Membrane-Targeted HrpN_{Ea} Can Modulate Apple Defense Gene Expression

E. Vergne,^{1,2,3} T. Dugé de Bernonville,^{1,2,3} F. Dupuis,^{1,2,3} S. Sourice,^{1,2,3} R. Cournol,^{1,2,3} P. Berthelot,^{1,2,3} M. A. Barny,⁴ M. N. Brisset,^{1,2,3} and E. Chevreau^{1,2,3}

¹INRA, UMR1345 Institut de Recherche en Horticulture et Semences, F-49071 Beaucouzé, France; ²Université d'Angers, UMR1345 Institut de Recherche en Horticulture et Semences, SFR 4207 QUASAV, PRES L'UNAM, F-49045 Angers, France; ³AgroCampus-Ouest, UMR1345 Institut de Recherche en Horticulture et Semences, F-49045 Angers, France; ⁴INRA, Laboratoire des Interactions Plantes-Pathogènes, F-75231 Paris, France

Submitted 9 October 2013. Accepted 15 October 2013.

Fire blight caused by *Erwinia amylovora* is the major bacterial disease of tribe *Maleae*, including apple. Among the proteins secreted by this bacterium, HrpN_{Ea}, also called harpin, is known to induce hypersensitive response in non-host plants and to form amyloid oligomers leading to pore opening in the plasma membrane and alteration of membrane homeostasis. To better understand the physiological effects of HrpN_{Ea} in the host plant, we produced transgenic apple plants expressing HrpN_{Ea} with or without a secretion signal peptide (SP). HrpN_{Ea} expressed with a SP was found to be associated within the membrane fraction, in accordance with amyloidogenic properties and the presence of transmembrane domains revealed by *in silico* analysis. Expression analysis of 28 apple defense-related genes revealed gene modulations in the transgenic line expressing membrane-targeted HrpN_{Ea}. While apple transgenic trees displaying a high constitutive expression level of SP-HrpN_{Ea} showed a slight reduction of infection frequency after *E. amylovora* inoculation, there was no decrease in the disease severity. Thus HrpN_{Ea} seems to act as an elicitor of host defenses, when localized in the host membrane.

Many gram-negative phytopathogenic bacteria use a type III secretion system (T3SS) encoded by a hypersensitive response and pathogenicity (*hrp*) gene cluster to manipulate the metabolism of their host and trigger susceptibility. Among the diverse type III secreted proteins, harpins are a class of helper proteins secreted into the apoplast and harboring shared common characteristics, such as high glycine content, lack of cysteine, high thermal stability, susceptibility to proteases, and acidic composition (Alfano and Collmer 1997; Kim and Beer 1998; Wei et al. 1992). When infiltrated into nonhost plants such as *Arabidopsis* or tobacco, harpins trigger the expression of defense

responses associated with disease resistance, such as the hypersensitive reaction (HR), accumulation of pathogenesis-related (PR) gene transcripts, and systemic acquired resistance (Baker et al. 1993; Dong et al. 1999; Galan and Collmer 1999; Gopalan et al. 1996; He et al. 1993; Strobel et al. 1996).

Current understanding of the biochemical mechanisms of HR elicitation by harpins in nonhost plants is far from complete. Earlier works suggested that several harpins act on plant membranes (Ahmad et al. 2001; Lee et al. 2001a; Pike et al. 1998; Racape et al. 2005). More recently, it was shown that HpaG, a harpin of a *Xanthomonas* sp., is able to form bioactive amyloid fibrils when infiltrated into tobacco intercellular spaces, leading to the HR of the infiltrated leaf area (Oh et al. 2007). Harpins share a high content in α -helical secondary structural motifs, among which are found coiled-coil (CC)- and leucine-zipper (LZ)-like motifs (Table 1) that have been shown to be responsible for the formation of dimeric or oligomeric structures in many α -helical proteins (Conway and Parry 1990; Gonzalez et al. 1996; Landschulz et al. 1988; Lupas and Gruber 2005; Lupas et al. 1991; O'Shea et al. 1989). These CC or LZ α -helical regions were found to correlate with HR-inducing regions in several harpins (Table 1), in accordance with their role in amyloid oligomer formation.

The first isolated harpin was HrpN_{Ea}, produced by *Erwinia amylovora* (Wei et al. 1992), and its structure-activity relationship has been quite well investigated on nonhost plants. HrpN_{Ea} protein was reported to cause depolarization of tobacco leaf cells (Pike et al. 1998), to inhibit mitochondrial functions in tobacco (Xie and Chen 2000), and to regulate the activity of different plasma membrane ion channels in *Arabidopsis* (El-Maarouf et al. 2001). Like HpaG and HrpZ, HrpN_{Ea} was more recently found to undergo amyloidogenesis in plant apoplast-like conditions (Oh et al. 2007), and three LZ-like and three HR-inducing motifs were found in its sequence (Table 1; Fig. 1, blocks 6 and 7) (Hao and Wei 2001; Sinn et al. 2008; Tarafdar et al. 2009). Among these motifs, the C-terminal one seems to be essential for virulence as well as for the HR-inducing activity (Sinn et al. 2008). In accordance with its shared characteristics with amyloid proteins, namely its pore-forming activity, HrpN_{Ea} was also found to be required for translocation of DspA/E, the essential pathogenicity effector of *E. amylovora*, into tobacco cells (Bocsanczy et al. 2008). Immunogold labeling revealed that HrpN_{Ea} proteins are secreted (Perino et al. 1999) and densely deposited along the Hrp pilus, which drives the type III effector proteins toward plant cells (Jin et al. 2001). HrpN-CyaA fusion experiments also indicated a weak translocation of HrpN_{Ea} into cells of infiltrated apple leaves (Boureau et al. 2011). However,

Current address for T. Dugé de Bernonville: EA2106 Biomolécules et Biotechnologies Végétales, UFR Sciences Pharmaceutiques, Université François Rabelais, 31 avenue Monge, 37200 Tours, France

Current address for S. Sourice: INSERM, Laboratoire d'Ingénierie Ostéo-articulaire et Dentaire, F-44042 Nantes, France

Corresponding author: E. Chevreau;
E-mail: elisabeth.chevreau@angers.inra.fr

*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary tables are published online.

direct evidence that HrpN_{Ea} inserts itself into the plasma membrane of plant cells to ensure its function is still lacking, on non-host as well as on host plants.

