The HrpN_{ea} Harpin from *Erwinia amylovora* Triggers Differential Responses on the Nonhost *Arabidopsis thaliana* Cells and on the Host Apple Cells

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Erwinia amylovora is a gram-negative necrogenic bacterium causing fire blight of the *Maloideae* subfamily of *Rosaceae* such as apple and pear. It provokes progressive necrosis in aerial parts of susceptible host plants (compatible interaction) and a hypersensitive reaction (HR) when infiltrated in nonhost plants (incompatible interaction). The HrpN_{ea} harpin is a type three secretion system effector secreted by *E. amylovora*. This protein is involved in pathogenicity and HR-eliciting capacity of *E. amylovora*. In the present study, we showed that, in nonhost *Arabidopsis thaliana* cells, purified HrpN_{ea} induces cell death and H₂O₂ production, two nonhost resistance responses, but failed to induce such responses in host MM106 apple cells. Moreover, HrpN_{ea} induced an increase in anion current in host MM106 apple cells, at the opposite of the decrease of anion current previously shown to be necessary to induce cell death in nonhost *A. thaliana* cells. These results suggest that HrpN_{ea} induced different signaling pathways, which could account for early induced compatible or incompatible interaction development.

Erwinia amylovora is a gram-negative necrogenic bacterium causing fire blight of the *Maloideae* subfamily of *Rosaceae* such as apple (*Malus* spp.) and pear (*Pyrus* spp.). It provokes progressive necrosis in aerial parts of susceptible host plants (compatible interaction) and a hypersensitive reaction (HR) when infiltrated in nonhost plants (incompatible interaction) (Wei et al. 1992). HR is a form of programmed cell death and is commonly considered as an efficient defense mechanism developed by plants to circumvent pathogen infections (Dangl et al. 1996). HR could be triggered by entire microorganisms or by microbe-associated products, referred to as elicitors. HrpN_{ea} is a harpin protein secreted by *E. amylovora* via its type III

secretion system (T3SS) into the plant intercellular space (Perino et al. 1999). A *hrpN_{ea}* mutant strain expressed a reduced capacity to trigger HR cell death when inoculated in nonhost tobacco leaf (Barny 1995; Wei et al. 1992) and provoked a reduced oxidative burst in tobacco suspension cells when compared with the wild-type strain (Baker et al. 1993). These data were confirmed by using purified proteins; purified HrpN_{ea} triggered a typical HR cell death requiring an active metabolism when infiltrated in nonhost tobacco leaf tissue (He et al. 1993, 1994; Wei et al. 1992) and an oxidative burst in tobacco suspension cells (Baker et al. 1993; Cessna and Low 2001; Chandra and Low 1997). Purified HrpN_{ea} also triggered a set of classical plant responses to pathogens, i.e., MAPK activation (Adam et al. 1997), cytosolic [Ca²⁺] elevation (Cessna et al. 2001; He et al. 1994; Pike et al. 1998), defense genes activation (Dong et al. 1999; Peng et al. 2003), and ion flux modulations (El Maarouf et al. 2001; Pike et al. 1998; Popham et al. 1995).

Effects of HrpN_{ea} in host plants remain more elusive. To our knowledge, there is no study showing the effect of purified HrpN_{ea} in host plant cells. Yet, studies using *E. amylovora* *hrpN_{ea}* mutant strains revealed that a *hrpN_{ea}* mutant expressed a reduced capacity to trigger necrotic lesions when inoculated in host plant tissue, suggesting that HrpN_{ea} is a determinant pathogenicity factor (Barny 1995; Wei et al. 1992). Such *hrpN_{ea}* mutants provoked a reduced oxidative burst when compared with wild-type strain (Venisse et al. 2003) suggesting that HrpN_{ea} is also involved in active oxygen species (AOS) production in the host plant. According to Venisse and associates (2001), pathogenicity of *E. amylovora* depends on its ability to trigger AOS production, thus suggesting that *E. amylovora* could take advantage of the lethal action of AOS on plant cells for a successful development and that AOS are a key factor in disease enhancement.

