

Antagonistic action of harpin proteins: HrpW_{ea} from *Erwinia amylovora* suppresses HrpN_{ea}-induced cell death in *Arabidopsis thaliana*

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Summary

Harpins are proteins secreted by the type-three secretion system of phytopathogenic bacteria. They are known to induce a hypersensitive response (HR) in non-host plant leaf tissue. *Erwinia amylovora*, the fire blight pathogen of pear and apple trees, secretes two different harpins, HrpN_{ea} and HrpW_{ea}. In the present study, we showed that an *Erwinia amylovora* *hrpW_{ea}* mutant induces stronger electrolyte leakages in *Arabidopsis thaliana* foliar disks than the wild-type strain, thus suggesting that HrpW_{ea} could function as a HR negative modulator. We confirmed this result by using purified HrpW_{ea} and HrpN_{ea}. HrpW_{ea} has dual effects depending on its concentration. At 200 nM, HrpW_{ea}, like HrpN_{ea}, provoked the classical defense response – active oxygen species (AOS) production and cell

death. However, at 0.2 nM, HrpW_{ea} inhibited cell death and AOS production provoked by HrpN_{ea}. HrpW_{ea} probably inhibits HrpN_{ea}-induced cell death by preventing anion channel inhibition, confirming that anion channel regulation is a determinant feature of the plant response to harpins. Collectively our data show that the HrpW_{ea} harpin can act antagonistically to the classical HrpN_{ea} harpin by suppressing plant defense mechanisms.

Supplementary material available online at
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Key words: *Arabidopsis*, Cell death, Harpin, Ion channel, Plant-microbe interaction

Introduction

Gram-negative pathogenic bacteria use protein secretion machinery, called the type-three secretion system (TTSS), to increase their virulence in host tissue. TTSSs were reported initially from the mammalian pathogen *Yersinia enterocolitica*, but have also been identified in various plant pathogenic bacteria such as *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Ralstonia* (Hueck, 1998). TTSSs allow delivery of specific effector proteins to host cell (Hueck, 1998). Bacterial TTSS effectors are highly diverse and collectively manipulate host cell metabolism to improve bacterial nutrition or to encounter plant cell defense mechanisms (Hauck et al., 2003; Abramovitch et al., 2003; DebRoy et al., 2004; Kim et al., 2005).

Harpins are TTSS-delivered proteins from plant pathogens and constitute a group of proteins that are secreted in the intercellular space of plant tissue during interaction, rather than being injected (Perino et al., 1999; Tampakaki and Panopoulos, 2000). Collectively, harpins could be involved in the release of nutrients from the host cells or they could facilitate the delivery of other bacterial proteins in the host cell (Holeva et al., 2004; Lindeberg et al., 2006). Harpins are not homologous in primary sequence but have common physiochemical characteristics: they are heat-stable, glycine rich, do not possess cysteine, and above all, trigger a hypersensitive response (HR) cell death,

requiring an active plant metabolism, in non-host plants (Wei et al., 1992; He et al., 1993; He et al., 1994; Arlat et al., 1994). The mechanisms by which harpins provoke HR cell death have been extensively studied. Harpins trigger closely related signal transduction mechanisms such as mitogen-activated protein kinase (MAPK) activation (Adam et al., 1997; Desikan et al., 1999), cytosolic [Ca²⁺] elevation (He et al., 1994; Pike et al., 1998; Blume et al., 2000; Cessna et al., 2001), active oxygen species (AOS) production (Baker et al., 1993; Desikan et al., 1996; Rebutier et al., 2007) and ion flux modulations (Desikan et al., 1999; Popham et al., 1995; El-Maarouf et al., 2001; Rebutier et al., 2005; Rebutier et al., 2007). Particular harpins possess a C-terminal domain that is homologous to class III pectate lyases. These HrpW harpins are widespread among phytopathogenic bacteria (Gaudriault et al., 1998; Kim and Beer, 1998; Charkowski et al., 1998). HrpW_{pst} from *Pseudomonas syringae* pv. tomato was shown to bind to pectate (Charkowski et al., 1998), but lacks detectable pectate lyase activity (Gaudriault et al., 1998; Charkowski et al., 1998). Purified HrpW harpins do not macerate plant tissue and elicit a HR on non-host plants (Gaudriault et al., 1998; Kim and Beer, 1998; Charkowski et al., 1998). Two harpins – HrpN_{ea} and HrpW_{ea} – have been characterized in *Erwinia amylovora*, the fire blight pathogen of pear and apple trees (Wei et al., 1992; Gaudriault et al., 1998; Kim and Beer, 1998). Mutant

analysis indicates that HrpN_{ea} is an important virulence factor involved in the generation of oxidative stress in planta (Wei et al., 1992; Barny, 1995), whereas HrpW_{ea} is not required for full virulence (Gaudriault et al., 1998). Furthermore, on non-host plants, such as tobacco, although *hrpN_{ea}* mutants elicited a weaker HR than the wild-type strain, *hrpW_{ea}* mutants elicited a stronger HR than the wild-type strain. HrpW_{ea} could thus function as a HR-negative modulator (Gaudriault et al., 1998). The aim of our study was to test this hypothesis. We first confirmed the phenotype of *E. amylovora hrpW_{ea}* and *hrpN_{ea}* on the model plant *A. thaliana*. Then, we tested the effects of purified HrpW_{ea} and HrpN_{ea} on *A. thaliana* cells in suspension to elucidate the putative role or control of early cellular mechanisms involved in cell death regulation.

