

Expression of *Pseudomonas syringae* type III effectors in yeast under stress conditions reveals that HopX1 attenuates activation of the high osmolarity glycerol MAP kinase pathway

Dor Salomon,^{1†} Eran Bosis,^{1†} Daniel Dar,^{1,2} Iftach Nachman² and Guido Sessa¹

Correspondence
Guido Sessa
guidos@post.tau.ac.il

¹Department of Molecular Biology and Ecology of Plants, Tel Aviv University, 69978 Tel Aviv, Israel

²Department of Biochemistry and Molecular Biology, Tel Aviv University, 69978 Tel Aviv, Israel

The Gram-negative bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) is the causal agent of speck disease in tomato. *Pst* pathogenicity depends on a type III secretion system that delivers effector proteins into host cells, where they promote disease by manipulating processes to the advantage of the pathogen. Previous studies identified seven *Pst* effectors that inhibit growth when expressed in yeast under normal growth conditions, suggesting that they interfere with cellular processes conserved in yeast and plants. We hypothesized that effectors also target conserved cellular processes that are required for yeast growth only under stress conditions. We therefore examined phenotypes induced by expression of *Pst* effectors in yeast grown in the presence of various stressors. Out of 29 effectors tested, five (HopX1, HopG1, HopT1-1, HopH1 and AvrPtoB) displayed growth inhibition phenotypes only in combination with stress conditions. Viability assays revealed that the HopX1 effector caused loss of cell viability under prolonged osmotic stress. Using transcription reporters, we found that HopX1 attenuated the activation of the high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway, which is responsible for yeast survival under osmotic stress, while other MAPK pathways were mildly affected by HopX1. Interestingly, HopX1-mediated phenotypes in yeast were dependent on the putative transglutaminase catalytic triad of the effector. This study enlarges the pool of phenotypes available for the functional analysis of *Pst* type III effectors in yeast, and exemplifies how analysis of phenotypes detected in yeast under stress conditions can lead to the identification of eukaryotic cellular processes affected by bacterial effectors.

Received 25 July 2012
Revised 28 August 2012
Accepted 10 September 2012

INTRODUCTION

The Gram-negative bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) is the causal agent of bacterial speck disease in tomato (Preston, 2000). The ability of *Pst* to promote disease largely depends on a type III secretion system, which delivers a suite of effector proteins across the bacterial and plant cell membranes into the host cell (Cunnac *et al.*, 2009). Type III effectors (T3Es) promote disease by manipulating host processes to the advantage of the pathogen and by interfering with host defence responses (Cunnac *et al.*, 2009). In resistant plants, certain

T3Es may also act as avirulence factors, if they are specifically recognized by a corresponding plant resistance protein (Martin *et al.*, 2003). This recognition often results in the induction of immune responses associated with a localized programmed cell death, known as the hypersensitive response (HR) (Mur *et al.*, 2008).

In recent years, a large number of *Pst* T3Es have been identified (Cunnac *et al.*, 2009). The *Pst* strain DC3000, for which the complete genome sequence is available (Buell *et al.*, 2003), encodes approximately 30 T3Es (Cunnac *et al.*, 2009). Molecular functions and targets have been assigned to only a few of these effectors (Block & Alfano, 2011). Among them, AvrPto and AvrPtoB inhibit the kinase activity of certain plant immune receptors that recognize pathogen-associated molecular patterns (Xiang *et al.*, 2008; Zeng *et al.*, 2012). AvrPtoB also contains an E3 ubiquitin ligase domain that ubiquitinates several host proteins involved in immune responses and targets them for

†These authors contributed equally to this work.

Abbreviations: CWI, cell wall integrity; HOG, high osmolarity glycerol; MAPK, mitogen-activated protein kinase; *Pst*, *Pseudomonas syringae* pv. *tomato*; T3E, type III effector; UPR, unfolded protein response.

Four supplementary figures and a supplementary table are available with the online version of this paper.

degradation (Gimenez-Ibanez *et al.*, 2009; Göhre *et al.*, 2008; Ntoukakis *et al.*, 2009; Rosebrock *et al.*, 2007). Similarly, HopM1 induces proteasome-mediated degradation of the ARF guanine exchange factor protein AtMIN7, which is required for robust immune responses against *Pst* (Nomura *et al.*, 2006). HopU1 is a mono-ADP-ribosyltransferase that ribosylates at least three chloroplast RNA-binding proteins and two glycine-rich RNA-binding proteins (Fu *et al.*, 2007). HopI1 is localized to the plant chloroplast, induces structural remodelling of the thylakoids, and suppresses salicylic acid accumulation (Jelenska *et al.*, 2007). HopG1 has been shown to localize to the plant mitochondria and to suppress plant immunity (Block *et al.*, 2010). HopF2 has been found to target mitogen-activated protein (MAP) kinase kinase 5 (MKK5) and to inactivate it via ADP-ribosylation (Wang *et al.*, 2010), as well as to target the *Arabidopsis* RIN4 protein to promote *Pst* virulence (Wilton *et al.*, 2010). More recently, the cysteine protease HopN1 has been shown to target and degrade PsbQ, a member of the oxygen evolving complex of photosystem II (Rodríguez-Herva *et al.*, 2012).

Attempts to uncover modes of action and host targets of many effectors have been undermined by functional overlap or redundancy in the large repertoire of effectors (Kvitko *et al.*, 2009), as recently underlined by the observation that a minimal set of eight T3Es is sufficient for wild-type *Pst* virulence levels (Cunnac *et al.*, 2011). In addition, the subtle effects that T3Es may exert to increase virulence and their detection by the plant surveillance system further complicate the functional characterization of T3Es. To expedite the discovery of targets and modes of action of *Pst* T3Es, Munkvold *et al.* (2008) employed the budding yeast *Saccharomyces cerevisiae* as a heterologous host. The use of yeast in the study of pathogenic bacteria relies on the observation that T3Es often target cellular processes that are conserved between yeast and other eukaryotes (Curak *et al.*, 2009; Siggers & Lesser, 2008; Valdivia, 2004). Indeed, when expressed in yeast, seven of the 27 *Pst* T3Es that were tested inhibited growth (Munkvold *et al.*, 2008). We have previously reported that expression of several *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) T3Es in yeast also results in growth inhibition (Salomon *et al.*, 2011). Remarkably, we found that certain effectors, which do not show visible phenotypes under normal yeast growth conditions, inhibit growth under stress conditions (Salomon *et al.*, 2011).

Here, we set out to study the virulence activities and eukaryotic targets of *Pst* T3Es using yeast grown under stress conditions as a heterologous host. To this aim, we screened 29 *Pst* DC3000 T3Es for their ability to inhibit yeast growth in the presence of various stressors. Out of the 29 effectors tested, five (HopX1, HopG1, HopT1-1, HopH1 and AvrPtoB) displayed growth inhibition phenotypes only in combination with certain stress conditions. Focusing our study on the effects of HopX1, we found that this effector severely inhibited yeast growth under osmotic stress. These results led us to hypothesize and subsequently

demonstrate that HopX1 attenuates the activation of the yeast high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway.

METHODS

Media, bacterial and yeast strains. Bacteria used in this study were: *Escherichia coli* strain DH12S and *Pst* strain DC3000. *E. coli* and *Pst* bacteria were grown in Luria–Bertani (LB) broth supplemented with the appropriate antibiotics at 37 and 28 °C, respectively. The following antibiotic concentrations were used for selection: ampicillin, 100 µg ml⁻¹; rifampicin, 100 µg ml⁻¹.

