Correction

PLANT BIOLOGY

The authors note that, because of a printer’s error, the affiliation superscripts and their related notes were incorrect in part. The corrected author line appears below.

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www.pnas.org/cgi/doi/10.1073/pnas.0914260107
Ca$^{2+}$, cAMP, and transduction of non-self perception during plant immune responses

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Edited by Jeffery L. Dangl, University of North Carolina, Chapel Hill, NC, and approved October 14, 2009 (received for review May 27, 2009)

Ca$^{2+}$ influx is an early signal initiating cytosolic immune responses to pathogen perception in plant cells; molecular components linking pathogen recognition to Ca$^{2+}$ influx are not delineated. Work presented here provides insights into this biological system of non-self recognition and response activation. We have recently identified a cyclic nucleotide-activated ion channel as facilitating the Ca$^{2+}$ flux that initiates immune signaling in the plant cell cytosol. Work in this report shows that elevation of cAMP is a key player in this signaling cascade. We show that cytosolic Ca$^{2+}$ elevation, nitric oxide (NO) and reactive oxygen species generation, as well as immune signaling, lead to a hypersensitive response upon application of pathogens and/or conserved molecules that are components of microbes and are all dependent on cAMP generation. Exogenous cAMP leads to Ca$^{2+}$ channel-dependent cytosolic Ca$^{2+}$ elevation, NO generation, and defense response gene expression in the absence of the non-self pathogen signal. Inoculation of leaves with a bacterial pathogen leads to cAMP elevation coordinated with Ca$^{2+}$ rise. cAMP acts as a secondary messenger in plants; however, no specific protein has been here-tofore identified as activated by cAMP in a manner associated with a signaling cascade in plants, as we report here. Our linkage of cAMP elevation in pathogen-inoculated plant leaves to Ca$^{2+}$ channels and immune signaling downstream from cytosolic Ca$^{2+}$ elevation provides a model for how non-self detection can be transduced to initiate the cascade of events in the cell cytosol that orchestrate pathogen defense responses.

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alnts are typically sessile creatures; when challenged with a stress, they cannot run or hide. In addition, vascular higher plants exist as multicellular organisms without a defense network of circulating mobile sentry cells equivalent to macrophages of the jawed vertebrate immune system. Therefore, cellular level recognition of pathogens as non-self by plants is a critical (and little understood) feature of system fitness. Non-self perception of pathogens initiates a signaling cascade leading to immune responses in plants. A key question underlying the rationale of the work reported here is the identification of components of the molecular mechanisms that allow for translation of non-self perception into a response signaling cascade in the plant cell; or as delineated by the Biblical-era scholar Hillel-the-Elder, “If I am not for myself, who will be for me?”

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One component of the repertoire of plant immune defense responses to non-self perception of a pathogen invader is the hypersensitive response (HR). HR involves rapid programmed cell death in the local region surrounding an infection site, limiting growth of the invading (avirulent) pathogen and arresting progression of disease symptoms (1). For well over a decade, it has been known that Ca$^{2+}$ is involved as an early signal in this defense response cascade (1). Much of the plant immune response characterization to date has involved application of pathogen cell extracts (or molecules purified from pathogens) to plant cell cultures (2–4; see refs. 5 and 6 for review). Specific evolutionarily conserved essential components of microbes, or pathogen associated molecular pattern (PAMP) molecules, elicit non-self perception and defense responses in plants under pathogenic attack. Early studies of pathogen-derived elicitor effects on cultured plant cells (7) identified an increase in cell-associated Ca$^{2+}$ as occurring within minutes after exposure to the pathogen-derived elicitor. Patch clamp studies suggested that elicitor-dependent activation of a cell membrane inwardly conducting Ca$^{2+}$-permeable ion channel contributed to the intracellular Ca$^{2+}$ elevation; elicitors increased channel open-state probability (8). Subsequent work with cell cultures and intact leaves confirmed that influx of Ca$^{2+}$ across the plasma membrane (PM) (and possibly efflux from intracellular stores as well) is an early event in plant immune signaling (6, 9–11). However, until only recently, no specific translation product of a plant ion channel gene was associated with Ca$^{2+}$ conductance leading to pathogen response signaling (or, for that matter, any other signal transduction system involving cytosolic Ca$^{2+}$ elevation in the plant cell).

