Endogenous plant elicitor peptides (Peps) can act to facilitate immune signaling and pathogen defense responses. Binding of these peptides to the Arabidopsis (Arabidopsis thaliana) plasma membrane-localized Pep receptors (PEPRs) leads to cytosolic Ca$^{2+}$ elevation, an early event in a signaling cascade that activates immune responses. This immune response includes the amplification of signaling evoked by direct perception of pathogen-associated molecular patterns by plant cells under assault. Work included in this report further characterizes the Pep immune response and identifies new molecular steps in the signal transduction cascade. The PEPR coreceptor BRASSINOSTEROID-INSENSITIVE1 Associated Kinase1 contributes to generation of the Pep-activated Ca$^{2+}$ signal and leads to increased defense gene expression and resistance to a virulent bacterial pathogen. Ca$^{2+}$-dependent protein kinases (CPKs) decode the Ca$^{2+}$ signal, also facilitating defense gene expression and enhanced resistance to the pathogen. Nitric oxide and reduced nicotinamide adenine dinucleotide phosphate oxidase-dependent reactive oxygen species generation (due to the function of Respiratory Burst Oxidase Homolog proteins D and F) are also involved downstream from the Ca$^{2+}$ pathogen. Nitric oxide and reduced nicotinamide adenine dinucleotide phosphate oxidase-dependent reactive oxygen species generation (due to the function of Respiratory Burst Oxidase Homolog proteins D and F) are also involved downstream from the Ca$^{2+}$ signal in the Pep immune defense signal transduction cascade, as is the case with BRASSINOSTEROID-INSENSITIVE1 Associated Kinase1 and CPK5, CPK6, and CPK11. These steps of the pathogen defense response are required for maximal Pep immune activation that limits growth of a virulent bacterial pathogen in the plant. We find a synergism between function of the PEPR and Flagellin Sensing2 receptors in terms of both nitric oxide and reactive oxygen species generation. Presented results are also consistent with the involvement of the secondary messenger cyclic GMP and a cyclic GMP-activated Ca$^{2+}$-conducting channel in the Pep immune signaling pathway.

Ca$^{2+}$ elevation within the cell cytosol is an early and critical step in the signal transduction cascades that lead to plant innate immunity against infectious pathogens (Lecourieux et al., 2006). One level of immune defense responses is initiated upon plant cell perception of evolutionarily conserved components of microbial invaders, pathogen (or microbe)-associated molecular patterns (PAMPs). Binding of PAMPs to plant cell pattern recognition receptors (PRRs) typically initiates a signaling cascade that evokes basal immune responses in plants. A well-characterized PAMP:receptor protein system in plants is the bacterial motility organ protein flagellin (typically studied by use of the active epitope peptide flag22) and its cognate PRR Flagellin Sensing2 (FLS2; Aslam et al., 2008, 2009; Jeworutzki et al., 2010).

Ca$^{2+}$-dependent protein phosphorylation, generation of reactive oxygen species (ROS), which can act as signaling and antimicrobial molecules, and defense gene expression occur as components of immune responses downstream from PAMP-induced Ca$^{2+}$ elevation (Nürnberger et al., 2004; Ali et al., 2007; Ronald and Beutler, 2010; Segonzac and Zipfel, 2011; Boudsocq and Sheen, 2013).

Another component of immune responses evoked by perception of PAMPs such as flag22 is the increased expression of some members of a family of Plant Elicitor Peptides (Pep1–Pep6). These peptide signals are referred to as elicitor peptides because they evoke defense responses in plants (Yamaguchi and Huffaker, 2011). They are also commonly included with such endogenous plant compounds as oligogalacturonides (structural components of plant cell walls that are released by plant cells under physical assault by pathogenic microbes) as plant compounds that are generated/released during the process of pathogenic infection and involved in activating defense programs (Lecourieux et al., 2006). Oligogalacturonides and Peps are collectively referred to as damage-associated molecular patterns (DAMPs; Boller and Felix, 2009). However, Peps are not necessarily only present at a site of infection due to host cell necrosis; they may be present at the site of infection without having been released from damaged...
cells. Thus, reference to Peps as DAMPs could be an incorrect categorization. However, because here we present an analysis of Pep peptide signaling within the context of what is known about PAMP signaling, we refer to these endogenous plant elicitors as DAMPs to distinguish them here as plant-derived signals that bind to PRRs and initiate defense signaling cascades.

Peps present initially at the site of infection, along with those released from damaged cells as well as subsequently synthesized peptides, and act (in a fashion similar to PAMPs) as extracellular signals that induce immune responses upon binding to plasma membrane-localized PRRs (Pep Receptors or PEPRs [Yamaguchi and Huffaker, 2011]). Pep binding to the PEPR1 or PEPR2 receptor activates a cyclic GMP (cGMP)-dependent Ca\(^2\+)

conduction ion channel (cyclic nucleotide gated channel2 [CNGC2]), leading to pathogen-associated cytosolic Ca\(^2\+)

production (Qi et al., 2010; Ma et al., 2012). Some evidence suggests that PEPRs have a cytosolic guanylyl cyclase (GC) domain that is responsible for cGMP production (Kwezi et al., 2007, 2011). Recent work from this lab supported this hypothesis; affinity-purified recombinant PEPR1-GC domain generated cGMP in vitro (Qi et al., 2010). Ma et al. (2012) extended this work by demonstrating that exogenous application of Pep1, a ligand for the PEPRs, led to in vivo cytosolic cGMP production in Arabidopsis (Arabidopsis thaliana) plants expressing a fluorescence indicator of cGMP generation. Furthermore, mutation of functional residues in the PEPR1-GC domain abolished Ca\(^2\+)dependent immune signaling activated by the Pep ligand.

Ca\(^2\+)dependent protein kinases (CPKs) are Ca\(^2\+) sensors that trigger downstream responses as components of plant immune defense systems (Lee and Rudd, 2002; Boudsocq and Sheen, 2013). In Arabidopsis, the cpl5 cpk6 cpk11 triple null mutant shows decreased flg22-induced gene expression, decreased flg22-induced ROS generation, and impairment of plant resistance to pathogens compared with the wild type and single CPK mutants (Boudsocq et al., 2010). CPKs regulate ROS production through phosphorylation of Respiratory Burst Oxidase Homolog proteins RBOHD and RBOHF (Boudsocq and Sheen, 2013); RBOH proteins are components of an NADPH oxidase that generates superoxide and concomitantly H\(_2\)O\(_2\). Both PAMPs and DAMPs can induce ROS generation (Qi et al., 2010; Schulze et al., 2010). The absence of RBOHD alone or both RBOHD and RBOHF does not influence DAMP- or PAMP-triggered early-stage membrane polarization and cytosolic Ca\(^2\+)

production (Krol et al., 2010). Thus, it can be concluded that RBOH-dependent ROS generation is downstream from Ca\(^2\+) signaling during pathogen defense responses (Segonzac and Zipfel, 2011). It should be noted, though, that Ranf et al. (2011) concluded that there were some feedback effects of ROS on Ca\(^2\+) signaling during pathogen responses. PAMP-triggered defense responses are impaired in rbohd/rbohf double mutant plants (Torres et al., 2002, 2005), suggesting that Ca\(^2\+)dependent ROS generation plays an important role in the signaling leading to innate immunity. Not much is currently known about plant innate immune cascade steps that link RBOH-dependent ROS production to defense gene activation and enhanced immunity.

Nitric oxide (NO) has been identified as a defense signal in plant disease resistance. An NO scavenger diminishes hypersensitive response (HR) to avirulent Pseudomonas syringae pv tomato (Pst), promoting bacterial growth and disease progression (Delledonne et al., 1998). Durner et al. (1998) showed that NO treatment enhances cGMP levels and plant defense gene expression; these effects are impaired by treatment of plants either with an NO scavenger or an inhibitor of NO synthase (NOS) thought to be responsible for NO generation during immune responses in plants. More recently, inoculation of Pst was shown to elevate leaf NO levels (Ma et al., 2008). Application of lipopolysaccharide (LPS), a PAMP that is a cell wall component of gram-positive bacteria, can trigger NO generation in Arabidopsis guard cells (Ali et al., 2007). Impairment of PAMP or avirulent pathogen-mediated NO generation in these studies occurred in the defense no death1 (dnd1; a mutant allele of CNGC2) and cml24-4 (loss of function of calmodulin [CaM]-like [CML] protein) mutants due to defective Ca\(^2\+) transport and NOS activation, respectively. Although NO has been identified as a plant immune defense signal for over a decade (Durner et al., 1998), its induction and function in plant immune responses downstream from flg22 or DAMPs such as Pep is still unknown.