Yeast strains used were: BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), $\Delta fus3$ (BY4741, $\Delta fus3::KanMX$), W303 (*MATa, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*), W303 $\Delta hog1$ and a Hog1-GFP strain (EY0986 ATCC 201388: *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, HOG1-GFP::HIS3MX6*). Genetic analysis estimated a 0.08 % nucleotide divergence between the BY4741 and W303 strains, and revealed seven deletions of at least 500 bp in BY4741 compared with W303 (Schacherer *et al.*, 2007). W303 $\Delta hog1$ was prepared by amplifying the KanMX4 cassette from BY4741 $\Delta hog1::kanMX4$ with primers flanking the deletion area and transforming it into W303. Correct integration was verified by colony PCR with a forward primer outside the amplified segment and a reverse primer within the KanMX4 cassette.

Yeast were grown at 30 °C in YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) or in selective synthetic complete medium (Salomon & Sessa, 2010) lacking leucine (–*leu*) or uracil (–*ura*), and supplemented with 2 % glucose, or 2 % galactose and 1 % raffinose. Yeast were transformed using the lithium acetate (LiAc) method, as previously described (Salomon & Sessa, 2010).

Plasmid construction. All *Pst* effector genes were PCR-amplified using Ex *Taq* DNA polymerase (Takara Bio) from genomic DNA of the *Pst* strain DC3000. Sequences of oligonucleotides used for amplification of effectors are listed in Table S1 available with the online version of this paper. Amplified effectors were inserted in-frame to a c-myc tag into the multiple cloning site of the pGML10 yeast expression vector (RIKEN) under the control of a galactose-inducible promoter.

Site-directed mutagenesis. A site-specific mutation was introduced into *hopX1* using the QuikChange kit (Stratagene). Sequences of oligonucleotides used for *hopX1* mutagenesis are: 5'-CCTCAACGATTGTCATGGCTCCGTGGTCAAATGGTTC-3' and 5'-GAACCATTTGACCACGGAGCCATGACAATCGTTGAGG-3'.

Yeast growth, viability, and pheromone sensitivity assays. Yeast growth inhibition and viability assays were performed as previously described (Salomon *et al.*, 2011).

Pheromone sensitivity halo assays were performed as described elsewhere (Hoffman *et al.*, 2002), using the strains of interest on selective inducing plates. The $\Delta fus3$ strain was used as a control.

Yeast β -galactosidase assays. W303 yeast strains containing the HOG 8xCRE-*lacZ* reporter plasmid (Tatebayashi *et al.*, 2006), the cell wall integrity (CWI) 2xRlm1-*lacZ* reporter plasmid (Jung *et al.*, 2002), the pheromone MAPK pathway P_{FUS1} -*lacZ* reporter (Tatebayashi *et al.*, 2007) or the unfolded protein response (UPR) UPR-*lacZ* reporter (Cox & Walter, 1996) were transformed with pGML10 plasmids encoding the indicated effectors. Cells were grown overnight in selective medium containing glucose (2 %). Cultures were washed, diluted and grown in 4 ml selective medium containing galactose (2 %) and raffinose (1 %) to OD₆₀₀ 0.5–0.8. Cultures were

then split into two tubes with 1 ml fresh selective medium containing galactose (2%) and raffinose (1%) with or without the respective stressing agent. To activate the HOG pathway, 0.5 M NaCl or 1 M sorbitol was added to the inducing medium for 1 h; to activate the CWI pathway, 7 mM caffeine was added to the inducing medium for 4 h; to activate the pheromone pathway, 5 μ M α -factor was added to the inducing medium for 2 h; to induce the UPR pathway, 2 mM DTT was added to the inducing medium for 4 h. Cells were then collected and subjected to a β -galactosidase activity assay using 0.2% sodium lauroyl sarcosinate as the permeabilizing agent (Kippert, 1995).

Hog1 nuclear translocation dynamics and protein expression levels. Hog1-GFP cells from the GFP tag yeast collection (Howson *et al.*, 2005) were transformed with either an empty or a HopX1-encoding pGML10 vector. Transformed cells were grown overnight in selective medium containing glucose (2%). Cultures were then washed, diluted and grown overnight in selective medium containing galactose (2%) and raffinose (1%). To monitor Hog1-GFP nuclear translocation dynamics, cells were placed in a custom-made polydimethylsiloxane (PDMS) microfluidic chamber and immobilized on a coverslip using concavalin A. Cells were kept at 30 °C with a steady flow of selective medium containing galactose (2%) and raffinose (1%) for 30 min, before switching to selective medium containing galactose (2%) and raffinose (1%) and 0.5 M NaCl. The cells in the flow chamber were visualized using a Nikon TiE epifluorescence microscope with an Andor Clara camera, at a $\times 100$ magnification. GFP emission was imaged with 1 s exposures at 25% UV lamp intensity. Images were obtained for 60 min at fixed 1 min intervals. To analyse the acquired images, differential interference contrast (DIC) images were used to detect single cells and determine their boundaries. Cells were then mapped between time points. Total cellular Hog1-GFP was quantified from the fluorescence images and was constant throughout the experiment. The brightest object in each cell was then detected in fluorescence images using local adaptive thresholding. This object represented the nuclear signal whenever it was above the cytoplasmic signal, or otherwise the cytoplasmic signal. Signal density was computed as total intensity divided by the number of pixels. Image analysis was performed using a custom-written software in MATLAB (Nachman *et al.*, 2007). To determine Hog1-GFP expression levels, cells were washed with double-distilled water to remove the growth medium and mounted on slides, and pictures were taken and analysed as described above.

RESULTS

Expression of seven *Pst* T3Es from a low-copy-number plasmid inhibits yeast growth

Seven out of 27 *Pst* strain DC3000 T3Es have been shown to inhibit growth when expressed in yeast grown under normal conditions (Munkvold *et al.*, 2008). Because *Pst* T3Es that were previously analysed in yeast were expressed from a high-copy-number plasmid, which may result in non-specific toxic effects (Siggers & Lesser, 2008; Slagowski *et al.*, 2008), we first set out to confirm phenotypes caused by *Pst* effectors by expressing 29 of them from a low-copy-number plasmid. This analysis also included the AvrE1 and HopR1 T3Es, which were not previously tested in yeast. Yeast cultures were serially diluted and plated onto repressing (2% glucose) and inducing (2% galactose and 1% raffinose) medium. As shown in Fig. 1, all yeast strains exhibited similar growth on repressing medium compared

with a control strain containing an empty vector. However, expression of seven T3Es inhibited yeast growth: AvrE1, HopAA1-1, HopAM1, HopU1 and HopAD1 completely inhibited yeast growth, while HopAO1 and HopD1 showed moderate growth inhibition. Results obtained for HopAA1-1, HopAM1, HopU1, HopAD1, HopAO1 and HopD1 were in agreement with the findings of Munkvold *et al.* (2008), but not the results for HopN1, which earlier was found to mildly inhibit yeast growth, but did not cause any phenotype in our experiments. Protein accumulation was confirmed by immunoblot analysis for all effectors, except for HopAA1-1, HopAD1, AvrE1, HopAA1-2, HopM1 and HopR1 (Fig. S1). HopAA1-1, HopAD1 and AvrE1 inhibited yeast growth upon induction (Fig. 1), suggesting that they were expressed, but below detection level. However, no evidence could be obtained for the expression of HopAA1-2, HopM1 and HopR1, which did not inhibit yeast growth. Taken together, these results indicate that at least seven *Pst* T3Es target cellular processes required for yeast growth, when expressed under normal conditions from a low-copy-number expression vector.

