Multiple *Xanthomonas euvesicatoria* Type III Effectors Inhibit flg22-Triggered Immunity

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Xanthomonas euvesicatoria is the causal agent of bacterial spot disease in pepper and tomato. *X. euvesicatoria* bacteria interfere with plant cellular processes by injecting effector proteins into host cells through the type III secretion (T3S) system. About 35 T3S effectors have been identified in *X. euvesicatoria* 85-10, and a few of them were implicated in suppression of pattern-triggered immunity (PTI). We used an *Arabidopsis thaliana* pathogen-free protoplast-based assay to identify *X. euvesicatoria* 85-10 effectors that interfere with PTI signaling induced by the bacterial peptide flg22. Of 33 tested effectors, 17 inhibited activation of a PTI-inducible promoter. Among them, nine effectors also interfered with activation of an abscisic acid-inducible promoter. However, effectors that inhibited flg22-induced signaling did not affect phosphorylation of mitogen-activated protein (MAP) kinases acting downstream of flg22 perception. Further investigation of selected effectors revealed that XopAJ, XopE2, and XopF2 inhibited activation of a PTI-inducible promoter by the bacterial peptide elf18 in *Arabidopsis* protoplasts and by flg22 in tomato protoplasts. The effectors XopF2, XopE2, XopAP, XopAE, XopH, and XopAJ inhibited flg22-induced callose deposition in planta and enhanced disease symptoms caused by attenuated *Pseudomonas syringae* bacteria. Finally, selected effectors were found to localize to various plant subcellular compartments. These results indicate that *X. euvesicatoria* bacteria utilize multiple T3S effectors to suppress flg22-induced signaling acting downstream or in parallel to MAP kinase cascades and suggest they act through different molecular mechanisms.

Plants utilize a complex detection and signaling network for early recognition of invading microbes and activation of immune responses (Jones and Dangl 2006). Pathogen recognition receptors (PRRs) localized on the external surface of the plant cell detect conserved pathogen-associated molecular patterns (PAMPs), such as fungal chitin or bacterial flagellin, elongation factor-Tu, lipopolysaccharides, and peptidoglycan (Macho and Zipfel 2014). Among the best characterized PRRs is the flagellin-sensing 2 (FLS2) receptor from *Arabidopsis thaliana*, a receptor like-kinase (RLK) with an extracellular leucine-rich domain (Gómez-Gómez and Boller 2000). FLS2 recognizes bacterial flagellin or its 22-amino acid peptide derivative flg22

in many plant species, including *Arabidopsis*, tomato, tobacco, and potato, but not in rice (Felix et al. 1999). PRRs are part of multiprotein complexes that include the receptor, coreceptors, regulatory proteins, and molecular components involved in intracellular signaling (Böhm et al. 2014; Macho and Zipfel 2014). Recognition of PAMPs by PRR complexes often results in phosphorylation and activation of receptor-like cytoplasmic kinases (RLCK) (Lin et al. 2013; Macho and Zipfel 2014). Downstream cellular events include ion fluxes, activation of mitogen-activated protein (MAP) kinase cascades and calcium-dependent protein kinases, production and release of reactive oxygen species, callose deposition at the cell wall, and accumulation of pathogenesis-related proteins (Boller and Felix 2009). Collectively, these immune responses are referred to as pattern-triggered immunity (PTI) and mediate plant resistance against a broad spectrum of microbial pathogens (Zhang and Zhou 2010).

A prerequisite for plant pathogens to colonize their host is the suppression of PTI. Many phytopathogenic gram-negative bacteria achieve this by injecting effector proteins into the plant cell through the type III secretion (T3S) system (Galán et al. 2014). Once inside the plant cell, T3S effectors manipulate important hubs of immune signaling making the host vulnerable to infection (Feng and Zhou 2012; Macho and Zipfel 2015). Several T3S effectors directly interfere with PRRs and their coreceptors to suppress PTI. For example, the *Pseudomonas syringae* pv. *tomato* AvrPtoB effector is a multidomain protein that acts both as E3 ligase to ubiquitinate and degrade PRRs and as an inhibitor of kinase activity of PRRs and their coreceptor BAK1 (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Shan et al. 2008; Zeng et al. 2012). PRR-associated RLCK and immunity-associated MAP kinase cascades are additional important targets for effectors that suppress PTI. Among the effectors that target RLCKs is AvrPphB from *P. syringae* pv. *phaseolicola*, which functions as a cysteine protease and cleaves multiple members of the BIK1 family, which are direct substrates of several PRRs (Zhang et al. 2010). A strategy used to interfere with PTI-associated MAP kinase cascades is exemplified by the action of the HopAI1 effector from *P. syringae* pv. *tomato*, which inactivates the MAP kinases MPK3, MPK4, and MPK6, through its phosphothreonine lyase activity (Zhang et al. 2007). Effectors can also suppress PTI by interfering with plant vesicle trafficking and protein secretion pathways that are required for secretion of immunity-associated molecules and for transport of immune receptors to the cell surface. For example, *Xanthomonas euvesicatoria* XopJ interferes with protein secretion to inhibit immunity-associated callose deposition at the cell wall (Bartetzko et al. 2009).

X. euvesicatoria is the causal agent of spot disease in pepper and tomato plants and its pathogenicity depends on the T3S system (Jones et al. 1998). To date, the pool of *X. euvesicatoria* T3S

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effectors includes about 35 proteins (Roden et al. 2004; Teper et al. 2016; Thieme et al. 2005). Several *X. euvesicatoria* effectors were reported to contribute to bacterial virulence by interfering with various PTI-associated responses (Bartetzko et al. 2009; Kim et al. 2008, 2009; Schulze et al. 2012; Singer et al. 2013; Stork et al. 2015). However, cellular targets and mechanisms of PTI inhibition are known for only a limited number of *X. euvesicatoria* effectors. The XopJ effector targets a proteasome subunit to suppress salicylic acid-mediated defense and protein secretion (Bartetzko et al. 2009; Ustün et al. 2013). The XopN effector targets the tomato 14-3-3 protein TFT1 to suppress PTI signaling (Kim et al.

