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

David Reboutier[1,2], Cécile Frankart[1], Joël Briand[1], Bernadette Biligui[1], Jean-Pierre Rona[1], Minna Haapalainen[3], Marie-Anne Barny[2] and François Bouteau[1,*]

[1]LEM, EA 3514, Université Paris Diderot, Case 7069, 2 place Jussieu, 75251 Paris cedex 5, France
[2]Laboratoire de Pathologie Végétale, UMR 217 INRA-INA-Paris VI, INA-PG, 16 rue Claude Bernard, 75231 Paris cedex 5, France
[3]General Microbiology, Department of Biological and Environmental Sciences, P.O.B. 56, 00014 University of Helsinki, Finland
*Author for correspondence (e-mail: francois.bouteau@univ-paris-diderot.fr)

Accepted 3 July 2007
Journal of Cell Science 120, 3271-3278 Published by The Company of Biologists 2007
doi:10.1242/jcs.011098

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
http://jcs.biologists.org/cgi/content/full/120/18/3271/DC1

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 [Ca^{2+}] 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; Reboutier et al., 2007) and ion flux modulations (Desikan et al., 1999; Popham et al., 1995; El-Maarouf et al., 2001; Reboutier et al., 2005; Reboutier 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 $[Ca^{2+}]$ 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-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). H_2O_2 production reached similar levels for the two harpins (Fig. 3B). Oxidative bursts reached their maximum between 30 minutes and 1 hour, then, H_2O_2 levels decreased and returned to control levels after 2 hours (Fig. 3A). In both cases, H_2O_2 production was blocked by the NADPH oxidase inhibitor DPI (10 μM) (Fig. 3B), suggesting that a plasma membrane NADPH oxidase was involved in H_2O_2 production.

To measure $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ peak reaching 2.5 μM, followed by a second slower transient $[Ca^{2+}]_{cyt}$ peak appearing within 1 minute and reaching 2 μM (Fig. 3C). However, neither $HrpW_{ea}$ nor $HrpN_{ea}$ triggered any $[Ca^{2+}]_{cyt}$ increase (Fig. 3C) over 60 minutes (data not shown). The absence of a Ca^{2+} response after addition of $HrpW_{ea}$ or $HrpN_{ea}$ indicated that $[Ca^{2+}]_{cyt}$ 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

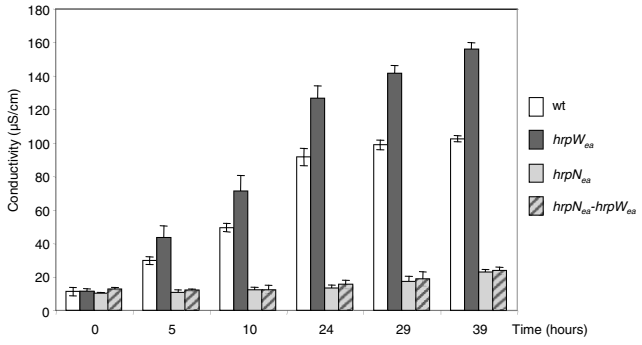

Fig. 1. Effects of *Erwinia amylovora* wild-type (wt), *hrpW*$_{ea}$, *hrpN*$_{ea}$ and *hrpN*$_{ea}$-*hrpW*$_{ea}$ mutant strains on electrolyte leakages from *A. thaliana* foliar disks. Disks were infiltrated with bacterial suspensions of 3×10^8 cells/ml. Values are means of three replicates and error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

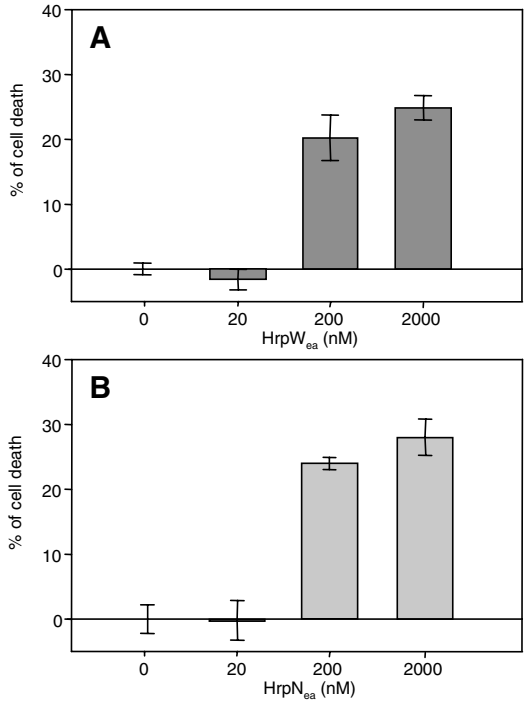

Fig. 2. Effect of increasing concentrations of $HrpW_{ea}$ (A), or $HrpN_{ea}$ (B), on cell death of *A. thaliana* suspension cells. Desalting buffer did not modify cell viability. Values are means of four replicates and error bars represent s.e.

