WtsE, an AvrE-family type III effector protein of *Pantoea stewartii* subsp. *stewartii*, causes cell death in non-host plants

JONG HYUN HAM^{1,2,†}, DORIS MAJERCZAK², SOPHIE EWERT¹,
MYSORE-VENKATARAU SREERAKHA¹, DAVID MACKKEY¹ AND DAVID COPLIN^{2,*}

Departments of ¹Horticulture and Crop Science and ²Plant Pathology, The Ohio State University, Columbus, OH 43210, USA

SUMMARY

Pantoea stewartii subsp. *stewartii* (*Pnss*) causes Stewart's bacterial wilt of sweet corn and leaf blight of maize. The pathogenicity of *Pnss* depends on synthesis of extracellular polysaccharide and an Hrp type III secretion system. WtsE, a type III secreted effector protein, is essential for the virulence of *Pnss* on corn. It belongs to the AvrE family of effectors, which includes DspA/E from *Erwinia amylovora* and AvrE1 from *Pseudomonas syringae*. Previously, WtsE was shown to cause disease-associated cell death in its host plant, sweet corn. Here, we examine the biological activity of WtsE in several non-host plants. WtsE induced cell death in *Nicotiana benthamiana*, tobacco, beet and *Arabidopsis thaliana* when it was transiently produced in plant cells following agroinfiltration or translocated into plant cells from *Pnss*, *Escherichia coli* or *Pseudomonas syringae* pv. *phaseolicola* (*Pph*). WtsE-induced cell death in *N. benthamiana*, tobacco and beet resembled a hypersensitive response and in *N. benthamiana* it was delayed by cycloheximide. Interestingly, WtsE strongly promoted the growth of *Pnss* in *N. benthamiana* prior to the onset of cell death. Deletion derivatives of WtsE that failed to induce cell death in *N. benthamiana* and tobacco also did not complement *wtsE* mutants of *Pnss* for virulence in sweet corn, indicating a correlation between the two activities. WtsE also induced cell death in *A. thaliana*, where it suppressed basal defences induced by *Pph*. Thus, WtsE has growth-promoting, defence-suppressing and cell death-inducing activities in non-host plants. Expression of WtsE also prevented the growth of yeast, possibly due to an innate toxicity to eukaryotic cells.

INTRODUCTION

Pantoea stewartii subsp. *stewartii* (*Pnss*) causes Stewart's bacterial wilt and leaf blight of sweet corn and maize (*Zea mays* L.). This disease is a serious problem in the north-central and eastern United States (Pateky, 2003). *Pnss* is transmitted by the corn flea beetle (*Chaetocnema pulicaria*) and it overwinters in the beetle's gut (Claflin, 1999). Bacteria grow in the xylem vessels of the corn plant causing wilt and in the intercellular spaces of the leaves causing 'water-soaked' lesions. An important virulence factor for wilting is extracellular polysaccharide (EPS) slime (Dolph *et al.*, 1988). The earliest symptom of infection is water-soaked lesions on young leaves. Water-soaking (Wts) results from the release of water and nutrients following cell membrane damage. Bacterial EPS holds the fluids in the leaf tissue so that the lesions appear water-soaked for several days before turning necrotic. Using inoculation procedures that distinguish between Wts and wilting, we have shown that the Hrp type III secretion system (TTSS) is needed to produce both symptoms. Other classical virulence factors, such as degradative enzymes, toxins and growth regulators, are not known to be produced by *Pnss*. At this point, WtsE is the only TTSS effector protein known to be required for virulence of *Pnss* (D. Majerczak and D. Coplin, unpublished data). WtsE belongs to the widespread and conserved AvrE-family of type III effectors (Frederick *et al.*, 2001, Ham *et al.*, 2006). In most cases, loss of an AvrE-family effector by an erwinia or pseudomonad plant pathogen greatly reduces virulence. This is especially true for *Pnss* WtsE, *Pantoea agglomerans* DspE (Mor *et al.*, 2001), *Erwinia amylovora* (*Ea*) DspA/E (Bogdanove *et al.*, 1998, Gaudriault *et al.*, 1997) and *Pseudomonas syringae* AvrE (Lorang *et al.*, 1994).

In a previous study (Ham *et al.*, 2006), WtsE was shown to induce cell death in susceptible corn seedlings when it was delivered by *Escherichia coli* MC4100 carrying the heterologous TTSS from *Dickeya dadantii* (*Dda*, formerly *Erwinia chrysanthemi*) (Ham *et al.*, 1998). The WtsF chaperone protein was needed for this delivery system, but was not required for secretion by *Pnss*. Similarly, other AvrE family proteins have been shown to cause symptoms in both host and non-host plants due to either virulence or avirulence activities. Boureau *et al.* (2006) have

*Correspondence: Tel.: +1 614 292 8503; Fax: +1 614 292 4455; E-mail: coplin.3@osu.edu

†Present address: Department of Plant Pathology and Crop Physiology, Louisiana State University AgCenter, Baton Rouge, LA 70803-1720, USA.

shown that transiently expressed *Ea* DspA/E induces necrosis in both apple and tobacco leaves. Oh *et al.* (2007) have reported that DspA/E causes programmed cell death in *Nicotiana benthamiana* when it is transiently expressed by agroinfiltration and that this response is dependent on the defence signalling protein SGT1. Surprisingly, despite inducing what appears to be a hypersensitive response (HR), DspA/E enhances *E. amylovora* growth in the first day following infection of *N. benthamiana*. Badel *et al.* (2006) reported that *Agrobacterium*-mediated transient expression of *P. syringae* pv. *tomato* AvrE1 elicits cell death in tobacco leaves as well as in the susceptible host, tomato. At this point, it is unclear if the response elicited in non-hosts by AvrE family effectors is always an HR and if susceptible and non-host responses involve the same mechanisms. For example, *Ea* DspE/A may act as a virulence factor to enhance bacterial growth in *N. benthamiana* prior to inducing a non-host HR (Oh *et al.*, 2007). (Here, the term 'virulence' will be used to designate the properties of effectors that promote bacterial growth, elicit disease-associated cell death or suppress host defences.)

