Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels

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A family of peptide signaling molecules (AtPeps) and their plasma membrane receptor AtPepR1 are known to act in pathogen-defense signaling cascades in plants. Little is currently known about the molecular mechanisms that link these signaling peptides and their receptor, a leucine-rich repeat receptor-like kinase, to downstream pathogen-defense responses. We identify some cellular activities of these molecules that provide the context for a model for their action in signaling cascades. AtPeps activate plasma membrane inwardly conducting Ca^{2+} permeable channels in mesophyll cells, resulting in cytosolic Ca^{2+} elevation. This activity is dependent on their receptor as well as a cyclic nucleotide-gated channel (CNGC2). We also show that the leucine-rich repeat receptor-like kinase receptor AtPepR1 has guanylyl cyclase activity, generating cGMP from GTP, and that cGMP can activate CNGC2-dependent cytosolic Ca^{2+} elevation. AtPep-dependent expression of pathogen-defense genes (*PDF1.2*, *MPK3*, and *WRKY33*) is mediated by the Ca^{2+} signaling pathway associated with AtPep peptides and their receptor. The work presented here indicates that extracellular AtPeps, which can act as danger-associated molecular patterns, signal by interaction with their receptor, AtPepR1, a plasma membrane protein that can generate cGMP. Downstream from AtPep and AtPepR1 in a signaling cascade, the cGMP-activated channel CNGC2 is involved in AtPep- and AtPepR1-dependent inward Ca^{2+} conductance and resulting cytosolic Ca^{2+} elevation. The signaling cascade initiated by AtPeps leads to expression of pathogen-defense genes in a Ca^{2+}-dependent manner.

calcium signaling | plant innate immunity | flagellin receptor 2 | brassinosteroid associated kinase 1

Pathogen recognition by host cells under assault occurs through the binding of extracellular warning molecules to cell membrane receptor proteins. The transmission of a nonself perception alarm to the cell interior occurs (at least in part) through the activation of cell membrane Ca^{2+} channels, leading to a cytosolic Ca^{2+} elevation that signals the threatened cell to mount an immune defense. These actions, (*i*) danger signal perception by sentry cell surface receptors and (*ii*) response of Ca^{2+} signaling, are two paramount and critical components of both animal and plant cell immunity. Much still remains unknown about the molecular steps linking these two vital components of the pathogen-defense response signal transduction system.

In the case of multicellular animals, a two decade-long search for the identity of the protein facilitating immune-related cell membrane inward Ca^{2+} channel currents ended recently in the cloning of a gene encoding Orai1 (1, 2). The name of this immune portal refers to the keepers of Heaven's gate in Greek mythology and bespeaks the significance of the work (3). A specific gene translation product [cyclic nucleotide-gated cation channel 2 (CNGC2)] has also been recently shown to be responsible for the cell membrane Ca^{2+} channel currents that facilitate downstream immune signaling, leading to pathogen-defense responses in plants (4). CNGC2 is a member of the CNGC protein family present in Arabidopsis and other plants (5). CNGCs are nonselective cation-conducting channels activated by cyclic nucleotides.

Pathogen recognition can occur through the binding of evolutionarily conserved essential molecular components of pathogens [pathogen-associated molecular pattern (PAMP) or microbe-associated molecular pattern (MAMP) molecules] to specific cell membrane receptors. In the model plant Arabidopsis, the bacterial flagellin protein and the bacterial elongation factor EF-Tu induce immune signaling responses; the receptors for these PAMPs are leucine-rich repeat receptor-like kinases (LRR-RLKs) (6). PAMP receptors in animals are also LRR-RLKs. It is currently presumed that plant LRR-RLKs such as the flagellin receptor (FLS2) act in pathogen-defense signaling cascades through phosphorylation events arising from their cytoplasmic kinase domains (6). Current models of FLS2 signaling identify another LRR-RLK, BAK1, as a coreceptor that physically and functionally interacts with FLS2; BAK1 loss of function (partially) impairs flagellin-induced FLS2-dependent immune responses (6).

In addition to PAMPs, some endogenous plant compounds that bind to cell surface receptors [danger-associated molecular pattern (DAMP) molecules] act in immune signaling to initiate intracellular signal transduction cascades leading to plant-defense responses to pathogens (6, 7). Among these DAMPs is a family of peptides (AtPep1–6) in Arabidopsis (8, 9). AtPep1 binds to the plant cell plasma membrane receptor AtPepR1, also a LRR-RLK (10). Because other AtPep peptides can compete with AtPep1 for binding to the receptor, it is speculated that AtPepR1 is a receptor for all AtPep peptides. Other than the interaction between AtPep peptides and their receptor, nothing is currently known about the molecular steps that link these DAMPs to downstream pathogen-defense responses such as altered gene expression (6, 7, 11).

