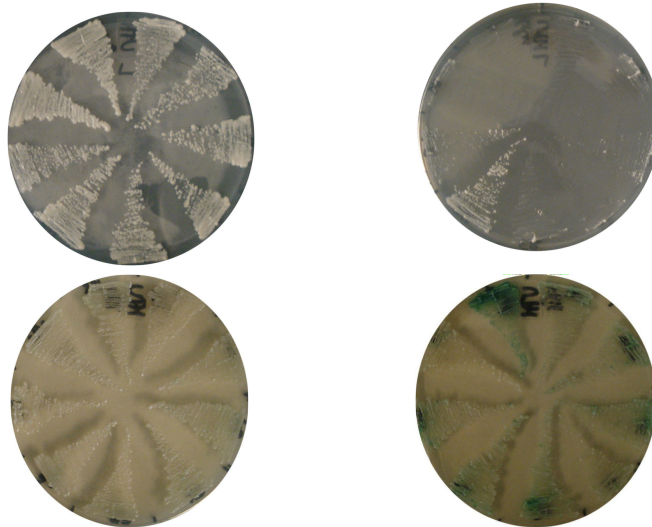
(B) The baits were co-transformed with pJK101 (the construction as described in the figure). The transformed yeast was dropped onto selection medium (UH)⁻ and grown for one week. The strain was then streaked onto Gal-Xgal-(UH)⁻ medium to detect repression of lacZ expression. The images were collected one week after streaking.

Figure II-2 Procedure of Y2H screening for proteins interacting with CERK1

(A) Testing interaction with the galactose-dependent LacZ reporter gene



(B) Testing individual interactors with K1



(C) Testing self-activation of putative interactors



Figure II-2 Procedure of Y2H screening for proteins interacting with CERK1

(A) Testing interaction with the galactose-dependent LacZ reporter gene

Colonies growing on selection medium Gal(UTHL)⁻ were streaked onto selection medium Gal-Xgal-(UTH)⁻ (left panel) and Glc-Xgal (UTH)⁻ (right panel) to test for the ability to activate galactose-dependent LacZ expression. Images were acquired one week after colony streaking. Eg. (1) and (2) did not turn blue on the Gal-Xgal-(UTH)⁻ medium, indicating that there is no protein interacting with K1 to activate LacZ expression. In addition, (3) is a false positive clone because the colony turned blue on Xgal medium regardless of carbon source (the LacZ gene is under a Gal promoter)

(B) Testing individual interactors with K1

Individual plasmids were re-transformed into the EGY48 strain containing the bait and reporter genes. Colonies growing on Gal(UTHL)⁻ were streaked onto selection medium (From left to right, top to bottom Gal(UTHL)⁻, Glc(UTHL)⁻, Gal-Xgal-(UTH)⁻ and Glc-Xgal-(UTH)⁻).

(C) Testing self-activation of putative interactors

Confirmed putative interactor plasmids were transformed into EGY48 containing reporter genes. Colonies growing on (UT)⁻ medium were streaked onto Gal(UTL)⁻ medium. The images were acquired one week after plating.

Table II-3 List of 54 proteins interacting with the AtCERK1 kinase domain as identified by LexA-Y2H screening

AGI number	Annotation (*)	Predicted location(*)	Number of clones	# of homozygous T-DNA insertion mutant lines
<u>AT1G07890</u>	Encodes a cytosolic ascorbate peroxidase APX1. Ascorbate peroxidases are enzymes that scavenge hydrogen peroxide in plant cells. Expression of the gene is down regulated in the presence of paraquat, an inducer of photooxidative stress.	cytosol	2	2
<u>AT1G08830</u>	Encodes a cytosolic copper/zinc superoxide dismutase CSD1 that can detoxify superoxide radicals. Its expression is affected by miR398-directed mRNA cleavage.	cytoplasm		2
<u>AT1G13930</u>	Involved in response to salt stress. Knockout mutants are hypersensitive to salt stress.	chloroplast	2	1

<u>AT1G14345</u>	Oxidoreductase; similar to hypothetical protein (<i>Vitis vinifera</i>) (GB:CAN74870.1); contains InterPro domain Aldo/keto reductase; (InterPro:IPR001395)	chloroplast thylakoid membrane	1	1
	encodes a cytosolic thioredoxin that reduces disulfide bridges of target proteins by the reversible formation of a disulfide bridge between two neighboring Cys residues present in the active site. Thioredoxins have been found to regulate a variety of biological reactions in prokaryotic and eukaryotic cells.			
<u>AT1G19730</u>		cytosol	1	3
<u>AT1G20020</u>	NADPH dehydrogenase	cytosol	1	1
	translation initiation factor/ FBR12, FUMONISIN B1-RESISTANT12, T24P13.1, T24P13_1	n/a	1	1
<u>AT1G31812</u>	Acyl coA binding protein	n/a	1	1
<u>AT1G42970</u>	Glyceraldehyde-3-phosphate dehydrogenase B subunit (GAPB)	n/a	1	1
<u>AT1G45230</u>	Defective chloroplasts and leaves protein-related / DCL protein-related	n/a	1	3
<u>AT1G50640</u>	A member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ATERF-3). The protein contains one AP2 domain.	n/a	1	1

AT1G52400	Beta-glucosidase		1	2
	Dormancy/auxin associated family protein; similar to dormancy/auxin associated protein-related (<i>Arabidopsis thaliana</i>) (TAIR:AT1G54070.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO17488.1); contains InterPro domain Dormancy auxin associated (InterPro:IPR008406)		1	3
AT1G56220		n/a	1	3
AT1G60850	DNA-dependent RNA polymerase	n/a	1	1
AT1G60950	Encodes a major leaf ferredoxin	chloroplast	1	1
	Mitochondrial transcription termination factor-related / mTERF-related; similar to mitochondrial transcription termination factor family protein / mTERF family protein (<i>Arabidopsis thaliana</i>) (TAIR:AT1G62085.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO40592.1); contains InterPro domain Mitochondrial transcription termination factor-related (InterPro:IPR003690)	mitochondrion	1	1
AT1G62120			1	1
	Epidermis-specific, encodes KCS10, a putative 3-ketoacyl-CoA synthase. probably involved in the synthesis of long-chain lipids found in the cuticle.	ER	1	1
AT2G26250			1	1
AT2G26280	ATP binding damaged DNA binding	n/a	1	1
AT2G28190	Encodes a chloroplastic copper/zinc superoxide dismutase CSD2 that can	chloroplast	1	1

	detoxify superoxide radicals. Its expression is affected by miR398-directed mRNA cleavage.			
<u>AT2G39840</u>	Encodes the catalytic subunit of a Type 1 phosphoprotein Ser/Thr phosphatase, expressed in roots, shoots and flowers.	system I phosphatase complex	1	1
	Microsomal signal peptidase 25 kDa subunit, putative (SPC25); Identical to Probable signal peptidase complex subunit 2 (<i>Arabidopsis thaliana</i>) (GB:P58684); similar to signal peptidase (<i>Arabidopsis thaliana</i>) (TAIR:AT4G04200.1); similar to unknown (<i>Populus trichocarpa</i>) (GB:ABK95467.1); contains InterPro domain Microsomal signal peptidase 25 kDa subunit; (InterPro:IPR009582)	ER	3	1
<u>AT2G41110</u>	A touch-inducible calmodulin that has higher affinity to kinesin-like calmodulin binding motor protein than CAM4 or CAM6	cytosol	1	1
<u>AT2G42540</u>	A cold-regulated gene whose product is targeted to the chloroplast and constitutive expression increases freezing tolerance in protoplasts in vitro and chloroplasts in vivo. NMR and x-ray diffraction studies suggest that COR15a alters the intrinsic curvature of the inner membrane of chloroplast envelope	chloroplast	1	2

	<p>IQD14; calmodulin binding; Identical to Protein IQ-DOMAIN 14 (IQD14) (<i>Arabidopsis thaliana</i>) (GB:Q8LPG9;GB:O22835); similar to IQD13 (IQ-domain 13), calmodulin binding (<i>Arabidopsis thaliana</i>) (TAIR:AT3G59690.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO46285.1); contains InterPro domain IQ calmodulin-binding region (InterPro:IPR000048)</p>	n/a	1	0
<p><u>AT2G43680</u></p>	<p>SPL3. Encodes a member of the SPL (squamosa-promoter binding protein-like) gene family, a novel gene family encoding DNA binding proteins and putative transcription factors. Contains the SBP-box, which encodes the SBP-domain, required and sufficient for interaction with DNA. It binds DNA, may directly regulate AP1, and is involved in regulation of flowering and vegetative phase change. Its temporal expression is regulated by the microRNA miR156. The target site for the microRNA is in the 3'UTR.</p>	nucleus	6	3
<p><u>AT2G33810</u></p>	<p>Predicted to encode a PR (pathogenesis-related) protein.</p>	n/a	1	0
<p><u>AT3G08770</u></p>	<p>Calmodulin, putative; similar to calmodulin, putative (<i>Arabidopsis thaliana</i>) (TAIR:AT2G41410.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO23336.1); contains InterPro domain EF-Hand type; (InterPro:IPR011992); contains InterPro domain Calcium-binding EF-hand; (InterPro:IPR002048)</p>	n/a	1	1
<p><u>AT3G10190</u></p>				

	<p>Protein kinase family protein; similar to protein kinase family protein (<i>Arabidopsis thaliana</i>) (TAIR:AT1G55200.1); similar to protein kinase family protein (<i>Arabidopsis thaliana</i>) (TAIR:AT5G56790.1); similar to hypothetical protein (<i>Vitis vinifera</i>) (GB:CAN76926.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO45720.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO65379.1); contains InterPro domain Protein kinase, core; (InterPro:IPR000719); contains InterPro domain Protein kinase-like (InterPro:IPR011009)</p>	n/a	1	1
<u>AT3G13690</u>	Ubiquitin-dependent peptidase		1	0
<u>AT3G14840</u>	<p>Leucine-rich repeat family protein / protein kinase family protein; similar to leucine-rich repeat family protein / protein kinase family protein (<i>Arabidopsis thaliana</i>) (TAIR:AT1G53440.1); similar to leucine-rich repeat transmembrane protein kinase, Putative (<i>Arabidopsis thaliana</i>) (TAIR:AT1G07650.1); similar to serine/threonine protein kinase-related (<i>Arabidopsis thaliana</i>) (TAIR:AT1G53420.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO39466.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO39457.1); similar to unnamed protein product (<i>Vitis vinifera</i>) (GB:CAO39469.1); contains InterPro domain</p>	plasma membrane	2	4