In the present study, we characterized the biological activities of WtsE in various non-host plants using different delivery systems. We found that, in addition to the host plant corn, WtsE-induced cell death (WICD) also occurs in non-host plants including tobacco, *N. benthamiana*, beet and *A. thaliana*. Furthermore, we demonstrate virulence activities for WtsE in both *N. benthamiana* and *A. thaliana*. Our results indicate that WtsE and other AvrE family members may have a similar target(s) that accounts for their virulence activity in susceptible hosts and their avirulence activity in resistant plants and that *N. benthamiana* and *A. thaliana* are relevant model systems to study the activities of WtsE.

RESULTS

Pnss causes cell death in non-host plants in a *wtsE*-dependent manner

In sweet corn, *Pnss* causes Wts within 12 h when introduced into corn leaves by vacuum infiltration at 2×10^8 CFU/mL. This response was dependent on WtsE (Frederick *et al.*, 2001, Ham *et al.*, 2006). However, in tobacco leaves, *Pnss* can only elicit cell death when the *hrp* genes are pre-induced, either by growth in an inducing medium that mimics the environmental conditions in plant apoplastic spaces or when *hrpS*, a transcriptional activator of the *hrp* genes, is constitutively expressed under a strong promoter in plasmid pRF205 (Frederick *et al.*, 1993). In this study, we characterized the ability of *Pnss* wild-type and mutant strains, all carrying pRF205 (*P_{lac}-hrpS⁺*), to induce cell death in a variety of non-host plants. When infiltrated into leaves at 2×10^8 CFU/mL, wild-type *Pnss* caused rapid, HR-like cell death in *N. benthamiana*,

tobacco and beet as soon as 12 h later and Wts in *A. thaliana* after 24 h (Fig. 1A–D). To determine the WtsE-dependence of *Pnss*-induced cell death in these plants, we compared wild-type *Pnss* (DC283) with a WtsE[−] strain (AA005) (Ham *et al.*, 2006). AA005 failed to cause cell death in *N. benthamiana*, beet and *A. thaliana*, but still caused cell death in tobacco (Fig. 1B). As AA005 also produces harpin (HrpN) that alone can elicit an HR in tobacco (Ahmad *et al.*, 2001), we tested an *hrpN* mutant (DM760) and a *wtsE hrpN* double mutant (DM2860). DM760 also caused cell death in tobacco (data not shown), but DM2860 did not (Fig. 1B). These results indicate that *Pnss*-induced cell death in *N. benthamiana*, beet and *A. thaliana* is dependent on WtsE, whereas cell death in tobacco can be elicited by either WtsE or harpin.

WtsE alone is sufficient for causing cell death in non-host plants and yeast

We previously showed that WtsE alone was capable of causing Wts in corn leaves in the absence of other possible TTSS effectors produced by *Pnss*. In these experiments, we used a heterologous delivery system to introduce WtsE into plant cells. This system consisted of *E. coli* MC4100 carrying cosmid pCPP2156 that contains the entire *hrp*-gene cluster from *Dda*. This Hrp TTSS is known to promiscuously secrete type III effectors from a number of plant and animal pathogens into culture supernatants and deliver them into plant cells (Anderson *et al.*, 1999, Ham *et al.*, 1998). We found that the associated WtsF chaperone protein was also needed for translocation of WtsE by this system. Here, we used this delivery system to determine the effect of WtsE on non-host plant cells. As shown in Fig. 1E,F, *E. coli* MC4100 (pCPP2156/pJH021 *wtsEF⁺*) caused rapid WICD in *N. benthamiana* and tobacco leaves, while *E. coli* MC4100 (pCPP2156) alone did not cause any symptoms in these plants. As described below, MC4100 (pCPP2156/pJH021) also caused electrolyte leakage in tobacco leaves. In contrast, MC4100 (pCPP2156/pJH021) did not cause WICD in beet leaves. Unfortunately, we were unable to assess the activity of WtsE in *A. thaliana* using this delivery system because MC4100 (pCPP2156) caused tissue collapse in *A. thaliana*, possibly due to the activity of harpin produced by the *Dda hrp* gene cluster (data not shown).

A second heterologous delivery system for introducing WtsE into non-host plants was transient expression mediated by *Agrobacterium tumefaciens* C58C1. We used the pBDex (Allen *et al.*, 2004) and pGD (Goodin *et al.*, 2002) binary vector systems to express WtsE and its derivatives. Infiltration of *Ag. tumefaciens* carrying the wild-type *wtsE* clone (pJH101) or a derivative lacking the first 236 amino acids (pJH028 or pJH102) induced WICD in tobacco (data not shown) and *N. benthamiana* leaves (Fig. 1G–H). Experiments with *Agrobacterium*-mediated transient expression of WtsE did not result in cell death in *A. thaliana* and beet.

Fig. 1 WtsE-induced cell death in a variety of non-host plants. (A–D) Wild-type or mutant *P. stewartii* strains carrying pRF205 were infiltrated at 2×10^8 CFU/mL into leaves of (A) *N. benthamiana*, (B) tobacco, (C) beet and (D) *A. thaliana*. (E,F) *E. coli* MC4100 (pCPP2156/pJH021) expressing the *D. dadantii* TTSS and WtsE or *E. coli* MC4100 (pCPP2156) without WtsE were infiltrated at 1×10^9 CFU/mL into leaves of (E) *N. benthamiana* and (F) tobacco. (G,H) Wild-type *wtsE* or derivatives were transiently expressed by infiltrating 10^9 CFU/mL of *Ag. tumefaciens* C58C1 into leaves of *N. benthamiana*. (I) *P. syringae* pv. *phaseolicola* NPS3121 expressing WtsE or carrying a control vector were infiltrated at 1×10^8 CFU/mL into leaves of 4-week-old *A. thaliana*. Pictures were taken at 24 h (A–F) or 48 h (H–I) after infiltration or 24 h after spraying with dexamethasone at 48 h after infiltration (G) and are representative of three or more separate experiments.