In *Maleae* host plants, *E. amylovora* causes fire blight, a devastating disease of worldwide economic incidence, particularly on apple and pear production. Infection occurs via natural openings such as nectaries in flowers or via wounds on leaves

or on succulent shoots. In susceptible cultivars, multiplication and progression of the bacteria in host parenchyma is followed by the progressive necrosis of the invaded tissues, whereas the necrosis is restricted to the infection site in resistant cultivars. While many studies investigated the HR-inducing activity of HrpN_{Ea} on nonhost plants, fewer works attempted to understand its effects during disease development on host plants.

Table 1. Correlation between coiled coil (CC)- and leucine zipper (LZ)-like regions and hypersensitive reaction (HR)-inducing regions in harpins of different bacteria

Harpin	HpaG	Hpa1	Hpa1	HpaXm	HrpZ	HrpZ	HrpN	HrpN
Bacteria	<i>Xanthomonas axonopodis</i> pv. <i>glycines</i>	<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>	<i>Xanthomonas oryzae</i> pv. <i>oryza</i>	<i>Xanthomonas citri</i> subsp. <i>malvacearum</i>	<i>Pseudomonas syringae</i> pv. <i>syringae</i>	<i>Pseudomonas syringae</i> pv. <i>phaseolicol</i>	<i>Erwinia pyrifoliae</i>	<i>Erwinia amylovora</i>
Protein size	133 aa ^a	137 aa	139 aa	133 aa	341 aa	345 aa	429 aa	403 aa
CC and LZ-like α -helical regions	41 to 59 (LZ) 91 to 109 (LZ)	38 to 57 (LZ)	36 to 53 (CC) 87 to 103 (CC)	39 to 52 (CC)	71 to 89 (LZ) 297 to 330 (LZ)	65 to 90 (LZ) 304 to 337 (LZ)	51 to 69 (LZ) 181 to 199 (LZ) 403 to 423 (LZ)	51 to 70 (LZ) 156 to 174 (LZ) 379 to 397 (LZ)
HR-inducing regions as infiltrated elicitor	35-53 N-terminal	38-57 N-terminal	36-53 N-terminal	39-52 N-terminal	109 first aa in N-terminal 216 last aa in C-terminal	200-345 C-terminal	43-70 and 165- 201 N-terminal	43-70 and 140- 176 N-terminal
HR inducing regions as secreted protein	–	–	–	–	–	–	–	255-403 C-terminal
References	Kim et al. 2004 Tarafdar et al. 2009	Wang et al. 2008	Ji et al. 2011	Miao et al. 2010	Alfano et al. 1996 Tarafdar et al. 2009	Lee et al. 2001b	Shrestha et al. 2008	Hao and Wei 2001 Sinn et al. 2008 Tarafdar et al. 2009

^a aa = amino acid

Fig. 1. Schematic summary of domains found in HrpN_{Ea} protein. Black circles above the first block are placed every 20 amino acids. In the first block are shown the secondary structures β -sheet (light gray box; 13 to 17) and α -helix (hard gray boxes; 52 to 70, 76 to 77, 102 to 103, 128 to 133, 157 to 173, 193 to 200, 202 to 210, 214 to 218, 251 to 261, 267 to 275, 292 to 306, 308 to 310, 329 to 335, 345 to 346, 348 to 362, 383 to 400) predicted on the I-TASSER server. In the seven following blocks, elements are represented by black boxes. The second to the fifth blocks represent transmembrane domain predictions and putative subcellular localization of the different segments obtained through, respectively, PRED-TMR (56 to 76), DAS (65 to 70, 213 to 221), MEMSAT (56 to 71) and PRED-TMBB (106 to 114, 162 to 172) servers. In the sixth block are given α -helical regions containing leucine zipper-like motifs (Tarafdar et al. 2009; 52 to 70, 157 to 173, 383 to 400). In the seventh block are assumed hypersensitive reaction (HR)-inducing regions (Hao and Wei 2001; 43 to 70, 140 to 176; Sinn et al. 2008; 255 to 403). The eighth block represents regions known to be required for interaction with an HIPM (HrpN_{Ea}-interacting protein from *Malus* spp.) interactor (Oh and Beer 2007; 0 to 101, 139 to 198).

HrpN_{Ea} in combination with DspA/E is involved in the induction of an oxidative burst during the compatible interaction between *E. amylovora* and pear, as *hrpN_{Ea}* mutants provoke a reduced oxidative burst when compared with the wild-type strain (Venisse et al. 2003). However, purified HrpN_{Ea} induces H₂O₂ production in nonhost *Arabidopsis* suspension cells but not in host apple suspension cells (Boureau et al. 2011; Reboutier et al. 2007). Thus the induction of the oxidative burst detected in pear is likely to be due to the DspA/E effector with the necessary participation of HrpN_{Ea} for the efficient translocation of the effector (Bocsanczy et al. 2008; Boureau et al. 2011). An interactor of HrpN_{Ea} protein was identified in apple using a yeast two-hybrid assay (Oh and Beer 2007) and was called HIPM (HrpN_{Ea}-interacting protein from *Malus* spp.). HIPM interacts with the N-terminal portion of HrpN_{Ea} in association with specific positions in plasma membranes. Its ortholog in *Arabidopsis* (AtHIPM) functions as a negative regulator of plant growth (Oh and Beer 2007). It is noteworthy that two LZ-like and HR-inducing regions in HrpN_{Ea} were also found to be required for interaction with HIPM (Fig. 1, block 8). Preliminary results suggest that the silencing of HIPM increases apple resistance to fire blight (Malnoy et al. 2008), but there is still no clear evidence that HIPM and its interaction with HrpN_{Ea} by the LZ-like and HR-inducing regions is required for fire blight disease development in apple. Interestingly, it was shown that overexpression of *hrpN_{Ea}* in planta improves pear resistance to fire blight on micropropagated plants (Malnoy et al. 2005), suggesting that HrpN_{Ea} may trigger the accumulation of plant host defenses effective against *E. amylovora*. Consistent with this hypothesis, HrpN_{Ea} has been shown to induce callose deposition in apple leaves (Boureau et al. 2011).