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

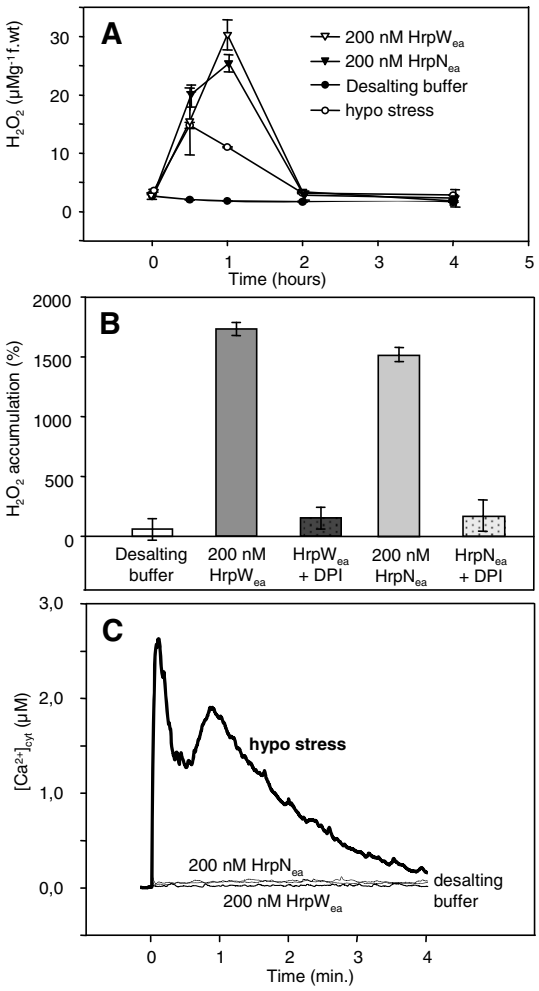

Fig. 3. Effect of 200 nM $HrpW_{ea}$ or $HrpN_{ea}$ on H_2O_2 production and on cytosolic Ca^{2+} level ($[Ca^{2+}]_{cyt}$) of *A. thaliana* suspension cells. (A) Time course of H_2O_2 accumulation in the medium of cells treated with 200 nM $HrpW_{ea}$, 200 nM $HrpN_{ea}$ or the corresponding volume of desalting buffer. Half dilution of cell suspension with distilled water triggered hypo-osmotic stress used as positive control. (B) Percentage of increase of H_2O_2 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 $[Ca^{2+}]_{cyt}$ were measured by using transgenic *A. thaliana* cells expressing aequorin protein. Cells suspended in culture medium containing 1 mM Ca^{2+}, 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 $HrpW_{ea}$