2009), whereas the XopD effector encodes a SUMO protease that inhibits host transcription to suppress defense hormone signaling (Canonne et al. 2011; Kim et al. 2013).

In this study, we used *Arabidopsis* mesophyll protoplasts, an established pathogen-free cell system for transient gene expression analysis (Fraiture et al. 2014; Schulze et al. 2012; Shan et al. 2008; Yoo et al. 2007), to identify the entire pool of *X. euvesicatoria* 85-10 T3S effectors that interfere with flg22 defense signaling. We identified 17 *X. euvesicatoria* effectors that inhibited the activation of a flg22-responsive promoter acting downstream or in parallel to MAP kinase posttranslational activation. Eight of these effectors only affected flg22-induced gene expression, while nine others also interfered with abscisic acid (ABA)-dependent gene expression. Subcellular localization studies and functional characterization for a group of inhibitors of flg22 signaling support the conclusion that they contribute to bacterial virulence by targeting different steps or pathways of PTI signaling.

RESULTS

A large group of *X. euvesicatoria* 85-10 T3S effectors inhibits flg22-inducible reporter gene activation in *Arabidopsis* protoplasts.

Suppression of plant immunity is a prerequisite for phytopathogenic bacteria to mount a successful infection in the host (Feng and Zhou 2012). To identify *X. euvesicatoria* T3S effectors that suppress PTI, we utilized an *Arabidopsis* leaf protoplast assay, a well-established system for PAMP signaling studies (Fraiture et al. 2014) and examined the ability of 33 effectors of *X. euvesicatoria* 85-10 (Supplementary Table S1) to inhibit activation of gene expression induced by the bacterial peptide flg22 (Asai et al. 2002). To this aim, each effector was transiently expressed in *Arabidopsis* mesophyll protoplasts and was tested for its ability to inhibit activation of the flg22-inducible *FRK1:LUC* promoter fused to the firefly luciferase gene (*pFRK1:LUC*) after application of flg22. Seven *X. euvesicatoria* effectors (XopX, XopL, XopD, XopS, XopB, XopN, and XopJ), which were previously reported to suppress PTI-associated responses (Bartetzko et al. 2009; Kim et al. 2008, 2009; Schulze et al. 2012; Singer et al. 2013; Stork et al. 2015), were tested again in this screen. AvrPto from *P. syringae* pv. *tomato* DC3000 was used as an additional positive control (He et al. 2006), while an empty vector was used as a negative control. Of the 33 effectors tested, 17 significantly reduced flg22-mediated activation of the *pFRK1:LUC* reporter gene (Fig. 1). These included six effectors (XopX, XopL, XopD, XopS, XopB, and XopN) that were already reported to interfere with PTI responses and 11 additional PTI inhibitor candidates (XopF2, XopM, XopAJ, XopV, XopAP, XopZ, XopAE, XopE2, XopH, XopF1, and XopI). Four effectors (XopK, XopE1, XopC, and XopJ3) increased flg22-mediated activation of the *pFRK1:LUC* reporter, while 12 other effectors (XopG, AvrBs1, XopAD, XopR, AvrBs2, XopP, XopAK, XopQ, XopJ, XopAW, XopAA, and XopO) did not display any significant effect (Fig. 1).

Next, we tested whether the 17 *X. euvesicatoria* effectors that reduced flg22-mediated activation of the *pFRK1:LUC* reporter only inhibit transcription of PTI-associated genes or also affect expression of genes involved in other cellular processes. To this aim, each effector was transiently expressed in *Arabidopsis* mesophyll protoplasts and was tested for its ability to inhibit activation of the ABA-inducible *RD29b* promoter (Christmann et al. 2005) fused to the luciferase gene (*pRD29b:LUC*). As shown in Figure 2, eight effectors (XopZ, XopH, XopAE, XopE2, XopF1, XopAP, XopB, and XopAJ) did not inhibit activation of the *pRD29b:LUC* reporter by ABA. The other nine effectors (XopF2, XopV, XopS, XopI, XopL, XopN, XopD, XopM and XopX) significantly inhibited activation of both

Fig. 1. Inhibition of flg22-inducible reporter gene activation by *Xanthomonas euvesicatoria* 85-10 type III secretion effectors. *Arabidopsis* mesophyll protoplasts were cotransformed with plasmids for the expression of the indicated effector or an empty vector (EV), the reporter gene *pFRK1:LUC*, and the normalizer gene *pUBQ10:GUS*. Twelve hours after transformation, protoplasts were treated with flg22 or water. Luciferase activity was measured for 8 h after flg22 treatment at 2-h intervals and was normalized by β -glucuronidase activity in flg22-treated and untreated samples. For each effector, induction of the reporter gene *pFRK1:LUC* by flg22 was calculated relative to that observed for the EV control (18.7 ± 2.6). Bars represent means $\pm$ standard error of at least three independent experiments measured at the time point of maximal induction. Asterisks indicate a significant difference (Student's *t* test, *P* value < 0.05) between the tested effector and the EV control.

pFRK1:LUC and *pRD29b:LUC* reporter genes by the respective inducer (Figs. 1 and 2). These results suggest that *X. euvesicatoria* bacteria employ a large part of their T3S effector repertoire to inhibit flg22 defense signaling either selectively or by simultaneously affecting additional cellular processes.

X. euvesicatoria T3S effectors interfere with flg22-induced signaling downstream of MPK3 and MPK6 activation.

The *Arabidopsis* MAP kinases MPK3 and MPK6 have been shown to participate in early flg22 signaling and to be activated by phosphorylation (Asai et al. 2002). Thus, we tested whether *X. euvesicatoria* effectors that inhibit activation of the *pFRK1:LUC* reporter interfere with flg22 signaling upstream or downstream of MPK3 and MPK6 activation. To this aim, hemagglutinin (HA)-tagged MPK3 or MPK6 were cotransfected into protoplasts with individual effectors and MPK3 or MPK6 activation by flg22 treatment was monitored by Western blot analysis using p44/42 MAP kinase antibodies, which specifically recognize phosphorylated forms of MAP kinases. An empty vector, the green fluorescent protein (GFP), and the *P. syringae* pv. *tomato* AvrPto effector, which was previously shown to

suppress flg22-induced activation of MPK3 and MPK6 by interfering with the activity of the FLS2 receptor (Xiang et al. 2008), were used as controls in these experiments. The XopX and XopM effectors were found to significantly inhibit general protein expression (data not shown) and were not included in this analysis. As shown in Figure 3, flg22 activated MPK3 and MPK6 phosphorylation in protoplasts transfected with an empty vector, while AvrPto suppressed this activation. Strikingly, none of the 15 *X. euvesicatoria* tested effectors affected MPK3 or MPK6 phosphorylation (Fig. 3, for XopF1; Supplementary Fig. S1, for the other effectors) suggesting that they inhibit flg22-induced reporter gene activation by affecting signaling mechanisms downstream or in parallel to MAP kinase activation.