Results

hrpW_{ea} induces strong electrolyte leakages in *Arabidopsis thaliana* foliar disks

To check whether, as already observed in tobacco (Gaudriault et al., 1998), HrpW_{ea} behaves as a negative HR modulator in *A. thaliana*, we quantified cell death induced by *E. amylovora* wild-type strain, *hrpN_{ea}*, *hrpW_{ea}* or double *hrpN_{ea}-hrpW_{ea}* mutants, by measuring electrolyte leakage of *A. thaliana* foliar disks. Although the *hrpN_{ea}* mutant was severely impaired in its ability to induce electrolyte leakage, the *hrpW_{ea}* mutant induced stronger electrolyte leakage than observed in the wild-type strain (Fig. 1). The double *hrpN_{ea}-hrpW_{ea}* mutant triggered electrolyte leakages similar to levels in the *hrpN_{ea}* mutant. Therefore, in *A. thaliana*, as in tobacco, HrpW_{ea} behaves in planta as a negative modulator of the cell death induced by *E. amylovora*. Furthermore, its effects are opposed to those induced by HrpN_{ea}, which appears to be necessary for *E. amylovora* to induce cell death in *A. thaliana*.

HrpW_{ea}, like HrpN_{ea}, induces cell death and a transient oxidative burst but no cytosolic [Ca²⁺]_{cyt} increase

The preceding result prompted us to check whether purified HrpW_{ea} was able to induce cell death in *A. thaliana* suspension cells, a convenient system that allows the identification of early physiological events induced by pathogen-derived elicitors on single cells and also allows us to monitor the behavior of large populations of cells. HrpW_{ea}, like HrpN_{ea}, provoked dose-

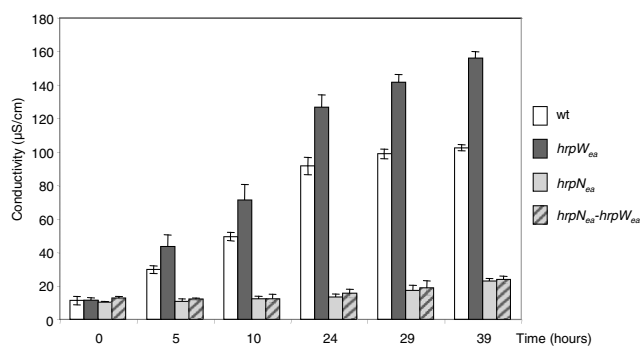


Fig. 1. Effects of *Erwinia amylovora* wild-type (wt), *hrpW_{ea}*, *hrpN_{ea}* and *hrpN_{ea}-hrpW_{ea}* mutant strains on electrolyte leakages from *A. thaliana* foliar disks. Disks were infiltrated with bacterial suspensions of 3×10^8 cells/ml. Values are means of three replicates and error bars represent s.e.

dependent cell death in *A. thaliana* cells (Fig. 2A,B). The cell death level was similar for the two harpins, and reached its maximum at a concentration near 200 nM. To have a better understanding of the HrpW_{ea}-induced cell death process, we compared the effects of purified HrpW_{ea} and HrpN_{ea} on physiological responses classically associated with cell death.

At 200 nM, both HrpW_{ea} and HrpN_{ea} induced large transient oxidative bursts sharing the same characteristics (Fig. 3A). H₂O₂ production reached similar levels for the two harpins (Fig. 3B). Oxidative bursts reached their maximum between 30 minutes and 1 hour, then, H₂O₂ levels decreased and returned to control levels after 2 hours (Fig. 3A). In both cases, H₂O₂ production was blocked by the NADPH oxidase inhibitor DPI (10 µM) (Fig. 3B), suggesting that a plasma membrane NADPH oxidase was involved in H₂O₂ production.

To measure [Ca²⁺]_{cyt} variations, we used transgenic *A. thaliana* cells expressing aequorin protein (Brault et al., 2004). Hypo-osmotic stress, used as a positive control, triggered an instantaneous and transient [Ca²⁺]_{cyt} peak reaching 2.5 µM, followed by a second slower transient [Ca²⁺]_{cyt} peak appearing within 1 minute and reaching 2 µM (Fig. 3C). However, neither HrpW_{ea} nor HrpN_{ea} triggered any [Ca²⁺]_{cyt} increase (Fig. 3C) over 60 minutes (data not shown). The absence of a Ca²⁺ response after addition of HrpW_{ea} or HrpN_{ea} indicated that [Ca²⁺]_{cyt} elevation is probably not involved in signal transduction mechanisms activated in *A. thaliana* in response to HrpN_{ea} or HrpW_{ea}.

HrpW_{ea} provokes ion channel modulations opposed to those induced by HrpN_{ea}

Recently, we showed that HrpN_{ea} probably triggers cell death

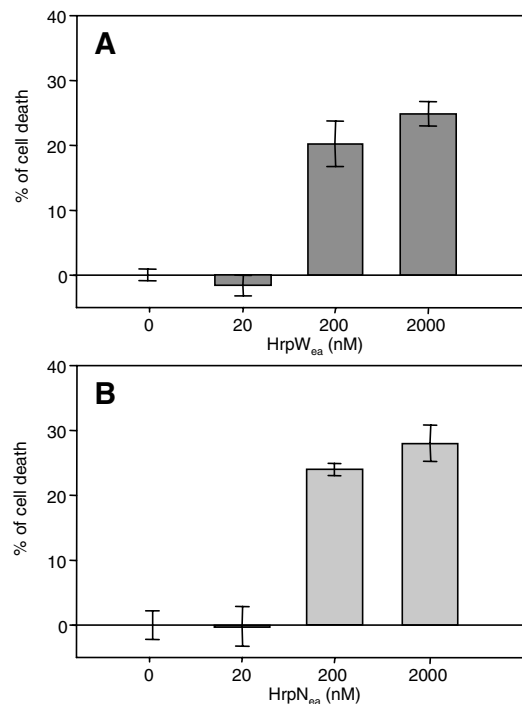


Fig. 2. Effect of increasing concentrations of HrpW_{ea} (A), or HrpN_{ea} (B), on cell death of *A. thaliana* suspension cells. Desalting buffer did not modify cell viability. Values are means of four replicates and error bars represent s.e.