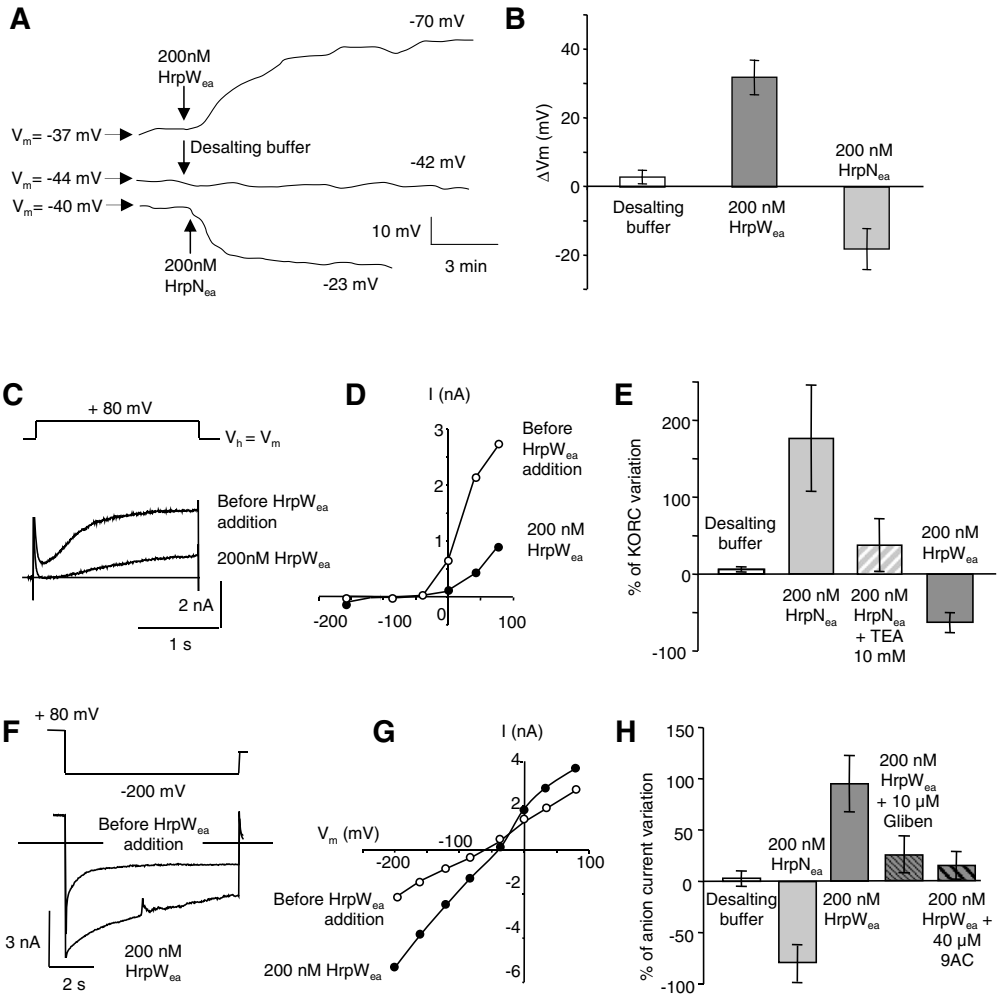

Fig. 4. Modulations of membrane potential (V_m), K^+ outward rectifying currents (KORCs) and anion currents of *A. thaliana* cells in response to 200 nM $HrpW_{ea}$ or $HrpN_{ea}$. (A) Free-running V_m observed in response to $HrpW_{ea}$, $HrpN_{ea}$ or the desalting buffer. (B) Mean values of the V_m variations. (C) Effect of 200 nM $HrpW_{ea}$ on KORCs recorded at +80 mV (leak substracted). 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 μM Glibenclamide or 40 μM 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

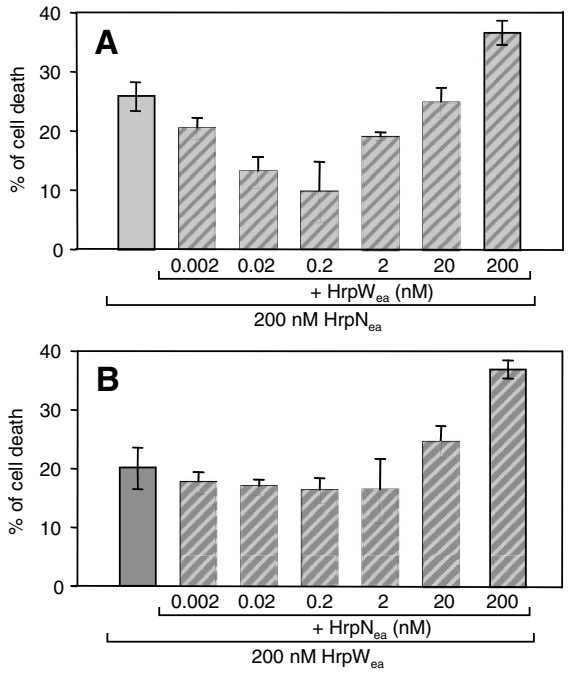

Fig. 5. Inhibitory effect of $HrpW_{ea}$ on cell death triggered by $HrpN_{ea}$. (A) Inhibition of $HrpN_{ea}$-induced cell death by increasing concentration of $HrpW_{ea}$ (from 0.002 nM to 200 nM) in *A. thaliana* suspension cells. (B) Increasing concentrations of $HrpN_{ea}$ (from 0.002 nM to 200 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.