The Arabidopsis dnd1 (“defense-no-death”) mutant lacks a functional cyclic nucleotide (cNMP) gated (non-selective) cation channel (CNGC2) and does not display an HR to avirulent pathogens (12). Prior work from this laboratory (13) has demonstrated that dnd1 cells lack a CAMP-activated inward PM Ca$^{2+}$ current, and that lack of this current is associated with impaired nitric oxide (NO) generation and plant immune responses, including HR (exogenous NO complements the dnd1 phenotype). NO has been referred to as the downstream “concert master” of innate immunity (14); orchestrating a suite of responses that includes defense gene expression and HR.

Little is currently known about how PAMP perception is linked to cytosolic Ca$^{2+}$ elevation during immune signaling in plant cells. The identification of a cAMP-activated, Ca$^{2+}$-conducting channel as a key component of plant immune signaling led to the work reported here that links the gating properties of this channel to upstream components of this signal transduction pathway.

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Results

cNMP, CNGCs, and Immune Signaling. A number of studies have demonstrated CNGC channel involvement in plant signaling cascades responding to pathogens (12, 13, 15). Patch clamp analysis of plant CNGCs in native (Arabidopsis) PM as well as upon expression in heterologous systems (13, 16–19) indicates that they are activated by cNMPs (most of the work has used...
cAMP), and that they can conduct Ca\(^{2+}\), K\(^{+}\), and, in some cases, Na\(^{+}\). We extend knowledge of CNGC function in plants here by showing that application of cAMP results in CNGC2-dependent elevation of cytosolic Ca\(^{2+}\) in leaves (Fig. 1A). The transitory cytosolic Ca\(^{2+}\) elevation initiated by this CNGC channel activating ligand in intact leaf cells of wild type (WT) plants occurs within minutes, and is absent in leaves of dnd1 (CNGC2 mutant) plants. In a fashion similar to these studies with leaves of Arabidopsis plants expressing recombinant aequorin, Volotovski et al. (20) have shown that application of cNMPs to isolated tobacco (Nicotiana tabacum) protoplasts resulted in elevation of cytosolic Ca\(^{2+}\). In this prior work, however, the elevation in protoplasm Ca\(^{2+}\) was not associated with a specific ion channel gene translation product, as we show here.

Perception of PAMPs by plant cells initiates basal-level, or “innate” immune responses (21). Lipopolysaccharide (LPS), a PAMP common to all Gram-negative bacteria (including Pseudomonas syringae), activates an inward cation current in Vicia faba (22) and 1,3-diazinane-2,4,5,6-tetrone (alloxan) are well characterized inhibitors of animal adenylyl cyclase (23–25), and block cAMP by inhibition of cNMP phosphodiesterase activity could not be associated with altered level of cAMP in plants. In consideration of this point, we took a pharmacological approach to manipulate endogenous cAMP. Dedioxyadenosine (DDA) and 1-methyl-3-(2-methylpropyl)-7-purine-2,6-dione (IBMX) are well characterized inhibitors of plant adenylyl cyclase and 1,3-diazinane-2,4,5,6-tetrone (alloxan) are well characterized inhibitors of plant adenylyl cyclase. A NO analysis was undertaken comparing the mean [Ca\(^{2+}\)] in the presence of 5% LPS and absence of alloxan. Mean values are shown for each treatment at 5–10 min after addition of LPS to the leaf tissue. Trees were totalized three times with similar results. For other fluorescence experiments monitoring in vivo generation of NO and ROS, quantitative analyses are shown in Fig. S4; see Fig. S4A for quantitative analysis of the experiment shown here in Fig. 3. (C) Analysis of the pathogen-induced cytosolic Ca\(^{2+}\) elevation in the presence and absence of LPS. For the experiments shown in Fig. 3 and the corresponding replications, mean [Ca\(^{2+}\)] during the Ca\(^{2+}\) spike was ascertained for leaves inoculated with Pst avrRpm1 and absence of alloxan. Bars with a * above indicate a significant effect of alloxan at P < 0.05 and bars with a ** above indicate a significant effect of alloxan at P < 0.01.