by inhibiting anion channel activity (Reboutier et al., 2005). Therefore, we checked the effects of HrpW_{ea} on ion channel modulation. Within the first minute of treatment, HrpW_{ea} (200 nM) decreased time- and voltage-dependent outward rectifying currents previously characterized as K⁺ outward rectifying currents (KORCs) (Reboutier et al., 2002) (Fig. 4C-E) and increased deactivating currents previously characterized as anion currents (Reboutier et al., 2002) (Fig. 4F-H). The

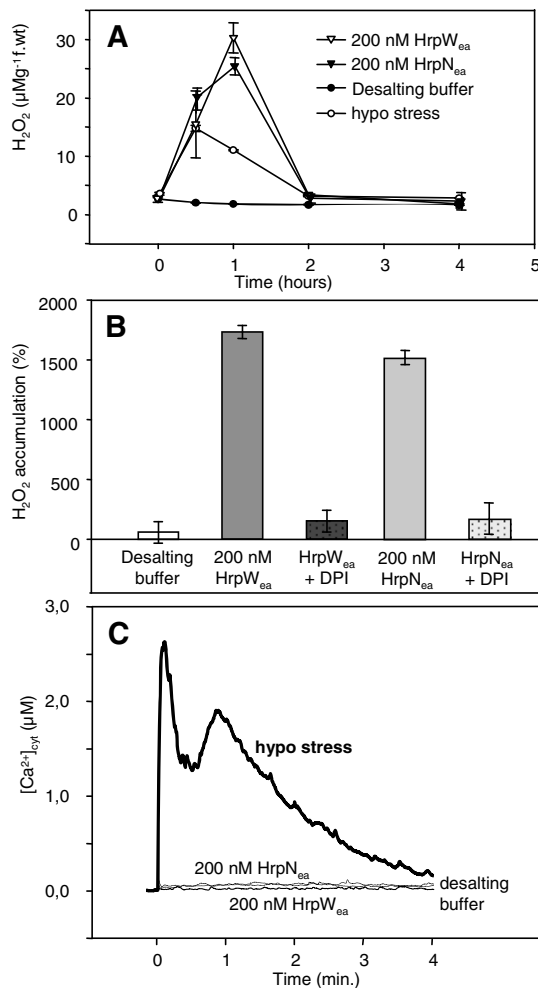


Fig. 3. Effect of 200 nM HrpW_{ea} or HrpN_{ea} on H₂O₂ production and on cytosolic Ca²⁺ level ([Ca²⁺]_{cyt}) of *A. thaliana* suspension cells. (A) Time course of H₂O₂ accumulation in the medium of cells treated with 200 nM HrpW_{ea}, 200 nM HrpN_{ea} or the corresponding volume of desalting buffer. Half dilution of cell suspension with distilled water triggered hypo-osmotic stress used as positive control. (B) Percentage of increase of H₂O₂ accumulation, at 1 hour, in the medium of cells treated with 200 nM HrpW_{ea} or HrpN_{ea}, alone or in combination with 10 µM DPI or by the corresponding volume of desalting buffer. Values are given as a percentage with respect to untreated cells (100%). Values are means of eleven replicates. Error bars represent s.e. (C) Changes in [Ca²⁺]_{cyt} were measured by using transgenic *A. thaliana* cells expressing aequorin protein. Cells suspended in culture medium containing 1 mM Ca²⁺, were treated with 200 nM HrpW_{ea} or HrpN_{ea} or the corresponding volume of desalting buffer. Hypo-osmotic stress induced by diluting the cell suspension 1:1 with distilled water was used as a positive control. Five independent experiments were carried out, with similar results.

HrpW_{ea}-induced increase in anion current was reduced after addition of 10 µM glibenclamide or 40 µM 9AC, two potent anion channel inhibitors (Fig. 4H), confirming the anionic nature of these currents. K⁺ and anion channel modulations led to depolarization of the plasma membrane [+32±5 mV (*n*=10)], from a mean membrane potential of -42±7 mV (*n*=72) (Fig. 4A,B). These effects were opposed to those triggered by 200 nM HrpN_{ea}, which increased KORC (Fig. 4E) and inhibited anion current (Fig. 4H), thus leading to hyperpolarization of the membrane [-18±6 mV (*n*=9)] (Fig. 4A,B). KORC increase induced by HrpN_{ea} was reduced after addition of the K⁺ channel inhibitor TEA (10 mM) (Fig. 4E) confirming the K⁺ nature of these currents.

Low concentrations of HrpW_{ea} counteracts cell death, AOS production and anion channel reduction

Since HrpW_{ea} had opposite effects to those triggered by HrpN_{ea} on anion channel modulation and because HrpN_{ea} induced cell death through anion channel activity decrease (Reboutier et al., 2005), we checked whether increasing concentrations of HrpW_{ea} could modify HrpN_{ea}-induced cell death in *A. thaliana* cells.

HrpW_{ea} inhibited HrpN_{ea} (200 nM)-induced cell death in a dose-dependent manner in the range 0.002 to 2 nM (Fig. 5A). The maximum inhibitory concentration was 0.2 nM. Concentrations greater than 0.2 nM did not inhibit HrpN_{ea}-induced cell death. On the contrary, addition of 200 nM HrpW_{ea} to 200 nM HrpN_{ea} increases cell death indicating that at high concentration HrpN_{ea} and HrpW_{ea} effects on cell death are additional. Reciprocally, we tested whether increasing concentrations of HrpN_{ea} could inhibit 200 nM HrpW_{ea}-induced cell death (Fig. 5B); however, HrpN_{ea} did not inhibit HrpW_{ea}-induced cell death. These results indicate that HrpW_{ea} effects are dual: HrpW_{ea} induces cell death at concentrations greater than 20 nM and inhibits HrpN_{ea}-induced cell death at 0.2 nM.

As the strongest inhibition of HrpN_{ea}-induced cell death was observed at 0.2 nM HrpW_{ea}, we checked the effect of addition of 0.2 nM HrpW_{ea} to 200 nM HrpN_{ea} on AOS production and ion channel modulation. HrpW_{ea} inhibited HrpN_{ea}-induced AOS increase (Fig. 6A). Furthermore, HrpW_{ea} totally prevented anion current decrease and partially inhibited the KORC increase induced by HrpN_{ea} (Fig. 6B,C). A control experiment with 0.2 nM HrpW_{ea} alone showed that it did not modify AOS production or ion channel activities (Fig. 6A-C).

