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The Involvement of Secondary Messengers in Plant Receptor-Like Kinase Signal Transduction

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The involvement of secondary messengers in plant receptor-like kinase signal transduction

Yichen Zhao

University of Connecticut (2013)

In plant signaling networks, plasmalemma localized leucine-rich repeat receptor-like kinases (LRR-RLKs) are involved in signal perception and initiation of cytosolic signal transduction cascades that alter cell function. Little is currently known about the molecular mechanisms involved in the translation of ligand binding to any LRR-RLK into the initiation of a cytosolic signal. This dissertation presents studies with BRI1, PEPR1, and FLS2; LRR-RLKs involved with hormone perception and plant immunity. The ligands for these receptors are the steroid hormone brassinosteroid (BR), an endogenous plant elicitor peptide ‘Peps’, and the bacteria flagellin (flg) protein, respectively. Binding of BR, Peps, and flg22 to their cognate receptors induced cytosolic Ca\(^{2+}\) elevation, which was responsible for ligand-dependent gene expression and phenotypes. The BRI1 and PEPR1 receptors share a cytosolic-localized guanylyl cyclase (GC) domain. A cGMP activated Ca\(^{2+}\) conducting ion channel cyclic nucleotide gated channel (CNGC) 2 was involved in their ligand-dependent Ca\(^{2+}\) elevation. BR can induce secondary messenger cGMP elevation \emph{in vivo}, and the treatments that either promote or prevent cytosolic cGMP generation mimicked or prevented the ligand-induced signaling cascades and gene expression. Ligand binding to the PEPR1 and FLS2 receptors initiated cytosolic Ca\(^{2+}\) signaling as well as NO and H\(_2\)O\(_2\) generation, and pathogen immunization. Both PEPR1 and FLS2 receptors are required to reach the maximal level of signaling and phenotype responses induced by either of the activating ligands. Therefore, there is interdependence between receptor PEPR1
and FLS2, and both of these receptors are required to initiate defense immune responses. Flg is different from BR and Peps, intracellular Ca\(^{2+}\) release into the cytosol may contribute to flg dependent Ca\(^{2+}\) signaling. Secondary messenger inositol triphosphate (IP\(_3\)) and IP\(_6\) (produced from IP\(_3\) phosphorylation) generation during plant cell signaling could lead to release of intracellular Ca\(^{2+}\) stores in plant cells. Experimental evidence suggesting that G proteins, and inositol phosphate elevations may be involved in translating perception of flagellin into release of intracellular Ca\(^{2+}\) stores, contributing to the cytosolic Ca\(^{2+}\) elevation that is a critical signal leading to immune responses in plant cells. Our results imply that secondary messengers are involved in the signal transduction of these receptors.
The Involvement of Secondary Messengers in Plant Receptor-Like Kinase Signal Transduction

Yichen Zhao

B.S., China Agricultural University, [2009]

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Submitted in Partial Fulfillment of the
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at the
University of Connecticut

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The Involvement of Secondary Messengers in Plant Receptor-Like Kinase Signal Transduction

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Chapter 1 Overview

The research work present in this dissertation is focused on Ca\(^{2+}\)-dependent signaling in plants and the involvement of secondary messengers in plant receptor-like kinase (RLK) signal transduction cascades. In plants, RLKs are used to transduce extracellular signals into the cell, and they are involved in many plant biological signal processes, such as pathogen defense, hormone perception and meristem regulation (Chae et al., 2009). The leucine-rich repeat (LRR) RLKs is the major group of plant RLKs. The extracellular LRR domains are known to be involved in protein-protein interaction or binding of the receptor to extracellular small molecules such as hormones or peptides (DeYoung and Innes, 2007; Qi et al., 2010; Ma et al., 2012). Some LRR-RLK signal transduction occurs through phosphorylation cascades that have already been reviewed in prior publications (Asai et al., 2002; Kim and Wang, 2010). Currently, there is accumulating evidence (including past and current work from the Berkowitz lab) that the secondary messenger Ca\(^{2+}\) is also involved in LRR-RLK receptor dependent signal transduction (Qi et al., 2010; Ma et al., 2012). The Ca\(^{2+}\) signaling is due to the changes of cytosolic Ca\(^{2+}\) concentration. Cytosolic Ca\(^{2+}\) concentration is altered when Ca\(^{2+}\) moves through plasma membrane channels after perception of the stimuli. Projects that I have done are focused on the role of Ca\(^{2+}\) and other secondary messengers in LRR-RLK receptor signal transduction cascades. This dissertation includes descriptions of these projects.

Chapter 2 introduces the possibility that the other signaling transduction pathway may exist in hormone brassinosteroid (BR) dependent signaling. BR related signaling is initiated upon BR binding with its receptor BR insensitive 1 (BRI1). BRI1 is a LRR-RLK receptor. The experimental results suggest that this process involve the secondary messenger cyclic guanosine 3'\,:5'-monophosphate (cGMP) and Ca\(^{2+}\). BRI1 contains a cytosolic guanylyl cyclase (GC)
domain, which can synthesize cGMP from GTP (Kwezi et al., 2007). Ca\(^{2+}\)-dependent BR signaling may occur from Ca\(^{2+}\) movement into the cytosol through a cyclic nucleotide gated channel (CNGC). CNGCs are plasma membrane Ca\(^{2+}\)-conducting channels; they are activated by cyclic nucleotides (cyclic adenosine 3′:5′-monophosphate (cAMP) and cGMP) and conduct Ca\(^{2+}\) into plant cells. The Ca\(^{2+}\) signaling that BR induced may lead to BR-dependent gene expression or affect plant phenotypes related to the action of this hormone. The Ca\(^{2+}\)-dependent expression of these BR-dependent genes may be independent from the phosphorelay cascade. On the other hand, some BR related gene expression that is not associated with cGMP and Ca\(^{2+}\) signaling occurs through a phosphorylation cascade and kinase signaling. The evidence shows a possible existence of two independent BR signaling pathways in BR signaling transduction cascades.

Chapters 3 is focused on the interdependence between two other LRR-RLK receptors, flagellin sensing 2 (FLS2) and plant elicitor peptides receptor (PEPR), these two receptors act upon perception of the pathogen-related flagellin peptide (studies using the peptide flg22) and plant defense related peptide Peps (an endogenous plant elicitor peptide involved in immune signaling), respectively. The signaling cascades downstream from both of these receptors involve cytosolic Ca\(^{2+}\) signaling, and there is some interaction between these two receptors. The research indicates that Pep/PEPR receptor signaling involves cGMP activated Ca\(^{2+}\) influx into the cytosol from extracellular pools while FLS2 signaling does not (Ma et al., 2012). However, both PEPR and FLS2 receptors are required for maximal flg22- and Pep-dependent defense signaling and immune responses. Further evidence also suggests that the Ca\(^{2+}\) sensors that are critical for flg22 associated immune signaling and FLS2 co-receptor BRI1-associated receptor kinase 1 (BAK1) are also required for Pep-induced immune defense response (Boudsocq et al., 2010; Schulze et al., 2010).
From the research work presented in Chapter 3, we find that in certain circumstances, flg22 induced defense gene expression may not require the presence of an extracellular Ca\(^{2+}\) source. Further evidence also suggests that flg22/FLS2 induced cytosolic Ca\(^{2+}\) signaling may involve another cytosolic secondary messenger, inositol triphosphate (IP3) and/or inositol hexaphosphate (IP6). The work presented in Chapter 4 extends the work from Chapter 3, still focusing on the flg22/FLS2 immune signaling pathway. The evidence from this chapter suggests that phospholipase C (PLC) signaling may be involved in flg22 associated defense gene expression and immune responses in plants.

In a fashion similar to cGMP, cAMP is also an important secondary messenger in plants. However, unlike in animals, there is no canonical adenylyl cyclase (AC) that has been identified in plants. No gene encoding an AC has yet been identified in plants. Chapter 5 is combination of two research projects that use screening methods to find candidate genes involved in the signaling pathways mention above. The purpose of one of the projects is to identify an AC gene in Arabidopsis through screening. This project involves an attempt to use an Arabidopsis cDNA expression library and an *Escherichia coli* mutant that lacks the ability to generate cAMP as a signal in a lactose fermentation pathway as a screening method to identify a plant gene encoding an AC. The other project described in Chapter 5 also involves a high-throughput genetic screen. In this case, Arabidopsis mutagenized seed are used. They are screened for suppression of a wild type phenotype of impaired root growth on medium containing a high concentration of the Pep (an endogenous plant elicitor peptide) peptide, a ligand for PEPR receptors. The goal of this work is to use thermal asymmetric interlaced (TAIL) PCR technology to sequence candidate genes encoding proteins that are involved downstream from the receptor in the Pep/PEPR signaling pathway. Some positive candidates been picked up from both of this two projects.
However, further research and more confirmation will be required to verify the positive candidates from these two screening projects.

The last chapter is work extended from Chapter 2. In Chapter 2, we still lack of the evidence to link the GC catalytic domain with the cGMP signaling involves in BR-dependent signal transduction pathway. In Chapter 6, the works were focused on using site-direct mutagenesis to point mutated the key residue that responsible for GC activity of BRI1. The plasmid contains BRI1 GC domain mutation was used to generate the transgenic mutant plants for further research.

The main research results from this dissertation research are focused on secondary messengers (e.g. Ca$^{2+}$, cGMP, IP3 and IP6), and their involvement in plant receptor like kinase signaling transduction. The signaling transduction pathways of these receptors are independent or interact with each other. During those signaling transduction cascades, secondary messengers play a very important role in hormone perception and pathogen defenses.
Chapter 2 Teaching an old hormone new tricks: Cytosolic Ca\textsuperscript{2+} elevation involvement in plant brassinosteroid signal transduction cascades

Abstract

Brassinosteroids (BRs) are hormones that control many aspects of plant growth and development, acting at the cell level to promote division and expansion. BR regulation of plant and plant cell function occurs through altered expression of many genes. Transcriptional reprogramming downstream from cell perception of this hormone is currently known to be mediated by a phosphorylation/dephosphorylation (‘phosphorelay’) cascade that alters stability of two master transcription regulators. Here we provide evidence that BR perception by their receptor also causes an elevation in cytosolic Ca\textsuperscript{2+}, initiating a Ca\textsuperscript{2+} signaling cascade in the plant (Arabidopsis) cell cytosol. BR-dependent increases in the expression of some genes (\textit{INDOLE-3-ACETIC ACID-INDUCIBLE 1} (IAA1) and \textit{PHYB ACTIVATION TAGGED SUPPRESSOR 1} (BAS1)) were impaired in wild type (WT) plants by a Ca\textsuperscript{2+} channel blocker and also in the “defense-no-death” (\textit{dnd1}) mutant, which lacks a functional cyclic guanosine 3’:5’-monophosphate (cGMP)-activated cell membrane Ca\textsuperscript{2+}-conducting channel. Alternatively, mutations that impair the BR phosphorelay cascade did not much affect BR-dependent expression of these genes. Similar effects of the Ca\textsuperscript{2+} channel blocker and \textit{dnd1} mutation were observed on a BR plant growth phenotype, de-etiolation of the seedling hypocotyl. Further evidence presented in this report suggests that a BR and BR receptor-dependent elevation in cGMP may be involved in the Ca\textsuperscript{2+} signaling cascade initiated by this hormone. Work presented
here leads to a new model of the molecular steps that mediate some of the cell responses to this plant hormone.
Introduction

Brassinolide and similar compounds, the ‘brassinosteroids’ (BRs), are a family of growth-promoting steroidal hormones that are ubiquitous in the plant kingdom. BRs have a positive effect on cell expansion and division, and therefore plants with mutations that impair BR signaling have a dwarf phenotype (Clouse, 2011). BRs regulate a broad range of physiological processes in plants, including reproduction and senescence programs, leaf development, root growth, vascular differentiation, and responses to light as well as other environmental cues, often in an integrated fashion with other hormones (Clouse, 2011; Witthöft and Harter, 2011; Ye et al., 2011). As detailed in a number of recent reviews (Kang et al., 2010; Clouse, 2011; Witthöft and Harter, 2011; Yang et al., 2011), the hormone is perceived at the cell surface upon binding to its receptor, brassinosteroid insensitive 1 (BRI1). BRI1 is a member of a large family of Leu-rich-repeat receptor-like kinases (LRR-RLKs). The global effects of the signaling cascade initiated upon BR binding to the BRI1 receptor on plant growth and development occur through the regulation by the steroid hormone of the expression of a wide array of genes.

Numerous studies, as summarized in the aforementioned reviews, have delineated steps in a protein phosphorylation/dephosphorylation (‘phosphorelay’) cascade as the basis for BR-mediated transcriptional reprogramming. Some of the steps involved in this phosphorelay system include the following. The BR receptor is maintained in an inactive state by binding of cytosolic BRI1 kinase inhibitor 1 (BKI1). Hormone binding to BRI1 releases BKI1 and recruits binding of the BRI1 co-receptor BRI1-associated receptor kinase 1 (BAK1) (leading to BRI1:BAK1 transphosphorylations). Downstream from receptor phosphorylation, the phosphorelay cascade involves phosphatase-dependent deactivation of the cytosolic kinase BR insensitive 2 (BIN2), a negative regulator of BR signaling. When activated, BIN2 phosphorylates two master
transcription factors (TFs), brassinazole resistant 1 (BZR1) and BRI1-EMS-suppressor 1 (BES1) (preventing their function in the nucleus). In their unphosphorylated, active state (i.e. in the presence of deactivated BIN2) these TFs move to (or are retained in) the nucleus and activate many genes, including other TFs, thus amplifying the BR signaling output.

The signal transduction cascade that links cell perception of extracellular BR to the control of gene expression, as well as the structure: function relationship of BRI1 and how the receptor acts through auto- and transphosphorylations to facilitate the BR response cascade is one of the best-studied signaling pathways in plants (Jaillais et al., 2011). And yet, in the wake of the flood of knowledge developed about BR/BRI1 signaling, some recent reviews point out a striking as-yet-unresolved issue regarding action of this hormone on plant cells. BR perception at the cell surface involves immediate effects on cell function that suggest a signaling cascade distinct from the phosphorelay system (Witthöft and Harter, 2011; Harter et al., 2012). Further, other recent studies suggest some BR-dependent plant phenotypes may not be mediated by phosphorelay signaling (Hacham et al., 2011).

Some time ago, Kwezi et al. (2007) identified a guanylyl cyclase (GC) activity associated with the cytosolic domain of (Arabidopsis) BRI1; this portion of the receptor (expressed as a recombinant protein in Escherichia coli and affinity-purified) generated cyclic guanosine 3’:5’-monophosphate (cGMP) from GTP in vitro. Prior studies from this lab (Qi et al., 2010) with another LRR-RLK (Plant Elicitor Peptide Receptor 1 (PEPR1)) that has a similar putative GC domain as BRI1 have demonstrated a similar level of in vitro GC activity and, further, provided evidence that PEPR1 signaling involves activation (possibly due to cGMP generation) of a Ca$^{2+}$-conducting cyclic nucleotide gated channel (CNGC) in vivo (Ma et al., 2012). The focus of the work presented here was to test the hypothesis that (some components of) BR:BRI1 signaling
involves cytosolic Ca\textsuperscript{2+} elevation, which is well known to act as a secondary messenger system in all cells (Dodd et al., 2010).

There are few reports of Ca\textsuperscript{2+} involvement in steps of the BR phosphorelay signaling cascade downstream from BRI1. DWARF1, an enzyme involved in BR synthesis is activated by Ca\textsuperscript{2+}/calmodulin (CaM); this would suggest that on a long-term basis, the Ca\textsuperscript{2+} status of cells might impact the generation or steady-state level of the steroidal hormone (Du and Poovaiah, 2005). A report on hormone signaling in *Triticum aestivum* (Singla et al., 2006) indirectly suggested that expression of a Ca\textsuperscript{2+} regulated auxin-responsive gene in wheat orthologous to Arabidopsis *IAA1* is also induced by BR. Working with Arabidopsis, we used genetic and biochemical approaches to evaluate if BR-dependent Ca\textsuperscript{2+} signaling is involved in regulation of *IAA1* (as well as other BR-responsive gene) expression, and plant phenotypes impacted by BR.

**Results**

**Hormone- and receptor-dependent cytosolic Ca\textsuperscript{2+} elevation.**

We evaluated BR-induced Ca\textsuperscript{2+} signaling by monitoring the effect of exogenous epibrassinolide (eBL; see Materials and Methods’) on the level of cytosolic Ca\textsuperscript{2+} in detached leaves of Arabidopsis plants expressing the Ca\textsuperscript{2+} reporter protein apoaequorin. Studies were undertaken using several wild type (WT) Arabidopsis ecotypes, as well as genetic mutants expressing the gene encoding this Ca\textsuperscript{2+} reporter protein. As shown in Figure 1, we observed an elevation in cytosolic Ca\textsuperscript{2+} initiating seconds after adding BR to leaves from (Wassilewskija (Ws-2) ecotype) WT plants. The *bri1-5* genotype has reduced sensitivity to BR (Wang et al., 2005) due to a mutation impairing the ability of BRII to bind the hormone ligand
Figure 1. Exogenous BR-dependent elevation of cytosolic Ca\(^{2+}\) in detached leaves from WT (Ws-2) and bri1-5 plants.

Ligand was added at time ‘0’; change in Ca\(^{2+}\) was calculated for each replicate by subtracting the value recorded at time 0 from all other measurements. Results are presented as mean values of Ca\(^{2+}\) increase (replicate number in parentheses) ± SE shown at 1 min intervals. A similar experimental design and data presentation were used for the experiments shown in Figures 2, 3 and 5. Note that the WT plants used for experiments shown in Figure 2, 3 and 5 were Col. Studies of BR signaling that involve exogenous application of eBL typically use concentrations of 100 nM to 1 µM (Li et al., 2010; Hacham et al., 2011). For the studies of Ca\(^{2+}\) elevation in aequorin (aeq)-expressing plants shown in this report, we used 100 nM eBL. We found a significantly greater increase in cytosolic Ca\(^{2+}\) when 1 µM was added to leaves of WT plants (data not shown).
As shown in Figure 1, the cytosolic Ca\textsuperscript{2+} elevation occurring upon addition of BR to WT leaves is impaired in the \textit{bri1-5} mutant; generation of the hormone-induced Ca\textsuperscript{2+} signal requires function of the BRI1 receptor.

BR signaling through the BRI1 receptor involves physical and functional interaction of BRI1 with its co-receptor BAK1 (Nam and Li, 2002). The \textit{bak1} mutant lacks expression of BAK1 mRNA and shows impairment of some aspects of BR signaling (Chinchilla et al., 2007; Kemmerling et al., 2007). Here, we found no effect of BAK1 null mutation on BR/BRI1-dependent Ca\textsuperscript{2+} signaling; hormone-induced cytosolic Ca\textsuperscript{2+} elevation was similar in (Columbia (Col) ecotype) WT and \textit{bak1} leaves (Figure 2). Some (but not all) functions of the BAK1 co-receptor involved with BR signaling can be replaced by other members of the BAK1 protein family such as BAK1-like 1 (BKK1) (He et al., 2007). The lack of an effect of BAK1 mutation on BR/BRI1 signaling could be due to either a) the possible replacement of BAK1 function by BKK1 (or a similar protein) in the \textit{bak1} mutant, or b) the molecular step(s) leading to BR-dependent Ca\textsuperscript{2+} signaling involve some aspect of BRI1 function independent of its interaction with a co-receptor.

As mentioned above, BRI1 has been shown to have \textit{in vitro} guanylyl cyclase activity independent of the presence of a co-receptor (Kwezi et al., 2007). If BRI1 GC activity is involved in BR-induced cytosolic Ca\textsuperscript{2+} elevation, the Ca\textsuperscript{2+} signal could be generated by opening of cGMP-activated Ca\textsuperscript{2+}-conducting ion channels in the cell membrane upon binding of BR to its receptor. We examined this hypothesis by monitoring BR-dependent cytosolic Ca\textsuperscript{2+} elevation in the ‘defense-no-death’ (\textit{dnd1}) mutant, which lacks a functional \textit{CNGC2} gene (Clough et al., 2000). \textit{CNGC2} is a plasma membrane localized, inwardly conducting Ca\textsuperscript{2+}-permeable channel (Ali et al., 2007) activated by cGMP (Qi et al., 2010). Results shown in Figure 3 indicate that, in
Figure 2. Exogenous BR-dependent elevation of cytosolic Ca$^{2+}$ in detached leaves from WT (Col) and bak1 plants.
Figure 3. Exogenous BR-dependent elevation of cytosolic Ca\textsuperscript{2+} in detached leaves from WT (Col) and \textit{dnd1} plants.
contrast to the lack of an effect of BAK1 null mutation (Figure 2), BR-dependent cytosolic Ca\(^{2+}\) elevation is impaired in plants that lack a functional CNGC2 gene.

