TECHNICAL ADVANCE

Methods to Study PAMP-Triggered Immunity Using Tomato and *Nicotiana benthamiana*

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Understanding the molecular basis of plant responses to pathogen-associated molecular patterns (PAMPs) is an active area of research in the field of plant-microbe interactions. A growing number of plant genes involved in various steps of PAMP-triggered immunity (PTI) pathways and microbial factors involved in the elicitation or suppression of PTI have been identified. These studies have largely relied on *Arabidopsis thaliana* and, therefore, most of the PTI assays have been developed and optimized for that model plant system. Although PTI is a conserved feature among plants, the response spectra vary across different species. Thus, there is a need for robust PTI assays in other pathosystems, such as those involving *Solanaceae* plant-pathogen interactions, which include many economically important plants and their diseases. We have optimized molecular, cellular, and whole-plant methods to measure PTI responses in two widely studied solanaceous species, tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana*. Here, we provide detailed protocols for measuring various PTI-associated phenotypes, including bacterial populations after pretreatment of leaves with PAMPs, induction of reporter genes, callose deposition, activation of mitogen-activated protein kinases, and a luciferase-based reporter system. These methods will facilitate limited genetic screens and detailed characterization of potential PTI-related genes in model and economically important *Solanaceae* spp.-pathogen interactions.

In recent years, many important discoveries have advanced our understanding of plant defenses that are activated when the host plant first senses the presence of a potential pathogen. These early host defenses involve recognition of conserved features of microbes, including flagellin, the major component of bacterial flagella; peptidoglycan (PGN) and chitin, components of the pathogen cell wall; and elongation factor Tu (EF-Tu) (Boller and Felix 2009). Collectively, these microbial

elicitors are termed pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). Similar to the Toll-like innate immune signaling pathways characterized in animals, in plants, PAMPs are perceived by pattern recognition receptors (PRR), which trigger a variety of downstream responses, including a calcium burst, activation of mitogen-activated protein kinase (MAPK) cascades, production of reactive oxygen species (ROS), expression of numerous defense-associated genes, production of ethylene, and callose deposition at the cell wall (Asai et al. 2002; Ausubel 2005; Boller and Felix 2009). This first line of defense, termed PAMP-triggered immunity (PTI), acts to inhibit pathogen growth. Current knowledge of PTI is mostly derived from studies performed with *Arabidopsis thaliana*. For example, the two best-characterized PRR, FLS2 and EFR, receptors of flagellin and EF-Tu, respectively, were identified and characterized in *Arabidopsis* (Gomez-Gomez and Boller 2000; Zipfel et al. 2006). Two other *Arabidopsis* protein kinases, BAK1 and BIK1, have been found to be involved in signaling associated with PAMP receptors (Chinchilla et al. 2006; Heese et al. 2007; Lu et al. 2010; Shan et al. 2008; Zhang et al. 2010).

Although PTI appears to be conserved across the plant kingdom, the specific responses and underlying molecular mechanisms may vary in different species. For instance, members of both the *Brassicaceae* and *Solanaceae* families perceive flagellin; however, they vary in their sensitivity to different peptides of the protein. Tomato, for example, recognizes a 15-amino-acid flagellin peptide from *Escherichia coli* whereas *Arabidopsis* does not (Felix et al. 1999; Meindl et al. 2000). Perception of bacterial EF-Tu appears to be conserved in the *Brassicaceae* family but has not been identified in any *Solanaceae* spp. examined (Kunze et al. 2004). Differences have been observed in PTI against *Pseudomonas syringae* pv. *tomato* DC3000 between *Arabidopsis* and *N. benthamiana*, including weaker growth of a DC3000 type III secretion mutant in *Arabidopsis*, possibly due to differences in PAMP perception between the two species (Hann and Rathjen 2007). These differences indicate that the study of PTI in diverse plant species will be important for a comprehensive understanding of the molecular basis of this important host defense system.

The majority of assays for investigating PTI in whole plants, protoplasts, or cell-culture systems have been developed and standardized for *Arabidopsis*. These assays include the detection or quantification of calcium production, ROS production, activation of MAPK, induction of defense-responsive genes, and cal-

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lose deposition (Chinchilla et al. 2006; Hauck et al. 2003; He et al. 2006; Zipfel et al. 2004). A few assays have been developed or optimized for *N. benthamiana* such as PTI-mediated suppression of host cell death, staining to detect PTI-associated vascular constriction, callose deposition, calcium burst, ROS, and nitric oxide production (Asai et al. 2008; Chakravarthy et al. 2009; Hann and Rathjen 2007; Heese et al. 2007; Kvitko et al. 2009; Oh and Collmer 2005; Oh et al. 2010). Some assays are readily transferable to other plant species whereas others require significant optimization due to differences in the host response, leaf morphology, or the age, size, and growth conditions of plants that are most conducive for the assay.