We recently proposed a model for the molecular steps linking perception of an extracellular pathogen-related DAMP (see discussion above about this term as it relates to Peps) ligand (Pep peptides) to Ca\(^2\+)mediated immune responses (Ma et al., 2012). This prior study focused on the PEPR receptors. Here, we expand the characterization of the Pep/PEPR signaling pathway. In this study, we used changes in mitogen-activated protein kinase (MPK3) and transcription factor WRKY33 transcript levels as read-outs to (1) further examine the contribution of the PEPR-GC domain to the signaling cascade, (2) evaluate the contribution of other proteins to the generation of the Ca\(^2\+) signal, and (3) delineate signaling steps downstream from Pep-induced Ca\(^2\+) elevation that mediate immune responses in Arabidopsis. We conclude that Pep-dependent gene expression is induced by this elicitor signal due to cGMP production (possibly by the PEPR receptor), a coreceptor may contribute to PEPR defense responses, and CPKs, NO production, and ROS (H\(_2\)O\(_2\)) generation contribute to downstream gene expression in this immune response signaling cascade.

RESULTS

PAMP-dependent activation of MPKs and WRKY transcription factors have been studied extensively (Wan et al., 2008; Park et al., 2012; Rasmussen et al., 2012); both of these classes of signal transduction
proteins are involved in downstream responses to cytosolic Ca\textsuperscript{2+} elevation induced by PAMPs. Similarly, Pep peptides can trigger cytosolic Ca\textsuperscript{2+} elevations in Arabidopsis wild-type plants (Qi et al., 2010; Ranf et al., 2011), leading to quick transcriptional activation or protein phosphorylation of downstream MPKs and WRKYs (Qi et al., 2010; Yamaguchi et al., 2010; Ranf et al., 2011; Ma et al., 2012). To understand what molecules are involved in the Pep-associated immune responses downstream from the critical early signal of cytosolic Ca\textsuperscript{2+} elevation, we investigated the alteration of MPK3 and WRKY33 transcript levels in response to Pep perception using a range of loss-of-function Arabidopsis mutants and by application of different pharmacological treatments to wild-type plants.

**CNGC2 and BAK1 Contribute to Pep-Induced Ca\textsuperscript{2+} Generation and Downstream Signaling**

All studies of Pep signaling included in this report used the Pep3 peptide (of the six known Pep peptides in Arabidopsis; Huffaker et al., 2006). Both Pep1 and Pep3 evoke pathogen defense responses (Huffaker et al., 2006; Huffaker and Ryan, 2007; Qi et al., 2010; Yamaguchi et al., 2010; Ma et al., 2012). However, Pep3 expression is increased by exposure of Arabidopsis plants to pathogens (and specifically *P. syringae*) as well as by PAMPs (including flg22), while Pep1 expression is not affected by these treatments (Huffaker et al., 2006). Prior studies from this lab also focused primarily on Pep3 as an activating ligand for study of immune signaling (Qi et al., 2010; Ma et al., 2012). In this prior work, Pep3-dependent cytosolic Ca\textsuperscript{2+} elevation was strongly impaired in the *dnd1* mutant, which lacks a functional CNGC2 channel. Here, we extend the study of CNGC2 involvement in Pep signaling leading to defense gene expression. As shown in Figure 1A, the Pep-dependent increase in expression of MPK3 found in wild-type seedlings is greatly reduced in the *dnd1* mutant. This result indicates that CNGC2-dependent Ca\textsuperscript{2+} signaling is important for Pep activation of defense genes.

**BRASSINOSTEROID-INSENSITIVE1 Associated Kinase1 (BAK1)** is a coreceptor for both FLS2 and BRASSINOSTEROID-INSENSITIVE1, which are involved in flagellin and brassinosteroid signaling, respectively (Russinova et al., 2004; Heese et al., 2007). BAK1 was recently found to physically interact with PEPRs (PEPR1 and PEPR2) in vitro (Postel et al., 2010). In vivo evidence indicates that BAK1 and PEPRs can phosphorylate each other after Pep1 treatment (Schulze et al., 2010). Thus, BAK1 may function as a coreceptor with PEPRs and contribute to Pep immune signaling leading to defense gene expression. Consistent with this possibility, we found that, similar to the *dnd1* mutant, Pep-dependent MPK3 expression is substantially impaired in the *bak1-4* null (Chinchilla et al., 2007) mutant (Fig. 1A). We find that BAK1 contributes to the generation of a Ca\textsuperscript{2+} signal in response to both the PAMP flg22 (Fig. 1B) and Pep (Fig. 1C); however, Pep-induced Ca\textsuperscript{2+} elevation is only modestly affected in the *bak1* mutant.
H₂O₂ and NO Production Is Impaired in fls2 and pepr1 Mutants

NO and ROS defense molecules including H₂O₂ are generated downstream from Ca²⁺ in immune signaling cascades (Levine et al., 1994; Delledonne et al., 1998; Ali et al., 2007; Ma et al., 2008, 2009; Moreau et al., 2010; Torres, 2010), and H₂O₂ generation is required for flg22-mediated immune responses (Torres, 2010). Furthermore, chelation of apoplastic Ca²⁺ in Arabidopsis leaves by the extracellular polysaccharide alginate derived from P. syringae strongly inhibits flg22-dependent H₂O₂ generation (Aslam et al., 2008), indicating that flg22-induced H₂O₂ generation is Ca²⁺-dependent. In prior studies from this lab using leaf tissue, an interdependence of flg22 and Pep signaling was noted; maximal signaling by either ligand required the presence of receptors for both the PAMP (FLS2) and the Pep elicitor (PEPR1/PEPR2; Ma et al., 2012). Included in this work were studies demonstrating that, when monitored on a leaf tissue basis, the maximal extent of flg22-dependent ROS generation required functional PEPR1/PEPR2. We speculated that this interdependence of PAMP and DAMP signaling might be due to an amplification of the different signals as perception of pathogen infection is spread from cell to cell, i.e. an enhancement of a ‘warning alarm’ initiated by PAMP perception through the activation of Pep signaling. Pep1-dependent ROS generation has been demonstrated on a whole-leaf (Huffaker et al., 2006) and leaf tissue (Roux et al., 2011) basis, and furthermore, Huffaker et al. (2006) have shown that preventing ROS generation impairs Pep1-dependent defense gene expression in detached, intact leaves. (As noted above, we used Pep3 in all studies.)

In the work reported here, we used a different experimental system to study interdependent flg22 and Pep immune responses. We also evaluated Pep effects on the defense signal NO for the first time. Ligand (either flg22 or Pep) effects on H₂O₂ and NO generation were monitored in individual leaf cells of wild-type, fls2 null mutant, or pepr1 null mutant seedlings (Fig. 2). Early immune signaling events such as membrane depolarization (associated with cytosolic Ca²⁺ elevation) induced by flg22 require the presence of a functional FLS2 receptor (Jeworutzki et al., 2010). Thus, it is not surprising that flg22-dependent H₂O₂ generation was inhibited in cells from fls2 plants compared with the wild type (Fig. 2A). However, maximal H₂O₂ generation in response to Pep also required the presence of both PEPR1 and FLS2 receptors in these single-cell assays (Fig. 2B). This interdependence of PAMP flg22 and DAMP Pep signaling on their respective receptors was
also evidenced with regard to NO generation (Fig. 2, C and D).