**BR signaling involves generation of the secondary messenger molecule cGMP.**

Changes in the level of the cytosolic secondary messenger molecule cGMP during myriad signaling cascades are monitored in animal cells using a non-Fluorescence Resonance Energy Transfer (FRET) based ratiometric **Fluorescence indicator** of cGMP (FlincG) protein. Nausch et al. (2008) developed a coding sequence for such a FlincG reporter by fusing the circularly permutated enhanced GFP sequence in tandem with a portion (including the cGMP binding domain) of the coding sequence for animal type-I cGMP-activated protein kinase. Isner and Maathuis (Isner and Maathuis, 2011) subcloned the FlincG coding sequence into a plant expression plasmid (along with the 35S Cauliflower Mosaic Virus promoter), generated FlincG-expressing Arabidopsis plants, and demonstrated that real-time increases in cytosolic [cGMP] could be monitored in roots of these plants upon addition of signaling molecules known to elevate the cyclic nucleotide secondary messenger. Here, we demonstrate that application of BR to seedlings leads to elevation of cGMP at the root tip (in the columella root cap and meristematic zone) that is not evidenced when water alone is added to roots (Figure 4D). This BR-dependent elevation in cytosolic cGMP could be responsible for the CNGC2-mediated, BR-induced Ca\(^{2+}\) signal (Fig. 3).

Results consistent with this model of BR signaling are shown in Figure 5. In this experiment, BR-dependent Ca\(^{2+}\) elevation was monitored in plants exposed to the GC inhibitor 6-anilino-5, 8-quinolinedione (LY83583). LY83583 has been used to block cGMP-mediated
Figure 4. Effects of exogenous BR on cell cytosolic cGMP in Arabidopsis seedlings expressing the fluorescent reporter protein FlineG.

After application of BR (or water as a control) to one end of the seedling chamber mounted on the stage of a confocal microscope, fluorescence images of the root tip were taken over time. (A-C) Images of the root tip at 0, 5, and 10 min after application of BR. Corresponding images of control-treated roots were dark to the eye at all time points. (D) Quantitative analysis of change in fluorescence over time is shown for BR-, and Control (water with 0.001% (v/v) DMSO) treated roots. Signals are shown as means (biological replication no., recorded from different seedlings, are in parentheses) ± SE. For each replicate recording, a series of fluorescence ratios at specific times after treatment were generated. The fluorescence ratios were signals recorded at a specific time relative to the signal at time 0 (F/F₀; see for comparison (Isner and Maathuis, 2011)). The difference in fluorescence change between seedlings treated with BR and water was not due to differences in initial fluorescence (F₀) of the seedlings, which was 374±35 and 397±59 for water-, and BR-treated seedlings, respectively. (E) BR-induced cGMP increase in WT (Col) seedlings in the absence (control, 0.04% (v/v) DMSO) and presence of a GC inhibitor. It should be noted that both treatments shown in E had higher levels of solvent than used in the experiment shown in A-D (0.001% (v/v) DMSO) due to use of the GC inhibitor in the experiment shown here.
Figure 5. Exogenous BR-dependent elevation of cytosolic Ca\textsuperscript{2+} in detached leaves from WT (Col) leaves in the absence (control) and presence of a GC inhibitor.

Detached WT- aeq Arabidopsis leaves exposed to a pretreatment involving incubation in water (0.04\% (v/v) DMSO) or GC inhibitor LY 83583 for ~15 min. Cytosolic Ca\textsuperscript{2+} elevation was monitored after exposure of the pretreated leaves to the BR (A) or glutamate (B). A) BR-dependent cytosolic Ca\textsuperscript{2+} elevation was reduced in leaves pretreated with GC inhibitor compared with leaves pretreated with water. B) Pretreatments are similar to those in A, and 1 mM glutamate (glu) was added at the time “1”. Cytosolic Ca\textsuperscript{2+} elevation caused by glu is dependent on the glu receptor and does not involve the GC activity. These results suggest that the inhibition of GC activity affects BR signaling. Results are presented as mean values of Ca\textsuperscript{2+} increase (replicate number in parentheses) + SE shown at 1 min intervals.
signaling in a number of plant systems (e.g. Salmi et al., 2007; Cousson, 2010; de Montaigu et al., 2010; Teng et al., 2010). BR-induced cytosolic Ca\(^{2+}\) elevation in WT plants was blocked in plants treated with the GC inhibitor (Figure 5). The utility of the GC inhibitor for probing the relationship between BR and Ca\(^{2+}\) signaling is supported by the results of a separate, control experiment. Glutamate (Glu) receptors are a second family, besides CNGCs, of Ca\(^{2+}\) conducting channels in plants. Glu receptors conduct Ca\(^{2+}\) in response to this amino acid ligand (Kudla et al., 2010); they are not activated by cGMP. Glu application to WT leaves causes an immediate increase in cytosolic Ca\(^{2+}\). Exposure of leaves to the GC inhibitor had no effect on this Glu-dependent Ca\(^{2+}\) signal; the Ca\(^{2+}\) elevation was as great or greater in the presence of the inhibitor as was observed when Glu was added to leaves without the inhibitor (Figure 5B). One possible explanation for why the GC inhibitor affected BR-dependent Ca\(^{2+}\) elevation and not Glu-dependent Ca\(^{2+}\) elevation is that BR-dependent generation of cGMP (Figure 4A-D) is impaired by the inhibitor. Results shown in Figure 4E support this contention. Recordings of cGMP-dependent fluorescence were made from WT (FlincG-expressing) seedlings exposed to BR after pretreatment with the GC inhibitor, or solvent alone (‘Control’). Exposure of seedlings to the inhibitor impaired BR-dependent cGMP generation. These results support a conclusion that the well documented action of LY83583 as a GC inhibitor blocks BR-induced Ca\(^{2+}\) elevations (Figure 1 and 5A) due to inhibition of BR-induced cGMP rise (Figure 4E); thus impairing activation of CNGC channels (Figure 3) and not other Ca\(^{2+}\)-conducting channels.

**Ca\(^{2+}\) signaling and brassinosteroid-dependent gene expression.**

Studies were undertaken to test the hypothesis that BR-dependent Ca\(^{2+}\) elevation impacts gene expression induced by the hormone. In a manner similar to that already demonstrated for orthologous genes in rice (Song et al., 2009) and wheat (Singla et al., 2006), we found that BR
application to Arabidopsis seedlings increased expression of IAA1 (INDOLE-3-ACETIC ACID-INDUCIBLE 1) in (Ws-2) WT plants (Figure 6A). In contrast to effects on WT plants, application of BR to bri1-5 plants had no significant effect on IAA1 expression. The bri1-5 has been used as a genotype with impaired BR signaling in many studies. Use of the bri1-5 mutant allowed us to develop aeq-expressing plants with a defective BRI1 receptor (i.e. Figure 1).

However, the bri1-5 allele is not a fully null (loss-of-function) mutation. Additional evidence consistent with the model developed here that BR perception by the BRI1 receptor is the mechanistic basis for generation of the Ca^{2+} signal as well as the downstream effects of the Ca^{2+} signal on IAA1 expression is presented in Figure 6B. The bri1-701 allele is a bona fide hormone receptor null mutation (Gou et al., 2012). We used the bri1-701 mutant to further evaluate if the BR-evoked signaling that is the focus of our work is dependent on the hormone receptor BRI1. Results shown in Figure 6B demonstrate that BR-dependent increases in expression of IAA1 is blocked in the bri1-701 mutant, as was the case when we tested BR-dependent IAA1 expression in the bri1-5 genotype (Figure 6).

The defective BRI1 receptor in the bri1-5 genotype presumably impairs the signaling cascade linking BR perception to downstream gene (IAA1) expression (Figure 6). Results presented in Figure 7 suggest that the Ca^{2+}-conducting channel CNGC2 may be involved in this BR signaling cascade. As was the case with the bri1-5 mutant, the BR-dependent IAA1 expression observed in WT (Col) plants is blocked in the dnd1 mutant. This result provides genetic evidence consistent with the possibility that at least in the case of IAA1, BR-dependent cytosolic Ca^{2+} elevation (which is impaired in the dnd1 mutant, Figure 3) can mediate (or at least is associated with) effects of the hormone on gene expression.
Figure 6. Comparison of BR-dependent IAA1 gene expression between WT and BRII mutant plants.

(A) Effect of BR application to WT (Ws-2) and bri1-5 plants on IAA1 expression. The bri1-5 mutant is a weak allele of BRII mutant. (B) Effect of BR application to WT (Col) and BR null mutant bri1-701 on IAA1. bri1-701 is a null mutant of BRII. Results are presented as means (for a given treatment and genotype) ± SE. For each genotype, IAA1 expression in the absence of added BR was normalized to 1. For each genotype, ANOVA analysis was undertaken to evaluate means separation between treatments (presence and absence of exogenous BR application). Asterisks above the bar representing the ‘+ BR’ treatment indicate significant differences (at P<0.05). A similar data presentation and ANOVA analysis of means separation was used for the experiments shown in Figures 7, 9, 10, 11 13).
Work presented in this report underlies the development of a novel model of a cellular signaling cascade initiated by perception of the hormone BR. BR binding to its receptor BRI1 leads to immediate (initiating at < 1 min) increases in the secondary cytosolic signaling molecules cGMP and Ca$^{2+}$. Generation of the BR and BRI1-dependent Ca$^{2+}$ signal is impaired in the dnd1 mutant. The function of CNGC2 as a component of a Ca$^{2+}$-conducting channel is required for optimal BR signaling that leads to increased expression of IAA1 (Figure 7), a gene known to be responsive to BR (Singla et al., 2006; Song et al., 2009). Further experiments examining IAA1 expression are consistent with this model (Figure 8). Prior studies have shown that elevation of cytosolic cGMP (by application of a lipophilic analog of cGMP to leaves) activates plasma membrane channels composed of CNGC2 subunits, resulting in elevation of cytosolic Ca$^{2+}$ (Ali et al., 2007; Qi et al., 2010). Here, we find that addition of a lipophilic analog of cGMP (dibromo-cGMP) to WT (Col) seedlings increased expression of IAA1 in the absence of added BR (Figure 8). In contrast to the effect of cGMP addition, application either a Ca$^{2+}$ channel blocker or a GC inhibitor completely blocked BR-dependent IAA1 expression (Figure 8). Consistent with results shown in Figure 7 using a genetic approach to affect BR signaling (i.e. by monitoring IAA1 expression in the dnd1 mutant), results presented in Figure 8 indicate that the signal transduction cascade linking BR perception by its receptor to downstream gene expression (at least in the case of IAA1) requires the BR/BRI1 dependent changes in the level of the secondary messenger molecules cGMP and Ca$^{2+}$. Further, elevation of the secondary messenger molecule cGMP alone might mimic effects of the hormone on IAA1 gene expression.

Additional studies were undertaken to further examine the novel finding that impairment of the phosphorelay cascade had little to no effect on BR-dependent gene expression, at least in the case of IAA1. BAS1 (PHYB ACTIVATION TAGGED SUPPRESSOR 1) encodes a protein
Figure 7. Effect of BR application to WT (Col), dnd1, bin2-1, and bri1-301 plants on IAA1 expression.
The expression of IAA1 in the absence of exogenous BR relative to the level in WT plants was 0.73, 0.65, and 0.86 in dnd1, bin2-1, and bri1-301 plants, respectively.
Figure 8. Manipulation of the cytosolic secondary messenger molecules Ca$^{2+}$ and cGMP impacts the effect of exogenous BR on \textit{IAA1} expression in WT (Col) plants.
The GC inhibitor (LY 83583) is added in 0.04\% (v/v) DMSO solvent. Therefore, in this experiment, all treatments (including the Control) had 0.04\% DMSO. Further experiments indicated that in the absence of any DMSO solvent, application of dibromo-cGMP still increased \textit{IAA1} expression as compared to the Control. Means separation was analyzed by ANOVA T-test comparisons of each treatment individually with the control. An asterisk over a bar representing a treatment indicates a significant (at P< 0.05) difference from the control.
involved in BR inactivation/catabolism (Turk et al., 2003; Turk et al., 2005; Thornton et al., 2011) and its expression is increased upon BR perception (Goda et al., 2002) as part of a feedback system that logically regulates action of this hormone on cells (Tanaka et al., 2005; Turk et al., 2005). We find that similar to BR-dependent IAA1 expression, BR-dependent BAS1 expression was blocked in the br1-701 mutant and impaired in the dnd1 mutant (Figure 9).

As reported above, in contrast to the lack of function of CNGC2 gene (Figure 3), BR-dependent cytosolic Ca\(^{2+}\) elevation is not effect in plants that has mutation on BRI1 co-receptor BAK1. Further evidence consist with the results shown in Figure 3 presented in here includes studies indicating that BR-dependent expression of the genes IAA1 and BAS1 requires the generation of a BR-dependent Ca\(^{2+}\) signal. Results shown in Figure 10 indicate that BR-dependent IAA1 and BAS1 expression is not compromised in either a bak1 mutant, or the bak1/bkk1 double mutant; these results suggest this component of BR signaling may not involve a co-receptor.

The central tenet of the work presented in our studies is the novel finding that BR perception causes an immediate elevation of cytosolic Ca\(^{2+}\) and generation of this Ca\(^{2+}\) signal contributes to at least some component of this hormone’s effects on plant cells. Evidence shown in Figure 8 suggests that BR-dependent IAA1 expression is diminished with the presences of Ca\(^{2+}\) channel blocker GdCl\(_3\). Further experimental evidence supporting this contention is presented in Figures 11. Either chelation of extracellular Ca\(^{2+}\) by prior exposure of Arabidopsis seedlings to EGTA (Aslam et al., 2008) or prevention of cytosolic Ca\(^{2+}\) elevation by application of the lipophilic Ca\(^{2+}\) chelator 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxyethyl ester) (BAPTA-AM) (Cousson, 2003; Cruz et al., 2012) prevented the BR-dependent increase in expression of both IAA (Figure 11A) and BAS1 (Figure 11B).
Figure 9. Effect of BR application to WT (Col) and BR null mutant *bri1-701* on *BAS1* expression.
Figure 10. Effect of BR application to WT (Col), bak1 null mutant and bak1, bkk1 double mutant plants on IAA1 and BAS1 expression.

The bak1-4 null mutant was used in this work. bak1-4,bkk1-1 double mutant plants are lethal at the seedling stage. Plants of the bak1-3,bkk1-1 are not true double ‘knock-outs’ because in the bkk1-1 homozygous background bak1 mRNA can be recovered from the bak1-3 allele. The bak1-5, bkk1-1 double mutant does not affect BR signaling. Therefore, along with WT ‘Col’, and bak1-4 null mutants, we used BAK1 bak1/bkk1 bkk1 heterozygous mutants in this experiment. Other work in our lab (Y. Ma, G. A. Berkowitz, unpublished data) indicates some BAK1-dependent gene expression is impaired in this genotype (i.e. the bkk1 null genetic background). An asterisk over a bar representing a treatment indicates a significant (at P< 0.05) difference from the control. Neither bak1 homozygous mutant nor bak1 heterozygous mutation in the bkk1 background had any effect on BR-dependent IAA1 or BAS1 expression as compared to WT.
Figure 11. Effects of Ca$^{2+}$ chelator on BR-induced *IAA1* and *BAS1* gene expression. Application of an extracellular Ca$^{2+}$ chelator (EGTA) or an intracellular Ca$^{2+}$ chelator (BAPTA-AM) along with BR prevents the BR-dependent increase in expression of both *IAA1* (A) and *BAS1* (B). An asterisk over a bar representing a treatment indicates a significant (at P< 0.05) difference from the control.
We undertook further experiments to compare the relative involvement of Ca\(^{2+}\) signaling and the BR-induced phosphorelay cascade in the effect of the hormone on \(IAA1\) transcript levels. The rationale underlying this work was as follows. Binding of BR to the BRI1 receptor; i.e. initial perception of the hormone, is required to activate both the phosphorelay signal cascade (Jaillais et al., 2011) and the BR-dependent Ca\(^{2+}\) signaling leading to gene expression (Figure 6-11).

We sought to use genotypes that are impaired in steps of the phosphorelay cascade other than mutants that have reduced binding of the hormone to the receptor (for example \(bri1\)-5). For this work, we used the \(bin2\)-1 and \(bri1\)-301 mutants. The \(bin2\)-1 mutant has a dominant, hypermorphic form of the negative regulator \(BIN2\) (Li et al., 2001; He et al., 2002; Li and Nam, 2002). It has been experimentally verified that BIN2-dependent phosphorylation of BES1 and BZR1 is increased in this genotype, resulting in increased degradation of these master transcription regulators and concomitant impairment of BR signaling through the phosphorelay cascade (He et al., 2002; Li and Nam, 2002; Yin et al., 2002). Mutations that result in a ‘kinase-dead’ BRI1 impair BR signaling, but binding of the hormone to the receptor is not necessarily impacted by such mutations (Wang et al., 2005). The \(bri1\)-301 genotype is such a mutant (Xu et al., 2008; Kang et al., 2010). In contrast to the \(dnd1\) mutant, which shown no BR-dependent increase in \(IAA1\) expression, the effect of BR addition on \(IAA1\) expression in these two mutant genotypes is similar to WT (Figure 7). Further evidence suggests that the similar patterns of BR-dependent increases in \(BAS1\) expression among the four genotypes tested as was determined for \(IAA1\) expression. As shown in Figure 12, the BR-dependent increase in \(BAS1\) transcript level found in WT plants was absent in \(dnd1\), while exogenous BR application increased \(BAS1\) expression in the \(bin2\)-1 and \(bri1\)-301 genotypes.
Figure 12. Effect of BR application to WT (Col), *dnd1*, *bin2-1*, and *bri1-301* plants on *BAS1* expression.

BR had no effect on *BAS1* level in *dnd1* plants. The 60% increase in *BAS1* expression observed in *bri-301* plants trended toward significance (P<0.06). The expression of *BAS1* in the absence of exogenous BR relative to the level in WT plants was 0.72, 0.86, and 1.0 in *dnd1*, *bin2-1*, and *bri1-301* plants, respectively.
Ca\textsuperscript{2+} signaling and the phosphorelay cascade represent two distinct BR response pathways.

BR perception by its receptor BRI1 is linked to downstream alteration in expression of at least some BR-responsive genes through a Ca\textsuperscript{2+} signaling cascade. Results presented in Figures 9-12 suggest that BR responsive genes regulated by Ca\textsuperscript{2+} signaling might not require activation of the master transcriptional regulators BZR1 and/or BES1 through the BR-dependent phosphorelay cascade. BZR1 and BES1 alter transcription of BR-responsive genes either directly (by binding to the promoter of specific genes), or indirectly, by modulating expression of transcription factors that then alter transcript levels of suites of other genes (Wang et al., 2002; He et al., 2005; Yin et al., 2005; Li et al., 2010).

SAUR-AC1 (SMALL AUXIN UP RNA 1 FROM ARABIDOPSIS THALIANA ECOTYPE COLUMBIA) is a BR-responsive gene that has been experimentally-confirmed to be activated by direct binding of BES1 to its promoter (Yin et al., 2005). Thus, it is hard to envision that in genotypes that have alterations in steps of the phosphorelay cascade resulting in degradation/inactivation of BES1, BR effects on SAUR-AC1 expression would not be affected. As shown in Figure 13, differences in BR effects on SAUR-AC1 expression amongst the four genotypes examined were markedly different than those found for either BAS1 (Figure 12) or IAA1 (Figure 9). In the case of SAUR-AC1, BR increased expression level in WT and \textit{dnd1} plants, while no effect of BR on SAUR-AC1 expression was observed with either the \textit{bin2-1} or the \textit{bri1-301} genotypes. These results suggest that some BR-responsive genes might be regulated by the phosphorelay system and others might be modulated (perhaps independently) by a Ca\textsuperscript{2+}-dependent signaling cascade.
Figure 13. Effect of BR application to WT (Col), dnd1, bin2-1, and bri1-301 plants on SAUR-AC1 expression.

The expression of SAUR-AC1 in the absence of exogenous BR relative to the level in WT plants was 0.91, 0.86, and 0.70 in dnd1, bin2-1, and bri1-301 plants, respectively.
 Ca$^{2+}$ signaling and BR phenotypes.

The end-point of hormone signaling cascades that involve transcriptional reprogramming is often altered plant phenotype. This typically involves changes in growth, form, function, or development of a plant, plant organ, or target cells in response to internal, developmental, or environmental cues. In the work presented here, we have identified a cytosolic Ca$^{2+}$ elevation as an immediate downstream signal generated upon BR perception by its receptor BRI1, and linked this signal to transcriptional regulation of several genes by this hormone. As mentioned above, BR signaling results in the potential reprogramming of many genes (Goda et al., 2002; Mussig et al., 2002). The demonstration here of BR-dependent Ca$^{2+}$ signaling as impacting transcript levels of a few genes does not necessarily link this signaling cascade to BR-dependent phenotypes of plants. Results presented in Figure 14 extend our analysis of BR Ca$^{2+}$ signaling to plant phenotypes. One documented quantitative measure of exogenous BR alteration of a plant phenotype is the effect of BR on hypocotyl length of etiolated Arabidopsis seedlings (Xu et al., 2008; Gou et al., 2012). Application of exogenous BR reduces hypocotyl length of seedlings grown in the dark. This effect is shown with WT seedlings in Figure 14. With WT seedlings, application of a Ca$^{2+}$ channel blocker along with BR completely prevented the BR-dependent phenotype. Additional studies were undertaken with dnd1 seedlings. In this case, BR had no effect; hypocotyl length was similar in the presence or absence of BR. Additionally, application of the Ca$^{2+}$ channel blocker to dnd1 seedlings had no effect. This result suggests that the effect of the Ca$^{2+}$ channel blocker in reversing the BR-dependent decrease in hypocotyl length is likely not due to some general, inhibitory effect of the channel blocker. Thus, the results of the experiment shown in Figure 14 provide pharmacological and genetic evidence that Ca$^{2+}$ signaling mediates this BR related phenotype.
Figure 14. Ca$^{2+}$ signaling is involved in the effect of BR on etiolated hypocotyl length of dark-grown Arabidopsis seedlings.