AtPep signaling has been likened to the systemin peptide wound signaling system operative in tomato (6). The brassinosteroid hormone receptor BRI1, another LRR-RLK, is a receptor (along with other unknown proteins) for systemin (12). It is well-known that at least one component of BRI1-dependent brassinosteroid signaling occurs through phosphorylation events dependent on the cytosolic kinase domain of the receptor, a signaling paradigm also suggested (see above) for FLS2.

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Surprisingly, a guanylyl cyclase (GC) domain was recently identified within the cytosolic kinase region of Arabidopsis (At) BRI1 and shown to have catalytic activity in vitro (13). Intriguingly, the cytosolic kinase region of the LRR-RLK AtPepR1 also contains a putative GC domain with the same functionally assigned residues in the catalytic center as AtBRI1 (13). Cyclic nucleotides such as cAMP and cGMP are potential activating ligands for plant CNGCs, including CNGC2. Thus, the Ca^{2+} conduction of the cyclic nucleotide-activated channel CNGC2, the role AtPep DAMPs play in pathogen-defense signaling cascades, and the possible guanylyl cyclase activity of the AtPep receptor AtPepR1 provide a potential paradigm for linking pathogen perception at the cell surface to intracellular Ca^{2+} signaling and immune responses in plants. The work presented in this report investigates this model.

Results

cGMP and Ca^{2+} Signaling in Arabidopsis. The electrophysiological properties of the Arabidopsis channel protein (At)CNGC2 have been characterized by expression of the coding sequence in a number of heterologous systems, including yeast (*Saccharomyces cerevisiae*) mutants (14–16), HEK cells (16–19), and frog (*Xenopus laevis*) oocytes (16, 17, 19). In these expression systems, homomeric channels formed by CNGC2 polypeptides conduct Ca^{2+} (among other cations) and are activated by cAMP as well as cGMP. However, it is still unclear (20) whether channels formed by any translation product of the 20 Arabidopsis *CNGC* genes and specifically, *CNGC2* form cGMP-, as opposed to cAMP-, activated channels in native plant cell membranes. CNGC2 is strongly expressed in leaves (21), and Ca^{2+} conduction by native CNGC2 channels has been associated with pathogen-defense response immune signaling (4, 22). Here, we investigated cyclic nucleotide activation of Ca^{2+}-conducting channels using plants expressing the cytosol-localized Ca^{2+}-dependent chemiluminescent protein aequorin. Within 1 min after application of dibromo-cGMP, a lipophilic analog of cGMP, to solutions containing detached whole leaves, an elevation of cytosolic Ca^{2+} in leaves of wild-type plants (expressing aequorin) was initiated (Fig. 1*A*), suggesting that native Ca^{2+}-conducting CNGC channel proteins are activated in planta by cGMP. As expected, application of (nonlipophilic) cGMP does not cause a cytosolic Ca^{2+} spike (Fig. S1*A*); this result is consistent with prior research indicating that the cyclic nucleotide binding domain of CNGC2 is localized in the cytosol and that cyclic nucleotide external to the cell cannot activate the channel (16).

The Arabidopsis *defense no death* (*dnd1*) mutant lacks a functional *CNGC2* coding sequence (22). We generated *dnd1* mutant plants expressing aequorin and found that cGMP-dependent cytosolic Ca^{2+} elevation was significantly impaired in this mutant (Fig. 1*A*). Control experiments indicated that the aequorin-expressing mutant genotypes generated for use in the work reported here [*dnd1* and *arabidopsis thaliana pep receptor 1* (*atpepr1*) null loss of function; see below] were capable of responding to signals that result in cytosolic Ca^{2+} elevations. As shown in Fig. S1*B*, these aequorin-expressing mutant lines responded to cold with a Ca^{2+} spike to an extent at least as great as wild-type plants expressing aequorin. Results in Fig. 1*A* suggest that a plant protein that generates cytosolic cGMP could act to activate CNGC2, leading to an influx of Ca^{2+} into the cell and an elevation in cytosolic Ca^{2+} and initiating an intracellular signal transduction cascade.

AtPepR1 Has GC Activity. We hypothesized that AtPepR1 has GC activity based on the following rationale. (*i*) Results shown in Fig. 1*A* show that cGMP can activate CNGC2, leading to cytosolic Ca^{2+} elevation. (*ii*) CNGC2-dependent Ca^{2+} currents contribute to plant innate immune responses (4). (*iii*) The LRR-RLK AtPepR1 likely plays a role in pathogen-defense signaling cascades (9). (*iv*) The AtPepR1 coding sequence contains a region homologous to the cytosolic GC domain of AtBRI1 that includes the conserved residues implicated in catalysis (Fig. S2) (13). To test the aforementioned hypothesis, we investigated the ability of AtPepR1 (specifically, the region of the protein with similarity to the GC domain of AtBRI1) to act as a GC. This region (AtPepR1-GC) of the AtPepR1 coding sequence (Met^{962} → Val^{1079}) was expressed in *Escherichia coli*. In a first series of experiments, we compared GC activities in *E. coli* extracts from six liquid cultures of bacteria transfected with either the empty pET28a vector or the pET28a plasmid harboring the AtPepR1-GC coding sequence. There was a modest, although consistent, increase in total cell cGMP in *E. coli* cultures expressing the recombinant AtPepR1-GC protein (Fig. S3). This suggested the possibility that the cytoplasmic region of the AtPepR1 receptor could function as a GC. Work was undertaken to further test this hypothesis.