Signaling and regulation of plant defenses mainly rely on a complex interplay of three phytohormones, salicylate (SA), jasmonate (JA), and ethylene (ET) (Pieterse et al. 2009). Subsequent expression of defense genes leads to cell-wall modifications (Hückelhoven 2007), accumulation of PR proteins (Van Loon et al. 2006) and antimicrobial secondary metabolites from diverse groups, such as phenolic compounds and, in particular, flavonoids (Cowan 1999; Treutter 2005), terpenoids (Cowan 1999), or derivatives of the cysteine sulfoxide, such as allicin from garlic (Harris et al. 2001). Candidate-gene and whole-genome approaches on the *E. amylovora*–apple interaction indicate that this bacterium rapidly triggers the expression of several PR protein-encoding genes (Baldo et al. 2010; Bonasera et al. 2006; Dugé de Bernonville et al. 2012; Milcevicova et al. 2010; Norelli et al. 2009; Sarowar et al. 2011; Venisse et al. 2002). All the accumulated data are in accordance with the fact that this interaction (compatible as well as incompatible) is characterized by a strong induction of the SA pathway. Remarkably, compatibility was found to be correlated with a strong downregulation of the JA signaling pathway. Both DspA/E and HrpN_{Ea} appeared to be the predominant actors modulating host

defense-gene expression in the apple–*E. amylovora* interaction, as a DspA/E-HrpN_{Ea} double mutant triggers responses similar to that observed with a T3SS mutant strain (Dugé de Bernonville et al. 2012). However, deciphering the specific role of HrpN_{Ea} during the interaction is difficult, because the phenotypes observed for HrpN_{Ea} deficient strains often overlap with phenotypes due to the absence of other effectors whose translocation requires a functional HrpN_{Ea}.

To overcome these overlapping phenotypes and directly analyze mechanisms associated with the perception or activity of HrpN_{Ea} in host plants, we transformed the susceptible ‘Galaxy’ apple genotype and produced transgenic lines expressing HrpN_{Ea} with or without a secretion peptide signal. Our results clearly indicate for the first time that HrpN_{Ea} can localize in the membrane fraction in host plants. Our work also showed that HrpN_{Ea} alone elicits host defenses when localized in the membrane fraction, independently of its function as a translocator of other *E. amylovora* type III effectors during infection.

RESULTS

Molecular and phenotypic characterization of apple *hrpN_{Ea}* transgenic lines.

In total, eight transgenic lines overexpressing *hrpN_{Ea}* under the control of the strong constitutive *Cauliflower mosaic virus* (CaMV)35S promoter were obtained in the ‘Galaxy’ genotype background. Ploidy level determined by flow cytometry detected two tetraploid lines, which were further eliminated (Table 2). All the transgenic lines were derived from adventitious buds arising from different leaf explants; therefore, they result from independent transformation events. The *hrpN_{Ea}* transgenes harbored either the tobacco PR1 secretion signal peptide (SP) (line 35SSP1) or not (line 35S1 to 35S5). The overall rate of transformation (0.1%) was low compared with previous experiments (1 to 1.4%) from the same laboratory (Faize et al. 2003, 2004), and viable transgenic lines expressing HrpN_{Ea} with a SP were particularly difficult to produce.

Fig. 2. Southern blot analysis of the six *hrpN_{Ea}* transgenic lines. The probe was an 800-bp polymerase chain reaction–amplified fragment of the *hrpN_{Ea}* gene. For each line, the number of bands estimated is in brackets.

Table 2. Characteristics of the eight transgenic lines obtained

Line	Transgene construction		Ploidy number
	35S- <i>hrpN_{Ea}</i> ^a	35S-SP- <i>hrpN_{Ea}</i> ^b	
35S1	x		2n
35S2	x		2n
35S3	x		2n
35S4	x		2n
35S5	x		2n
35S6	x		4n
35S7	x		4n
35SSP1		x	2n

^a 35S = *Cauliflower mosaic virus* promoter.

^b SP = secretion signal peptide.

Polymerase chain reaction (PCR) analyses detected the presence of the *hrpN_{Ed}* transgene and the absence of contaminant agrobacteria in all analyzed lines (data not shown). The integration of the *hrpN_{Ed}* gene in each line was analyzed by Southern blot (Fig. 2). All analyzed lines contained at least one inserted transgene and presented distinct hybridization patterns. The number of insertions within each line could be estimated to vary between one and six copies.

Constitutive (T0) *hrpN_{Ed}* gene expression was determined in transgenic lines in two to three biological repeats by quantitative real-time PCR (Fig. 3). All lines were constitutively expressing *hrpN_{Ed}* (conversely to the control, 'Galaxy', showing

no specific amplification; data not shown). However the transgene expression level was greatly variable between lines, with 35SSP1 exhibiting the highest expression, followed by 35S1 and 35S2.

Fire blight resistance of the transgenic lines was evaluated by inoculation of greenhouse plants grown on their own root system. At 21 days after inoculation, 100% of the susceptible control shoots were infected with high severity (necrosis reaching 68% of shoot length), whereas less than 8% of resistant control shoots were infected with very limited severity (2% of shoot length) (Fig. 4). A significant reduction of infection frequency (82% against 100% for the control) was only detected

Fig. 3. Constitutive expression of the *hrpN_{Ed}* transgene in the six *hrpN_{Ed}* transgenic lines. Relative expression ratios were calculated using mean of line 35S3 as a calibrator. Histograms represent the mean of two to three biological repeats times two technical repeats $\pm$ standard deviation.

Fig. 4. Evaluation of fire blight resistance of the six transgenic lines, the wild-type 'Galaxy' and the resistant cultivar Evereste 21 days after inoculation of 24 to 52 shoots per genotype with the virulent strain CFBP1430. Black histograms represent infection frequency = infected/inoculated shoots in percentage. White histograms represent disease severity = necrosis length/total shoot length in percentage. N = number of inoculated shoots. Bars indicate confidence intervals at $\alpha = 0.05$. Black star: significant difference in infection frequency between 35SSP1 and 'Galaxy', using Fisher's exact test with $\alpha = 0.05$ (P value 0.021). Gray stars: significant differences in disease severity between 35S5 or 35SSP1 and 'Galaxy', using Student test and Bonferroni correction with $\alpha = 0.05$ (P value for 35S5 and 35SSP1, respectively, 0.015 and 0.038).

for the 35SSP1 line. On the other hand, this line exhibited a significant increase in disease severity (necrosis reaching 78% of shoot length against 68% for the control), which was also observed in transgenic line 35S5.