Validation of the biological relevance of the *Arabidopsis* protoplast system for the identification of *X. euvesicatoria* effectors that suppress PTI.

Despite the fact that *Arabidopsis* is a *X. euvesicatoria* nonhost plant, we chose the *Arabidopsis* protoplast system to identify *X. euvesicatoria* effectors that suppress PTI because this system is much more robust than its tomato counterpart and is highly responsive to flg22 elicitation. The choice of *Arabidopsis* as the model plant and the PAMP flg22 as the inducer of PTI signaling

Fig. 2. Inhibition of abscisic acid (ABA)-inducible reporter gene activation by *Xanthomonas euvesicatoria* 85-10 type III secretion effectors. *Arabidopsis* mesophyll protoplasts were cotransformed with plasmids for the expression of the indicated effector or an empty vector (EV), the reporter gene *pRD29b:LUC*, and the normalizer gene *pUBQ10:GUS*. Twelve hours after transformation, protoplasts were treated with ABA or a mock solution. Luciferase activity was measured for 8 h after ABA treatment at 2-h intervals and was normalized by β -glucuronidase activity in treated and untreated samples. For each effector, induction of the reporter gene *pRD29b:LUC* by ABA was calculated relative to that observed for the EV control (26.8 ± 3.6). Bars represent means $\pm$ standard error of at least three independent experiments at the time point of maximal induction. Asterisks indicate a significant difference (Student's *t* test, *P* value < 0.05) between the tested effector and the EV control.

Fig. 3. The XopF1 effector does not affect activation of mitogen-activated protein (MAP) kinase. *Arabidopsis* protoplasts were cotransformed with plasmids for the expression of **A**, hemagglutinin (HA)-tagged MPK3 or **B**, MPK6, and XopF1, AvrPto, green fluorescent protein (GFP), or an empty vector (EV). Twelve hours later, protoplasts were treated with flg22 (+) or mock (–) and were harvested after 5 min. MAP kinase phosphorylation and expression of HA-tagged MAP kinases were monitored by immunoblot analysis using α :p44/42 MAP kinase (α :P-MAPK) and α :HA antibodies, respectively. Coomassie staining served as loading control. The experiment was repeated three times with similar results.

was based on the assumptions that i) *X. euvesicatoria* effectors target components of PTI signaling pathways that are conserved in *Arabidopsis* and tomato plants and ii) components of PTI signaling targeted by *X. euvesicatoria* effectors rely on the signal activated by recognition of flg22 and other PAMPs as well. To confirm the correctness of the first assumption, we tested whether *X. euvesicatoria* effectors that inhibited flg22-induced gene expression in *Arabidopsis* protoplasts have a similar effect in tomato protoplasts. To verify the second assumption, we tested whether they inhibit gene expression induced by the bacterial PAMP elf18 (Kunze et al. 2004) in *Arabidopsis* protoplasts. This analysis included six genes encoding effectors that inhibited reporter gene activation induced by flg22 but not by ABA (i.e., XopAP, XopF1, XopAJ, XopAE, XopH, and XopE2), the XopF2 effector, which was a very strong inhibitor of flg22-induced reporter gene activation but a weak inhibitor of ABA-induced gene expression, and *P. syringae* pv. *tomato* AvrPto as a positive control. Four of the seven *X. euvesicatoria* effectors tested (i.e., XopF2, XopE2, XopAE, and XopAJ) and AvrPto significantly inhibited flg22-induced activation of the *pFRK1:LUC* reporter gene in tomato protoplasts (Fig. 4). In addition, three *X. euvesicatoria* effectors (i.e., XopF2, XopE2, and XopAJ) and AvrPto significantly reduced elf18-induced activation of the *pFRK1:LUC* reporter gene in *Arabidopsis* protoplasts (Fig. 5). These results confirm that the *Arabidopsis* protoplast system in combination with flg22 elicitation is reliable for the identification and further characterization of *X. euvesicatoria* effectors that suppress PTI.

XopF2, XopE2, XopAP, XopAE, XopH, and XopAJ decrease callose deposition at the cell wall and enhance disease symptoms.

To evaluate the contribution of XopF1, XopF2, XopE2, XopAP, XopAE, XopH, and XopAJ to *X. euvesicatoria* 85-10 virulence, we inactivated the corresponding genes by deletion

Fig. 4. Inhibition of flg22-inducible reporter gene activation by *Xanthomonas euvesicatoria* 85-10 type III secretion effectors in tomato. Tomato mesophyll protoplasts were cotransformed with plasmids for the expression of the indicated effector or an empty vector (EV), the reporter gene *pFRK1:LUC*, and the normalizer gene *pUBQ10:GUS*. Twelve hours after transformation, protoplasts were treated with flg22 or water. Luciferase activity was measured for 6 h after flg22 treatment at 2-h intervals and was normalized by average β -glucuronidase activity in flg22-treated and untreated samples. For each effector, induction of the reporter gene *pFRK1:LUC* by flg22 was calculated relative to that observed for the EV control (1.8 ± 0.1). Bars represent means $\pm$ standard error of at least three independent experiments measured at the time point of maximal induction. Asterisks indicate a significant difference (Student's *t* test, *P* value < 0.05) between the tested effector and the EV control.

or insertion mutagenesis. The mutant strains were used to infect susceptible pepper plants by syringe-infiltration at a titer of 1×10^5 CFU/ml. Bacterial growth of the mutant strains was monitored for 8 days in the infected plants, but it did not differ from that observed for wild-type bacteria (Supplementary Fig. S2).