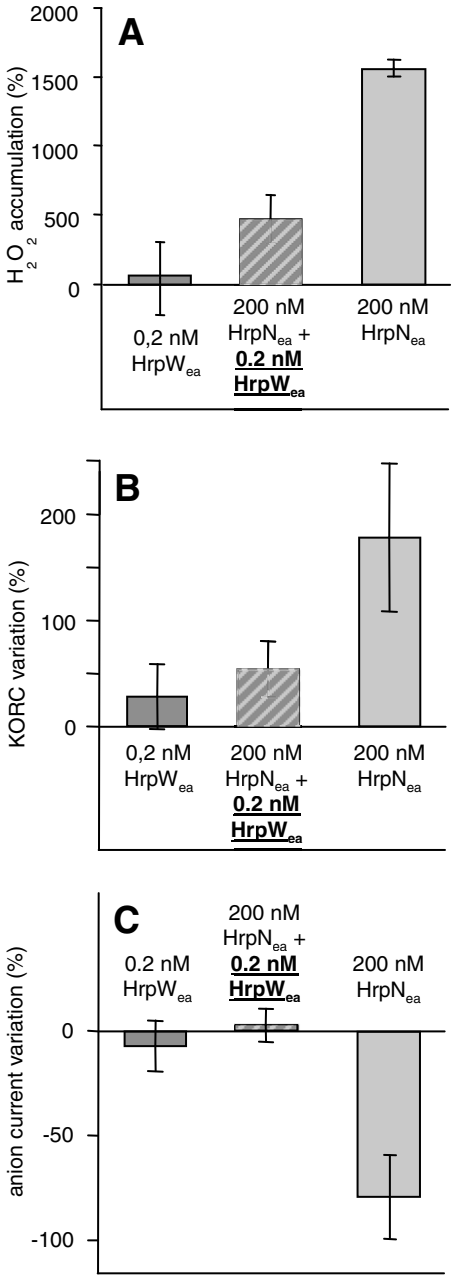

Fig. 6. Inhibitory effect of $HrpW_{ea}$ on H_2O_2 production and ion channel modulations triggered by $HrpN_{ea}$. (A) Inhibition of 200 nM $HrpN_{ea}$-induced H_2O_2 production by 0.2 nM $HrpW_{ea}$. Values are given as a percentage with respect to untreated cells (100%). Values are means of four replicates. (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.

et al., 1997) showing that $HrpN_{ea}$ could activate AOS production without triggering any $[Ca^{2+}]_{cyt}$ variation. It is possible that the use of diverse plant species, such as *Nicotiana tabaccum* (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 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

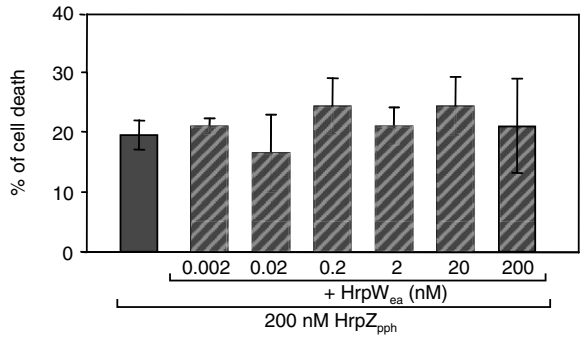

Fig. 7. Increasing concentrations of $HrpW_{ea}$ (from 0.002 nM to 200 nM) do not modify 200 nM $HrpZ_{pph}$-induced cell death in *A. thaliana* suspension cells. Values are means of three replicates and error bars represent s.e.

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 H_2O_2 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 H_2O_2 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 apoplasm. 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 apoplasm 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, bacause 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 $HrpZ_{pph}$-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 apoplasm. 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 apoptotis 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 $CaCl_2$, 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 NO_3^- (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 AAACTCGAGTTAAGC-CGCGCCCAGCTTGCC for $HrpN_{ea}$; AAGGATCCTAGTCAATTCTTACGC-TTTAAC and AAACTCGAGTTATTGGCATCTTCGCTGTG 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 *HrpNea* and *HrpWea*, 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 Prescission 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 KNO_3, 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 $CaCl_2$, 0.5 mM K_2SO_4 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 pharmocological 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 H_2O_2 in culture medium

H_2O_2 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 $K_3Fe(CN)_6$ 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 Ca^{2+} 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 Ca^{2+}) 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 $CaCl_2$ (final concentration). The resulting luminescence was used to estimate the total amount of aequorin in each experiment. Calibration of the Ca^{2+} 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.