Five *Pst* T3Es inhibit growth when expressed in yeast under stress conditions

We hypothesized that additional *Pst* effectors target eukaryotic cellular processes that are required for yeast growth only under stress conditions. To test this hypothesis, we expressed the 29 *Pst* effectors described above in yeast in the presence of various stressors: caffeine, a purine analogue that elicits pleiotropic effects in yeast and is highly toxic to mutants of the CWI MAPK pathway and to mutants of the Tor pathway (Kuranda *et al.*, 2006; Parsons *et al.*, 2004); sorbitol, an osmotic stressor that is highly toxic to mutants of the HOG MAPK pathway (Gustin *et al.*, 1998; Hohmann, 2002); NaCl, an osmotic and ionic stressor that is highly toxic to mutants of the HOG MAPK pathway as well as to mutants defective in ion homeostasis (Auesukaree *et al.*, 2009; Gustin *et al.*, 1998; Hohmann, 2002); tunicamycin, an inhibitor of protein *N*-glycosylation that is highly toxic to mutants of the UPR pathway and to mutants with cell wall defects (Parsons *et al.*, 2004); and 37 °C heat stress, which causes pleiotropic effects in yeast (Auesukaree *et al.*, 2009). Yeast cultures containing vectors for the expression of *Pst* effectors were serially diluted and plated onto repressing or inducing medium in the presence or absence of the stressors at levels that minimally impact yeast growth (Salomon *et al.*, 2011): caffeine (7 mM), NaCl (0.5 M), sorbitol (1 M), tunicamycin (0.12 μ g ml⁻¹) and temperature of 37 °C. Growth of yeast expressing *Pst* effectors was monitored and scored based on the highest dilution in which colonies were detected. As summarized in Table 1, combining the expression of effectors with stress conditions enhanced the growth inhibition phenotypes detected for certain effectors under normal growth conditions. For example, phenotypes induced by expression of HopAO1 were significantly enhanced in the presence of caffeine, NaCl,

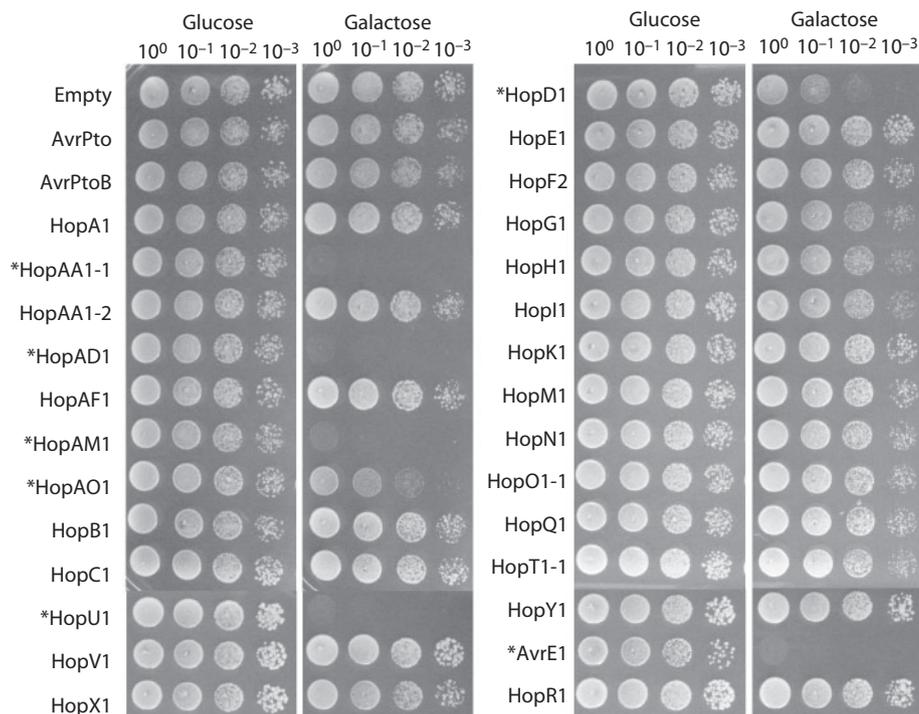


Fig. 1. Yeast growth is inhibited by expression of seven *Pst* T3Es. BY4741 yeast strains carrying the yeast expression vector pGML10, either empty or encoding the indicated galactose-inducible *Pst* effectors with a C-terminal myc tag, were grown overnight in repressing medium (2% glucose). Cultures were then normalized to OD₆₀₀ 1.0, and serial 10-fold dilutions were spotted onto repressing or inducing medium (2% galactose and 1% raffinose). Photographs were taken after 2 and 3 days of growth at 30 °C for yeast growing in repressing and inducing medium, respectively. An asterisk indicates effectors showing a growth inhibition phenotype. Data shown are representative of three independent experiments for each effector.

sorbitol and at 37 °C. Similarly, growth inhibition phenotypes caused by the expression of HopD1 were enhanced in the presence of NaCl, sorbitol, tunicamycin and at 37 °C. More importantly, in the presence of various stressors, growth inhibition phenotypes were detected for HopX1, HopG1, HopT1-1, HopH1 and AvrPtoB, which did not affect growth under normal conditions. Notably, expression of HopX1 strongly inhibited growth in the presence of the osmotic stressors NaCl and sorbitol, and had a milder effect in the presence of caffeine and at 37 °C. Altogether, the employment of stressors enabled us to identify phenotypes for five additional *Pst* T3Es that target yeast cellular processes required for yeast growth only under stress conditions.

HopX1 attenuates the activation of the HOG MAPK pathway in yeast

Yeast sensitivity to osmotic stress might indicate improper activation of the HOG MAPK pathway, which is of critical importance for yeast survival under osmotic stress (Hohmann, 2002; Yoon *et al.*, 2003). Because our results indicated that expression of HopX1, and to a lesser extent of HopG1 and HopT1-1, inhibited yeast growth in the presence of the osmotic stressors NaCl and sorbitol, but

not under normal growth conditions (Table 1), we tested whether the sensitivity to osmotic stress conferred by these effectors was a result of improper activation of the HOG pathway. To this end, we monitored activation by osmotic stress of a *lacZ* reporter driven by the 8xCRE HOG responsive element (Tatebayashi *et al.*, 2006). Expression of HopX1, HopG1 and HopT1-1 was first induced in W303 yeast cultures containing a HOG-responsive reporter, and 0.5 M NaCl or 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. It should be noted that as observed in the BY4741 yeast strain, the W303 strain was sensitive to osmotic stress upon expression of HopX1, HopG1 and HopT1-1 (Fig. S2), indicating that these effectors have a similar qualitative effect on the two yeast strains, as opposed to the differential effects previously observed for other bacterial effectors (Salomon *et al.*, 2011). As shown in Fig. 2, expression of HopX1 significantly attenuated the activation of the HOG reporter under both NaCl and sorbitol stress by ~50%, while HopG1 and HopT1-1 had mild effects (~10–15%) under NaCl and sorbitol stress. These results correlated with the different severities of growth inhibition phenotypes detected when these effectors were expressed in yeast under

Table 1. Yeast growth inhibition phenotypes induced by *Pst* T3Es under various stress conditions

BY4741 yeast strains carrying plasmids for the expression of the indicated effectors or containing an empty vector were grown overnight in repressing medium (2% glucose). Cultures were then washed and normalized to OD₆₀₀ 1.0, and 10-fold serial dilutions were spotted onto repressing medium or onto inducing medium (2% galactose and 1% raffinose), and grown under the following stress conditions: in the presence of 7 mM caffeine, 0.5 M sodium chloride (NaCl), 1 M sorbitol, 0.12 µg ml⁻¹ tunicamycin (Tm), or at a temperature of 37 °C. None: growth under normal conditions without stress. Two to four days after spotting, the highest dilutions in which colony growth was visible were determined for each strain, and reported according to the growth scale below. Data shown are representative of three independent experiments for each effector. Degree of growth: ----, no growth in any dilution; ---, up to the 10⁰ dilution; --, up to the 10⁻¹ dilution; -, up to the 10⁻² dilution; no sign, up to the 10⁻³ dilution.