However, agroinfiltration seems inefficient at least in wild-type *A. thaliana* due to its resistance to *Ag. tumefaciens* (Zipfel *et al.*, 2006).

In addition, WtsE activity in yeast cells was examined by cloning *wtsE* in a conditional expression vector derived from pMyr (Stratagene) and expressing it under control of the *GAL1* promoter, which is induced by galactose. As shown in Fig. 2A, the *Saccharomyces cerevisiae* strain cdc25H expressing full-length WtsE did not grow in galactose medium, while yeast cells expressing the N- or C-terminal halves of WtsE grew normally. The fact that the clones encoding the two halves of WtsE have been used to identify specific interacting proteins by yeast two-hybrid assays indicates that these derivatives are expressed and stable in yeast. In addition, we introduced the *wtsE* gene into the chromosome of strain Y7092 and found that it also inhibited growth when induced by galactose (Fig. 2B). This activity of WtsE in yeast cells indicates that it may be toxic to eukaryotic cells in general.

WtsE-induced cell death in non-hosts is delayed by cycloheximide

Our previous work demonstrated that WICD in corn differs in nature from defence-related cell death. Specifically, cycloheximide

(CHX) inhibited a gene-for-gene HR elicited by *Xanthomonas oryzae* pv. *oryzae* with *avrRxo1* in B73 maize, but failed to inhibit *Pnss*-induced WICD in sweet corn (Ham *et al.*, 2006). In this study, we tested the sensitivity of WICD in non-host plants to CHX. In tobacco, *P. syringae* pv. *tomato* strain DC3000 induces a non-host HR that was inhibited by CHX (Bozso *et al.*, 1999). Similarly, macroscopic necrosis induced by MC4100 (pCPP2156/pAA008), expressing the *Dda* TTSS and WtsE, was delayed and significantly reduced by CHX (data not shown). To quantify the CHX-induced attenuation of WICD, we measured electrolyte leakage (EL) at various times after infiltration of tobacco with bacteria and CHX. WtsE-induced EL increased up to 19 h and then levelled off (Fig. 3 and data not shown). EL was reduced two-fold by CHX between 6 and 19 h, but at 24 h, CHX alone caused considerable EL. Although visible symptoms were not seen in the MC4100 (pCPP2156) controls, this strain did cause significant EL. This may be due to the production and secretion of harpin by the *Dda hrp* genes, as this EL was also inhibited two-fold by CHX. Similar results were obtained in *N. benthamiana*, but CHX was somewhat toxic to this plant and this resulted in high variability (data not shown). WICD caused by *Pnss* DC283 (pRF205) in beet was likewise inhibited by coinfiltration with CHX. In contrast, we did not see an effect of CHX on WICD in *A. thaliana*. However,

Fig. 2 WtsE inhibited the growth of yeast. *S. cerevisiae* strains cdc25H (A) or Y7092 (B) did not proliferate when conditional expression of wild-type *wtsE* was induced by galactose. Expression of the N-terminal (N-WtsE, amino acids 1–964) or C-terminal (C-WtsE, amino acids 964–1835) halves of WtsE (A) or of WtsE-Δaa1831–1834 (B) had no detrimental effect on yeast growth. Growth on suppressive glucose media demonstrated the viability of uninduced yeast.

Fig. 3 WtsE-induced cell death in tobacco was inhibited by cycloheximide (CHX). Electrolyte leakage was used quantitatively to measure cell death caused by *E. coli* MC4100 (pCPP2156/pJH021), expressing the *D. dadantii* type III secretion system and *wtsE*, or *E. coli* MC4100 (pCPP2156) without *wtsE* in tobacco leaves. Leaves were infiltrated with bacteria (1×10^9 CFU/mL) $\pm$ 100 μ g/mL CHX. Infiltrated leaf discs were placed in water and conductivity was measured over time. For each treatment, the conductivity value of the corresponding negative control (buffer or buffer with CHX) was subtracted. Then each value was converted to a percentage of the maximum leakage obtained with MC4100 (pCPP2156/pJH021) at 19 h in the same experiment; the later values ranged from 18.5 to 60.3 μ S/cm. Values shown are the mean of five experiments $\pm$ SD.

interpretation of this result is difficult as CHX also failed to inhibit the HR induced by known gene-for-gene activation of RPM1 and RPS2 by AvrRpm1 and AvrRpt2, respectively (data not shown).

Deletion analysis revealed important portions of WtsE for both cell death and virulence activities

WtsE contains several putative sequence motifs, including a leucine zipper, a coiled-coil domain, a nuclear localization signal (NLS), and two endoplasmic reticulum membrane retention/retrieval signals (ERMRS) (Ham *et al.*, 2006). Among these motifs, only the C-terminal ERMRS and an NLS are present in most AvrE family proteins. However, the location of the NLS is not conserved. We previously reported that the C-terminus of WtsE, which contains an ERMRS motif (LKKEGFEMKS) predicted by PSORTII, is important for the virulence function of WtsE. This was based on the non-pathogenic phenotype of a miniTn5gus insertion in *wtsE* that removes the last 12 amino acids (Ham *et al.*, 2006). In this study, we have precisely deleted the penultimate four amino acids (1831–1834, FEMK) from the putative ERMRS. When delivered by the *E. coli* MC4100 (pCPP2156) system this mutant protein (WtsE-Δaa1831–1834) did not cause WICD in tobacco (Table 1) and its activity was greatly reduced when highly expressed after agroinfiltration in *N. benthamiana* (Fig. 1G). In yeast, the WtsE-Δaa1831–1834 protein was not toxic and the yeast strain grew normally (Fig. 2). Finally, the WtsE-Δaa1831–1834 protein did not restore the virulence of *Pnss wtsE* mutants in sweet corn, when it was used to complement *Pnss* DM5101 *wtsE::miniTn5gus* (data not shown) and AA005 *wtsE::aphT* (Table 1). These findings confirm the importance of the C-terminus of WtsE for its ability to induce cell death in plants and yeast.