To circumvent the possibility that the contribution of the effectors to *X. euvesicatoria* virulence is too subtle to be detected in the original bacterial context or is masked by functional redundancy, we introduced the effectors into the attenuated *P. syringae* pv. *tomato* DC3000 Δ CEL, which lacks the AvrE and HopM1 effectors encoded by the conserved effector locus (CEL) and contains a different effector repertoire than *X. euvesicatoria* (Alfano et al. 2000). This strain fails to suppress callose deposition at the cell wall in *Arabidopsis* plants (DebRoy et al. 2004), a defense response triggered by PAMPs in infected host leaves, and it is less virulent than the wild-type *P. syringae* pv. *tomato* DC3000 in tomato plants (Badel et al. 2003). Individual *X. euvesicatoria* effectors introduced into *P. syringae* pv. *tomato* DC3000 Δ CEL strains were tested for their effect on callose deposition in *Arabidopsis* and on bacterial growth and development of disease symptoms in tomato. For their expression in *P. syringae* pv. *tomato* Δ CEL and translocation into host cells, the *X. euvesicatoria* effectors were fused downstream of the T3S translocation signal sequence of the *P. syringae* pv. *tomato* effector AvrRpt2 (located between amino acids 1 and 100; AvrRpt2₁₋₁₀₀ [Kim et al. 2009]). The *P. syringae* pv. *tomato* effector HopM1, which was previously shown to suppress PTI in *Arabidopsis* (DebRoy et al. 2004) and to contribute to *P. syringae* pv. *tomato* virulence in tomato by enhancing disease symptoms without affecting bacterial growth (Badel et al. 2003), was also introduced in the *P. syringae* pv. *tomato* DC3000 Δ CEL strain and served as a positive control in these experiments. To test the effect of

Fig. 5. Inhibition of elf18-inducible reporter gene activation by *Xanthomonas euvesicatoria* 85-10 type III secretion effectors. *Arabidopsis* mesophyll protoplasts were cotransformed with plasmids for the expression of the indicated effector or an empty vector (EV), the reporter gene *pFRK1:LUC*, and the normalizer gene *pUBQ10:GUS*. Twelve hours after transformation, protoplasts were treated with elf18 or water. Luciferase activity was measured for 8 h after elf18 treatment at 2-h intervals and was normalized by β -glucuronidase activity in elf18-treated and untreated samples. For each effector, induction of the reporter gene *pFRK1:LUC* by elf18 was calculated relative to that observed for the EV control (8.8 ± 2.5). Bars represent means $\pm$ standard error of at least three independent experiments measured at the time point of maximal induction. Asterisks indicate a significant difference (Student's *t* test, *P* value < 0.05) between the tested effector and the EV control.

effectors on callose deposition, *Arabidopsis* Col-0 leaves were hand-infiltrated with a suspension (2×10^8 CFU/ml) of *P. syringae* pv. *tomato* Δ CEL expressing HopM1, AvrRpt2₁₋₁₀₀, or AvrRpt2₁₋₁₀₀-effector fusions and were stained for callose after 16 h. As expected, *Arabidopsis* leaves infected with *P. syringae* pv. *tomato* Δ CEL expressing the HopM1 effector had fewer callose deposits as compared with leaves infiltrated with *P. syringae* pv. *tomato* Δ CEL expressing AvrRpt2₁₋₁₀₀ (Fig. 6). Similarly, fewer callose deposits accumulated in leaves infected with *P. syringae* pv. *tomato* Δ CEL expressing all the tested AvrRpt2₁₋₁₀₀-effector fusions except for AvrRpt2₁₋₁₀₀-XopF1 (Fig. 6).

Next, to test the contribution of the *X. euvesicatoria* effectors to *P. syringae* pv. *tomato* Δ CEL virulence, tomato plants were infiltrated with a mock solution or with a suspension (1×10^5 CFU/ml) of *P. syringae* pv. *tomato* Δ CEL bacteria expressing HopM1, AvrRpt2₁₋₁₀₀, or an AvrRpt2₁₋₁₀₀-effector fusion and development of disease symptoms and bacterial growth were monitored for 3 days postinoculation (dpi). *P. syringae* pv. *tomato* Δ CEL expressing HopM1, AvrRpt2₁₋₁₀₀ fused to XopF2, XopE2, XopAP, XopAE, XopH, and XopAJ, but not XopF1 caused more severe disease symptoms than *P. syringae* pv. *tomato* Δ CEL expressing AvrRpt2₁₋₁₀₀ (Fig. 7A and B). Despite the effect of the tested effectors on disease symptoms, bacterial populations were similar for all the strains at 3 dpi (Supplementary Fig. S3). As assessed by Western blot analysis (Supplementary Fig. S4), HopM1 and the XopF1, XopF2, XopE2, XopAP, and XopAJ effectors fused to AvrRpt2₁₋₁₀₀ were detected and expressed at different levels in *P. syringae* pv. *tomato* Δ CEL strains, while expression of the XopAP and XopH effectors fused to AvrRpt2₁₋₁₀₀ was below detection levels. These results confirmed that effectors that inhibited flg22- but not ABA-inducible reporter gene activation in protoplasts act as PTI inhibitors in planta and contribute to development of disease symptoms.

Fig. 6. Inhibition of flg22-induced callose deposition by *Xanthomonas euvesicatoria* type III secretion effectors in *Arabidopsis* plants. *Arabidopsis* leaves were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 Δ CEL strains (1×10^8 CFU/ml) expressing the indicated *X. euvesicatoria* effectors or an empty vector (EV). Sixteen hours after inoculation, leaves were cleared and stained with aniline blue, and callose deposits were visualized in four fields (0.6 mm^2) by fluorescence microscopy and were counted. In each experiment, the number of callose deposits counted in mock-inoculated leaves was used as background and was subtracted from the mean of each treatment. Callose deposition is reported as the percentage of that observed in leaves inoculated with the *P. syringae* pv. *tomato* DC3000 Δ CEL strain carrying an EV (172.4 ± 13.2 deposits/ 0.6 mm^2). Data are the means $\pm$ standard error of at least three independent biological replicates. Asterisks indicate statistically significant differences (Student's *t* test, *P* value < 0.05) between the tested effectors and the EV control.