NO has been referred to as the concert master of the HR and plant innate immunity (Dangl, 1998), and PAMP-dependent NO generation requires the critical and early Ca\(^{2+}\) elevation in the signaling pathway (Delledonne et al., 1998; Ali et al., 2007). Thus, the results with H\(_2\)O\(_2\) and NO shown in Figure 2 suggest that the functional interaction between FLS2 and PEPR signaling with regard to cytosolic Ca\(^{2+}\) elevation impacts downstream steps of the immune response. The interdependent immune responses we identify here occur when signaling is monitored in individual cells (Fig. 2) and assayed minutes after addition of ligand. This suggests that the functional interaction between the PAMP (flg22) and DAMP elicitor peptide (Pep) signals and the cognate receptors is not just occurring due to amplification of defense responses within the leaf as signals are spread from cell to cell or due to increased expression of PROPEPs upon application of flg22 over several hours (Huffaker and Ryan, 2007). The mechanistic basis for this interdependence of PAMP and plant DAMP signaling at the cellular level is not delineated here; however, it is also not without precedent. Human innate immune responses have been also recently found to include the synergistic amplification of flagellin signaling by an endogenous DAMP peptide; the signaling pathways downstream from the cell membrane flagellin and LL-37 peptide receptors include steps that interact and amplify the immune responses evoked by the individual ligands (Nijnik et al., 2012).

### Immune Pathway Steps That Affect Pep-Induced Ca\(^{2+}\)-Dependent Signaling

Protein phosphorylation and MPK cascades are involved in PAMP signaling (Jeworutzki et al., 2010; Park et al., 2012; Rasmussen et al., 2012); little is known at present about involvement of protein phosphorylation in DAMP signaling (Schulze et al., 2010). Boudsocq et al. (2010) identified some specific CPK isoforms that act as Ca\(^{2+}\) sensors in plant immune signaling cascades and demonstrated that the cpk5 cpk6 cpk11 triple mutant showed compromised immune response to flg22. In their detailed and thorough analysis of CPK involvement in flg22 immune signaling, Boudsocq et al. (2010) also demonstrated that flg22 application to Arabidopsis plants increased endogenous CPK activity and that flg22-dependent increases in defense gene expression were impaired in the cpk5 cpk6 cpk11 triple mutant and by the kinase inhibitor K252a. Here, we find that Pep immune signaling is also compromised in this genotype, as evaluated by monitoring MPK3 and WRKY33 expression (Figs. 3A and 4A). These results suggest that CPK-dependent phosphorylation is involved in the Pep signaling cascade. We also evaluated the contribution of protein phosphorylation as a step in the Pep/PEPR immune signaling pathway by investigating the effect of the kinase inhibitor K252a on Pep-dependent MPK3 and WRKY33 expression (Figs. 3B and 4B). Results with the kinase inhibitor are consistent with the effect of the cpk5 cpk6 cpk11 mutant on Pep signaling; protein phosphorylation is clearly involved in Pep/PEPR immune signaling in a fashion similar to that linked to flg22 signaling.

It has been proposed that NO and H\(_2\)O\(_2\) are involved in many common signaling pathways (for review, see Moreau et al., 2010), and both are involved in pathogen defense signaling as delineated earlier. Both signaling molecules are also generated in response to flg22 and Pep (Fig. 2), suggesting their involvement in Pep/PEPR immune signaling. Results of our gene expression analyses support this contention. NO Associated1 is a gene associated with NO generation; noa1 null mutants

![Figure 3: Genotype and treatment effects on Pep3-dependent MPK3 expression.](image-url)

**Figure 3.** Genotype and treatment effects on Pep3-dependent MPK3 expression. A, MPK3 expression was monitored in wild-type seedlings (with and without exposure to Pep3 for 30 min) and in seedlings with mutations affecting CPK signaling (cpk5 cpk6 cpk11). NO generation (noa1), or ROS production (rbohD/rbohF). Results shown are means \pm SE (n = 4) of transcript levels normalized to the level in wild-type seedlings in the absence of Pep3. Asterisks indicate significant differences (P < 0.05) between the wild type and mutants with Pep treatment. B, MPK3 expression in wild-type seedlings treated with protein kinase inhibitor (K252a), an NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [cPTIO]), or a GC inhibitor (LY83583). Seedlings were pretreated with these compounds prior to the addition of Pep3 for 30 min. MPK3 expression level was normalized to that in the control without inhibitor or Pep added. Results shown are means of three biological replications \pm SE. Asterisks indicate significant differences (P < 0.05) between the levels of expression with inhibitor (and Pep3 added) compared with the control (with Pep added).
Figure 4. Genotype and treatment effects on Pep3-dependent WRKY33 expression. A, WRKY33 expression was monitored in wild-type seedlings (with and without exposure to Pep3 for 30 min) and in seedlings with mutations affecting CPK signaling (cpk5 cpk6 cpk11), NO generation (noa1), ROS production (rbohD/rbohF), or CNGC2 channel function (dnd1). Results shown are means ± se (n = 4) of transcript levels normalized to the level in wild-type seedlings in the absence of Pep. Asterisks indicate significant differences (P < 0.05) between the wild type and mutants with Pep treatment. B, WRKY33 expression in wild-type seedlings treated with protein kinase inhibitor (K252a), an NO scavenger (cPTIO), or a GC inhibitor (LY83583). Seedlings were pretreated with these compounds prior to addition of Pep3 for 30 min. WRKY33 expression level was normalized to that in the control without inhibitor or Pep added. Results shown are means of three biological replications ± se. Asterisks indicate significant differences (P < 0.05) between the levels of expression with inhibitor (and Pep added) compared with the control (with Pep added).

exhibit less NO production than wild-type plants and lack some NO-dependent stress responses (Guo et al., 2003; Moreau et al., 2008). NO has been recognized to be involved in plant disease resistance for over a decade; this mobile and reactive molecule regulates gene expression in both biotic and abiotic stress signaling pathways (Guo et al., 2003; Moreau et al., 2010). We find a similar level of inhibition (compared with wild-type plants) of Pep-dependent MPK3 and WRKY33 expression in the noa1 mutant as observed in the cpk5 cpk6 cpk11 mutant (Figs. 3A and 4A). We also find that application of the NO scavenger cPTIO inhibits Pep-dependent defense gene expression (Figs. 3B and 4B). This pharmacological approach supports the work with the NO mutant; NO generated from Pep binding to its receptor (Fig. 2) is likely an intermediary step in the downstream signaling pathway of this peptide elicitor.

Binding of Pep to its receptor leads to ROS generation as well (Fig. 2; Ma et al., 2012). Pep-evoked ROS generation occurs downstream from cytosolic Ca2+ elevation in this signaling cascade. We used the rbohD/rbohF double mutant to evaluate whether Pep-dependent ROS generation is an important step in the immune signaling initiated by the plant elicitor peptide. A significant reduction of Pep-dependent MPK3 and WRKY33 expression compared with the wild type was observed in this mutant (Figs. 3A and 4A). The level of Pep-dependent WRKY33 expression in the rbohD/rbohF, noa1, and cpk5 cpk6 cpk11 mutants were similar in the experiment shown in Figure 4A.

The primary goal of our studies was to delineate steps in the Pep/PEPR signaling pathway. However, we note that both Pep and flg22 evoke NO and ROS generation in a manner that is independent on their respective PRR receptors (Fig. 2). We did some further work within this context to investigate if some of the steps involved in the Pep signaling pathway were similarly involved in flg22-dependent gene expression. As noted above, the involvement of CPK5, CPK6, and CPK11 in flg22 signaling leading to defense gene expression is well characterized (Boudsocq et al., 2010). The involvement of NO and ROS in the signaling pathway leading to flg22-dependent defense gene expression is less clarified. Dubiella et al. (2013) recently showed that flg22-dependent ROS generation is impaired in the rbohD single mutant, but localized (at the site of treatment) defense gene expression was similar in flg22-treated leaves of wild-type and rbohD mutant plants. These authors concluded that RBOHD-dependent ROS generation was not involved in localized activation of defense genes but rather acted in cell-to-cell communication to activate defense genes in tissues distal to the site of PAMP perception. Macho et al. (2012) also found no effect (at the site of PAMP application) of RBOHD null mutation on flg22-dependent defense gene expression. Here, we extended these studies by examining flg22-dependent WRKY33 and MPK3 expression in the rbohD/rbohF double mutant (Fig. 5). In contrast to a marked difference in Pep-dependent gene expression in the wild type and rbohD/rbohF mutants, flg22 signaling, at least in terms of MPK3 and WRKY33 expression, is not reduced from the wild type in the double mutant.