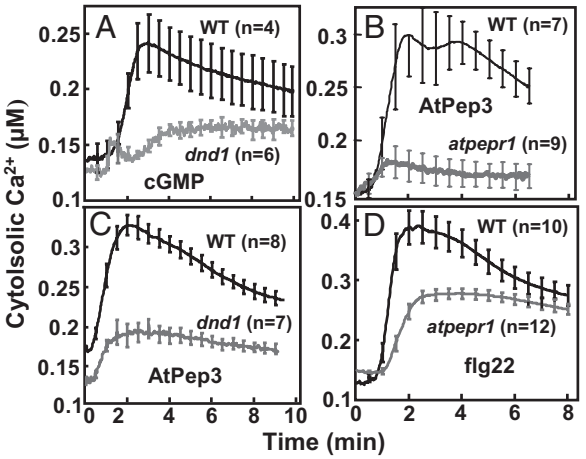

Fig. 1. Ligand-induced cytosolic Ca^{2+} elevation in leaves of aequorin-expressing Arabidopsis plants. Ligand was added at time 0 to wild-type (WT; darker lines) or mutant (lighter lines) plants as noted in *A*–*D*. (*A*) A lipophilic analog of cGMP. (*B*) AtPep3. (*C*) AtPep3. (*D*) flg22. The signals shown are averages generated from biological replicates (replicate numbers are in parentheses); leaves for each replicate were taken from different plants. At 0.5-min time intervals, SE was calculated for each mean; SE values are portrayed as error bars. Results similar to those shown here are presented in Fig. 4*A* and Figs. S6 and S7 for whole leaves or roots of various genotypes of aequorin-expressing Arabidopsis plants. The experimental design, analysis, and presentation of the work shown in these other figures are similar to that shown here.

Recombinant AtPepR1-GC protein (expressed with a six-His tag at either the carboxyl or amino terminus) was affinity-purified from *E. coli* cultures (Fig. S4). AtPepR1-GC protein was evaluated for GC activity; results in Fig. 2 indicate that the cytoplasmic domain of AtPepR1 can function as a GC in vitro.

Animal GCs require a metal cofactor for maximal activity, presumably for binding to the nucleophilic polyphosphates of the GTP nucleotide substrate. All animal GCs studied to date show higher catalytic activity in the presence of Mn^{2+} compared with Mg^{2+} (23). GC activity of the affinity-purified recombinant (plant) AtBRI1 protein is increased by both Mn^{2+} and Mg^{2+}, although activity is higher in the presence of Mg^{2+} (13). In the case of affinity-purified AtPepR1-GC, Mn^{2+} seems to be the optimal metal cofactor. With Mn^{2+} added to the reaction, we observed significant GC activity of recombinant AtPepR1-GC, irrespective of the His-tag at either the amino or carboxyl terminus (Fig. 2). It should be noted that the in vitro GC activity that we show for the cytosolic GC domain of AtPepR1 is at least as high or higher than the activity shown by the corresponding cytosolic domains of other plant GCs that have been expressed as recombinant proteins in *E. coli* (13), but it is substantially

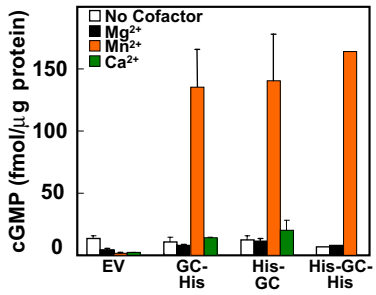

Fig. 2. Affinity-purified AtPepR1-GC has GC activity. Details of AtPepR1-GC cloning, expression in *E. coli*, purification, and use with cGMP ELISAs can be found in *SI Materials and Methods*. GC assays were performed on empty vector protein (EV) or with protein affinity-purified from *E. coli* transfected with the cloned AtPepR1-GC region coding sequence expressed with a six-His tag fused to either the carboxyl terminus (GC-His), the amino terminus (His-GC), or both ends of the protein (His-GC-His). GC assays were performed in the absence of any added cofactor (no cofactor) or with 5 mM Mg^{2+}, Mn^{2+}, or Ca^{2+} added to the assay. Results from several different experiments are shown in this figure. An individual experiment represented assays from different *E. coli* genotypes (with different assay cofactor additions as noted in the figure) performed in parallel. For an individual experiment, 3–4 replicate measurements of cGMP production were made (i.e., three to four wells of an ELISA plate). These replicates were averaged to yield one value of cGMP production for a given genotype and assay condition for an experiment. Results are shown in the figure for three different experiments (i.e., individual evaluations of cGMP generation using three different ELISA plates) for cultures expressing GC-His and His-GC; means of cGMP values obtained in the three experiments are shown ±SE. In the case of His-GC-His protein, only one experiment (using one ELISA plate) was performed, and the four measurements were averaged to generate one replicate; no SE bars are shown for this genotype.