Effector	None	Caffeine	NaCl	Sorbitol	Tm	37 °C
Empty	----	----	----	----	----	----
HopAA1-1*	----	----	----	----	----	----
HopAM1	----	----	----	----	----	----
HopU1	----	----	----	----	----	----
AvrE1*	----	----	----	----	----	----
HopAD1*	----	----	----	----	----	----
HopAO1	---	---	---	---	---	---
HopD1	----	----	----	----	----	----
HopX1	----	----	----	----	----	----
HopG1	----	----	----	----	----	----
HopT1-1	----	----	----	----	----	----
HopH 1	----	----	----	----	----	----
AvrPtoB	----	----	----	----	----	----
AvrPto	----	----	----	----	----	----
HopA1	----	----	----	----	----	----
HopAA1-2*	----	----	----	----	----	----
HopAF1	----	----	----	----	----	----
HopB1	----	----	----	----	----	----
HopC1	----	----	----	----	----	----
HopE1	----	----	----	----	----	----
HopF2	----	----	----	----	----	----
HopI1	----	----	----	----	----	----
HopK1	----	----	----	----	----	----
HopM1*	----	----	----	----	----	----
HopN1	----	----	----	----	----	----
HopO1-1	----	----	----	----	----	----
HopQ1	----	----	----	----	----	----
HopV1	----	----	----	----	----	----
HopY1	----	----	----	----	----	----
HopR1*	----	----	----	----	----	----

*Indicates effectors whose expression was not detected in immunoblot analysis.

osmotic stress (Table 1, Fig. S2). Because HopX1 caused more pronounced phenotypes than HopG1 and HopT1-1, we focused our subsequent analyses on this effector.

HopX1 has been identified as a putative member of the transglutaminase superfamily (Nimchuk *et al.*, 2007). Putative catalytic triad residues of this effector have been reported to be required for avirulence activity on a resistant bean host and for virulence activity in *Arabidopsis* plants (Nimchuk *et al.*, 2007). Interestingly, the ability of HopX1 to inhibit yeast growth and to attenuate activation of the HOG pathway was dependent on an intact putative catalytic triad, as expression of an HopX1 mutant with a substitution in the putative catalytic residue D236 (HopX1^{D236A}) did not cause these phenotypes (Fig. 3). Immunoblot analysis confirmed that HopX1 and its mutant HopX1^{D236A} were expressed at similar levels in yeast (Fig. S3). These results suggested that the phenotypes caused by HopX1 expression in yeast were dependent on the putative enzymic activity of this effector.

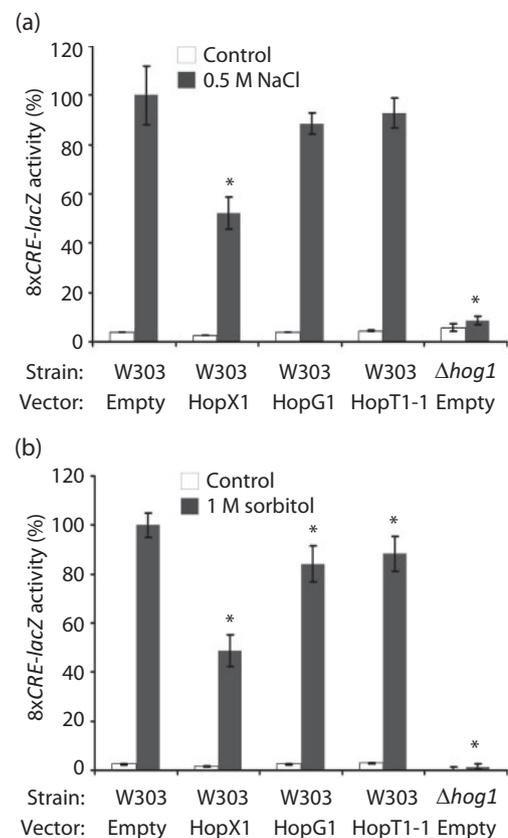


Fig. 2. HopX1 attenuates activation of the HOG pathway. Activation of an 8xCRE-regulated *lacZ* reporter monitored following incubation of wild-type or $\Delta hog1$ W303 yeast strains containing an empty vector or expressing HopX1, HopG1 or HopT1-1 with 0.5 M NaCl (a) or 1 M sorbitol (b). Data are the means of four independent yeast transformants \pm SD and represent reporter activity relative to the treated W303 empty strain. The assays were repeated three times with similar results. An asterisk indicates a statistically significant difference in mean values as compared with the wild-type strain carrying an empty vector by an unpaired, two-tailed Student's *t* test (P value < 0.05).

HopX1 expression in yeast results in loss of cell viability under prolonged osmotic stress

To further characterize the effect of HopX1 in yeast under osmotic stress, we tested whether growth inhibition caused by expression of HopX1 under osmotic stress was a result of cytotoxicity or arrest of cell growth. To this aim, we performed cell viability assays for yeast strains expressing

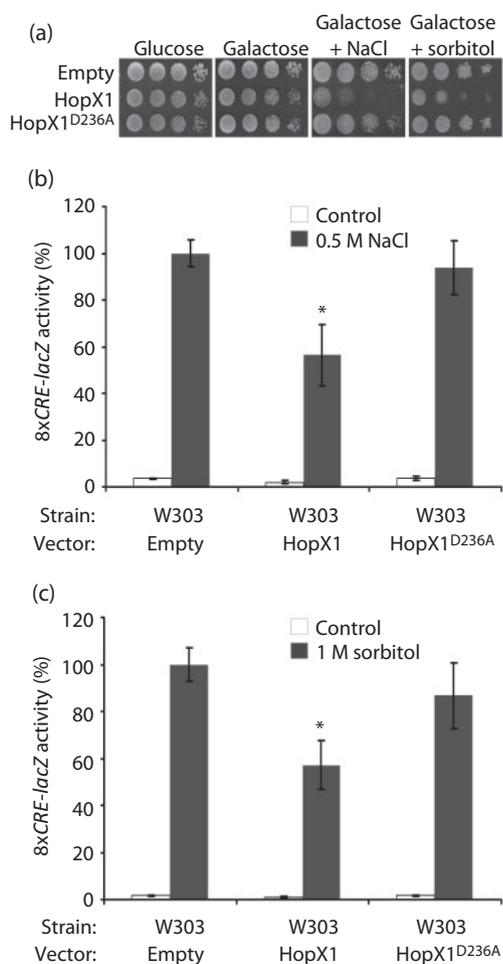


Fig. 3. HopX1 effects on yeast depend on putative catalytic activity. (a) Growth of yeast expressing HopX1 or HopX1^{D236A} under osmotic stress. BY4741 yeast strains carrying a vector either empty or for the expression of HopX1 or HopX1^{D236A} were spotted in 10-fold serial dilutions on repressing or inducing medium to determine growth inhibition in the presence of 0.5 M NaCl and 1 M sorbitol. (b, c) Effect of HopX1 and HopX1^{D236A} on activation of the 8xCRE-regulated *lacZ* reporter measured following incubation of W303 yeast with 0.5 M NaCl (b) or 1 M sorbitol (c). Data are the means of four independent yeast transformants \pm SD and represent reporter activity relative to the treated W303 empty strain. The assays were repeated three times with similar results. An asterisk indicates a statistically significant difference in mean values as compared with the wild-type strain carrying an empty vector by an unpaired, two-tailed Student's *t* test (*P* value < 0.05).