Collectively, these data suggest that HrpN_{ea} could have the same effects on nonhost and on host plants. Yet, the direct involvement of HrpN_{ea} in host plants remains unclear, since purified HrpN_{ea} has never been tested. In this study, by using susceptible MM106 apple suspension cells (host), we aimed to investigate the effects of purified HrpN_{ea} on cell death and AOS production, to compare them with those triggered in *Arabidopsis thaliana* suspension cells (nonhost). We showed differential behaviors for MM106 apple suspension cells and *A. thaliana* suspension cells in response to HrpN_{ea} concerning cell death, AOS production, and ion channel modulation, an

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early prerequisite to plant cell-death induction (Reboutier et al. 2005). Our results suggest that HrpN_{ea} induced different signaling pathways, which could account for early induced compatible or incompatible interaction development.

RESULTS

Comparison of the effects triggered by HrpN_{ea} on cell death and AOS production in the host MM106 and in the nonhost *A. thaliana* suspension cells.

A. thaliana and MM106 apple suspension cells viability was quantified by using fluorescein diacetate (FDA). HrpN_{ea} did not alter viability of MM106 apple cells up to 1 μ M (Fig. 1) but induced a dose-dependent cell death (Fig. 1) in *A. thaliana* cells. Maximum cell death was obtained for 200 nM HrpN_{ea}.

To quantify AOS generation in response to HrpN_{ea}, we measured H₂O₂ production. In MM106 suspension cells, 200 nM HrpN_{ea} did not trigger any significant H₂O₂ production when compared with the control (Fig. 2A and C), even after 24-h exposure, whereas in *A. thaliana* suspension cells, 200 nM HrpN_{ea} induced a rapid transitory increase of H₂O₂ production (Fig. 2B and C). The H₂O₂ level increased during the first 30 min and then decreased and returned to control level (Fig. 2A and B). Interestingly, hypoosmotic shock, used as positive control, triggered a much stronger transitory oxidative burst in MM106 than in *A. thaliana* suspension cells (Fig. 2A and B), thus suggesting a high AOS production potential from apple cells when compared with *A. thaliana* cells.

Effect of HrpN_{ea} on ion currents.

In previous studies, we showed that HrpN_{ea} modulated ion channels and triggered cell death by inhibiting anion channel activity (El Maarouf et al. 2001; Reboutier et al. 2005). To determine whether ion channel modulation could participate in the differential effect of HrpN_{ea} on cell death, we investigated effects of purified HrpN_{ea} on K⁺ and anion channel in apple cells and compared them with those triggered by HrpN_{ea} in *A. thaliana* cells. Under control conditions, successful impalements were obtained in turgid cultured cells of *A. thaliana* and MM106. Previous electrophysiological and pharmacological studies made on *A. thaliana* cells (Reboutier et al. 2002) allowed the identification of two main ion channel currents in the plasma membrane: K⁺ outward rectifying currents (KORC) and slow deactivating anion currents. Ion currents recorded on

MM106 cells showed the same outline as those of *Arabidopsis* cells. A first MM106 cell population displayed predominant currents characterized by a slow deactivation (Fig. 3A), as described for slow-type anion channels (Schroeder and Keller 1992). The cells with these predominant currents had a plasma membrane potential of -31 ± 12 mV ($n = 11$), being close to the equilibrium potential estimated for NO₃⁻ ($E_{\text{NO}_3^-} \approx -20$ mV, $[\text{NO}_3^-]_{\text{out}} \approx 11$ mM with $[\text{NO}_3^-]_{\text{in}}$ estimated at 5 mM). These currents were sensitive to potent anion channel blockers: 9-anthracene-carboxylic acid (9AC, 40 μ M) (Fig. 3B and C) and glibenclamide (10 μ M) as the anion current described in *A. thaliana* suspension cells (Table 1). Although, the inhibitors have less effect on apple cells than on *A. thaliana* cells, probably due to different species sensitivity as previously reported (Forestier et al. 1998), these data suggest that anion channels are good candidates for the mediation of these large deactivating currents in MM106 cells.