We recognized a particular need for robust PTI assays for studying the interactions between model species in the *Solana ceae* family and their microbial pathogens. This plant family contains approximately 100 genera and more than 2,500 species, including economically important crops such as potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), eggplant (*S. melongena*), pepper (*Capsicum* spp.), and tobacco (*Nicotiana* spp.). It is the third most valuable crop family in the world and the most valuable among vegetable crops (Olmstead et al. 2008). Solanaceous plants, such as tomato and *N. benthamiana*, also serve as important model systems for understanding plant immunity at both the organismal and molecular level. The advantages of these model systems include the availability of vast natural variation in the form of wild species, the large number of important and diverse pathogens that attack them, and their amenability to *Agrobacterium*-mediated transformation and virus-induced gene silencing (VIGS) (Goodin et al. 2008).

Here, we present detailed protocols we have either optimized based on existing PTI assays or developed ourselves for molecular, cellular, and whole-plant methods to measure PTI responses in tomato and *N. benthamiana*. These methods will hopefully aid in the effort to characterize potential PTI-related genes and facilitate the study of plant innate immunity in the economically important interactions between solanaceous plants and their pathogens.

RESULTS AND DISCUSSION

Preinoculation of *N. benthamiana* leaves with a nonpathogen to induce PTI and enhance differences in bacterial growth between wild-type and PTI-compromised plants.

One of the most convincing forms of evidence to support a role in PTI of a given host gene is to discover an enhancement of bacterial growth in plants that are compromised for expression of the gene compared with wild-type plants. However, in most cases, such differences in bacterial growth are extremely small and special conditions are required for their detection. For instance, an increase in growth of *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* plants with a mutation in the flagellin receptor FLS2 compared with wild-type plants was only detectable when plants were spray inoculated and younger leaves were assayed (Zipfel et al. 2004).

It has been shown previously in *N. benthamiana* that preinoculation of a leaf with a nonpathogen followed several hours later by inoculation with a virulent or avirulent bacterium abolishes or significantly delays host cell death associated with disease or the hypersensitive response (HR), respectively (Klement et al. 2003; Oh and Collmer 2005; Oh et al. 2010). We have also shown recently that *N. benthamiana* plants silenced for *Fls2* are specifically compromised in their response to flg22 using this cell-death-based PTI assay (Chakravarthy et al. 2010). Therefore, we tested whether preinduction of PTI by a nonpathogen prior to inoculation with pathogenic bacteria would enhance the bacterial growth dif-

ference between PTI-compromised and control plants. *N. benthamiana* leaves silenced or not for *Fls2* were syringe infiltrated with either 10 mM MgCl₂ alone or with the non-pathogenic saprophyte *P. fluorescens* 55 at 10⁹ CFU/ml. Seven hours later, the plants were syringe infiltrated with the pathogenic strains DC3000 Δ *hopQ1-1* or DC3000 Δ *hopQ1-1*/ Δ *avrPto*/ Δ *avrPtoB* at a low titer (2 $\times$ 10⁵ CFU/ml). DC3000 Δ *hopQ1-1* lacks the major effector (HopQ1-1) that is recognized by *N. benthamiana*, making it pathogenic on this plant species (Wei et al. 2007), whereas DC3000 Δ *hopQ1-1*/ Δ *avrPto*/ Δ *avrPtoB* lacks two additional effectors that are required for full virulence (Kvitko et al. 2009). In plants pretreated with only MgCl₂, we were unable to detect any population difference for either *P. syringae* pv. *tomato* strain in *Fls2*-silenced compared with empty-vector control plants (Fig. 1). However, when leaf tissue was pretreated with *P. fluorescens*, we observed a statistically significant increase in the population of both pathogenic bacteria in *Fls2*-silenced plants compared with control plants (Fig. 1). The population of DC3000 Δ *hopQ1-1*/ Δ *avrPto*/ Δ *avrPtoB* in *Fls2*-silenced plants was approximately 100-fold more than in the empty vector control plants three and one-half days postinoculation (Fig. 1A). The population difference of DC3000 Δ *hopQ1-1* in the silenced plants compared with the control plants, albeit smaller (sixfold), was still statistically significant (Fig. 1B). The smaller growth difference observed with the DC3000 Δ *hopQ1-1* strain is due to the presence of the *AvrPto*/*AvrPtoB* effectors which are known to be strong suppressors of PTI (Lin and Martin 2005; He et al. 2006; Shan et al. 2008). Therefore, prior induction of PTI with a live nonpathogen effectively enhances the difference in bacterial growth between PTI-compromised plants and control plants, which could otherwise not be detected.

Preinduction of PTI in tomato leaves by using individual PAMPs for inhibition of pathogenic bacterial growth.