	<p>Serine/threonine protein kinase; (InterPro:IPR002290); contains InterPro domain Protein kinase, core; (InterPro:IPR000719); contains InterPro domain Protein kinase-like (InterPro:IPR011009); contains InterPro domain Serine/threonine protein kinase, active site; (InterPro:IPR008271); contains InterPro domain Leucine-rich repeat; (InterPro:IPR001611); contains InterPro domain Tyrosine protein kinase; (InterPro:IPR001245)</p>			
<u>AT3G26840</u>	<p>Esterase/lipase/thioesterase family protein</p>		1	4
<u>AT3G49470</u>	<p>NACA2 (NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX SUBUNIT ALPHA-LIKE PROTEIN 2); Identical to Nascent polypeptide-associated complex subunit alpha-like protein 2 (<i>Arabidopsis thaliana</i>) (GB:Q94IX9;GB:Q9S7G0); similar to nascent polypeptide associated complex alpha chain protein, putative / alpha-NAC, putative (<i>Arabidopsis thaliana</i>) (TAIR:AT4G10480.1); similar to nascent polypeptide associated complex alpha chain protein, putative / alpha-NAC, putative (<i>Arabidopsis thaliana</i>) (TAIR:AT4G10480.2); similar to unknown (Populus trichocarpa) (GB:ABK93574.1); contains InterPro domain Nascent polypeptide-associated complex NAC (InterPro:IPR002715); contains InterPro domain Nascent polypeptide-associated complex, alpha subunit (InterPro:IPR016641); contains InterPro</p>	n/a	1	3

	domain Ubiquitin-associated/translation elongation factor EF1B, N-terminal (InterPro:IPR000449)			
<u>AT3G51895</u>	Sulfate transmembrane transporter		1	3
<u>AT3G54890</u>	Chlorophyll binding protein	n/a	1	0
	Similar to unknown protein (<i>Arabidopsis thaliana</i>) (TAIR:AT1G33450.1); similar to RNA polymerase II complex component SRB7 protein-like (<i>Oryza sativa</i> (japonica cultivar-group)) (GB:BAD03057.1); contains domain FAMILY NOT NAMED (PTHR13381); contains domain gb def: Hypothetical protein C24H11.9 (PTHR13381:SF2)			
<u>AT4G04780</u>		n/a	1	2
	Member of the plasma membrane intrinsic protein PIP. functions as aquaporin. Salt-stress-inducible MIP	plasmamembrane	1	1
<u>AT4G35100</u>			1	1
<u>AT4G37450</u>	Arabinogalactan protein	n/a	1	2
	Mitochondrial glycoprotein family protein / MAM33 family protein; similar to mitochondrial glycoprotein family protein	mitochondria	1	
<u>AT5G02050</u>			1	
	Polyubiquitin 3 (UBQ3). UBQ3 encodes different number of ubiquitins in different ecotypes. UBQ3 transcript level is modulated by UV-B and light/dark treatments.	cytoplasm	1	3
<u>AT5G03240</u>			1	3

<u>AT5G04750</u>	F1F0-ATPase inhibitor protein, putative	n/a	1	0
<u>AT5G07530</u>	Glycine rich lipid binding protein	n/a	1	0
<u>AT5G11720</u>	alpha glucosidase	n/a	1	4
<u>AT5G12960</u>	Putative glycosyl hydrolase of unknown function (DUF1680)	Endo membrane system	1	1
<u>AT5G15140</u>	Aldose 1-epimerase protein	Endo membrane system	1	2
<u>AT5G15960</u>	Cold and ABA inducible protein KIN1, possibly functions as an anti-freeze protein. Transcript level of this gene is induced by cold, ABA, dehydration and osmoticum (mannitol). However, protein activity of GUS fused to the promoter of this gene is inhibited by cold treatment, suggesting an inhibition of the protein by increased transcript level.	n/a	1	0
<u>AT5G19510</u>	Translation elongation factor EF1B	cytosol	1	0
<u>AT5G20240</u>	Floral homeotic gene encoding a MADS domain transcription factor. Required for the specification of petal and stamen identities.	cytoplasm/ nucleus	1	1

<u>AT5G20500</u>	Putative glutaredoxin	ER	1	2
<u>AT5G24770</u>	Has acid phosphatase activity dependent on the presence of divalent cations (Mg ²⁺ , Co ²⁺ , Zn ²⁺ , Mn ²⁺) and anti-insect activity. Insects fed with the protein show a retarded development. Induced in response to abscisic acid, jasmonic acid, salt, water deficiency and wounding.	ER	1	1
<u>AT5G47210</u>	RNA binding	n/a	1	3
<u>AT5G48485</u>	DIR1, encodes a putative apoplastic lipid transfer protein that is involved in systemic acquired resistance. Mutants in this gene exhibit wild-type local resistance to avirulent and virulent <i>P. syringae</i> , but pathogenesis-related gene expression is abolished in uninoculated distant leaves and fail to develop SAR to virulent <i>Pseudomonas</i> or <i>Peronospora parasitica</i> .	endomembrane system	1	0
<u>AT5G50030</u>	Invertase/pectinesterase inhibitor	ER	1	2
<u>AT5G51280</u>	Putative DEAD-box protein involved in ATP catabolic process	n/a	2	1
<u>AT5G54270</u>	Light harvesting complex	n/a	1	4

(*) The annotation and predicted location were extracted from The Arabidopsis Information Resource (TAIR) website (Arabidopsis.org)

Figure II-3 Screening for AtCERK1 interactors responsive to chitin based on ROS production

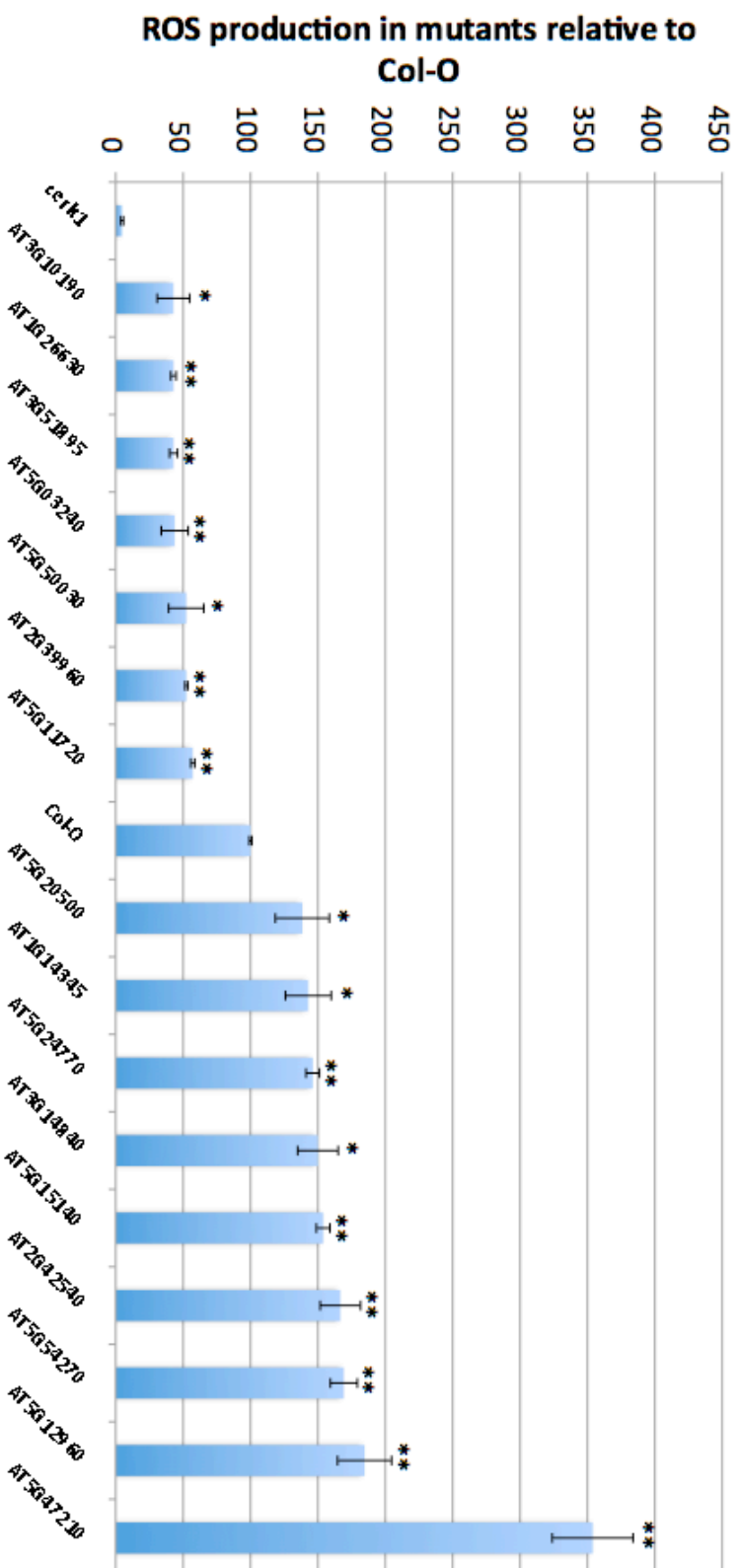
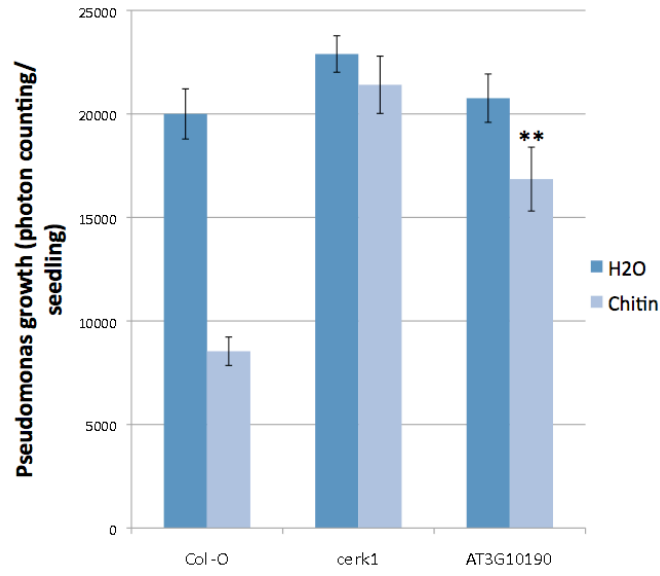


Figure II-3 Screening for AtCERK1 interactors responsive to chitin based on ROS production

ROS production by selected T-DNA insertion mutants after treatment with purified chitin. Signal was measured 20 seconds before and after the maximum signal recorded. The data are the average ratio (%) of the ROS signal from 3 biological replicates (for each replicates, 16 seedlings of each mutant lines were used) compared with the Col-0 wild-type (100%). Bars represent Standard errors. Student T-test compared between mutants and Col-O wild-type (*) P<0.05, (**) P<0.01

Figure II-4 Alteration of chitin signaling affects pathogen resistance

A



B

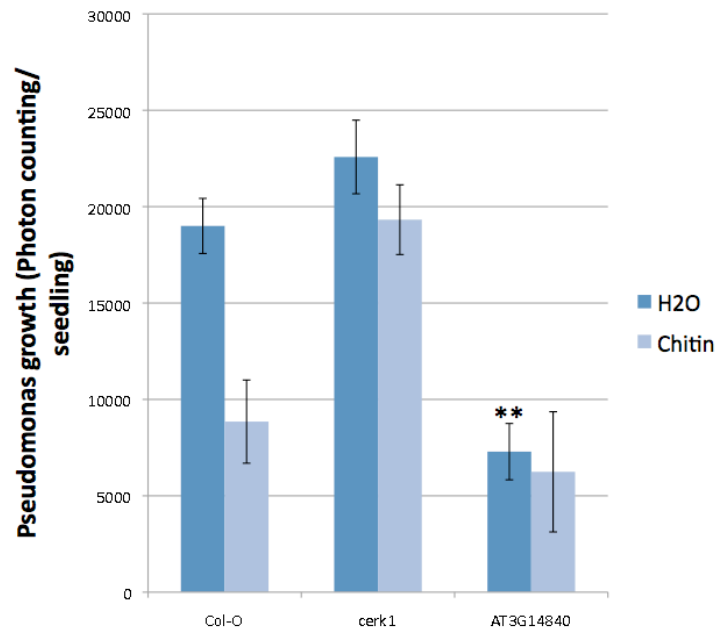


Figure II-4 Alteration in chitin signaling affects pathoge defense

Colonization by *P. syringae* DC3000 on plants containing a T-DNA insertion in AT3G10190 encoding a putative Calmodulin binding protein (A) or AT3G14840 encoding a putative LRR-RLK protein (B) 10 day old seedlings were pre-treated with purified chitin for 24 hours, or water as control. Seedlings were inoculated with DC3000 *lux DCABE* as described in Materials & Methods. The luminescent signal was measured 24 hrs after inoculation at intervals of 40 seconds. The data represent the average Lux signal recorded from 16 seedlings. These experiments were performed three times, and each replicate gave similar results. Bars represent standard error. Student T-test compared between mutants and Col-O wild-type in the same treatment (**) $P < 0.01$

4. Discussion

DupLEXA Yeast 2 Hybrid screening system provides a good tool for screening proteins interacting with AtCERK1

Using Y2H screening methods, various interactions between receptor like kinases (RLKs) and their regulators have been identified. For example, two ethylene receptors, Ethylene Response 1 (ETR1) and Ethylene Response Sensor (ERS), were shown to interact with a negative regulator of ET signaling, Constitutive Triple Response 1 (CTR1), using Y2H screening (101). Interestingly, BAK1, which is known to interact with both FLS2 and EFR, was first identified in a Y2H screening as an interactor of the brassinosteroid receptor, Brassinosteroid insensitive 1 (BRI1). In this study, Y2H screening was used as to identify proteins that interact with the intracellular kinase domain of AtCERK1.