As previously reported in *A. thaliana* for HrpN_{ea} purified as cell-free elicitor preparation (El Maarouf et al. 2001; Reboutier et al. 2005), the affinity chromatography-purified HrpN_{ea} (200 nM) decreased anion currents in about 2 min in *A. thaliana* suspension cells (Fig. 3E). However, these currents were increased in MM106 (Fig. 3D and E). The desalting harpin buffer used as negative control had no significant effect whatever the cell-suspension type (Fig. 3E). We could not determine any concentration-dependent effect of HrpN_{ea} on anion channel regulation in the 0.02 to 2 μ M range for *A. thaliana* and MM106 suspension cells (data not shown).

The second MM106 cell population presented predominant time- and voltage-dependent outward rectifying currents activated by depolarizing pulses (Fig. 4A and B), thus sharing characteristics of KORC (Schroeder, 1988). These cells with predominant time- and voltage-dependent outward rectifying currents were polarized at -53 ± 16 mV ($n = 9$) and, thus, closed to the equilibrium potential estimated for K⁺ in our conditions ($E_{\text{K}^+} \approx -60$ mV, assuming $[\text{K}^+]_{\text{out}} \approx 10$ mM with $[\text{K}^+]_{\text{in}}$ estimated at 100 mM). Tail current analysis indicated reversal potentials of -56 mV, also close to the estimated E_{K^+} . BaCl₂ at 5 mM, a potent K⁺ channel inhibitor, reduced drastically these time-dependent outward rectifying currents (Fig. 4A and B), as observed for KORC of *A. thaliana* cells (Table 1). All these data support the hypothesis of currents being due to K⁺ efflux, equivalent to KORC from *A. thaliana* suspension cells (Reboutier et al. 2002).

Fig. 1. Effect of HrpN_{ea} (0.01 to 1 μ M) on cell viability of *Arabidopsis thaliana* and MM106 suspension cells. Data correspond to mean values $\pm$ SE and were obtained from at least three independent experiments.

Fig. 2. Effect of 200 nM HrpN_{ea} on production of H₂O₂ of *Arabidopsis thaliana* and MM106 suspension cells. Time course of H₂O₂ accumulation in the medium of **A**, MM106 or **B**, *A. thaliana* cells. Suspension cells were treated by 200 nM HrpN_{ea} (○) or the corresponding volume of desalting buffer (▽). Half dilution of cell suspension with distilled water triggered hypoosmotic stress used as positive control (▼). Data correspond to mean values $\pm$ standard error and were obtained from at least six independent experiments. **C**, Percentage of variation of H₂O₂ accumulation, at 1 h, in the medium of *A. thaliana* or MM106 cells treated by HrpN_{ea} or by the corresponding volume of desalting buffer. Values are given as percentage with respect to untreated cells. Values are mean of six replicates. Error bars correspond to standard error.

As previously reported for HrpN_{ea} purified as cell free elicitor preparation (El Maarouf et al. 2001), the affinity chromatography-purified HrpN_{ea} (200 nM) increased *A. thaliana* KORC activities, when the desalting harpin buffer had no significant effect on ion channel regulation (Fig. 4D). No significant changes were observed for KORC in MM106 suspension cells (Fig. 4C and D). As for anion currents, we couldn't determine any concentration-dependent effect of HrpN_{ea} on KORC regulation in the 0.02 to 2 μ M range, as in *A. thaliana* suspension cells and MM106 (data not shown).