Pretreatment of *Arabidopsis* with PAMPs such as flg22, elf18, and lipopolysaccharide (LPS) induces PTI, which effectively restricts the growth of pathogenic bacteria inoculated subsequently (Kunze et al. 2004; Mishina and Zeier 2007; Newman et al. 2002). We tested the effectiveness in tomato leaves of preinduction of PTI on bacterial growth inhibition using two PAMPs, PGN (from *Staphylococcus aureus*) and flg22. Tomato plants pretreated with 1 μ M flg22 12 h prior to bacterial inoculation supported a population of DC3000 Δ *avrPto*/ Δ *avrPtoB* that was significantly lower compared with plants pretreated with buffer only at both 2 and 4 days after inoculation (Fig. 2A). Pretreatment with PGN (100 μ g/ml) caused a statistically significant reduction of *P. syringae* pv. *tomato* growth compared with the buffer-treated control plants at 4 days postinoculation (Fig. 2A). Although PGN has been reported to act as a PAMP in plants (Gust et al. 2007; Erbs et al. 2008), this appears to be the first report that pretreatment with PGN can restrict bacterial growth in plants. We note, however, that it is possible this commercial PGN preparation contains other contaminating PAMPs in addition to PGN. Previously, we counted the number of necrotic lesions (specks) in a defined leaf area as another measure of bacterial virulence and host immunity (Lin and Martin 2005). By using that approach, we observed significantly fewer specks 4 days after pathogen inoculation on plants pretreated with either PGN or flg22 compared with buffer-pretreated leaves (Fig. 2B and C). Together, these methods will be useful in tomato for demonstrating the effect on bacterial growth of specific PAMPs and in evaluating the effect of knocking down the expression of host genes putatively involved in PTI.

Fig. 1. Pretreatment of plants with a nonpathogenic bacterium enhances the difference observed in pathogenic bacterial populations in *Nicotiana benthamiana* plants compromised for pathogen-associated molecular pattern-triggered immunity. **A**, Bacterial populations of DC3000Δ*avrPto*/Δ*avrPtoB*/Δ*hopQ1-1* (ΔΔΔ) or DC3000Δ*hopQ1-1* (Q1-1) in *Fls2*-silenced or nonsilenced control plants that were infiltrated with 10 mM MgCl₂ 7 h before inoculation with the pathogenic bacteria (2×10^5 CFU/ml). **B**, Bacterial populations of DC3000Δ*avrPto*/Δ*avrPtoB*/Δ*hopQ1-1* (ΔΔΔ) in *Fls2*-silenced or nonsilenced control plants that were inoculated with *Pseudomonas fluorescens* 55 at 1×10^9 CFU/ml 7 h before inoculation with the DC3000 strain at 2×10^5 CFU/ml. **C**, Bacterial populations of DC3000Δ*hopQ1-1* (Q1-1) in *Fls2*-silenced or nonsilenced plants that were inoculated with *P. fluorescens* 55 at 1×10^9 CFU/ml 7 h before inoculation with the pathogenic bacteria at 2×10^5 CFU/ml. Error bars in A, B, and C indicate $\pm$ standard error ($n = 4$). Significantly different populations based on a pairwise Student's *t* test ($P \leq 0.05$) are indicated with an asterisk.

Development of reporter genes

for assaying PTI activation in tomato and *N. benthamiana*.

Gene expression profiling studies of *Arabidopsis* cell cultures and seedlings treated with flg22 have revealed a large number of genes that are rapidly induced by this PAMP (Navarro et al. 2004). These genes encompass a wide range of functional classes, including receptor-like kinases, transcription factors, and genes involved in protein turnover (Navarro et al. 2004). Similar studies using other PAMP treatments revealed that there is a high degree of similarity in transcrip-

Fig. 2. Preinduction of pathogen-associated molecular pattern (PAMP)-triggered immunity by purified PAMPs inhibits the growth and disease symptoms of pathogenic bacteria in tomato leaves. **A**, Bacterial growth assay on tomato plants (Rio Grande-prf3) pretreated with 1 μ M flg22, peptidoglycan (PGN) at 100 μ g/ml, or buffer control (10 mM MgCl₂) by syringe infiltration 12 h prior to inoculation with DC3000Δ*avrPto*/Δ*avrPtoB* at 10^4 CFU/ml by vacuum infiltration. Bacterial populations were measured at day 0, 2, and 4 postinoculation. Significantly different population of bacteria compared with the buffer control based on a pairwise Student's *t* test is indicated with either one or two asterisks (* and ** indicate $P \leq 0.05$ and 0.001, respectively; $n = 4$). **B**, Specks were counted using Adobe Photoshop CS4. Significantly different numbers of specks per leaf area compared with the buffer control based on a pairwise Student's *t* test are indicated with either one or two asterisks (* and ** indicate $P \leq 0.05$ and 0.001, respectively; $n = 6$). Error bars in both A and B indicate $\pm$ standard error. **C**, Photographs of representative leaves from the same experiment as A, 4 days postinoculation. More specks are observable on the MgCl₂ control than with PAMP pretreatment.

tional changes that occur in response to different PAMPs such as elf26, flg22, and PGN, which indicates that some of these differentially expressed genes might be useful as reporters for PTI induction (Gust et al. 2007; Zipfel et al. 2006). In fact, recent studies with *Arabidopsis* have effectively used expression of certain genes as reporters for PTI in protoplast systems and plant tissues (Shan et al. 2008).