X. euvesicatoria effectors that inhibited flg22 defense signaling localize to different subcellular compartments.

The subcellular localization of effectors in the host may provide important clues about the effector molecular functions. To investigate whether *X. euvesicatoria* effectors that suppress

Fig. 7. The XopF2, XopE2, XopAP, XopAE, XopH and XopAJ effectors contribute to disease symptoms caused by *Pseudomonas syringae* pv. *tomato* DC3000 Δ CEL bacteria in tomato plants. Tomato leaves were mock inoculated (Mock) or vacuum-infiltrated with suspensions (1×10^5 CFU/ml) of *P. syringae* pv. *tomato* DC3000 Δ CEL strains expressing an empty vector (EV), **A**, the *P. syringae* pv. *tomato* effector HopM1, or **B**, the indicated *Xanthomonas euvesicatoria* effectors. Inoculated leaves were monitored for the appearance of disease symptoms and photographed at 3 days postinoculation. The experiment was repeated three times with similar results.

PTI target similar or different molecular mechanisms, we determined subcellular localization for a group of *X. euvesicatoria* effectors that were identified in this study as inhibitors of flg22 defense signaling. In this group were the XopF1, XopAJ,

Fig. 8. Subcellular localization of the XopAE, XopH, XopF1, XopAJ, and XopF2 effectors. *Agrobacterium* strains expressing the indicated effectors fused to the N-terminus of yellow fluorescent protein (YFP) were infiltrated in leaves of *Nicotiana benthamiana* plants. In the upper panel, the fluorescence (YFP), light transmitted, and merged images are shown for each effector; in the lower panel, fluorescence distribution of XopF2-YFP and G-ck, a cyan fluorescent protein (CFP) marker of the Golgi apparatus. YFP, CFP, and merged fluorescence images are shown. Distribution of YFP fluorescence was determined at 24 h (for YFP, XopAE, XopH, and XopF1, whose expression is driven by the 35S *Cauliflower mosaic virus* promoter) or 36 h (XopAJ, XopF2, and G-ck, whose expression is driven by the pER8 system) postinoculation, by confocal microscopy. Scale bars in each panel represent 20 μ m.

XopAE, and XopH effectors that inhibited reporter gene activation induced by flg22 but not by ABA and the XopF2 effector, which is a very strong inhibitor of flg22-induced reporter gene activation but a weak inhibitor of ABA-induced gene expression. The yellow fluorescent protein (YFP) was fused to the C-terminus of the effectors and expression of the fusion proteins was driven either by the *Cauliflower mosaic virus* (CaMV) 35S promoter (for XopF1, XopAE, and XopH) or by an estradiol-inducible expression system (for XopF2 and XopAJ). The obtained fusion proteins and a YFP control were transiently expressed via *Agrobacterium tumefaciens* in leaves of *N. benthamiana* plants, and their subcellular localization in lower epidermal cells was determined by confocal laser scanning microscopy. YFP, XopAE-YFP, and XopH-YFP showed a similar subcellular localization, and their fluorescence was detectable in the cytoplasm and in the nucleus (Fig. 8, upper panel). Fluorescence of XopF1-YFP and XopAJ-YFP was detected in the cytoplasm but not in the nucleus (Fig. 8, upper panel). To confirm cytoplasmic localization for XopAE-YFP, XopH-YFP, XopF1-YFP, and XopAJ-YFP, the effector fusions were coexpressed with the cyan fluorescent protein (CFP), which localizes to the cytoplasm and the nucleus (Kruse et al. 2010). As expected, XopAE-YFP and XopH-YFP showed complete colocalization with CFP, while XopF1-YFP and XopAJ-YFP colocalized with CFP in the cytoplasm but not in the nucleus (Supplementary Fig. S5). XopF2-YFP fluorescence was confined to small structures in the cytoplasm and to a few larger structures, mainly surrounding the nucleus. Because this pattern is consistent with localization to the Golgi apparatus, we transiently coexpressed XopF2-YFP with G-ck, a CFP marker for the Golgi apparatus (Nelson et al. 2007). Colocalization of the two proteins confirmed that the XopF2 effector localizes to the Golgi apparatus (Fig. 8, lower panel). These results demonstrate that *X. euvesicatoria* effectors sharing the ability to inhibit flg22 defense signaling localize to various compartments of the host cells, implying that individual effectors target different steps or pathways of PTI signaling.

DISCUSSION

In this study, we employed an *Arabidopsis* protoplast transient expression system for identifying the repertoire of *X. euvesicatoria* 85-10 effectors that interfere with PTI. The PAMP used in our investigation is flg22, a 22-amino acid peptide corresponding to the highly conserved amino terminus of flagellin that is sufficient to trigger immune responses in several plant species (Felix et al. 1999). The cell-based protoplast system provides a fast and reliable experimental platform allowing testing of each individual effector independently of the original bacterial context that may mask subtle or redundant contribution of the effector to PTI suppression. Similar screens were performed in protoplasts to identify *P. syringae* pv. *tomato* and *Phytophthora infestans* effectors that interfere with flg22 defense signaling (He et al. 2006; Zheng et al. 2014) and to provide evidence that the *X. euvesicatoria* effectors XopB, XopS, and XopL inhibit flg22- and elf18-induced activation of a PAMP-inducible promoter (Schulze et al. 2012; Singer et al. 2013).

Of the 33 *X. euvesicatoria* 85-10 effectors tested in our analysis, 17 (approximately 50%) inhibited and four enhanced the induction of a flg22-inducible reporter gene construct. It is tempting to speculate that effectors that enhanced reporter induction are recognized in *Arabidopsis* cells and prime immune signaling. Effectors that inhibited reporter induction represent a large proportion of the known effector pool of the *X. euvesicatoria* 85-10 bacterial strain, indicating that suppression of PTI is a primary role of *X. euvesicatoria* effectors. This finding together with the observation that several *X. euvesicatoria* effectors (i.e., AvrBsT,

XopE1, XopM, XopQ, AvrBs1, AvrXv4) were previously shown to inhibit cell death associated with effector-triggered immunity (ETI) (Szczesny et al. 2010; Teper et al. 2014, 2016) support the notion that suppression of different branches of plant immunity at different levels is a fundamental requirement for *X. euvesicatoria* bacteria to colonize their host. In line with this observation, multiple effectors of the same pathogen were previously implicated in suppression of plant immunity. For example, a large group of *P. syringae* pv. *tomato* effectors was reported to suppress PTI and ETI responses (Guo et al. 2009; He et al. 2006; Jamir et al. 2004). Similarly, eight of 33 tested *Phytophthora infestans* effectors were shown to inhibit flg22-induced gene activation (Zheng et al. 2014).