To determine if the NLS is also involved in cell death or virulence, the amino acid residues KKHK, which constitute the

Table 1 Summary of results for cell-death and virulence activities of wild-type WtsE and its derivatives.

Derivative	Cell death in <i>N. benthamiana</i> and tobacco using the <i>E. coli</i> delivery system*	Cell death in <i>N. benthamiana</i> by agroinfiltration†	Complementation of <i>Pnss</i> AA005 <i>wtsE</i> for virulence§
no WtsE	–	–	0.1 ± 0.1
wild-type WtsE	+	+	2.4 ± 0.6
WtsE-Δaa51-236‡	+	+	2.4 ± 0.8
WtsE-Δaa51-492‡	–	–	0.5 ± 0.5
WtsE-Δaa1831-1834	–	–	0.7 ± 0.6
WtsE NLS' (KKHK1358-61AAAA)	+	not done	2.4 ± 0.6

Results for cell death when the indicated WtsE derivatives (or empty vector, no WtsE) were delivered from **E. coli* MC4100 (pCPP2156) or †*A. tumefaciens*, as described in Fig. 1.

‡For agroinfiltration, derivatives of WtsE were Δaa1-236 and Δaa1-492.

§Derivatives of WtsE (or empty vector, no WtsE) were tested for their ability to restore the virulence of *Pnss* AA005 *wtsE::aphT* on sweet corn. Six-day-old seedlings were inoculated by placing the inoculum into the whorls and disease symptoms were rated 4 days later. Results of the representative experiment shown are the average and standard deviation from 18–22 plants. The experiment was done four times.

NLS of WtsE, were changed to AAAA and the resulting mutant plasmid, pJH049, was tested for WICD activity in the same manner. This mutation affected neither the ability of WtsE to cause WICD using the MC4100 (pCPP2156) delivery system in tobacco and *N. benthamiana* nor its ability to complement AA005, which has an aphT insertion in the middle of *wtsE*, for virulence in sweet corn (Table 1). Intriguingly, the NLS mutant did not consistently complement DM5101, which has a mini-Tn5 insertion at the beginning of *wtsE* (data not shown).

A region near the N-terminus of WtsE was also required for WICD in both sweet corn and non-hosts. Using agroinfiltration, we found that a derivative of WtsE lacking the N-terminal 236 amino acids (pJH102) still produced WICD in *N. benthamiana*. However, a deletion removing the first 492 amino acids (pJH103) did not (Fig. 1H). This indicates that the region of WtsE between A237 and F492 is required for WICD. This finding was confirmed using the MC4100 delivery system. As Hrp secretion signals are typically at the N-terminus (Petnicki-Ocwieja *et al.*, 2002), we added back the first 50 amino acids to these two N-terminal deletion mutants. Plasmids pJH041 *wtsE*-Δaa51-236 and pJH043 *wtsE*-Δaa51-492 were constructed in pBluescript SK and introduced into *E. coli* MC4100 (pCPP2156). WtsEΔ51-236 caused WICD confirming the agroinfiltration result and indicating that this protein was indeed translocated. In contrast, WtsE-Δaa51-492 did not cause WICD, confirming the importance of amino acids 237–492 (Table 1). To determine if this portion of WtsE is also required for the virulence of *Pnss*, the internal deletion mutations were cloned into a broad host range and low copy number plasmid, pRK415, and introduced into *Pnss* AA005 *wtsE* for complementation tests in sweet corn. Similarly, WtsE-Δaa51-236, but not WtsE-Δaa51-492, restored the virulence of the *wtsE* mutant (Table 1). Overall, the ability of the above mutants to cause WICD in non-hosts was positively correlated with their ability to complement a *Pnss wtsE* mutant for virulence in sweet corn.

WtsE has virulence activity in non-host plants

Although the response of *N. benthamiana* to WtsE superficially resembles an HR, our results do not preclude that WtsE also carries out virulence functions in this non-host. Oh *et al.* (2007) showed that DspA/E promotes the early growth of *Ea* in *N. benthamiana*, despite later inducing a non-host HR. We have similarly compared the growth of wild-type and *wtsE Pnss* strains in *N. benthamiana* (Fig. 4). The population of the wild-type strain increased over one log during the first 12 h after infiltration, but declined after the onset of necrosis at 24 h. In contrast, the *wtsE* mutant declined steadily after only 6 h. Thus, WtsE has a virulence activity that can promote the growth of *Pnss* in *N. benthamiana* prior to inducing HR-like necrosis.

We also sought to determine if WtsE can suppress basal defences in *A. thaliana*, where it causes a Wts-like necrosis. WtsE was translocated into *A. thaliana* leaf cells by *P. syringae* pv. *phaseolicola* (*Pph*) NPS3121. In ecotype Col-0, this *Pph* strain causes minimal cell death and induces strong basal defence responses, including induction of the defence protein PR-1 and deposition of two morphologically distinct types of callose (small and big callose) (Ham *et al.*, 2007). When infiltrated into Col-0 leaves at 1×10^8 CFU/mL, NPS3121 (pJH082 *wtsEF*⁺) caused water-soaked lesions (Fig. 1I). In addition to causing symptoms, WtsE also suppressed early *Pph*-induced defence responses. Induction of PR1 was significantly reduced when *Pph* expressed WtsE (Fig. 5A). Also, prior to the appearance of symptoms (15 h after infiltration) formation of big callose deposits was almost completely suppressed and formation of small callose deposits was significantly reduced (Fig. 5B).