In planta localization of the HrpN_{Ea} protein.

HrpN_{Ea} localization was analyzed by Western blot in different protein fractions in wild-type ‘Galaxy’ (negative control) and in the most-expressing lines 35S1 and 35SSP1 (Fig. 5A). The Coomassie blue staining tests revealed proteins in each sample (Fig. 5B). The G6PDH (EC 1.1.1.49) enzymatic activity was used to verify the low level of cytosolic contamination in microsomal and intercellular washing fluids (IWF) fractions (Supplementary Table 1). For each genotype, in microsomal fraction and IWF, there was not enough G6PDH to allow for NADP+ reduction. This indicates that microsomal and IWF fractions were free from cytosolic contaminants. Two bands were observed after Western blot using a rabbit anti-HrpN_{Ea} polyclonal serum. The protein displaying the higher molecular mass (48 kDa) corresponds to the HrpN_{Ea} fused with the tobacco PR1 SP, whereas the second (44 kDa) to the harpin alone. As expected in total extract, we detected only the lower band in the 35S1 line, in which *hrpN_{Ea}* is not fused with the secretion SP sequence, and both bands in the 35SSP1 line, which con-

tains the secretion SP. In the 35S1 line, HrpN_{Ea} protein (lower band) was detected in total extract only and in neither microsomal nor in IWF fractions. Thus in 35S1, HrpN_{Ea} lacking a SP seems to be maintained in the cytoplasm. Concerning the 35SSP1 line, HrpN_{Ea} was detected both in total extract and microsomal fractions, but not in IWF.

We investigated *in silico* the secondary structure and transmembrane domain predictions for HrpN_{Ea} protein (Fig. 1, block 1 to 5). Like others harpins (Tarafdar et al. 2009), HrpN_{Ea} is predicted to be predominantly composed of α-helices (12 in total). According to the different prediction methods used, it seems that the most likely transmembrane domain in HrpN_{Ea} is the second α-helix (amino acids 56 to 76) and that at least the C-terminal half of the protein is cytoplasmic. Prediction of this transmembrane domain is in good accordance with the detection of HrpN_{Ea} in the microsomal fraction.

Defense-related and signaling gene expression analysis.

The expression of 28 apple marker genes (Table 3) was analyzed by quantitative real-time PCR before (T0) and after (24 h postinfection [hpi]) infection by *E. amylovora* in wild-type ‘Galaxy’ and transgenic lines 35S1 and 35SSP1. Two biological repeats (BR) were performed. The set of genes included PR protein-coding genes and genes related to the antioxidant

Fig. 5. Localization of HrpN_{Ea} protein by Western blot staining. HrpN_{Ea} protein was assayed in different protein extracts in wild-type ‘Galaxy’ (negative control) and in the two highest expressing *hrpN_{Ea}* transgenic lines. **A**, Western blot staining. The black arrow points to the HrpN_{Ea} protein without signal peptide in the microsomal fraction of the line 35SSP1. **B**, Coomassie blue staining. Protein quantities in wells from left to right are 7, 2.5, 1, 1.15, 0.705, 2.6, 6.1, and 2.9 µg.

system, secondary metabolism, and cell-wall modification, as well as key markers of the SA, JA, or ET pathways (Brisset and Dugé de Bernonville 2011).

The gene expression of every sample was normalized to the mean expression level of the corresponding genes from the two BR of 'Galaxy' lines at T0. Hierarchical clustering of the 12 line/time/BR expression profiles (Fig. 6; Supplementary Table 2) showed that expression data was consistent between the two biological repeats. It also revealed a very strong similarity between the expression profiles of the three lines 24 hpi. The downregulation of genes from the JA biosynthesis/signaling pathway (*JAR*, *LOX*) and from the phenolic biosynthesis pathways (*PAL*, *DFR*, *ANS*, *CHS*) as well as the strong induction of a polyphenol oxidase (*PPO*) were observed. Concerning cell wall modification-related genes, the expression of two markers (*CAD*, *CAL_S*) did not vary, whereas that of *PECT* was repressed. The ET biosynthesis/signaling pathway (*ACCO*, *EIN3*) was globally induced, as well as the terpenoids (*HMGR*, *FAR*) and cysteine sulfoxide derivatives (*CSL*) biosynthesis pathways

or PR protein-coding genes (*PR1*, *PR2*, *PR4*, *PR5*, *PR8*, and *PR14*). Most of the genes from the antioxidant system were also induced (*POX*, *GST*, *PR15*) apart from *APOX*, which was repressed. Finally, concerning the SA biosynthesis/signaling pathway, the gene coding for a WRKY transcription factor was strongly induced, whereas the gene encoding the EDS1 regulator was repressed.

By contrast, expression profile of line 35SSP1 clearly differed from the two other lines at T0 and displayed a stronger similarity with the expression profiles of the three lines after infection. In fact, association testing for paired samples indicated a strong and significant correlation between 35SSP1 at T0 and 'Galaxy' at 24 hpi ($P = 3.03e-05$, Spearman's rank correlation *rho* statistic), and between 35SSP1 at T0 and 35S1 at 24 hpi ($P = 8.94e-06$), between 35SSP1 T0 and 35SSP1 24 hpi ($P = 4.02e-05$). Concerning line 35S1, its expression profile showed a significant similarity with that of the control 'Galaxy', in spite of the downregulation of several genes in BR1 at T0. In addition, no significant association was found between 35S1 at

Table 3. Gene name, function, GenBank accession, National Center for Biotechnology Information unigene, and primers of genes analyzed in this study