PAMP Signaling to NO Mediated by Endogenous cNMP. Genes encoding enzymes that synthesize (adenyl cyclase) and break down (cNMP phosphodiesterase) cAMP in plant leaves have not yet been identified (22); translational arrest of a specific gene has not been associated with altered level of cAMP in plants. In consideration of this point, we took a pharmacological approach to manipulate endogenous cAMP. Dedioxyadenosine (DDA) and 1,3-diazinane-2,4,5,6-tetrone (alloxan) are well characterized inhibitors of animal adenylyl cyclase (23–25), and block cAMP-dependent signaling in plants (26–29). Results presented in Fig. 2C and Fig. S1A indicate that both of these adenylyl cyclase inhibitors block LPS-dependent generation of NO in leaf tissue from WT plants.

cNMP phosphodiesterase enzymatic activity is present in plant cells (22, 26, 30) and can act to catabolize cAMP and maintain homeostatic levels in the cytosol after changes that occur in the level of this secondary messenger during signaling cascades (4). We hypothesized that blocking the breakdown of endogenous cAMP by inhibition of cNMP phosphodiesterase activity could therefore affect NO generation in the plant cell. Animal and plant cNMP phosphodiesterase is sensitive to the inhibitor 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione (IBMX) (31, 32). Application of IBMX to leaf epidermal peels from WT plants evokes NO generation in the absence of a pathogen signal (i.e., the PAMP LPS) (Fig. S1B). IBMX induction of NO response cascades (13), cAMP-dependent NO generation was also impaired in dnd1 cells compared to WT (Fig. 2A and B).
generation is impaired in dnd1 cells (Fig. S1B), and blocked by chelation of extracellular Ca2+ using EGTA (Fig. S1C). These results, demonstrating that IBMX induction of NO generation is dependent on the presence of free extracellular Ca2+, and a functional (cAMP-activated) PM Ca2+-conducting channel (CNGC2) are consistent with the aforementioned assertion that IBMX acts through activating cNMP-dependent Ca2+ current to evoke NO generation.

**HR and cNMPs In Plants.** Results presented in Figs. 1 and 2 and Fig. S1 provide evidence consistent with a link between cAMP, cytosolic Ca2+ elevation, and innate immune signaling in plant cells. Results presented in Figs. 2D and 3–5 extend this link to the plant HR to pathogens. We used the well-studied in planta interaction between Arabidopsis and the bacterial pathogen *P. syringae* p.v. *tomato* DC3000 (*Pst*) (33). Inoculation of WT Arabidopsis plants with *Pst* harboring the avirulence (avr) genes *avrRpm1* or *avrRpt2* results in HR, while in *dnd1* mutant plants lacking a PM inwardly conducting Ca2+-permeable channel this immune response is impaired (12, 13). Here, we focus on examining the possibility that cAMP acts as a signaling molecule to gate Ca2+ conductance through CNGCs during immune signaling leading to HR.

The Ca2+-dependent luminescence of recombinant aequorin has been used to demonstrate pathogen-associated cytosolic Ca2+ elevations in leaves of Arabidopsis plants exposed to *Pst* (11). Grant et al. (11) demonstrated that inoculating *Arabidopsis* with *Pst* harboring *avrRpm1* (but not *avrRpt2*) resulted in biphasic cytosolic Ca2+ elevations in leaf tissue; the first Ca2+ spike lasted approximately 15 min and peaked at approximately 8–10 min post-inoculation and a second, broader Ca2+ transient initiated at approximately 60 min post-inoculation and peaked at approximately 105 min (11). Grant et al. concluded that the first Ca2+ spike occurred in response to *Pst* whether or not the pathogen harbored an *avr* gene.

Here, we used a similar experimental system to examine whether coinfiltration of an adenylyl cyclase inhibitor with pathogen affected the pathogen-induced cytosolic Ca2+ elevation in *Arabidopsis* leaves (Fig. 3). A cytosolic Ca2+ elevation occurred between approximately 10 and 20 min post-inoculation of *Arabidopsis* leaves with *Pst avrRpm1* (Fig. 3 A and B). However, we note only a modest, slow second rise in Ca2+ initiating an approximate 60-min post-inoculation that did not result in a discernable peak much above that occurring with mock inoculum (either by ~105 min post-inoculation, or thereafter) (e.g., Fig. 3A). In our studies, inoculation of leaves with the pathogen *Pst* either with, or without *avr* genes led to a cytosolic Ca2+ elevation (with a peak at ~10–15 min post-inoculation) above that observed in leaves treated with mock inoculum (Fig. 3 B–D); a result similar to that found previously (11). The objective of these experiments was to determine if adenylyl cyclase inhibitor affected pathogen-associated cytosolic Ca2+ elevation. As shown in Fig. 3, coinfiltration of pathogen with inhibitor reduced the cytosolic Ca2+ elevation; this occurred with *Pst avrRpm1* (Fig. 3 A and B), *Pst avrRpt2* (Fig. 3C), or *Pst avrRpm1−, avrRpt2* (Fig. 3D); also see Fig. 2D.