HopX1 or an empty vector under osmotic stress conditions. Yeast cultures grown in the presence or absence of 0.5 M NaCl were sampled at different time points after induction, and serial 10-fold dilutions were plated onto repressing medium to determine the number of viable cells present in the cultures. A sharp decrease in the number of viable cells starting at 10 h after exposure to stress was observed for yeast expressing HopX1 in the presence of NaCl (Fig. 4). These results suggest that expression of HopX1 interfered with the ability of yeast to adapt and survive under prolonged osmotic stress.

HopX1 does not affect Hog1 expression or nuclear entry dynamics

We next tested whether HopX1 attenuated the HOG pathway by affecting the Hog1 MAPK. To this aim, we used a yeast strain expressing Hog1-GFP from the *S. cerevisiae* GFP tag collection (Howson *et al.*, 2005) to monitor the expression level of Hog1 and its nuclear entry following osmotic stress (Ferrigno *et al.*, 1998). The Hog1-GFP-expressing strain was transformed with the pGML10 vector either empty or encoding HopX1. To determine whether the attenuation of the HOG pathway by HopX1 was mediated by manipulation of Hog1 expression levels, we quantified the fluorescence levels of Hog1-GFP cells expressing HopX1 or an empty vector. As shown in Fig. 5(a), the expression of HopX1 did not affect Hog1-GFP levels.

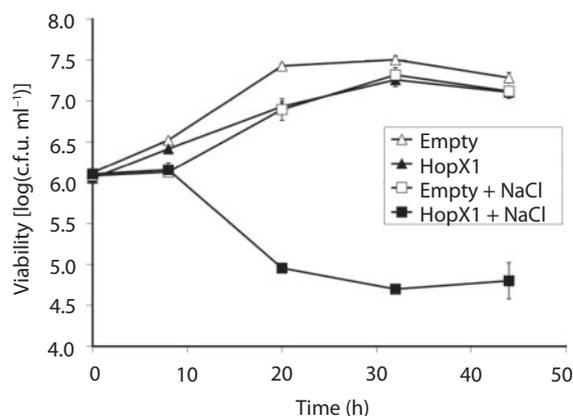


Fig. 4. Expression of HopX1 in yeast induces loss of cell viability in the presence of osmotic stress. BY4741 yeast strains carrying plasmids for expression of HopX1, or containing an empty vector, were grown overnight in repressing medium. Cultures were diluted to OD₆₀₀ 0.5 and allowed to recover in fresh repressing medium for 2 h prior to washes, normalization to OD₆₀₀ 0.2, and incubation in inducing medium with or without 0.5 M NaCl. For each time point, an aliquot was removed from the cultures and serial 10-fold dilutions were spotted onto repressing medium to assess the number of viable cells. Data represent the mean \pm SEM (*n* = 3). The assay was repeated three times with similar results.

During osmotic stress, the Hog1 MAPK is phosphorylated and subsequently enters the cell nucleus to activate transcription of target genes (Hohmann, 2002). To determine whether HopX1 affects Hog1 nuclear entry dynamics, Hog1-GFP-expressing cells were grown overnight in repressing medium, washed, diluted and grown overnight in inducing medium. Induced cells were then visualized over time using a fluorescence microscope to monitor the dynamics of Hog1 translocation into the nucleus following exposure to osmotic stress (Ferrigno *et al.*, 1998). As shown in Fig. 5(b, c), Hog1-GFP translocation from the cytoplasm into the nucleus peaked 2 min after exposure to 0.5 M NaCl, and gradually decreased during the following 60 min, consistent with previous dynamic measurements (Hersen *et al.*, 2008). The expression of HopX1 did not affect the dynamics of Hog1-GFP translocation into the nucleus (Fig. 5b, c), even though HopX1 expression did result in sensitivity to osmotic stress when expressed in the Hog1-GFP strain (data not shown). Taken together, these results indicate that the HopX1 attenuation of the HOG pathway activation was not mediated by manipulation of Hog1 expression levels or nuclear translocation dynamics.

Effects of HopX1 on the activation of other MAPK pathways in yeast

The dramatic effect of HopX1 on the activation of the HOG MAPK pathway prompted us to investigate whether HopX1 can also affect the activation of other MAPK pathways in yeast. First, we monitored the effect of HopX1 on the activation of the CWI pathway-specific 2xRlm1-*lacZ* reporter in response to caffeine, which was previously shown to activate the CWI pathway (Bosis *et al.*, 2011; Jung *et al.*, 2002). As shown in Fig. 6(a), HopX1 had a mild effect (~20% reduction) on the activation of the CWI pathway, which was not statistically significant (P value <0.05).

We next monitored the effect of HopX1 on the activation of the P_{FUS1} -*lacZ* reporter, which is induced by the pheromone MAPK pathway in response to α -factor (Tatebayashi *et al.*, 2007). HopX1 had a mild effect (~27% reduction) on the activation of the pheromone pathway (Fig. 6b). We then performed a halo assay to determine whether the effect of HopX1 on the pheromone MAPK pathway has an impact on yeast sensitivity to α -factor (Hoffman *et al.*, 2002). Cells expressing HopX1 or containing an empty vector were able to produce haloes of similar size in response to α -factor, as opposed to cells in which Fus3, the MAPK of the pheromone-regulated pathway, was deleted (Fig. S4). These results suggest that the effect of HopX1 on the activation of the pheromone pathway was not sufficient to interfere with the functionality of the pathway.

Finally, to determine whether HopX1 has a general effect on gene expression in yeast that could account for its effects on MAPK pathways, we tested the effect of HopX1

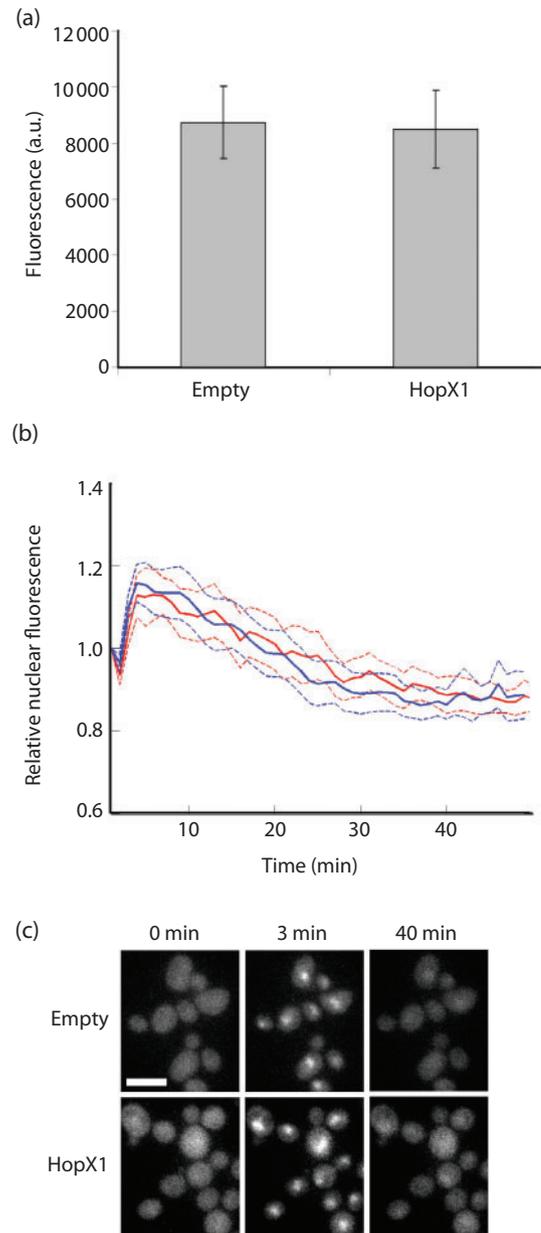


Fig. 5. HopX1 does not affect Hog1 expression and nuclear entry dynamics. (a) Hog1-GFP cells expressing HopX1 ($n=302$) or an empty vector ($n=420$) were monitored for fluorescence intensity. Data are means \pm SD of fluorescence reported in arbitrary units (a.u.). (b) Induced Hog1-GFP cells containing pGML10 either empty or encoding HopX1 were immobilized in a microfluidic chamber. Fluorescence intensity in the nucleus was monitored after replacing inducing medium with inducing medium containing 0.5 M NaCl, for a period of 60 min. Results are displayed as relative nuclear fluorescence (with no background subtraction) compared with the nuclear fluorescence at $t=0$ min. Continuous lines represent the mean of the sampled cells, and dashed lines represent the SD. Cells containing the empty expression vector are represented by the red lines ($n=133$), and cells expressing HopX1 are represented by the blue lines ($n=211$). The assay was repeated twice with similar results. (c) Fluorescence images at different time points after addition of NaCl. Bar, 5 μ m.