Previous studies showed that the longer the cloned insert used as bait, the higher the possibility to obtain false positive clones, with an ideal length of no more than 2kb (102). This observation may reflect the potential for longer cloned inserts to recombine with a yeast transcription activation domain sequence, leading to expression of reporter genes (102). In the current study, an insert of 947 bp, encoding the AtCERK1 intracellular kinase domain was used as the bait. Previous research suggest that an ideal Y2H screen against an Arabidopsis cDNA library should use $\sim 10^6$ transformants (103). In the present study, 4.5×10^6 transformants were obtained to screen, which represent

saturation. In order to optimize the ability to obtain interactors involved in chitin signaling, the cDNA library used was derived from chitin treated Arabidopsis seedlings. The Y2H screen identified a total of 54 potential interactors out of 600 original transformants that grew on selection media. Thus, the DupLEXA Y2H screening system is sensitive and powerful for a large-scale screening for protein-protein interaction.

Before utilizing the DupLEXA system, I also experimented with the Matchmaker GAL4 two-hybrid system 3 (Clontech laboratories, Inc. Mountain View, CA). Two screens were performed with this system, again using the AtCERK1 intracellular kinase domain as bait. Although many positive clones were obtained from both screens, sequencing of the clones showed that all were derived from different fragments of the same Zinc finger protein (data not shown). I assume that these were not true, physiological interactions. Other authors have also noted that many interactors detected in DupLEXA system are not detected using the Matchmaker GAL4 system and vice versa (104, 105). The choice of Y2H bait vectors used can also affect the diversity of interactors that are found (106). In the current study, the DupLEXA system appears to provide a diverse and repeatable list of putative interactors that I then sought to confirm using other methods.

Further studies of AtCERK1 putative interactors

Given the high number of interactors identified from the screening, a detailed study of each gene would be too laborious. Moreover, the purpose of my study was to

identify components of the chitin signaling pathway that are important for PTI. In addition, with a very high number of interactors detected based on my saturated screen, it is likely that false positive identifications are among the list of 54 interactors found. Based on the annotations of the putative interactor genes, nuclear and organellar proteins are respectively composed of 4% and 23% of the total interactors while AtCERK1 is a transmembrane protein. Therefore, it is likely that at least 27% of the interactors are false positive. I utilized additional screens to winnow the list of genes to those that showed measurable phenotypes associated with chitin-induced PTI. As discussed in chapter 3, one of the genes identified from the screen, AT3G14840, was subsequently chosen for detailed analysis. However, there are clearly other putative AtCERK1 interacting proteins that warrant further study.

One of the interesting potential AtCERK1 interactors is AT3G08770, which encodes Lipid Transfer Protein6 (LTP6) (107). This protein belongs to the lipid transfer protein (LTP) family, which is composed of small, cationic, cysteine-rich peptides with the ability to perform the transfer of phospholipids between membranes (108, 109). All of the proteins in the Arabidopsis LTP family are known to possess anti-microbial activity and, indeed, LTP genes are predicted to be pathogenesis-related (PR) genes (109, 110). Interestingly, using a purified tobacco membrane system, wheat LTP1 was shown to bind to receptors for cryptogein (111), which is one of the *Phytophthora cryptogea* elicitors with the ability to trigger a hypersensitive response (HR), as well as system acquired resistance (SAR), in some hosts (112). However, at high concentration, wheat

LTP1 is an antagonist of cryptogein, competitively binding to receptors of the elicitor, suppressing plant responses (111). It was also reported that the formation of the complex elicitor/sterol is the initial step triggering plant responses (113). Interestingly, a later study showed that rice LTP2 binds a sterol molecule, suggesting that the complex LTP2/sterol could interact with a membrane-associated receptor to mediate defense responses (114).

The putative interaction of LTP6 with AtCERK1, the hypothesized mechanism of LTPs working together with other receptors to regulate pathogen defense make AT3G08770.1 (LTP6) a likely candidate to play a role in PTI. Unfortunately, there is no available T-DNA insertion line in this gene and, therefore, I was unable to test whether mutations in this gene affected chitin signaling or pathogen resistance.

Another interesting candidate is AT5G48485, which encodes the Defective in Induced Resistance 1 (DIR1) protein (115). A *dir1-1* mutant was found to be impaired in systemic acquired resistance (SAR) (115). Similar to LTP6, DIR1 is also a lipid transfer protein, also called LTP2-like resistance signaling protein due to their structural similarity (107, 116). However, a more recent study about the protein structure of DIR1 showed that although quite similar to LTP2, DIR1 represents a new type of lipid transfer protein; possessing specific features such as a large cavity for binding of two molecules of long-chain fatty acid derivatives, an unusual acidic pI, and especially a long PxxPxxP proline-rich motif on the surface (117). Notably, proline-rich regions were reported to be involved in protein-protein interaction (118). Therefore, DIR1 might interact with

other proteins, likely mediating defense responses. One possibility is that the LTP activity of DIR1 could mediate the transfer a lipid molecule as a signal to activate SAR (115). Unfortunately, the mutation in *DIR1* was one of those for which I was unable to recover homozygous mutant lines. Indeed, The Arabidopsis Information Resource (TAIR) indicates that the *dir1-1* mutant exhibits reduced viability.

A final example of an AtCERK1 interactor that warrants further study is AT3G10190, which is predicted to encode a putative calcium-binding protein, calmodulin (CaM). Mutants in this gene responded only weakly to chitin, showing a ROS production level lower than the wild-type (Figure II-3). The AT3G10190 mutant was also significantly more susceptible to bacterial infection, even after chitin treatment. These data suggest that the AT3G10190 encodes a gene required for innate immunity. Interestingly, the Arabidopsis S-locus calcium-binding receptor like kinase1 (CBRLK1) was shown to interact with calmodulin (119). This protein was subsequently shown to be a negative regulator of plant defense against bacterial pathogens (120). Chitin elicitation results in a rapid and pronounced elevation of intracellular calcium levels (121). Hence, it would not be surprising to find that a calcium binding protein plays a role in the early events of chitin induced PTI.

III. PAMP RESPONSIVE PROTEIN KINASE 1 (PRPK1) IS INVOLVED IN PAMP-TRIGGERED IMMUNITY

1. Introduction

Chitin is an important component of the fungal cell wall (9, 60, 78). It is one of the best-studied PAMPs and is capable of eliciting basal defense responses against fungal pathogens. Recently, the receptor for chitin was identified in Arabidopsis, LYK1/CERK1 (9, 28, 78). However, little is known about the molecular basis of chitin perception or how chitin elicitation is translated into a cellular signal, ultimately leading to pathogen resistance.

The results from Y2H screening for proteins interacting with AtCERK1 (Chapter 2) indicated that a Leucine-rich repeat receptor-like kinase interacts with AtCERK1. Further investigation indicated that T-DNA insertion mutants of this kinase were hypersensitive to chitin, as evidenced by higher ROS production upon chitin treatment. The kinase was named PAMP-responsive protein kinase 1 (PRPK1) since the protein is likely involved in not only chitin signaling but also the response to flagellin, another well-studied PAMP. Although the interaction between PRPK1 and AtCERK1 was first identified using the Y2H approach, my attempts to confirm this interaction using other methods, such as split YFP, split luciferase and co-immunoprecipitation were unsuccessful. However, PRPK1 T-DNA insertion mutants showed increased resistance to the hemibiotrophic pathogen, *P. syringae*, but increased susceptibility to the necrotrophic fungal pathogen, *Sclerotinia*

sclerotiorum. Therefore, PRPK1 is clearly important to pathogen resistance in *Arabidopsis*.

2. Materials and methods

Table III-1 Primers used for genotyping and gene expression analysis

Primer	Sequences	Purpose
Lb1.3	ATTTTGCCGATTTTCGGAAC	Left border primer for the T-DNA insertion SALK lines
PRPK1-1 LP	CAGCCAGCAAAGACCAATAAC	Identify the genotype of SALK_091588
PRPK1-1 RP	CGCCGTTACTTGTAAGTCTC	Identify the genotype of SALK_091588
PRPK1-3 LP	TTGTTCTCAGCTTTCTACGGC	Identify the genotype of SALK_040386
PRPK1-3 RP	TTGCAGGATCAAAGTTATCCG	Identify the genotype of SALK_040386
PRPK1-4 LP	TTGTTCTCAGCTTTCTACGGC	Identify the genotype of SALK_040387
PRPK1-4 RP	CTTCTCCGATCTTGTTTGCAG	Identify the genotype of SALK_040387
PRPK1-F	ACGGTACCTGGAGAGGCTGCTTAAGACCC	Assess the expression of PRPK1 by RT-PCR
PRPK1-R	ACGGATCCTTAAGTTCTAGTGTTCCAATAGGC	Assess the expression of

		PRPK1 by RT-PCR
SqPRPK1-F	TGCAAACAAGATCGGAGAAG	Semi qRT-PCR of PRPK1
SqPRPK1-R	CAGAGCCGAAATCATAGCAA	Semi qRT-PCR of PRPK1
UBQ5-F	CTTCAGCAGCCGTTGCCTCA	Amplifying UBQ5 control in RT-PCR
UBQ5-R	CTGGTAAACGTAGGTGAGTCC	Amplifying UBQ5 control in RT-PCR
qACO2-F	ACAACCCGGG AAGCGATGCG G	qRT-PCR of ACO2
qACO2-R	TGCATAAAGCTTCATGTAGTC	qRT-PCR of ACO2
qJAR1-F	GCAACTGTTTAGACCGAGCA	qRT-PCR of JAR1
qJAR1-R	GCTCCAAGGCTCCAATAGTC	qRT-PCR of JAR1
qCOI1-F	CTTCTTTGGTGTGTCGGAGA	qRT-PCR of COI1
qCOI1-R	GTAGCAAAGCGCCATAGTCA	qRT-PCR of COI1

Plant germination and growth conditions

As described in Materials and Methods of chapter I.

PAMP treatments

As described in Materials and Methods of chapter I.

Reactive oxygen species assay

As described in Materials and Methods of chapter I.

Pathogen strains and culture conditions

As described in Materials and Methods of chapter I.

Pathogen assay

P. syringae DC3000 *lux* DCABE (100) inoculation assays were performed as described in the Materials and Methods sections of the two previous chapters.

***Sclerotinia sclerotiorum* disease assay**

Two leaves were detached from 4-week-old Arabidopsis plants (with six biological replicates for each genotype) and transferred onto damp Whatman paper in Petri dishes. A 2mm-diameter agar disc of *S. sclerotium* was then placed upside down onto each leaf. Eighteen hours after inoculation, the fungal discs were removed and the lesion size was measured.

Total RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

As described in the Materials and Methods of chapter I.

In order to measure the PRPK1 gene expression level in the T-DNA insertion mutant lines, one microliter of RT-PCR product was used in a polymerase chain reaction (PCR) consisting of 2.5 mM MgCl₂, 1mM dNTPs, 0.5 units of Ex-Taq DNA Polymerase (Takara, Madison WI) using gene-specific primers as listed in Table III-1. As an internal control, gene-specific primers of *ubiquitin 5* (UBQ5) were used together with specific primers in each PCR reaction. To analyze expression of specific genes, 6 µL of the PCR reaction was used for agarose gel electrophoresis.