The involvement of NO in PAMP signaling beyond the studies mentioned above linking LPS application to NO generation is not clarified. Mur et al. (2008) provided indirect evidence that NO is involved in signaling associated with HR response to avirulent pathogens but does not influence some aspects of PAMP-triggered immunity (associated with flg22 application) evaluated in their study. Here, we find that flg22-dependent gene expression was impaired in the noa1 mutant (Fig. 5) in a fashion similar to what occurred when Pep signaling was evaluated in this mutant (Figs. 3A and 4A).
Proteins Involved in CNGC Activation Affect Defense Gene Expression

CaM physically interacts with CNGCs; the CaM binding site on plant CNGCs is in a cytosolic region of the channels and overlaps with the cyclic nucleotide-binding domain (Köhler et al., 1999; Köhler and Neuhaus, 2000; Hua et al., 2003; Ali et al., 2006; Abdel-Hamid et al., 2010). CaM binding diminishes cyclic nucleotide-dependent activation of CNGCs (Hua et al., 2003) and reduces CNGC-dependent Ca\(^{2+}\) influx into cells (Ali et al., 2006). Exposure of Arabidopsis protoplasts to W7, a CaM antagonist, can result in spontaneous CNGC current (Ali et al., 2007; Ma et al., 2008). Prior exposure to W7 increased the cytosolic Ca\(^{2+}\) elevation associated with inoculation of Arabidopsis leaves with *Pst* harboring the avirulence gene *AvrRpt2* (Ma et al., 2008), and prior exposure to W7 increased the PAMP (LPS)-dependent inward current through Ca\(^{2+}\)-conducting channels in patch-clamp studies of Arabidopsis protoplasts (Ali et al., 2007). These W7 effects were attributed to the prevention of CaM blockage of CNGC channel currents. In the presence of a CaM antagonist, CNGC channels might activate at basal levels of cytosolic cyclic nucleotides (i.e. in the absence of a signal that would cause elevation of these secondary messenger molecules). Consistent with these previous observations, incubation of seedlings with W7 alone significantly increased both MPK3 and WRKY33 transcript levels (Fig. 6) in the absence of added Pep. Interpretation of the results with W7 shown in Figure 6 should be made with a degree of caution as well as within a context due to the following points. W7 is not a specific inhibitor of CaM, but rather also binds to and inhibits other proteins with CML domains such as CPKs (although the concentrations required to fully inhibit CPKs are substantially greater than we used here; Syam Prakash and Jayabaskaran, 2006). Of course, CPKs are involved in Pep signaling (Figs. 3 and 4). Thus, W7 effects on CPKs should be considered when extrapolating from the effects of W7 (added in the absence of Pep) on Pep-responsive genes such as WRKY33 and MPK3 shown in Figure 6. However, genetic and pharmacological inhibition of CPK activity reduces Pep-dependent expression of pathogen defense genes (Figs. 3 and 4). Therefore, the increases in WRKY33 and MPK3 transcript levels caused by W7 shown in Figure 6 are not consistent with these responses being mediated by W7 effects on CPKs. The effects of W7 shown here are consistent with the aforementioned effects of W7 on CaM, CNGCs, and Ca\(^{2+}\) currents associated with pathogen signaling mentioned above. Nonetheless, interpretation of effects of inhibitors should always be made with due caution regarding their nonspecificity. In the specific case of W7, general nonspecific effects of this compound can be checked by comparing effects with the W7 analog N-(6-aminohexyl)-1-naphthalene sulphonamide (W5; Syam Prakash and Jayabaskaran, 2006). W5 has reduced affinity for CaM but does mimic the nonspecific effects of W7 on biological systems, such as interaction with membranes. In further experiments, we found that W5 (at 50 μM, the concentration at which W7 was used) had no significant effect on transcript levels of either MPK3 or WRKY33 (data not shown). These results are consistent with the possibility that W7 acts to increase expression of Pep-responsive pathogen defense genes by mimicking the effect of Pep on CNGCs.

![Figure 5](image_url)

**Figure 5.** Genotype effects on flg22-dependent MPK3 and WRKY33 expression. Experimental design and analysis were similar to the work shown in Figures 3A and 4A, except 1 μM flg22 was used as an activating ligand.

![Figure 6](image_url)

**Figure 6.** Effect of a CaM antagonist (W7) and an inhibitor of cGMP breakdown (3-isobutyl-1-methylxanthine [IBMX]) on WRKY33 and MPK3 expression. Wild-type seedlings were treated with W7 or IBMX for 30 min prior to measurement of WRKY33 or MPK3 expression. Results shown are means ± se (n = 3) of transcript levels normalized to the level in wild-type seedlings without treatment. Asterisks indicate significant differences (*P* < 0.05) between the control (water) and treatment with W7 or IBMX.
IBMX is a cyclic nucleotide phosphodiesterase (PDE) inhibitor that prevents the breakdown of cyclic nucleotides in plant and animal cells (Ma et al., 2009). A number of prior studies have shown that (1) PDE activity is relatively high in leaf mesophyll cells of plants (Li et al., 1994), (2) altering PDE activity in plant cells through IBMX application increases signaling dependent on cytosolic levels of cyclic nucleotide (Li et al., 1994; Volotovski et al., 1998; Ma et al., 2009), (3) the resting state level of cyclic nucleotides in plants cells is influenced by PDE activity (Kurosaki and Kaburaki, 1995), and (4) alterations in the level of cyclic nucleotides in plants during signaling cascades are associated with alterations in PDE activity (Cook et al., 1994; Assmann, 1995). This body of research suggests that the turnover of cyclic nucleotides in plant cells is rapid enough to be influenced by effects of IBMX application on PDE activity and that application of a PDE inhibitor such as IBMX could affect basal levels of cyclic nucleotides and initiate signaling downstream from this secondary messenger molecule.

In a fashion similar to W7, exposure of seedlings to IBMX increased WRKY33 expression in the absence of the activating Pep ligand (Fig. 6), possibly due to the increased cAMP or cGMP levels in the cytosol. The results shown in Figure 6 suggest that the activation of CNGCs (by blocking CaM effects or the inhibition of cyclic nucleotide hydrolysis) increases defense gene expression. Conversely, the inhibition of cyclic nucleotide generation should attenuate Pep-induced defense gene expression. Exposure of seedlings to the guanylyl cyclase inhibitor LY83583 impaired Pep-dependent defense gene expression. Exposure of seedlings to the guanylyl cyclase inhibitor LY83583 impaired Pep-dependent increases in MPK3 and WRKY33 expression (Figs. 3B and 4B). These results with the CaM antagonist and IBMX in the absence of Pep and the GC inhibitor in the presence of Pep are consistent with the genetic evidence in Figure 1 (with the dnd1 mutant) and suggest that CNGCs and cyclic nucleotides may play important roles in the Pep/PEPR signaling pathway leading to defense gene activation.

Pep-Triggered Immunity against *Pst* DC3000 Is Compromised in *cpk* and *bak1* Mutants

Prior exposure of plants to the PAMP flg22 or Pep can lead to an immune response; subsequent bacterial inoculation of plants pretreated with either peptide leads to a reduction (compared with untreated plants) in growth of the virulent pathogen *Pst* DC3000 (Zipfel et al., 2004; Ma et al., 2008; Boudsocq et al., 2010; Yamaguchi et al., 2010; Ma et al., 2012). Here, we extend the characterization of the immune responses triggered by the elicitor peptide Pep signal to determine if some of the steps involved in Pep-dependent increases in expression of MPK3 and WRKY33 are critical to the ultimate end point of the signal transduction cascade, i.e. the development of an immune response. Compared with wild-type plants, the *cpk5* *cpk6* *cpk11* and *bak1* mutants demonstrate reduced defense gene expression upon Pep treatment (Figs. 1A, 3A, and 4A), suggesting that the immunization response might be compromised by the impairment of the Pep signal transduction cascade in these plants. We examined this hypothesis in the experiment shown in Figure 7A by monitoring growth of *Pst* DC3000 in these mutant genotypes. Application of Pep led to an immunization response in wild-type plants; *Pst* DC3000 bacteria recovered from Pep-treated plants 3 d after inoculation was reduced by 75% compared with water-treated wild-type plants (Fig. 7A). The extent to which Pep pretreatment restricted bacterial growth was impaired in the *cpk5* *cpk6* *cpk11* and *bak1* mutants compared with the level of immunization displayed by wild-type