The effect we find of the adenylyl cyclase inhibitor on plant response to pathogen cannot be attributed to a direct effect of the inhibitor on the bacterium. *P. syringae* growth on solid culture medium was unaffected by the adenylyl cyclase inhibitor (Fig. S2). Thus, we conclude that blocking cAMP synthesis during immune signaling in plants exposed to the bacterial pathogen *P. syringae* impairs pathogen-associated cytosolic Ca2+ elevation; specifically the initial Ca2+ spike occurring at approximately 10–15 min.

We also found that inhibition of the enzyme responsible for cAMP synthesis in plants had corresponding effects on HR as well as other steps in the immune signal transduction pathway (Fig. 4). Adenylyl cyclase inhibitor blocked HR in WT plants (Fig. 4A). In contrast to the effect of the adenylyl cyclase inhibitor, blocking the breakdown of cAMP had the converse effect on HR. Examination of leaves at an early time point post-inoculation indicated that coinfiltration of the cNMP phosphodiesterase inhibitor IBMX along with avirulent pathogen potentiated plant response, hastening onset of HR (Fig. 4B). In prior studies of Ca2+ signaling and HR from this lab (13), we monitored HR visually in ethanol-bleached leaves. In our current work, we note that development of HR can be ascertained at an earlier time (post-inoculation), and in a more sensitive and quantitative manner by monitoring ion leakage as was done in the work shown in Fig. 4 A and B. In other experiments similar to those shown in these figures, we found similar treatment effects using photography of ethanol-bleached leaves to monitor HR-related tissue necrosis. In these additional experiments, we found that treatment of leaves with the adenylyl cyclase inhibitor DDA blocked HR-related necrosis (black regions of ethanol-bleached leaves), and treatment of leaves with IBMX hastened onset of HR to avirulent pathogen.

Generation of reactive oxygen species (ROS) is known to occur downstream from cytosolic Ca2+ elevation in immune responses to pathogens. This point has been demonstrated with signaling cascades leading to HR of (*Arabidopsis*) plants inoculated with *avr* pathogen (6, 11), and also with ROS generation by cultured plant cells responding to pathogen elicitors/PAMPs (2, 34, 35). Results of
the experiment shown in Fig. 4D provide genetic and pharmacological evidence that Ca\(^{2+}\) as well as cNMPs are upstream from PAMP-dependent ROS generation. Application of the PAMP LPS led to ROS generation in plant cells; this pathogen signaling cascade was inhibited either by addition of an adenylyl cyclase inhibitor or in the absence of a functional Ca\(^{2+}\)-conducting CNGC2 channel in dnd1 cells (Fig. 4D).

Results presented here document effects of adenylyl cyclase inhibitors on a series of steps in immune signaling cascades; including those linking pathogen perception to cytosolic Ca\(^{2+}\) elevation, steps leading from PAMP recognition to NO and ROS generation, and plant HR to avirulent pathogens. The effects of the pharmacological agents (DDA and alloxan) on pathogen defense responses in plants could be due to a specific inhibition of cNMP synthesis, or potential nonspecific effects of these compounds on plant cells. Results presented in Fig. 4C are consistent with a specific inhibition of cAMP synthesis as mediating effects on immune signaling. If the pharmacological agent alloxan blocked the plant immune signaling cascade due to a specific effect on cAMP synthesis, then addition of exogenous cAMP should reverse the block. As shown in Fig. 4C, addition of cAMP did complement the effect of this pharmacological agent.