Six of the 17 *X. euvesicatoria* effectors that were found to inhibit flg22 reporter gene activation (i.e., XopL, XopX, XopB, XopS, XopN, and XopD) were previously shown to interfere with various PTI responses. XopN was reported to reduce PAMP-induced gene expression and callose deposition in tomato plants (Kim et al. 2009), whereas XopD was shown to interfere with activation of defense gene expression induced by the plant hormones salicylic acid and ethylene in tomato (Kim et al. 2008). In addition, the XopB, XopS, and XopL effectors were found to inhibit PAMP-induced activation of the *NHL10* promoter in *Arabidopsis* protoplasts (Schulze et al. 2012; Singer et al. 2013). Finally, XopX was recently shown to suppress flg22-induced production of reactive oxygen species but to promote transcriptional activation of PTI-associated genes (Stork et al. 2015). Our findings provide further evidence for the role of these effectors in modulation of plant immunity. Moreover, the detection of a phenotype for effectors that were already linked to suppression of PTI responses in the host plant validated the use of the *Arabidopsis* protoplast transient expression system for the functional analysis of the entire pool of *X. euvesicatoria* effectors. The XopJ effector was previously shown to interfere with cell wall-associated defense responses (Bartetzko et al. 2009) but did not inhibit flg22-induced reporter gene activation in our analysis. It is possible that XopJ affects a pathway of PTI signaling that does not lead to *FRK1* transcriptional activation or that it targets molecules involved in the execution rather than in signaling of immune responses.

Eleven *X. euvesicatoria* effectors that inhibited flg22-inducible reporter gene activation (i.e., XopF2, XopM, XopAJ, XopV, XopAP, XopZ, XopAE, XopE2, XopH, XopF1, and XopI) have not been previously linked to suppression of plant immunity and their molecular mode of action is still largely unknown. Of the 17 effectors that inhibited flg22-inducible reporter gene activation, eight effectors inhibited only PTI, while the other nine also interfered with ABA-dependent activation of a reporter gene driven by an ABA-inducible promoter. It is interesting to note that the effectors identified in this study as inhibitors of flg22 reporter gene activation did not affect flg22-induced MAP kinase phosphorylation, indicating that none of them interfered with the function of the FLS2 receptor or components of the FLS2 receptor complex. This is different from what has been observed in similar screens for *P. syringae* pv. *tomato* and *Phytophthora infestans* effectors (He et al. 2006; Zheng et al. 2014). The *P. syringae* pv. *tomato* effectors AvrPto and AvrPtoB were first shown to suppress flg22 defense signaling upstream to the activation of MAP kinase cascades (He et al. 2006), and follow-up studies demonstrated that they interfere with the function of PRR receptors and coreceptors (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Shan et al. 2008; Xiang et al. 2008, 2011; Zeng et al. 2012). Similarly, three *Phytophthora* effectors (SFI5, SFI6, and SFI7) were found to inhibit flg22-induced phosphorylation of MAP kinases (Zheng et al. 2014). However, it is still possible that certain *X. euvesicatoria* effectors affect the functions of PRRs other than FLS2 or that of early signaling components that do not participate in flg22 defense signaling. In addition, it is

possible that certain *X. euvesicatoria* effectors interfere with the function of early signaling components that are specific to the host plants pepper and tomato and are absent or not sufficiently conserved in the nonhost *Arabidopsis*. In support of this hypothesis, the *Phytophthora* effectors SFI5, SFI6, and SFI7 attenuated flg22-induced phosphorylation of MAP kinases in tomato but not in *Arabidopsis* protoplasts (Zheng et al. 2014).

Mutations in individual genes encoding effectors that inhibited flg22-induced PTI but not ABA-inducible gene expression (i.e., XopE2, XopF1, XopF2, XopH, XopAP, XopAE, and XopAJ) did not cause reduction of *X. euvesicatoria* virulence in pepper plants. This is not unexpected and is probably due to the functional redundancy often observed within the pool of T3S effectors of a given bacterial strain. However, when performing gain-of-function experiments through heterologous expression in the attenuated *P. syringae* pv. *tomato* Δ CEL strain, the expression of all the effectors tested, except for XopF1, inhibited callose deposition at the plant cell wall in *Arabidopsis* and enhanced development of disease symptoms in tomato. Despite the redundancy in their contribution to PTI suppression, the various effectors that inhibited flg22-induced PTI appear to act at different levels and by different mechanisms, as they were found in this and previous studies to localize to various cellular compartments (Bartetzko et al. 2009; Schulze et al. 2012; Thieme et al. 2007). Noteworthy is the localization of XopF2 to the Golgi apparatus. A similar localization was observed for the XopJ and XopB effectors that interfere with PTI-associated responses and inhibit vesicle trafficking (Bartetzko et al. 2009; Schulze et al. 2012). It remains to be established whether these effectors share redundant functions by affecting similar cellular processes.

In conclusion, this study identified 11 *X. euvesicatoria* 85-10 effectors as PTI inhibitors, providing molecular and physiological phenotypes for further investigation of their molecular function and interference with plant immunity.

MATERIALS AND METHODS

Bacterial strains and plant material.