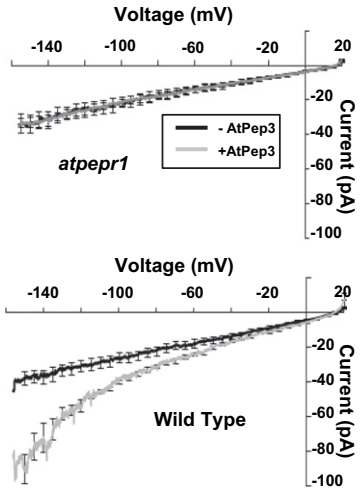

Fig. 3. Patch clamp recordings from mesophyll protoplasts isolated from leaves of WT and *atpepr1* mutant plants show an AtPepR1-dependent activation of an inwardly rectified Ca^{2+}-conducting plasma membrane channel by AtPep3. Ramp recordings (whole-cell configuration) made in the presence (gray lines) and absence (black lines) of 200 nM AtPep3 are shown for mesophyll protoplasts isolated from leaves of WT and *atpepr1* mutant plants. Results shown are mean current values calculated from multiple recordings made from WT (n = 6) and *atpepr1* mutant (n = 5) protoplasts. The multiple recordings used to generate the mean values shown here are biological replications; individual recordings were made from different protoplast preparations. Results are presented as means with SE calculated at 5-mV intervals. As is convention (20), Ba^{2+} was used as a charge carrier to monitor Ca^{2+}-conducting channels. In the pipette and bath solutions used, the Ba^{2+}, Cl^-, and K^+ Nernst equilibrium potentials (E_{Ba}, E_{Cl}, and E_K; after correcting for ion activities) (24) are calculated to be +26, −31, and −75 mV, respectively. The reversal potential for the WT protoplasts in the presence of AtPep3 ligand was +20 mV; this value is close to E_{Ba} and distant from E_{Cl} and E_K, indicating that the charge was primarily carried by Ba^{2+}. The addition of AtPep3 increased inward current at hyperpolarizing membrane potentials with WT protoplasts; no effect of the ligand was observed on currents recorded from *atpepr1* protoplasts.

lower than the GC activity of animal GC enzymes either in native membranes or expressed as recombinant proteins in heterologous (eukaryotic) expression systems. This could be because of the possibility that the plant proteins (including AtPepR1-GC) do not fold optimally after expression in a prokaryotic system and reconstituted in vitro or else they require other unknown factors present in vivo within the plant cell cytosol for maximal activity. GC activity of AtPepR1-GC, with preference of Mn^{2+} over Mg^{2+} as cofactor, was confirmed with MS (Fig. S5).

Further investigations were undertaken to determine if the AtPep peptide ligands for this LRR-RLK receptor as well as the receptor itself function in the plant to activate CNGC2 and generate cytosolic Ca^{2+} spikes. As mentioned above, cytosolic Ca^{2+} elevation is a key upstream component of pathogen-response signaling cascades in the cytosol of plant cells.

AtPep Peptides Activate Inward Plasma Membrane Ca^{2+} Currents. AtPep3 expression is increased in response to plant pathogens, including *Pseudomonas syringae*, and PAMPs, including a peptide (flg22) corresponding to the active component of the bacterial flagellin protein (8). It is speculated that AtPep3 binds to AtPepR1 (10). Application of AtPep3 ligand to Arabidopsis leaves results in AtPepR1-dependent cytosolic Ca^{2+} elevation (Fig. 1*B*). For this experiment, wild-type (WT) plants and *atpepr1* null mutants expressing the Ca^{2+}-sensing aequorin protein in the cytoplasm were used. Application of AtPep3 resulted in an elevation of cytosolic Ca^{2+} in leaves of WT plants that was not evident in the *atpepr1* mutant. These results suggest that the LRR-RLK ligand AtPep3 activates a plasma membrane inwardly conducting Ca^{2+} current in mesophyll cells through the action of AtPepR1. Confirmation of this point can be seen in Fig. 3. Results of patch clamp experiments with mesophyll protoplasts prepared from leaves of WT and *atpepr1* mutant plants are shown. Application of the ligand AtPep3 activated an inwardly rectified Ca^{2+}-conducting channel in WT protoplasts. This did not occur with protoplasts isolated from *atpepr1* plants.

