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Five *Xanthomonas* type III effectors suppress cell death induced by components of immunity-associated MAP kinase cascades

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Mitogen-activated protein kinase (MAPK) cascades play a fundamental role in signaling of plant immunity and mediate elicitation of cell death. *Xanthomonas* spp. manipulate plant signaling by using a type III secretion system to deliver effector proteins into host cells. We examined the ability of 33 *Xanthomonas* effectors to inhibit cell death induced by overexpression of components of MAPK cascades in *Nicotiana benthamiana* plants. Five effectors inhibited cell death induced by overexpression of MAPKKKα and MEK2, but not of MAP3Kε. In addition, expression of AvrBs1 in yeast suppressed activation of the high osmolarity glycerol MAPK pathway, suggesting that the target of this effector is conserved in eukaryotic organisms. These results indicate that *Xanthomonas* employs several type III effectors to suppress immunity-associated cell death mediated by MAPK cascades.

Plants have developed complex recognition systems and signaling pathways to defend themself against microbial pathogens. A first layer of plant immune responses confers resistance to a broad range of microorganisms and relies on the recognition of conserved microbial molecules known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs).[1] These responses are collectively referred to as PAMP-triggered immunity (PTI). A second layer of plant immunity, which is effective against host-adapted pathogens, is activated by the specific recognition of pathogen effector proteins and is termed effector-triggered immunity (ETI). ETI is usually associated with the hypersensitive response (HR), a rapid cell death in the infected plant tissues.[2] Mitogen-activated protein kinase (MAPK) cascades play a fundamental role in signaling pathways of both layers of plant immunity.[3] The MAP3Kα and MAP3Kε MAPKKKs are involved in signaling pathways mediating the development of ETI-associated HR in *N. benthamiana* plants.[4,5] Moreover, MAP3Kα and MAP3Kε were shown to be required for tomato disease resistance to bacterial pathogens.[4,5] The MEK2 MAPKK was reported to be a key regulator of the HR elicited by multiple *R/avr* gene pairs in *N. benthamiana* plants and to act downstream of both MAP3Kα and MAP3Kε, and upstream of the SIPK and WIPK MAPKs.[4-8] Interestingly, *N. benthamiana* SIPK and WIPK and their *Arabidopsis thaliana* homologs MPK6 and MPK3, respectively, are also important regulators of PAMP-triggered immunity (PTI).[9,10] Similarly, the *Arabidopsis thaliana* MKK4 and MKK5, which act upstream of MPK6 and MPK3, were shown to be involved in the activation of PTI.[9]

Xanthomonas is a genus of plant pathogenic bacteria that cause disease in hundreds of plant crops.[11] The ability of most *Xanthomonas* spp to cause infection and colonize their hosts largely depends on a type III secretion (T3S) system.[11] This secretion apparatus translocates a suite of effecter proteins directly into the cytosol of the host cell.[12] Inside the host cell, effector proteins contribute to pathogenesis by suppressing immune responses and manipulating host metabolism and hormone signaling.[13-17] Several effectors of *Xanthomonas* and other phytopathogenic bacteria were reported to suppress cell death caused by activation of immune responses. For example, the XopQ, XopN, XopZ and XopX effectors of *Xanthomonas oryzae* were shown to suppress cell death induced by recognition of damage-associated molecular patterns (DAMPs) in rice.[18] The AvrBsT and XopQ effectors of *Xanthomonas euvesicatoria* were reported to attenuate ETI-mediated cell death in resistant cultivars of pepper and tomato plants.[19,20] Several effectors of *Pseudomonas syringae* were found to suppress the HR-like cell death induced by the effector HopPsyA in tobacco and Arabidopsis.[21] In this study, we performed a functional screen to identify *Xanthomonas* type III effectors that suppress cell death induced by overexpression of components of immunity-associated MAP kinase cascades.