In order to optimize reporter genes for PTI in tomato, we used quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to examine the expression in leaves of 26 genes in response to inoculation with the type III secretion mutant DC3000 Δ hrcQ_bRSTU (referred to hereafter as DC3000 Δ hrcQU) (Supplementary Table S1). The genes tested were either tomato homologs of *Arabidopsis* genes known to be induced by PAMPs (18 genes) or tomato genes known to be induced in response to virulent or avirulent pathogens (8 genes) (Mayrose et al. 2006; Navarro et al. 2004; Thara et al. 1999). Some of these genes were used previously as markers for PTI in the interaction of

tomato cv. VF36 with *Xanthomonas campestris* pv. *vesicatoria* (Kim et al. 2009). For each gene, the calculated efficiency for primer pairs was >1.8 and product size was $\leq$ 150 bp (Udvardi et al. 2008). Melting curve analysis was performed at the end of each run to ensure that unique products were obtained. Expression of three of the tomato genes (*SlLrr22*, *SlGras2*, and *SlPti5*) was induced significantly upon inoculation with the nonpathogen *P. fluorescens* or the DC3000 Δ hrcQU mutant (Fig. 3A). *SlWrky28* was also significantly induced in similar experiments (data not shown) (Kim et al. 2009). *SlPti5* expression, in particular, was markedly enhanced during either *P. fluorescens* or DC3000 Δ hrcQU-induced PTI, with its expression being >15-fold over that of mock-inoculated leaves (Fig. 3A).

We also optimized reporter genes for use in *N. benthamiana*. In this case, qRT-PCR was used to examine the expression in leaves of five *N. benthamiana* homologs of pathogen- or PAMP-induced genes from *Arabidopsis* and tomato after inoculation with the type III secretion mutant DC3000 Δ hrcC mutant or the nonpathogen *P. fluorescens*. Expression of two of these genes, *NbPti5* and *NbGras2*, was significantly increased by either DC3000 Δ hrcC or *P. fluorescens* (Fig. 3B). We also developed primers and optimized PCR conditions to analyze the expression of *NbACRE31*, a gene previously shown to be induced by flg22 in *N. benthamiana* (Fig. 3B) (Heese et al. 2007).

Callose deposition assay in *N. benthamiana*.

Deposition of callose at the cell wall is a plant response to microbes that has been widely used as a marker for PTI in *Arabidopsis* and *N. benthamiana* (Adam and Somerville 1996; Hann and Rathjen 2007; Hauck et al. 2003). *Arabidopsis* plants with *pmr4* (callose synthase) and *npr1* or *pad4* mutations support higher growth of a *P. syringae* pv. *phaseolicola* strain expressing *hopM1*_{PtoDC3000}, suggesting that formation of callose deposits can inhibit bacterial growth (Ham et al. 2007). We compared the timing of callose formation in *Arabidopsis* and *N. benthamiana* in response to buffer only (5 mM morpholineethanesulfonic acid [MES]), flg22, DC3000, or DC3000 Δ hrcQU (Badel et al. 2006). In *Arabidopsis*, flg22 and DC3000 Δ hrcQU induced numerous callose deposits 6 h postinoculation (hpi), whereas buffer-only or wild-type DC3000 did not induce callose formation (Fig. 4A). At 12 hpi, DC3000 Δ hrcQU induced formation of twofold more callose deposits than flg22, which probably reflects the action of numerous PAMPs present in this bacterium. Flg22 and DC3000 Δ hrcQU induced callose formation to a similar degree in leaves of *N. benthamiana*, although it was not observable until later (at 24 hpi) than in *Arabidopsis*. As expected, neither buffer-only nor wild-type DC3000 caused callose formation in *N. benthamiana* (Fig. 4B).

These experiments revealed that callose deposition occurs in markedly different ways in *Arabidopsis* and *N. benthamiana*. First, flg22 and the type III secretion system (T3SS)-deficient DC3000 strain induced callose formation much faster in *Arabidopsis* than in *N. benthamiana*. Second, the number of callose deposits induced by flg22 was approximately fivefold higher in *Arabidopsis* than in *N. benthamiana*. Third, the T3SS mutant induced twofold more callose deposits in *Arabidopsis* than did flg22 12 hpi, whereas flg22 and the T3SS mutant induced similar numbers of callose deposits in *N. benthamiana*. Together, these observations raise the possibility that the role of callose deposition might be fundamentally different in these two model plants and, in particular, are consistent with the possibility of greater importance of Fls2-mediated detection of flagellin in *N. benthamiana*, which lacks the EFR pattern recognition receptor for the EF-Tu PAMP (Zipfel et al. 2006).