Results of experiments shown in Figs. 1*B* and 3 indicate that AtPep3 and its receptor AtPepR1 may act upstream from CNGC2 in signaling cascades, possibly because of cGMP activation of the channel (Fig. 1*A*) and the GC activity of the receptor (Fig. 2 and Fig. S3). Results of experiments with AtPep3 and *dnd1* plants shown in Fig. 1*C* are consistent with this model. AtPep3-dependent cytosolic Ca^{2+} elevation found in leaves of WT plants is significantly impaired when the ligand is applied to leaves of *dnd1* mutant plants lacking a functional CNGC2. These results identify the plasma membrane inwardly rectified, Ca^{2+}-conducting cyclic nucleotide-activated channel CNGC2 (25) as facilitating AtPep3- and AtPepR1-dependent cytosolic Ca^{2+} elevation. We also checked for AtPep3-dependent cGMP elevation in leaves. However, when monitored on a whole-leaf basis, we never observed a consistent AtPep3-dependent rise. We speculate that AtPepR1 GC activity in vivo may cause cGMP increase in localized domains near the plasma membrane in close proximity to the ion channels activated by this ligand.

Further support for the contention that CNGCs act downstream from AtPep3 is shown in Fig. S6*A*. Lanthanides such as Gd^{3+} and La^{3+} block plant plasma membrane Ca^{2+} channels (26). Gd^{3+} has been specifically shown to block inwardly rectified cyclic nucleotide-activated Ca^{2+} channels in the plasma membrane of Arabidopsis leaf cells (20). Gd^{3+} has also been shown to prevent the programmed cell death occurring during the hyper-

sensitive response to pathogens that is dependent on CNGC-mediated cytosolic Ca^{2+} elevation (4, 27). Results of the experiment shown in Fig. S6*A* indicate that Gd^{3+} blocks AtPep3-dependent cytosolic Ca^{2+} as well, a result consistent with AtPep3 (and AtPepR1) acting upstream from a CNGC2-dependent plasma membrane Ca^{2+} current. Evidence presented in Figs. 1 *B* and *C* and 3 and Fig. S6*A* is consistent with AtPepR1 receptor and CNGC2 as downstream from AtPep3 in signaling pathways, leading to cytosolic Ca^{2+} elevation.

Application of a plasma membrane Ca^{2+} channel blocker completely blocked AtPep3-dependent cytosolic Ca^{2+} elevation (Fig. S6*A*), whereas the addition of the ligands cGMP (Fig. 1*A*) or AtPep3 (Fig. 1*C*) to leaves of *dnd1* plants (lacking a functional CNGC2 channel) results only in impaired (but not completely blocked) cytosolic Ca^{2+} elevation compared with responses of WT plants. These results are similar in nature to other immune signaling responses in the *dnd1* mutant; we also found PAMP-induced nitric oxide generation (dependent on movement of external Ca^{2+} into the cell) only partially inhibited from WT in this genotype (4). As discussed in more detail in prior work from one of our labs, indirect evidence as well as threading quaternary models of CNGC2 through known crystal structures suggest that plant CNGC channel complexes are heterotetramers comprised of more than one CNGC gene product (4, 17, 25, 28). In the *dnd1* mutant, loss-of-function mutation of *CNGC2* could result in assembly of nonnative, partially functional channel complexes comprised of other CNGC polypeptides. Refs. 25 and 28 elaborate on this point.

Inositol 1,4,5-triphosphate (IP3) acts as a secondary messenger in signal transduction pathways involving cytosolic Ca^{2+} elevations in plants (29). In these signaling pathways, cytosolic IP3 generation on perception of an external stimulus leads to cytosolic Ca^{2+} elevation because of activation of IP3-sensitive tonoplast-localized Ca^{2+} channels (30); the resulting cytosolic Ca^{2+} spike occurs because of release of intracellular Ca^{2+} stores. Using plants in which signal-induced IP3 generation is blocked (31), we found no impairment of AtPep3-mediated cytosolic Ca^{2+} elevation; these results suggest that IP3-activated release of vacuolar Ca^{2+} does not contribute to AtPep- and AtPepR1-mediated Ca^{2+} signaling.

AtPepR1-Dependent Phenotypes. Indirect evidence suggests that AtPepR1 is involved in AtPep-dependent cell signaling pathways and pathogen-response signal transduction. Alkalinization of external medium on application of elicitor molecules derived from pathogens, PAMPs, or DAMPs to plant cell cultures has been used to show involvement of the activating ligand in plant pathogen-defense signaling (6, 9). Tobacco cell cultures do not respond to application of AtPep peptides. Expression of AtPepR1 in tobacco cell cultures results in AtPep-dependent alkalinization of the medium (10). However, phenotypes associated with AtPepR1 loss of function in Arabidopsis (other than the AtPep-dependent inward Ca^{2+} currents leading to the cytosolic Ca^{2+} elevation reported here) have not been heretofore identified. Thus, we include in the work here an investigation of AtPepR1 phenotypes and provide some evidence that AtPepR1 function in planta could be related to the presence of AtPep peptides as activating ligands as well as AtPep-dependent Ca^{2+} signaling.