DISCUSSION

In the present study, our aim was to investigate, in host MM106 apple cells, effects of purified HrpN_{ea} on cell death and AOS production, typical nonhost resistance responses, to compare them with those triggered in nonhost *A. thaliana* cells. HrpN_{ea} triggered cell death in *A. thaliana* suspension cells, whereas it did not trigger cell death in apple suspension cells. In the same way, HrpN_{ea} provoked H₂O₂ production in *A. thaliana* suspension cells, as previously reported on tobacco cells (Baker et al. 1993), but not in MM106 apple cells. These data are in accordance with the hypothesis that host plant cells do not raise defense against a compatible pathogen or raise a response too weak or too delayed to stop the pathogen. However, HrpN_{ea} was described as involved in AOS production and necrotic symptoms triggered by *E. amylovora* in host plants (Venisse et al. 2001, 2003). Our results then suggest that HrpN_{ea} involvement in oxidative burst and cell death triggered by *E. amylovora* is likely to be indirect and that HrpN_{ea} could participate to the effect of another effector more directly involved in AOS production than in cell death. In *Erwinia carotovora*, HrpN is considered more likely to be a T3SS-assisting protein than a true effector (Holeva et al. 2004). DspA/E is another T3SS effector involved in cell death and oxidative burst triggered by *E. amylovora* in apple (Boureau et al. 2006; Venisse et al. 2003). *dspA/E* mutants are systematically more affected in their capacity to induce an oxidative burst than *hrpN_{ea}* mutants and a *hrpN_{ea}-dspA/E* double mutant no longer triggered AOS production (Venisse et al. 2003). Collectively, these data suggest that, in host cells, DspA/E could be a good candidate for oxidative burst elicitation and cell death in response to *E. amylovora* and that HrpN_{ea} could participate to DspA/E effects. Other harpins such as HrpZ_{Psp} or PopA were shown to insert into biological membranes to form ion-conducting pores (Lee et al. 2001; Racapé et al. 2005). HrpN_{ea}, sharing the same physicochemical characteristics as HrpZ_{Psp} or PopA, could also be inserted into the plasma membrane and form pores. Such pore-forming capacity could be involved in forming a protean structure similar to bacterial type III translocator, which allows type III effectors translocation (i.e., DspA/E translocation) across the host plasma membrane, as it was shown for YopB, YopD, and LcrV from *Yersinia* spp. (Buttner and Bonas 2002). If HrpN_{ea} assists DspA/E in entering the host cells, this would explain the in planta results indicating that a *hrpN_{ea}* mutant is defective in the ability to cause disease. Some nonhost plants are able to recognize HrpN_{ea} and respond with programmed cell death, whereas the host plants are probably missing a guarding receptor able to recognize the harpin and give the alarm. Host plants may also have many other traits that make them more susceptible. For example, genes encoding proteins that directly interact with DspA/E of *E. amylovora* were found only in the host plants but not in the nonhost plants, including *Arabidopsis* (Meng et al. 2006).

Unexpectedly, H₂O₂ levels produced by apple suspension cells in response to the control hypoosmotic stress were much higher (about 20 times) than H₂O₂ levels produced by *A.*

thaliana suspension cells. H_2O_2 levels reached in apple plant tissue in response to *E. amylovora* were never determined and could thus not be compared with H_2O_2 levels reached in response to hypoosmotic stress. Nevertheless, the fact that apple cells produced high amounts of AOS could contribute to the strategy, used by *E. amylovora*, consisting of the induction of an oxidative burst to colonize host plants and to trigger disease (Venisse et al. 2003). This hypothesis is in accordance with the fact that *E. amylovora* tolerates more than 10 times higher concentrations of H_2O_2 than *P. syringae* pv. *tabaci* (Venisse et al. 2003) and possesses enzymes such as catalases and peroxidases that can directly break down H_2O_2 (Keck et al. 1997).

In apple suspension cells, $HrpN_{ea}$ did not modify K^+ channel activity and provoked an increase in anion channel activity as opposed to anion channel decrease triggered by $HrpN_{ea}$ in non-host *A. thaliana*. The fact that this effect was not dependent on $HrpN_{ea}$ concentration (within the range tested) suggests that the effect is more likely to be indirect. Increase in anion channel activity is probably not linked to the ability of *E. amylovora* to provoke electrolyte leakage (Venisse et al. 2001). Electrolyte leakages were observed in nonhost as well as in susceptible host tissues and seemed to be dependent on AOS accumulation (Venisse et al. 2001). The authors suggest that the electrolyte leakage, a later response as compared with anion channel increase, could be due to an alteration of the membranes through lipid peroxidation. Although only regulation of K^+ channel ac-

tivities was reported as a component of a signaling pathway during compatible interaction (cf9/Avr9; Blatt et al. 1999), the increase in anion channel activity of MM106 cells is likely due to the function of $HrpN_{ea}$ as a pathogenicity factor. Such an increase in anion channel activity was already reported to be a necessary step of different signaling pathways induced in response to cryptogin (Wendehenne et al. 2002) or the phytohormone abscisic acid (Ghelis et al. 2000). This result showed that $HrpN_{ea}$ is perceived by apple cells, although it did not trigger