Quantitative RT-PCR: As described in Materials and Methods of Chapter I.

Callose deposition assay

Five-day old seedlings were pre-treated with chitoctase for 24 hours prior to the callose deposition assay. The assay was performed using the protocol in (122) and quantified with a Leica M205 FA Stereo microscope (Molecular Cytology Core, University of Missouri, Columbia, MO)

Molecular modeling of proteins

As described in Materials and Methods of chapter I.

3. Results

Identification of the PAMP responsive protein kinase 1 (PRPK1) gene

In chapter 2, a Y2H screen was performed to identify proteins interacting with the AtCERK1 intracellular kinase domain using an Arabidopsis chitin-pretreated seedling cDNA library. This screen identified 54 putative interactors. T-DNA insertion mutants were acquired for the majority (43) of these interactors and the mutants were subsequently screened for their ability to produce reactive oxygen species upon chitin elicitation. This secondary screen led to the identification of 16 mutant lines that produced either more or less ROS production upon chitin elicitation. These 16 mutant lines were subsequently challenged with the bacterial pathogen, *P. syringae*, leading to the identification of two mutants with an altered pathogen response. One of these genes, AT3G10190.1, is predicted to encode a putative calcium-binding protein. The second gene, AT3G14840.2, is predicted to encode a leucine rich repeat receptor like kinase (LRR-RLK). The AT3G10190.1 mutant was significantly more susceptible to pathogen infection. A total of four unique T-DNA insertion mutant lines were obtained for AT3G14840.2. All four of these mutant lines showed enhanced ROS production upon chitin treatment and were significantly more resistant to infection by *P. syringae*. Although originally isolated due to their altered response to chitin treatment, the AT3G14840.2 mutant lines were subsequently found to also react more strongly (based on ROS production) to flagellin elicitation. In contrast to their response to the hemibiotroph *P. syringae*, the AT3G14840.2 mutant lines were more susceptible to the

necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. These data suggest a critical role for AT3G14840.2 in PAMP-triggered immunity and, hence, this gene was named PAMP-responsive protein kinase 1 (PRPK1).

The PRPK1 gene spans 5952 bp, and consists of 23 introns and 24 exons. The gene model and predicted protein structure suggest that the gene encodes a 1021 amino acid long protein that contains an extracellular LRR domain, a transmembrane domain and an intracellular Ser/Thr kinase domain (Fig III-1 A). The four insertion mutant lines were named *prpk1-1*, *prpk1-2*, *prpk1-3* and *prpk1-4* with the insertions located in intron 2, intron 13, exon 18 and exon 18, respectively. A primer pair was designed to amplify the kinase domain of PRPK1. RT-PCR analysis with the primer amplified the expected fragment from mRNA obtained from wild-type seedlings but no band was obtained for any of the four mutant lines (Fig III-1B). This indicates that the transcription of the gene was blocked in all mutant plants.

Early PAMP signaling responses are partially blocked in *prpk1* mutants

As an initial test to determine if PRPK1 is involved in chitin signaling, a ROS assay was performed to measure the early responses of the *prpk1* T-DNA lines to chitooctose treatment (Figure III-2 A) Compared with the wild-type, ROS production in all four mutant lines was significantly higher upon chitin treatment.

Although PRPK1 was originally identified by screening for genes involved in chitin signaling, I also tested whether the *prpk1* mutants might also be affected in their response to other PAMPs, particularly flg22 and elf18. As shown in Figure II-2 B, C, treatment of the various mutant plants with flg22 resulted in a significantly stronger ROS response, as compared to the wild-type. In contrast, the mutants responded similarly to the wild-type when treated with elf18. Therefore, PRPK1 likely acts not only in the chitin signaling pathway, but also in response to flagellin elicitation but is not involved in all PAMP signaling responses (i.e., not in response to EF-Tu).

Callose deposition, a late PAMP-triggered response, is impaired in the *prpk1* mutants

The production of ROS in response to PAMP elicitation is considered a relatively rapid cellular response. Therefore, I also examined the production of callose, which is a PAMP response that develops significantly later. Callose deposition was measured on 5-day old seedlings of both wild-type and mutants either mock-treated or treated with various PAMPs (chitin, flg22, elf18) for 24 hours (Figure III-3). In contrast to wild-type

plants, no callose was detected in the *prpk1-1* or *prpk1-4* mutants when treated with either chitin or flg22 (Figure III-3A). In contrast, both the wild-type and *prpk* mutant plants produced similar levels of callose when challenged with elf18 or pep1 (Fig III-3B). Again, these data suggest a specific role for PRPK1 in the chitin and flagellin PAMP response pathway.

The *prpk1* mutants are more resistant to the hemibiotrophic pathogen *P. syringae*

The data above clearly indicate that PRPK1 is involved in the chitin and flagellin PAMP responses and, therefore, it would be expected that *prpk1* mutants would show alterations in PAMP-triggered immunity. In order to confirm this prediction, wild-type and mutant plants were challenged with the hemibiotrophic pathogen *P.syringae* pv. tomato DC3000. Figure III-4 shows that 2 days after inoculation (2 DAI), the ability of the pathogen to penetrate into seedlings of the various mutant lines was significantly reduced compared to the wild-type. Therefore, the *prpk1* mutants were more resistant to the bacterial pathogen.

PRPK1 mutants are more susceptible to the necrotrophic pathogen *S. sclerotiorum*

Previous studies have shown that the plant response to necrotrophic and hemibiotrophic pathogens involves distinct pathways. For example, the hypersensitive response (HR) is quite effective in stopping invasion by hemibiotrophic pathogens but enhances infection by necrotrophic pathogens (66). The enhanced resistance shown by PRPK1 mutants suggest that PRPK1 functions as a negative regulator of the defense pathways responding to hemibiotrophic pathogens. In order to characterize the response of these mutants to a necrotrophic pathogen, wild-type and mutant plants were infected with the fungal pathogen, *Sclerotinia sclerotiorum*. The data shown in

Figure III-5 indicate that the *prpk1* mutant plants were significantly more susceptible to this pathogen, as indicated by the larger lesion sizes on the mutant plants compared to wild-type. These data suggest that PRPK1 acts as a positive regulator of pathways involved in defense against necrotrophic pathogens.

Expression of several genes in the JA/ET pathway are reduced in *prpk1* mutants

Two parallel hormone pathways are known to mediate distinct mechanisms of defense against either hemibiotrophic or necrotrophic pathogens. The salicylic acid (SA)-dependent signaling pathway primarily targets hemibiotrophs; whereas the jasmonic acid (JA) and ethylene (ET)-dependent signaling pathways primarily mediate resistance to necrotrophic pathogens (66). As discussed above, analysis of the PRPK1 mutant lines suggests that PRPK1 normally suppresses the SA pathway (consistent with the greater resistance of the mutants to *P. syringae*) and activates JA and/or ET pathways (consistent with the greater susceptibility of the mutants to *S. sclerotiorum*). In order to test this hypothesis, the expression of key genes in the SA and JA/ET pathways was examined in wild-type and *prpk1* mutant plants either mock-treated or treated with PAMPs. Figure III-7 shows that the expression of *JAR1*, *COI1* and *ACO2* as measured by qRT-PCR. *ACO2* encodes ACC oxidase 2, a vital enzyme involved in ET synthesis. In *prpk1* mutants, the gene is expressed at a consistently low level relative to wild-type. Expression of *JAR1*, the JA receptor, is also low in *prpk1* mutants, indicating suppression of the JA signaling pathway in the mutant. *COI1* is a critical gene required for bioactive

JA expression. In *prpk1* mutants, *COI1* expression was consistently lower than in the wild-type. In contrast to these results, measurements of gene expression of key SA pathway genes, such as *PR1*, *PR2* and *SID2*, showed no effect of the *prpk1* gene mutations (data not shown). Therefore, the data support the postulated role of PRPK1 as a positive regulator of the JA and ET pathways. However, the gene expression results do not support the hypothesis that PRPK1 negatively regulates SA signaling. However, it is known that the JA/ET and SA pathways act in opposition (14, 66) and, hence, defects in the JA/ET pathway could lead to enhancements in the resistance to hemibiotrophic pathogens, but not reflected in changes in the gene expression of *PR1*, *PR2* and *SID2*.

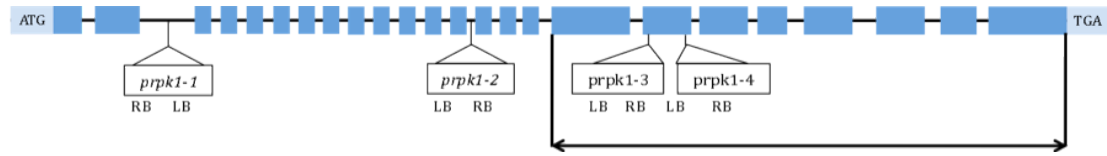
PRPK1 protein modeling structure is similar to BAK1

PRPK1 protein is predicted to be a LRR-RLK. Many LRR-RLKs were identified as PAMP receptors and thus directly involved in PTI as the very first perception step (123). Therefore, I also want to study the structure PRPK1 protein as a LRR-RLKs to determine if PRPK1 protein is similar to LRR-RLK PAMP receptors. Blasting the amino acid sequence of PRPK1 with those of other 231 Arabidopsis LRR-RLK, it turned out that BAK1 is the best template for PRPK1 protein structure modeling. The alignment based on kinase domain protein sequence showed that PRPK1 aligns best to BAK1, with identity of 42.8%, and similarity of up to 60.1% (Figure III-8). Although BAK1 is not the most similar LRR-RLK to PRPK1, BAK1 is the most known structure with highest similarity score. Therefore, a model of the kinase domain of PRPK1 protein the kinase domain of PRPK1 to that of BAK1. This modeling approach was performed by Cuong The Nguyen, a Ph.D.

student in Dr. Gary Stacey lab. Consequently, the secondary and tertiary structures of kinase domain of PRPK1 are highly similar to that of BAK1 (Figure III-9 and Figure III-10).

Figure III-1 Analysis of *PRPK1* insertion mutants

A



B



C

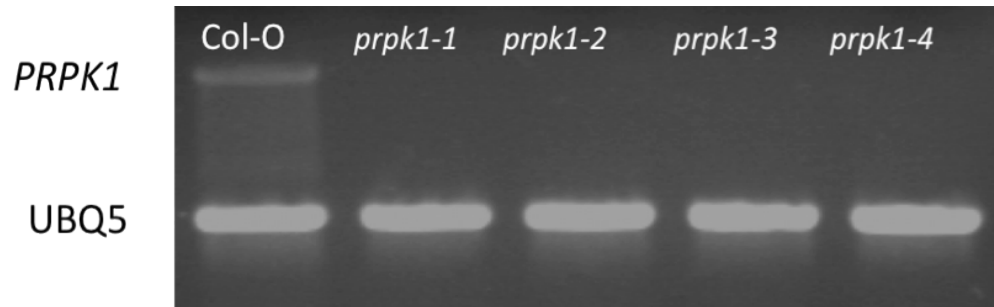


Figure III-1 Analysis of *prpk1* insertion mutants

(A) A diagram of the PRPK1 ORF showing the locations of the various T-DNA insertion mutations (*prbk1-1*, *prbk1-2*, *prbk1-3* and *prbk1-4*) (not drawn to scale).

(B) Predicted structure of the PRPK1 protein, including the signal peptide, leucine-rich repeat domain, transmembrane domain, kinase domain and polythreonine tract.

(C) semi qRT-PCR analysis of the PRPK1 expression in wild-type and insertion mutant from 12-day-old seedling plants. Primers were designed to amplify the kinase domain of PRBK1 (as described in the Materials & Methods).