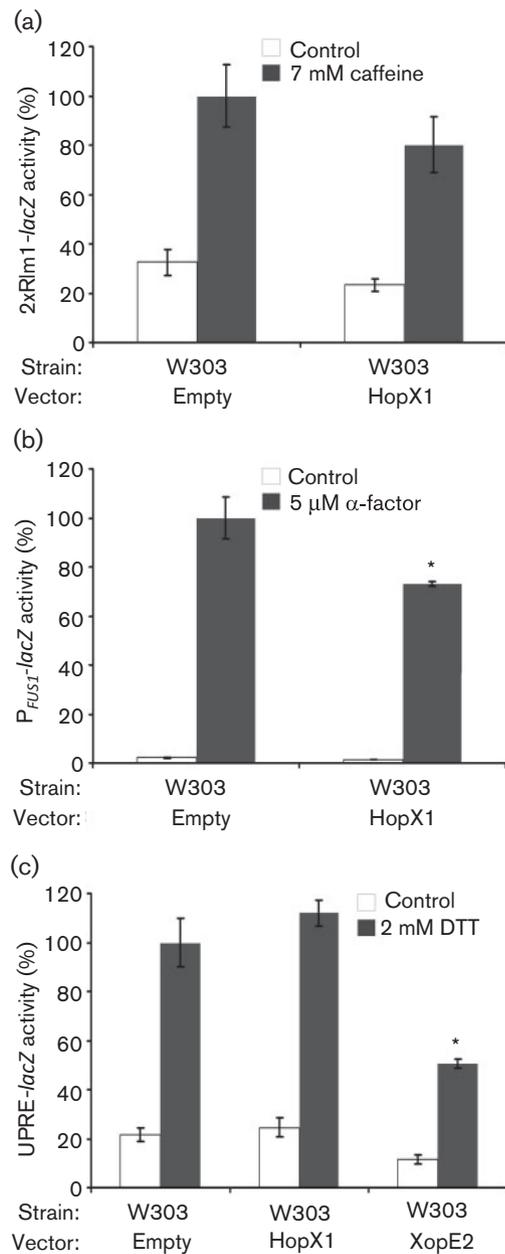


Fig. 6. HopX1 mildly reduces the activation of the CWI and pheromone-responsive MAPK pathways. (a) The activation of a 2xRlm1-regulated *lacZ* reporter was monitored following incubation of W303 yeast strains containing an empty vector or expressing HopX1 in the presence of 7 mM caffeine. (b) The activation of a *P_{FUS1}*-regulated *lacZ* reporter was monitored following incubation of W303 yeast strains containing an empty vector or expressing HopX1 in the presence of 5 μM α-factor. (c) DTT-mediated activation of a UPRE-regulated reporter. HopX1 or XopE2 was expressed in W303 cells containing a UPRE-*lacZ* reporter that were treated with 2 mM DTT, and activation of the reporter was measured. Data are the means of four independent yeast transformants ± SD and represent reporter activity relative to the treated W303 empty strain. The assays were repeated three times with similar results. An asterisk indicates a statistically significant difference in mean values as compared with the wild-

type strain carrying an empty vector by an unpaired, two-tailed Student's *t* test (*P* value <0.05).

on a non-MAPK pathway. To this aim, we monitored the expression of the UPR-specific UPRE-*lacZ* reporter in response to DTT (Bosis *et al.*, 2011). As shown in Fig. 6(c), HopX1 had no effect on the activation of the UPR pathway, as opposed to the previously reported effect of the *Xanthomonas* effector XopE2 on activation of this pathway (Bosis *et al.*, 2011). Therefore, HopX1 appears to specifically affect the activity level of MAPK pathways in yeast, and in particular that of the HOG MAPK pathway.

DISCUSSION

In this study, we screened a group of *Pst* DC3000 T3Es for their ability to inhibit yeast proliferation under various growth conditions. By using a low-copy-number vector for expression of 29 *Pst* effectors in yeast under normal growth conditions, we confirmed phenotypes observed earlier for six of these effectors (HopAA1-1, HopAM1, HopU1, HopAD1, HopAO1 and HopD1) that were expressed from a high-copy-number plasmid (Munkvold *et al.*, 2008). The HopN1 effector, which was reported to inhibit yeast growth (Munkvold *et al.*, 2008), did not cause any detectable phenotype in our system. This discrepancy may derive from the higher protein expression levels of the system employed by Munkvold and colleagues. However, a recent study has shown that HopN1 localizes to plant chloroplasts and targets a plant-specific protein involved in photosynthesis (Rodríguez-Herva *et al.*, 2012), suggesting that the toxic phenotype reported by Munkvold and colleagues was non-specific.

Our screen included the HopR1 and AvrE1 effectors, which were not previously tested in yeast. While HopR1 did not result in any growth inhibition phenotype and its expression was below detection levels, the expression of AvrE1 caused severe inhibition of yeast growth even though its expression was not detected. Interestingly, the *Pantoea stewartii* T3E WtsE, which belongs to the AvrE family, has been reported to inhibit yeast growth (Ham *et al.*, 2008). The finding that expression of both AvrE1 and WtsE severely inhibited yeast growth suggests that members of the AvrE family share an activity that targets a conserved eukaryotic process.

The employment of stress conditions in our screen enabled us to significantly enlarge the pool of yeast phenotypes available for *Pst* effectors. Growth inhibition phenotypes were identified for HopX1, HopG1, HopT1-1, HopH1 and AvrPtoB in yeast grown under various stress conditions, providing new tools to investigate *Pst* effectors that do not induce growth phenotypes in yeast under normal conditions. In future investigations, the growth inhibition phenotypes identified in this study will assist in the search for eukaryotic targets of *Pst* effectors.

Three of the effectors that inhibited growth only under stress conditions (i.e. HopX1, HopG1 and HopT1-1) did

so in the presence of NaCl and sorbitol, which are both osmotic stressors. The strongest growth inhibition phenotype under osmotic stress was caused by HopX1, while HopG1 and HopT1-1 caused a mild phenotype. Because yeast adaptation and survival under osmotic stress are largely mediated by the HOG MAPK pathway (Hohmann, 2002), we hypothesized and subsequently showed that HopX1 attenuated the activation of the HOG MAPK pathway. Conversely, HopG1 and HopT1-1 did not significantly affect the activation of the HOG pathway in the presence of NaCl, and had only a mild effect in the presence of sorbitol. Moreover, HopX1 caused a loss of cell viability under prolonged osmotic stress that was apparent only 10 h after exposure. This result is in agreement with a recent study which reported that yeast with an inactive HOG pathway are able to survive the first hours of exposure to osmotic stress, but lose their viability upon longer exposure (Maayan & Engelberg, 2009). Therefore, our results suggest that attenuation of the HOG MAPK pathway may account for the HopX1-mediated loss of cell viability under osmotic stress. However, HopX1 did not affect Hog1 expression levels or its nuclear translocation dynamics following exposure to osmotic stress. It is possible that HopX1 targets Hog1 and manipulates it in a manner that does not interfere with its nuclear translocation or stability, but hampers its activity or ability to bind to or modify downstream components of the pathway, such as transcriptional regulators (Hohmann, 2002). Alternatively, HopX1 may affect the activation of the HOG pathway by targeting a component of the pathway downstream of Hog1 (Hohmann, 2002).