Fig. 3. Induction of reporter genes upon pathogen-associated molecular pattern (PAMP)-triggered immunity activation in tomato and *Nicotiana benthamiana*. **A**, Expression of reporter genes *Gras2*, *Lrr22*, and *Pti5* in tomato (Rio Grande-prf3) leaves 6 h after inoculation with the nonpathogenic bacterium *Pseudomonas fluorescens* or DC3000 Δ hrcQU (10^9 or 2×10^8 CFU/ml, respectively). Fold-induction levels compared with mock-inoculated samples, considered as 1, are shown. Data presented are the average of values from three plants (three biological replicates with three technical replicates of each) $\pm$ standard error (SE). **B**, Expression of reporter genes *Acre31*, *Pti5*, and *Gras2* in *N. benthamiana* leaves 6 h after inoculation with the nonpathogenic bacterium *P. fluorescens* or DC3000 Δ hrcC (1×10^9 or 2×10^8 CFU/ml, respectively). Fold-induction levels compared with mock-inoculated samples, considered as 1, are shown. Data presented are the average of values from five plants (five biological replicates with three technical replicates of each) $\pm$ SE. Significantly different induction levels are indicated with one or two asterisks (* and ** indicate $P \leq 0.05$ and 0.001, respectively).

Development of a *SlPti5::LUC* reporter for assaying PTI in protoplasts of tomato and *N. benthamiana*.

Plant protoplasts have been used successfully as transient expression systems to dissect signal transduction pathways in response to specific PAMPs (Asai et al. 2002; He et al. 2006; Sheen 2001). One of the features of the *Arabidopsis* protoplast system is the use of luciferase (LUC) as a reporter to quantify activation of specific gene promoters (Asai et al. 2002). *SlPti5* is a transcription factor that was identified as a Pto-interactor in a yeast two-hybrid library screen (Zhou et al. 1997). As shown above, the *SlPti5* gene is strongly induced in leaves upon inoculation with the DC3000 Δ *hrcQU* strain (Fig. 3A). The promoter region of *Pti5* has been characterized and found to contain four W boxes, a G box, and a putative TGA1-binding site (H. Nakayashiki and G. Martin, unpublished). A 1-kb fragment encompassing this upstream region was fused to a LUC reporter gene to generate a *SlPti5::LUC* reporter construct.

Tomato protoplasts were transformed with this reporter construct and a constitutively expressed β -glucuronidase (GUS) expression vector (*Cauliflower mosaic virus* [CaMV] 35S::GUS) which served as a control for successful transformation. The protoplasts were then treated with buffer only or with 100 nM flg22 and *SlPti5* promoter activity measured as a LUC/GUS ratio (GUS activity was unaffected by flg22 treatment) (data not shown). The addition of flg22 increased *SlPti5* promoter activity approximately sevenfold above the control (Fig. 5A). The T3SS effector AvrPto has been shown previously to suppress flg22-induced PTI responses in *Arabidopsis* (He et al. 2006), and we found that this effector also suppressed expression of the *SlPti5::LUC* reporter gene (Fig. 5A). We also tested the *SlPti5::LUC* construct for monitoring PTI in *N. benthamiana* protoplasts. *SlPti5* promoter activity was induced approximately threefold in *N. benthamiana* protoplasts upon exposure to flg22 and approximately twofold in response to fungal chitin (Fig. 5B).

Fig. 4. Kinetics of callose deposition in *Arabidopsis* and *Nicotiana benthamiana*. Leaves of **A**, *Arabidopsis* or **B**, *N. benthamiana* were syringe infiltrated with flg22, DC3000, DC3000 Δ *hrcQ_bRSTU* (CUCPB5113), or buffer (10 mM MgCl₂) and then assayed for callose deposition at the indicated times (hpi = hours postinoculation) using aniline blue and epifluorescence microscopy. Callose deposits of 8.5 to 85 μ m in diameter were counted in captured images using CellProfiler software. Results shown are the mean number of callose deposits per square millimeter of leaf tissue and standard deviation from 16 microscopy fields from four samples for each treatment $\pm$ standard error. Significantly different callose deposits compared with the mock inoculation based on comparisons of each pair using a Student's *t* test ($P \leq 0.05$) are indicated with an asterisk. The experiment was repeated two times for *Arabidopsis* and three times for *N. benthamiana* with similar results.

Fig. 5. Activation of *SlPti5* promoter activity by pathogen-associated molecular patterns in tomato and *Nicotiana benthamiana* protoplasts. **A**, *SlPti5* reporter gene is induced by flg22 and AvrPto suppresses this induction in tomato protoplasts; EV = empty vector. Tomato Rio Grande-prf3 protoplasts were transfected with a construct containing the *SlPti5* promoter with a luciferase (LUC) reporter (*pPti5::LUC*) and pCaMV35S: β -glucuronidase (GUS). Six hours after transfection, the protoplasts were treated with 100 nM flg22 for 3 h. **B**, *SlPti5* reporter gene is induced in *N. benthamiana* protoplasts. *N. benthamiana* protoplasts were transfected with *pPti5::LUC* and incubated for 6 h before being treated with either 100 nM flg22 or chitin at 50 μ g/ml for 3 h. Promoter activity in A and B was calculated as the LUC/GUS ratio and converted to fold-induction compared with buffer-only control samples, which were set at 1. Error bars indicate $\pm$ standard error (A, $n = 4$ and B, $n = 3$). Significantly different level of promoter activity compared with the buffer-only control based on a Tukey highly significant difference test ($P \leq 0.05$) is indicated with an asterisk.