DISCUSSION

AvrE family effector proteins are important for the full virulence of most Gram-negative plant pathogenic bacteria in the

Fig. 4 WtsE promotes early growth of *P. stewartii* in *N. benthamiana*. Wild-type (solid triangles) and *wtsE* (open squares, strain DM5101) *Pnss* were hand-infiltrated at 5×10^8 CFU/mL and viable cell counts were monitored over the course of 2 days. Both strains carried pRF205, which induces Hrp gene expression. The experiment has been done three times with similar results. The bacterial populations $\pm$ SD shown are the combined data from two of the experiments. Similar results were obtained with strain AA005 *wtsE* (not shown).

Enterobacteriaceae and Pseudomonadaceae (reviewed in Ham *et al.*, 2006), but they were first discovered due to their ability to induce an HR in non-hosts. The prototype for this group, *P. syringae* pv. *tomato* AvrE, was initially identified as an avirulence gene in soybean. Later, this family was shown to cause both cell death in susceptible hosts and the HR in many non-hosts. For example, Badel *et al.* (2006) reported that transient expression of AvrE1 elicited cell death in tobacco and *N. benthamiana* leaves, as well as susceptible tomato leaves. Likewise, *Ea* DspA/E, an essential pathogenicity factor, was reported to induce cell death in host (apple) and non-host (tobacco) plants when transiently expressed (Boureau *et al.*, 2006). We likewise reported that WtsE alone can cause Wts in sweet corn (Ham *et al.*, 2006). Plant responses to DspA/E and AvrE effectors have not been well studied in susceptible hosts because they have not been associated with a disease of an ideal model plant and/or the pathogen has other effectors with a redundant function, e.g. HopM1 in *P. syringae* pv. *tomato* DC3000 (DebRoy *et al.*, 2004).

Here, we show that WtsE alone has cell death activity in a variety of non-host plants. During the course of these studies, we developed one or more delivery systems for these non-hosts so that we will be able to study the mode of action of WtsE in several

Fig. 5 WtsE suppressed basal defence responses in *A. thaliana*. (A) WtsE suppressed *P. syringae* pv. *phaseolicola* (*Pph*)-induced accumulation of PR-1 protein. Buffer or *Pph* expressing either WtsE (from pJH082) or carrying a control vector were infiltrated into *A. thaliana* leaves at 1×10^8 CFU/mL. After 48 h, total protein extracts were prepared from infiltrated leaves and subjected to anti-PR-1 Western blotting. The lower panel shows Ponceau-S staining to control for protein loading. Results are representative of three or more experiments. (B) WtsE suppressed *Pph*-induced callose deposition. Leaves infiltrated as in (A) were collected 15 h after infiltration and callose was stained with aniline blue. Both small (<20 μm) and big (>20 μm) callose deposits were significantly reduced when *Pph* expressed *wtsE*. Both types of callose deposits were counted in representative areas of five leaves for each treatment from three separate experiments and the average and standard deviation from all counts are shown.

model plants. First, *Pnss* strains constitutively expressing *hrpS* caused cell death in *N. benthamiana*, *A. thaliana* and beet in a *wtsE*-dependent manner. In tobacco, however, both *wtsE* and *hrpN* *Pnss* mutants induced cell death, but a *wtsE hrpN* double mutant did not, indicating that either WtsE or harpin can induce cell death in this plant. In contrast, *N. benthamiana* and *A. thaliana* were not responsive to the amount of harpin produced by *Pnss* in planta. Second, *Agrobacterium*-mediated transient expression of *wtsE* elicited WICD in tobacco and *N. benthamiana*. Third, translocation of WtsE from *E. coli* MC4100 (pCPP2156 *hrpDda*⁺) resulted in WICD in *N. benthamiana* and tobacco, but not in beet

leaves. Unfortunately, the *E. coli* delivery system could not be used in *A. thaliana* because MC4100 (pCPP2156) alone induced tissue collapse and agroinfiltration did not work in *A. thaliana* Col-0 and some plantings of tobacco did not respond well. However, WtsE did cause Wts symptoms in *A. thaliana* when it was delivered by a fourth system, *Pph* NPS3121. From these results, we conclude that expression of WtsE alone is sufficient to cause cell death in a variety of host and non-host plants. Conditional expression of WtsE also arrested the growth of yeast cells, indicating it is toxic to a broad spectrum of eukaryotic cells.

In non-hosts, WICD could either be an HR due to the avirulence activity of WtsE or be similar to the disease-associated Wts response in corn. These responses are difficult to distinguish based solely on symptoms and timing. Moreover, the mechanisms needed to elicit either one could be similar if R-proteins are guarding one or more of the intended cellular targets of WtsE. In our previous study, we demonstrated that WtsE-induced Wts in corn seedlings was not inhibited by CHX, which blocks *de novo* protein synthesis in eukaryotic cells (Ham *et al.*, 2006). In the same study, a control HR in corn seedlings, derived from a known gene-for-gene interaction, was inhibited by CHX, indicating the susceptible Wts response differs from a defence-associated HR. In an attempt to determine whether or not WICD in non-host plants involves an HR, we again tested for inhibition by CHX. Interestingly, WICD was delayed but not fully inhibited in the leaves of *N. benthamiana*, tobacco and beet treated with CHX. Thus, we hypothesize that WtsE is capable of inducing two types of cell death in non-host plants. First, some hosts, such as tobacco and beet, may respond with an HR typified by CHX-sensitive, dry, collapsed necrosis. Second, they may exhibit disease-associated WICD, if an HR is inhibited by CHX (or corresponding R-genes are absent). Thus, the delayed appearance of mild necrosis in CHX-treated tobacco, *N. benthamiana* and beet may have been due to the unmasking of disease-associated WICD, once an HR was blocked. Further work is needed to characterize these responses. However, the ability of wild-type, but not *wtsE*, strains of *Pnss* to grow initially in *N. benthamiana* suggests that the initial response of this plant may be disease-associated cell death that later triggers an HR. In *A. thaliana*, however, our efforts to use CHX to determine the nature of WICD were uninformative because it failed to inhibit both WICD and known HRs. At this point, we favour the hypothesis that the response in *A. thaliana* represents disease-associated cell death because, when delivered by *Pph*, it induces tissue collapse that is phenotypically distinct from known HRs and resembles the disease-associated cell death induced by AvrE1 and HopM1 when they are delivered by *Pph* (data not shown).