WT (Col) and *dnd1* seedlings were grown on solid medium with water (Control), BR, or BR and GdCl$_3$ added. Means separation was analyzed by ANOVA T-test comparisons within each genotype of each treatment individually with the Control. For each genotype, asterisks over bars representing treatment means indicate a significant (at P< 0.05) difference from the control (water addition) for that treatment.
Discussion

Work included in this report supports a revised model of BR signaling. The central tenet of this model is that BR perception by its receptor BRI1 leads to an immediate (i.e. < 1 minute) increase in cytosolic Ca$^{2+}$. At the BR concentration used in our studies of its effect on Ca$^{2+}$ elevation (100 nM), the BR-dependent Ca$^{2+}$ signal is of a similar magnitude as that occurring in other cell signaling pathways (e.g. Pep (Qi et al., 2010) and Elongation factor Tu 18 (Ranf et al., 2011)), and at least in some cases, is responsible for BR-dependent changes in gene expression as well as a BR-related phenotype.

There are a number of other conclusions that can be formulated based on the experimental results in this report. Exogenous BR application also causes in vivo cGMP elevation. This step of the newly-delineated BR signaling cascade might occur due to GC activity of the hormone receptor. Exogenous application of lipophilic cGMP was found to mimic the effect of the BR hormone on IAA expression; this result is consistent with the involvement of cGMP production by BRI1 in the signaling pathway. Further support for this speculation could involve demonstration that the GC catalytic domain of BRI1 (as opposed to kinase activity of the receptor, for example) is specifically required for initiation of the Ca$^{2+}$ signaling cascade. Experiments along these lines are not included in this report. However, a recent study from this lab of Ca$^{2+}$ signaling associated with PEPR1, an LRR-RLK with a similar GC domain as BRI1, did examine the GC domain of PEPR1. Mutation of critical amino acids in the GC domain of PEPR1 thought to be involved in GTP binding and catalysis (S1014, G1016 and R1027 in PEPR1 corresponding to S1071, G1073 and R1084 in BRI1) did not alter the deduced structure of the PEPR1 kinase domain but did abolish ligand-dependent Ca$^{2+}$ signaling by this receptor (Ma et al., 2012). These amino acids are conserved in the GC domain of BRI1. Results of the GC
inhibitor work reported here are consistent with the mutation studies, suggesting that BR-dependent cGMP elevation is required for both the generation of the Ca\(^{2+}\) signal, as well as BR-dependent gene expression (in the case of \textit{IAA1}). BR-dependent cGMP elevation likely activates a CNGC channel comprised (at least in part) by the CNGC2 translation product; a known subunit of plasma membrane-localized Ca\(^{2+}\) conducting channels activated by cyclic nucleotide.

As mentioned above, there is no BR phosphorelay signaling step downstream from the BRI1 receptor that is currently known to be affected by Ca\(^{2+}\). However, a recent report has demonstrated a physical and functional interaction between BRI1 and the Ca\(^{2+}\) binding protein CaM. Oh et al. (Oh et al., 2012) have shown that Arabidopsis CaM7 displays Ca\(^{2+}\)-dependent binding to a peptide corresponding to the cytosolic domain of BRI1. The exciting work of Oh et al. (2012) also demonstrated that co-expression of CaM with the cytosolic kinase domain of BRI1 in a heterologous system (\textit{E. coli}) resulted in an impairment of BRI1 auto-, and transphosphorylation (in this case, of \textit{E. coli} proteins). This work raises the intriguing possibility that elevation of cytosolic Ca\(^{2+}\) might act, through Ca\(^{2+}\)-dependent binding of CaM to BRI1, to shut down the BRI1-dependent phosphorylations that initiate the phosphorelay cascade. Perhaps, then, the BRI1 receptor and activation of a CNGC channel are the source of the Ca\(^{2+}\) signal that shuts down BRI-dependent phosphorelay signaling. It may be also germane to this evolving model of BR signaling that conductance by CNGC channels is down regulated by CaM (Hua et al., 2003). Thus, we envision a feedback loop that allows for modulation of both BR signaling pathways.

Results in this report indicate that BR-dependent Ca\(^{2+}\) signaling and the well-characterized phosphorelay cascade might act independently to activate different BR-responsive genes. Figure 15 shows a new model of BR signaling transduction pathway. Based on the
Figure 15. Two BR dependent signal transduction pathways. One is involves in phosphorylation signaling (Left). The other is activates Ca\(^{2+}\) channel via secondary messenger cGMP (Right).
hypothesis present in this work, we suggest that independent from well-characterized BR-dependent phosphorylation signaling pathway, BR-dependent cytosolic Ca\(^{2+}\) signal transduction with the involvement of secondary messenger cGMP. However, the specific pathway between BR-dependent Ca\(^{2+}\) signaling to its related gene expression is remain unclear and full elucidation of this model awaits the analysis of more BR-responsive genes.

In prior work from this lab characterizing the role of CNGCs in signal transduction pathways (Qi et al., 2010), we speculated that the Ca\(^{2+}\)-conducting channel was present along with a receptor protein that generated the ligand that activates the channel as a multi-enzyme protein complex. In this manner, microdomains may exist where the localized concentration of a cytosolic messenger molecule (such as cyclic nucleotide) is elevated in proximity to the channel that is activated by the molecule. When the recent work of Oh et al. (Oh et al., 2012) is considered along with results presented here, the notion of a multi-enzyme microdomain could provide an explanation for how BRII, the Ca\(^{2+}\)-conducting CNGC channel, and CaM act in concert to initiate a finely-regulated signal. It is germane to note that in animal cells, CNGCs as well as enzymes that activate the channels and receptors are envisioned to operate as multimolecular complexes existing as membrane ‘signalosomes’ (Trudeau and Zagotta, 2003; Wang and Malbon, 2011; Bankston et al., 2012; Ostrom et al., 2012).

BR-dependent cytosolic Ca\(^{2+}\) elevation could be involved in the immediate responses of cells to BR perception, as mentioned in the Introduction. In other words, aside from the well known Ca\(^{2+}\) signal regulation of gene expression (Finkler et al., 2007; Doherty et al., 2009), a rise in cytosolic Ca\(^{2+}\) could also alter conductance of K\(^{+}\) and Cl\(^{-}\) channels, altering membrane potential (Li et al., 2006). Further, an elevation in cytosolic Ca\(^{2+}\) could have immediate impact on cell function through activation of Ca\(^{2+}\)-dependent protein kinases (Cheng et al., 2002). Of
course, these speculations await testing in future studies. Here, the novel effect of BR on
cytosolic Ca\textsuperscript{2+} is documented, raising new questions about cell signaling pathways associated
with this hormone.

**Materials and Methods**

**Plant material**

All Arabidopsis lines used in the reported work are in the Columbia (Col-0) background except *bri1-5* (AT4g29400) (Noguchi et al., 1999); the *bri1-5* mutation is in the Wassilewskija (Ws-2) background. Col-0 or Ws-2 plants were used as controls as appropriate. The aequorin-expressing lines Col-aeq, Ws2-aeq, *dnd1*-aeq (Qi et al., 2010) and *bri1-5*-aeq were used to monitor treatment effects on cytosolic Ca\textsuperscript{2+} concentration. Col plants expressing the cGMP reporter protein δ-FlincG (Isner et al., 2011) were used for *in vivo* cGMP measurement. Arabidopsis seeds were surface-sterilized by first washing the seeds in 70% (v/v) ethanol, 20% (v/v) bleach and 0.02% (v/v) Triton X-100, shaking at 300 rpm for 10 min, and then rinsing with 95% (v/v) ethanol 3 to 4 times.

For all measurements except the quantitative real time PCR (qPCR) assay of gene expression, seeds were first planted on \(\frac{1}{2}\) strength Murashige and Skoog (MS) medium (Caisson Labs, Logan, UT) supplemented with 1% (w/v) Suc and solidified with 1% (w/v) agar. Seeds were stratified (4 °C) for 2 to 3 d after plating to break dormancy. Plants used for cytosolic Ca\textsuperscript{2+} measurements were grown as follows. Seeds were grown on agar plates for 7 to 10 d at 16 h light/(\~100 mol m\textsuperscript{-2} s\textsuperscript{-1})/8 h dark and 25 °C, and then transplanted into pots containing artificial LP5 mix (Sun Gro, Bellevue WA). The pots were put in an EGC growth chamber (Chagrin Falls, OH) at 12 h light/(\~100 mol m\textsuperscript{-2} s\textsuperscript{-1})/12 h dark and 22 °C. Healthy, nonsenseeating leaves
were used after 2 to 3 weeks growth in pots. For in vivo cGMP measurement, the seeds were set vertically on square plates containing ½ MS medium for 5 d. For measurement of etiolated hypocotyls lengths, square plates containing seed were placed in 24 h dark for 14 d at 22 °C. For qPCR measurement of gene expression, seeds were grown in tubes containing 3 mL liquid ½ strength MS medium (1 seed/tube) with shaking (180 rpm) for 14 d at an illumination of 24 h light (~90 mol m\(^{-2}\) s\(^{-1}\)) and 22 °C.

**Hormone and reagent treatment**

Over 60 different BR compounds have been identified in plants. Brassinolide (BL) and epibrassinolide (eBL) are the most biologically active of these compounds, and are typically used in tissue culture applications to study BR effects on plants (Ferrie et al., 2005). In our work, synthetic eBL (Sigma-Aldrich, St Louis, MO) was applied to plants, seedlings, and detached leaves to test effects of exogenous BR on plant tissue. Stock solutions of eBL were made in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich). For all experiments except q-PCR studies, the eBL was added at a final concentration of 100 nM. For gene expression analysis, the final concentration of the eBL used was 1 μM (see Li et al., 2010 for example).

In some cases, a lipophilic analog of cGMP (8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt, Sigma-Aldrich) was added to plants (at 150 μM final concentration) in the same manner as the eBL treatment. For these experiments, water (i.e. no solvent) was added to the control treatment. Some experiments involved use of the GC inhibitor LY-83583 (6-anilino-5, 8-quinolinequinone; Enzolife Sciences; Farmingdale, NY). The inhibitor was added to solutions containing leaves or seedlings (20 μM final concentration) 10 min before plants were treated with eBL. For these experiments, control treatments included application of 0.04% (v/v) DMSO in place of the inhibitor. Stock solutions of 50 mM LY-83583 were made up in 100%
DMSO. In some cases, the Ca\(^{2+}\) blocker GdCl\(_3\) (at 150 µM final concentration) (Sigma-Aldrich) was used; the channel blocker was added to the plants 10 min before the eBR ligand was applied. Water was used as control for this work. All experiments were repeated at least twice.

**Cytosolic Ca\(^{2+}\) Measurements**

The method from Qi et al. (Qi et al., 2010) was used with slight modification for cytosolic Ca\(^{2+}\) measurements using aeq-expressing plants. See Qi et al. (Qi et al., 2010) for details of methods used to generate aeq-expressing genotypes: Col-aeq, Ws-aeq, dnd1-aeq and bri1-5-aeq used here. Leaves used for experiments were cut from 3 to 4 week old plants expressing cytosol-localized Ca\(^{2+}\) dependent chemiluminescent apoaequorin protein reconstituted with coelenterazine-cp (CTZ-cp, AAT bioquest inc, Sunnyvale, CA).

Individual detached leaves were placed in a capless 2 mL centrifuge tube containing 500 µL control buffer (1 mM KCl, 1 mM CaCl\(_2\)•6H\(_2\)O, and 10 mM MgCl\(_2\)•6H\(_2\)O, adjusted to pH 5.7 with Tris-base). For each tube, 1 µL CTZ-cp was added (10 µM final concentration in 0.2% (v/v) ethanol). The leaf in the control buffer was vacuum infiltrated for 15 s and incubated at room temp. in the dark for 1 to 2 h to allow coelenterazine incorporation into leaves. As the CTZ-cp is a light sensitive reagent, all preparatory steps after adding the CTZ-cp were carried out in dark; tubes were covered with foil paper.

A luminometer (TD-20/20, Tuner Design; Sunnyvale, CA) was used for measurement of the luminescence level. The centrifuge tubes were placed in the luminometer, and left for 2 to 3 min to allow leaves to recover from touch induced Ca\(^{2+}\) spikes induced by handling the tubes. Luminescence level was measured every second and ligand (eBL) was added only after background luminescence of the leaves was stable. When background luminescence was stable, 500 µL of control buffer containing ligand (at 2 folds final concentration) was added to the tubes.
containing leaves by gently pipetting the solution against the interior wall of the centrifuge tube. For these experiments, eBL (100 nM final concentration) was delivered to the leaves in DMSO solvent (0.001% [v/v] final concentration). The GC inhibitor LY 83583 in DMSO (0.04% [v/v] final concentration) was added to the leaves 10 min before adding eBL. Control leaves were treated with 0.04% DMSO 10 min before adding eBL. All the treatments were added at “0” minutes; results shown in figures are mean values calculated from a minimum of at least 3 biological replicates. For each replication, leaves were cut from different plants. After recording luminescence from a treatment replicate, the remaining aeq (i.e. not bound to Ca\(^{2+}\)) in an assay tube was discharged by adding 800 \(\mu L\) Ca\(^{2+}\) release buffer (2 M CaCl\(_2\)•6H\(_2\)O in 30% ethanol) with continued recording for ~10 min, until the instantaneous luminescence level dropped below 2000. Values obtained for aeq discharge were used to convert luminescence readings to cytosolic Ca\(^{2+}\) concentration for each treatment replicate using an algorithm as described by Qi et al. (Qi et al., 2010). Results are presented in Figures as BR-dependent increase in cytosolic Ca\(^{2+}\); this value represents the difference between the Ca\(^{2+}\) level recorded at any time point and the basal level of Ca\(^{2+}\) measured at time ‘0’ (i.e. prior to BR application). The basal level of cytosolic Ca\(^{2+}\) for the experiments reported here ranged from 0.20 to 0.24 \(\mu M\) and averaged 0.220 ± 0.004 \(\mu M\).

**In vivo cGMP measurements**

Individual 7-d-old (Col plants expressing the cGMP reporter FlincG) seedlings were placed in 60 \(\mu L\) water on a 24 X 40 mm cover glass and covered with a 22 X 22 mm cover slip. The small cover slip was taped (on two opposite sides) onto the larger one (forming a chamber) and then the chamber with a seedling inside was taped onto the stage of a confocal microscope (Nikon A1R, Nikon; Melville, NY). Measurements of fluorescence were made prior to addition.
of ligand; seedlings with roots having high levels of GFP expression (i.e. GFP fluorescence with just water in the chamber) were chosen for use. The root tip was located under bright field illumination, observation of background GFP fluorescence was made, and after adjustment of the perfect focus, 150 μL water containing eBL in 0.001% (v/v) DMSO was delivered to one open end of the chamber at time ‘0’ min. The control ‘water only’ treatment contained just DMSO. A filter paper was kept in contact with the chamber solution at the opposite side of the chamber to wick water as the ligand was delivered. Time-lapse changes of fluorescence were captured every 30 s using 480/20-nm excitation wavelength using the microscope’s high-performance optical offset (Perfect Focus System) to facilitate real-time correction of focus drift. Fluorescence intensity at the root tip was quantified as relative brightness (within a defined range of 256 shades of gray per unit area) using NIH ImageJ processing/analysis software.

**qPCR Analysis**

q-PCR analyses were performed to study genotype and treatment effects on the expression level of some BR-responsive genes. Growth of Arabidopsis seedlings in ½ strength MS liquid medium was performed according to Qi et al (Qi et al., 2010). The seedlings were grown in separate tubes containing 3 mL liquid medium for 14 d on a shaker (180 rpm) with 24 h illumination (~90 mol m⁻² s⁻¹) at 22 °C. The ligand eBL (or lipophilic cGMP) was added directly to the growth medium and the seedlings were incubated for a further 8 h. Water containing 0.001% (v/v) DMSO was given to the seedling for the control treatment. For experiments involving the addition of inhibitors, LY83583 (20 μM) or GdCl₃ (150 μM) were added to the growth medium containing plants 10 min prior to addition of eBL. Seedlings were kept on the shaker in the light during inhibitor pretreatment and exposure to eBL. After incubation, the seedlings were collected and frozen immediately with liquid N₂, and stored at -80 °C for future
use. Total RNA were extracted from the whole liquid-grown seedlings using the Plant RNA Extraction Kit (Macherey-Nagel; Bethlehem, PA). During the RNA extraction process, tissue extraction was treated with rDNase (Macherey-Nagel) to remove potential genomic DNA from samples.

After extraction, 500 ng of total RNA was used for reverse transcription by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (ABI); Carlsbad, CA). The synthesized cDNA was diluted 1:10 (v/v) in water, and 1 μL of the diluted cDNA was used for each qPCR reaction. qPCR assays were performed using the ABI 7900 HT Real-time PCR system with the SYBR Green gene expression assay (ABI). Treatment effects on the expression level of *IAA1* (AT4G14560), *BAS1* (AT2G26710), and *SAUR-AC1* (AT4G38850) were examined. 18s rRNA was used as an endogenous control. The primers used for these analyses were as follows: *IAA1* (Forward) 5’- AGTCACCAATGGGCTTAACC-3’, *IAA1* (Reverse) 5’- CTGTTGAGTCTGTTGTTCTTGC-3’; *BAS1* (Forward) 5’- CCCGTTGGCTTCATACCGT-3’, *BAS1* (Reverse) 5’- TTACAGCGAGTGAATTTGGC-3’; *SAUR-AC1* (Forward) 5’- AGGAGTTTCTTTGGGTCTAAG-3’, *SAUR-AC1* (Reverse) 5’- CATAGACGCCATGAATCTC-3’; and 18s rRNA (Forward) 5’- CGGCTACCACATCAAAGGAA-3’, 18s rRNA (Reverse) 5’- GCTGGAATTACCAGCGCT-3’.

For each analysis, 3 mechanical replications were tested on one plate, and each treatment mean was generated from analysis of at least 3 biological replications from separate RNA isolated from different individual seedling. ANOVA of corresponding threshold cycle (CT) values was used for evaluation of means separation amongst treatments in an experiment (as noted in Figure legends), and to generate standard errors of the means for control treatments (Schmittgen and Livak, 2008). In many cases, experiments involving qPCR analysis of gene expression involved
the evaluation of eBL effects on different genotypes. In these cases, gene expression in the absence of added eBL was normalized to ‘1’ for each genotype in a given experiment (as noted in figures). In these cases, the relative amount of gene expression in the absence of eBL for each mutant line used in an experiment (i.e. in a given figure) is compared to the expression level in WT plants for that experiment. This information is provided in the corresponding Figure legend.