This work is dedicated to Prof. Claude Grignon on the occasion of his retirement. We are grateful to A. Pugin and O. Lamotte for help with some experiments and S. V. Beer for kindly providing the *E. amylovora* mutant strains. We thank A. Pugin, H. Keller, J. M. Frachisse, S. V. Beer and C. S. Oh for fruitful discussions. This work was supported by funds from the MENESR (EA 3514 and ACI 5078) and INRA. D.R. was supported by a grant from MENESR.

References

Abramovitch, R. B., Kim, Y. J., Chen, S., Dickman, M. B. and Martin, G. B. (2003). Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J.* **22**, 60-69.

Adam, A. L., Pike, S., Hoyos, M. E., Stone, J. M., Walker, J. C. and Novacky, A. (1997). Rapid and transient activation of a myelin basic protein kinase in tobacco leaves treated with harpin from *Erwinia amylovora*. *Plant Physiol.* **115**, 853-861.

Amano, T., Hirasawa, K., O'Donohue, M. J., Pernolle, J. C. and Shioi, Y. (2003). A versatile assay for the accurate, time-resolved determination of cellular viability. *Anal. Biochem.* **314**, 1-7.

Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C. and Boucher, C. A. (1994). PopA1, a protein which induces a hypersensitivity-like response on specific Petunia genotypes, is secreted via the Hrp pathway of Pseudomonas solanacearum. *EMBO J.* **13**, 543-553.

Axtell, M. J. and Staskawicz, B. J. (2003). Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* **112**, 369-377.

Baker, C. J., Orlandi, E. W. and Mock, N. M. (1993). Harpin, an elicitor of the HR in tobacco caused by *E. amylovora*, elcits active oxygen production in suspension cells. *Plant Physiol.* **102**, 1341-1344.

Barny, M. A. (1995). *Erwinia amylovora hrp*N mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco. *Eur. J. Plant Pathol.* **101**, 333-340.

Blume, B., Nurnberger, T., Nass, N. and Scheel, D. (2000). Receptor mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell* **12**, 1425-1440.

Bouizgarne, B., El-Maarouf-Bouteau, H., Frankart, C., Reboutier, D., Madiona, K., Pennarun, A. M., Monestiez, M., Trouverie, J., Amiar, Z., Briand, J. et al. (2006). Early physiological responses of *Arabidopsis thaliana* cells to fusaric acid: toxic and signalling effects. *New Phytol.* **169**, 209-218.

Bradley, D. J., Kjellbom, P. and Lamb, C. J. (1992). Elicitor- and wound-induced oxidative cross-linking of a praline rich plant cell wall protein: a novel, rapid defense response. *Cell* **70**, 21-30.

Brault, M., Amiar, Z., Pennarun, A. M., Monestiez, M., Zhang, Z., Cornel, D., Dellis, O., Knight, H., Bouteau, F. and Rona, J. P. (2004). Plasma membrane depolarization induced by abscisic acid in Arabidopsis suspension cells involves reduction of proton pumping in addition to anion channel activation, which are both Ca^{2+} dependent. *Plant Physiol.* **135**, 231-243.

Bretz, J. R., Mock, N. M., Charity, J. C., Zeyad, S., Baker, C. J. and Hutcheson, S. W. (2003). A translocated protein tyrosine phosphatase of Pseudomonas syringae pv. tomato DC3000 modulates plant defence response to infection. *Mol. Microbiol.* **49**, 389-400.

Cessna, S. G., Messerli, M. A., Robinson, K. R. and Low, P. S. (2001). Measurement of stress-induced Ca^{2+} pulses in single aequorin-transformed tobacco cells. *Cell Calcium* **30**, 151-156.

Chandra, S., Stennis, M. and Low, P. S. (1997). Measurement of Ca^{2+} fluxes during elicitation of the oxidative burst in aequorin-transformed tobacco cells. *J. Biol. Chem.* **272**, 28274-28280.

Charkowski, A. O., Alfano, J. R., Preston, G., Yuan, J., He, S. Y. and Collmer, A. (1998). The Pseudomonas syringae pv. tomato HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. *J. Bacteriol.* **180**, 5211-5217.

DebRoy, S., Thilmony, R., Kwack, Y. B., Nomura, K. and He, S. Y. (2004). A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. USA* **101**, 9927-9932.