Growth inhibition of Arabidopsis seedlings by the bacterial PAMP flagellin (i.e., flg22) was used to show function of the flagellin receptor FLS2 (6). We used a similar strategy here to identify an (Arabidopsis) plant phenotype associated with functional AtPepR1. As was the case with leaves (Fig. 1*B*), we found an AtPepR1-dependent cytosolic Ca^{2+} elevation in Arabidopsis roots on application of AtPep3 (Fig. 4*A*). Promotion of cytosolic Ca^{2+} spikes in roots of Arabidopsis seedlings by application of several different ligands has been shown to inhibit primary root growth (26, 32). In addition, we have found that some members

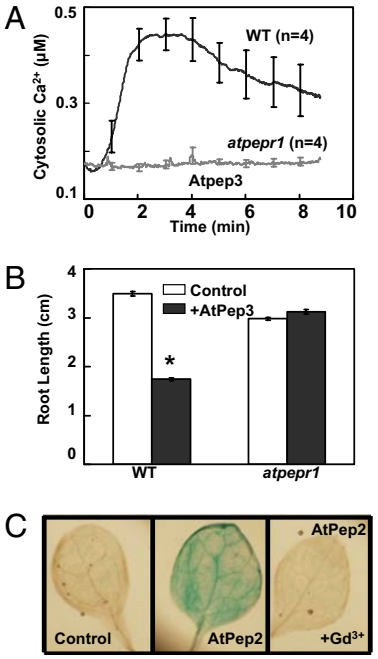

Fig. 4. AtPep ligand and AtPepR1 receptor effects on root growth and pathogen-defense gene activation are associated with Ca^{2+} signaling. (*A*) AtPep3 (20 nM) application to Arabidopsis roots results in AtPepR1-dependent cytosolic Ca^{2+} elevation in a manner similar to that occurring in leaves (Fig. 1*B*). (*B*) Root length of WT and *atpepr1* mutant (7-d-old) seedlings grown (on solid agar medium) in the absence (open bars) and presence (filled bars) of 20 nM AtPep3. An asterisk above a filled bar indicates that the mean ($n = 10$) root length of seedlings of the specific genotype (WT or *atpepr1*) was significantly different ($P \leq 0.01$) when seedlings were grown in the presence and absence of AtPep3. (*C*) AtPep2 effects on *PDF1.2*::*GUS* expression in the absence and presence of the Ca^{2+} channel blocker Gd^{3+} (2 mM).

of the CNGC protein family are expressed in roots, contribute to Ca^{2+} uptake, and facilitate Ca^{2+}-mediated inhibition of root elongation under some conditions (33). Here, we find that application of nanomolar levels of AtPep3 resulted in an inhibition of primary root length in WT Arabidopsis seedlings (Fig. 4*B*). However, the ligand AtPep3 had no effect on root length when applied to *atpepr1* loss-of-function mutants (Fig. 4*B*). Results shown in Fig. 4 *A* and *B*, in addition to those shown in Figs. 1 and 3, document AtPepR1-dependent phenotypes in Arabidopsis plants and further show that AtPepR1 not only acts as a receptor for AtPep peptides but is also the cause of at least some of the plant cell signaling initiated by these ligands.

Ca^{2+} Signaling May Mediate AtPep Peptide and AtPepR1 Receptor Involvement in Plant Immune Responses. AtPep peptides have been shown to be involved in plant immune responses from several lines of evidence (8, 11). AtPep peptide application to leaves results in generation of hydrogen peroxide, an important component of the pathogen-defense response in plants. AtPep peptide (particularly AtPep2 and AtPep3) expression is induced by PAMPs and pathogens. AtPep application results in expression of pathogen-resistance genes. Finally, constitutive overexpression of an AtPep peptide ameliorates the pathogen-induced inhibition of Arabidopsis root growth. One could speculate that these AtPep phenotypes are mediated by binding of these ligands to the AtPepR1 receptor, although, as mentioned above, direct involvement of the receptor AtPepR1 in most of these immune responses has not been shown in prior work.