Optimization of a MAPK activation PTI assay for tomato and *N. benthamiana* protoplasts.

In *Arabidopsis*, the MAPK AtMPK3 and AtMPK6 have been found to be specifically activated by flg22 (Asai et al. 2002). These kinases were further used to study the activation of PTI in *Arabidopsis* by specific PAMPs as well as the suppression of certain components of PTI pathways by bacterial effector proteins (He et al. 2006). We relied on these studies to modify this assay for use in tomato protoplasts. Genes encoding the tomato homologs of AtMPK3 and AtMPK6, namely SIMpk1 and SIMpk3, respectively (Holley et al. 2003; Pedley and Martin 2004), were cloned into an expression vector, pTEX, and used for protoplast transformation. Similar to their *Arabidopsis* homologs, these MAPK were activated by flg22 and their activation was suppressed by *P. syringae* pv. *tomato* T3SS effector AvrPto (Fig. 6A). In addition, *N. benthamiana* homologs of AtMPK3 and AtMPK6, NbSIPK and NbWIPK, respectively (Sharma et al. 2003), were cloned into a protoplast expression vector, pJD301 (Promega Corp., Madison, WI, U.S.A.). These MAPK were also activated by flg22 (Fig. 6B) and other PAMPs, including PGN (50 µg/ml) and chitin (50 µg/ml) (Yeaman et al. 2010) (data not shown).

In conclusion, we have presented a series of assays for measuring plant responses to PAMPs that have been optimized for tomato and *N. benthamiana*. Some of these assays have been developed in our laboratory while others were adapted and optimized from published protocols for *Arabidopsis* or *N. benthamiana*. We believe that these methods will be useful for the community studying molecular mechanisms underlying plant innate immunity. Our results also fur-

ther demonstrate that, despite many similarities, there exist differences in the nature of PTI responses among different plant species. These differences highlight the need for the development and optimization of PTI assays for other plant-microbe systems, especially those involving economically important crop species, such as members of the *Poaceae* (grass) and *Fabaceae* (legume) families.

MATERIALS AND METHODS

Virulence assays.

To prepare the bacterial inoculum, a lawn of *Pseudomonas* spp. was grown overnight at 30°C on King's medium B plates with the appropriate antibiotics. The cells were scraped from the plates into 10 mM MgCl₂ and adjusted to the desired CFU per milliliter.

Seven-week-old *N. benthamiana* plants were used for the bacterial population assays. One day before the experiment, plants were transferred to a room with continuous light, 23°C temperature, and approximately 40% relative humidity (in our experience, these conditions are optimal, but researchers should test for themselves the conditions that work best in their facilities). *P. fluorescens* at 1×10^9 CFU/ml or 10 mM MgCl₂ was infiltrated with a needleless syringe into well-expanded leaves of *N. benthamiana* and, 7 h later, DC3000 Δ hopQ1-1 or DC3000 Δ hopQ1-1/ Δ avrPto/ Δ avrPtoB at 2×10^5 CFU/ml were syringe infiltrated into an area overlapping the initial *P. fluorescens* infiltration. For tomato, PAMPs (1 µM flg22 or PGN at 100 µg/ml) were infiltrated into well-expanded leaves of 4-week-old plants (Rio Grande-prf3; RG-prf3, *Pto/Pto prf/prf*). At 12 h after inoculation, the whole plants were vacuum infiltrated with DC3000 Δ avrPto/ Δ avrPtoB (5×10^4 CFU/ml). To assess bacterial populations, three 0.79-cm² leaf discs were harvested from inoculated areas and ground in 1 ml of 10 mM MgCl₂, diluted, and plated to determine the CFU/cm² (Anderson et al. 2006). The flg22 peptide (TRLSSG LKINSKDDAAGLQIA from DC3000; >85% purity) was obtained from GenScript. The PGN, derived from *S. aureus*, was obtained from Sigma-Aldrich (no. 77140-25MG).

For enumeration of speck numbers, photographs of individual detached leaves from each treatment (4 days after inoculation) were analyzed using Adobe Photoshop CS4. Speck numbers were counted manually using the Count Tool. The leaf area was calculated for each leaf using the Analysis Tool. It is important to align a ruler to each leaf when taking the photographs in order to account for any differences in magnification that may occur during the analysis.

VIGS.

VIGS was performed as previously described (Velasquez et al. 2009). All *N. benthamiana* plants were kept in a growth chamber with a 16-h day length and 50% relative humidity before they were used for the assays.

Protoplast isolation and protoplast-based assays.