Desikan, R., Hancock, J. T., Coffey, M. J. and Neill, S. J. (1996). Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by NADPH oxidase-like enzyme. *FEBS Lett.* **382**, 213-217.

Desikan, R., Reynolds, A., Hancock, J. T. and Neill, S. J. (1998). Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *A. thaliana* suspension cultures. *Biochem. J.* **330**, 115-120.

Desikan, R., Clark, R., Atherfold, P., Hancock, J. T. and Neill, S. J. (1999). Harpin induces mitogen-activated protein kinase activity during defence responses in *Arabidopsis thaliana* suspension cultures. *Planta* **210**, 97-103.

El-Maarouf, H., Barny, M. A., Rona, J. P. and Bouteau, F. (2001). Harpin, a hypersensitive response elicitor from *Erwinia amylovora*, regulates ion channel activities in *Arabidopsis thaliana* suspension cells. *FEBS Lett.* **497**, 82-84.

Espinosa, A., Guo, M., Tam, V. C., Fu, Z. Q. and Alfano, J. R. (2003). The Pseudomonas syringae type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. *Mol. Microbiol.* **49**, 377-387.

Gaudriault, S., Malandrin, L., Paulin, J. P. and Barny, M. A. (1997). DspA, An essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of

Pseudomonas syringae, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol. Microbiol.* **26**, 1057-1069.

Gaudriault, S., Brisset, M. N. and Barny, M. A. (1998). HrpW of *Erwinia amylovora*, a new Hrp-secreted protein. *FEBS Lett.* **428**, 224-228.

Gauthier, A., Lamotte, O., Reboutier, D., Bouteau, F., Pugin, A. and Wendehenne, D. (2007). Cryptogein-induced anion effluxes: electrophysiological properties and analysis of the mechanisms through which they contribute to the elicitor-triggered cell death. *Plant Signal. Behav.* **2**, 89-98.

Hauck, P., Thilmony, R. and He, S. Y. (2003). A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. *Proc. Natl. Acad. Sci. USA* **100**, 8577-8582.

He, S. Y., Huang, H. C. and Collmer, A. (1993). *Pseudomonas syringae* pv. *Syringae* harpinPss: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**, 1255-1266.

He, S. Y., Bauer, D. W., Collmer, A. and Beer, S. V. (1994). Hypersensitive response elicited by *Erwinia amylovora* harpin requires active plant metabolism. *Mol. Plant Microbe Interact.* **7**, 289-292.

Holeva, M. C., Bell, K. S., Hyman, L. J., Avrova, A. O., Whisson, S. C., Birch, P. R. and Toth, I. K. (2004). Use of a pooled transposon mutation grid to demonstrate roles in disease development for Erwinia carotovora subsp. atroseptica putative type III secreted effector (DspE/A) and helper (HrpN) proteins. *Mol. Plant Microbe Interact.* **17**, 943-950.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* **62**, 379-433.

Ichinose, Y., Andi, S., Doi, R., Tanaka, R., Taguchi, F., Sasabe, M., Toyoda, K., Shiraishi, T. and Yamada, T. (2001). Generation of hydrogen peroxide is not required for harpin-induced apoptotic cell death in tobacco BY-2 cell suspension culture. *Plant Physiol. Biochem.* **39**, 771-776.

Jabs, T., Tschope, M., Colling, C., Hahlbrock, K. and Scheel, D. (1997). Elicitor-stimulated ion fluxes and O^{2-} from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc. Natl. Acad. Sci. USA* **94**, 4800-4805.

Kim, J. F. and Beer, S. V. (1998). HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. *J. Bacteriol.* **180**, 5203-5210.

Kim, M. G., da Cunha, L., McFall, A. J., Belkhadir, Y., DebRoy, S., Dangl, J. L. and Mackey, D. (2005). Two Pseudomonas syringae type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. *Cell* **121**, 749-759.

Kim, J. A., Kang, Y. S., Lee, S. H., Lee, E. H., Yoo, B. H. and Lee Y. S. (1999) Glibenclamide induced apoptosis through inhibition of CFTR-Cl-channels and intracellular Ca2+ release in HepG2 human hepatoblastoma cells. *Biochem. Biophys. Res. Commun.* **261**, 682-686.

Knight, H., Trewavas, A. J. and Knight, M. R. (1996). Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* **8**, 489-503.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583-593.