**Measurement of etiolated hypocotyl length**

This assay of a BR-dependent plant phenotype allows for quantitative evaluation of exogenously added BR effects on seedlings, and was adopted from Xu et al. (Xu et al., 2008; Müssig et al., 2002). Arabidopsis seedlings were grown vertically on ½ strength Murashige and Skoog medium in square plates containing 1% (w/v) agar at 22 °C in the dark for 14 d. eBL (100 nM) and/or GdCl\(_3\) (150 μM) were added to the solid growth medium before pouring the medium into the Petri plates. For the control treatment, plants were grown on medium containing 0.001% (v/v) DMSO. The hypocotyls length of seedlings was measured after 14 days.
Chapter 3. Interdependence between two pattern recognition receptors PEPR1 and FLS2 in pathogen defense

Abstract

Little is known about molecular steps linking perception of pathogen invasion by cell surface sentry proteins acting as pattern recognition receptors (PRRs) to downstream cytosolic Ca\(^{2+}\) elevation, a critical step in plant immune signaling cascades. Some PRRs recognize molecules (such as flagellin) associated with microbial pathogens (pathogen-associated molecular patterns, PAMPs), whereas others bind endogenous plant compounds (damage-associated molecular patterns, DAMPs) such as peptides released from cells upon attack. This work focuses on the possible cross linkage between the PAMP Flagellin sensing2 (FLS2) and DAMP Plant Elicitor Peptide Receptor (PEPRs) and their cognate activating ligands in Arabidopsis. Some differences were identified between Pep (an endogenous plant elicitor peptide)/PEPR signaling and the Ca\(^{2+}\)-dependent immune signaling initiated by the flagellin (study using flg22). Flg22/FLS2 signaling may have a greater requirement for intracellular Ca\(^{2+}\) stores and inositol phosphate signaling, whereas Pep/PEPR signaling is facilitated only by extracellular Ca\(^{2+}\). Maximal FLS2 signaling requires a functional Pep/PEPR system. This interdependence was evidenced as a requirement for functional PEPR receptors for maximal flg22-dependent Ca\(^{2+}\) elevation, NO and H\(_2\)O\(_2\) generation and flg22/FLS2-dependent impairment of pathogen growth. In a corresponding fashion, FLS2 loss of function mutant also impaired Pep signaling. The evidence suggests the possibility that these two signaling molecule patterns can function independently but also function interdependently to cause ligand-dependent Ca\(^{2+}\) responses, NO generation, H\(_2\)O\(_2\) generation and immunization against pathogen growth.
Introduction

Signals are received and processed through cell surface receptors in all living organisms. These processes are mediated by receptor kinases (RKs) in animals. A RK usually contains three functional domains: an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. The signal transduction process starts with the ligand binding to the extracellular domain of the receptor, activation of the intracellular kinase domain, which leads to kinase phosphorylation of target proteins within the cell. In plants, receptor-like kinases (RLKs) similarly transduce extracellular signals into the cell. Plant RLKs form a monophyletic gene family related to animal RKs. However, different from RKs, almost all plant RLKs phosphorylate serine/threonine residues instead of the predominantly tyrosine kinase activity in the animal RKs. RLKs are involved in many plant biological signal processes. RLKs are involved in processes as follows: pathogens defense (FLAGELLIN SENSING 2 (FLS2), EF-Tu Receptor (EFR)), hormone perception (brassinosteroid insensitive 1 (BRI1), BRI1-associated receptor kinase 1 (BAK1)), meristem regulation (CLAVATA 1 (CLV1), Barely Any Meristem 1-3 (BAM1-3)), phytosulforkine signaling (Phytosulforkine Receptor (PSKR)), cell expansion (Wall Associate Kinase (WAK2), WAK4), vascular development (Phloem Intercalated with Xylem (PXY)), symbiosis (Nod Factor Receptor (NORK)), abscisic acid responsiveness (Receptor-like Protein Kinase (RPK)), cell proliferation (ERECTA (ER), ERECTA-LIKE (ERL1)), cell fate (CRINKLY 4 (CR4)) and root and nodule growth (Hypernodulation Aberrant Root (HAR1)) (see reviews of RLKs by (Shiu and Bleecker, 2001; Chae et al., 2009).

Based on the structure of the extracellular domain, the RLKs can be categorized into many groups, such as S-domain group, epidermal growth factor (EGF)-like repeat group and leucine-rich repeat (LRR) group. The characterization of the different groups of the RLKs has
already been reviewed in prior publications. The S-domain group was the first RLK group that identified; their extracellular region is similar to the S-locus glycoproteins that function in the self incompatibility response (Pastuglia et al., 1997). The EGF-like repeat group is another subfamily of RLKs. In Arabidopsis, WAKs (WAK1-WAK4) have extracellular EGF-like repeats (He et al., 1996; Miller et al., 1997). Genetic experiments suggest that WAKs may be involved in pathogen responses (He et al., 1998). The LRR-RLKs is the major group of plant RLKs among all three groups. In the Arabidopsis genome, there are a total of 417 genes encoding RLKs and more than half of them belong to the LRR-RLK family. The LRR domains are known to be involved in protein-protein interaction or small molecule binding, such as hormone or peptides (Chinchilla et al., 2006; DeYoung and Innes, 2007; Qi et al., 2010; Yamaguchi et al., 2010; Ma et al., 2012). LRR-RLKs contains imperfect repeats of a 24 amino acid leucine-rich motif in the extracellular part of receptor for ligand binding. The LRR motif is composed of a consensus core of L-x-x-L-x-L-x-x-N (L=Leucine, N=Asparagine). Each repeat contains an α-helix and β-sheet hairpin with a β-sheet, forming the surface for protein-protein interaction (Kobe and Kajava, 2001). Based on the copy number and arrangement of LRR motifs in the extracellular domain, the LRR-RLKs can be further identified into 13 subfamilies (LRR I – LRR XIII). (Kajava, 1998; Kobe and Kajava, 2001)

A critical early component of signaling cascades linking pathogen perception by plants to intracellular immune responses involves a temporary elevation in cytosolic Ca$^{2+}$, a Ca$^{2+}$ “burst” or elevation. Plant responses to pathogens include an innate-, or basal-immune response; defense gene expression is activated and antimicrobial molecules are generated. Innate immune responses occur upon recognition of evolutionarily conserved essential components of pathogens: pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs).
The pattern-recognition receptor (PRR) FLS2 is a receptor that belongs to LRR-RLK family (Boller and Felix, 2009). Flagellin:FLS2 signaling cascade is a well studied PAMP:receptor protein system in plants. FLS2 and its cognate associate pattern, the flagellin peptide (study using flg22), have been widely used to study plant innate immunity signaling. Binding of flg22 to FLS2 induces a cytosolic Ca\(^{2+}\) elevation, which is required for downstream innate immune signaling leading to defense responses (Aslam et al., 2008; Aslam et al., 2009; Jeworutzki et al., 2010). FLS2 can also interact with a brassinosteroid co-receptor BAK1 (Chinchilla et al., 2007). BAK1 is a co-receptor for FLS2 and many other LRR-RLKs. BAK1 belongs to protein kinase group LRRII. It only contains five extracellular LRR repeats. BAK1 is also named SERK3; it is also a member of the somatic embryogenesis-related kinase (SERK) family. BAK1 was first identified as a co-receptor for BR signaling. It can form a ligand dependent complex \textit{in vivo} with the LRR-RLK BRI1. Published reports suggest that BAK1 associates with the other LRR-RLK receptor FLS2 upon stimulation with flg22 and is required for responses triggered by flg22 (Chinchilla et al., 2007; Boller and Felix, 2009).

Plant elicitor peptides receptor 1 (PEPR1) and PEPR2 are two receptors that both belong to the LRR-RLK XI family. They are receptors for the Plant Elicitor Peptides (Peps) family. Atpep1 (\textit{Arabidopsis thaliana} Pep 1) was the first peptide identified in this family; it represents a small portion of a cytoplasmic protein encoded by PROPEP1. PROPEP1 is a gene induced by wounding, methyl-jasmonate, flg22 and Pep1 itself (Boller and Felix, 2009). Similar with the system in tomato, Pep1 represents an endogenous signal for stress and wounding, and acts as a damaged associate molecular pattern (DAMP) (Boller and Felix, 2009). In Arabidopsis, exogenous peptides can induce cytosolic Ca\(^{2+}\) elevation, and the Pep/PEPR signaling cascade
can lead to the increase expression of defense genes and increase resistance against pathogens (Qi et al., 2010; Yamaguchi et al., 2010; Yi et al., 2012).

The work report in this chapter indicates a possible linkage between the DAMP Peps/PEPR signaling and the PAMP flg22/FLS2 signaling. The work suggests that these receptors and their ligands act synergistically to generate a Ca\(^{2+}\) signal and Ca\(^{2+}\) related NO and H\(_2\)O\(_2\) generation. These ligand/receptor signaling cascades also play interdependent roles in Ca\(^{2+}\)-triggered pathogen responses and the immunization to a bacterial pathogen.

**Results**

**FLS2, PEPR receptors and Ca\(^{2+}\) signaling.**

It is known that application of flg22 peptides to leaves of aequorin (aeq)-expressing Arabidopsis WT plants (WT-aeq) result in a cytosolic Ca\(^{2+}\) elevation. The early work in the lab has also demonstrated that application of Pep peptides to leaves of Arabidopsis WT-aeq plants also results in a cytosolic Ca\(^{2+}\) elevation involved in immune responses that is impaired in leaves of pepr1 null mutants expressing aeq (pepr1-aeq) (Qi et al., 2010). Pep-dependent pathogen defense programs are facilitated by PEPR1 and PEPR2. PEPR1 can responds to Peps 1-6 and PEPR2 only responds to Pep1 and 2. Our prior work (Qi et al., 2010) focused on Pep3 activating PEPR1.

PEPR1 and FLS2 are both PRRs, extracellular peptide ligands bind to these receptor and cytosolic signaling is initiated. Both PEPRs and FLS2 are responsible for pathogen defense signals. The previous work has shown that the alternation of the intracellular Ca\(^{2+}\) concentration triggered by flg22 is impaired in some mutants, such as pepr1 and pepr2, the mutant had insertions on the genes encoding the PEPR1 or PEPR2 receptor (Qi et al., 2010). Moreover, the Ca\(^{2+}\) signal induced by Pep3 is also impaired in Arabidopsis leaves that are pretreated with flg22.
for 1 h (Figure 16). Flg22 pretreatment of plant results in endocytotic degradation of FLS2, cause the plant to completely lose the FLS2 receptor from the plasma membrane (Robatzek et al., 2006). Since FLS2 null mutant aeq plants were not available at the time of my experiment, flg22 pretreatment to cause endocytosis of FLS2 were used instead of fls2-aeq to test the role of FLS2 in Pep-induced Ca²⁺ elevation. Thus, the result may indicate that when cells lack the FLS2 receptor, Pep dependent cytosolic Ca²⁺ elevation is reduced. Cytosolic Ca²⁺ elevation caused by glutamate (glu) is dependent on the glu receptor and does not involve the FLS2 or PEPR1 receptors. For comparison, Ca²⁺ conduction through glu receptors is not affected by the endocytotic degradation of FLS2 (Figure 16).

**Differences in Ca²⁺ Signaling Associated with PEPR and FLS2 Immune Cascades.**

Previous work in the lab had demonstrated that Pep/PEPR signaling requires an extracellular source of Ca²⁺, the work also indicated that different from the Pep/PEPR signaling cascade, the flg22/FLS2 signaling cascade could involve cytosolic Ca²⁺ elevation occurring due to release of intracellular Ca²⁺ stores (e.g., from the vacuolar and/or endoplasmic reticulum). However, the evidence only suggested the involvement of intracellular Ca²⁺ sources in flg22/FLS2 signaling, but did not preclude the involvement of extracellular Ca²⁺ (Ma et al., 2012); both intracellular and extracellular sources are likely involved in the signaling pathway. There is also some evidence shown in the lab that similar to the BRI1 receptor, the PEPR receptor contains a guanylyl cyclase (GC) catalytic domain. Application of Pep1 to Fluorescence indicator of cGMP (FlincG) WT seedling roots resulted in a time-dependent elevation of in planta [cGMP] (Ma et al., 2012). This evidence suggests that Pep/PEPR induced Ca²⁺ elevation occurs due to the GC
Figure 16. Maximal Pep3-dependent Ca\textsuperscript{2+} elevation requires the flagellin receptor FLS2. Incubation of detached Arabidopsis leaves in flg22 results in endocytotic degradation of FLS2; within 1 h of constant exposure to flg22, a complete loss of FLS2 receptor from the plasma membrane occurs (Robatzek et al., 2006). Here, we exposed leaves of WT aequorin-expressing plants to a pretreatment involving incubation in water or flg22 for \~2.5 h (A-C). Cytosolic Ca\textsuperscript{2+} elevation was monitored after exposure of the pretreated leaves to the ligand Pep3 (A, B) or glutamate (C). (A) Pep3-dependent cytosolic Ca\textsuperscript{2+} elevation was reduced in leaves pretreated with flg22 compared with leaves pretreated with water. Leaves pretreated with flg22 had a higher [Ca\textsuperscript{2+}] at 0 min, possibly because of a residual effect of a Ca\textsuperscript{2+} elevation occurring during the flg22 pretreatment. (B) Unconverted luminometer output is shown for one leaf exposed to either water (Left) or flg22 (Right) pretreatment for 2.5 h before Pep3 addition. Initiation of Ca\textsuperscript{2+} release is indicated by an arrow. The rapid and large rise in luminescence on initiation of Ca\textsuperscript{2+}
release indicates that, after both pretreatments, there was a large pool of undischarged aequorin still present in the leaves. The difference in Pep3-dependent signal occurring after the two pretreatments cannot be attributed to discharge of the available pool of aequorin during the flg22 pretreatment. (C) Pretreatments are similar to those in A, and 1 mM glutamate (glu) was added at the time indicated by an arrow. Cytosolic Ca\(^{2+}\) elevation caused by glu is dependent on the glu receptor and does not involve the FLS2 or PEPR1 receptors. These results suggest that the absence of the FLS2 receptor specifically affects Peps (and flg22) signaling. Results are presented as mean values of Ca\(^{2+}\) increase (replicate number in parentheses) + SE shown at 1 min intervals.
activity of the PEPR receptors. The cyclic nucleotide gated channel (CNGC) are activated by the elevation of the cGMP upon Pep binding to PEPR receptors, which finally results in the elevation of cytosolic Ca\(^{2+}\) elevation. Further evidence also shown that in the presence of a GC inhibitor, the Pep induced cytosolic Ca\(^{2+}\) elevation was completely blocked. In addition, the mutation of key residues in the GC catalytic domain of PEPR1 abolished Pep signaling (Ma et al., 2010). All the evidence together indicates that Pep/PEPR1 signaling involves a functional GC domain of this receptor protein. Recent studies with Phosphatidylinositol-Specific Phospholipase C (PI-PLC) inhibitors suggest that inositol triphosphate (IP3) and/or inositol hexaphosphate (IP6) signaling leading to vacuolar Ca\(^{2+}\) release may contribute to flg22-dependent cytosolic Ca\(^{2+}\) bursts (Kwaaitaal et al., 2011), although the results were equivocal. In this same work, inhibitor studies suggested that rather than CNGC channel, which acts in Pep/PEPR signaling cascades, a plasma membrane Ca\(^{2+}\)-conducting GLR channel acts downstream from flg22/FLS2 to facilitate extracellular Ca\(^{2+}\) influx; this result is consistent with the assertion that flg22-dependent membrane depolarization is not impaired in the dnd1 mutant (Jeworutzki et al., 2010). Membrane depolarization is an indirect measurement of Ca\(^{2+}\) influx into the cell (Jeworutzki et al., 2010).

In order to examine the differences in the pathway between flg22 and Pep induction of cytosolic Ca\(^{2+}\) signaling, we used aeq expressing Arabidopsis plants that also express mammalian type I inositol polyphosphate 5-phosphatase (IP5-pta-aeq) (Perera et al., 2008). The mammalian phosphatase expressed in these plants impairs PI-PLC-dependent generation of IP3 and IP6; levels of both signaling molecules are reduced and cytosolic Ca\(^{2+}\) elevation that operate through PI-PLC signaling are inhibited in these plants (Perera et al., 2010). Other than this, the aeq expressing dnd1 mutants were also used for the comparison of the difference in
flg22 and Pep induced Ca\(^{2+}\) signaling pathways. The results are shown in Figure 17 A-D. The results show that impairment of IP3 and IP6 generation (in IP5-ptase-aeq plants) substantially inhibited the cytosolic Ca\(^{2+}\) elevation induced by flg22 (Figure 17 A), but this impairment of IP3 and IP6 generation causes no significant changes in the cytosolic Ca\(^{2+}\) signaling that responds to Peps (Figure 17 B). On the other hand, with null mutation of CNGC2, dndl-aeq plants show an effect on Pep related cytosolic Ca\(^{2+}\) elevation, but had no effect on flg22 induced cytosolic Ca\(^{2+}\) elevation (Figure 17 C and D).

The results indicate that the plasma membrane-localized cGMP–activated Ca\(^{2+}\) channel CNGC2 is involved in Pep3 signaling but not flg22 signaling. The PI-PLC-dependent vacuolar Ca\(^{2+}\) channel is involved in flg22 but not Pep3 signaling. Thus, we identify two different pathways leading to Pep- and flg22-induced Ca\(^{2+}\) signaling. One of them is PI-PLC signaling pathway in flg22 (and not in Pep) signaling, and the other is CNGC related pathway in Pep (and not in flg22) signaling.

**ROS and NO production is impaired in fls2 and pepr1 mutants**

The reactive oxygen species (ROS) defense molecules nitric oxide (NO) and H\(_2\)O\(_2\) are generated downstream from Ca\(^{2+}\) in immune signaling cascades (Levine et al., 1994; Delledonne et al., 1998; Ali et al., 2007; Ma et al., 2008; Ma et al., 2009; Moreau et al., 2010; Torres, 2010), and H\(_2\)O\(_2\) generation is required for flg22-mediated immune responses (Torres, 2010). Furthermore, chelation of apoplastic Ca\(^{2+}\) in Arabidopsis leaves by the extracellular polysaccharide alginate derived from *Pseudomonas syringae* strongly inhibits flg22-dependent H\(_2\)O\(_2\) generation (Aslam et al., 2008), indicating that flg22 induced H\(_2\)O\(_2\) generation is Ca\(^{2+}\) dependent. There is evidence suggesting that cytosolic Ca\(^{2+}\) elevation affects downstream
Figure 17. Pep and flg22-dependent cytosolic Ca$^{2+}$ elevation (A-D).
Comparisons of peptide-dependent cytosolic Ca$^{2+}$ elevation in WT-aeq plants with either dnd1-aeq or ip5-ptase-aeq plants. The absolute cytosolic Ca$^{2+}$ level was recorded prior to, and after ligand addition (at time ‘0’). In all cases, results are shown as the change in cytosolic Ca$^{2+}$ from the minimum Ca$^{2+}$ recorded at or near time ‘0’. In all four experiments, recordings were made from WT-aeq (‘Col’) plants (black lines). Also shown are changes in Ca$^{2+}$ calculated for ip5-ptase-aeq plants (A and B) and dnd1-aeq plants (C and D). Flg22 (1 µM) was used in (A) and (C). Pep3 (20 nM) was used in (B) and (D). The measurement of Pep3 induced cytosolic Ca$^{2+}$ elevation is performed by Dr. Yi Ma in Berkowitz lab.
immune responses. Based on the hypothesis that Pep- and flg22-dependent signaling accesses different pools of Ca\(^{2+}\). I speculated that Pep and flg22 might have an additive and/or synergistic effect on Ca\(^{2+}\)-dependent immune responses. Pep1-dependent ROS generation has been demonstrated on a whole leaf (Huffaker et al., 2006) and leaf tissue (Schwessinger et al., 2011) basis and further, Huffaker et al. (2006) have shown that preventing ROS generation impairs Pep1-dependent defense gene expression in detached, intact leaves. (Pep3 was used here).

In the work reported here, we used a different experimental system to study interdependent flg22 and Pep immune responses. We also evaluated DAMP (Pep) effects on the defense signal NO for the first time. Ligand (either flg22 or Pep3) effects on H\(_2\)O\(_2\) (the graduated student Robin Walker performed the experiment involving H\(_2\)O\(_2\) measurement in the Berkowitz lab) and NO generation were monitored in individual leaf cells of WT, fls2 null mutant, or pepr1 null mutant leaf tissues (Figure 18A-D). Early immune signaling events such as membrane depolarization (associated with cytosolic Ca\(^{2+}\) elevation) induced by flg22 require the presence of a functional FLS2 receptor (Jeworutzki et al., 2010). Thus, it is not surprising that flg22-dependent H\(_2\)O\(_2\) generation was inhibited in cells from fls2 plants compared to WT (Figure 18 A). However, flg22-dependent H\(_2\)O\(_2\) generation was also impaired when lack of PEPR1 receptor. Furthermore, the maximal H\(_2\)O\(_2\) generation in response to Pep also required the presence of both PEPR1 and FLS2 receptors in these single cell assays (Figure 18 A and B). This interdependence of PAMP flg22 and DAMP Pep3 signaling on their respective receptors was also evidenced with regard to NO generation (Figure 18 C and D). NO has been referred to as the ‘concert master’ of the hypersensitive response and plant innate immunity (Dangl, 1998), and PAMP-dependent NO generation requires the critical and early Ca\(^{2+}\) elevation in the signaling pathway.
Figure 18. FLS2 and PEPR receptor involvement in flg22- and Pep3-dependent NO and H$_2$O$_2$ generation in leaf guard cells.
Ligands (flg22, Pep3) were added to reaction buffer containing epidermal peels which had been previously incubated in NO- and H$_2$O$_2$-specific fluorescent dyes. Peels were prepared from WT, fls2, and pepr1 null mutant plants. (A) Flg22-dependent H$_2$O$_2$ generation. (B) Pep3-dependent H$_2$O$_2$ generation. (C) Flg22-dependent NO generation. (D) Pep3-dependent NO generation. (E) i, iii, v and (F) i, iii, v shows the individual guard cell image of NO and H$_2$O$_2$ generation when given flg22 on WT, fls2 and pepr1 plant; (E and F) ii, iv, vi shows the individual guard cell image of NO and H$_2$O$_2$ generation when given Pep3 on WT, fls2 and pepr1 plant. Mean maximal fluorescence of guard cells (occurring ~5-10 min after ligand addition) in a peel was ascertained exactly as described previously (Ma et al., 2009) and averaged to generate one biological replicate. Signals shown (as bars) are means (±SE) of three replicate peels prepared from leaves of different plants; fluorescence of approximately 6 guard cells in an epidermal peel was averaged for one replicate. Significant differences (at P<0.05) between tissue from a mutant (either fls2 or pepr1) and WT are indicated by a ‘*’ above the bar representing the mean value recorded from peels obtained from mutant plants.
Thus, the results with H$_2$O$_2$ (Figure 18 A and B) and NO (Figure 18 C and D) indicate 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 (Figure 18 A-D), and assayed minutes after addition of ligand, suggesting that the functional interaction between the PAMP (flg22) and DAMP (Pep3) 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 this two receptors related 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).