Notably, the effect of HopX1 on the HOG pathway was more pronounced than on two other yeast MAPK pathways (i.e. CWI and pheromone-responsive pathways), while no effect was observed on the non-MAPK UPR pathway. The mild decrease in the activation of the CWI and pheromone-responsive pathways could result from a non-specific effect on a homologue of the HopX1 target, as components of MAPK pathways are highly similar (Gustin *et al.*, 1998). It is also possible that the mild effect on these pathways is indirect and stems from the cross-talk between MAPK pathways (García *et al.*, 2009; Patterson *et al.*, 2010; Rodríguez-Peña *et al.*, 2010).

The HopX1 intact putative transglutaminase catalytic triad was required for inhibiting yeast growth under osmotic stress and for attenuating the activation of the HOG pathway. Similarly, the intact catalytic triad of HopX1 was required for its virulence activity in plants (Nimchuk *et al.*, 2007). These results imply that the catalytic activity of HopX1 is responsible for the phenotypes observed in yeast and plants, thus supporting our hypothesis that the virulence activities of bacterial T3Es (and specifically of HopX1) can be studied and characterized in yeast.

MAPK pathways are conserved and also found in plants (Rodríguez *et al.*, 2010). Therefore, the ability of HopX1 to affect a yeast MAPK pathway is intriguing, as several plant

MAPK pathways have been shown to play a role in plant immunity against bacterial pathogens (Asai *et al.*, 2002; del Pozo *et al.*, 2004; Melech-Bonfil & Sessa, 2010; Tena *et al.*, 2011). Moreover, several bacterial T3Es, among them *Pst* effectors, have been shown to target plant MAPK pathways (Feng & Zhou, 2012; Shan *et al.*, 2007). However, it should be noted that while components of MAPK pathways are conserved between yeast and plants, the roles of the various pathways are not. Therefore, the effect of HopX1 on the yeast HOG MAPK pathway, which is responsible for osmotic adaptation, does not necessarily imply that HopX1 would affect a plant MAPK pathway that plays a similar role under osmotic stress.

ACKNOWLEDGEMENTS

We thank M. Kupiek (Tel Aviv University), C. Lesser (Massachusetts General Hospital and Harvard Medical School), R. Schekman (University of California, Berkeley) and H. Saito (University of Tokyo) for plasmids. This work was supported by a research grant from the Israel Science Foundation (ISF; grant no. 326/10). I.N. is an Alon fellow and a faculty fellow of the Edmond J. Safra Center for Bioinformatics at Tel Aviv University.

REFERENCES

- Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L., Gomez-Gomez, L., Boller, T., Ausubel, F. M. & Sheen, J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415**, 977–983.
- Auesukaree, C., Damnernsawad, A., Kruatrachue, M., Pokethitiyook, P., Boonchird, C., Kaneko, Y. & Harashima, S. (2009). Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. *J Appl Genet* **50**, 301–310.
- Block, A. & Alfano, J. R. (2011). Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? *Curr Opin Microbiol* **14**, 39–46.
- Block, A., Guo, M., Li, G., Elowsky, C., Clemente, T. E. & Alfano, J. R. (2010). The *Pseudomonas syringae* type III effector HopG1 targets mitochondria, alters plant development and suppresses plant innate immunity. *Cell Microbiol* **12**, 318–330.
- Bosis, E., Salomon, D. & Sessa, G. (2011). A simple yeast-based strategy to identify host cellular processes targeted by bacterial effector proteins. *PLoS ONE* **6**, e27698.
- Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S. & other authors (2003). The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci U S A* **100**, 10181–10186.
- Cox, J. S. & Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* **87**, 391–404.
- Cunnac, S., Lindeberg, M. & Collmer, A. (2009). *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. *Curr Opin Microbiol* **12**, 53–60.
- Cunnac, S., Chakravarthy, S., Kvitko, B. H., Russell, A. B., Martin, G. B. & Collmer, A. (2011). Genetic disassembly and combinatorial reassembly identify a minimal functional repertoire of type III