Low concentrations of HrpW_{ea} did not counteract HrpZ_{pph}-induced cell death

Since HrpW_{ea} could counteract the effect of HrpN_{ea}, we checked the ability of HrpW_{ea} to inhibit cell death triggered by HrpZ_{pph}, a harpin similar to HrpN produced by *Pseudomonas syringae* pv. *Phaseolicola* (Li et al., 2005). HrpZ_{pph} induced cell death in *A. thaliana* suspension cells (Fig. 7), yet HrpW_{ea} had no significant effect on HrpZ_{pph}-induced cell death (Fig. 7).

Discussion

Here, we studied the effects of HrpW_{ea} on non-host *A. thaliana*. The effect of HrpW_{ea} on cell death appeared dual because 200 nM HrpW_{ea} triggered cell death, as already reported for tobacco (Kim and Beer, 1998), whereas 0.2 nM HrpW_{ea}

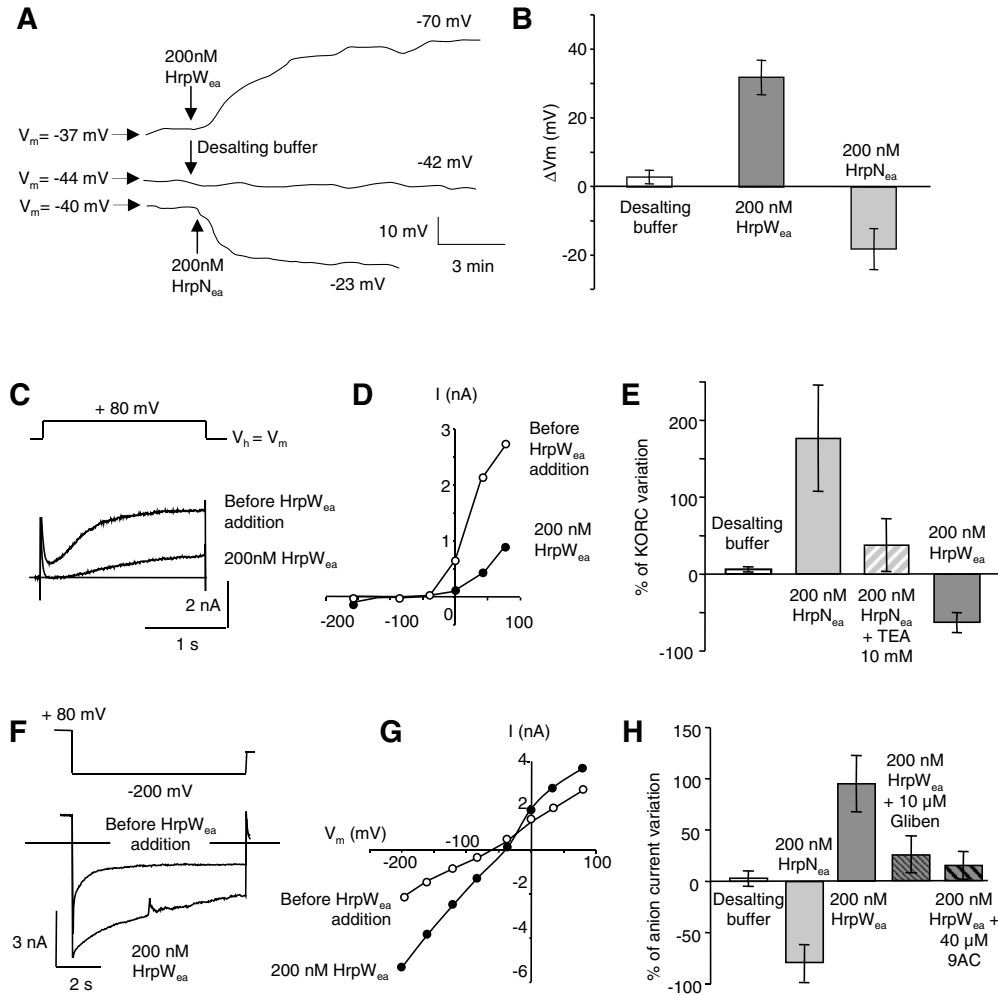


Fig. 4. Modulations of membrane potential (V_m), K^+ outward rectifying currents (KORCs) and anion currents of *A. thaliana* cells in response to 200 nM HrpW_{ea} or HrpN_{ea}. (A) Free-running V_m observed in response to HrpW_{ea}, HrpN_{ea} or the desalting buffer. (B) Mean values of the V_m variations. (C) Effect of 200 nM HrpW_{ea} on KORCs recorded at +80 mV (leak subtracted). The activated currents were measured when the maximum effect of HrpW_{ea} was reached. V_h , holding potential; V_m , membrane potential. (D) Current-voltage relationships. The steady-state current amplitudes were measured at membrane potentials ranging from -200 to +80 mV. (E) Mean amplitudes of steady state KORCs (at 80 mV) in response to 200 nM HrpW_{ea}, HrpN_{ea} alone, HrpN_{ea} and 10 mM TEA or the corresponding desalting buffer volume. Values are given as a percentage with respect to current value before treatment. (F) Effect of 200 nM HrpW_{ea} on anion current recorded at -200 mV. Currents were measured when the maximum effect of HrpW_{ea} was reached. (G) Corresponding current-voltage curves. (H) Mean amplitudes of anion currents in response to 200 nM HrpW_{ea} alone, HrpW_{ea} and 10 μ M Glibenclamide or 40 μ M 9AC, 200 nM HrpN_{ea} or the corresponding desalting buffer volume. Values are given as a percentage with respect to current value before treatment. All values are mean of at least five independent experiments. Error bars represent s.e.

inhibited cell death provoked by 200 nM HrpN_{ea}. This result confirmed the data we obtained in planta, since an *hrpW_{ea}* mutant induced stronger electrolyte leakages compared with those observed in the wild-type strain, suggesting that HrpW_{ea}, when delivered to *A. thaliana* leaves by the bacteria, rather acts as a cell death negative modulator. Such a dual effect was not observed with the classical harpin HrpN_{ea}, for which cell death induced by the purified protein correlates with a *hrpN_{ea}* mutant altered in its cell-death-inducing capacity.