Table 1. Effect of Ba^{2+} , 9-anthracen carboxylic acid (9-AC), and glibenclamide^a

Suspension cell type	MM106	<i>A. thaliana</i>
KORC		
By 5 mM $BaCl_2$	$97 \pm 7\%$ ($n = 3$)	$83 \pm 5\%$ ($n = 15$)
Anion currents		
By 40 μ M 9-AC	$28 \pm 6\%$ ($n = 5$)	$61 \pm 10\%$ ($n = 5$)
By 10 μ M g of libenclamide	$40 \pm 3\%$ ($n = 5$)	$63 \pm 20\%$ ($n = 7$)

^a Inhibition by Ba^{2+} , a K^+ channel blocker, on steady state K^+ outward rectifying currents (KORC) activated at +80 mV of MM106 suspension cells and *Arabidopsis thaliana* and effect of 9-AC and glibenclamide, two anion channel blockers, on anion currents of MM106 suspension cells and *A. thaliana* recorded at -200 mV after 2 s of deactivation. Inhibitions are given as a percentage with respect to the control level. Data correspond to mean values $\pm$ standard deviation and n is the number of independent experiments.

Fig. 3. $HrpN_{ea}$ induced changes in anion currents of MM106 and *Arabidopsis thaliana* suspension cells. **A**, MM106 slow relaxation currents in response to a hyperpolarizing voltage steps from -200 to 40 mV after a prepulse of +40 mV. **B**, Currents measured at -200 mV in control conditions and after addition of 40 μ M 9-anthracen carboxylic acid (9-AC), an anion channel inhibitor (V_h = holding potential, V_m = membrane potential). **C**, Corresponding current-voltage relationship. **D**, Currents measured at -200 mV in control conditions and after addition of 200 nM $HrpN_{ea}$. **E**, Maximal anion current variation recorded at -200 mV in response to $HrpN_{ea}$ and the desalting buffer used as negative control for MM106 and *A. thaliana* suspension cells. Data correspond to mean values $\pm$ standard error in percentage of the control level. Data were obtained from at least five independent experiments.

cell death or AOS production. In *A. thaliana*, anion channel inhibition was shown to participate to HrpN_{ea}-induced cell death (Reboutier et al. 2005). The fact that HrpN_{ea} did not provoke the same anion channel modulation in apple and in *A. thaliana* cells showed that HrpN_{ea} did not induce the same signaling pathways in these two plants. Moreover, it might explain that HrpN_{ea} did not trigger cell death in apple cells. Taken together these results confirm that anion channels are a determinant feature of plant response to harpins and could contribute to the determination of the issue leading to cell death or cell survival and, thus, to incompatibility or compatibility.

To conclude, in *A. thaliana* suspension cells, HrpN_{ea} immediately triggers cell death and AOS production, defense responses that putatively allow plant cells to circumvent infection. On the opposite hand, in apple cells, HrpN_{ea} is probably recognized, since anion channel activity is increased, but does not trigger the cell death and AOS defense response. The absence of such appropriate responses could explain apple susceptibility to *E. amylovora*.

MATERIALS AND METHODS

Cell culture conditions.

Arabidopsis thaliana L. (ecotype Columbia) and *Malus* sp. (MM106) suspension cells were grown at $24 \pm 2^\circ\text{C}$ under continuous white light ($40 \mu\text{E m}^{-2} \text{s}^{-1}$) with rotation shaking, in a 1-liter round-bottom flask containing 350 ml of Gamborg culture medium (Reboutier et al. 2002). For MM106 cell suspen-

sion, 100 μl of 2.4D (1 mg/ml) and 100 μl of kinetin (1 mg/ml) were added. The pH of the culture medium was adjusted at 5.8. Cells were subcultured weekly by a 10-fold dilution.