The delivery systems we developed were used to determine the effects of various mutations in *wtsE* on virulence in *Pnss* and on non-host cell death. Deletion analysis revealed that the first 236 amino acids of WtsE were dispensable for the cell death activity when WtsE was transiently expressed in *N. benthamiana*

following agroinfiltration (Fig. 1H). This was confirmed by the ability of WtsE lacking amino acids 51–236 to induce cell death comparable with wild-type WtsE in *N. benthamiana* when delivered by *E. coli* MC4100 (pCPP2156) and to fully complement a *wtsE* strain of *Pnss* (Table 1). In addition, these results indicate that the entire secretion signal is probably contained within the first 50 amino acids of WtsE. Extending this deletion to amino acids 492 disrupted the ability of WtsE to cause cell death and to complement a *wtsE* strain of *Pnss*, indicating that amino acids 237–492 are important for both the avirulence and the virulence activities of WtsE. In contrast, mutation of the putative NLS did not significantly affect the cell death or virulence activities of WtsE (Table 1). Our preliminary finding that the putative ERMRS is needed for virulence (Ham *et al.*, 2006) was confirmed by a more precise deletion of the penultimate four amino acids at the C-terminus of WtsE, which removes the right half of this motif. This change resulted in a large reduction of both cell death and virulence activities. It is unusual that WtsE would have this retention signal, as it lacks an apparent ER targeting motif. An interesting possibility is that WtsE provides the retention signal *in trans* to an ER-resident protein with which it interacts. Meng *et al.* (2006) have shown that *Ea* DspA/E interacts with several leucine-rich-repeat receptor-like kinases (LRR RLKs) from apple in the yeast two-hybrid system and we have similar preliminary data for WtsE (J. Ham *et al.*, unpublished). As these potential defence signalling molecules are probably trafficked from the ER to their active site at the plasma membrane, it may be that WtsE blocks their function by retaining them in the ER. This may be an important defence-suppressing activity, as the expression of numerous LRR RLKs is up-regulated during plant basal defences (Navarro *et al.*, 2004; Thilmony *et al.*, 2006).

It is significant that mutations in WtsE have so far failed to separate the induction of HR-like cell death in non-hosts from its virulence activities in sweet corn. This suggests that elicitation of both an HR and disease-associated WICD involve the same mechanism. As the AvrE family effectors are conserved in many plant pathogenic bacteria and essential for their virulence, it is reasonable that plants would have evolved a corresponding family of R-proteins. Our finding is consistent with the guard hypothesis, which proposes that plant defence systems indirectly detect effector proteins by recognizing perturbation of a host target(s) associated with their virulence activity. In response, the very large AvrE family effectors appear to have also incorporated the ability to suppress some host defences. AvrE1 has been shown to suppress defences in *A. thaliana* (DebRoy *et al.*, 2004) and we have shown the same for WtsE here. This may simply be a second function of WtsE that disarms basal resistance, but it also raises the possibility that the Wts caused by WtsE in corn and *A. thaliana* may be a form of controlled, partially suppressed programmed cell death. In conclusion, we have demonstrated that WtsE elicits cell death in *N. benthamiana*, tobacco, *A. thaliana* and beet, that

it carries out both growth-promoting and defence-suppressing virulence functions in *N. benthamiana* and *A. thaliana*, and that it is toxic to yeast. Thus, the WtsE delivery systems that we have developed for the model plants, *A. thaliana* and *N. benthamiana*, may be valuable for studying the functions of an important family of bacterial effectors proteins that is conserved in many bacterial pathogens of plants.

EXPERIMENTAL PROCEDURES

Bacterial and yeast strains and their growth conditions

Bacterial and yeast strains and plasmids used in this study are described in Table 2. *E. coli*, *Pnss* and *Ag. tumefaciens* strains were routinely grown and maintained in Luria–Bertani (LB) broth or agar (Sambrook *et al.*, 1989) at 28 °C for *Pnss* and *Ag. tumefaciens* strains and at 37 °C for *E. coli* strains. *P. syringae* pv. *phaseolicola* strains were grown in King's B agar (Schaad *et al.*, 2001) at 28 °C. Appropriate antibiotics were supplied as needed at the following concentrations (µg/mL): ampicillin (Ap), 200; chloramphenicol (Cm), 34; kanamycin (Km), 100 for *P. s.* pv. *phaseolicola* and 50 for *Pnss* and *E. coli*; nalidixic acid (Nal), 20; spectinomycin (Sp), 50; rifampicin (Rif), 100; and tetracycline (Tc), 25. *S. cerevisiae* strains were grown and maintained in yeast extract-peptone-dextrose (YPD) agar or appropriate complete minimal (CM) dropout media (Ausubel *et al.*, 1995).

General DNA manipulation techniques

DNA isolation, digestion with restriction enzymes and ligation were done following standard protocols (Sambrook *et al.*, 1989). Purification of DNA fragments from agarose gels or PCR was performed using appropriate Qiagen kits (Qiagen, Valencia, CA). Cloning of PCR products was done with Platinum® *Taq* DNA polymerase High Fidelity and TOPO® vectors (Invitrogen, Carlsbad, CA) and all clones of PCR products were confirmed by DNA sequencing with fluorescent dye terminator chemistry and the ABI 3700 capillary electrophoresis sequencer (PE-Applied Biosystems, Wellesley, MA) at The Ohio State University Plant-Microbe Genomics Facility. Plasmids were introduced into bacterial cells by either electroporation using a GenePulser™ (Bio-Rad Laboratories, Hercules, CA) apparatus set to 200 Ω and 1.5 kV or triparental mating as previously described (Merighi *et al.*, 2003). Yeast transformation was conducted following a standard protocol using lithium acetate (Ausubel *et al.*, 1995).