Tomato RG-prf3 or *N. benthamiana* leaves, 3 to 4 weeks old, which were fully expanded and not too thick, were used for isolation of protoplasts. The abaxial side of leaflets was rubbed gently with Carborundum and serrated with a sharp razor blade without cutting through the leaflet. The leaflets were rinsed with water before placing their abaxial sides down in a petri dish containing 15 ml of the enzyme solution (recipe below). After an overnight (for tomato) or 3-h (for *N. benthamiana*) incubation at 30°C in the dark, protoplasts were released from the leaflets by gently swirling the plate by hand. The mixture was poured through a 45- to 100-µm sieve to collect protoplasts that were then divided (7.5 ml)

Fig. 6. Activation of mitogen-activated protein kinases (MAPK) by flg22 in tomato and *Nicotiana benthamiana* protoplasts. **A**, Activation of MAPK in tomato protoplasts by flg22 and suppression of MAPK activity by *Pseudomonas syringae* pv. *tomato* effector AvrPto. Hemagglutinin (HA)-tagged AvrPto or an empty vector (EV) control plasmid was co-expressed with HA-tagged SIMPK1 or SIMPK3 in tomato Rio Grande-prf3 protoplasts. Transfected protoplasts were incubated for 6 h before treatment with 100 nM flg22 for 10 min. Anti-HA antibodies were used for the immunoprecipitation of SIMPK1/3 and AvrPto. In vitro kinase assay was used to detect kinase activity (upper panel). Lower panel shows a blot for protein expression of SIMPK1/3 and AvrPto using anti-HA antibodies. **B**, Activation of MAPK activity in *N. benthamiana* protoplasts. HA-tagged NbSIPK or NbWIPK was expressed in *N. benthamiana* protoplasts. Transfected protoplasts were subjected to similar treatments and assay as described above.

into two 15-ml culture tubes. Viable protoplasts were isolated by sucrose gradient centrifugation as follows. The protoplast mixture was gently overlaid with 1 ml of W5 solution (recipe below) and centrifuged without mixing at $400 \times g$ for 3 min (0 brake and 0 acceleration). The layer containing viable green protoplasts was transferred to a new tube (the gradients are as follows, from top to bottom: W5, viable green protoplasts, brownish green protoplasts, and a pellet of dead protoplasts and cell debris). Viable protoplasts were washed once with fresh W5 and then allowed to recover on ice in fresh W5 for at least 1.5 h in the dark with good aeration (protoplasts in the solution were gently suspended every 15 min to prevent pellet formation). Protoplasts were quantified using a hemacytometer under a light microscope. After the incubation period, protoplasts were collected by centrifugation ($100 \times g$ for 1 min) and resuspended in MMG buffer (recipe below) at a density of 2×10^5 protoplasts/ml. Protoplasts were maintained on ice (up to 3 h). Polyethylene-glycol mediated transformation of protoplasts was performed as described by He and associates (2006) (or on the website of the Jen Sheen laboratory). Six hours after transformation, PAMP treatments were performed for 10 min (MAPK suppression assay) or 3 h (LUC assay) before collecting the protoplasts by centrifugation. The treated protoplasts were kept at -80°C until use for the assays.

Recipes.

Protoplast isolation medium: K3 + 0.4 M sucrose + 1% cellulase + 0.15% macarozyme

For 1 liter: K3 (100 ml of stock I, 5 ml of stock II, 2 ml of stock III, 10 ml of stock IV, and 10 ml of stock V), 136.8 g of sucrose, 3 g of cellulase R10, and 1.5 g of macarozyme.

Stock I (1 liter): 1.5 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 9.0 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 g of KNO_3 , 2.5 g of NH_4NO_3 , and 1.3 g of $(\text{NH}_4)_2\text{SO}_4$.

Stock II consisted of 1% EDTA ferric sodium salt.

Stock III (1 liter) consisted of 12.5 mg of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ and 12.5 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$.

Stock IV (1 liter) consisted of 75 mg of KI, 1 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 200 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 300 mg of H_3BO_3 , 25 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

Stock V (1 liter) consisted of 1 g of Thiamine HCl, 100 mg of nicotinic acid, 100 mg of pyridoxine HCl, 10 g of m-inositol, and 25 g of xylose.

W5 (1 liter): 9.0 g of NaCl, 1.0 g of glucose, 18.4 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.4 g of KCl.

MMG buffer: 0.4 M mannitol, 15 mM MgCl_2 , and 4 mM MES (pH 5.7).

Promoter reporter, MAPK, and AvrPto constructs.

Each hemagglutinin-tagged *SIMP*K gene was expressed in protoplasts using the pTEX CaMV 35S promoter expression cassette. *NbSIPK/WIPK* and the AvrPto gene were expressed from an enhanced CaMV 35S promoter in vector pJD301 (Promega Corp.). In total, 10 μg of pTEX::*SIMP*Ks or 7 μg of pJD301 plasmid was used in each transformation. Full-length *SIPti5* promoter was subcloned into *pFRK1::LUC* nos c vector using *Bam*HI (5') and *Nco*I (3') sites, which remove the entire *FRK1* cassette. The recombinant *pSIPti5::LUC* nos c (10 μg) was used in each transformation.