Bacteria used were *Escherichia coli* DH12S, *Agrobacterium tumefaciens* GV2260, *X. euvesicatoria* 85-10 (Thieme et al. 2005), and *Pseudomonas syringae* pv. *tomato* DC3000 Δ CEL (Alfano et al. 2000). Bacteria were grown in Luria Bertani broth supplemented with the appropriate antibiotics at the following temperatures: *E. coli* at 37°C, *A. tumefaciens*, *X. euvesicatoria*, and *P. syringae* pv. *tomato* at 28°C. Antibiotics were used at the following concentrations (μ g/ml): ampicillin, 100; kanamycin, 50; rifampicin, 100; spectinomycin, 50; streptomycin, 100; nitrofurantion, 50; and gentamicin, 10. Bacterial growth assays were performed as described (Teper et al. 2014). Bacterial strains were injected with a needleless syringe into pepper leaves or vacuum-infiltrated into tomato leaves at a concentration of 1×10^5 CFU/ml in 10 mM $MgCl_2$, and bacterial growth was monitored at different times postinoculation.

Plant cultivars used were *Arabidopsis thaliana* ecotype Col-0, pepper (*Capsicum annuum*) 'ECW-30R' (Minsavage et al. 1990), tomato (*Solanum lycopersicum*) 'Hawaii 7981' (Scott et al. 1995), and *Nicotiana benthamiana* (Goodin et al. 2008). *Arabidopsis* plants were grown in a phytochamber in short day conditions (8 h light and 16 h dark) at 22°C with 40 to 60% humidity and approximately $120 \mu E m^{-2} s^{-1}$ light intensity. Tomato, pepper, and *N. benthamiana* plants were grown in a greenhouse in long-day conditions (16 h light and 8 h dark) at 25°C.

Plasmid constructions.

For expression in protoplasts, the *X. euvesicatoria* effector genes were polymerase chain reaction (PCR)-amplified, using Ex-Taq DNA polymerase (Takara Bio, Madison, WI, U.S.A.)

from *X. euvesicatoria* 85-10 genomic DNA. Sequences of oligonucleotides used in this study for PCR amplification are available upon request. Amplified effectors were inserted in frame to a c-myc tag into the multiple cloning site of the pGML10 vector (RIKEN, Tokyo). C-myc tagged effector genes were then excised from the pGML10 vector using restriction enzymes or were PCR-amplified and were inserted into the pTEX vector (Frederick et al. 1998) under the control of the CaMV 35S promoter.

For subcellular localization studies, an optimized version of the YFP (Griesbeck et al. 2001) and CFP (Nelson et al. 2007) were PCR-amplified and were cloned into the pGML10 vector either alone or in frame to the 3' end of the *xopH*, *xopAJ*, *xopF1*, *xopF2*, *xopAP*, and *xopAE* genes. The effector-YFP gene fusions and CFP were then excised from the pGML10 vector using restriction enzymes or were PCR-amplified and were inserted into the pBTEX binary vector under the control of the CaMV 35S promoter (Frederick et al. 1998) or into the pER8 binary vector for estradiol-inducible expression (Zuo et al. 2000).

For generation of *X. euvesicatoria* 85-10 deletion mutant strains of the *xopH*, *xopF1*, *xopF2*, *xopAP*, and *xopE2* genes, a 1-kb fragment upstream and a 1-kb fragment downstream of each effector gene were PCR-amplified from the *X. euvesicatoria* 85-10 genome and were cloned into the pGML10 vector. The gentamicin resistance cassette was then PCR-amplified from the pBBR1MCS-5 plasmid (Kovach et al. 1995) and was cloned into pGML10 between the two fragments. The obtained cassettes were excised from the pGML10 vector and were cloned into the pVIK165 suicide vector (Kalogeraki and Winans 1997). For generation of *X. euvesicatoria* 85-10 insertion mutant strains of the *xopAJ* and *xopAE* genes, a 250-bp fragment of the effector gene (nucleic acids 50 to 300) was PCR-amplified from the *X. euvesicatoria* 85-10 genome and was cloned into the pVIK165 suicide vector. Suicide vectors were mobilized from *E. coli* into *X. euvesicatoria* 85-10 by triparental mating (Rott et al. 1996). Mutants were selected with the appropriate antibiotics and were verified by PCR.

For expression of *X. euvesicatoria* effectors in the *P. syringae* pv. *tomato* DC3000 Δ CEL strain, a HA epitope tag was cloned into the pVSP61(*avrRpt2*₁₋₁₀₀) vector (Kim et al. 2009) creating pVSP61(*avrRpt2*₁₋₁₀₀:HA). Effector genes were then PCR-amplified and were cloned into pVSP61(*avrRpt2*₁₋₁₀₀:HA) in frame to *avrRpt2*₁₋₁₀₀ and the HA tag, resulting in pVSP61(*avrRpt2*₁₋₁₀₀:effector:HA). For complementation of the *P. syringae* pv. *tomato* DC3000 Δ CEL strain, the *hopM1* gene was PCR-amplified and was cloned into pVSP61(*avrRpt2*₁₋₁₀₀:HA), replacing the *avrRpt2*₁₋₁₀₀ fragment and in frame to the HA tag, resulting in pVSP61(*hopM1*:HA). The obtained vectors were mobilized from *E. coli* into *P. syringae* pv. *tomato* DC3000 Δ CEL by triparental mating.

Protoplast preparation and transfection.

The preparation of *Arabidopsis* mesophyll protoplasts was performed as described by Fraiture et al. (2014), with minor changes. Briefly, leaf stripes were dipped into 1.5% cellulose 'Onozuka' R10 and 0.4% macerozyme R10 solution (Yakult Pharmaceutical Industry, Tokyo), were vacuum-infiltrated for 30 min, and were digested for 3 h at 20°C in the dark. Next, protoplasts were filtered through a 45 to 100- μ m nylon mesh, were washed twice with W5 buffer, and were suspended in MMG buffer (4 mM MES, pH 5.7, 0.4 M mannitol, 15 mM MgCl₂) to a density of 2×10^5 cells/ml. Polyethylene glycol-mediated transfection was performed at a total DNA concentration of 100 μ g of plasmid DNA per milliliter of protoplast suspension. Protoplast samples were then incubated in W1 buffer at 20°C in the dark for 12 to 14 h, allowing plasmid gene expression.