**Interdependence of Peps and flg22 Immunization**

As the results shown above, both flg22- and Peps-dependent NO and H$_2$O$_2$ generation is effected by the loss of either one of the receptors. This indicates that there is a possible interaction between these two receptors or their downstream signaling. The paramount downstream effect of pathogen defense signaling cascades that involve the FLS2 and PEPR1/2 receptors and their cognate ligands is an increase in host fitness and decrease in pathogen virulence. There is much evidence that application of either flg22 or Pep peptides can 'immunize' the plant against pathogens (Boudsocq et al., 2010; Yamaguchi et al., 2010). This immunization
is demonstrated in Figure 19; when given virulent *Psudomonas syringae* pv. tomato (*Pst* DC3000) to plants pretreated with either Pep3 or flg22, bacterial growth was reduced on WT plants compared with plants pretreated with water. However, in the absence of either of the FLS2 or PEPR receptors (*fls2* or *pepr1/2* mutants), the reduction of bacterial growth caused by the flg22 or Pep3 pretreatment is comprised. These results indicate that both the FLS2 and PEPR1/2 receptors are required for the plants to reach the maximal immunization with either flg22 or Pep3. Furthermore, in the absence of the pretreatment, there is a slight but significant increase in bacterial growth in either *pepr1/2* or *fls2* mutant plants compared with WT plants. This suggests that PEPR1, PEPR2 and FLS2 receptors are all important in plant basal pathogen resistance.

**Peps trigged immunity against DC3000 is compromised in cpk5,6,11 and bak1 mutants**

*Ca²⁺*-dependent protein kinases (CPKs) are *Ca²⁺* sensors recently characterized to trigger downstream responses as components of plant immune defense systems (Lee and Rudd, 2002; Boudsocq and Sheen, 2012). In Arabidopsis, CPK4, 5, 6 and 11 were shown to be critical for flg22 associated PAMP signaling; the *cpk5,6* double mutant, *cpk5,6,11* triple mutant and the *cpk4,5,6,11* quadruple mutant show progressively decreased flg22 induced gene expression, ROS generation and impairment of pathogen resistance (Boudsocq et al., 2010). As mentioned above, BAK1 is a co-receptor for both FLS2 and BRI1, it is involved in both flg22 and BR signaling. However, a recent study shows that BAK1 also physically interact with PEPRs in vitro (Postel et al., 2010). There is also recent evidence that shows that BAK1 and PEPRs can phosphorylate each other when treated with Pep1 *in vivo* (Schulze et al., 2010). Thus, BAK1 may also act as co-receptor with PEPRs in Pep-dependent signaling pathway. Both *cpk5,6,11* and *bak1* mutants are compromised in resistance against bacterial growth when pretreated with flg22 (Boudsocq et al.,
Figure 19. Signaling mediated by the PEPR receptors and FLS2 have interdependent effects on growth of a virulent pathogen.

Proliferation of (virulent) *Pst* DC3000 on leaves of WT, *pepr1/2*, and *fls2* mutant plants pretreated with water, *flg22*, or Pep3. Plants were pretreated one day prior to inoculation with *Pst* and bacterial growth was evaluated three days after inoculation. Results shown (note log scale of ordinate axis) are mean values of *Pst* recovered from leaves (n=4) ± SE. A ‘*’ above a bar representing bacterial growth in leaves of either *pepr1/2* or *fls2* genotypes indicates that for that genotype, bacterial growth in leaves subjected to the *flg22* (or Pep3) pretreatment was significantly different (at P<0.01) than the level found in WT leaves (for that pretreatment). Separately, a Tukey-Kramer Multiple Comparisons Test was used to evaluate means separation for plants of the three genotypes subjected to the water pretreatment; bars with different letters above them were significantly different (at P<0.05).
Pep3 induced immunization may also be compromised in these mutants; we examined this hypothesis by monitoring growth of *Pst* DC3000 bacteria in these mutant genotypes. The results are shown in Figure 20. As shown before in Figure 19, pretreatment of Pep3 increases the immunity of WT plants, resulting in the restriction of bacterial growth on WT plants. However, as shown in Figure 20, the inhibition of bacterial growth by Pep3 pretreatment in WT plants is compromised in both *cpk5,6,11* and *bak1* mutants. The application of Pep3 does slightly restrict the bacterial growth in the *bak1* mutant compared to the plants treated with water controls. The loss-of-function of BAK1 does not completely prevent Pep3 immunization. The results indicate that CPKs and BAK1 are involved in both flg22 and Pep3 induced immune signaling.

**Discussion**

Pep3/PEPR and flg22/FLS2 signaling cascades both contribute to pathogen defense. These two receptors lead to cytosolic Ca\(^{2+}\) elevation upon ligands binding to their receptors. However, in the case of Pep3, the cGMP activated CNGC channels are involved in Pep3-dependent Ca\(^{2+}\) signaling (Qi et al., 2010). The PEPR receptors contain a GC catalytic domain, which can cause cGMP elevation upon application of Pep peptide (Ma et al., 2012). Inhibition of GC activity inhibited the increase of both cGMP and cytosolic Ca\(^{2+}\) level. The loss of function of CNGC2 also impairs the cytosolic Ca\(^{2+}\) elevation induced by Pep3. In the case of flg22, flg22/FLS2 signaling is not related to cGMP as a cytosolic secondary messengers; the FLS2 receptor does not contain a putative GC catalytic domain. The evidence shown here suggests that flg22 induced cytosolic Ca\(^{2+}\) signaling may involve PI-PLC dependent IP3 and/or IP6 signaling. Decreasing the generation of IP3 or IP6 results in the impairment of flg22 induced cytosolic Ca\(^{2+}\) elevation. However, even though there are differences between Pep/PEPR and flg22/FLS2 signaling cascades, interactions between these two receptors may exist. The results here
Figure 20. Pep3 induced immunity to virulent pathogen is compromised in *cpk5,6,11* and *bak1* mutant plants.

Proliferation of (virulent) *Pst* DC3000 on leaves of WT, *cpk5,6,11* and *bak1* mutant plants pretreated with water or Pep3. Plants were pretreated one day prior to inoculation with *Pst* and bacterial growth was evaluated three days after inoculation. Results shown (note log scale of ordinate axis) are mean values of *Pst* recovered from leaves (n=4) ± SE. A ‘*’ above a bar representing bacterial growth in leaves of either *cpk5,6,11* or *bak1* genotypes indicates that for that genotype, bacterial growth in leaves subjected to Pep3 pretreatment was significantly different (at P<0.01) than the level found in WT leaves (for that pretreatment). Separately, a Tukey-Kramer Multiple Comparisons Test was used to evaluate means separation for plants of the three genotypes subjected to the water pretreatment; bars with different letters above them were significantly different (at P<0.05).
demonstrate that there is a functional interaction between Pep-, and flg22-dependent signaling. Both FLS2 and PEPR receptors were required for the maximal cytosolic Ca\(^{2+}\) elevation in response to either Pep3 or flg22. More evidence supports this interaction between Pep and flg22 signaling was regard to downstream steps in the Ca\(^{2+}\) immune signaling cascades, including NO and H\(_2\)O\(_2\) generation. The absence of either one receptor results in the impairment of both flg22- and Pep- induced NO and H\(_2\)O\(_2\) generation. However, this interdependence of PAMP peptide and DAMP peptide signaling on a cellular level also occurs in animal cells. In the case of at least some animal DAMP peptides and flagellin, the cell signaling cascades responding to the perception of these peptides share many molecular steps and act synergistically (Nijnik et al. 2012).

The biological function of immune signaling is an enhancement of host system fitness in the face of a pathogenic challenge. The results here provide some new insights into flg22- and Pep effects on system fitness. Application of either flg22 or Pep3 prior to inoculation of plants with \textit{Pst. DC3000} resulted in a decrease in growth of bacterial in the plants, these results indicate that both flg22 and Pep3 can immunize the plant against bacteria. However, this immunization by either flg22 or Pep3 was compromised when there is absence of either one of two receptors. One explanation for this result is that the action of each receptor amplifies the activation of defense responses by the signaling cascade evoked by the other receptor. There is also evidence suggesting that flg22 induced immunization is impaired in \textit{cpk5,6,11} and \textit{bak1} mutants (Boudsocq et al., 2010). The results here suggest that Pep3 induced immunization is also impaired in the \textit{cpk5,6,11} and \textit{bak1} mutants.

The work presented in this chapter focused on the interaction between flg22 and Pep3 induced signaling. The evidence suggest that PEPRs and FLS2 receptors are required for full
activity of flg22- or Pep3-dependent cytosolic Ca\textsuperscript{2+} signaling, downstream NO or H\textsubscript{2}O\textsubscript{2} generation, and the plant immunization. Some of the points raised in this chapter, such as an explanation for the molecular basis of the interdependence of FLS2 and PEPRs signaling require further study. The main new point of understanding from the work in this chapter is that there is an important interaction between immune signaling cascades evoked by perception of a PAMP or an endogenous plant peptide acting as a DAMP.

**Materials and Methods**

**Plant Material**

All Arabidopsis lines used in the reported work are in the Columbia (Col-0) background. The aequorin-expressing lines Col-aeq, *dnl1*-aeq (Qi et al., 2010) and IP-5-Ptase-aeq (Pererar et al., 2010) were used to monitor treatment effects on cytosolic Ca\textsuperscript{2+} concentration. Arabidopsis Col-0, *fls2* (Heese et al, 2007) *pepr1* (Yamaguchi et al, 2010) were used for NO and H\textsubscript{2}O\textsubscript{2} measurement. Arabidopsis Col-0, *pepr1/2* (Yamaguchi et al, 2010), *fls2* (Heese et al., 2007), *bakl-4* (Chinchilla et al., 2007) and *cpk5,6,11* (Boudsocq et al., 2010) were used for bacterial growth measurement. Seeds were surface sterilized and spread on Petri dishes containing half-strength Murashige and Skoog (MS) salts with macro- and micronutrients (Caisson), 2.6 mM (2-(N-morpholino)-ethanesulfonic acid (MES) (adjusted to pH 5.7 with Tris), 1% sucrose, and 0.8% agar (4), and grown in a growth chamber with a day (80–100 μmol·m\textsuperscript{-2}·s\textsuperscript{-1} illumination)/night cycle of 12/12 h at 25 °C. About 10 d after germination, seedlings were transplanted into pots containing LP5 soilless mix containing starter fertilizer (Sun Gro) at 12 h light (100 μmol·m\textsuperscript{-2}·s\textsuperscript{-1} illumination)/12 h dark (72% relative humidity) and 22 °C. Seeds were stratified at 4 °C in the dark for 2 d before use. During growth, plants were irrigated with Jack’s Professional Peat-
Lite 20:10:20 (NP<sub>2</sub>O<sub>5</sub>-K<sub>2</sub>O) solution (at 2 g/L) one to two times to provide supplementary fertilizer.

**Cytosolic Ca<sup>2+</sup> Measurements**

The method from Qi et al. (2010) was used with slight modification for cytosolic Ca<sup>2+</sup> measurements using aeq-expressing plants. Leaves used for experiments were cut from 3-4 week old plants expressing cytosol-localized Ca<sup>2+</sup> dependent chemiluminescent apoaequorin protein reconstituted with coelenterazine-cp (CTZ-cp, AAT bioquest inc, Sunnyvale, CA). Individual detached leaves were placed in a capless 2 mL centrifuge tube containing 500 μL control buffer (1 mM KCl, 1 mM CaCl<sub>2</sub>•6H<sub>2</sub>O, and 10 mM MgCl<sub>2</sub>•6H<sub>2</sub>O, adjusted to pH 5.7 with Tris-base). For each tube, 1 μL CTZ-cp was added (10 μM final concentration in 0.2% (v/v) ethanol). The leaf in the control buffer was vacuum infiltrated for 15 s and incubated at room temp. in the dark for 1-2 h to allow coelenterazine incorporation into leaves. As the CTZ-cp is a light sensitive reagent, all preparatory steps after adding the CTZ-cp were carried out in dark; tubes were covered with foil paper.

A luminometer (TD-20/20, Tuner Design; Sunnyvale, CA) was used for measurement of the luminescence level. The centrifuge tubes were placed in the luminometer, and left for 2-3 min to allow leaves to recover from touch induced Ca<sup>2+</sup> spikes induced by handling the tubes. Luminescence level was measured every second and ligand (Pep3 or flg22) was added only after background luminescence of the leaves was stable. When background luminescence was stable, 500 μL of control buffer containing ligand (at 2X final conc.) was added to the tubes containing leaves by gently pipetting the solution against the interior wall of the centrifuge tube. For these experiments, pep3 (20 nM final conc.) and flg22 (1 µM final conc.) was delivered to the leaves in water. In the experiment with flg22 pretreatment, flg22(1 µM final conc.) were added to the
leaves 1 h before adding the ligand, control leaves were add with same volume of the water. The GC inhibitor LY 83583 in DMSO (0.1% [v/v] final conc.) was added to the leaves 10 min before adding. Control leaves were treated with 0.1% DMSO 10 min before adding Pep3 and flg22. All the treatments were added at “0” minutes; results shown in figures are mean values calculated from a minimum of at least 3 biological replicates. For each replication, leaves were cut from different plants. After recording luminescence from a treatment replicate, the remaining aeq (i.e. not bound to Ca$^{2+}$) in an assay tube was discharged by adding 800 µL Ca$^{2+}$ release buffer (2 M CaCl$_2$$\cdot$6H$_2$O in 30% ethanol) with continued recording for ~10 min, until the instantaneous luminescence level dropped below 2000. Values obtained for aeq discharge were used to convert luminescence readings to cytosolic Ca$^{2+}$ conc. for each treatment replicate using an algorithm as described by Qi et al. (10).

**NO and H$_2$O$_2$ production in guard cells**

The method for NO detection in guard cells using the NO-specific fluorescent dye diaminofluorescein–2 diacetate (DAF-2DA; Invitrogen, Carlsbad, CA) was adapted from Ali et al (2007). The method for H$_2$O$_2$ detection in guard cells using the H$_2$O$_2$-specific fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA; Invitrogen) was adapted from Gerber et al. (2004). Rosette leaves of WT, fls2, pepr1, and pepr1/2 4-6 wk old 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 CaCl$_2$$\cdot$6H$_2$O, at pH 6.15) for 1-2 h. For assays of NO generation, peels were then placed in 3 mL buffer B (NO synthase reaction kit buffer; Sigma-Aldrich) which contained 50 µM DAF-2DA to load cells with NO-specific dye. For assays of H$_2$O$_2$ generation, peels were alternatively placed in 3 mL buffer A which contained 50 µM H$_2$DCF-DA for 30 min. After loading NO- and H$_2$O$_2$- specific dye in the tissue, peels were
washed three times in buffer A and incubated in 3 mL of buffer A (for H$_2$O$_2$ assays) or buffer B (for NO assays) containing flg22 or Pep3 for 5-10 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 H$_2$O$_2$- dependent fluorescence were monitored over time. Mean maximal fluorescence of guard cells (occurring ~5-10 min after ligand addition) in a peel was ascertained exactly as described previously (Ma et al., 2009) and averaged to generate one biological replicate. Maximal fluorescence measurements of approximately 6 guard cells in an epidermal peel were averaged for one replicate. For each treatment, captured images at the maximum fluorescence intensity for treatment replicates were used to calculate data represented as means. Quantitative analyses of the NO- and H$_2$O$_2$- 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 pre-exposure of plants to Pep3 and subsequent inoculation of leaves with virulent pathogen was adapted from Yamaguchi et al (Yamaguchi et al., 2010). Flg22, Pep3 and water were separately syringe injected into (attached) rosette leaves from WT, fls2, pepr1/2, cpk5,6,11, or bak1 plants 24 h prior to bacterial inoculation. Peptides were delivered to a leaf from a blunt end 1 mL syringe in ~0.5 mL water with peptide. *P. syringae* pv. tomato ‘DC3000’ (*Pst DC3000*) was grown at 28 °C on low-salt (1 g/L NaCl) Luria-Bertani (LB) (Fisher Scientific; Pittsburg, PA) 2% (w/v) agar medium containing 100 mg/L 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 MgCl$_2$•6H$_2$O to a concentration of 3 x 10$^8$ cfu/mL. The resuspended bacteria were applied (in 0.01% Silwet L-77 surfactant) by spraying on leaves (previously treated with water or peptides) from 4-5 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 days post inoculation. For each biological replicate, 0.7 cm diam. leaf discs were cut from leaves of two different plants and ground together in 100 µL of 10 mM MgCl$_2$•6H$_2$O 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 LB agar medium containing 100 mg/L rifampicin. Plates were left at 25 °C for 2 d; after this incubation period colonies on plates were counted.
Chapter 4 Inositol polyphosphates, nitric oxide, G proteins, and intracellular Ca^{2+} release are involved in flagellin dependent immune responses.

Abstract

Flagellin-sensing 2 (FLS2) is a receptor protein that translates the binding of the ligand flagellin (flg) (studied using the peptide flg22) into cytosolic signals that initiate plant immune responses to bacterial pathogens. A critical step in this immune signal transduction pathway is cytosolic Ca^{2+} elevation, which leads to an oxidative burst and flg22 related defense gene expression. Although flg/FLS2 signaling is thought to primarily involve influx of extracellular Ca^{2+}, recent work from this lab has shown that flg22 induced defense gene expression can occur in the absence of extracellular Ca^{2+}. Intracellular Ca^{2+} release into the cytosol may contribute to flg22 dependent Ca^{2+} signaling. Inositol tri-phosphate (IP3) is a cytosolic secondary messenger produced by phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate. IP3 and inositol hexaphosphate (IP6, produced from IP3 phosphorylation) generation during plant cell signaling is thought to cause release of vacuolar Ca^{2+}; IP3 involvement in signaling cascades could lead to release of an intracellular Ca^{2+} stores in plant cells. Nitric oxide (NO) is another important signaling molecule involved in immune defense responses. We find that flg22 causes NO generation, and this flg22-dependent NO generation (and flg22-dependent increases in expression of some defense genes) is inhibited in Arabidopsis genotypes that have impaired IP3 and IP6 signaling. G proteins are thought to be upstream from PLC signaling. Mutation of G-protein α and β subunits impairs flg22 dependent gene expression,
suggesting that a G-protein may activate PLC signaling as part of the flg22-induced immune response. In plants, flg22 can activates Mitogen-activated protein kinase (MAPK), induce MAPK phosphorylation. The IP3 and IP6 are both required for flg22-related MPK phosphorylation. This work provides evidence suggesting that G proteins, and inositol phosphate elevations may be involved in translating perception of flagellin into release of intracellular Ca\(^{2+}\) stores, contributing to the cytosolic Ca\(^{2+}\) elevation that is a critical signal leading to immune responses in plant cells.
Introduction

$Ca^{2+}$ may represent the most influential ion in eukaryotic organisms. It is involved as a secondary cytosolic signal in almost all aspects of animal and plant development and participates in many regulatory processes. The changes of cytosolic $Ca^{2+}$ in the green algae is the first published examples of $Ca^{2+}$ functioning as a secondary messenger in plants (Williamson and Ashley, 1982). $Ca^{2+}$ was known as an important secondary messenger involved in many cell signaling cascades in animals and plants. The elevation of cytosolic $Ca^{2+}$ concentration is a signaling step that related to many physiological processes, including responses to abiotic and biotic stimuli, such as hormones, oxidative stress and pathogens (Dodd et al., 2010). It has also been reported that the changes of the cytosolic $Ca^{2+}$ concentration were triggered by cellular secondary messengers, for example, cGMP, nicotinic acid adenine dinucleotide phosphate (NAADP), sphingosine-1-phosphate, and cyclic ADP ribose (cADPR) etc. (Dodd et al., 2010). It is also known that the alteration of cytosolic $Ca^{2+}$ concentration results from the movement of $Ca^{2+}$ through plasma membrane $Ca^{2+}$ channels after perception of the stimuli.

$Ca^{2+}$ channels are important in signal transductions, the opening of $Ca^{2+}$ channel results in changes of cytosolic $Ca^{2+}$ level. A $Ca^{2+}$ channel can be defined as a channel permeable to $Ca^{2+}$ whose physiological role is to mediate rapid $Ca^{2+}$ transport across cellular membranes. In the plant, $Ca^{2+}$ channels have been characterized in the plasma membrane, endoplasmic reticulum (ER), tonoplast, nuclear and plastid membranes of the plant cell (White, 2000).