Name	Function ^a	Accession	Unigene	Forward primers	Reverse primers
PR1	PR protein 1	AF507974	Mdo.3966	F AGCACACGAGTTTCGACTCATAA	R CACAAAACACTACGCCAACCAA
PR2	PR protein 2 (glucanases)	AF494404	Mdo.2984	F AATTGTTATATCGGAGAGTG	R TGGCAAAGATGTAAGTTTC
PR4	PR protein 4 (hevein-like)	CN877594	Mdo.2382	F GAAGGTGCCTCTTGTTG	R CGTCGGTGTCAATTTGG
PR5	PR protein 5 (thaumatin-like, osmotin)	DR998561	Mdo.999	F AAAGGGGCTCGCATTTGG	R CTTGGCATTTGGAGGACACC
PR8	PR protein 8 (class III chitinase)	DQ318214	Mdo.3935	F CCAAGCCCCTGTCCTAAACCTC	R CAACTTGCCTTGCCATCATCAGC
PR14	PR protein 14 (lipid transfer protein)	CV656658	Mdo.12217	F AGTGTTCCTTATTGTGATAGC	R CAACTACAAGCAATTCTCC
PR15	PR protein 15 (oxalate oxidase)	GO500607	None	F GCCTAAACCCTCCCCACACTC	R CATCTCCCTTGTTCACACCTTGG
APOX	Ascorbate peroxidase	CN928974	Mdo.1894	F GCTTCCAACGTACAAGGCTCTTC	R CGCACAGGCATCGGCTTC
POX	Peroxidase	CN913385	Mdo.11566	F TGTCATTATTATTCCTTCTGTG	R AACCAGAGCATCACATCC
GST	Glutathion <i>S</i> -transférase	FE969955	Mdo.12372	F GAGCCCTTCATATCCCTCAATCC	R GCCTCCACCTCCGACCAC
PAL	Phenylalanine ammonia-lyase	AF494403	Mdo.1787	F GGATCTCCTCAAAGTTGTCG	R CTCACTCTCGCCATTTGTC
CHS	Chalcone synthase	AF494401	Mdo.6113	F ATTGGTGCCGACCCAGTGC	R AATCCAGAAGAGTGAGTTCCAGTCC
DFR	Dihydroflavonol reductase	AF494390	Mdo.13736	F CGGTTGGATGTACTTCG	R TGTGAGAAAGGCAGAGG
ANS	Anthocyanidin synthase	DQ156905	Mdo.2932	F GCCTCAAGTTCCACCATCG	R GAAGTAGTCTCCACTCAAGC
PPO	Polyphenol oxidase	L29450	Mdo.2905	F TGCCCCGCCGCTTCCAC	R GCTCCATCGCTTTGTAGTATTTGTC
HMGR	Hydroxymethyl glutarate-CoA reductase	AY043490	Mdo.2960	F AACAGGTCAAGCAGATTTC	R TTGTCAGAGCAGTAGTTG
FPPS	Farnesyl pyrophosphate synthase	AY083165	Mdo.2964	F TCTTGTTGGTGGTGAAAG	R CTGCCTCTGTAAATCTTGC
FAR	(E,E)-alpha-farnesene synthase	EB111255	Mdo.3011	F GATATTTTAGATGAGGCGAAAGC	R GCGTTGATTTGCCATTTGAC
CSL	Cystein-S-lyase	AY347795	Mdo.12560	F CACCCTGTCTGTTTCCAATCG	R AGCAATCTTTATCTTCTCCCTCTC
PECT	Pectin methyl esterase	CV628630	Mdo.15511	F GGAGGGAGGGTCAACTGG	R CAAACAACCTTCTCCACAACC
CAD	Cinnamyl alcohol dehydrogenase	AF053084	Mdo.2625	F GGAGCCCAAGACAGACTTCAAC	R CGCCTTCACAACCCTCAACAG
CAL_S	Callose synthase	CN496203	Mdo.12945	F TGCCTTCATGCCAACGGGATG	R CGGAAACAAACGGAAACCAAGCC
EDS1	Disease resistance protein EDS1	CN949066	Mdo.1759	F TGGAGAAAGTGATTTTGGAGAAGC	R AGAACCAGATTGTGACAAACGC
WRKY	WRKY transcription factor 30	AY347836	None	F AATATACATTGGGAGCAAAAGAGTC	R AGAGTTTCAGCATGGAAAGCG
JAR	Jasmonate resistant 1	CN879199	Mdo.2326	F CGTTCCCTTCTCCACAATCC	R GGGTTCTTGGTGAATGTTGC
LOX	Lipoxygenase AtLOX2	CN941066	Mdo.10456	F GTTGCGTATGGGAAGGAATGG	R GGTAGTAGTGGTTACATAGTCAGTG
EIN3	EIN3-binding F box protein 1	CV082047	Mdo.12601	F CGTTATGGCGTTGGTTAGG	R CAGAAATGGAGAGGACTTGC
ACCO	1-aminocyclopropene-1-carboxylate oxidase	AB086888	Mdo.3241	F GTTGGTGCTGGGTAGATCAC	R ACGGAGAGTGGGTTGATGTG
ACTIN	Actin 7	CV151413	Mdo.701	F CAACCTCTCGTCTGTGATAATG	R GCATCCTTCTGTCCCATCC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	CN494000	Mdo.1683	F GCTGCCAAGGCTGTTGGAA	R CAGTCAGGTCAACAACGGAAAC
TUA	Tubulin alpha-1 chain	CO065788	Mdo.3499	F GTTCAATGCTGTTGGTGGTG	R CTGCGGAGAAGGATAGATGG
HrpN _{EA}	HrpN _{EA}	M92994	None	F ACCAGCCAGTATGGAGCAGT	R AGCTTGCCAAGTGCCATATT

^a PR = pathogenesis related.

T0 and the other samples collected at 24 hpi ($P > 0.05$, Spearman's rank correlation ρ statistic).

DISCUSSION

Our present results add new evidence about the biological activity of HrpN_{Ea}, an important secreted protein of *E. amylovora*. More precisely, our results strongly suggest that HrpN_{Ea} can localize in the host plasma membrane. This is in accordance with its amyloidogenic properties (Oh et al. 2007), its indispensable role in type III effector translocation (Bocsanczy et al. 2008; Boureau et al. 2011), and the *in silico* analysis of the HrpN_{Ea} protein sequence (Fig. 1).