We hypothesized that certain *Xanthomonas* effectors interfere with plant immunity by targeting components of MAP kinase cascades or other downstream signal proteins that are involved in the elicitation of the HR. To test this hypothesis, we screened

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a group of T3S effectors of *Xanthomonas euvesicatoria* and *Xanthomonas perforans* bacteria for their ability to suppress cell death induced by overexpression of known regulators of cell-death signaling associated with plant immunity. These included MAP3Kα,[4] the kinase domain of MAP3Kε (MAP3KεKD)[5] or a constitutively active form of MEK2 ($MEK2^{DD}$).[8] Thirty-3 effectors were transiently co-expressed via *Agrobacterium* in *N. benthamiana* leaves with each one of the cell death inducers or an empty vector (**Table 1**). Expression of the effectors was driven by the CaMV 35S promoter, while that of MAP3Kα, MAP3KεKD, and $MEK2^{DD}$ was under the control of an estradiol-inducible system.[22] Leaves were visually monitored for the development of cell death during 7 days after estradiol application. Expression of certain effectors with an empty vector caused chlorosis (yellowing

Table 1. Inhibition of cell death by *Xanthomonas* type III effectors. *N. benthamiana* leaves were co-inoculated with *Agrobacterium* strains expressing the indicated effector protein from *Xcv* strain 85-10 and either MAP3Kα, MAP3KεKD, $MEK2^{DD}$ or an empty vector control (EV). Scores of +++, ++ and + indicate the appearance of cell death at 3, 5 and 7 days post-inoculation, respectively. A minus sign (–) indicates that no cell death was observed up to 7 days after inoculation. C indicates the appearance of leaf chlorosis in the infiltrated area 5 days after inoculation.

Type III effector	EV	MAP3Kα	MAP3KεKD	$MEK2^{DD}$
Empty	–	+++	+++	+++
XopB	++	+++	+++	+++
XopC	–	+++	+++	+++
XopD	C	+++	+++	+++
XopE1	C	+	+++	++
XopE2	C	+++	+++	+++
XopF1	–	+++	+++	+++
XopF2	+	++	++	+++
XopG	–	+++	+++	+++
XopH	C	++	++	+++
XopI	–	+++	++	+++
XopJ	C	+++	+++	+++
XopK	–	+++	+++	+++
XopL	+	++	+++	+++
XopM	C	+	+++	++
XopN	++	+++	+++	+++
XopO	–	+++	+++	+++
XopQ	C	C	+++	+
XopR	–	+++	+++	+++
XopS	C	+++	+++	+++
XopV	C	++	+++	+++
XopX	++	+++	+++	+++
XopZ	–	+++	+++	+++
XopAD	–	+++	+++	+++
XopAE	–	+++	+++	+++
XopAK	–	+++	+++	+++
Ecf	C	+++	+++	+++
AvrBs1	–	–	+++	+
AvrBs2	–	+++	+++	+++
AvrRxo1	+	+++	+++	+++
AvrBsT[a]	+++	+++	+++	+++
AvrBs3[b]	C	+++	+++	+++
AvrXv3[c]	–	+++	+++	+++
AvrXv4[c]	C	+	+++	++

[a]Effector derived from the *Xcv* 75–3 strain.
[b]Effector derived from the *Xcv* 116 strain.
[c]Effector derived from the *Xcv* 91–118 strain.