Phospholipids are emerging as novel secondary messengers in plant cells. They are membrane components and naturally locate with membrane receptors (Meijer and Munnik, 2003). Upon perception of stimuli by a receptor, receptor activation rapidly initiates a cell response involving phospholipids as secondary messengers. This responses translate directly or
indirectly (e.g. via G-protein involvement) into effector enzyme activity, and resulted in conversion of lipids to signaling molecules. The most well-known lipid-signaling pathway is associated with phospholipase C (PLC) signaling. PLC signaling was discovered in the 1980s in animals (Rebecchi and Pentyala, 2000). When the extracellular receptor is activated by the stimuli, PLC utilizes phosphatidylinositol 4, 5-bisphosphate (PtdIns (4, 5) P2) and produces inositol tri-phosphate (IP3) and diacylglycerol (DAG). IP3 in the cytosol initiates release of intracellular Ca\(^{2+}\) (Munnik and Testerink, 2009; Andreeva et al., 2010; Munnik and Nielsen, 2011). The increase of the cytosolic Ca\(^{2+}\) concentration can trigger a variety of changes in downstream signaling, impacting various cell biological processes. Thus, IP3 is known as an important secondary messenger (Berridge, 1993; Mueller-Roeber and Pical, 2002). In animal cells, IP3 can mediate Ca\(^{2+}\) release from the endoplasmic reticulum (ER) to the cytosol (Clapham, 2007). It is known that IP\(_3\) induces cytosolic Ca\(^{2+}\) elevations in animal. PLC activity has also been detected in plant cells (Munnik and Testerink, 2009) and PLC signaling has been associated with cytosolic Ca\(^{2+}\) elevation (Ma et al., 2012). Plants respond to pathogen attack by activating various defense mechanisms. IP3 signaling is known to play a role in triggering these responses. Elicitor treatments activate PLC, change the IP3 level in plant tissue and affect their pathogen defense (Legendre et al., 1993; Toyoda et al., 1993). However, even now, there is no gene encoding an IP3 receptor yet identified in plants. Early experiments reported that IP\(_3\) could facilitate Ca\(^{2+}\) release from the vacuole across the tonoplast.

Inositol hex-phosphate (IP6), which is generated by further phosphorylation on IP3, also can evoke internal store Ca\(^{2+}\) release. Patch clamp studies with isolated vacuoles have confirmed that both IP3 and IP6 activate vacuolar Ca\(^{2+}\) channels (Lemtiri-Chlieh et al., 2003). IP6, also referred to as phytate; is a significant signaling molecular in the plant cell, especially in guard
cells (Murphy et al., 2008). IP6 is synthesized through a phospholipase C-dependent pathway in which IP3 is sequentially phosphorylated to IP6 by action of two inositol polyphosphate kinases (IPKs), *IPK2* and *IPK1* (Stevenson-Paulik et al., 2002). IP6 is known to increase defense responses to pathogens in Arabidopsis and potato, there is evidence suggesting that basal resistance to plant pathogens is seriously impaired when IP6 level is reduced. (Murphy et al., 2008). Thus, IP6 may also act as a secondary messenger, which acts in signaling cascades by increasing cytosolic Ca$^{2+}$ to influence many plants biological process.

It is known that IP3 can cause a cytosolic Ca$^{2+}$ elevation and it is also known that different stimuli cause IP3 increase, like abscisic acid (ABA) (Perera et al., 2008). It is already been known that environmental signals cause IP3 elevation and Ca$^{2+}$ increase (Meijer and Munnik, 2003). IP6 is known to be generated from IP3, the enzyme and pathway are identified (Stevenson-Paulik et al., 2002; Munnik and Nielsen, 2011). So we know IP3 increase can lead to IP6 increase. Thus during a response to a stimuli, the increase in IP3 that occurs could lead to IP6 increase (Munnik and Vermeer, 2010; Munnik and Nielsen, 2011). PI-PLC inhibitors, which prevent generation of IP3- and IP3-dependent IP6 production, reduce cytosolic Ca$^{2+}$ elevation associated with PAMPs (Lecourieux et al., 2002; Kwaaitaal et al., 2011), and pathogens (Lecourieux et al., 2006; Murphy et al., 2008). The evidence with PI-PLC inhibitors suggests that IP3 and/orIP6 signaling leading to vacuolar Ca$^{2+}$ release may contribute to flg22-dependent cytosolic Ca$^{2+}$ bursts (Kwaaitaal et al., 2011). Previous work in this lab found that flg22 induced cytosolic Ca$^{2+}$ increase is impaired in the mutant that reduced level of IP3 or/and IP6 (Ma et al., 2012). The impairment of the flg22-dependent cytosolic Ca$^{2+}$ elevation and pathogen defenses in plants have impaired IP3 (or/and IP6) signaling may suggests that IP3 and IP6 signaling may be involved in the biological cascades induced by flg22 and pathogen perception. The work report
in this chapter indicates that there is possible connection between flg22 induced signaling and IP3/IP6 signaling molecules.

**Results**

**Flg22-dependent NO production is impaired when lack of IP3 and IP6 signaling molecules.**

The reactive oxygen species defense molecule NO is generated downstream from Ca\(^{2+}\) in immune signaling cascades (Levine et al., 1994; Delledonne et al., 1998; Ali et al., 2007; Ma et al., 2008; Ma et al., 2009; Moreau et al., 2010; Torres, 2010). There is evidence shown in Chapter 2 that we detect an impairment of flg22 induced cytosolic Ca\(^{2+}\) elevation in aequorin (aeq) expressing Arabidopsis IP5-Ptases plants. Mammalian type I IP5-ptase is an enzyme that specifically metabolize IP3 in animals (Laxminarayan et al., 1993; Majerus et al., 1999). The transgenic Arabidopsis plants that constitutively express the IP 5-ptase can have impaired IP3 signaling in response to some stimuli (Perera et al., 2008). We know that NO generation is downstream from Ca\(^{2+}\), the impairment of flg22 related cytosolic Ca\(^{2+}\) burst in IP5-Ptase-aeq plants maybe leads to an inhibition of flg22-depednent NO generation.

In Figure 21A, flg22 effects on NO generation were monitored in individual leaf cells of Col-aeq and IP5-Ptase-aeq plants. The results show an impairment of flg22-dependent NO generation in IP5-Ptase-aeq plants. From this experiment, we find that when an increase of IP3 signaling is prevented or reduced in the cell, the flg22 induced NO generation is also reduced. The evidence suggests an involvement of IP3 molecules in the flg22 signaling pathway. However, we know that IP3 is required for the synthesis of IP6, the IP5-Ptases plant not only may have impairment in IP3 signaling, but also may have decrease in IP6 signaling. Thus, IP6 could also be involved in the flg22 signaling pathway.
Figure 21. IP3 and IP6 signaling in flg22 induced NO generation.

NO is generated downstream of the initial Ca$^{2+}$ elevation in immune signaling. NO is another important signaling molecule involved in immune defense responses. 1 μM flg22 were added to reaction buffer containing epidermal peels which had been previously incubated in NO specific fluorescent dyes. Peels were prepared from Col, Col-aeq, ip5-ptase and ipk1 plants. (A) Comparison of WT (Col-aeq) and transgenic Arabidopsis plants that constitutively express the IP5-ptase. (IP5-ptase is an enzyme that specifically metabolize IP3 in animals (Laxminarayan et al., 1993; Majerus et al., 1999). Plants that constitutively express the IP5-ptase can have impaired IP3 signaling in response to some stimuli.). Ip5-ptase transgenic plants are generated with Col-aeq plants. Col-aeq is used as WT plant for the comparison of ip5-ptase. (B) Comparison of WT (Col) and ipk1, which is impaired in IP6 synthesis, result in the impairment in IP6 signaling. Flg22 causes NO generation, and this flg22-dependent NO generation is inhibited in Arabidopsis genotypes that have impaired IP3 and IP6 signaling. Signals shown (as bars) are means (± SE) of four replicate peels prepared from leaves of different plants; fluorescence of approximately 3 guard cells in an epidermal peel was averaged for one replicate. The maximum fluorescence intensity that could be recorded from the peels was 256 fluorescence units/pixel. Significant differences (at P<0.05) between fluorescence recorded from a mutant and WT are indicated by a ‘*’ above the bar representing the mean value recorded from peels obtained from mutant plants.
IP6 synthesis occurs from phosphorylation of IP3 through IPK2 and IPK1, and IPK1 functions in the final step of IP6 biosynthesis. Thus IPK1 is essential for IP6 synthesis in plants. In order to test if IP6 effect flg22 signaling, we tested flg22 induced NO generation in individual leaf cells of ipk1 mutants (Figure 21B). The ipk1 mutant contains a T-DNA insertion in the IPK1 gene, which impairs of IP6 synthesis in plants (Stevenson-Paulik et al., 2005). In Figure 21B, we can find that similar to effects of IP5-Ptase, ipk1 mutation also impairs flg22 induced NO generation.

We have known that IP3 effects on flg22 induced cytosolic Ca\(^{2+}\) signaling. IP3 is important molecules to synthesis IP6, impairment of IP3 signaling may also result in the impairment of IP6 signaling. Ipkl blocks the synthesis pathway of IP6 from IP3, result in the decrease of IP6 basal level and IP6 signaling, which effects the flg22 induced NO generation. Since result shows that ip5-ptase and ipk1 have similar inhibition in flg22 induced NO generation. There is possibility that the inhibition of flg22 induced NO generation in ip5-ptase leaves may due to the impairment of IP6 signaling. However, there is no clear evidence suggest this speculation at this point, thus we cannot exclude the possibility that IP3 signaling and IP6 signaling are both required for flg22 associated immune signaling.

**The role of IP3 and IP6 in flg22 induced gene expression**

It has been proposed that IP3 and IP6 are involved in flg22-associated Ca\(^{2+}\) elevation and NO generation. 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). Treatment of (Arabidopsis) plants with flg22 results in the induction of many defense genes. Such global transcriptional reprogramming occurs at least in part through flg22 activation of transcriptional factors.
WRKY29 and WRKY33 are two defense related transcriptional factors. Both WRKY29 and WRKY33 can be induced by flg22 (Schikora et al., 2011; Ma et al., 2012). Here, we examine the flg22-induction of WRKY29 and WRKY33 expression in the absence or presence of the IP3 signaling (Figure 22). In Figure 22A we can find that when plants lack IP3 signaling, flg22-induced WRKY29 expression is impaired. Similar results are found with flg22-dependent WRKY33 expression (Figure 22B). IP3 signaling is required for flg22-induced defense gene expression, in a fashion similar to this requirement; IP6 and G-protein are also involved in flg22-dependent gene expression (Figure 23). With application of flgg22 to the Arabidopsis seedlings, we can find flg22 associated WRKY33 expression is impaired in IP6 null mutant ipk1 and G-protein α and β subunit null mutant gpa1agb1.

G-proteins and G protein signaling are well studied in animals. G-proteins are among the most important intracellular molecular switches, transducing external signaling from an activated transmembrane G-protein-coupled receptor to downstream effectors within cells, thereby playing an important role in signal transduction (Gilman, 1987; Mishra et al., 2007). In plants, G-protein cascades have been studied but much has not yet been deduced. In the model plant Arabidopsis, G-proteins contain only a signal canonical Gα gene, GPA1, one Gβ gene, AGB1, and two Gγ genes (Mishra et al., 2007; Tuteja, 2009). G-proteins are thought to be upstream of PLC-signaling, among all five subfamilies of PLC, PLCβ, PLCδ and PLCε are either activated (perhaps indirectly) by G-proteins or the activation is dependent on its interaction with G-proteins (Ortega et al., 2005). The results shown in Figure 23 suggest that G-proteins are also required in flg22-dependent defense gene expression. This result also indicates that in the pathway of flg22-induced defense signaling, there is a possible involvement of PI-PLC signaling.
Figure 22. Genotype effects on flg22-dependent WRKY33 and WRKY29 expression.
WRKY29 and WRKY33 are two defense related transcriptional factors. (A) WRKY29 expression was monitored in WT (Col-aeq) seedlings (with, and without exposure to flg22 for 30 min), and in ip5-ptase seedlings. (B) WRKY33 expression in WT and ip5-ptase seedlings when treat with flg22. With the impairment on IP3 signaling, Flg22-related WRKY29 (A) and WRKY33 (B) expression are inhibited. Results shown are means ± SE (n=4) of transcript levels normalized to the level in WT seedlings in the absence of flg22. Asterisks indicate significant differences (P<0.05) between WT and mutants with flg22 treatment.
**Figure 23** Genotype effects on flg22-dependent *WRKY33* expression.

*WRKY33* expression was monitored in WT (Col) seedlings, and in seedlings with mutations affecting IP6 signaling (*ipk1*) or G-protein signaling (*gpa1agb1*). The expression of *WRKY33* is inhibited in *ipk1* mutant, *ipk1* have impaired IP6 signaling. Result shown in figure suggests that IP6 signaling is involved in flg22-associated *WRKY33* expression. G-protein double mutant *gpa1agb1* have impairment on G-protein signaling. Result shown in figure suggests that G-protein signaling is required for flg22-related *WRKY33* expression. Results shown are means ± SE (n=5) of transcript levels normalized to the level in WT seedlings in the absence of flg22. Asterisks indicate significant differences (P<0.05) between WT and mutants with flg22 treatment.
in flg22-dependent immune signaling response.

**G-protein and IP6 signaling molecules are required for flg22-dependent MAPK phosphorylation**

In plants, a major mechanism linking environmental stress perception to cellular responses involves signaling through Mitogen-activated protein kinase (MPK) cascades (Nakagami et al., 2005). MPK cascades are eukaryotic signaling modules that involve as a minimum of a MPK kinase kinase (MPKKK), a MPKK and a MPK, which via a phosphorelay system serve both signal transduction and amplification. Flg22 is known to induce MPK phosphorylation (Kwaaitaal et al., 2011). In this phosphorelay cascade, MPK phosphorylation occurs as the signaling protein is activated. Therefore one way to evaluate MPK signaling/activation is to monitor MPK phosphorylation (Popescu et al., 2009). We evaluated flg22 effects on MPK signaling cascades by evaluating phosphorylation.

The experiment shown in Figure 24 compared MPK phosphorylation induced by flg22 in WT and ip5-ptase plants. Results indicated that MPK3 and MPK6 phosphorylation is impaired in plants with impaired IP3 signaling. The evidence suggests that when flg22 induces the MAPK phosphorylation, IP3 signaling is involved. In Figure 25, further evidence also suggest that both IP6 signaling and G-proteins may be required for flg22 dependent MAK phosphorelay signaling. This result together with the gene expression results (Figure 22 and Figure 23) indicates that PI-PLC signaling and G-proteins upstream from PLC activation could be involved in flg22-dependent immune signaling cascades. These results also consistent with the result shown in Figure 26, the flg22-dependent protection on the affect of bacteria *Pto* DC3000 in the WT plants is impaired in mutants with impaired IP6 and G-proteins signaling.
Figure 24. Flg22 induced MPK phosphorylation in WT(Col-aeq) and ip5-ptase plants.
In this experiment, Col-aeq was used as WT plant. The WT and ip5-ptase transgenic plant seedlings were treated with 1 μM flg22. Seedlings were treated with flg22 at time ‘0’, and the flash frozen in liquid nitrogen 5, 15 and 30 min after treatment. Samples were subjected to immunoblot analysis with antibodies against phospho-p44/p42 (α-pTEpY).
Figure 25. Flg22 induced MPK phosphorylation in WT (Col), ipk1 and gpa1agb1 double mutant.

Seedlings were treated with 1 μM flg22 at time ‘0’, and the flash frozen in liquid nitrogen 5, 15 and 30 min after treatment. Samples were subjected to immunoblot analysis with antibodies against phospho-p44/p42 (α-pTEpY).
Figure 26. Flg22 immunity to virulent pathogen is compromised in *ipk1* and *gpa1agb1* mutant plants.

Proliferation of (virulent) *Pst* DC3000 on leaves of WT, *ipk1* and *gpa1agb1* mutant plants pretreated with water or flg22. Plants were pretreated one day prior to inoculation with *Pst* and bacterial growth was evaluated three days after inoculation. Results shown (note log scale of ordinate axis) are mean values of *Pst* recovered from leaves (n=4) ± SE. A ‘*’ above a bar representing bacterial growth in leaves of either *ipk1* and *gpa1agb1* genotypes indicates that for that genotype, bacterial growth in leaves subjected to flg22 pretreatment was significantly different (at P<0.05) than the level found in WT leaves (for that pretreatment).
Discussion

Flg22 can induce cytosolic Ca\(^{2+}\) signaling and lead to pathogen defense gene expression in plants. The signal molecules IP\(_3\) and IP\(_6\) are also known to induce increased cytosolic Ca\(^{2+}\) concentration (Lemtiri-Chlieh et al., 2003). The cytosolic Ca\(^{2+}\) elevation that occurs in response to IP3 and IP6 has been shown to occur from release intracellular Ca\(^{2+}\) stores, for example, facilitated by tonoplast localized Ca\(^{2+}\) channel (Lemtiri-Chlieh et al., 2003). Unlike IP\(_3\) and IP\(_6\), Peps and BR mobilized extracellular Ca\(^{2+}\) (Ma et al., 2012). The evidence shows that Pep-, or BR-dependent cytosolic Ca\(^{2+}\) elevation is impaired in the dnd1 mutant, however, flg22 induced cytosolic Ca\(^{2+}\) signal is not (Ma et al., 2012). As reported above, the cytosolic Ca\(^{2+}\) change triggered by flg22 is impaired in the ip5-ptase transgenic plant that attenuates IP\(_3\) signaling in response to stimuli.

IP3 and IP6 are involved in flg22 dependent Ca\(^{2+}\) elevation. Furthermore, both IP3 and IP6 are required for flg22 dependent NO generation, defense related and gene expression (Figure 21 and 22). Ca\(^{2+}\) and NO are important signaling molecules in immune response pathways. Flg22 had been wildly used to study the plant innate immune signaling. Results reported here suggest the possible involvement of IP3 and IP6 in flg22 induced immune responses.

IP3 and IP6 are both generated from the PI-PLC signaling pathway. The fact that the impairment of IP3 or IP6 signaling inhibits defense related transcriptional factor gene expression and MPK phosphorylation (Figure 22-25) suggests that IP3 and IP6 are involved in flg22 induced defense signal transduction. Moreover, upstream from PLC signaling, G-proteins may also be required for this flg22 associated immune response (Figure 23 and 25). However, the specifics of how PLC signaling is involved in flg22 signal transduction is not elucidated yet.
It is well known that extracellular Ca\(^{2+}\), cell membrane receptors and channels are involved in flg22 induced immune responses (Kwaaitaal et al., 2011). It is also known that intercellular Ca\(^{2+}\) sources can be released from PI-PLC signaling pathways. The results here provide a possibility to link PI-PLC signaling pathway and flg22 induced signaling. The results suggest that PLC signaling stimulates intracellular Ca\(^{2+}\) release that may also contribute to flg22 induced immune response in addition to the well characterized systems that facilitate extracellular Ca\(^{2+}\) involvement in a flg22/pathogen signaling.

**Materials and Methods**

**Plant Material**

All Arabidopsis lines used in the reported work are in the Columbia (Col-0) background. The aequorin-expressing lines Col-aeq was used as wild type for comparing No generation, gene expression and MPK phosphorylation with ip5-ptase-aeq (Pererar et al., 2010). Seeds were surface sterilized and spread on Petri dishes containing half-strength Murashige and Skoog (MS) salts with macro- and micronutrients (Caisson), 2.6 mM (2-(n-morpholino)-ethanesulfonic acid (MES) (adjusted to pH 5.7 with Tris), 1% sucrose, and 1% agar, and grown in a growth chamber with a day (80–100 \(\mu\)mol·m\(^{-2}\)·s\(^{-1}\) illumination)/night cycle of 12/12 h at 25 °C. For the NO experiment, seeds were transplanted to soil about 10 d after germination. Seedlings were transplanted into pots containing LP5 soilless mix containing starter fertilizer (Sun Gro) at 12 h light (100 \(\mu\)mol·m\(^{-2}\)·s\(^{-1}\) illumination)/12 h dark (72% relative humidity) and 22 °C. Seeds were stratification at 4 °C in the dark for 2 d before use. During growth, plants were irrigated with Jack’s Professional Peat-Lite 20:10:20 (NP\(_2\)O\(_5\)-K\(_2\)O) solution (at 2 g/L) one to two times to provide supplementary fertilizer.
**NO production in guard cells**

The method for NO detection in guard cells using the NO-specific fluorescent dye diaminofluorescein–2 diacetate (DAF-2DA; Invitrogen, Carlsbad, CA) was adapted from Ali et al (2007). Rosette leaves of 4-6 wk old WT, ipkl and ip5-ptase transgenic plant were detached from plant 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 1-2 h. Peels were then placed in 3 mL buffer B (NO synthase reaction kit buffer; Sigma-Aldrich) which contained 50 µM DAF-2DA to load cells with NO-specific dye. After 30 min incubation to load the NO-specific dye in the tissue, peels were washed three times in buffer A and incubated in 3 mL buffer B containing ligand flg22 (1 µM) for 5 min. The epidermal peels were placed underneath a cover slip on a microscope slide with several drops of buffer B. NO-dependent fluorescence was monitored over time. Mean maximal fluorescence of guard cells (occurring ~5-10 min after ligand addition) in a peel was ascertained exactly as described previously (Ma et al., 2009) and averaged to generate one biological replicate. Maximal fluorescence measurements of approximately 3 guard cells in an epidermal peel were averaged for one replicate. For each treatment, captured images at the maximum fluorescence intensity for treatment replicates were used to calculate data represented as means. Quantitative analyses of the NO-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.