MAPK assay.

Activation and suppression of PAMP-induced MAPK in tomato and *N. benthamiana* protoplasts was performed as described by He and associates (2006). The amount of flg22 used was 100 nM.

LUC assay.

LUC activity assays were performed as described by He and associates (2006) using the Luciferase Assay System kit (Promega Corp.). LUC activity was calculated as the ratio of LUC/GUS.

Callose deposition assay.

Callose deposition assays for *Arabidopsis* and *N. benthamiana* were modified as follows from previously described procedures (Ham et al. 2007; Hann and Rathjen 2007; Hauck et al. 2003). Leaves of 6- and 8-week-old *N. benthamiana* and *Arabidopsis* ecotype Columbia plants, respectively, were infiltrated with 40 μM flg22 or a bacterial suspension of 2.2×10^8 CFU/ml with a needleless syringe. After 6, 12, and 24 h, leaf disks from infiltrated areas were excised with a cork borer from *Arabidopsis* (5 mm in diameter) and *N. benthamiana* (10 mm in diameter). The collected leaf disks were placed in wells of a 12-well microtiter plate containing 2 ml of 95% ethanol and then incubated at 37°C until the leaf disks were cleared of chlorophyll, with the ethanol being replaced as necessary until the clearing was complete. The incubation time for leaf clearing was 3 h for *Arabidopsis* and 6 h for *N. benthamiana*. The cleared leaf disks were washed two times with 70% ethanol and then three times with distilled water. The leaf disks were then placed in the wells of a 12-well plate and immersed in 1% aniline blue in 150 mM K_2HPO_4 (pH 9.5/KOH) and incubated in the dark 30 min for *Arabidopsis* and 1 h for *N. benthamiana*. The stained leaf disks were mounted with 60% glycerol on glass slides and observed from the adaxial surface of the disk by epifluorescence microscopy using ultraviolet light. Images of 1-mm² microscopy fields, avoiding areas close to wounds generated by inoculation or by sample excision, were captured using an Olympus digital camera and its software (DP2-BSW). The numbers of callose deposits of 8.5 to 85 μm in diameter, detected by aniline blue fluorescence, were counted in complete fields using CellProfiler cell image analysis software.

qRT-PCR for marker gene expression.

Four-week-old RG-prf3 tomato plants were vacuum infiltrated with a suspension of DC3000 Δ *hrcQU* at 2×10^8 CFU/ml in 10 mM MgCl_2 containing 0.002% Silwet or *P. fluorescens* at 1×10^9 CFU/ml, or mock inoculated with 10 mM MgCl_2 containing 0.002% Silwet. Five-week-old *N. benthamiana* plants were syringe infiltrated on one half of a well-expanded leaf with a suspension of either DC3000 Δ *hrcC* or *P. fluorescens* at 2×10^8 CFU/ml in 10 mM MgCl_2 . As a control, 10 mM MgCl_2 was syringe infiltrated on the other half of the leaf. Leaf tissue was harvested at 6 h after infiltration for tomato and *N. benthamiana*. Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.) and treated with RQ1 DNase (Promega Corp.), followed by column purification using the RNeasy mini kit (Qiagen). One microgram was used to prepare cDNA using Superscript III and oligo dT primer (Invitrogen, Carlsbad, CA, U.S.A.). qRT-PCR was performed using 150 to 400 nM sequence-specific primers and SYBR Green (Applied Biosystems Inc, Foster City, CA, U.S.A.) with the ABI Prism 7900 HT Sequence Detection System. Primers were designed using the PrimerQuest software of IDT (Integrated DNA Technologies, Coralville, IA, U.S.A.). The efficiency of all primer pairs used was calculated to be ≥ 1.8 using the formula $10^{(-1/\text{slope})}$ (Pfaffl 2001).

A mock cDNA reaction lacking Superscript III was performed for each RNA sample and checked in a qRT-PCR reaction with ATPase control primers to check for DNA contamination. Cycling conditions during qRT-PCR were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C

for 1 min. Each reaction was performed in triplicate. Dissociation curve analysis was performed at the end of each run to ensure that unique products were amplified. Data was normalized to the ATPase control gene. Fold-induction of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Pfaffl 2001). The ΔC_t (cycle threshold) values for gene-of-interest (buffer-pathogen) and the ATPase control (buffer-pathogen) were calculated for all samples and used to compute the significance of induction levels observed. A pairwise Student's *t* test was performed to obtain the *P* values indicated in the figure. The plasmids and bacterial strains described in this article are available as a consolidated "Solanaceae PTI assay kit".

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CellProfiler, cell image analysis software: www.cellprofiler.org
 GenScript website: www.genscript.com
 SOL Genomics Network (SGN): solgenomics.net
 Sheen Lab, Massachusetts General Hospital and Harvard Medical School: genetics.mgh.harvard.edu/sheenweb
 Tomato Genetics Resource Center: tgrc.ucdavis.edu