WtsE constructs

Genomic DNA of *Pnss* DC283 was used as the template for the PCRs used to generate WtsE constructs, if not otherwise stated. *wtsE* clones and mutants are described in Table 2. These were

prepared by standard PCR, restriction digestion and ligation procedures cited above and each construction usually involved multiple cloning steps. Details are given in the supporting information. For expression of *wtsE* in yeast, we modified Stratagene's pMyr vector, by replacement of the multicloning site and coding region for the Myr fusion protein with the multicloning site and GAL4 DNA binding protein from pAS1. Alternatively, *wtsE* was introduced into the chromosome of yeast strain Y7092 (Alto *et al.*, 2006). For delivery by *E. coli* MC4100 (pCPP2156) constructs were made in pBluescript SK(+), for delivery by *Pph* NS3121 they were made in pDSK519, and for agroinfiltration they were made in the pGD and pBDex binary vectors (Allen *et al.*, 2004; Goodin *et al.*, 2002).

Plants

Sweet corn seedlings (*Zea mays* cv. Seneca Horizon), tobacco (*N. tabaccum* cv. Bottom Special), *N. benthamiana* and beet (*Beta vulgaris* cv. Red Ace Hybrid) were grown and maintained in growth chambers set to 30 °C and an 18/6-h day/night cycle. *A. thaliana* (ecotype Col-0) plants were grown in a growth room at 23 °C for 8 h in the light and at 16 °C for 16 h in the dark.

Virulence and cell death assays

Virulence assays for *Pnss* mutants in sweet corn seedlings were performed as previously described (Frederick *et al.*, 2001). Briefly, bacterial suspensions containing 0.2% Tween 40 were added to the whorls of 8-day-old sweet corn seedlings without wounding and disease severity was rated on a 0 to 3 scale (0 = no symptoms, 1 = a few lesions but no ooze, 2 = many lesions and some ooze, and 3 = confluent lesions and ooze). *Agrobacterium*-mediated transient expression experiments were conducted following Goodin *et al.* (2002). Procedures for preparation and infiltration of bacterial suspensions were as described previously, except needleless syringes were used to infiltrate bacterial suspensions into tobacco, *N. benthamiana*, beet and *A. thaliana* (Ham *et al.*, 2006).

Electrolyte leakage assays

Bacteria were grown overnight on plates, resuspended in fresh LB broth, and were further cultured at 28 °C with vigorous shaking (150 r.p.m.) until OD₆₀₀ values reached c. 0.6 for *E. coli* strains. They were then washed and resuspended in 0.01 M potassium phosphate buffer (pH 7.0) at c. 1×10^9 CFU/mL. CHX was added to some treatments at 100 µg/mL. *N. benthamiana* or tobacco leaves were sampled directly after infiltration, using an 0.8-cm-diameter cork borer. Two leaf discs were removed from the infiltrated area from each of four leaves from two different plants, briefly washed in deionized water, and placed into a plastic tube

Table 2 Strains and plasmids used in this study.