Figure III-2 Enhanced ROS production in *prpk1* mutants upon PAMP treatment

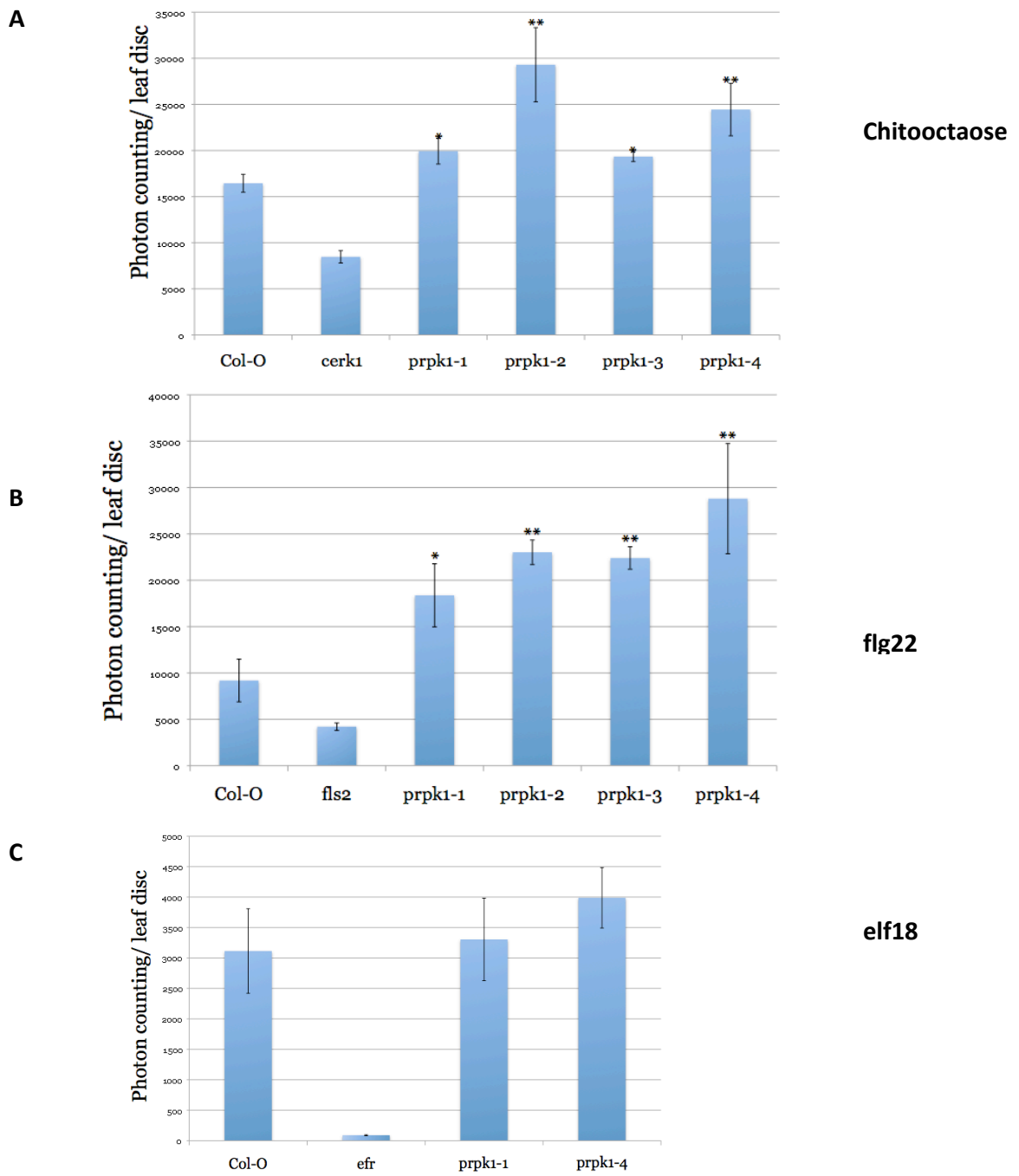


Figure III-2 Increasing ROS production upon PAMP treatment

ROS generation in *prpk1* mutant lines treated with chitooctaose (A), flg22 (B) or elf18 (C). ROS was analyzed 20 seconds before and after the maximum signal observed. The data are the average of 12 leaf discs punched from six 4-week-old plants. (Bars represent standard errors). Student T-test (*) <0.05

Figure III-3 Suppression of callose deposition in *prpk1* mutants in response to chitin and flg22

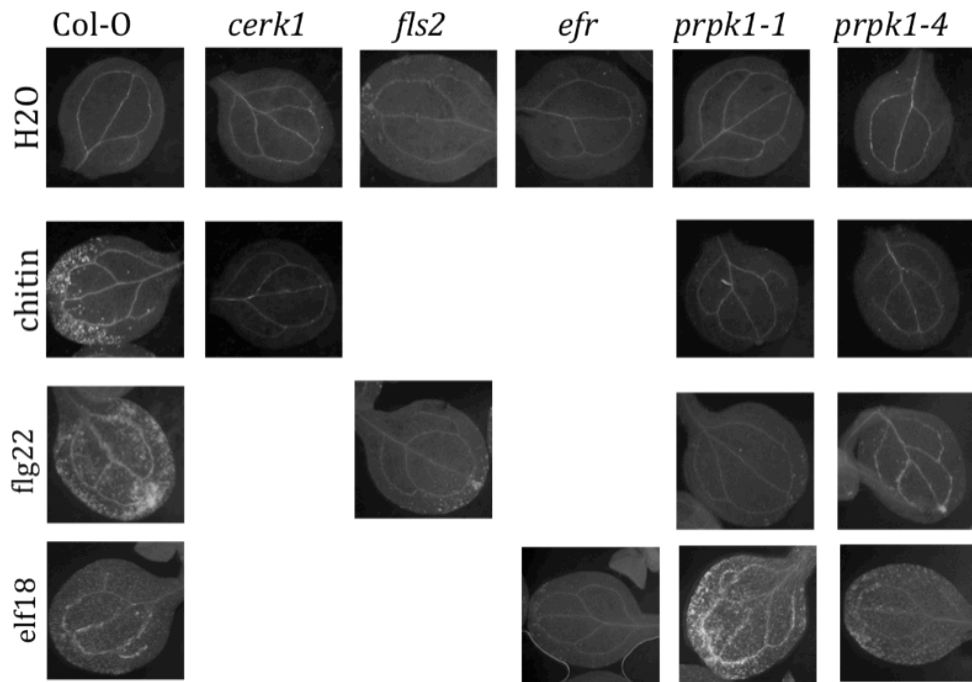
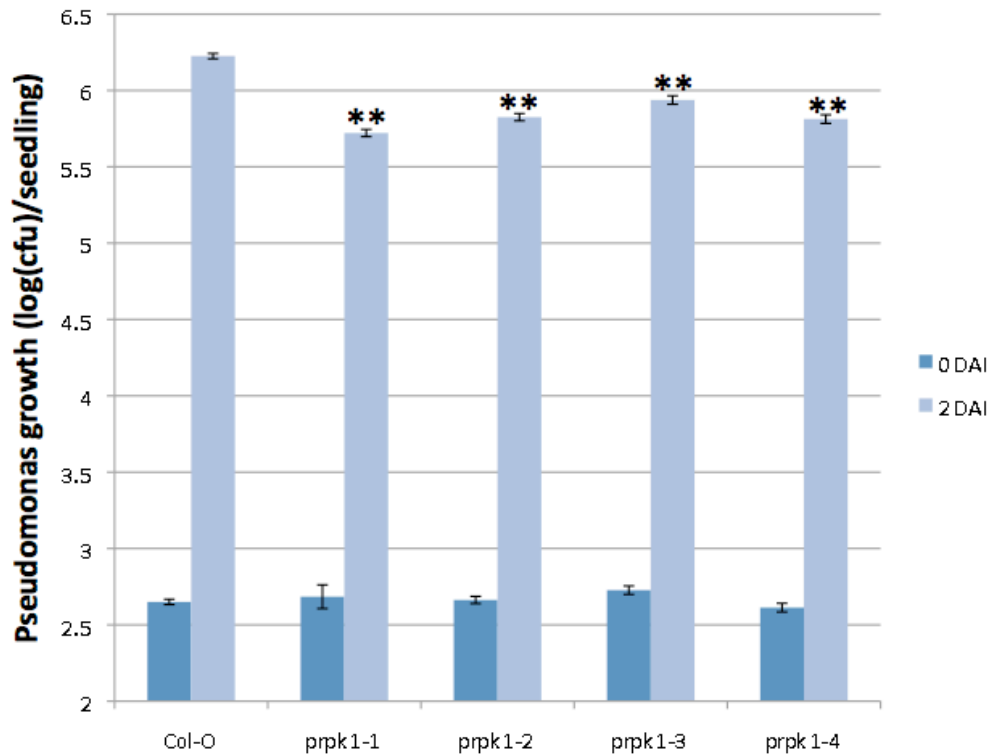


Figure III-3 Suppression of callose deposition in *prpk1* mutants in response to chitin and flg22

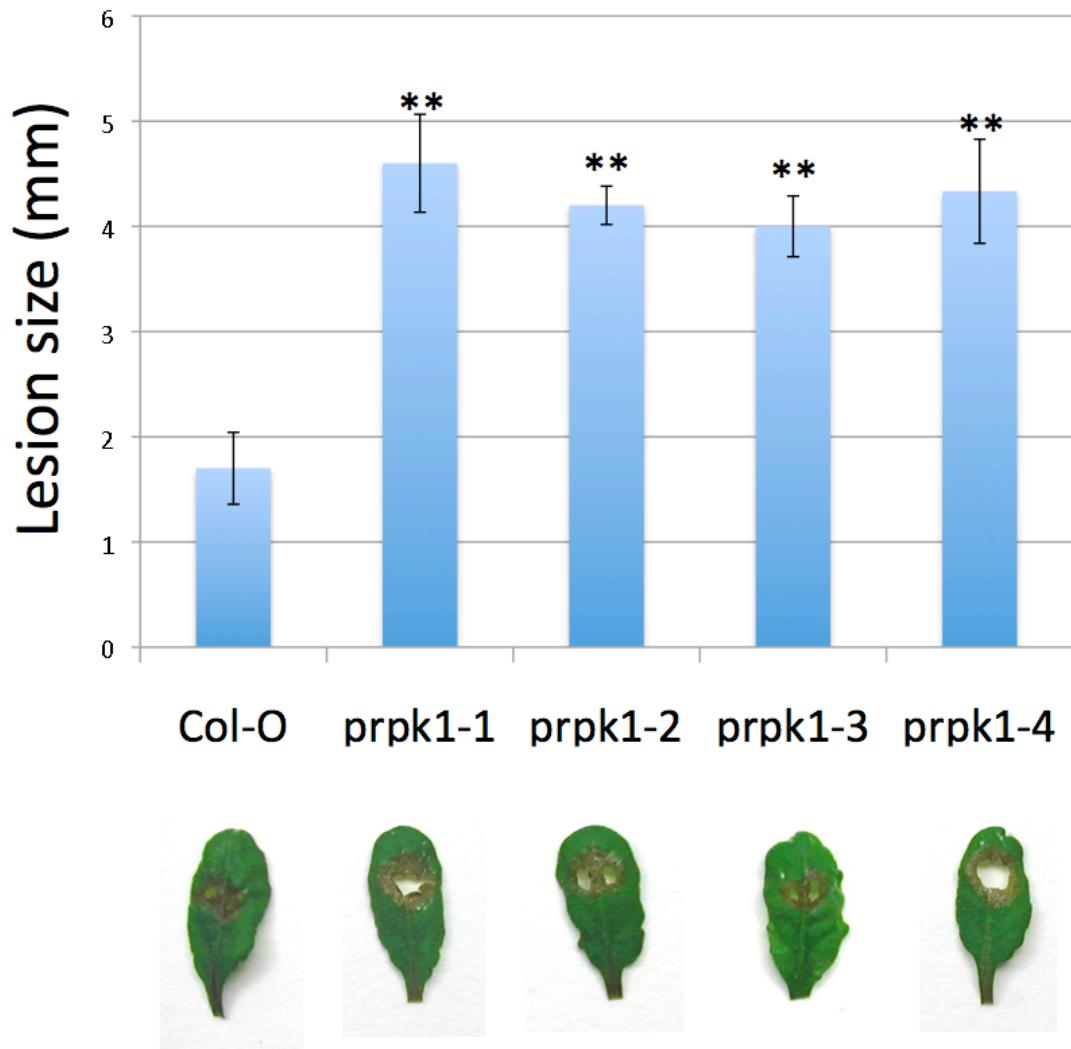
Callose deposition in 5-day old leaves of *prpk1* mutant plants, compared to the wild-type and the negative control *cerk1* after treatment with 1 μ M of chitooctase, or other PAMPs, flg22 or elf18 for 24 hours. Each treatment was performed on 15 seedlings with similar results.