In the 35SSP1 line, through the tobacco PR1 SP, HrpNEa was predicted to be targeted to the endoplasmic reticulum (ER) and co-translationally translocated across the ER membrane into the lumen of the ER (Denecke et al. 1990). The SP is theoretically immediately cleaved from the protein once it has been translocated into the lumen of the ER, but during translocation, the nascent protein with its SP is inserted into translocons of ER membrane (Johnson et al. 1999; Paetzel et al. 2002; Rapoport 2007). In the 35SSP1 line HrpN_{Ea}+SP was found in the microsomal fraction as expected (Fig. 5A). Whereas most soluble and transmembrane secretory proteins are actively sorted from the ER into COPII vesicles via export signals within their sequences, proteins without export signals can also exit the ER via a nonspecific process known as 'bulk-flow.' This bulk-flow seems not to be relatively efficient and contributes in a minor way to protein secretion (Malkus et al. 2002). So in the 35SSP1 line, if HrpN_{Ea} is not a transmembrane protein and in the absence of information in its sequence liable to target it to other organelles or to retain it in the ER lumen, it should have joined intercellular spaces, through the bulk flow, once the SP was cleaved. But we did not find HrpN_{Ea} in IWF of the 35SSP1 line, and we found it in microsomes of this line (Fig. 5A). These results suggest either that, once the SP is cleaved, HrpN_{Ea} binds the ER membrane or that, as a transmembrane protein, HrpN_{Ea} integrates the ER membrane during translocation (Rapoport 2007). Through the bulk flow, we could hypothesize that this membrane-associated HrpN_{Ea} should finally join the plasma membrane.

To better understand the biological activity of HrpN_{Ea} in a host plant and to investigate if this activity requires its insertion into the plant cell membrane, we monitored the expression of several marker genes previously reported to be differentially expressed during the interaction between apple and *E. amylovora*. In the susceptible control genotype 'Galaxy', we found expression patterns similar to those previously reported in other susceptible genotypes (Baldo et al. 2010; Bonasera et al. 2006; Dugé de Bernonville et al. 2012; Milcevicova et al. 2010; Norelli et al. 2009; Sarowar et al. 2011; Venisse et al. 2002), i.e., induction of the SA/ET pathway and of several PR-related genes and downregulation of the JA marker genes (Fig. 6). Contrary to the well-known downregulation of genes involved in the biosynthesis of flavonoid, we report here that other secondary metabolites (terpenoids and derivatives from the cysteine sulfoxide) have their biosynthesis pathways activated in an apple-*E. amylovora* susceptible interaction. However, given the outcome of the interaction, it does not seem that these activations produce compounds quantitatively or qualitatively able to disturb *E. amylovora* in its spread. Interestingly, the presence of HrpN_{Ea} (line 35SSP1 at T0) in the membrane fraction mimics to a certain extent the infection by *E. amylovora*. Indeed, gene expression profiles in the 35SSP1 line at T0 exhibited strong similarities (more than 60% of genes with similar expression profiles) with that of the wild-type 'Galaxy' genotype 24 hpi, although to a lesser amplitude. In the transgenic line with cytoplasmic HrpN_{Ea}

(35S1), nearly half of the tested genes were weakly repressed at T0, at least in one repetition. However, at 24 hpi, the expression profile in this line as well as its phenotype (Fig. 4) appeared similar to those of the wild-type line. Thus the effects of constitutive cytoplasmic HrpN_{Ea} proteins on host gene transcription were likely to be counterbalanced during infection. On the other hand, full eliciting activity of HrpN_{Ea} is likely to require its integration into the host plasma membrane. This is probably achieved naturally when the protein is delivered by the bacteria during infection. Whether this process requires interaction with HIPM remains to be elucidated. This also happened constitutively in transgenic line 35SSP1, through the effect of the SP. A similar result was already pointed out by Tampakaki and Panopoulos (2000) in their work on nonhost tobacco hypersensitive cell death elicitation by intra- or extracellularly transgenic expression of *hrpZ* from *Pseudomonas syringae* pv. *phaseolicola*.

The constitutive defense elicitation due to the integration of HrpN_{Ea} in cytoplasmic membranes of line 35SSP1 could explain, at least partly, the reduction of infection frequency observed (18%; Fig. 4), as if the starting disease was in some shoots rapidly stopped by the preformed defense arsenal. On the other hand, it seems that if the bacteria were able to overcome these preformed host defenses in other shoots, then the disease severity slightly exceeded the wild-type level (10%; Fig. 4). These fire blight susceptibility results on transgenic apple lines do not confirm previous results on transgenic pear lines, in which constitutive expression of *hrpN_{Ea}* (from pCambia35S-*hrpN_{Ea}*) led to a significant reduction of fire blight susceptibility (Malnoy et al. 2005). However, these experiments on pear were done on *in vitro* micropropagated shoots. When assayed in the greenhouse, a subset of these transgenic pears, expressing the *hrpN_{Ea}* gene in the same range as the transgenic apples studied here (data not shown), were as susceptible as the control (data not shown).

Interestingly, in spite of the fact that HrpN_{Ea} constitutively triggers in 35SSP1 gene expression changes similar to that observed in 'Galaxy' and 35S1 during the infection, there was no overall additional overexpression of these genes in 35SSP1 during the infection, as compared with 'Galaxy' and 35S1. Indeed expression levels postinfection in the 35SSP1 line did not exceed those in the 'Galaxy' control genotype postinfection. Thus, it is likely that the infection triggers maximal gene expression thresholds that cannot be overcome whatever the constitutive expression level.

In conclusion, this study demonstrates for the first time that HrpN_{Ea} can localize in the membrane fraction in host plants. Furthermore, ectopic expression of HrpN_{Ea} demonstrates its ability to modulate apple defense-related genes in the absence of other effectors when localized in a membrane fraction. However, the precise mechanism of action of membrane-localized HrpN_{Ea} during natural infection remains to be determined, in particular, whether full activity of HrpN_{Ea} requires its interaction with HIPM.

MATERIALS AND METHODS

Production of transgenic plants.