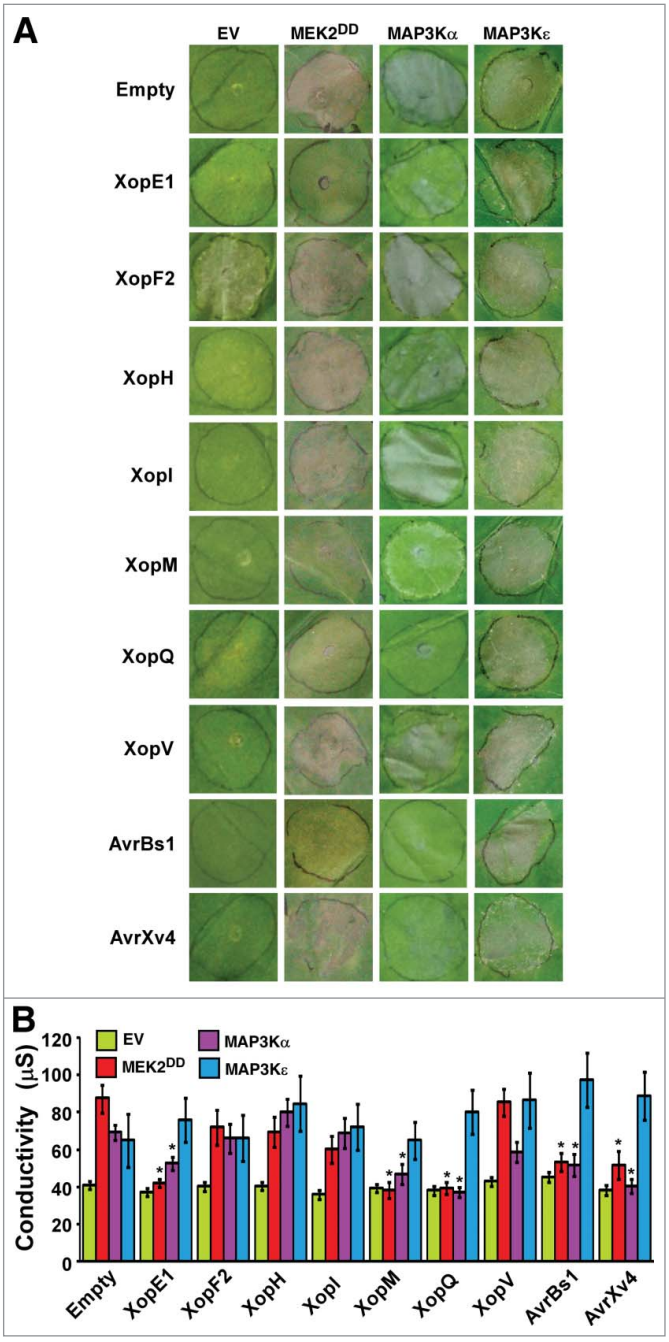

Figure 1. Inhibition of cell death by *Xanthomonas* type III effectors. An empty vector (EV), a constitutively active form of MEK2 ($MEK2^{DD}$), MAP3Kα or the kinase domain of MAP3Kε (MAP3KεKD), driven by the estradiol-inducible XVE expression system, were co-expressed via *Agrobacterium* in *N. benthamiana* leaves with the indicated effector protein driven by the CaMV 35S promoter. Expression of $MEK2^{DD}$, MAP3Kα and MAP3KεKD was induced by 17β-estradiol at 24 h after agro-infiltration. (**A**). Pictures of the infiltrated leaves at 5 days after 17β-estradiol treatment. (**B**). Quantification of cell death in leaves by measuring electrolyte leakage at 36 h after 17β-estradiol treatment. Values are the mean conductivity ± SE for leaf samples from at least 10 plants. Asterisks indicate a significant difference (Student's t test, $P < 0.05$) as compared to the empty vector control.

of the leaf tissue without apparent necrotic damage) or various degrees of cell death (necrotic damage) in the infiltrated area (**Table 1**). These phenotypes can be ascribed either to the virulence activity of the effectors in plant cells, or to their recognition by the plant surveillance system. As shown in **Fig. 1A** and **Table 1**, the XopE1, XopF2, XopH, XopI, XopM, XopQ, XopV, AvrBs1 and AvrXv4 effectors partially or fully inhibited cell death triggered by at least one of the cell death inducers. To quantify the inhibition of cell death for these 9 effectors, ion leakage from the infiltrated areas was measured at 36 h after estradiol application (**Fig. 1B**). A significant reduction in ion leakage induced by expression of $MEK2^{DD}$ and MAP3Kα was observed in leaves expressing XopE1, XopM, XopQ, AvrBs1 and AvrXv4, but not other effectors (**Fig. 1B**). Ion leakage induced by MAP3KεKD was not affected by any of the tested effectors (**Fig. 1B**).