Figure III-4 The *prpk1* mutants are more resistant to *P. syringae* pv. tomato DC3000



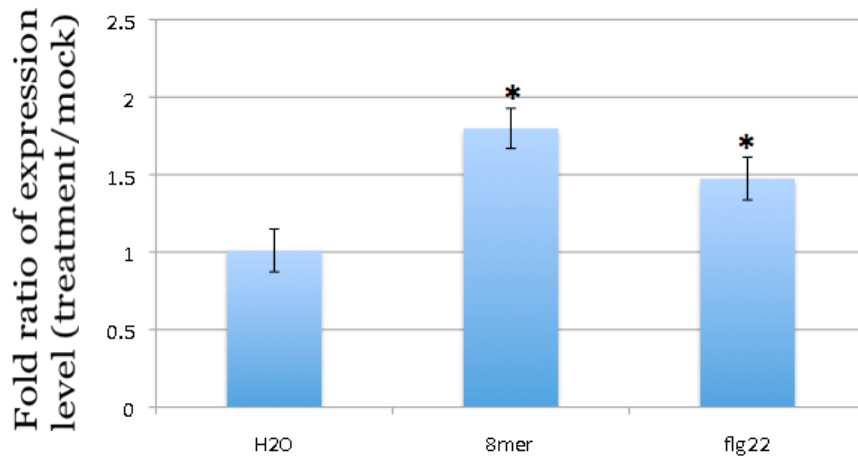
Ten-day-old seedlings of mutants and wild-type were inoculated with *P. syringae* DC3000 at the concentration of 0.5×10^4 cfu/mL by soaking in a bacterial suspension for 3 hours. The bacterial solution was then removed and the seedlings were washed three times with ddH₂O. Bacterial growth was measured by grinding the seedlings and then plating the resulting extracts on YPD medium with rifampicin and kanamycin as selection. The data are represented as the log₁₀ of colony forming units (3 hours and 48 hours after inoculation) per seedling. The data are the average of 18 seedlings. Bars represent standard error. Student T-test (**) $P < 0.01$. The experiment was done in triplicate, each with similar results. Bars represent standard errors.

Figure III-5 *prpk1* mutants are more susceptible to *Sclerotinia sclerotiorum*



Four-week-old mature leaves were inoculated with *Sclerotinia sclerotiorum* by attaching 2mm diameter agar discs of *S. sclerotiorum* onto detached leaves. Lesion size was measured 16 hours after inoculation. The data represent the average lesion size of 18 leaves from 6 plants. The experiments were performed in triplicate, each with similar results. Bars represent standard errors. Student T-test (***) $P < 0.01$. The pictures were taken 24 hours after inoculation. Bars represent standard error.

Figure III-6 *PRPK1* expression is induced by both chitin and flg22



Ten-day-old seedlings were treated with 1 μ M of chito-octaose (8mer), flg22 or ddH₂O or DMSO as mock for 30 minutes. The data are the average of the ratio of the gene expression comparing mock treated and PAMP treatments of three biological replicates. Bars represent standard error. Student T-test (*) P<0.05, (**) P<0.01

Figure III-7 The expression of marker genes of the JA/ET pathway is significantly reduced in the *PRPK1* mutants

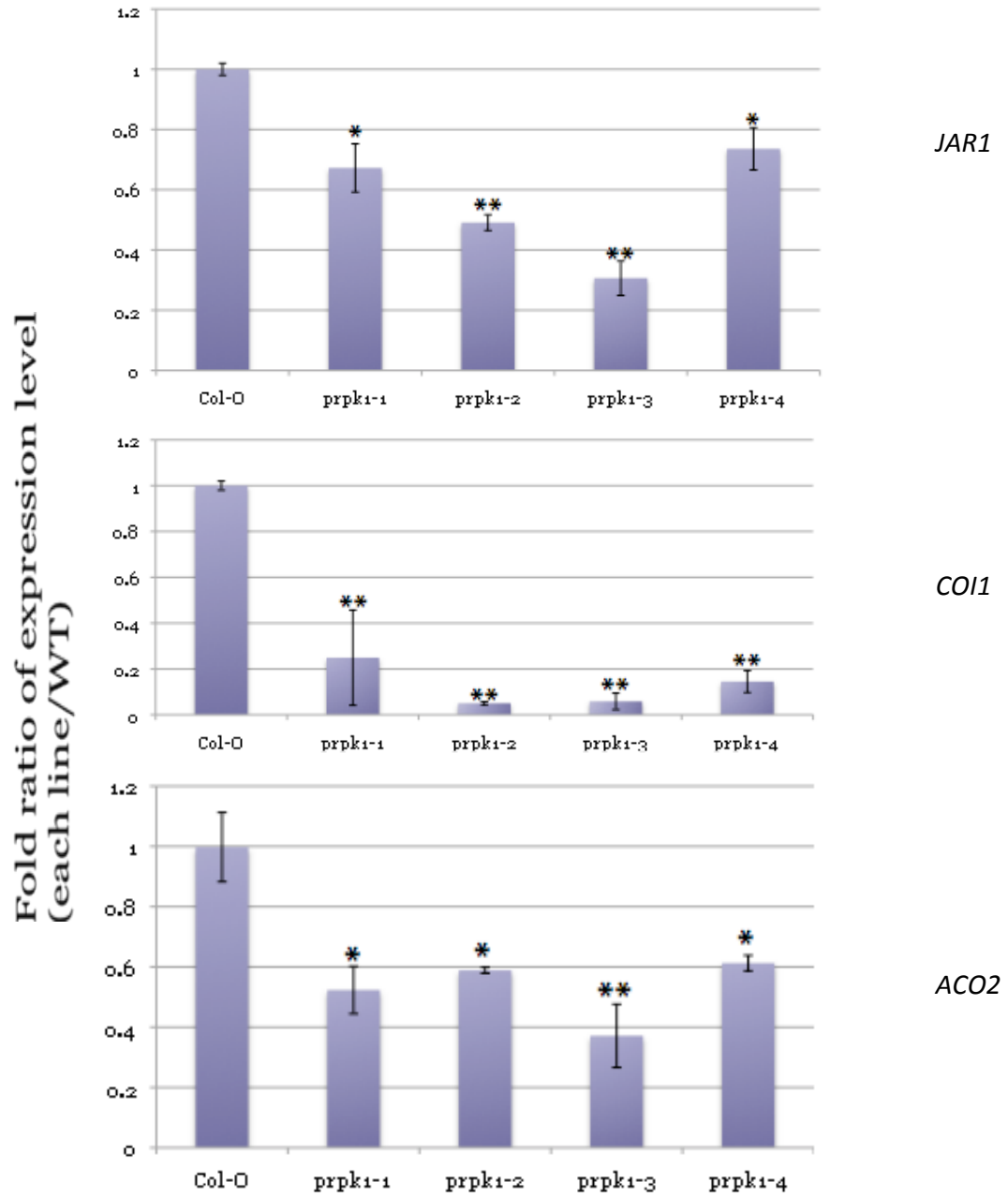
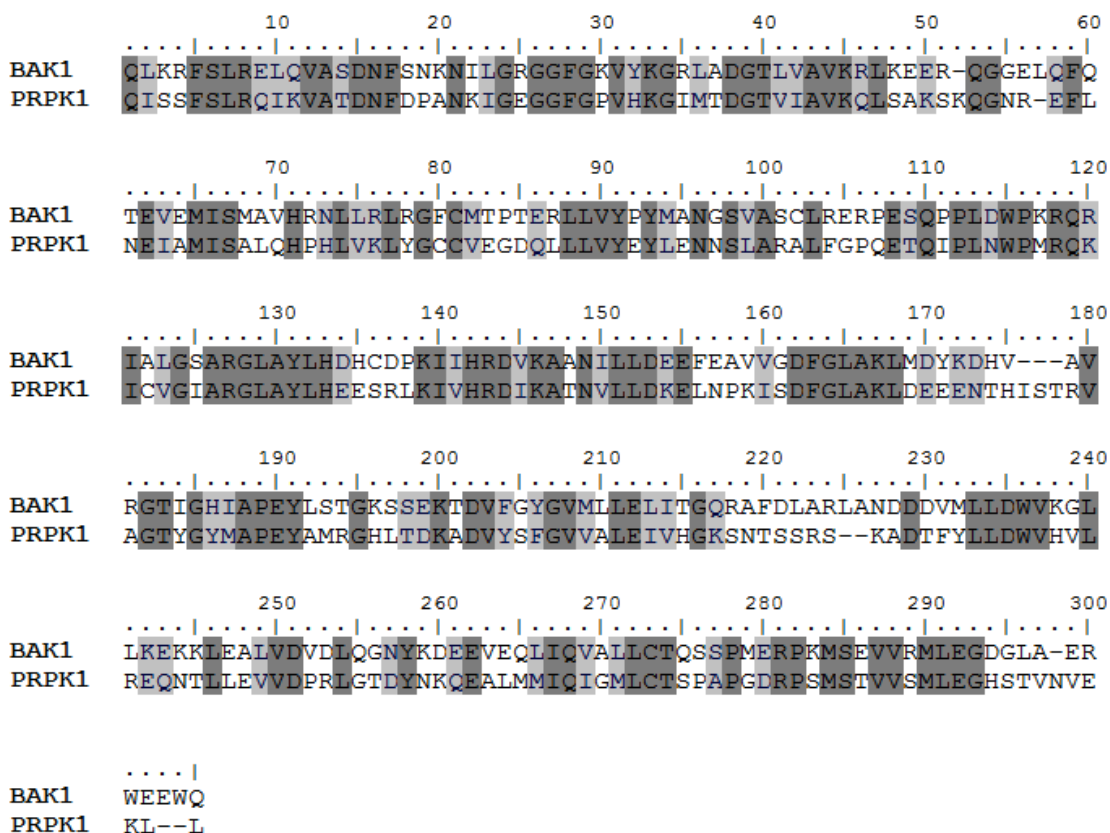


Figure III-7 The expression of marker genes of the JA/ET pathway is significantly reduced in the *PRPK1* mutants

RNA isolated from 10-day-old seedlings were used for quantitation of gene expression by qRT-PCR. The genes chosen represent key genes in the JA and ET signaling pathways. The data represent the average of the ratio of gene expression in the mutants compared to the wild-type for three biological replicates. Bars represent standard errors. Student T-test (*) $P < 0.05$, (**) $P < 0.01$