To gain a better understanding of the dual effects of HrpW_{ea}, we compared the effects of purified HrpW_{ea} with the well-characterized effects of HrpN_{ea} on physiological responses classically associated with cell death. At 200 nM, purified HrpW_{ea} or HrpN_{ea} had strictly the same effects on

AOS production and $[Ca^{2+}]_{cyt}$ variation. Both harpins triggered a strong transient H_2O_2 production probably through plasma membrane NADPH-oxidase activation. Neither 200 nM HrpW_{ea} nor 200 nM HrpN_{ea} provoked $[Ca^{2+}]_{cyt}$ variation in *A. thaliana* cells. Moreover, Ca^{2+} -channel inhibitors or Ca^{2+} surrogates were inefficient at reducing harpin-induced cell death and harpin-induced effects on ion channels (not shown). These data indicated that $[Ca^{2+}]_{cyt}$ elevation is probably not involved in signal transduction mechanisms activated in response to these harpins in *A. thaliana*. These results differ from previous data suggesting that Ca^{2+} was involved in signal transduction mechanisms triggered by harpins (He et al., 1993; He et al., 1994; Blume et al., 2000; Cessna et al., 2001), but are in accordance with data from Chandra et al. (Chandra

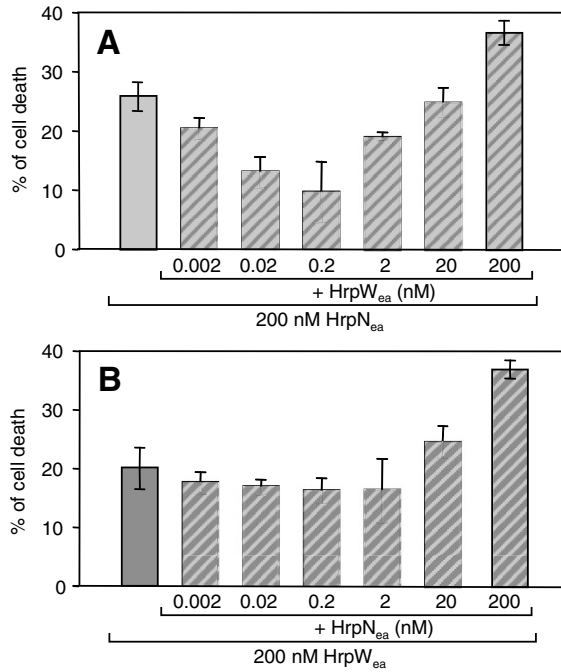


Fig. 5. Inhibitory effect of HrpW_{ea} on cell death triggered by HrpN_{ea}. (A) Inhibition of HrpN_{ea}-induced cell death by increasing concentration of HrpW_{ea} (from 0.002 nM to 200 nM) in *A. thaliana* suspension cells. (B) Increasing concentrations of HrpN_{ea} (from 0.002 nM to 200 nM) do not modify HrpW_{ea}-induced cell death in *A. thaliana* suspension cells. Values shown in both A and B are means of four replicates and error bars represent s.e.

et al., 1997) showing that HrpN_{ea} could activate AOS production without triggering any [Ca²⁺]_{cyt} variation. It is possible that the use of diverse plant species, such as *Nicotiana tabacum* (He et al., 1993; He et al., 1994; Cessna et al., 2001), *Nicotiana plumbaginifolia* (Chandra et al., 1997) or *A. thaliana* (the present study) would be responsible for the discrepancy observed. Interestingly, purified HrpW_{ea} provoked opposed KORC and anion channel modulations when compared with those triggered by HrpN_{ea}: 200 nM HrpW_{ea} decreased KORC activity and strongly increased anion channel activity. Because HrpN_{ea} was previously shown to trigger cell death by decreasing anion channel activity (Reboutier et al., 2005), it suggested that HrpW_{ea} and HrpN_{ea} provoke cell death by two distinct mechanisms. Moreover, it allowed us to hypothesize that HrpW_{ea} could counteract HrpN_{ea}-induced cell death, by having an opposed effect on anion channel modulation. This hypothesis was confirmed when 0.2 nM HrpW_{ea} was found to inhibit HrpN_{ea}-induced cell death. Although the PopA_{rs} harpin was recently found to form oligomers through the conserved GxxxG amino acid motif (Racape et al., 2005), this inhibition is unlikely to be the result of the direct interaction between HrpW_{ea} and HrpN_{ea} because GST pull-down technology did not show any interaction between HrpW_{ea} and HrpN_{ea} (data not shown). In addition, the fact that the most effective concentration of HrpW_{ea} to inhibit HrpN_{ea}-induced cell death was one-thousandth of that of HrpN_{ea}, also constitutes evidence against a direct protein-protein interaction. Inhibition of HrpN_{ea}-induced cell death by HrpW_{ea} more probably acts through