Li, C. M., Haapalainen, M., Lee, J., Nurnberger, T., Romantschuk, M. and Taira, S. (2005). Harpin of Pseudomonas syringae pv. phaseolicola harbors a protein binding site. *Mol. Plant Microbe Interact.* **18**, 60-66.

Lindeberg, M., Cartinhour, S., Myers, C. R., Schechter, L. M., Schneider, D. J. and Collmer, A. (2006). Closing the circle on the discovery of genes encoding Hrp regulon members and type III secretion system effectors in the genomes of three model Pseudomonas syringae strains. *Mol. Plant Microbe Interact.* **19**, 1151-1158.

Mackey, D., Belkhadir, Y., Alonso, J. M., Ecker, J. R. and Dangl, J. L. (2003). Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* **112**, 379-389.

Mehdy, M. C. (1994). Active oxygen species in plant against pathogens. *Plant Physiol.* **105**, 467-472.

Okada, Y. and Maeno, E. (2001) Apoptosis, cell volume regulation and volume-regulatory chloride channels. *Comp. Biochem. Physiol. Mol. Integr. Physiol.* **130**, 377-383.

Perino, C., Gaudriault, S., Vian, B. and Barny, M. A. (1999). Visualization of harpin secretion in planta during infection of apple seedlings by *Erwinia amylovora*. *Cell. Microbiol.* **1**, 131-141.

Pike, S. M., Ádám, A. L., Pu, X. A., Hoyos, M. E., Laby, R., Beer, S. V. and Novacky, A. (1998). Effects of *Erwinia amylovora* harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from perturbations caused by inoculated *E. amylovora*. *Physiol. Mol. Plant Pathol.* **53**, 39-60.

Popham, P. L., Pike, S. M. and Novacky, A. (1995). The effect of harpin from *Erwinia amylovora* on the plasmalemma of suspension-cultured tobacco cells. *Physiol. and Mol. Plant Pathol.* **47**, 39-50.

Racape, J., Belbahri, L., Engelhardt, S., Lacombe, B., Lee, J., Lochman, J., Marais, A., Nicole, M., Nurnberger, T., Parlange, F. et al. (2005). Ca^{2+}-dependent lipid binding and membrane integration of PopA, a harpin-like elicitor of the hypersensitive response in tobacco. *Mol. Microbiol.* **58**, 1406-1420.

Reboutier, D., Bianchi, M., Brault, M., Roux, C., Dauphin, A., Rona, J. P., Legue, V., Lapeyrie, F. and Bouteau, F. (2002). The indolic compound hypaphorine produced by ectomycorrhizal fungus interferes with auxin action and evokes early responses in nonhost *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* **15**, 932-938.

Reboutier, D., Frankart, C., Vedel, R., Brault, M., Duggleby, R. G., Rona, J. P., Barny, M. A. and Bouteau, F. (2005). A CFTR chloride channel activator prevents HrpN(ea)-induced cell death in *Arabidopsis thaliana* suspension cells. *Plant Physiol. Biochem.* **43**, 567-572.

Reboutier, D., Frankart, C., Briand, J., Biligui, B., Laroche, S., Rona, J. P., Barny, M. A. and Bouteau, F. (2007). The $HrpN_{ea}$ Harpin from *Erwinia amylovora* triggers differential responses on the nonhost *Arabidopsis thaliana* cells and on the host apple cells. *Mol. Plant Microbe Interact.* **20**, 94-100.

Samuel, M. A., Hall, H., Krzymowska, M., Drzewiecka, K., Hennig, J. and Ellis, B. E. (2005). SIPK signaling controls multiple components of harpin-induced cell death. *Plant J.* **42**, 406-416.

Tampakaki, A. P. and Panopoulos, N. J. (2000). Elicitation of hypersensitive cell death by extracellularly targeted HrpZPsph produced in planta. *Mol. Plant Microbe Interact.* **13**, 1366-1374.

Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A. and Beer, S. V. (1992). Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**, 85-88.

Wendehenne, D., Lamotte, O., Frachisse, J. M., Barbier-Brygoo, H. and Pugin, A. (2002). Nitrate efflux is an essential component of the cryptogein signaling pathway leading to defense responses and hypersensitive cell death in tobacco. *Plant Cell* **14**, 1937-1951.

Xie, Z. and Chen, Z. (2000). Harpin induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. *Mol. Plant Microbe Interact.* **13**, 183-190.