**qPCR Analysis**
q-PCR analyses were performed to study genotype and treatment effects on the expression level of some BR-responsive genes. Growth of Arabidopsis seedlings in ½ strength MS liquid medium was performed according to Qi et al (Qi et al., 2010). The seedlings were grown in separate tubes containing 3 mL liquid medium for 14 d on a shaker (180 rpm) with 24 h illumination (~90 mol m⁻² s⁻¹) at 22 °C. The ligand flg22 (1 µM) was added directly to the growth medium and the seedlings were incubated for 0.5 h. Water was given to the seedling for the control treatment. After incubation, the seedlings were collected and frozen immediately with liquid N₂, and stored at -80 °C for future use. Total RNA were extracted from the whole liquid-grown seedlings using the Plant RNA Extraction Kit (Macherey-Nagel; Bethlehem, PA). During the RNA extraction process, tissue extraction was treated with rDNase (Macherey-Nagel) to remove potential genomic DNA from samples.

After extraction, 500 ng of total RNA was used for reverse transcription by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (ABI); Carlsbad, CA). The synthesized cDNA was diluted 1:10 (v/v) in water, and 1 µL of the diluted cDNA was used for each qPCR reaction. qPCR assays were performed using the ABI 7900 HT Real-time PCR system with the SYBR Green gene expression assay (ABI). Treatment effects on the expression level of WRKY29 (AT4G23550) and WRKY33 (AT2G38470) were examined. UBQ10 (AT4G05320) was used as an endogenous control. The primers used for these analyses were as follows: WRKY29 (Forward) 5' - ATCCAACGGATCAAGAGCTG-3', WRKY29 (Reverse) 5' - GCGTCCGACACAGATTCTC-3'; WRKY33 (Forward) 5' - CCCGTTGGCTTCATACCG T-3', WRKY33 (Reverse) 5' - TTACAGCGAGTGTCAATTTGGC-3'; and UBQ10 (Forward) 5' - CGGCTACCACATCCAAGGAA-3', UBQ10 (Reverse) 5' - GCTGGAATTACCGCGGCT-3'.
For each analysis, 3 mechanical replications were tested on one plate, and each treatment mean was generated from analysis of at least 3 biological replications from separate RNA isolated from different individual seedling. ANOVA of corresponding threshold cycle (CT) values was used for evaluation of means separation amongst treatments in an experiment (as noted in Figure legends), and to generate standard errors of the means for control treatments (Schmittgen and Livak, 2008). In many cases, experiments involving qPCR analysis of gene expression involved the evaluation of flg22 effects on different genotypes. In these cases, gene expression in the absence of added flg22 was normalized to ‘1’ for each genotype in a given experiment (as noted in figures). In these cases, the relative amount of gene expression in the absence of flg22 for each mutant line used in an experiment (i.e. in a given figure) is compared to the expression level in WT plants for that experiment.

**MPK phosphorylation**

The MPK phosphorylation assay was adapted from the method described by Keinath et al., (2011). Seedlings grown in ½ strength MS liquid medium for 14 d were used for MPK phosphorylation measurement. The seedlings were grown in a 24 well plate, individual seedlings were grown separately in each well containing 1 mL liquid medium for 14 d on a shaker (180 rpm) with 24 h illumination (~90 mol m⁻² s⁻¹) at 22 °C. The ligand flg22 and the control solvent (water) were added directly into each well. The seedlings were kept in the medium with the treatment for 0, 5, 15 and 30 min. Seedlings were then blotted dry and frozen in liquid nitrogen. For time point “0 min”, seedlings were immediately move out of the assay well, dried and frozen in liquid nitrogen after the ligand were added into the medium. For the protein extraction of the seedlings, seedlings were ground with 1 ml of extraction buffer (10 mM HEPEs, pH7.5, 100 mM
NaCl, 1 mM EDTA, 10% Glycerol, 0.5% Triton X-100 and protease inhibitor cocktail from Roche) (Lu et al., 2010).

Extracted protein was added to 6× SDS (Sodium dodecyl sulfate) dye, and denatured by incubation at 95 °C for 5 min. The proteins were loaded onto a 12% SDS-PAGE (sodium dodecyl sulfate polysacrylamide gel electrophoresis) gel. After electrophoresis, proteins were transferred from the gel to a western blotting membrane. After being blocked with 5% BSA (Bovine serum albumin; Research Product International, Prospect IL), the membrane was incubated with the primary antibody anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000 dilution in 5% (w/v) BSA, 1× TBS (Tris-buffered saline), 0.1 % Tween-20 (Bio-Rad, Hercurles, CA)), Cell Signaling Technology Inc. distributed by New England Biolabs, Frankfurt, Germany) at 4 °C overnight. After incubation, the membrane was washed with 1× TBS 3 times. The membrane was then incubated with secondary antibody, anti-Rabbit IgG (Immunoglobulin G; 1:1000 dilution in 5% (w/v) BSA, 1× TBS, 0.1 % Tween-20; GE Healthcare, Buckinghamshire, UK) at room temperature for 1 h.

The chemiluminescent HRP (horseradish peroxidase) substrates (also known as ECL reagents, Thermo Scientific, Rockford, IL) are used in the detection of Western blots. The chemiluminescent substrate is added to the membrane and incubated for 1 min. Detect the activity of MPK phosphorylation by expose the luminescent on the film for 5 min in the dark room.
Chapter 5. Use of a cDNA library expressed in a heterologous system for functional assay to identify a gene involved in AC synthesis and screening of an collection of DNA insertional mutant seed to identify genes involved in Pep2 signaling

Introduction

Project 1 Identification of an adenylyl cyclase gene in Arabidopsis using the *cyA* *Escherchia coli* mutant

Cyclic nucleotide function in plants is well documented over the past decades. It is now clear that these secondary metabolites play vital roles in plant biological processes (Newton et al., 1999). Cyclic nucleotides 3’,5’-cyclic AMP (cAMP) and 3’,5’-cyclic GMP (cGMP) have been implicated in many important plant processes, such as stomatal function, response to pathogen attack, seed germination, pollen tube growth and chloroplast development. (Cooke et al., 1994; Martines-Atienza et al., 2007). cAMP and cGMP can also activate cyclic nucleotide gated channel (CNGC), and initiate Ca²⁺ entry into the cytosol (Ma et al., 2009; Qi et al., 2010). The increased cytosolic Ca²⁺ concentration leads to ligand related plant actions, such as NO generation, ligand-related gene expression and pathogen defense (Ali et al., 2007). Thus, both cAMP and cGMP are known as important secondary messenger is plant signal transduction cascades (Volotovski et al., 1998). Assmann had surmised that in the higher plant cAMP is a naturally occurring component (Assmann, 1995). cAMP synthesis in animals occurs due to adenylyl cyclases (ACs), it is known that ACs catalyze the conversion of ATP to cAMP and pyrophosphate (Gehring, 2010). However, there is no gene encoding a canonical protein in plants
that corresponds to an animal guanylyl cyclases (GC) or AC. Currently, there is evidence that suggests the presence of GCs in plants, and that they are involved in signal transduction (Ma et al., 2012). On the other hand, no AC has yet been identified in plants, with the exception of a partial gene sequence encoding a pollen tissue specific signaling protein PSiP, which has competence for production of cAMP when expressed in Escherchia coli (E. coli) and is considered to be a soluble AC (Moutinho et al., 2001).

Beyond its function in the plant, cAMP also is important for bacterial cell activities. As introduced above, cAMP synthesis occurs due to adenylyl cyclase. In the bacterium E. coli, three putative AC genes from the complete genome sequence have been identified. These three genes were named as cyaA, cyaB and cyaC, and they were responsible for encoding the Class I, Class IV and Class III adenylyl cyclases (Charania et al., 2009). Shah and Peterkofsky (Shah and Peterkofsky, 1991) reported they had generated a new E. coli strain with a complete cya gene deletion. This new E.coli strain had an impairment of cAMP synthesis (Shah and Peterkofsky, 1991). After this strain became available in the E.coli Genetic stock center, people started to use this strain as a tool to determine the function of AC genes from other bacterial species. This tool was used to demonstrate that the Prevotella ruminicola D31d gene was an adenylyl cyclase. After transforming the Prevotella ruminicola D31d into the cyaA E. coli mutant, the Prevotella ruminicola D31d complemented the cyaA mutation (Cotta et al., 1998). cAMP is known to be essential for lactose utilization inside the cell. E. coli that lack AC activity could not synthesize cAMP, which led to the inability to ferment lactose. MacConkey agar is a culture medium designed to grow Gram-negative bacteria and differentiate them for lactose fermentation (MacConkey, 1905). When the cell culture fermented lactose on MacConkey agar medium, the result suggests that this cell has AC activity (Cotta et al., 1998). This report provides the
rationale for using the *E. coli cyaA* deletion mutant as a screening tool to identify AC genes in Arabidopsis. The Arabidopsis genome has already been fully sequenced. Arabidopsis cDNA expression libraries are available (Elledge et al., 1991) and can be used to transform genes into the *E. coli* mutant for screening.

**Project 2. Identification of gene products involved in Pep2 signaling downstream from the PEPR receptors**

As mentioned in Chapter 3, flagellin (studied using the peptide flg22) is perceived by plant cells upon binding to its receptor flagellin sensing 2 (FLS2). This binding induces cytosolic Ca\(^{2+}\) elevation which is an important element of innate immune signaling in plants (Chinchilla et al., 2006; Boudsocq et al., 2010). Flg22 is known to affect expression of many pathogen defense related genes (Boudsocq et al., 2010; Denoux et al., 2010). In addition to the gene expression, higher concentrations of flg22 cause a strong inhibition of Arabidopsis seedling growth. The root length of Arabidopsis seedlings grown on the medium containing flg22 is shorter than when seedlings are grown on normal medium (Gómez-Gómez et al., 1999). By using this characteristic of flg22 effects on seedlings, the Arabidopsis ecotype Wassilewskija (Ws-0) was shown to be completely insensitive to the flg22 peptide. This phenotype led to the identification of a locus that conferred sensitivity of flg22 in Arabidopsis plants: *FLS 1*. This locus in other ecotypes like Col-0 (Columbia) and Landsberg erecta (La-er) was suggested in early studies to code for the putative receptor for flg22 or for another positive regular acting in the signal transduction pathway induced by flg22 (Gómez-Gómez et al., 1999).

After *FLS1* was identified (Gómez-Gómez et al., 1999), the same group isolated another flagellin-insensitive mutant. They used map-based cloning to identify the corresponding locus of this mutant, and named it FLS2. FLS2 is involved in flagellin recognition. This mutant was
identified by screening the La-er ethyl methane sulfonate mutagenized seed with flg22. A mutant that does not respond to the flg22 peptide was selected and identified (Gómez-Gómez and Boller, 2000).

Pep (an endogenous plant elicitor peptide) and its receptors PEPR1 (Plant Elicitor Peptide Receptor) and PEPR2 have similar effects in Arabidopsis as flg22 (Krol et al., 2010). There is evidence that shows that the root length of Arabidopsis wildtype seedlings are inhibited when grown on the medium with addition of Pep1 (Krol et al., 2010). However, when there is a mutation of either PEPR1 or PEPR2, the root length of seedlings was not affected by Pep1 (Krol et al., 2010). My lab also found that Pep2 and Pep3 could also inhibit the growth of Arabidopsis seedlings just like flg22 and Pep1 (Qi et al., 2010; Qi and Berkowitz, unpublished data). The wild type Col-0 root length of the seeding is inhibited in the presence of 20 nM of Pep2 or Pep3. This effect is reversed when growing the pepr1, pepr2 and pepr1/2 double mutant. Among all these three Pep peptides, Pep2 showed the strongest effect (Qi and Berkowitz, unpublished data). This project involved the screening of T-DNA insertion Arabidopsis seeds on growth medium containing Pep2 to identify genetic suppress of the root growth inhibition phenotype displayed by wildtype (Col) Arabidopsis.

Materials and Methods

Preparation of cyaA mutant E. coli competent cells

The cyaA mutant E. coli strain was obtain from the Coli Genetic Stock Center in Yale University (Coil Genetic Stock Center, New Heaven, CT). The E. coli strain was spread on Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1% Agar) plate that contains 50 μ g/mL kanamycin, the plate was incubated at 37 °C for 12-16 h. After incubation, a single colony was picked from the plate and grown in 5 mL LB liquid medium. This liquid
culture was incubated at 37 °C with shaking at 250 rpm for 16 h. After 16 h, 1 ml of the liquid culture was transferred into the 100 ml fresh LB medium, and shaken at 37 °C for 2 h. The culture grown with continuous shaking until absorbance at 600 nm reached 0.4. After the shaking, the culture was placed on ice for 10 min.

The cell culture was transferred into a pre-chilled tube and centrifuged at 4 °C, 3000 X g for 10 min. The cell pellet from the culture was resuspended in 1.6 ml pre-chilled CaCl₂ (100 mM). After adding the CaCl₂, the cell culture was incubated on ice again for 30 min, and centrifuged at 4 °C, 3000 X g for 10 min after incubation. The cell pellet was resuspended again in 5 ml 100 mM CaCl₂, 15% glycerol (v/v). The resuspended cell culture was dispensed in 1.5 mL microcentrifuge tubes (500 μL/tube) and store at -80 °C.

**Plasmid Recovery**

The Arabidopsis cDNA expression library was generated by Dr. Elledge (Elledge et al., 1991). The cDNA library was expressed on the λ YES vector (Elledge et al., 1991). This vector is capable of replicating as a λ phage, a plasmid lysogen in *E. coli*, or as a centromere plasmid in yeast (Elledge et al., 1991). The plasmid can be looped out from the vector by site specific recombination of the cre protein and lox sites in the vector (Elledge et al., 1991). The method from the U.S patent of Richard Gaber (Gaber, 1993) was used to recovery the plasmid from the vector.

*E. coli* strain BNN132 was grown overnight in LB liquid medium containing maltose (0.2% (w/v)) and kanamycin (50 μ g/ml). The cells were harvested and resuspend in λ dilution buffer (contained 10 mM Tris-HCl buffer, 5 mM MgSO₄, 200 mM NaCl and 0.1% (w/v) gelatin, at pH 7.4). The λ phage library was added to the cell culture, and the cell culture with the phage library was incubated at 37 °C for 20 min. After incubation, the cells were grown in LB medium
for 30 min. The cell culture was then plated on the LB ampicillin plate (ampicillin 100 μ g/ml), the plates with the cell culture were incubated overnight at 37 °C. The plasmid recovered from the phage had resistance to ampicillin, which can be used to select for cells that are infected with the λ phage from the ones that are not. After the overnight incubation, scrape the plates and extract the plasmid from the cells harvested from the plate.

**Screening for the mutants that recover the AC activity**

A plasmid library transformed with an Arabidopsis cDNA library was transfected into the *cyA* mutant competent cells. The transformed cells were incubated overnight at 37 °C on MacConkey agar (Becton, Dickinson and Company, Sparks, MD) plates that contained kanamycin (50 μ g/ml) and ampicillin (100 μ g/ml). After incubation, colonies that complement the *cyA* mutation can be identified from the MacConkey agar plate by color. A colony that can ferment lactose shows bright red and the one that cannot shows white.

**Screening for Arabidopsis mutants that are not sensitive to Pep2**

The T-DNA insertion mutant sets (CS76506, set of 100 pools of random T-DNA insertion mutants) were purchased from ARBC stock center (Ohio State University, Columbus, OH). Arabidopsis seeds were surface-sterilized by first washing the seeds in 70% (v/v) ethanol, 20% (v/v) bleach and 0.02% (v/v) Triton X-100, shaking at 300 rpm for 10 min, and then rinsing with 95% (v/v) ethanol 3 to 4 times. After sterilization, seeds were planted on the ½ strength MS plate (contain Murashige and Skoog (MS) salt (Caisson Labs, Logan, UT), 1% (w/v) Suc and 1% (w/v) agar) that contained 20 nM Pep2. The seeds were planted in lines in the 10 cm×10 cm square plate. After planting, the seeds were stratified (4 °C) for 2 to 3 d to break dormancy, and grown vertically at 22 °C for 10 d. Mutants that are insensitive to Pep2 can be identified from the longer root length.
After 10 d, seedlings with longer root lengths were selected for analysis. Seedlings were then transplanted into pots containing artificial LP5 mix (Sun Gro, Bellevue WA). The pots were put in an EGC growth chamber (Chagrin Falls, OH) at 12 h light (~100 mol m\(^{-2}\) s\(^{-1}\))/12 h dark and 22 °C. The seeds from each individual plants were collected, and re-planted on the ½ strength MS square plates that contain 20 nM Pep2. The seedlings that are still insensitive to Pep2 in this recheck step were picked out to sequence for the flanking DNA of the insertion.

**Sequencing the flanking DNA at the site of the T-DNA insertion**

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is a tool for the recovery of DNA fragments adjacent to known sequences. TAIL-PCR utilizes three nested primers in consecutive reactions together with an arbitrary degenerate (AD) primer having a lower melting temperature (Tm) so that the relative amplification frequencies of specific and non-specific products can be thermally controlled (Liu et al., 1995). The details for this PCR reaction and the cycle condition are based on Singer and Burke (Singer and Burke, 2003). Three nested primers for this TAIL-PCR reaction are designed based on the left boarder of the pROK2 vector and the arbitrary degenerate primers were described in Singer and Burke (2003). The primers that were used are the following: LB1 5′ - GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC -3′ ; LB2, 5′ - GCTTCCTATTATATCTTCCAAATTACCAATA-3′ ; LB3, 5′ - TAGCATCTGAATTTCTATAACCAATCTCGATACAC -3′ and AD 5′ - NGTCGASWGANAWGAA -3′. The genomic DNA of mutant plants was used as template for the TAIL-PCR. Genomic DNA was extracted from the whole seedlings using the Plant DNA Extraction Kit (QIAGEN inc., Valencia, CA).

**Results**
Identification of an Arabidopsis adenylyl cyclase gene candidate

As mentioned in the introduction, inside the *E. coli* cell, cAMP plays an important role in lactose utilization. The mutant genotype we used as a host cell in this experiment lacks endogenous AC activity. Thus, these *E. coli* cells are unable to synthesize cAMP, and this result in the inability to undertake lactose fermentation or growth on MacConky medium (Cotta et al., 1998; Moutinho et al., 2001). Cells that cannot utilize lactose will appear white instead of bright red on the MacConky agar plate (Macconkey, 1905). By using this characteristic of MacConkey agar plates, the cell colony that can’t utilize the lactose can be easily identified. The colony that had been identified in this manner was re-streaked on a fresh MacConky agar plate for confirmation. The picture shown in figure 27 shows the growth of a positive candidate colony on the MacConky medium. The bright red color of the colony on the plate indicates that these cells had the ability to ferment the lactose (Figure 27). These mutant cells loss their lactose fermentation ability because of the deletion of the *cyaA* gene. It is a reasonable possibility to suggest that the transformed mutant cell which recovers its lactose utilization activity may have a plasmid that has Arabidopsis DNA encoding an AC gene. Thus, the cAMP is re-synthesized in the mutant cell. The sequence of the plasmid insertion is required for confirmation and further research. Continuation of this project is beyond the scope of my dissertation research.

Identification of a gene that may be involved in Pep2 signaling

The T-DNA random insert mutant seeds were grown on ½ MS medium that contained 20 nM Pep2. As per our hypothesis, the mutant that contains a mutation in a gene involved in Pep2 signaling may be insensitive to Pep2. Mutant seeds that did not respond to the presence of Pep2 in the growth medium were selected and transplanted to soil for further analysis. In Figure 28, the growth of WT, *pepr1-1* the unknown candidate mutant (labeled as X1) on ½ MS plate
Figure 27. The transformed *E. coli* cells that can ferment lactose were grown on the MacConky medium plate. After the red colony was selected from the screening plate, the cell was cultured in LB liquid medium (contained 50 µg/ml kanamycin and 100 µg/ml ampicillin) overnight at 37 °C. The cell culture was restreaked onto the fresh MacConky plate for confirmation. The colonies that can ferment lactose appear as red on the MacConkey plate.
Figure 28 The effect of Pep2 on the seedling root length is impaired in the candidate mutant X1.
A) Growth of seedlings (WT, pepr1-l and X1) on ½ strength MS plates containing 20 nM Pep2.  
B) Quantitative root length comparison of WT, pepr1-l and X1 mutant plant grown on ½ strength MS plates contained 20 nM Pep2 and water. Results shown are mean values of root length of each individual seedling (n=8) ± SE. For each genotype, asterisks over bars representing a significant (at P< 0.05) difference between the presence of Pep2 and the control (water addition).
containing 20 nM Pep2 is shown. As introduced earlier, the root length of WT seedlings was inhibited when grown on medium containing exogenous Pep1, pepr2 and pepr1/2 double mutant do not show this phenotype (Krol et al., 2010). However, the inhibition of root growth still occurs in the pepr1 mutant (Krol et al., 2010). Pep2 had similar effects on the seedling root growth as Pep1. The effects of Pep2 on root length can be reversed in pepr2 and pepr1/2 double mutant (Qi and Berkowitz, unpublished data), but still remains on pepr1 mutant. The experiment shown in Figure 28 compared the root growth among WT, pepr1 mutant and the candidate mutant X1. Candidate mutant X1 had obvious longer root than WT and pepr1 mutant under the effects of Pep2 (Figure 28A). Quantitative root length comparison between the WT, pepr1-1 and X1 mutant shown in Figure 28B also indicates that the root length of X1 is significantly different than WT and pepr1-1 mutant when grown on medium containing Pep2.