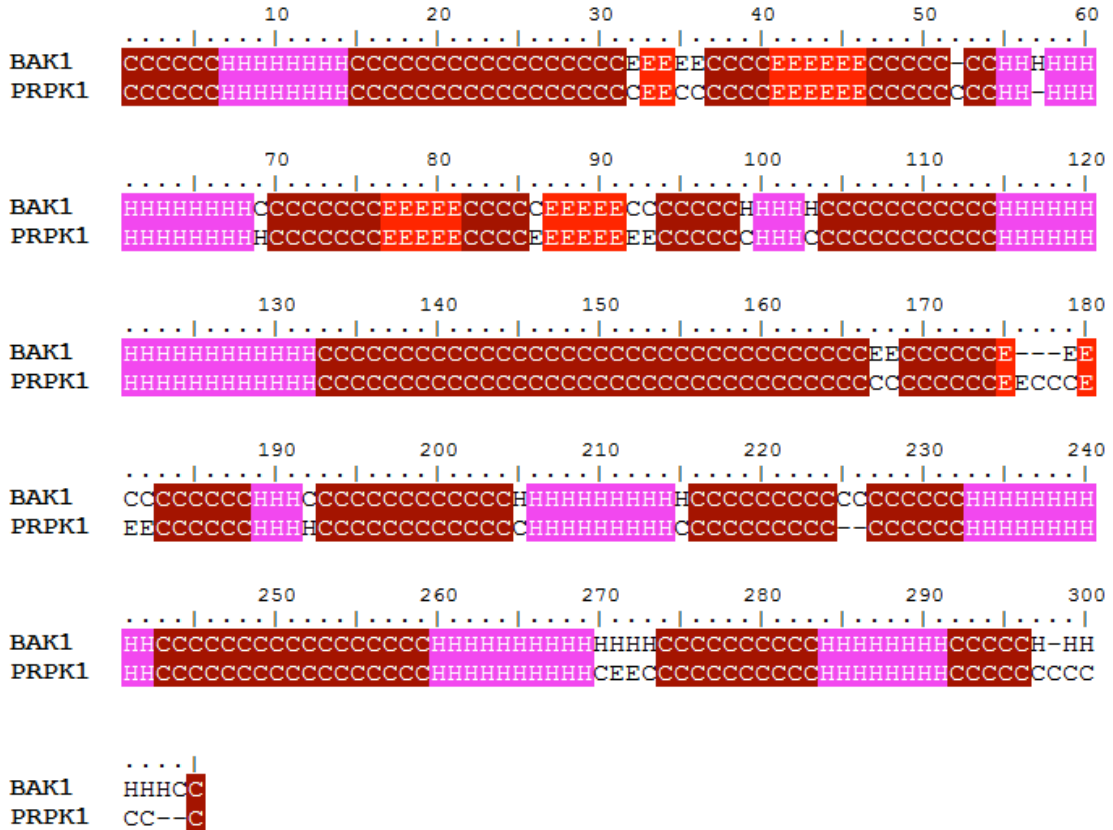
Figure III-8 Alignment of amino acid sequences of the PRPK1 and BAK1 kinase domains



The best alignment (identity: 42.8% and similarity: 60.1%, small gaps and less number of gaps as compared to other alignments) of the target and template kinase domains used for modeling tertiary structure of the prpk1 protein. Dark gray columns: same/conserved amino acids, light gray columns: similar amino acids, -: gaps. The alignment was made between the 656-956 fragment of the prpk1 kinase domain and the 272-576 fragment of the template structure (PDB code: 3uim -chain A, (124)).

The figure was generated by Cuong The Nguyen, a Ph.D. student in Dr. Gary Stacey lab.

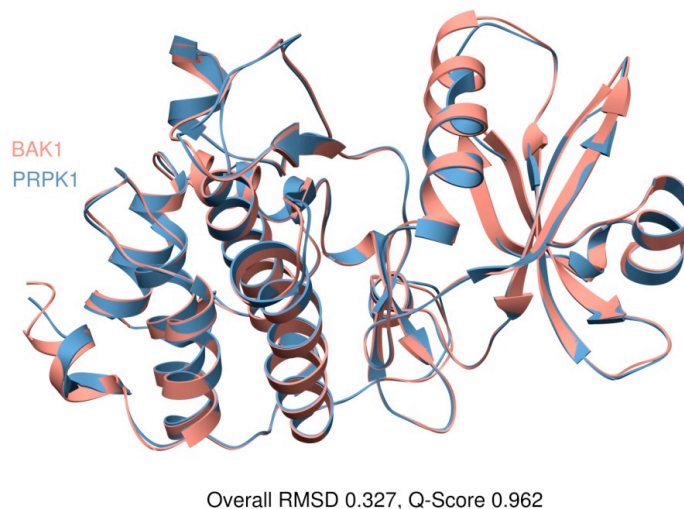
Figure III-9 The secondary structure alignment between the PRPK1 kinase domain (as target) and the template BAK1 kinase domain



The secondary structure alignment between PRPK1 kinase and BAK1 kinase. The secondary structure of the target protein was predicted by PSIPRED (125). C: Coil; E: Beta Sheet; H: Alpha Helix. Columns with same colors are conserved in secondary structure between two proteins.

The figure was generated by Cuong The Nguyen, a Ph.D. student in Dr. Gary Stacey lab

Figure III-10 Superimposition of the PRPK1 kinase domain structure and the template BAK1 kinase domain structure.



Superimposing of the tertiary structure of kinase domain of PRPK1 (blue) using the best alignment template BAK1 (salmon).

The figure was generated by Cuong The Nguyen, a Ph.D. student in Dr. Gary Stacey lab.

4. Discussion

PRPK1 is a key component in plant innate immunity via regulating expression of the JA/ET hormone signaling pathway.

My interest in PRPK1 originated from its identification as a putative interactor of AtCERK1 from the Y2H study described in chapter II. Unfortunately, my repeated attempts to confirm this interaction using a variety of other methods failed. Hence, at this time, I cannot conclude that PRPK1 actually does interact with AtCERK1 in planta. However, I continued my focus on PRPK1, primarily due to the fact that it showed a significant pathogen response difference when compared to the wild-type, which suggested that PRPK1 does play an important role in pathogen defense. Also interesting is that, although originally identified through studies of chitin signaling, mutants in PRPK1 also showed altered response to flagellin, but not other PAMPs that were tested. These data suggest a possible functional link between chitin and flagellin signaling that warrants further investigation.

Mutations in *PRPK1* differentially affect plant susceptibility to hemibiotrophic and necrotrophic pathogens. The plants are more resistant to the former and more susceptible to the latter. This result led us to examine the expression of key genes in the SA pathway (critical for resistance to hemibiotrophs) and the JA/ET pathways (critical to resistance to necrotrophs). The data support the hypothesis that PRPK1 functions as a positive regulator of the JA/ET pathways. However, *prpk1* mutations had no effect on

the three SA pathway genes whose expression I measured. Hence, I cannot conclude that PRPK1 functions directly to repress the expression of the SA pathway. However, it is known that the JA/ET and SA pathways act in opposition (126). Hence, defects in the JA/ET pathway could lead to enhancement in the resistance to hemibiotrophic pathogens. In addition, this observation is also supported by the fact that the difference between the wild-type and *prpk1* mutants challenged with the hemibiotrophic pathogen *P. syringae* was not as dramatic as the response to the necrotrophic fungus *Sclerotinia*.

PRPK1 is a downstream signaling component shared among hormone signaling and PAMP signaling pathways

Chitin treatment of known mutants defective in either the SA or JA/ET pathway showed that the early responses to this PAMP (e.g., ROS production and early gene expression) were not affected. Hence, chitin signaling, similar to other PAMPs that have been studied, is independent of well established defense pathways (at least in early steps) (9, 34). However, PAMP treatments do lead to upregulation of both SA and JA/ET pathway components but this is generally viewed as a later event (14).

A study from our own lab provides one example. Son et al. (85, 86) identified a complex network of transcription factor (TF) interactions that mediate the response to chitin and likely other PAMPs. Among the transcription factors in this network was the Ethylene Response Factor ERF5. The expression of ERF5 is rapidly and highly induced by chitin (60). Son et al. chose ERF5 for further study since it was a nodal transcription

factor within the TF interacting network (86). Similar to my results with PRPK1, ERF5 mutants showed increased susceptibility to necrotrophic fungal pathogen infection and increased resistance to *P. syringae*. This phenotype was mirrored when the expression of key JA/ET and SA pathway genes were examined in the wild-type and ERF5 mutant plants. Therefore, if PRPK1 is essential for chitin signaling, mutations in this gene would block the activation of the downstream TF network. The net effect would be increased susceptibility to necrotrophic pathogens and increased resistance to *P. syringae*, as measured in the *prpk1* mutants.

My data also identified PRPK1 as a regulator in flg22 signaling, which leads to a hormone-independent PTI and a hormone regulating pathway. In the first, flg22 triggered immunity is independent of the SA and JA/ET signaling pathways, indicated by the consistent flg22-induced resistance to pathogens in mutants defective in SA and JA/ET signaling (6). However, in the other parallel pathway, it was shown that flg22 signaling can enhance SA accumulation (68) as well as induce the release of ERF104 from MPK6 via ET signaling (69). PRPK1 being involved in both flg22 signaling pathway and JA/ET signaling pathway suggests that PRPK1 is likely a component in flg22-triggered hormone regulating pathway. Furthermore, unlike the *fls2* mutant, which lost the flg22-induced resistance to *P. syringae* (6, 127), *prpk1* mutants are constitutively resistant to *P. syringae*. Hence, PRPK1-regulated flg22 signaling (likely the branch of flg22-triggered hormone regulating signaling) is also independent of flg22-triggered immunity. In conclusion, my data support the report that flg22 signaling triggers both hormone

signaling and flg22-triggered immunity and the former signaling is independent from the latter signaling.

PRPK1 regulates two independent responses in PAMP-triggered signaling, ROS production and callose deposition

Mutations in *PRPK1* lead to increased ROS production, as well as decreased callose deposition. Since both are thought to be indicative of PTI, these results seem to be contradictory. However, similar findings were previously reported from a study of mutants in the *stomatal cytokinesis-defective 1* (*SCD1*) gene (128). In that study, the *scd1-1* mutant decreased ROS production but induced callose deposition without any PAMP treatment. Hence, ROS production and callose deposition, although normally thought to be associated with plant defense, are clearly independent responses and not necessarily linked. ROS production is also a very early response in PTI, while callose deposition occurs much later (1, 3, 129).

The increase in ROS production and decrease in callose deposition in *prpk1* mutants may promote infection by necrotrophic fungi

Although ROS production has antimicrobial activity (58, 64), excessive ROS production can also facilitate necrotrophic infection since it promotes programmed cell death (130). This hypothesis would be consistent with the finding that *prpk1* mutants generate significantly more ROS upon PAMP treatment and show increased susceptibility to the necrotrophic fungal pathogen *S. sclerotiorum*. In addition, the lower

callose deposition by the *prpk1* mutants may also promote fungal infection. The accumulation of callose deposition was shown to block fungal colonization by promoting the deposition of lignin-rich material between the plant cell wall and membrane at the site of fungal penetration (131, 132).

PRPK1 is a common downstream regulator in chitin and flg22 signaling

A variety of published reports have shown that each PAMP possesses its own cognate receptor and may trigger unique responses (4, 5). However, clearly some PAMP receptors share interacting partner proteins (e.g., BAK1, BIK1) (43, 45, 48). There is also compelling evidence that the PAMP signaling pathways share many downstream steps (9, 34, 35), including the MAPK cascade (56) and WRKY transcription factors (79), converging on a largely common set of PAMP induced genes (e.g., 441 genes commonly up-regulated by chitin, flg22 and elf18) (9). My data suggest that PRPK1 is also a signaling component shared between the chitin and flg22 signaling pathways.