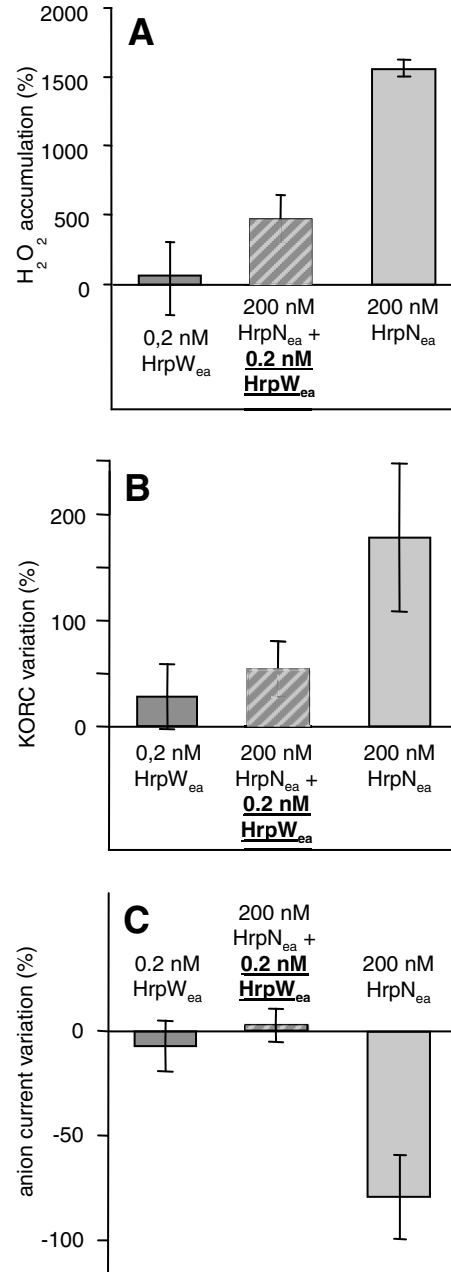


Fig. 6. Inhibitory effect of HrpW_{ea} on H₂O₂ production and ion channel modulations triggered by HrpN_{ea}. (A) Inhibition of 200 nM HrpN_{ea}-induced H₂O₂ production by 0.2 nM HrpW_{ea}. Values are given as a percentage with respect to untreated cells (100%). Values are means of four replicates. (B) Inhibition of 200 nM HrpN_{ea}-induced KORC activation by 0.2 nM HrpW_{ea}. (C) Inhibition of 200 nM HrpN_{ea}-induced anion current decrease by 0.2 nM HrpW_{ea}. Values are given as a percentage with respect to currents recorded before treatment. Values are means of at least four replicates. Error bars represent s.e.

integration of the two signal transduction pathways triggered by HrpW_{ea} or HrpN_{ea}.

Because anion channel inhibition was shown to be involved in HrpN_{ea}-induced cell death (Reboutier et al., 2005; Reboutier et al., 2007), we checked the effect of addition of 0.2 nM HrpW_{ea} to 200 nM HrpN_{ea} on ion channel modulation. At 0.2

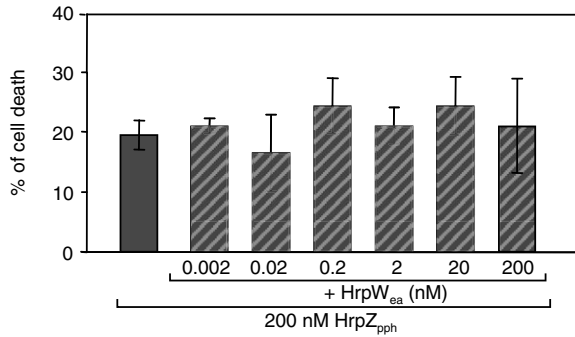


Fig. 7. Increasing concentrations of HrpW_{ea} (from 0.002 nM to 200 nM) do not modify 200 nM HrpZ_{p_{ph}}-induced cell death in *A. thaliana* suspension cells. Values are means of three replicates and error bars represent s.e.

nM, HrpW_{ea} was able to reduce KORC activation and totally prevented anion channel inhibition provoked by HrpN_{ea}. The fact that HrpW_{ea} could prevent anion channel inhibition provoked by HrpN_{ea} probably explains the decrease in cell death. Taken together, all these results confirmed that anion channels are a determinant feature of the plant response to harpins and participate in the decisions leading to cell death.

At 0.2 nM, HrpW_{ea} was also able to strongly decrease AOS production provoked by 200 nM HrpN_{ea}. Anion channels were shown to be involved in cell death and AOS production in response to cryptogein (Wendehenne et al., 2002). Consequently, we checked the effect of anion channel modulators, which regulate cell death in *A. thaliana* (Reboutier et al., 2005), on H₂O₂ production in response to HrpW_{ea} and HrpN_{ea}. Anion channel modulators did not modify HrpW_{ea}- or HrpN_{ea}-induced AOS production (see supplementary material Fig. S1A,B). These results suggested that the H₂O₂ production induced by HrpN_{ea} or HrpW_{ea} does not depend on anion current modulation. Moreover, the anion channel activator bromotetramisole, which was shown to inhibit HrpN_{ea}-induced cell death (Reboutier et al., 2005), did not decrease AOS production (see supplementary material Fig. S1B). This result indicated that AOS production in response to HrpN_{ea} was not sufficient to trigger cell death. Ion channel modulations and AOS production in response to harpins are more likely to be concomitant than linked. Many studies suggest that AOSs are involved in cell death observed in response to pathogens (Levine et al., 1994) or elicitors such as the HrpZ_{pss} harpin (Desikan et al., 1996; Desikan et al., 1998). However, data are controversial and other studies propose that AOSs are not involved in harpin-induced cell death (Ichinose et al., 2001; Xie and Chen, 2000). More generally, AOS production appears to be a generic defense response triggered by pathogens or elicitors (Desikan et al., 1998; Mehdy, 1994; Bradley et al., 1992; Samuel et al., 2005; Jabs et al., 1997), which could participate in limiting the spread of pathogens.

HrpW_{ea} inhibited cell death triggered by 200 nM HrpN_{ea} at a very low concentration (0.2 nM). Yet, HrpW_{ea} and HrpN_{ea} are roughly secreted in the same amounts by the bacteria (Gaudriault et al., 1998). It is possible that free concentrations of each protein differ in the plant cell apoplast. Indeed HrpW proteins have been reported to bind to pectate through to their C-terminal domain homologous to pectate lyase (Gaudriault et

al., 1998; Kim and Beer, 1998; Charkowski et al., 1998), whereas classical harpin proteins, such as HrpN_{ea}, did not. Furthermore, we cannot exclude the fact that each protein could be present in the apoplast at different amounts because of differential proteolytic degradation in planta.