![Figure 7. Evaluation of Pep3-induced immunity to a virulent pathogen in leaves of wild-type plants and in genotypes that have mutations in genes that affect Pep-dependent expression of defense genes. A, Proliferation of (virulent) *Pst* DC3000 on leaves of wild-type, *cpk5* *cpk6* *cpk11*, and *bak1-4* mutant plants pretreated with water (control) or Pep3. Plants were pretreated 1 d prior to inoculation with *Pst* and bacterial growth was evaluated 3 d after inoculation. Results shown (note log scale of ordinate axis) are mean values of *Pst* recovered from leaves (*n* = 4 ± se on day 3. Measurement of bacteria recovered from leaves on day 0 (immediately after inoculation) indicated that there was no significant difference between any of the treatments (data not shown). B, In another experiment, effect of Pep3 pretreatment on suppression of *Pst* growth in *rbohD/*rbohF and *noa1* plants was compared with the effect of Pep on *Pst* growth in wild-type plants. Experimental design and analysis were similar to the work shown in A.](image-url)
null mutation of FLS2 affected both flg22- and Pep-dependent ROS expression and immune signaling were reported by Ranf et al. (2011). Effects of CPKs, CaM, and/or CML proteins. f, Ca2+ activation of CPKs results in modulation of defense gene expression. i, Ca2+/CaM or a CML protein can lead to NO generation by an unidentified NOS-type enzyme (Ma et al., 2008). NO can act as a cell-to-cell signal and also has effects on defense gene expression. h, Ca2+/CaM or a CML protein can lead to NO generation by an unidentified NOS-type enzyme (Ma et al., 2008). NO can act as a cell-to-cell signal and also modulate defense gene expression. i, Ca2+/CaM or a CML protein can feedback on CNGC currents and prevent further activation by cyclic nucleotides (Fig. 5; Hua et al., 2003). j, Flagellin/FLS2 interaction leads to immune responses separately from Pep/PEPR signaling (but as noted above, there is a functional interaction between the two pathways). Experimental results included in this report probed steps of the Pep signal transduction cascade (and by kinase inhibitors). Null mutation of these CPK genes also impaired the ability of Pep to immunize the plant against growth of virulent bacteria. The cpk5 cpk6 cpk11 triple null mutant (and by kinase inhibitors). Null mutation of these CPK genes also compromised the ability of Pep to immunize the plant against growth of virulent bacteria. The cpk5 cpk6 cpk11 triple null mutant also affects flg22 defense signaling. 6, Pep-dependent defense gene expression and immunization against virulent bacterial growth was compromised in the noa1 mutant, which lacks the ability to generate NO in response to external stimuli. Defense gene expression down-stream from flg22 perception was affected by the noa1 mutation in a fashion similar to effects on Pep/PEPR signaling. 7, Pep-dependent defense gene expression and Pep-induced immunization of the plant against growth of virulent bacteria were compromised in the rbohD/rbohF double mutant. In contrast to Pep/PEPR signaling, the rbohD/rbohF double mutation did not affect flg22-dependent defense gene expression. 8, Application of W7 leads to increased expression of pathogen defense gene expression without Pep application; this could be due to W7 antagonism of CaM function, leading to increased CNGC currents at ambient levels of cyclic nucleotide. 9, Application of the GC inhibitor LY83538 impairs Pep-dependent defense gene expression. 10, Application of the cyclic nucleotide PDE inhibitor IBMX results in increased expression of WRKY33 without Pep application; this might be due to an increase in ambient cell cGMP because breakdown of the cyclic nucleotide is impaired. 11, Application of the NO scavenger cPTIO impairs Pep-dependent defense gene expression. 12, Application of the kinase inhibitor K252a impaired Pep-dependent defense gene expression, possibly due to inhibition of CPKs or other kinases involved in the signaling pathway. The kinase inhibitor K252a also blocks flg22 signaling (Boudsocq et al., 2010; Jaworutski et al., 2010).

**DISCUSSION**

The desired physiological outcome of immune signaling in the face of a pathogenic challenge is an enhancement of host system fitness (as opposed to the fate of individual cells at the infection site that may or may not survive a skirmish; they could undergo programmed cell death to limit infection spread). Studies included in this report identified some specific steps involved in the Arabidopsis Pep signal transduction cascade and demonstrated in several cases (BAK1, CPK, NO, and ROS) that these molecules contribute to the positive effect the signaling pathway has on plant immunity. As indicated earlier, many of the steps in the Pep signal transduction cascade downstream from generation of the early Ca2+ signal are also involved in the evocation of defense programs upon perception of PAMPs such as flagellin. We show here that, on an individual cell basis, there is an interdependence of flg22 and Pep signaling; maximal generation of NO and ROS and NO generation. 4, Null mutation of PEPR1 impaired both pep- and flg22-dependent ROS and NO generation. 5, Pep-dependent gene expression was impaired in the cpk5 cpk6 cpk11 triple null mutant (and by kinase inhibitors). Null mutation of these CPK genes also compromised the ability of Pep to immunize the plant against growth of virulent bacteria. The cpk5 cpk6 cpk11 triple null mutant also affects flg22 defense signaling. 6, Pep-dependent defense gene expression and immunization against virulent bacterial growth was compromised in the noa1 mutant, which lacks the ability to generate NO in response to external stimuli. Defense gene expression downstream from flg22 perception was affected by the noa1 mutation in a fashion similar to effects on Pep/PEPR signaling. 7, Pep-dependent defense gene expression and Pep-induced immunization of the plant against growth of virulent bacteria were compromised in the rbohD/rbohF double mutant. In contrast to Pep/PEPR signaling, the rbohD/rbohF double mutation did not affect flg22-dependent defense gene expression. 8, Application of W7 leads to increased expression of pathogen defense gene expression without Pep application; this could be due to W7 antagonism of CaM function, leading to increased CNGC currents at ambient levels of cyclic nucleotide. 9, Application of the GC inhibitor LY83538 impairs Pep-dependent defense gene expression. 10, Application of the cyclic nucleotide PDE inhibitor IBMX results in increased expression of WRKY33 without Pep application; this might be due to an increase in ambient cell cGMP because breakdown of the cyclic nucleotide is impaired. 11, Application of the NO scavenger cPTIO impairs Pep-dependent defense gene expression. 12, Application of the kinase inhibitor K252a impaired Pep-dependent defense gene expression, possibly due to inhibition of CPKs or other kinases involved in the signaling pathway. The kinase inhibitor K252a also blocks flg22 signaling (Boudsocq et al., 2010; Jaworutski et al., 2010).
by either peptide requires both cognate receptors. The molecular basis for this is not delineated. However, this interdependence of PAMP and DAMP signaling on a cellular level also occurs in animal cells. In the case of at least some animal DAMP peptides and flagellin (as discussed above), the cell signaling cascades responding to the perception of these peptides share many molecular steps and act synergistically (Nijnik et al., 2012). Our work with the double NADPH peroxidase mutant rhoD/rhoF notes a difference in the involvement of these ROS-generating proteins in Pep/PEPR and flagellin/FLS2 signaling leading to defense gene expression.

Results in this report indicate that BAK1 and the Ca\(^{2+}\) channel CNGC2 contribute to Pep-dependent generation of the Ca\(^{2+}\) signal. However, the impact of BAK1 loss of function on Pep-dependent cytosolic Ca\(^{2+}\) elevation is modest, perhaps less severe than corresponding effects on flg22-dependent Ca\(^{2+}\) elevation (Fig. 1). Nonetheless, BAK1 loss of function did impair the immune response stimulated by application of exogenous Pep (Fig. 7).

Within the context of prior work in the area of innate immune signaling associated with cytosolic Ca\(^{2+}\) elevation upon pathogen perception, we conclude that CPKs are involved in transmitting the early Ca\(^{2+}\) signal to downstream steps such as defense gene expression. Use of a kinase inhibitor supported our conclusions based on use of the cphk5 cphk6 cphk11 mutant. However, it should be noted that some evidence suggests that protein phosphorylation might also be involved in early steps of flagellin and Pep peptide signaling, in addition to the downstream role of CPKs in the signal transduction pathway (Jeworutzki et al., 2010; Schulze et al., 2010). The kinase inhibitor studies here would not discriminate between these different signaling steps.