Results presented here (Figs. 1 *A*–*C* and 3) show that AtPep peptides and AtPepR1 are upstream from (CNGC2-dependent)

Ca^{2+} signaling. An elevation in cytosolic Ca^{2+} is a critical early signal involved in transduction of pathogen perception to initiate plant immune responses. Thus, we undertook work to evaluate a possible link between AtPep peptide- and AtPepR1-mediated immune responses and Ca^{2+} signaling. Defense activation by the PAMP flagellin (flg22) is mediated by flg22-induced cytosolic Ca^{2+} elevation (34). Here, we find that flg22 application to leaves of WT plants results in cytosolic Ca^{2+} elevation and that, in leaves of *atpepr1* mutant plants, this PAMP-induced cytosolic Ca^{2+} elevation is at least partially impaired (Fig. 1*D*). We also found a reciprocal sensitivity of AtPep3-dependent Ca^{2+} signaling to FLS2 presence (Fig. S7); absence of the flagellin receptor FLS2 partially impairs AtPep3-dependent cytosolic Ca^{2+} elevation. These results link the LRR-RLK receptor AtPepR1, Ca^{2+} signaling, and flagellin-mediated immune responses in plants. Models of flagellin receptor FLS2-signal transduction events (6) speculate that binding of the PAMP facilitates FLS2 receptor association with other LRR-RLKs (such as BAK1) as a necessary step leading to defense responses. Here, we do not present a model for why null mutation of the LRR-RLK AtPepR1 impairs FLS2-dependent signaling (Fig. 1*D*) or why absence of the FLS2 receptor impairs AtPep3 signaling (Fig. S7), but we speculate that AtPepR1 may impact PAMP signaling by binding to other pathogen defense-related LRR-RLKs.

Results presented in Fig. 4*C* link Ca^{2+} signaling to AtPep peptide-mediated plant-defense responses. Application of AtPep peptides, particularly AtPep2, results in an increase in expression of the pathogen-defense gene *PDF1.2* in leaves of Arabidopsis plants (11). In experiments similar to that shown in Fig. 1*B* with AtPep3, we found that application of AtPep2 to leaves of WT plants results in a similar cytosolic Ca^{2+} elevation (Fig. S6*B*). We monitored effects of AtPep2 peptide on *PDF1.2* expression in the experiment shown in Fig. 4*C* using Arabidopsis plants transformed with the β-glucuronidase (*GUS*) reporter gene under control of the Arabidopsis *PDF1.2* promoter. Application of AtPep2 to leaves of these *PDF1.2*::*GUS* plants did lead to *GUS* expression. Significantly, AtPep-dependent *GUS* expression was blocked in leaves treated with the plasma membrane Ca^{2+} channel blocker Gd^{3+} in the experiment shown in Fig. 4*C*. PAMPs such as flg22 induce expression of defense-related genes such as *MPK3* and *WRKY33* (35). Similar to results shown in Fig. 4*C*, we find that treatment of leaves with the Ca^{2+} channel blocker reduced AtPep-dependent expression of these defense-related genes as well (Fig. S8). Thus, these results tie the function, identified in this report, of AtPep peptides and their receptor AtPepR1 as initiators of CNGC-dependent Ca^{2+} signaling to downstream Ca^{2+}-mediated immune signaling in plants.

Discussion

Much attention is currently focused on unraveling the molecular mechanisms involved in plant immune signal transduction cascades (6, 7, 35–37). However, at present, the steps upstream from Ca^{2+} influx into the cytosol linking pathogen perception to the initiation of cellular defense response programs in plants remain a source of speculation (6, 38). Work presented here provides insights into this biological paradigm, specifically with regard to the peptide signaling ligands AtPeps and their LRR-RLK receptor AtPepR1. At present, involvement of these molecules in plant immune responses is characterized as follows. Initial perception of invading pathogenic cells and/or the PAMPs associated with them is known to initiate a suite of defense responses, including expression of AtPep peptides. As the integrity of a cell under assault by a pathogen is compromised, AtPep peptides could be released to the apoplast and act as a DAMP signal to cells neighboring the initial infection site. It is noteworthy that recent work (28) links cAMP elevation at the initial infection site to initiation of pathogen defense-related cytosolic Ca^{2+} signaling, implying that both cAMP and cGMP have specific roles in plant-defense signal cascades. However, the specific roles of these second messengers in early defense responses remain to be elucidated.

We show here that AtPep outside of a plant cell at nanomolar concentrations leads to activation of a plasma membrane-localized cyclic nucleotide-gated Ca^{2+}-conducting channel and CNGC-dependent cytosolic Ca^{2+} elevation. Activation of plasma membrane CNGC-dependent inward Ca^{2+} current by the ligand AtPep3 requires the presence of a functional receptor, AtPepR1, for the ligand. In the presence of the activating ligand, the receptor causes downstream activation of CNGCs. We also identified an intriguing cross-sensitivity of Ca^{2+} signaling induced by the PAMP flagellin and DAMP AtPep3 to the presence of their corresponding LRR-RLK receptors FLS2 and AtPepR1. FLS2 and AtPepR1 are pattern recognition receptors (PRRs) that initiate PAMP-triggered immunity (PTI) (6, 7). This cross-sensitivity raises the possibility that these PRR sentry proteins that translate and/or amplify perception of nonself somehow integrate their signaling during PTI to evoke pathogen-defense responses. These PRRs might form multiprotein complexes with each other (for example, along with the accessory LRR-RLK protein, BAK1).