PRPK1 as a BAK1-like Leucine-rich repeat receptor-like kinase, is likely the missing part of Arabidopsis chitin receptor complex

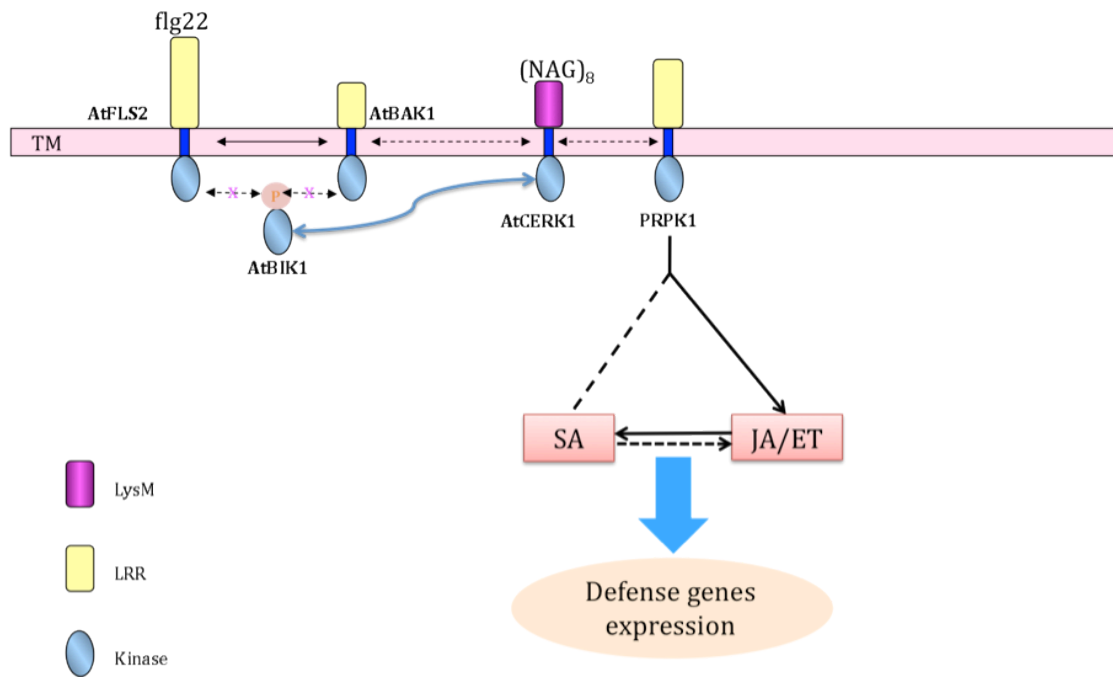
PRPK1, predicted to be a LRR-RLK with a transmembrane domain, was first identified from a Y2H screening as an interactor with the AtCERK1 intracellular kinase domain. Aligning the sequence and secondary structure of the kinase domain of PRPK1 with those of other LRR-RLKs revealed the best template BAK1 for modeling PRPK1 protein structure (Figure III-9 and Figure III-10). We modeled the tertiary structure of

PRPK1 kinase, highly similar to BAK1 kinase (Figure III-11). This is an interesting finding since both BAK1 and the BAK1 homolog BAK1-like1 are known to interact directly with other PRRs and regulate PTI (43, 44, 47, 123).

BAK1 was initially identified as a protein interacting with BRI1, the receptor of the phytohormone brassinosteroid in a ligand-inducible manner (133-135). Later, BAK1 was shown to interact with FLS2 upon the recognition of flagellin by the PRR (44). Furthermore, a recent study using co-immunoprecipitation and mass spectrometry revealed that in addition to BAK1, the BAK1-like1/SERK4 also interact with both FLS2 and EFR (43). Notably, all these interactions only occurred in the presence of the specific ligand (flg22 or elf18) (43). The interaction of BAK1 with FLS2 is critical to flg22-triggered immunity (43, 44). Mutations defective in *BAK1* and *BAK1-like1* result in a loss of response to both elf18 and flg22, including decreased ROS and ethylene production, as well as a reduction in defense gene expression (43). Apart from PRRs, BAK1 was reported to interact with two other LRR-RLKs, which are AtPEPR1 (136) and AtPEPR2 (137). Both are functionally redundant receptors of PEP1 and PEP2; which are plant peptides in Arabidopsis (138-141). The peptides are produced upon wounding, PAMP treatment, or pathogen infection (142) and capable of triggering innate immune responses and enhanced resistance to *Pythium irregulare* infection (138, 139). Similar to the model of FLS2/BAK1 and EFR/BAK1, the interaction between BAK1 and AtPEPR1/AtPEPR2 occurs upon perception of the ligands AtPEP1 and AtPEP2 and is crucial for AtPEP1 and AtPEP2-triggered immunity (136, 137, 141). Taken together, BAK1

is a regulator of PTI through forming receptor complex with FLS2, EFR and AtPEPR1/AtPEPR2 (123). Despite the core role of BAK1 in innate immunity by means of heteromerizing with other RLKs upon ligand recognition, BAK1 does not interact with CERK1. In *Nicotiana benthamiana* it was revealed that BAK1 does not interact with AtCERK1 even with or without chitin treatment (47). However, BAK1 was reported to interact with an AtCERK1 interactor, BIK1 (46). Notably, in the FLS2/BAK1-mediated flg22 signaling, BIK1 is a very early signal component, crucial for flg22-triggered immunity (46, 48). Besides, in *bik1* mutants, some chitin responses are also impaired (48). Therefore, it is possible that PRPK1 as a BAK1-like LRR-RLK plays the role of co-receptor or co-adaptor in the receptor complex of chitin. In this role, PRPK1 could be thought of as analogous to BAK1, which is known to interact directly with other PRRs and regulate PTI (43, 44, 47).

Figure III-11 Model of PRPK1 functioning in chitin signaling and PTI



PRPK1 might interact with AtCERK1 as a co-receptor or co-adaptor to form a receptor complex in perceiving of chitin. PRPK1 positively regulates JA/ET signaling pathway. The hormone signaling pathway, in turn might affect SA signaling pathway. Activation of hormone signaling pathways regulates expression of defense genes, leading to corresponding responses to different kinds of pathogens.

SUMMARY

Chitin, a polymer of β -1,4-N-acetyl glucosamine is an important component of the fungal cell wall. It is one of the best-studied PAMPs and is capable of eliciting basal defense responses against bacterial and fungal pathogens. Recently, the Arabidopsis receptor for chitin was identified as LYK1/CERK1 (9, 28, 36, 38, 81). However, little is known about the molecular basis of chitin perception and how chitin elicitation is translated into a cellular signal.

The goal of my study was to identify novel components involved in chitin signaling and pathogen defense. To accomplish this goal, I employed a variety of molecular and genetic approaches, including forward genetic screening, yeast two-hybrid based protein-protein interaction screening, as well as molecular, genetic and pathology techniques.

I utilized an EMS mutagenesis population with a mutation efficiency of 60% to screen for mutants defective in chitin signaling using as an initial test the ability to produce ROS upon chitin treatment. This screen identified seven, independent mutants. Subsequent tests showed that six of these mutants were specifically affected in their response to chitin, while the remaining mutant showed differences in the response to both chitin and flagellin treatment (Figure I-1 and Figure I-2). In additions to differences in ROS production, the six, chitin-specific mutants also differences in chitin-induced gene expression (Figure I-3) and resistance to bacterial pathogen infection. (Figure I-4)

As was expected, two of the six mutants were identified as new mutant alleles of *AtCERK1* alleles: *Atcerk1-4* (J4-7) and *Atcerk1-5* (M7-2) (Table I-1 and Figure I-6). These new alleles may be valuable for future studies since, unlike the *Atcerk1-1*, *Atcerk1-2* and *Atcerk1-3*, which produce no measurable AtCERK1 protein (9, 28, 87), the two *AtCERK1* alleles in my study express AtCERK1 protein with a modified or severely truncated kinase domain. Previously Miya et al. (28) demonstrated that AtCERK1 possesses a functional kinase domain, which was assumed to be critical for AtCERK1 function. My study of the *Atcerk1-4* and *Atcerk1-5* mutants provided strong support that AtCERK1 kinase activity is essential for chitin signaling and PTI.

Since AtCERK1 is a critical component of chitin-induced PTI, I sought to extend our understanding of the mechanism of this receptor by identifying proteins that interact with the intracellular kinase domain. I employed a LexA-based Yeast 2 Hybrid screen to screen a cDNA library of 4.5×10^6 transformants derived from seedlings treated with chitin. The 54 proteins identified were annotated as both transmembrane and intracellular proteins, including endoplasmic reticulum proteins, transcription factors and kinases (Table II-3). With the high number of transformants screened, it is likely that some are false positives. Therefore, potential interactors were carefully investigated for their involvement in chitin signaling based on their ability to produce ROS upon chitin elicitation, as well as their response to pathogen infection. Genotyping of 110 T-DNA insertion mutant lines of the 54 putative AtCERK1 interactors, 81 homozygous lines for 43 genes were identified. Screening of these 81 mutant lines identified 16 lines that

showed an altered response to chitin, as measured by ROS production (Figure II-3). Subsequent screening of these 16 mutant lines showed that only two, AT2G14840 and AT3G10190, showed an altered response to infection by the bacterial pathogen *P. syringae* (Figure II-4). Those mutants in the AT2G14840 genes were constitutively resistant *P. syringae*, while the AT3G10190 mutant plants were more susceptible to *P. syringae* even when pre-treated with chitin.

AT3G10190 is predicted to encode a calmodulin protein. There are studies showing the involvement of calmodulin proteins in plant innate immunity. For example, calmodulin was shown to bind to a receptor-like kinase, also a negative regulator of plant defense against bacteria (119). The binding of Ca²⁺/Calmodulin to the receptor was predicted to suppress the negative role of the receptor, activating basal defenses in the presence of pathogens (120). The importance of Ca²⁺ in defense signal transduction is further supported by the demonstration that Ca²⁺ chelation by bacterial exopolysaccharides is a virulence strategy used by pathogens to overcome PTI (143).

The AT2G14840 gene is predicted to encode a leucine-rich repeat receptor like kinase, which became the focus of a more detailed analysis. This gene was later named PAMP-responsive protein kinase 1 (PRPK1). PRPK1 is interesting since mutations in this gene alter the response to chitin and flg22 but not elf18 (Figure III-2 and Figure III-3). Subsequent studies showed that *prpk1* mutants showed increased resistance to the hemibiotroph *P. syringae* but were significantly more susceptible to infection by the necrotrophic, fungal pathogen *S. sclerotiorum* (Figure III-4 and Figure III-5). Given the

importance of the JA/ET signaling to defense against necrotrophic pathogens, these results suggested that PRPK1 may act as a positive regulator of the JA/ET pathways. Consistent with this hypothesis, key genes involved in JA and ET signaling were expressed at a significantly lower level in the *PRPK1* mutant lines (Figure III-7).

The JA/ET and SA defense pathways act in opposition to control defense responses to necrotrophic and hemibiotrophic pathogens, respectively. My data suggests that PRPK1 positively regulates JA/ET signaling and, hence, likely indirectly negatively impacts SA signaling pathways. This explanation is supported by the differential response of PRPK1 mutants to infection by *P. syringae* and *S. sclerotiorum*. The recent paper from our lab by Son et al (86) is also consistent with this interpretation. In this case, ERF5, part of a transcription factor interaction network involved in PAMP signaling, were found to differentially regulate the response to hemibiotrophic and necrotrophic pathogens. The expression of *ERF5*, similar to *PRPK1*, is induced by chitin treatment. Unfortunately, although PRPK1 was first identified as a putative interactor with the AtCERK1 kinase domain, I was unable to confirm this interaction using independent methods. However, I continue to believe that this interaction is likely. I based this largely on the analogy between the proposed AtCERK1 receptor complex and what is known about other pattern recognition receptors, especially the well studied FLS2. FLS2 is known to form a complex with BAK1 (43, 44), an LRR-RLK similar to PRPK1, and the cytoplasmic kinase BIK1 (46, 48). BIK1 is also a known interactor of AtCERK1 (48). However, BAK1 does not interact with AtCERK1 (47). Given

the sequence and structural similarity of PRPK1 to BAK1, I propose that PRPK1 plays the role of an adaptor or co-receptor (like BAK1) in the AtCERK1 receptor complex forming a structure very analogous to that involved in flagellin perception. The testing of this hypothesis is a current research priority.

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