We speculate, based on numerous prior studies with these pharmacological agents as well as work included in this report, that the cyclic nucleotide PDE inhibitor IBMX, the CaM antagonist W7, and the GC inhibitor LY83583 affected the Pep signaling pathway upstream from Pep-induced cytosolic Ca\(^{2+}\) elevation. The results of these studies suggested the following trend: inhibition of cGMP generation (LY83583) impaired the Pep signaling pathway, and agents that possibly raise cytosolic cGMP (IBMX) or might allow activation of a Ca\(^{2+}\) channel in the absence of cGMP elevation (W7) mimic the effect of Pep and initiate the signaling pathway in the absence of added exogenous ligand. (We note, however, that application of IBMX resulted in a significant increase in WRKY33 expression but did not increase MPK3 expression [Fig. 6].) The work presented in this report focused on innate immune signaling downstream from Pep3 (referred to here, as well as in the literature, as a DAMP), a PAMP (flg22), and a virulent pathogen that, when inoculated onto plants, evokes defense pathways dependent on the PAMP and DAMP.

In summary, the work presented in this report extends our knowledge of the signal transduction cascade evoked by Peps. These peptides, and molecules involved in the signal transduction pathway evoked by these signals, contribute to defense responses of plants to microbial pathogens. The Pep/PEPR pathogen defense signal transduction pathway developed from the work presented in this report, as well as from prior studies from this lab and others, is represented in the model presented in Figure 8.

**MATERIALS AND METHODS**

**Plant Materials**

In addition to wild-type Columbia plants, the following mutant genotypes (all previously characterized) were used in this work: the cphk5 cphk6 cphk11 triple mutant (Boudsocq et al., 2010), nox1 (Durner et al., 1998), rhoD/rhoF double mutant (Torres et al., 2002), dhu1 (Ali et al., 2007), and two alleles of baki, baki-1 (Chinchilla et al., 2007) and baki-4 (Gou et al., 2012). The baki-1 genotype was originally characterized as lacking expression of any BAK1 mRNA (Chinchilla et al., 2007). We used the baki-1 genotype to generate baki mutant plants expressing the Ca\(^{2+}\) reporter protein apoaequorin (see below). However, recent studies (Gou et al., 2012) have presented a more nuanced analysis of this BAKI allele; although the transcript level of BAK1 in not detectable in baki-1, it is a leaky mutant, and in the baki-1 like genetic background, baki-3 does not display null phenotypes. Gou et al. (2012) presented an analysis of the baki-4 allele and confirmed that it is a true baki null mutant. We therefore used baki-4 mutant plants for subsequent experiments. We conclude that the use of both baki alleles in our work is not a problem based on the following point. Randl et al. (2011) demonstrated that the level of cytosolic Ca\(^{2+}\) elevation in response to both flg22 and Pep is similar (and not significantly different \(P < 0.05\) ) in leaves of baki-1 and baki-3 plants.

Seeds of Arabidopsis (Arabidopsis thaliana) genotypes were surface sterilized and spread on petri dishes containing one-half-strength Murashige and Skoog medium (Caissios), 2.6 mM MES (adjusted to pH 5.7 with Tris), 1% (w/v) Suc, and spread on petri dishes containing one-half-strength Murashige and Skoog medium (Caisson), 2.6 mM MES (adjusted to pH 5.7 with Tris), 1% (w/v) Suc, and 0.8% (w/v) agar (Ma et al., 2012). These seeds were germinated and grown in a growth chamber for 1 week with a day (80–100 μmol m\(^{-2}\) s\(^{-1}\) illumination)/night cycle of 12 h/12 h at 25°C. The seedlings were then transferred to pots containing LPS potting mix with starter fertilizer (Sun Gro) and were grown in a growth chamber at 16-h light (100 μmol m\(^{-2}\) s\(^{-1}\) illumination)/8-h dark (72% relative humidity) and 22°C (see below for liquid-grown seedlings used for gene expression analysis). Seeds were incubated at 4°C in the dark for 2 d prior to use to facilitate stratification and increase germination. During growth, plants were irrigated with Jack’s Professional Peat-Lite 20:10:20 (N-P\(_2\)O\(_5\)-K\(_2\)O) solution (at 2 g L\(^{-1}\) ) one to two times to provide supplementary fertilizer.

**RNA Isolation and Complementary DNA Synthesis**

Growth of seedlings in one-half-strength Murashige and Skoog liquid medium was performed according to Yamaguchi et al. (2010). Liquid-grown 10-d-old wild-type and mutant genotype seedlings (one seedling grown in a separate tube with 3 mL medium was used as a genotype and treatment replicates) were used for experiments. Wild-type seedlings were treated with 50 μM W7 (Enzo Life Sciences; for use of IBMX, see Ma et al., 2009) in the absence of Pep for 5 min prior to collection for analysis of gene expression. In other experiments, seedlings were treated with 2 μM K2536 (Sigma; Zottini et al., 2007), 500 μM 4’DPTO (Enzo Life Sciences; Zottini et al., 2007), 20 mM LY83583 (Enzio Life Sciences), or water as a control for 5 min with shaking at 180 rpm, followed by addition of either 20 nm Pep (this concentration was used for all experiments) or water for a further 30 min with shaking prior to collection. For comparison of gene expression in wild-type and mutant genotypes, seedlings were treated for 30 min with 20 μM Pep (or water as a control) prior to collection. After treatments, seedlings were collected and frozen immediately in liquid nitrogen and processed for RNA extraction or stored at −80°C for future use. Total RNA from a whole liquid-grown seedling was isolated using the Plant RNA Extraction Kit (Macherey-Nagel) according to the manufacturer’s manual.

After preparation, 500 ng of total RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer’s protocol. The synthesized complementary DNA was made into a 1:5 dilution, and 1 μL of the diluted complementary DNA was used for the subsequent quantitative real-time PCR.
Quantitative Gene Expression Assay

The quantitative PCR was performed using the ABI 7900 HT Real-Time PCR system. The TaqMan gene expression assay (Applied Biosystems) method was used, and the expression level of WRYK33 (At1g38470) and MPK3 (At3g45640) were examined. Ubiquitin (UBQ5, At3g26250) was employed as an internal control. The primers used were WRYK33-a (forward; 5'-GCAAACAAATGGGTGCGGTTG-3') and WRYK33-b (reverse; 5'-TGTTTGTCACATTCCTTCG-3'). MPK3-a (forward; 5'-ATGCCCTATACGAGGTT-3') and MPK3-b (reverse; 5'-TACAGGCTTGTTAACACTG-3'). UBQ5-a (forward; 5'-AGATCAAGCACAACTGTT-3') and UBQ5-b (reverse; 5'-CAGATACGCTTACACTCT-3'). ANOVA of corresponding threshold cycle values was used for means separation among treatments in an experiment and to generate $s_2$ of the means for control treatments (for details, see Ma et al., 2012).

Cytosolic Ca²⁺ Measurements

This method was adapted from Qi et al. (2010) with slight modifications (for complete details of how aequorin [aq] expressing lines were generated, leaves were handled, and how luminometer recordings were used to calculate cytosolic Ca²⁺ levels, see Qi et al., 2010). For the work reported here, we used a line of bak1-3 mutant plants expressing aequorin by crossing the mutant with a wild-type line expressing aequorin. The T3 progeny of the cross were checked for absence of a full-length BAK1 gene and optimized for aequorin expression using methods described previously (Qi et al., 2010). Pep3 (20 nM) and flg22 (1 μM) ligand effects on cytosolic Ca²⁺ levels were evaluated in whole detached leaves of 3- to 4-week-old plants expressing cytosol-localized Ca²⁺-dependent chemiluminescent aequorin protein re-constituted with coelenterazine-cp. The concentration of flg22 used in our studies matched that typically used to compare the effects of this PAMP with other PAMPs (Rand et al., 2011). flg22 concentrations as low as 0.1 nM have been shown to cause cytosolic Ca²⁺ elevations in Arabidopsis (Aslam et al., 2009; Jeswurutski et al., 2010). However, Aslam et al. (2009) found that increasing the concentration of flg22 even up to 0.5 μM (higher levels were not checked in this study) caused increasingly greater Ca²⁺ elevations, supporting the logic of using such a high concentration of ligand. We used 20 nM Pep3 here because prior work from this lab (Qi et al., 2010) indicated that repeatable PEPR1-dependent elevations in cytosolic Ca²⁺ could be obtained from Arabidopsis leaves with this concentration of the elicitor. Other studies of Pep signaling used similar concentrations of activating ligand (Yamaguchi et al., 2010). Although the concentrations of activating ligands used here suggest different relative potencies, we did not investigate this point in our work.