	Description	Source/Reference
<i>Agrobacterium tumefaciens</i>		
C58C1	Vir ⁺ , prototrophic strain, Rif ^r	(Van Larebeke <i>et al.</i> , 1974)
<i>Escherichia coli</i>		
MC4100	<i>araD139 Δ(argF-lac)205 λ⁻ flbB5301 ptsF25 relA1 rpsL150 deoC1</i>	CGSC* (Casadaban, 1976)
DB3.1 TM	F ⁻ <i>gyrA462 endA1 Δ (sr1-recA) mcrB mrr hsdS20(r_B⁻, m_B⁻) supE44 ara-14 galK2 lacY1 proA2 rpsL20(Sm^R) xyl-5 λ⁻ leu mtl1</i>	Invitrogen
<i>Pantoea stewartii</i> subsp. <i>stewartii</i>		
DC283	A Nal ^r derivative of wild-type strain SS104	(Coplin <i>et al.</i> , 1986)
DM5101	DC283 <i>wtsE::miniTn5gus</i> , Km ^r ; <i>miniTn5gus</i> insertion at amino acid 43	(Ham <i>et al.</i> , 2006)
AA005	A <i>wtsE::aphT</i> derivative of DC283, Km ^r ; <i>aphT</i> insertion at amino acid 962	(Ham <i>et al.</i> , 2006)
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>		
NPS3121	Wild-type strain, Rif ^r	(Lindgren <i>et al.</i> , 1986)
<i>Saccharomyces cerevisiae</i>		
cdc25H	<i>MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 cdc25-2 GAL⁺</i>	(Petitjean <i>et al.</i> , 1990)
Y7092	<i>MATα can1Δ::STE2pr-SpΔhis5 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lyp1Δ trp1Δ::GAL-lpgB1-URA3</i>	(Alto <i>et al.</i> , 2006)
Plasmids		
pAA008	Clone of the 8.9-kb <i>EcoRI/BamHI</i> fragment carrying <i>wtsE</i> and <i>wtsF</i> in pBluescript SK, Ap ^r	(Ham <i>et al.</i> , 2006)
pAS1	Bait vector for yeast two-hybrid system, <i>P_{ADH1}</i> , ColE1 origin and Ap ^r marker for use in <i>E. coli</i> and 2 μ origin and <i>LEU2</i> selectable marker for use in <i>S. cerevisiae</i>	ABRC†, (Fan <i>et al.</i> , 1997)
pBDex	A derivative of pTA7002 (Aoyama and Chua, 1997) with the Basta plant selection marker replaced by the hygromycin selection marker, Km ^r	(Allen <i>et al.</i> , 2004)
pBluescript KS and SK (+)	ColE1 <i>α-lacZ</i> , Ap ^r	Stratagene
pCPP2156	pCPP19 cosmid carrying the <i>D. dadantii</i> <i>hrp</i> cluster, Sp ^r	(Ham <i>et al.</i> , 1998)
pCR®4-TOPO®	TOPO® vector for cloning PCR products, Ap ^r , Km ^r	Invitrogen Corp
pDSK519	Broad-host-range cloning vector; Km ^r	(Keen <i>et al.</i> , 1988)
pENTR/D-TOPO®	An entry vector for the Gateway® cloning system, Km ^r	Invitrogen
pENTR::N-WtsE	Clone of the 0.5-kb 5'-end portion of <i>wtsE</i> in pENTR/D-TOPO®, Km ^r	This study
pENTR::WtsE	Wild type <i>wtsE</i> clone in pENTR/D-TOPO®, Km ^r	This study
pENTR::WtsE-ΔERMRS	ΔERMRS derivative of <i>wtsE</i> clone in pENTR/D-TOPO®, Km ^r	This study
pGD	Binary vector for agroinfiltration, Km ^r	(Goodin <i>et al.</i> , 2002)
pJH001	pCR®4-TOPO® clone with a 6.4-kb <i>EcoRI/BamHI</i> fragment containing the <i>wtsEF</i> operon and its native promoter, Ap ^r , Km ^r	This study
pJH003	Clone of the 6.4-kb <i>EcoRI/BamHI</i> fragment from pJH001 in pRK415 carrying the <i>wtsEF</i> operon and its native promoter, Tc ^r	This study
pJH021	pBluescript SK(+) clone of the 6.4-kb <i>EcoRI/BamHI</i> fragment from pJH001 carrying the <i>wtsEF</i> operon and its native promoter, Ap ^r	This study
pJH023	pJH021 with <i>wtsE-Δaa1831-1834</i> , Ap ^r	This study
pJH024	pJH003 with <i>wtsE-Δaa1831-1834</i> , Tc ^r	This study
pJH028	pBDex:: <i>wtsE-Δaa1-236</i> , Km ^r	This study
pJH040	pJH021 with <i>wtsE-Δaa51-236</i> , Ap ^r	This study
pJH041	pJH003 with <i>wtsE-Δaa51-236</i> , Tc ^r	This study
pJH042	pJH021 with <i>wtsE-Δaa51-492</i> , Ap ^r	This study
pJH043	pJH003 with <i>wtsE-Δaa51-492</i> , Tc ^r	This study
pJH048	pJH021 with <i>wtsE</i> NLS ⁻ (KKHK1358-61AAAA), Ap ^r	This study
pJH049	pJH003 with <i>wtsE</i> NLS ⁻ (KKHK1358-61AAAA), Tc ^r	This study
pJH082	Clone of the 6.4-kb <i>EcoRI/BamHI</i> fragment from pJH001 in pDSK519 carrying the <i>wtsEF</i> operon and its native promoter, Km ^r	This study
pJH087	Derivative of pJH028 with <i>Δaa1831-1834</i> in WtsE, Km ^r	This study
pJH101	pGD:: <i>wtsE</i> , Km ^r	This study
pJH102	pGD:: <i>wtsE-Δaa1-236</i> , Km ^r	This study
pJH103	pGD:: <i>wtsE-Δaa1-492</i> , Km ^r	This study
pMyr	Prey vector for CytoTrap TM yeast two-hybrid system, <i>P_{GAL1}</i> , ColE1 origin and Cm ^r marker for use in <i>E. coli</i> and 2 μ origin and <i>URA3</i> selectable marker for use in <i>S. cerevisiae</i>	Stratagene

Table 2 *Continued*

	Description	Source/Reference
pMyr-BD	A modified pMyr vector (Stratagene); the GAL4-BD and multicloning site of pAS1 was substituted for the Myr fusion protein and multicloning site of pMyr. Cm ^r , URA3.	This study
pMyr-BD::C-WtsE	pMyr-BD expressing the C-terminal half of WtsE (amino acids 964–1835) in <i>S. cerevisiae</i> . Cm ^r , URA3.	This study
pMyr-BD::N-WtsE	pMyr-BD expressing N-terminal half of WtsE (amino acids 1–964) in <i>S. cerevisiae</i> . Cm ^r , URA3.	This study
pMyr-BD::WtsE	pMyr-BD expressing the full-length WtsE in <i>S. cerevisiae</i> . Cm ^r , URA3.	This study
pPGA	Modified destination vector for the Gateway [®] cloning of <i>wtsE</i> . Cm ^r	Dr. C. Boone, Univ. of Toronto
pPGA::WtsE	Wild type <i>wtsE</i> construct in pPGA for the integration of <i>GAL1-wtsE-URA3</i> into the Y7092 genome	This study
pPGA::WtsE-ΔERMRS	ΔERMRS derivative <i>wtsE</i> construct in pPGA for the integration of <i>GAL1-wtsE-URA3</i> into the Y7092 genome	This study
pRF205	pVK100 with a 1.7-kb <i>HindIII</i> fragment containing P _{lac} - <i>hrpS</i> ⁺	(Frederick <i>et al.</i> , 1993)
pRK415	IncP α - <i>lacZ</i> , Tc ^r	(Keen <i>et al.</i> , 1988)

**E. coli* Genetic Stock Center.

†Arabidopsis Biological Resource Center at The Ohio State University.

containing 15 mL of deionized water, which was then held in the light at room temperature. At intervals the conductivity of the fluid in each tube was measured using a WTW model Cond 330i conductivity meter with a TetraCon 325 probe (WTW, Weilheim, Germany).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Text S1 Additional experimental procedures.

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