- effectors in *Pseudomonas syringae*. *Proc Natl Acad Sci U S A* **108**, 2975–2980.
- Curak, J., Rohde, J. & Stagljar, I. (2009). Yeast as a tool to study bacterial effectors. *Curr Opin Microbiol* **12**, 18–23.
- del Pozo, O., Pedley, K. F. & Martin, G. B. (2004). MAPKKK α is a positive regulator of cell death associated with both plant immunity and disease. *EMBO J* **23**, 3072–3082.
- Feng, F. & Zhou, J. M. (2012). Plant–bacterial pathogen interactions mediated by type III effectors. *Curr Opin Plant Biol* **15**, 469–476.
- Ferrigno, P., Posas, F., Koepf, D., Saito, H. & Silver, P. A. (1998). Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *EMBO J* **17**, 5606–5614.
- Fu, Z. Q., Guo, M., Jeong, B. R., Tian, F., Elthon, T. E., Cerny, R. L., Staiger, D. & Alfano, J. R. (2007). A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity. *Nature* **447**, 284–288.
- García, R., Rodríguez-Peña, J. M., Bermejo, C., Nombela, C. & Arroyo, J. (2009). The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. *J Biol Chem* **284**, 10901–10911.
- Gimenez-Ibanez, S., Hann, D. R., Ntoukakis, V., Petutschnig, E., Lipka, V. & Rathjen, J. P. (2009). AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. *Curr Biol* **19**, 423–429.
- Göhre, V., Spallek, T., Häweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J. W. & Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr Biol* **18**, 1824–1832.
- Gustin, M. C., Albertyn, J., Alexander, M. & Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **62**, 1264–1300.
- Ham, J. H., Majerczak, D., Ewert, S., Sreerekha, M. V., Mackey, D. & Coplin, D. (2008). WtsE, an AvrE-family type III effector protein of *Pantoea stewartii* subsp. *stewartii*, causes cell death in non-host plants. *Mol Plant Pathol* **9**, 633–643.
- Hersen, P., McClean, M. N., Mahadevan, L. & Ramanathan, S. (2008). Signal processing by the HOG MAP kinase pathway. *Proc Natl Acad Sci U S A* **105**, 7165–7170.
- Hoffman, G. A., Garrison, T. R. & Dohlman, H. G. (2002). Analysis of RGS proteins in *Saccharomyces cerevisiae*. *Methods Enzymol* **344**, 617–631.
- Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66**, 300–372.
- Howson, R., Huh, W. K., Ghaemmaghami, S., Falvo, J. V., Bower, K., Belle, A., Dephoure, N., Wykoff, D. D., Weissman, J. S. & O’Shea, E. K. (2005). Construction, verification and experimental use of two epitope-tagged collections of budding yeast strains. *Comp Funct Genomics* **6**, 2–16.
- Jelenska, J., Yao, N., Vinatzer, B. A., Wright, C. M., Brodsky, J. L. & Greenberg, J. T. (2007). A J domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. *Curr Biol* **17**, 499–508.
- Jung, U. S., Sobering, A. K., Romeo, M. J. & Levin, D. E. (2002). Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. *Mol Microbiol* **46**, 781–789.
- Kippert, F. (1995). A rapid permeabilization procedure for accurate quantitative determination of β -galactosidase activity in yeast cells. *FEMS Microbiol Lett* **128**, 201–206.
- Kuranda, K., Leberre, V., Sokol, S., Palamarczyk, G. & François, J. (2006). Investigating the caffeine effects in the yeast *Saccharomyces cerevisiae* brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. *Mol Microbiol* **61**, 1147–1166.
- Kvitko, B. H., Park, D. H., Velásquez, A. C., Wei, C. F., Russell, A. B., Martin, G. B., Schneider, D. J. & Collmer, A. (2009). Deletions in the repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion effector genes reveal functional overlap among effectors. *PLoS Pathog* **5**, e1000388.
- Maayan, I. & Engelberg, D. (2009). The yeast MAPK Hog1 is not essential for immediate survival under osmotic stress. *FEBS Lett* **583**, 2015–2020.
- Martin, G. B., Bogdanove, A. J. & Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. *Annu Rev Plant Biol* **54**, 23–61.
- Melech-Bonfil, S. & Sessa, G. (2010). Tomato MAPKKK ϵ is a positive regulator of cell-death signaling networks associated with plant immunity. *Plant J* **64**, 379–391.
- Munkvold, K. R., Martin, M. E., Bronstein, P. A. & Collmer, A. (2008). A survey of the *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion system effector repertoire reveals several effectors that are deleterious when expressed in *Saccharomyces cerevisiae*. *Mol Plant Microbe Interact* **21**, 490–502.
- Mur, L. A., Kenton, P., Lloyd, A. J., Ougham, H. & Prats, E. (2008). The hypersensitive response; the centenary is upon us but how much do we know? *J Exp Bot* **59**, 501–520.
- Nachman, I., Regev, A. & Ramanathan, S. (2007). Dissecting timing variability in yeast meiosis. *Cell* **131**, 544–556.
- Nimchuk, Z. L., Fisher, E. J., Desveaux, D., Chang, J. H. & Dangl, J. L. (2007). The HopX (AvrPphE) family of *Pseudomonas syringae* type III effectors require a catalytic triad and a novel N-terminal domain for function. *Mol Plant Microbe Interact* **20**, 346–357.
- Nomura, K., Debroy, S., Lee, Y. H., Pumphlin, N., Jones, J. & He, S. Y. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science* **313**, 220–223.
- Ntoukakis, V., Mucyn, T. S., Gimenez-Ibanez, S., Chapman, H. C., Gutierrez, J. R., Balmuth, A. L., Jones, A. M. & Rathjen, J. P. (2009). Host inhibition of a bacterial virulence effector triggers immunity to infection. *Science* **324**, 784–787.
- Parsons, A. B., Brost, R. L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G. W., Kane, P. M., Hughes, T. R. & Boone, C. (2004). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat Biotechnol* **22**, 62–69.
- Patterson, J. C., Klimenko, E. S. & Thorner, J. (2010). Single-cell analysis reveals that insulation maintains signaling specificity between two yeast MAPK pathways with common components. *Sci Signal* **3**, ra75.
- Preston, G. M. (2000). *Pseudomonas syringae* pv. *tomato*: the right pathogen, of the right plant, at the right time. *Mol Plant Pathol* **1**, 263–275.
- Rodríguez, M. C., Petersen, M. & Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. *Annu Rev Plant Biol* **61**, 621–649.
- Rodríguez-Herva, J. J., González-Melendi, P., Cuartas-Lanza, R., Antúnez-Lamas, M., Río-Alvarez, I., Li, Z., López-Torrejón, G., Díaz, I., Del Pozo, J. C. & other authors (2012). A bacterial cysteine protease effector protein interferes with photosynthesis to suppress plant innate immune responses. *Cell Microbiol* **14**, 669–681.
- Rodríguez-Peña, J. M., García, R., Nombela, C. & Arroyo, J. (2010). The high-osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways interplay: a yeast dialogue between MAPK routes. *Yeast* **27**, 495–502.

- Rosebrock, T. R., Zeng, L., Brady, J. J., Abramovitch, R. B., Xiao, F. & Martin, G. B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. *Nature* **448**, 370–374.
- Salomon, D. & Sessa, G. (2010). Identification of growth inhibition phenotypes induced by expression of bacterial type III effectors in yeast. *J Vis Exp* (37).
- Salomon, D., Dar, D., Sreeramulu, S. & Sessa, G. (2011). Expression of *Xanthomonas campestris* pv. *vesicatoria* type III effectors in yeast affects cell growth and viability. *Mol Plant Microbe Interact* **24**, 305–314.
- Schacherer, J., Ruderfer, D. M., Gresham, D., Dolinski, K., Botstein, D. & Kruglyak, L. (2007). Genome-wide analysis of nucleotide-level variation in commonly used *Saccharomyces cerevisiae* strains. *PLoS ONE* **2**, e322.
- Shan, L., He, P. & Sheen, J. (2007). Intercepting host MAPK signaling cascades by bacterial type III effectors. *Cell Host Microbe* **1**, 167–174.
- Siggers, K. A. & Lesser, C. F. (2008). The yeast *Saccharomyces cerevisiae*: a versatile model system for the identification and characterization of bacterial virulence proteins. *Cell Host Microbe* **4**, 8–15.
- Slagowski, N. L., Kramer, R. W., Morrison, M. F., LaBaer, J. & Lesser, C. F. (2008). A functional genomic yeast screen to identify pathogenic bacterial proteins. *PLoS Pathog* **4**, e9.
- Tatebayashi, K., Yamamoto, K., Tanaka, K., Tomida, T., Maruoka, T., Kasukawa, E. & Saito, H. (2006). Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway. *EMBO J* **25**, 3033–3044.
- Tatebayashi, K., Tanaka, K., Yang, H. Y., Yamamoto, K., Matsushita, Y., Tomida, T., Imai, M. & Saito, H. (2007). Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J* **26**, 3521–3533.
- Tena, G., Boudsocq, M. & Sheen, J. (2011). Protein kinase signaling networks in plant innate immunity. *Curr Opin Plant Biol* **14**, 519–529.
- Valdivia, R. H. (2004). Modeling the function of bacterial virulence factors in *Saccharomyces cerevisiae*. *Eukaryot Cell* **3**, 827–834.
- Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., Chen, S., Tang, X. & Zhou, J. M. (2010). A *Pseudomonas syringae* ADP-ribosyltransferase inhibits *Arabidopsis* mitogen-activated protein kinase kinases. *Plant Cell* **22**, 2033–2044.
- Wilton, M., Subramaniam, R., Elmore, J., Felsensteiner, C., Coaker, G. & Desveaux, D. (2010). The type III effector HopF2Pto targets *Arabidopsis* RIN4 protein to promote *Pseudomonas syringae* virulence. *Proc Natl Acad Sci U S A* **107**, 2349–2354.
- Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L. & other authors (2008). *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr Biol* **18**, 74–80.
- Yoon, S., Liu, Z., Eyobo, Y. & Orth, K. (2003). *Yersinia* effector YopJ inhibits yeast MAPK signaling pathways by an evolutionarily conserved mechanism. *J Biol Chem* **278**, 2131–2135.
- Zeng, L., Velásquez, A. C., Munkvold, K. R., Zhang, J. & Martin, G. B. (2012). A tomato LysM receptor-like kinase promotes immunity and its kinase activity is inhibited by AvrPtoB. *Plant J* **69**, 92–103.

Edited by: G. Preston