The apple cultivar Galaxy was chosen because of its high susceptibility to fire blight. Fully expanded leaves were excised from *in vitro*-growing shoots three weeks after subculture. Transformation experiments were carried out according to the procedure published by Norelli and associates (1996), using *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993) containing pCambia35S-*hrpN_{Ea}* or pCambia35S-SP-*hrpN_{Ea}* binary expression vectors. The binary vector pCambia35S-*hrpN_{Ea}* was constructed by cloning a *hrpN_{Ea}* gene (accession number

M92994; Wei et al. 1992) without signal sequence under the control of the CaMV35S promoter into the pCambia2300 plasmid. The binary vector pCambia35S-SP-hrpN_{Ea} was constructed by cloning the *hrpN_{Ea}* fused to the tobacco PR1 secretion SP (Denecke et al. 1990) into the pCambia2300 plasmid. Eight independent transformation experiments were performed (on a total of 2,600 explants) with each vector. PCR analysis was performed to check the presence of the transgene *hrpN_{Ea}* and the absence of agrobacteria in transgenic lines. These lines and control plants were propagated in vitro and were acclimatized in a greenhouse as previously reported (Faize et al. 2003). Before acclimatization, the ploidy level of transgenic lines was checked by flow cytometry, as described in Chevreau and associates (2011), and tetraploid lines were eliminated.

***E. amylovora* growth conditions, inoculum preparation, inoculation procedures, and phenotype analyses.**

The wild-type strain CFBP1430 (Paulin and Samson 1973) was grown overnight at 26°C on solid King's medium B (King

et al. 1954). Inocula were prepared in sterile water and were adjusted to the concentration of 10⁸ CFU/ml. Grafted greenhouse-grown plants used for defense-related gene expression analysis were inoculated by vacuum infiltration as described in Pontais and associates (2008) with bacterial suspension or water as control.

Fire blight resistance was evaluated on 28 to 52 greenhouse-grown plants per transgenic line. The youngest not fully expanded leaf of each actively growing shoot was wounded with scissors previously dipped into bacterial suspension. Only actively growing shoots were inoculated. Symptoms (frequency and disease severity) were assessed 21 days after inoculation. The infection frequency was calculated as the ratio between infected and inoculated shoots and was expressed in percentages. The disease severity was calculated as the ratio between necrosis length and total shoot length calculated on infected shoots only and was expressed in percentages. Nontransformed 'Galaxy' genotype was used as susceptible control (30 plants) and the 'Evereste' genotype was used as resistant control (24 plants).

Fig. 6. Heatmap and hierarchical clustering of gene expression analysis in HrpN_{Ea} overexpressing transgenic lines 35S1, 35SSP1, and the 'Galaxy' control. Expression was observed before infection (T0) and 24 h postinfection (24 hpi). Color coded log₂ normalized expression levels are presented for each biological repeat (BR1 and BR2). Before normalizing, raw expression levels for each gene were calibrated to the corresponding 'Galaxy' mean T0 value (then setting the 0 value). Complete-linkage hierarchical clustering analysis based on the euclidian distances of the 12 line/time/BR expression profiles. Approximately unbiased probabilities (in red) and classical bootstrap probabilities (in green) for hierarchical clustering were computed as described in Suzuki and Shimodeira (2011).

DNA extraction and Southern blot analysis.

Plant genomic DNA was isolated from shoots of 4-week-old greenhouse-grown transformed lines and untransformed 'Galaxy' control according to Ishaq and associates (1990).

A total of 10 µg of DNA was digested for 2 h in a total volume of 200 µl of reaction mix with 120 U of *Eco*RI, 15 µg of acetylated bovine serum albumin, and $1/10$ of total volume of Buffer H from Promega (Madison, WI, U.S.A.). Digestion was then continued for 12 h after the addition of 60 U of *Eco*RI and 3 µl of buffer H. The digested DNA was then precipitated with sodium acetate and resuspended in 35 µl of buffer TE (10 mM Tris, pH 8.0, 1 mM EDTA). The digested DNA was separated on a TBE 0.5X 1% agarose gel for 12 h at 30 volts and then transferred on a positively charged nylon membrane (Hybond XL Amersham, GE Healthcare, Piscataway, NJ, U.S.A.). For preparing the probe, an 800-bp PCR-amplified fragment of the *hrpN_{Ea}* gene was purified by Wizard SV gel and PCR clean up system from Promega. The probe was then labeled with radioactive (P^{32}) desoxycytosine 5'-triphosphate (dCTP) and was purified with Probe Quant G50 column from Amersham. For the hybridization, we used standard hybridization buffer with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5×Denhardt, 0.5× SDS, and 100 mg of salmon sperm DNA per liter. Prehybridization was carried out at 65°C for 1 h, and then, hybridization was carried out at 65°C for 24 h. After hybridization, the membrane was washed three times at 60°C: i) two times for 10 min in 2× SSC, ii) two times for 10 min in 1× SSC, then iii) one time for 5 min in 0.5× SSC. The membrane was then exposed to an X-ray film for three days at room temperature and was read with a Molecular Imager FX scanner from BioRad (Hercules, CA, U.S.A.).

Protein extraction, quantification, and Western blot analysis.

For total protein extraction, leaf tissues from greenhouse-grown plants (1 g of fresh weight) were homogenized in 10 ml of ice-cold buffer, as previously reported (Venisse et al. 2001), but with Tris buffer instead of sodium phosphate buffer. Homogenates were centrifuged at $16,000 \times g$ for 20 min at 4°C, and supernatants were immediately used, partly for glucose-6-phosphate dehydrogenase (G6PDH) activity measurement and partly for microsomal fraction separation, or were stored at -20°C in a denaturing loading buffer (0.2 M Tris HCl, 0.5 M saccharose, 0.01% bromophenol blue, 5 mM EDTA, 3% SDS) until HrpN_{Ea} immunodetection.

For microsomal fraction separation, 2 ml of total protein extract were passed through a metallic screen (pore size, 40 µm) and were centrifuged at $25,000 \times g$ for 60 min. The pellet was washed in 1 ml of 50 mM Tris buffer (pH 7.5) and was then centrifuged at $25,000 \times g$ for 60 min. The pellet was suspended in 150 µl of a PSK buffer (3 mM KCl, 0.3 M saccharose, 5 mM KH₂PO₄, pH 7.8, 5 mM K₂HPO₄, pH 7.8) as reported by Wendehenne and associates (1995) and was immediately used for G6PDH activity measurement or stored at -20°C, as described above.

IWF were extracted by centrifugation (at $10,000 \times g$ for 20 min) of leaves previously vacuum-infiltrated with 100 mM Tris buffer, pH 7, containing 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 0.01% (vol/vol) Triton X-100 and were immediately used for G6PDH activity measurement or stored at -20°C, as described above.