The T-DNA that was inserted in the genome was carried on the pROK2 vector, the sequence of this T-DNA insertion is known. TAIL-PCR is a technique that can amplify the genomic region adjacent to a known sequence. During the amplification, the primers were designed based on the sequence of the T-DNA insertion (Liu et al., 1995; Sessions et al., 2002). After the region adjacent to the insertion was amplified, the flanking DNA at the sides of the insertion can be sequenced. With this gene mutation, the plant is insensitive to Pep2 (Figure 28).

The mutant that is insensitive to Pep2 is picked from the plates, and the flanking DNA of the insertion is sequenced, the sequence of the adjacent region is compared with the Arabidopsis whole genome sequence using BLAST analysis. The results from this BLAST analysis indicated that X1 had 98% similarity with Arabidopsis PEPR2 gene, suggesting that this mutant had the T-DNA insertion in the PEPR2 gene. It is already known that PEPR2 is an important receptor for Pep2 signaling. This result suggests that this method is able to identify genes that are involved in
Pep2 signaling. In order to find a novel gene in the Pep2 signaling pathway, genes that are known to be involved in Pep2 signaling (e.g. *PEPR1, PEPR2*) needs to be excluded by genotyping the seedling after the screening. Prior to TAIL-PCR, RT-PCR was run so as to exclude the *PEPR1* and *PEPR2* genes as candidates for the unknown cDNA. The RT-PCR results excluded the possibility of *PEPR1* and *PEPR2* gene at that time. However, after sequencing the TAIL-PCR product, the sequencing result confirmed that the insertion of X1 mutant is in fact the *PEPR2* gene. After finding out that the unknown cDNA encoded *PEPR2*, it became evident that there was an error that occurred in the RT-PCR procedure that caused the PCR genotyping result to be inaccurate; thus we failed to exclude the known candidate. After comparing the sequence of the unknown gene with the Arabidopsis genome data base, we concluded that the gene identified from this project is *PEPR2*.

**Discussion**

cAMP is an important secondary messenger in animals and plants. However, unlike in animals, no canonical AC have been identified in plants yet. One of the research projects discussed in this chapter involves screening for the gene that is responsible for AC activity in Arabidopsis. In the experiment, the *E. coli* mutant failed to utilize the lactose because of the deletion of its AC genes. The experimental plan is to find an Arabidopsis cDNA insertion that can allow recovery of lactose utilization by the *E. coli* mutant, which we assume may be due to the cAMP synthesis from AC activity of the insertion. However, this experiment does not directly test cAMP production in the transformed mutant cells. We cannot exclude the possibility that the gene we transformed and expressed in the *E. coli* cell may not be involved in AC activity, but is related to lactose fermentation. Thus, we still lack evidence to prove that the cDNA insertion we identified from this experiment is a gene that is involved in Arabidopsis AC
activity. For further confirmation, synthesis of cAMP in candidate cells needs to be measured in order to prove the AC activity is really increased in the cell. An AC ELISA assay needs to be used to evaluate cAMP formation by the candidate cell (Macdonald-Fyall et al., 2004), and the cAMP synthesis level needs to be compared between the candidate cell with the \textit{E. coli cya} mutant.

The other research that is described in this chapter shows a method to identify genes that may be involved in Pep2 signaling downstream from the PEPR receptor. However, the candidate mutant obtained from this project was confirmed to be a \textit{PEPR2} mutant, which is a gene that is known to be involved in Pep2 signaling. To avoid this problem, the method needs to be optimized in the future. The mutant candidate needs to be genotyped before the TAIL-PCR amplification and sequence analysis. However, the fact that \textit{PEPR2} mutant is screened out from the T-DNA insertion seed pools suggests that this method is a practical method to identify genes that are involved in Pep2 signaling in Arabidopsis.
Chapter 6. Cloning and site-directed mutagenesis of BRI1

Introduction

Guanylyl cyclase (GC) activity has been detected in animals and plants for many years (Ludidi and Gehring, 2003; Shiga and Suzuki, 2005). The enzyme activity catalyzes the formation of a 3’,5’-cyclic guanosine 5’-monophosphate (cGMP) from GTP (Durner et al., 1998). cGMP acting in animal cells signals through receptor activity on the plasma membrane (Newton and Smith, 2004). In higher plants, cGMP has been implicated in responses to abiotic and biotic stress (Cooke et al., 1994; Martines-Atienza et al., 2007). As mentioned in Chapter 2, brassinosteroid insensitive 1 (BRI1) may contain a GC catalytic domain on its intracellular region (Kwezi et al., 2005). In Chapter 2, we describe cyclic nucleotide gated cation channel 2 (CNGC2) involvement in some BR-dependent signal transduction. It is also suggested that both cGMP and cytosolic Ca^{2+} are involved in some of the BR-dependent signaling pathway. However, in the previous chapter, the work lacked evidence to link this putative BRI1 GC catalytic domain directly with the cGMP signaling involved in the BR-dependent signal transduction pathway.

Currently, there is evidence that suggests the GC domain of Arabidopsis plant elicitor peptide receptor 1 (PEPR1) receptor is important in Pep-dependent signaling (Ma et al., 2012). The report shows that mutation of key residues (S1014A, G1016A and R1027Q) of the GC catalytic domain on the PEPR receptor abolishes Pep dependent signaling in protoplasts (Ma et al., 2012). During the alignment of the PEPR1 and BRI1 receptor, we can find that the BRI1 GC catalytic domain shares a similar 14 amino acid sequence with the PEPR1 GC catalytic domain. The protein sequence alignment is shown in Figure 29. In Figure 29, we can find that the protein
sequence of the GC domain of PEPR1 (S1014-R1027), PEPR2 (S981-R994) and BRI1 (S1071-R1084) is conserved. The key residues that are important for the GC activity in Pep-dependent signaling may correspond to the same residues in the BRI1 catalytic GC domain. This analysis supports the rationale underlying the work shown in this chapter which is focused on mutating the key residues in the BRI1 receptor, and subcloning the mutated BRI1 receptor back into a plasmid that expresses the BRI1 receptor under control of the BRI1 native promoter: pBIB-BRI1-FLAG-HYG (Oh et al., 2009). After obtaining the plasmid with the BRI1 GC mutation, the reconstructed plasmid pBIB-BRI1m-FLAG-HYG will be transfected into the bri1-5 mutant to generate BRI1m-FLAG transgenic plants that can be used for further research.

Results

Cloning the BRI1 gene into a Zero-Blunt End TOPO vector

The BRI1 receptor under control of its native promoter has already been cloned into the pBIB-HYG vector which can be expressed in plants (Oh et al., 2000). The size of the pBIB-HYG vector is 12.9 kb. Due to the large size of the vector, the site-direct mutagenesis efficiency may be decreased. In order to obtain the correct construct with BRI1 GC mutations, the BRI1 gene should be cloned into a smaller vector to increase the efficiency of the site directed mutagenesis. In order to clone the BRI1 gene from the plasmid and ligate it back, two primers containing appropriate restriction sites were designed. The pBIB-BRI1-FLAG-HYG vector contains a BRI1 gene flanked by SalI and KpnI sites. The BRI1 DNA coding sequence also contains a SmaI site on position 486. The restriction sites SmaI and KpnI were chosen for ligation-independent cloning (LIC) that is desired for the plasmid recombination. The BRI1 DNA fragment that includes the GC domain within it was amplified with the sense primer BRI1-
Figure 29 The over-all structure and GC catalytic domain alignment of leucine-rich repeat receptor-like kinases (LRR-RLKs) PEPR1, PEPR2, and BRI1.

This analysis is adapted from that of Ma et al., (2012). In the structure model, the extracellular domain includes a signal peptide (SP) for targeting to the plasma membrane a region of LRRs. These proteins have a single-pass transmembrane domain (TM). The cytosolic region of these receptors includes a kinase domain (azure) and within it, a putative guanylyl cyclase (GC) catalytic domain (green). The sequence of the 14 residues of the putative GC catalytic domain of PEPR1, PEPR2 and BRI1 are shown surrounded by a purple box. To the immediate right of protein name is the amino acid position of the first residue included in the alignment. Functionally assigned residues in this region of the proteins are highlighted in red within the GC domain.
F-Sma1 5’-CCCGGGCATCTCTATGATC-3’ and the antisense primer BRI1-R-Kpn1 5’-GGTACCTAATTTTCCTTCAGGAAC-3’. The underlined sequences represent vector-compatible ligation-independent cloning overhangs for cloning. The gene-specific primer sequences resulted in amplification of BRI1 encoding amino acids 163 through 1196, the carboxy-terminal amino acid. The BRI1 gene was amplified with Phusion High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA) in a PCR reaction to form a blunt end PCR product. After pre-incubation at 94 °C for 5 min, 35 cycles (of 94 °C, 1 min; 58 °C, 1 min; 72 °C, 3 min (1 min/kb)) were performed followed by a 10 min extension at 72 °C. The PCR product was confirmed by running on a 1% agarose gel. The size of the BRI1 DNA fragment amplified from the PCR reaction should be 3.2 kb. The gel picture used to confirm the size is shown in Figure 30. The confirmed PCR product was purified with a PCR purification kit (Macherey-Nagel; Bethlehem, PA). The purified PCR product was transformed into the pCR-Blunt vector (Invitrogen, Carlsbad, CA). The plasmid construct generated was sequenced to verify the fidelity of the BRI1 sequence in this construct. The BRI1 sequence was cloned into the pCR-Blunt vector, with a total size of about 7 kb.

**Site-directed mutagenesis on BRI1**

The goal of this experiment is to mutate the three key residues S1071, G1073 and R1084 that are located in positions 1071, 1073 and 1084 respectively of the BRI1 sequence. These three residues were suggested to be the key functional residue for BRI1 GC catalytic activity (Kwezi, 2007). The previous cloned plasmid that contains the BRI1 DNA coding sequence was used as the template for site-directed mutagenesis with the Quick Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Constructs containing the three mutations were generated with following substitutions: S-1071-A, G-1073-A and R-1084-Q. The primers that were used for
Figure 30. The size of BRI1 DNA fragment from PCR amplification was confirmed in agarose gel electrophoresis. The size of this PCR product is 3.2 kb. In the picture, the bands were slightly above the 3 kb band in the DNA ladder, indicating that the product obtained from PCR reaction is correct, in terms of its size.
mutagenesis were: F 5’- GAGTAGCTCGAGTAAGACCACAGCGTAAGCATAAACGTCCTCC -‘3 and R 5’- F: R>Q : 5’- CTCGAGCTACTCAGGTAACACGCAACGGATTCACC -‘3. The DNA construct generated was sequenced to verify specific mutations by sequencing the GC region sequence.

**Generation of BRI GC mutation transgenic plants**

The BRII\textsuperscript{m} DNA fragment was obtained by digesting the pCR-Blunt-BRII\textsuperscript{m} plasmid with Smal and Kpn1 restriction enzymes. The restriction digestion product was run on a 1% agarose gel and purified using a gel extraction kit (Macherey-Nagel; Bethlehem, PA). The gel picture shown in Figure 31 is taken after double digestion of pCR-Blunt-BRII\textsuperscript{m} plasmid with Smal and Kpn1 restriction enzymes. The upper bands just above 3 kb are the target bands representing the DNA fragment of BRI\textsuperscript{m}. The BRII\textsuperscript{m} DNA fragment was treated with alkaline phosphatase (Calf-Alkaline Phosphatase, Invitrogen) to prevent self-ligation of the blunt end site. The DNA sequence needs to be ligated back into the pBIB-HYG plant expression vector in order to generate a construct that can then be used to generate transgenic mutant plants. The vector pBIB-BRI1-FLAG-HYG was also digested with Smal and Kpn1 restriction enzymes and gel purified. The pBIB-HYG vector (60 fmol) and the BRII\textsuperscript{m} DNA fragment (540 fmol) were added to a single tube together with T4 DNA ligase (Agilent Technologies, Santa Clara, CA). After 16 h ligation reaction at 15 °C, reconstructed plasmid were transformed into One Shot TOPO10 E. coli cells (ABI Life Technology, Bedford, MA) and the cell cultures were store in glycerol at -80 °C. A schematic diagram representing the procedure used to generate the BRII GC domain mutation plasmid is shown in Figure 32.
Figure 31. Restriction enzyme digest analysis of pCR-blunt-BRI1 plasmid using SmaI and Kpn1.
The BRI1 DNA fragment (3.2 kb) is located slightly above the 3 kb band in the DNA ladder. The pCR-blunt vector were digested into 2 pieces (1.5 kb and 2 kb) due to the Kpn1 restriction site located on the vector.
Figure 32. A schematic representing the procedure used to generate the BRI GC domain mutation plasmid pBIB-BRI\textsuperscript{m}-FLAG-HYG. The red color area represents the BRI\textsubscript{I} receptor or the BRI\textsubscript{I} DNA fragment. The pBIB-HYG vector is represented in yellow. The blue line located on the BRI\textsubscript{I} receptor represents the GC domain of BRI\textsubscript{I}. The pCR-blunt vector that was used for site-directed mutagenesis is showed in green.
The method used to transform Arabidopsis plants with pBIB-BRI1<sup>m</sup>-FLAG-HYG plasmid was modified from the protocol described by Clough and Bent (Clough and Bent, 1998). The pBIB-BRI1<sup>m</sup>-FLAG-HYG plasmid was extracted from an overnight grown cell culture. The plasmid was then transformed into the competent GV3010 (pMP90) <i>Agrobacterium tumefaciens</i> cells. The transformed cells were grown on LB liquid medium with appropriate antibiotics (50 µg/mL gentamycin, 50 µg/mL kanamycin and 50 µg/mL hygromycin). The cell culture was harvested and resuspended in inoculation buffer (1/2 MS liquid medium, 40 nM 6-benzylamino purine (BAP), 5% (w/v) sucrose, 0.005% Silwet L-77, pH 5.7). The cell culture in the buffer was dipped onto Arabidopsis flower buds. After the dipping, transfected plants were covered with plastic wrap in order to maintain the flowers a moist environment. The plastic wrap was removed 2 d after the transfection. The seeds of the transfected plants were harvested after about 2 weeks. To identify the plants that contains reconstructed plasmid, Arabidopsis seeds were grown on ½ strength MS medium plates containing 50 µg/mL hygromycin. Seeds were surface-sterilized by first washing the seeds in 70% (v/v) ethanol, 20% (v/v) bleach and 0.02% (v/v) Triton X-100, shaking at 300 rpm for 10 min, and then rinsing with water for 3 to 4 times. After sterilization, distilled water that contains 0.1% agar were add to the seeds, the seeds were pipetted out and placed on ½ MS plants that contained hygromycin. Initially, 11 T1 plants were selected from the ½ MS plate after 14 d. The seedlings were transplanted into the pots containing artificial LP5 mix (Sun Gro, Bellevue WA). The pots were put in an EGC growth chamber (Chagrin Falls, OH) at 12 h light (~100 mol m<sup>−2</sup> s<sup>−1</sup>)/12 h dark and 22 °C. The seeds from those Arabidopsis seedlings were harvested and re-grown again on the selection plates that contained the same antibiotic for further confirmation. of the presence of antibiotic resistance carried by the transfected plasmid.
Discussion

Secondary messenger cGMP and cytosolic Ca\(^{2+}\) may play an important role in BR-dependent signaling. Exogenous BR application can cause *in vivo* cGMP elevation, and we suspect that this BR signaling cascade might occur due to GC activity of the hormone receptor (see detail in Chapter 2). However, till now, there is no direct evidence to prove that the GC domain located on the receptor BRI1 is important for BR-dependent signaling. The purpose of the work shown in this chapter is to generate a new tool to find the evidence about the GC domain and BR-induced signaling. In the earlier chapter, we have shown the work involved with GC activity inhibitor suggested that BR-dependent cGMP elevation is required for both the generation of the Ca\(^{2+}\) signaling (Figure 5), as well as BR-dependent gene expression (Figure 8). However, there is no evidence to directly prove the cytosolic Ca\(^{2+}\) increase, the cGMP elevation and the downstream gene expression induced by exogenous BR is due to the GC catalytic domain on the receptor. Thus, whether the GC activity of BRI1 is responsible for BR induced Ca\(^{2+}\) signaling and downstream gene expression is not clear yet. In this work described in this chapter, three critical amino acids in the GC domain of BRI1 were mutated, and BRI1 (S1071A, G1073A, R1084Q)-FLAG transgenic plants were generated by expressing the mutated BRI1\(^{\text{m}}\)-FLAG plasmid in *bri1-5* mutant plants.

Since *bri1-5* mutant plants lack the ability to perceive the hormone BR, generation of *bri1-5* mutants expressing the BRI1-FLAG coding sequence will provide a biological tool useful for evaluating if the point mutations affect the proposed BR signaling pathway (Figure 15). The amino acids in the GC domain of BRI1 are conserved with the amino acids in the GC domain of PEPR1. Current work in the lab shows that mutation of critical amino acids in the GC domain of PEPR1 thought to be involved in GC activity did not alter the deduced structure of the PEPR1
kinase domain but did abolish Pep dependent signaling by this receptor (Ma et al. 2012). With the mutation of the GC domain of the BRI receptor, this BRI1\textsuperscript{m} transgenic plant can be used as a negative control in further research about the involvement of GC activity in BR dependent signaling cascades. In the early chapter, we have shown that cGMP and cytosolic Ca\textsuperscript{2+} elevation are required for the expression of some BR-dependent genes. Application of exogenous BR to the BRI1\textsuperscript{m} transgenic plant and measurement of BR-dependent gene expression may provide evidence to evaluate the relationship between the GC domain of BRI1 and BR-dependent signaling. Furthermore, a new aequorin (aeq)-expressing plant BRI\textsuperscript{m}-aeq can be generated by crossing the WT-aeq with the BRI1\textsuperscript{m} transgenic plants. This new aeq-expressing mutant can be used to monitor whether the GC domain mutations affects the BR-induced cytosolic Ca\textsuperscript{2+} changes inside the plant.

It is well known that phosphorelay cascades and kinase activity are important in the BR-dependent signaling pathway. The model introduced in Chapter 2 (Figure 15) suggests that the BR-dependent phosphorelay signaling cascade might be independent from BR-dependent Ca\textsuperscript{2+} signaling in terms of activation of different BR-responsive genes. To verify the model, the phosphorylation activity of BRI1 GC mutant plant can be measured with the application of exogenous BR. It helps us to understand the relationship between these two BR-dependent signaling pathways. Furthermore, it is known that a specific tyrosine residue locate in the intracellular domain of the BRI1 receptor is important for BR dependent phosphorelay signaling (Oh et al., 2009). The mutation of both the GC domain residues and the tyrosine residue should strongly block the BR induced signaling in both BR-dependent phosphorelay signaling pathway and cGMP activated Ca\textsuperscript{2+} signaling pathway. Those experiments may help us gain more information about BR signal transduction cascades in the plant.
The purpose of the work shown in this chapter is to generate a new BRI1 mutant to facilitate future research to further test models of BR signaling developed from the research presented in Chapter 2. The mutant plasmid contains three point mutations on the GC domain of the BRI1 receptor. Due to the big size of the binary vector pBIB-HYG, the BRI1 DNA coding sequence was amplified from the original plasmid with restriction enzyme sites and subcloned into a smaller vector. This step helps to increase the efficiency of site-directed mutagenesis. The mutated BRI1 receptor was ligated back to the binary vector pBIB-HYG after the mutations were confirmed. The SmaI site used for the ligation in this experiment is a blunt end site, thus the ligation requires longer reaction time (about 16 h) for a higher ligation rate. The size of the BRI\textsuperscript{m} DNA fragment (~ 3 kb) that needs to be inserted back into the vector is much smaller than the size of the vector (~14.9 kb), thus the insert DNA fragment tended to self-ligate during the ligation. In order to prevent this problem, the BRI\textsuperscript{m} DNA fragment was treated with alkaline phosphatase before the ligation. Alkaline phosphatase catalyzes the removal of 5’ phosphate group from DNA. Since alkaline phosphatase treated fragments lack the 5’ phosphoryl termini required by ligases, this prevents the self-ligation of the fragment (Seeburg et al., 1977). In order to successfully generate the BRI1 GC domain mutant, the plasmid reconstruction was used in this project and the details for each step were modified to successfully generate the BRI1\textsuperscript{m} construct with the long term goal transform plants with this coding sequence.
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