Results presented in Fig. 4B (and Fig. S1B and C) are consistent with IBMX acting to alter pathogen signaling by activating (through inhibition of cAMP breakdown) PM Ca\(^{2+}\)-conducting CNGC channels. IBMX potentiates the signaling cascade, leading to NO generation in the absence of a pathogen signal (Fig. S1B) and a hastening of HR in leaves inoculated with avr pathogen (Fig. 4B). Chelation of free extracellular Ca\(^{2+}\) in the assay medium prevents IBMX induction of NO generation (Fig. S1C). IBMX induction of NO generation is also impaired in dnd1 cells (Fig. S1B). Thus, these results suggest that IBMX acts through effects on cNMP mediated Ca\(^{2+}\) channel activation (as opposed to a nonspecific or unknown mechanism), and support a model of cAMP acting upstream from Ca\(^{2+}\) in the pathogen response signaling cascade. These results also are consistent with IBMX potentiation of HR in plants exposed to an avirulent pathogen (Fig. 4B) due to specific effects on these components of the pathogen response signaling cascade.

Further support for this model is presented in Fig. 5. In a series of experiments, cAMP levels in leaves during pathogen response signaling were monitored. During pathogen response signaling cascades we observe a cytosolic Ca\(^{2+}\) spike occurring approximately 10–20 min post-inoculation (Fig. 3). If this cytosolic Ca\(^{2+}\) elevation was caused by a pathogen-induced increase in cAMP, then the elevation in cAMP should occur in concert with, or before the onset of the influx of Ca\(^{2+}\) across the PM. As shown in Fig. 5A–C, we observe a rise in leaf cAMP 5–10 min after inoculation with pathogen. This cAMP elevation above ambient levels did not occur in mock-inoculated leaves (Fig. 5A and C). Coinfiltration of leaves with pathogen and adenylyl cyclase
inhibitor abolished the pathogen-associated cAMP rise (Fig. 5 A and B), while addition of the adenylyl cyclase inhibitor to leaves in the absence of pathogen had no effect (Fig. 5 B). As shown in Fig. 5 C, the pathogen-associated cAMP elevation (occurring at 10 min in this experiment) was potentiated by coinfiltration of IBMX with pathogen; in the presence of IBMX the cAMP elevation occurred sooner and was increased.

Results in Fig. 5 support the aforementioned assertion that the pharmacological agents act through corresponding inhibition of cAMP synthesis or breakdown during the signaling cascade. In addition, results shown in Fig. 5 provide evidence documenting a rise in cAMP in pathogen-inoculated leaves during plant immune signaling, and provide evidence consistent with a central tenant of the model developed here; that is, that elevation of cytosolic cAMP is a key step in the transduction of pathogen perception to downstream defense signaling cascades in plants. Further support for this model is provided in Fig. S3. Application of exogenous cAMP to Arabidopsis plants led to the expression of PRI, a gene known to be induced during pathogen response signaling leading to plant defense responses (36). The PAMP LPS has been shown in this report to activate a number of CNGC2- (and cAMP-) dependent steps of the pathogen response signaling cascade, including cytosolic cAMP elevation (17–19). The prior work is limited to studies of cultured cell response to extracts of pathogenic fungi containing elicitors. Application of fungal extracts induced elevation of endogenous cAMP level in plant cell cultures (4, 37, 38). However, when cAMP elevation in response to fungal elicitor was monitored specifically in plants, no pathogen-induced changes were found (4). The level of pathogen-induced cAMP elevation we find in Arabidopsis leaves (~3-fold increase over background; Fig. 5) was of a similar magnitude as that demonstrated in some of these previous studies with elicitor-induced elevations in cell cultures (4). The level of cAMP elevation we find here in response to pathogen was also of a similar magnitude as that found in prior studies associating cNMP signaling with plant response to abiotic (salinity and osmotic) stress (39).

In addition to the aforementioned prior studies linking pathogens to altered endogenous level of cNMP in cultured plant cells, other work indicates that application of exogenous cAMP can in some cases activate components of the pathogen response signaling cascade in cultured plant cells. Bindschedler et al. found that application of exogenous cAMP led to generation of H2O2 in French bean cells (3), but Davies et al. found no effect of exogenous cAMP on H2O2 generation in cultured Arabidopsis cells (2). The work here expands on these studies by linking pathogen perception to changes in cAMP and cAMP-dependent Ca2+ signaling during immune responses in plants.

At present, it is unclear what specific gene products encode the proteins responsible for synthesis and catalysis of cAMP in plant cells. A number of cAMP phosphodiesterase activity is present in plants (4, 27). Intriguingly, application of fungal extracts to plant cell cultures affects the level of extractable activities of cyclase (i.e., rising in the first few minutes) and phosphodiesterase (declining during this time) in a manner consistent with generation of transient cAMP elevations in a plant cell upon perception of a pathogen (4).