Harpin preparation.

hrpN_{ea} was polymerase chain reaction (PCR)-amplified with high fidelity TAQ (Roche) using pMAB 40 as a template and the following primers: AACGGATCCATGAGTCTGAATAC AAGTGGG and AAACCTCGAGTTAAGCCGCGCC CAGCTT GCC. The PCR-amplified products were digested with *Bam*H1 and *Xho*1, were cloned into the pGEX6P vector (Amersham, Tokyo), and were sequenced. The plasmid containing hrpN_{ea}, named pMAB164, was used to transform *Escherichia coli* BL21. *Escherichia coli* strain was grown up to an optical density of 0.5 at 600 nm, and the overproduction of GST-HrpN_{ea} was induced by 1 mM isopropyl- β -D-thiogalactoside. Bacterial cells were harvested, were resuspended in 12 ml of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.3), and were sonicated for 5 min. After centrifugation, the supernatant was injected into the affinity chromatography column (GST-trap FF 1 ml; Amersham). The GST tag was removed by using the Prescission protease enzyme (Amersham). Then, HrpN_{ea} was eluted and desalted with a Hi-Trap desalting column (Amersham). By using this type of column, the protein buffer is changed to 5 mM KNO₃, 1 mM morpholineethanesulfonic acid, pH 5.8. This desalting buffer was used as control in all experiments.

Fig. 4. HrpN_{ea} induced changes in KORC (K⁺ outward rectifying currents) of *Arabidopsis thaliana* but not in KORC of MM106 suspension cells. **A**, MM106 time-dependent currents in response to a depolarizing voltage step of +40 mV (leak subtracted), measured in control conditions and after addition of 5 mM BaCl₂, a K⁺ channel inhibitor (V_h = holding potential, V_m = membrane potential). **B**, Corresponding current-voltage relationship. **C**, Currents measured at +80 mV in control conditions and after addition of 200 nM HrpN_{ea}. **D**, Steady state KORC increase recorded at +80 mV in response to HrpN_{ea} and the desalting buffer used as negative control for MM106 and *A. thaliana* suspension cells. Data correspond to mean values $\pm$ standard error in percentage of the control level. Data were obtained from at least five independent experiments.

Cell-death quantification.

Cell death was quantified using the FDA spectrofluorimetric method according to Amano and associates (2003). Briefly, 4-day-old *Arabidopsis thaliana* or MM106 suspension cells were collected and washed by filtration in a suspension buffer containing 175 mM mannitol, 0.5 mM CaCl_2 , 0.5 mM K_2SO_4 , and 10 mM HEPES (H10 medium), adjusted to pH 5.8 (with KOH). A culture of 1 ml of cell suspension was incubated in the presence or absence of HrpN_{ea}. When the incubation time was up (after 24 h), 500 μl of the suspension was diluted in 1.5 ml of H10 medium in a quartz cuvette (final cell density was 1×10^5 cells/ml). 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 over a 120-s time period, using a Hitachi F-2000 spectrofluorimeter. The slope of fluorescence increase, representing cell viability, was calculated for each treatment and directly compared with nontreated cells. Cell death was calculated in percent with respect to the cell death standard level, corresponding to nontreated cells and 0% of cell death. Cell death was calculated as follows: % of cell death = (slope of treated cells/slope of nontreated cells) $\times$ 100.

Quantification of H_2O_2 in the culture medium.

H_2O_2 release in the medium culture was quantified, as described in Bouizgarne and associates (2006), by measuring the chemiluminescence of luminol (Sigma, St. Louis) reacting with H_2O_2 . Briefly, 1.5 ml of the cell suspension (stabilized for 4 h in H10 medium) was inoculated with HrpN_{ea}. Before each measurement, 200 μl of the medium culture was added to 600 μl of phosphate buffer (50 mM, pH 7.9) prior to addition of 100 μl of luminol 1.1 mM. Then, 100 μl of $\text{K}_3\text{Fe}(\text{CN})_6$ 14 mM was added as electron acceptor. Chemiluminescence was monitored at 30-min intervals with a FB12-Berthold luminometer (signal integrating time of 0.2 s).

Electrophysiology.

Experiments were conducted at $22 \pm 2^\circ\text{C}$ on 4-day-old cultures. Single electrode voltage clamp recording was performed as previously described (Bouizgarne et al. 2006; El-Maarouf et al. 2001; Rebutier et al. 2002). The main ions in the medium after 4 days of *A. thaliana* cell culture were 9 mM K^+ and 11 mM NO_3^- (Rebutier et al. 2002).

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