Tomato mesophyll protoplasts were prepared as described by Nguyen et al. (2010), with minor changes. The lower epidermis

of fully expanded leaflets was gently rubbed with Carborundum and was rinsed with sterile water and leaf strips were floated on enzyme solution containing 2% cellulose 'Onozuka' R10, 0.4% macerozyme R10 (Yakult Pharmaceutical Industry) and 0.4 M sucrose in K3 medium. After 30 min of vacuum-infiltration and 3 h of incubation at 30°C in the dark, the enzyme-protoplast mixture was filtered through a 45 to 100- μ m nylon mesh. Viable protoplasts were collected by sucrose gradient centrifugation and were washed in W5 buffer. After recovery on ice for 2 h, protoplasts were harvested by centrifugation and were suspended to a density of 6×10^5 cells/ml in MMG buffer, prior to polyethylene glycol-mediated transfection that was performed at a total DNA concentration of 100 μ g of plasmid DNA per milliliter of protoplast suspension. Protoplast samples were then incubated in W1 buffer at 25°C in the dark for 12 to 14 h, allowing plasmid gene expression.

Luciferase and β -glucuronidase (GUS) assays.

Luciferase and GUS assays were performed as described by Zheng et al. (2014), with minor changes. Protoplasts were cotransfected with the reporters *pFRK1:LUC* (inducible by flg22 and elf18 [Asai et al. 2002; Albert et al. 2010]) or *pRD29b:LUC* (inducible by ABA [Christmann et al. 2005]), *pUBQ10:GUS* (for normalization of transformation efficiency, viability, and protein expression) and a pTEX vector containing a *35S:effector* gene construct, an empty pTEX vector (negative control), or a p2FGW7 vector containing a *35S:avrPto* gene construct (positive control) (Zheng et al. 2014). For the luciferase assay, luciferin was added to a 600- μ l transfected protoplast solution to a final concentration of 200 μ M. Protoplasts (100 μ l) were transferred to six wells in an opaque 96-well plate. For each sample, a PAMP (flg22 or elf18) or ABA was added to three wells to a final concentration of 500 nM or 50 μ M, respectively. The remaining three wells were mock-treated with 1 μ l of water or ethanol as control of the PAMP and ABA treatment, respectively. The luminescence reflecting the luciferase activity was measured every 2 h for 8 h, using a Turner Biosystems Veritas luminometer (Promega, Madison, WI, U.S.A.). For the GUS assay, 50 μ l of transfected protoplast solution for each sample was treated with 500 nM PAMP or 50 μ M ABA, and 50 μ l were mock-treated. Protoplast pellets were collected 4 (tomato) or 6 h (*Arabidopsis*) after elicitation. The cells were lysed in 100 μ l of cell culture lysis reagent solution (Promega). For each sample, three technical replicates of 10 μ l were incubated with 90 μ l of MUG substrate (1 mM 4-methyl-umbelliferyl- β -D-glucuronide, 100 mM Tris-HCl, pH 8.0, 2 mM MgCl₂) for 30 min at 37°C. The reaction was stopped with 90 μ l of 0.2 M Na₂CO₃. The fluorescence was monitored in an opaque plate, using a Turner Designs 20e luminometer 96-well plate reader with λ_{ex} = 360 nm and λ_{em} = 460 nm. For *Arabidopsis* protoplast assays, induction rate was calculated as (elicitor luciferase/elicitor GUS)/(mock luciferase/mock GUS) in each biological repeat. For tomato protoplast assays, induction rate was calculated as (elicitor luciferase/mock luciferase) in each biological repeat and was normalized by average GUS ratio (elicitor GUS/mock GUS).

Detection of phosphorylated MAP kinase.

Arabidopsis mesophyll protoplasts were cotransformed with pUC19 containing a *35S:MPK3* or *35S:MPK6* gene construct along with a pTEX vector containing a *35S:effector* gene construct, pTEX empty vector, a p2FGW7 vector containing a *35S:GFP* gene construct (Fraiture et al. 2014), or a p2GW7 vector containing a *35S:avrPto* gene construct (Fraiture et al. 2014). For each effector tested, 1 ml of protoplasts was distributed into two tubes and were treated with 500 nM flg22 or water for 5 min. Pellets of protoplasts were collected and were

denatured in protein-loading buffer. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were blotted onto a polyvinylidene difluoride membrane. Phosphorylation of MAPKs was monitored by immunoblot analysis using an antiphospho-p42/44 MAP kinase antibody (Cell Signaling Technology, Danvers, MA, U.S.A.).

Agrobacterium-mediated transient expression and localization assay.

pBTEX and pER8 plasmids carrying genes encoding either YFP or an *X. euvesicatoria* effector-YFP fusion were transformed into *A. tumefaciens* GV2260 by electroporation. *Agrobacterium* strains were grown overnight at 28°C, were diluted to an optical density at 600 nm = 0.1 in MgCl₂ and were syringe-infiltrated into leaves of six-week-old *N. benthamiana* plants. Leaf areas infiltrated with *A. tumefaciens* carrying the pER8 plasmids were treated with estradiol at 24 h after infiltration. Protein localization was visualized by a confocal laser scanning microscope (LSM 510 META; Zeiss, Heidelberg, Germany). Images were processed with AxioVision software (Zeiss). The Golgi marker G-ck labeled with CFP was used for colocalization experiments (Nelson et al. 2007). YFP and chlorophyll were excited with an Argon laser at 488 nm, while CFP was excited with a Diode laser at 405 nm. Emission was detected with a spectral detector set between 420 and 490 nm for CFP, between 505 and 550 nm for YFP, and between 635 and 750 nm for chlorophyll.

Callose deposition assay.

P. syringae pv. *tomato* DC3000 ΔCEL strains carrying the pVSP61 vector for expression of effector genes were syringe-infiltrated at a concentration of 1×10^8 CFU/ml into *Arabidopsis* leaves. Callose deposition assays were performed as described (Kim and Mackey 2008). At 16 h after infiltration, leaves were cleared of chlorophyll, callose deposits were stained with an aniline blue solution (0.01%) and were visualized by a fluorescence microscope with Axio and AxioVision softwares (Zeiss), with excitation at 365 nm and emission at 445 nm. For each treatment, a leaf from each of three plants was inoculated and four fields (0.6 mm²) were photographed for each leaf. The number of callose deposits (9.75 to 188.5 μm in diameter) in the photographed fields was automatically counted, using the Icy software. At least three independent biological repeats were carried out for each effector.

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