Based on the assumption that MAP kinase cascades are conserved in eukaryotic organisms, we used a heterologous yeast system to examine if the effectors that suppressed cell death in plants target a conserved signaling components. To address this question, we tested the ability of the effectors to inhibit activation of the yeast high osmolarity glycerol (HOG) MAPK pathway.[23] To this aim, we monitored activation by osmotic stress of a *lacZ* reporter driven by the 8x*CRE* HOG responsive element in the presence of XopE1, XopM, XopQ, AvrBs1 or AvrXv4.[24] Expression of the effectors was first induced in yeast cultures containing the HOG-responsive reporter and 1 M sorbitol was then added to activate the HOG pathway. Following 1 h incubation, cultures were subjected to a β-galactosidase assay to monitor the activation of the reporter. As shown in **Fig. 2**, expression of AvrBs1, but not that of the other effectors, significantly attenuated the activation of the HOG reporter by approximately 70%. These results suggest that AvrBs1 directly targets a component of the MAPK cascade or a downstream signaling protein, which is conserved in plants and yeast.

MAPK cascades play a central role in immune signaling and activation of the ETI-associated HR cell death. Therefore, components of these cascades or their downstream proteins represent convenient targets for bacterial effectors. Indeed, effectors of multiple pathogens were reported to manipulate MAPK signaling: for example, the PexRD2 effector of *Phytophthora infestans* was reported to directly target MAPKKKε to promote disease in *N. benthamiana* plants.[25] In addition, the *Pseudomonas syringae* effectors HopF2 and HopAI1 suppress PTI by inhibiting MAP kinase cascades: HopF2 inactivates Arabidopsis MKK5 by ADP-ribosylation and HopAI1 dephosphorylates Arabidopsis MPK3 and MPK6 inhibiting their kinase activity.[26,27] In this study, we identified 5 *Xanthomonas* effectors that significantly inhibit cell death induced by MAPKKKα and a constitutively active form of MEK2. However, none of the 33 tested effectors suppressed the cell death induced by MAPKKKε, suggesting that the target of the 5 cell death suppressors probably participate in signaling rather than in the execution of cell death. We utilized a heterologous MAPK reporter system in yeast to examine whether the target of these effectors is conserved in other eukaryotic organisms. Among the 5 effectors that inhibited cell death, AvrBs1 was able to suppress activation of the HOG MAPK pathway in yeast. This is in agreement with previous findings showing that expression of AvrBs1 in yeast causes sensitivity to NaCl and sorbitol that are known inducers of the HOG pathway.[28]

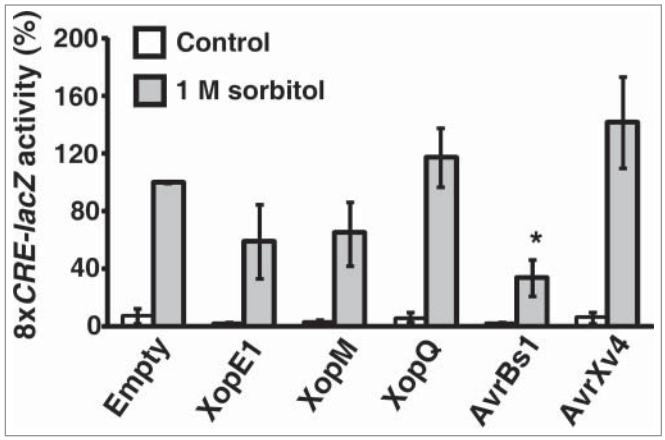

Figure 2. AvrBs1 attenuates activation of the yeast HOG pathway. Activation of an 8x*CRE*-regulated *lacZ* reporter monitored upon incubation of yeast strains containing an empty vector or the indicated effectors with or without 1 M sorbitol. Activity was normalized to that of yeast containing an empty vector and treated with sorbitol. Values represent the means ±SE of the relative activation 1 h after sorbitol addition in 3 biological repeats. Asterisks indicate a significant difference (Student's t test, $P < 0.05$) as compared to yeast containing an empty vector and treated with sorbitol.

In conclusion, our screen identified 5 *Xanthomonas* effectors that suppressed cell death induced by components of immunity-associated MAP kinase cascades. Furthermore we found that AvrBs1 suppresses activation of the HOG MAPK pathway in yeast, suggesting that this effector targets a signaling component that is conserved in eukaryotic organisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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