Within the context of work presented here, the molecular structure and functional properties of plant CNGCs provides a basis to refine our current model of immune signaling cascades. Plant CNGCs are PM localized and are ligand, as well as voltage gated (17–19). At the (inside negative) membrane potentials present across the plant cell PM, a rise in cytosolic cAMP would activate inwardly rectified cation current (17–19). CNGCs conduct monovalent cations and Ca2+; but in the presence of Ca2+ [i.e., at the millimolar concentrations likely present in the apoplast (41), monovalent cation conductance through CNGCs is restricted (18). Cytosolic calmodulin (CaM) binds to CNGCs at a region of the protein overlapping with the cNMP binding region; CaM binding prevents cAMP activation of CNGC current (42). Cytosolic Ca2+ is required for CaM block of current (42). In the presence of an exogenous supply of cAMP, CNGC current is non-inactivating (17). However, sustained PAMP (LPS) activation of inward Ca2+ current through CNGCs only occurs in the presence of a CaM antagonist (13).

Experimental evidence is lacking, but threathing quaternary models of plant CNGCs through known crystal structures of ion channels suggests they are tetramers: all native (animal) CNGCs are heterotetramers comprised of more than one CNGC gene product (43). Thus, the role CNGCs play in the pathogen response signal cascade can be envisioned as follows. Due to their likely heterotetrameric structure in native membranes, translational arrest of one plant CNGC gene alone could alter Ca2+ conductance and impair pathogen signaling leading to HR. Consistent with this point, loss-of-function mutants of either CNGC2 or CNGC4 (both are expressed in leaves) have impaired HR (12, 13, 16). Perhaps loss-of-function mutation of one CNGC gene (for example, CNGC2 in the ndd1 mutant) prevents assembly of the native channel complex but leads to formation of non-native (partially functional) channels in mutant plants. We find that Ca2+ -dependent (see ref. 13) NO generation in response to either cAMP (Fig. 2 A) or PAMPs (Fig. 13) is impaired, but not completely inhibited in ndd1 cells; results consistent with this model of channel assembly.

Cytosolic cAMP rise mediated by PAMP binding to an (as-yet-unidentified) PAMP receptor (or a step downstream from this receptor) could initiate and/or amplify pathogen perception-mediated cytosolic immune signaling by activating inward CNGC Ca2+ current. Resultant cytosolic Ca2+ elevation could then impact downstream steps of the signal cascade leading to NO generation through increased binding of cytosolic Ca2+ to CaM (44). Elevated cytosolic Ca2+ /CaM could also block sustained inward Ca2+ conducttion through CNGCs, leading to a transitory cytosolic Ca2+ spike associated with pathogen/PAMP perception (Figs. 2 D and 3 A, also see refs. 11 and 13). The initial cAMP-dependent activation of inward Ca2+ current and concomitant cytosolic Ca2+ spike (occurring minutes after pathogen recognition) can be thus envisioned to be temporally separated from the pathogen defense responses mediated through a rise in NO occurring over hours (44). The critical and early CNGC-mediated Ca2+ conductance occurring during plant immune signaling cascades could be initiated through PAMP perception and be similar during plant defense responses to virulent pathogens and HR invoked by plant recognition of avirulence factors in pathogens. The question of what factors initiated by avr gene product recognition augment this “basal” Ca2+ signal to induce HR remains unanswered here). This model of CNGC-mediated Ca2+ signaling is informed by, and consistent with the experimental results presented here. This work elucidates an example of a plant signaling...
pathway involving cAMP and that identifies a specific protein target of this important secondary messenger.

Materials and Methods

For details, see SI Text.

**Plant Material and Pathogen Inoculation.** Arabidopsis WT [Columbia (Col) ecotype], dnd1 (12), and aerin-transformed plants of these genotypes were used for all experiments. Avirulent Pst (avrRpt2* or avrRpm1*1) and virulent (avrRpt2* and avrRpm1*1) strains of Pst were used for syringe-injection or vacuum infiltration inoculation exactly as described in ref. 44.

Reagents.

Unless otherwise noted, all reagents were purchased from Sigma.

The membrane permeable lipophilic cAMP analog dibutyryl-cAMP was used in all cases.

**In Vivo NO and ROS Analysis.** NO and ROS generation were evaluated in guard cells of epidermal peels using fluorescent dyes as described (13, 35).

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