We report here for the first time that HrpW_{ea}, a protein from the harpin family, inhibits cell death. The question remains why *E. amylovora*, considered as a necrotroph, possesses the cell death inhibitor HrpW_{ea}? Bretz et al. (Bretz et al., 2003) suggested that HopPtoD2, an injected TTSS effector of *P. syringae*, acts as an inhibitor of defense responses by suppressing cell death and AOS production. When delivered by *E. amylovora*, HrpW_{ea} could in the same manner be considered not only as a cell death inhibitor but, more widely, as a defense response inhibitor. HrpW_{ea} seemed not be required for pathogenicity, because a mutant strain was as pathogenic as the wild-type strain on apple trees (Gaudriault et al., 1998); however, HrpW_{ea} could be required for survival in non-host plants during the epiphytic period. The fact that HrpW_{ea} was not able to inhibit HrpZ_{p_{ph}}-induced cell death in *Arabidopsis* suggests that HrpW_{ea} cell death inhibition is specific for HrpN_{ea}-hypersensitive reaction, thus confirming the high specificity acquired by secreted proteins from plant pathogens during evolution. Several injected TTSS effectors were shown to inhibit cell death (Abramovitch et al., 2003; Axtell and Staskawicz, 2003; Espinosa et al., 2003; Mackey et al., 2003) when injected into plant cells. To our knowledge, HrpW_{ea} is the first TTSS-delivered protein that can inhibit plant cell defense mechanisms when secreted in the plant cell apoplast. These results suggest that inhibitors of defense mechanisms could act when secreted in the extracellular medium.

In conclusion, this work highlights the role of anion channels in programmed cell death. Using CFTR modulators, we recently proposed that anion current decrease was a prerequisite to HrpN_{ea}-induced cell death (Reboutier et al., 2005), as previously described with glibenclamide in animal cells (Kim et al., 1999). However, the signaling pathway(s) leading to this cell death remain largely unknown. HrpW_{ea}-induced cell death could be compared with the mechanisms described during apoptotic volume decrease (AVD) in animal cells (Okada and Maeno, 2001) or cryptogein-induced cell death in tobacco cells (Wendehenne et al., 2002; Gauthier et al., 2007), which involve strong anion efflux through the activation of anion channels. Our work also proposes a new role for the HrpW_{ea} harpin that was mainly described as a cell death inducer. Here, we show by using in planta and cellular approaches that this harpin is more likely to be a plant defense inhibitor. These new data bring new tracks concerning modulation of plant defenses by phytopathogenic bacteria.

Materials and Methods

Electrolyte leakage determination

A. thaliana (ecotype Columbia) 5-week-old leaves were infiltrated with bacterial suspension Ea321 (WT *Erwinia amylovora*), Ea321-T5 (*hrpN_{ea}* mutant), Ea321-G204 (*hrpW_{ea}* mutant) and Ea321-T5-G204 (double *hrpN_{ea}-hrpW_{ea}* mutant) (supplementary material Table S1) of 3×10^8 cells/ml in assay medium (0.5 mM MES, 0.5 mM CaCl₂, pH 6). Electrolyte leakage assays were initiated by removing six infiltrated leaf samples using a boring tool with an inner diameter of 0.5 cm. These samples were washed in deionized water to remove surface-adhered electrolytes. Then they were placed in a test tube and vacuum-infiltrated in 10 ml distilled water. Samples were incubated under light conditions, at 22°C, with continuous rotation shaking. Conductivity was measured at 0, 5, 10, 24, 29 and 39 hours after bacteria infiltration with a SevenMulti conductimeter (Mettler Toledo, France).

Cell culture

A. thaliana (ecotype Columbia) suspension cells were cultured as described (Reboutier et al., 2005). Main ions after 4 days of culture were 9 mM K⁺, 11 mM NO₃⁻ (Reboutier et al., 2002). Experiments were conducted on 4-day-old cultures.

Harpin preparations

HrpN_{ea} and *HrpW_{ea}* were PCR amplified with high-fidelity TAQ (Roche) using pMAB 40 (Gaudriault et al., 1997) as a template with the following primers: AACGGATCCATGAGTCTGAATACAAGTGGG and AAACGAGTTAAGC-CGCGCCAGCTTGCC for *HrpN_{ea}*; AAGGATCCTAGTCAATTTACGC-TTTAAC and AAACGAGTTATTGGCATCTTCGCTGTG for *HrpW_{ea}*. The PCR-amplified products were digested with *Bam*HI and *Xho*I, cloned into the pGEX6P vector (Amersham) and sequenced. The plasmid containing *HrpN_{ea}* and *HrpW_{ea}*, respectively named pMAB164 and pMAB165 were used to transform *Escherichia coli* BL21. *E. coli* strains were grown to an optical density of 0.5 at 600 nm and the overproduction of GST-HrpW_{ea} or GST-HrpN_{ea} was induced with 1 mM IPTG. Bacterial cells were harvested, resuspended in 12 ml PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, DTT 1 mM, PMSF 1 mM, pH 7.3) and sonicated for 5 minutes. After centrifugation, the supernatant was injected into an affinity chromatography column (GST-trap FF, 1 ml, Amersham). The GST-tag was removed by using the Precision Protease enzyme (Amersham). Then HrpW_{ea} or HrpN_{ea} were eluted and desalted with a Hi-Trap desalting column (Amersham). For this column, the protein buffer was changed to 5 mM KNO₃, 1 mM MES (pH 5.8). This desalting buffer was used as a control in all experiments.