The receptor AtPepR1 is also shown here to have GC activity, and it is conceivable that AtPepR1 could activate CNGC2 through this cytosolic activity. This point remains speculative and is not experimentally confirmed by the work here. AtPep3 application (to either WT or *atpepr1* plants) did not cause measurable increases in whole-leaf cGMP. We speculate that localized elevations of cGMP generation within the cell can occur, leading to signaling specifically in microdomains near the plasma membrane and thereby activating the channels. Such microdomain signaling events occur in animal nucleotidyl cyclases and animal cell cyclic nucleotide signaling (39). Some Ca^{2+} channels are present in multienzyme protein complexes with proteins that generate their activating ligands (1). It is intriguing to note that Ca^{2+} signaling involving the Ca^{2+} channel Orai1 that acts in animal immune signaling involves Orai1:adenylyl cyclase protein complexes and microdomains of cyclic nucleotide elevation (40, 41). Alternatively, the receptor could act through cytosolic kinase activity to activate channels and initiate downstream CNGC-dependent Ca^{2+} signaling cascades. In either case, we show that involvement of both the AtPep ligands and their receptors in pathogen-defense signaling is associated with the initiation of Ca^{2+} signals in the cytosol; thus, the work here provides a model underlying their involvement in this signal transduction cascade. In addition, the studies in this report provide evidence linking a plant protein (AtPepR1) that has GC activity (shown here in vitro) and its activating ligand (AtPep3) to a specific cGMP-sensitive molecule (CNGC2) acting downstream from the GC and ligand in a signal transduction pathway. There may be numerous GCs in plants (13); however, in planta cGMP level has not yet been shown to be affected by translational arrest of any gene product.

Materials and Methods

For details, see *SI Materials and Methods*.

Plant Material. Arabidopsis WT [Columbia (Col) ecotype], WT Col plants transformed with mammalian type I inositol polyphosphate 5-phosphatase (31), *dnd1* mutant (22), and *atpepr1* null (loss-of-function) mutant (Salk 059281) (10) as well as aequorin-transformed plants of these genotypes were used for experiments. The *atpepr1* homozygous null mutant and *dnd1* mutant were crossed with WT plants expressing aequorin to generate *atpepr1*–aequorin and *dnd1*–aequorin plants. RT-PCR was performed on progeny of the *atpepr1* cross to confirm that the transfer-DNA insertion was homozygous and prevented accumulation of full-length transcripts. Homozygous *dnd1* plants expressing aequorin were identified using derived cleaved amplified polymorphic sequence analysis and an MboI restriction site (42). Seeds of Arabidopsis plants expressing the reporter protein GUS under control of the *PDF1.2* promoter (*PDF1.2*::*GUS*) were obtained from the Arabidopsis Biological Resource Center (stock no. CS6506) at Ohio State Univer-

sity (Columbus, OH). Aequorin-expressing plants in which signal-induced IP3 generation is blocked were provided by Dr. I. Perera (North Carolina State University, Raleigh, NC).

Reagents. Unless otherwise noted, all reagents were purchased from Sigma. The membrane permeable lipophilic cGMP analog dibromo-cGMP (at 1 mM) was used in all cases. For experiments using aequorin-expressing plants to measure cytsolic Ca^{2+}, the ligands AtPep3 (20 nM), flg22 (1 μM), and AtPep2 (20 nM) were added at time 0 min.

GC Enzymatic Activity of Recombinant AtPepR1-GC Protein. A 354-bp fragment of the AtPepR1 coding sequence corresponding to a 118-residue polypeptide (Met^{962} → Val^{1079}) was cloned and expressed in *E. coli*, and the encoded recombinant AtPepR1-GC protein was purified and used for determination of GC activity as described in *SI Materials and Methods*.

Cytosolic Ca^{2+} Measurements. Ligand effects on cytosolic Ca^{2+} level were evaluated in whole detached leaves of 3- to 4-wk-old plants or detached whole root systems of 2-wk-old plants expressing cytosol-localized Ca^{2+}-dependent chemiluminescent apoaequorin protein reconstituted with coelenterazine-cp (CTZ-cp). These plants (referred to as WT, *dnd1* mutant, or *atpepr1* mutant plants expressing aequorin) had similar responses to cold treatment; these genotypes gave similar luminescence signals and have a single copy of the apoaequorin gene under control of the *35S* promoter (43).

Note Added in Proof. During review of this manuscript, AtPepR1 was shown to physically associate with the LRR-RLK BAK1 (44); this recent publication, then, confirms a point of speculation made here. Another recent article (45) confirms that AtPep peptides generate a cytosolic Ca^{2+} elevation in wild-type plants.

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