Ligands were added at time 0, and signals shown are averages of biological replicates from different plants. A single leaf was cut at the petiole for each treatment replicate. Detached leaves were vacuum infiltrated with 10 μM coelenterazine-cp in 500 μL control buffer (in a 2-mL centrifuge tube) containing 1 mM KCl, 1 mM CaCl₂, 200 mM MgCl₂·6H₂O, and 10 mM MgCl₂·6H₂O for 10 s and kept in darkness at room temperature for 1 h to allow coelenterazine incorporation into the leaves. For luminometer recordings, the centrifuge tube was placed into a luminometer (TD-20/20, Turner Designs) and kept in the dark for 2 to 3 min for recovery from handling and dissipation of touch-induced Ca²⁺ spikes; ligands were added to leaves only after they displayed a stable level of luminescence. Ligands were added (at 2× final concentration) in 500 μL of control buffer. Discharge of the remaining aequorin after luminescence measurements was done by adding 800 μL 30% ethanol containing 2 mM CaCl₂·6H₂O. se was calculated every minute.

NO and H₂O₂ Production in Guard Cells

The method for NO detection in guard cells using the NO-specific fluorescent dye diaminofluorescein-2 diacetate (Invitrogen) was adapted from Ali et al. (2007). The method for H₂O₂ detection in guard cells using the H₂O₂-specific fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (Invitrogen) was adapted from Gerber et al. (2004). Rosette leaves of 4- to 6-week-old wild-type, bs2, and pep71 plants were detached from plants and used to make 2-mm-wide epidermal peels. Epidermal peels were incubated in buffer A (10 mM KCl, 25 mM MES-KOH, and 0.1 mM CaCl₂·6H₂O at pH 6.15) for 1 to 2 h. For assays of NO generation, epidermal peels were transferred to 3 mL of buffer A containing 50 μM diaminofluorescein-2 diacetate and incubated for 30 min to load cells with NO-specific dye. For assays of H₂O₂ generation, peels were alternatively placed in 3 mL buffer A, which contained 50 μM 2',7'-dichlorodihydrofluorescein diacetate for 30 min. After loading NO- and H₂O₂-specific dye in the tissue, peels were washed three times in buffer A and incubated in 3 mL of buffer A (for H₂O₂ assays) or buffer B (for NO assays) containing flg22 (1 μM) or Pep (20 nM) for 5 to 10 min. The epidermal peels were placed underneath a cover slip on a microscope slide with several drops of buffer A (or B as appropriate, with ligands added). Both NO- and H₂O₂-dependent fluorescence were monitored over time.

Mean maximal fluorescence of guard cells (occurring approximately 5 to 10 min after ligand addition) in a peel was ascertained exactly as described previously (Ma et al., 2009). Fluorescence and bright-field images were captured using an inverted Olympus IX70 microscope and green fluorescence protein excitation and emission filters. Digitized images were acquired using a MagnaFire CCD camera and software. Maximal fluorescence measurements of approximately six guard cells in an epidermal peel were averaged for one replicate. Representative guard cell fluorescence images recorded from leaf epidermal strips prepared from wild-type, bs2, and pep71 plants are shown in Figure 2. Captured images at the maximum fluorescence intensity for treatment replicates were used to calculate data represented as means. Quantitative analyses of the NO- and H₂O₂-dependent fluorescence in guard cell pairs were undertaken using ImageJ software as described in Ali et al. (2007). The digitized image showing maximum fluorescence for guard cell pairs from an epidermal peel represented a genotype replicate; a minimum of three epidermal peels were analyzed for each genotype.

Growth of Virulent Pseudomonas syringae in Plants

The following method for precrop of plants to Pep and subsequent inoculation of leaves with virulent pathogen was adapted from Yamaguchi et al. (2010). Pep (or water as a control) was syringe injected into (attached) rosette leaves from wild-type, cpk6 cpk6 cpk11, bak1, rbohD/rbohD, or noa1 plants 24 h prior to bacterial inoculation. Peptides were delivered to the leaves from a blunt-end 1-mL syringe in approximately 0.5 mL water. Pseudomonas syringae pv tomato DC3000 was grown at 28°C on low-salt (1 g L⁻¹ NaCl) Luria-Bertani (Fisher Scientific) 2% (v/v) agar medium containing 100 mg L⁻¹ rifampicin for about 2 d. Colonies from these plates were used for liquid overnight cultures (medium composition was the same as the plates except no agar was used). Bacteria were isolated from overnight liquid cultures and resuspended in sterile 10 mM MgCl₂·6H₂O to a concentration of 3 × 10⁹ colony forming units mL⁻¹. The resuspended bacteria were applied (in 0.01% [v/v] Silwet L-77 surfactant) by spraying on leaves (previously treated with water or peptides) from 4- to 5-week-old plants. After spray inoculation, plants were covered with a plastic lid and placed in a growth chamber for 3 d. Leaf tissue was collected for measurement of bacterial titer on the day of inoculation (day 0) and 3 d post inoculation. For each biological replicate, 0.7-cm-diameter leaf discs were cut from leaves of two different plants and ground together in 100 μL of 10 mM MgCl₂·6H₂O in a 1.5-mL tube. The samples were thoroughly vortexed in 900 μL of water and diluted 1:10 serially. Then 10 μL of the samples (and serial dilutions) were spread on plates containing low-salt Luria-Bertani agar medium containing 100 mg L⁻¹ rifampicin. Plates were left at 25°C for 2 d; after this incubation period, colonies on plates were counted.

ACKNOWLEDGMENTS

We thank Jen Sheen (Harvard University) for providing cpk6 cpk6 cpk11 seeds, June M. Kwak (University of Maryland) for providing rbohD/rbohD seeds, Nigel M. Crawford (University of California, San Diego) for providing noa1 seeds, and Johannes Stratmann (University of South Carolina) for providing bak1 seeds.

Received August 8, 2013; accepted August 27, 2013; published September 9, 2013.

LITERATURE CITED

Abdel-Hamid H, Chin K, Shahinas D, Moeder W, Yoshioka K (2010) Calmodulin binding to Arabidopsis cyclic nucleotide gated ion channels. Plant Signal Behav 5:1147–1149

Ali R, Ma W, Lemtiri-Chlieh F, Tsalts D, Leng Q, von Bodman S, Berkowitz GA (2007) Death don’t have no mercy and neither does calcium: Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell 19:1081–1095

Ali R, Zielinski RE, Berkowitz GA (2006) Expression of plant cyclic nucleotide-gated cation channels in yeast. J Exp Bot 57:125–138

Aslam SN, Erbs G, Morrissie KL, Newman MA, Chinchilla D, Boller T, Molinaro A, Jackson RW, Cooper RM (2009) Microbe-associated...
molecular pattern (MAMP) signatures, synergy, size and charge: influences on perception or mobility and host defence responses. Mol Plant Pathol 10: 375–387

Aslam SN, Newman MA, Erbs G, Morrissey KL, Chinchilla D, Boller T, Jensen TT, De Castro C, Ierano T, Molinaro A, et al (2008) Bacterial polysaccharides suppress induced innate immunity by calcium chelation. Curr Biol 18: 1078–1083

Assmann SM (1995) Cyclic AMP as a second messenger in higher plants. Plant Physiol 100: 878–889

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60: 379–406

Boudsocq M, Sheen J (2013) CDPKs in immune and stress signaling. Trends Plant Sci 18: 50–60

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng S-H, Sheen J (2010) Differential innate immune signalling via Ca²⁺ sensor protein kinases. Nature 464: 418–422

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones J, Daldrop U, Seybold H, Durian G, Komander E, Lassig R, Witte CP, Schulze WX, Romeo T (2013) Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. Proc Natl Acad Sci USA 110: 8744–8749

Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci USA 95: 10328–10333

Gerber B, Zeidler D, Durner J, Dubery IA (2004) Early perception responses of Nicotiana tabacum cells in response to lipopolysaccharides from Burkholderia cepacia. Planta 218: 647–657

Gou X, Yin H, He K, Du J, Yi J, Xu S, Lin H, Clouse SD, Li J (2012) Genetic evidence for an indispensable role of somatic embryogenesis receptor from Medicago sativa oxidase synthase gene involved in hormonal signaling. Science 330: 1546–1549

Huffaker A, Pearce G, Ryan CA (2006) An endogenous peptide signal in Arabidopsis activates components of the innate immune response. Proc Natl Acad Sci USA 103: 10098–10103

Huffaker A, Ryan CA (2007) Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response. Proc Natl Acad Sci USA 104: 10732–10736

Ji D, Hu B, Wassenberg KM, Tschaplinski TJ, Beck SC, Rathjen JP (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proc Natl Acad Sci USA 104: 12217–12222

Köhler C, Neuhauß G (1998) Characterisation of a novel gene family of putative cyclic nucleotide- and calmodulin-regulated ion channels in Arabidopsis thaliana. Plant J 18: 97–104

Köhler C, Neuhauß G (2000) Characterisation of calmodulin binding to cyclic nucleotide-gated ion channels from Arabidopsis thaliana. FEBS Lett 471: 133–136

Krol E, Mentzel T, Chinchilla D, Boller T, Felix G, Kemmerling B, Postel S, Arens M, Hewurzuki E, Al-Rasheid KAS, et al (2010) Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. J Biol Chem 285: 13471–13479

Kurosaki F, Kaburaki H (1995) Phosphodiesterase isoenzymes in cell extracts of cultured carrot. Phytochemistry 40: 685–689

Kwezi L, Meier S, Mengur L, Ruzvidzo O, Irving H, Gehring C (2007) The Arabidopsis thaliana brassinosteroid receptor (BRI1) contains a domain that functions as a guanylyl cyclase in vitro. PLoS ONE 2: e449

Kwezi L, Ruzvidzo O, Wheeler JJ, Govender K, Iacuone S, Thompson PE, Gehring C, Irving HR (2011) The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signalling in plants. J Biol Chem 286: 22580–22588

Krol E, Mentzel T, Chinchilla D, Boller T, Felix G, Kemmerling B, Iacuone S, Thompson PE, Gehring C, Irving HR (2011) The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signalling in plants. J Biol Chem 286: 22580–22588

Krol E, Mentzel T, Chinchilla D, Boller T, Felix G, Kemmerling B, Iacuone S, Thompson PE, Gehring C, Irving HR (2011) The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signalling in plants. J Biol Chem 286: 22580–22588

Lecouereaux D, Ranjeva P, Pugin A (2006) Calcium in plant defence-signalling pathways. New Phytol 171: 249–259

Lee J, Rudd JJ (2002) Calcium-dependent protein kinases: versatile plant signalling components necessary for pathogen defence. Trends Plant Sci 7: 97–98

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 78: 583–593

Li W, Luan S, Schreiber SL, Assmann SM (1994) Cyclic AMP stimulates K⁺ channel activity in mesophyll cells of Vicia faba L. Plant Physiol 106: 957–961

Ma W, Qi Z, Smigiel A, Walker RK, Verma R, Berkowitz GA (2009) Ca²⁺, cAMP, and transduction of non-self perception during plant immune responses. Proc Natl Acad Sci USA 106: 20967–20970

Ma W, Smigiel A, Tsai YC, Beem AJ, Berkowitz GA (2008) Innate immunity signalling: cytosolic Ca²⁺ elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. Plant Physiol 148: 818–828

Ma Y, Walker RR, Zhao Y, Berkowitz GA (2012) Linking ligand perception by PEPR pattern recognition receptors to cytosolic Ca²⁺ elevation and downstream immune signalling in plants. Proc Natl Acad Sci USA 109: 19852–19857

Macho AP, Boutrot F, Rathjen JP, Zipfel C (2012) Aspartate oxidase plays an important role in Arabidopsis stomatal immunity. Plant Physiol 159: 1845–1856

Moreau L, Lee GI, Wang Y, Crane BR, Klessig DF (2008) AINOS/AINOA1 is a functional Arabidopsis thaliana GTPase and not a nitric oxide-mediated phase II. J Biol Chem 283: 32957–32967

Moreau L, Lindermayr C, Durner J, Klessig DF (2010) NO synthesis and signalling in plants—where do we stand? Physiol Plant 138: 372–383

Mur LJA, Laarhoven LJF, Harren FJM, Hall MA, Smith AR (2008) Nitric oxide interacts with salicylate to regulate biphasic ethylene production during the hypersensitive response. Plant Physiol 148: 1537–1546

Nijink A, Pisticci J, Filewod NCJ, Hancock REW (2012) Signalling pathways mediating chemokine induction in keratinocytes by cathelicidin LL-37 and flagellin. J Innate Immun 4: 377–386

Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals striking similarities and obvious differences. Immunol Rev 198: 249–266

Park CJ, Caddell DF, Ronald PC (2012) Protein phosphorylation in plant immunity: insights into the regulation of pattern recognition receptor-mediated signalling. Front Plant Sci 3: 777

Pastor S, Küffer I, Beuter C, Mazzotta S, Schwedt A, Borlotti A, Halter T, Kemmerling B, Nürnberger T (2010) The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity. Eur J Cell Biol 89: 169–174

Qi Z, Verma R, Gehring C, Yamaguchi Y, Zhao Y, Ryan CA, Berkowitz GA (2010) Ca²⁺ signaling by plant Arabidopsis thaliana Pep peptides depends on AtPEPR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca²⁺ channels. Proc Natl Acad Sci USA 107: 21193–21198

Ranf S, Eschen-Lippold I, Pecher P, Lee J, Scheel D (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. Plant J 68: 100–113

Rasmussen MW, Roux M, Petersen M, Mundy J (2012) MAP kinase cascades in Arabidopsis innate immunity. Front Plant Sci 3: 169

Ronald PC, Beutler B (2010) Plant and animal sensors of conserved microbial signatures. Science 330: 1061–1064

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton L, Malinovsky FG, Tör M, de Vries S, Zipfel C (2011) The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BAK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. Plant Cell 23: 2440–2455

Rusinova E, Borse J, Kwaaitaal M, Cara-Delgado A, Yin Y, Chory J, de Vries SC (2004) Heterodimerization and endocytosis of Arabidopsis brassinosteroid receptors BRI1 and ASEK3 (BAK1). Plant Cell 16: 3216–3229

Plant Physiol. Vol. 163, 2013
Schulze B, Mentzel T, Jehle AK, Mueller K, Beeler S, Boller T, Felix G, Chinchilla D (2010) Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. J Biol Chem 285: 9444–9451

Segonzac C, Zipfel C (2011) Activation of plant pattern-recognition receptors by bacteria. Curr Opin Microbiol 14: 54–61

Syam Prakash SR, Jayabaskaran C (2006) Heterologous expression and biochemical characterization of two calcium-dependent protein kinase isoforms CaCPK1 and CaCPK2 from chickpea. J Plant Physiol 163: 1083–1093

Torres MA (2010) ROS in biotic interactions. Physiol Plant 138: 414–429

Torres MA, Dangl JL, Jones JDG (2002) Arabidopsis gp91phox homologues AtBbohD and AtBbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci USA 99: 517–522

Torres MA, Jones JDG, Dangl JL (2005) Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in Arabidopsis thaliana. Nat Genet 37: 1130–1134

Volotovski ID, Sokolovsky SG, Molchan OV, Knight MR (1998) Second messengers mediate increases in cytosolic calcium in tobacco protoplasts. Plant Physiol 117: 1023–1030

Wan J, Zhang XC, Stacey G (2008) Chitin signaling and plant disease resistance. Plant Signal Behav 3: 831–833

Yamaguchi Y, Huffaker A (2011) Endogenous peptide elicitors in higher plants. Curr Opin Plant Biol 14: 351–357

Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA (2010) PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. Plant Cell 22: 508–522

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428: 764–767

Zottini M, Costa A, De Michele R, Ruzzene M, Carimi F, Lo Schiavo F (2007) Salicylic acid activates nitric oxide synthesis in Arabidopsis. J Exp Bot 58: 1397–1405