Cell death quantification

Cell death was quantified using the fluorescein diacetate (FDA) spectrofluorimetric method (Amano et al., 2003). Four-day-old *A. thaliana* cells were collected and washed by filtration in a medium containing 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄ and 10 mM HEPES (H10 medium) adjusted with KOH to pH 5.8. 1 ml of cell suspension was incubated in the presence of HrpW_{ea} and/or HrpN_{ea} alone or with the appropriate pharmacological effector. After 24 hours of treatment, 500 µl suspension was diluted in 1.5 ml H10 medium in a quartz cuvette. Cells were gently stirred with a magnetic stirrer. Then, FDA was added at a final concentration of 12 µM and the fluorescence increase was monitored for 120 seconds using a F-2000 spectrofluorimeter (Hitachi, Japan). We verified the linearity between the percentage of dead cells and FDA-detected esterase activity by melting different amounts of control living cells and heated dead cells. A 100% esterase activity corresponds roughly to 100% living cells. 0% esterase activity corresponds to 100% dead cells. Cell death was thus calculated as follows: % of cell death=(slope of treated cells/slope of non-treated cells) × 100.

Quantification of H₂O₂ in culture medium

H₂O₂ release in the culture medium was quantified as described (Bouizgarne et al., 2006). Briefly, 1.5 ml of the cell suspension (stabilized for 4 hours in H10 medium) was inoculated with HrpW_{ea} and/or HrpN_{ea} alone or with the appropriate chemical effector. Before each measurement, 200 µl cell culture was added to 600 µl phosphate buffer (50 mM, pH 7.9) before addition of 100 µl of 1.1 mM luminol. Then 100 µl of 14 mM K₃Fe(CN)₆ was added as an electron acceptor. Chemiluminescence was monitored at 30-minute intervals with a FB12-Berthold luminometer (signal integrating time 0.2 second).

Aequorin luminescence measurements

Cytoplasmic Ca²⁺ variations were recorded with an *A. thaliana* cell suspension expressing the aequorin gene (Brault et al., 2004). Aequorin was reconstituted by overnight incubation of the cell suspension in Gamborg medium (containing 1 mM Ca²⁺) supplemented with 30 g l⁻¹ sucrose and 2.5 µM native coelenterazine. Cell-culture aliquots (250 µl) were transferred carefully to a luminometer glass tube and the luminescence was recorded continuously, at 0.2-second intervals, with a FB12-Berthold luminometer (Berthold Technologies, Bad Wildbad, Germany). Treatments were performed by addition of harpin directly into the luminometer tube. At the end of each experiment, the residual aequorin was discharged by the addition of 10% (v/v) ethanol and 1 M CaCl₂ (final concentration). The resulting luminescence was used to estimate the total amount of aequorin in each experiment. Calibration of the Ca²⁺ measurement was performed by using the equation $pCa = 0.332588(-\log k) + 5.5593$, where k is a rate constant equal to luminescence counts per second divided by the total remaining counts (Knight et al., 1996).

Electrophysiology

For electrophysiological measurements, cells were impaled in the culture medium as previously described (Reboutier et al., 2005; Bouizgarne et al., 2006). Individual cells were voltage clamped using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Voltage and current were digitized with a personal computer fitted with a Digidata 1320A acquisition board (Axon Instruments, USA). The electrometer was driven by pClamp software (pCLAMP8, Axon Instruments, USA). All experiments were performed at 22±2°C.

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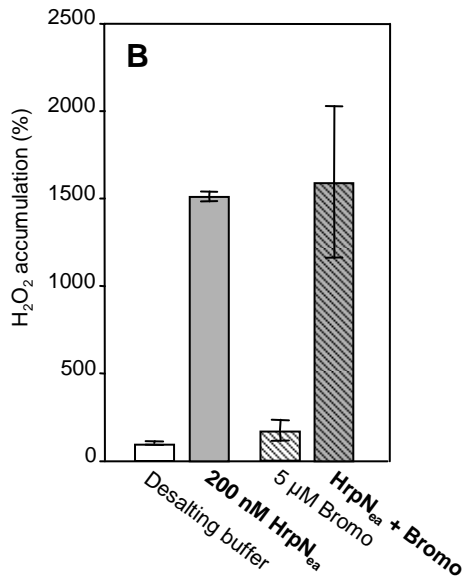
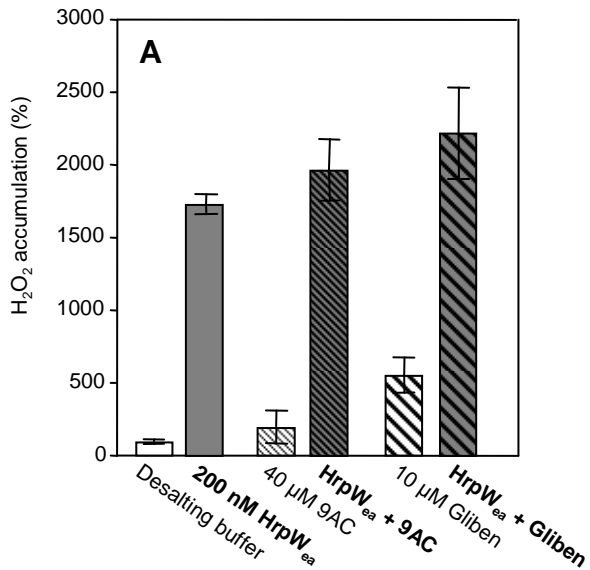


Table S1. *Erwinia amylovora* strains used for electrolyte leakage experiments.

<i>E. amylovora</i> strain	Relevant property	Genotype	Source or reference
Ea321	Wild type	<i>HrpN_{ea}, HrpW_{ea}</i>	CNPB 1367
Ea321-T5	Tn5-induced mutant in <i>HrpN_{ea}</i>	<i>hrpN_{ea}</i>	Wei et al., 1992
Ea321-G204	TnPhoA insertion in <i>HrpW_{ea}</i>	<i>hrpW_{ea}</i>	Kim and Beer, 1998
Ea321-T5-G204	Tn5-induced mutant in <i>HrpN_{ea}</i> and TnPhoA insertion in <i>HrpW_{ea}</i>	<i>hrpN_{ea}, hrpW_{ea}</i>	Kim and Beer, 1998