Protein content in the extracts was determined according to the method of Bradford (1976), using the Coomassie protein assay reagent (ThermoScientific, Rockford, IL, U.S.A.). The G6PDH (EC 1.1.1.49) cytosolic marker enzymatic activity was used to confirm the low level of cytosolic contaminants in microsomal and IWF fractions (Larsson et al. 1994). G6PDH

activity was assayed spectrophotometrically by measurement of NADPH formation at 340 nm (extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction volume was 850 µl, with considered protein extracts complemented with 50 mM Tris buffer (pH 7.5), and G6P and NADP⁺ were added to obtain, respectively, 2 and 0.65 mM final concentrations. For HrpN_{Ea} immunodetection, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described by Laemmli (1970). Coomassie blue staining and Western blots were performed according to Sambrook and associates (1989). A rabbit anti-HrpN_{Ea} polyclonal serum and a goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase were used for Western blots.

RNA extraction, reverse transcription, and gene expression analyses.

RNA from leaves from micropropagated plants used for transgene expression studies or from grafted greenhouse grown plants used for defense-related gene expression analysis were extracted as described by Venisse and associates (2002). Purity and concentration of the samples were assayed with a Nanodrop spectrophotometer (ThermoScientific). Reverse transcription was performed as described by Promega.

Quantitative PCR was used to quantify cDNA in samples. Briefly, 5 µl of the appropriately diluted samples (at least 50-fold) was mixed in a final volume of 25 µl with 6.25 µl of quantitative PCR mastermix (MasterMix Plus for SYBR Green I with fluorescein; Eurogentec EGT GROUP, Seraing, Belgium), with 0.5 µl of each primer and with 12.75 µl of water. Primers designed with Beacon Designer v7.2 (PREMIER Biosoft International, Palo Alto, CA, U.S.A.) were added according to their optimal concentration (determined for reaction efficiency near to 100%, calculated as the slope of a standard dilution curve; Pfaffl 2001). Accessions and primer sequences are indicated in Table 3. Reaction was performed with an ICycler iQ real-time PCR detection system (Bio-Rad, Munich) using the following program: 50°C, 2 min; 95°C, 10 min; 35 cycles comprising 95°C for 15 s, 60°C for 45 s, and 72°C for 30 s, with real-time fluorescence monitoring. Melt curves were performed at the end of each run to check the absence of primer-dimers and nonspecific amplification products. Data were acquired and analyzed with ICycler iQ optical system software 5.1 (Bio-Rad). Gene expression levels were calculated using the delta delta cycle threshold method and were corrected as recommended by Vandesompele and associates (2002), with three reference genes—*Actin* (accession CV151413), *Gapdh* (accession CN494000), and *Tua* (accession CO065788)—used for the calculation of a normalization factor.

Bioanalyses.

The secondary structures β-sheet and α-helix were predicted on the I-TASSER server. Transmembrane domain predictions and putative subcellular localization of the different segments were obtained through PRED-TMR, DAS, MEMSAT, and PRED-TMBB servers.

Statistical analysis.

Statistical computations were performed with R v2.15 (R Core Team 2012) except when indicated. Concerning the inoculation assay, the 'infection frequency' data were compared between transgenic lines and susceptible control 'Galaxy' using the Fisher's exact test; the 'disease severity' data were compared between transgenic lines and susceptible control 'Galaxy' using the Student test (bilateral, homoscedastic, $\alpha = 0.01$) in Excel and the Bonferroni correction. For hierarchical clustering, distances between each sample corresponded to absolute values of dissimilarity measures (1-absor[X,Y]), and the dendro-

grams using these distances were constructed with an average linkage algorithm. The heatmap was built using the 'gplots' package (Warnes 2012). Approximately unbiased (AU) *P* values and bootstrap probabilities (BP) were calculated using the 'pvclust' package (Suzuki and Shimodeira 2011). AU *P* value, which is computed by multiscale bootstrap resampling, is a better approximation to unbiased *P* value than BP value computed by normal bootstrap resampling. *P* values were calculated with 10,000 permutations. As indicated in pvclust, for a cluster with an AU *P* value > 0.95, the hypothesis that "the cluster does not exist" is rejected with significance level 0.05. Tests for association between paired samples were performed using gene expression measurements to investigate correlations between apple lines. Spearman's *rho* statistic was used to estimate a rank-based measure of association.

ACKNOWLEDGMENTS

We gratefully acknowledge the team responsible for the greenhouses (INEM) and especially N. Dousset and L. Leclout for excellent technical assistance. We gratefully acknowledge S. Bourque for microsomal fraction extraction protocols. This work was supported by a grant from the Conseil Général du Maine de Loire and funding from the Région Pays de Loire (COSAVE program) and from ISAFRUIT EU project (6th PCRD).

LITERATURE CITED

- Ahmad, M., Majerczak, D. R., Pike, S., Hoyos, M. E., Novacky, A., and Coplin, D. L. 2001. Biological activity of harpin produced by *Pantoea stewartii* subsp. *stewartii*. Mol. Plant-Microb. Interact. 14:1223-1234.
- Alfano, J. R., and Collmer, A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: Trafficking harpins, Avr proteins, and death. J. Bacteriol. 179:5655-5662.
- Alfano, J. R., Bauer, D. W., Milos, T. M., and Collmer, A. 1996. Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. Mol. Microbiol. 19:715-728.
- Baker, C. J., Orlandi, E. W., and Mock, N. M. 1993. Harpin, an elicitor of the HR in tobacco caused by *Erwinia amylovora*, elicits active oxygen production in suspension cells. Plant Physiol. 102:333-340.
- Baldo, A., Norelli, J. L., Farrell, R. E., Jr., Bassett, C. L., Aldwinckle, H. S., and Malnoy, M. 2010. Identification of genes differentially expressed during interaction of resistant and susceptible apple cultivars (*Malus × domestica*) with *Erwinia amylovora*. BMC Plant Biol. 10:1.
- Bocsanczy, A. M., Nissinen, R. I., Oh, C. S., and Beer, S. V. 2008. HrpN of *Erwinia amylovora* functions in the translocation of DspA/E into plant cells. Mol. Plant Pathol. 9:425-434.
- Bonasera, J. M., Kim, J. F., and Beer, S. V. 2006. PR genes of apple: Identification and expression in response to elicitors and inoculation with *Erwinia amylovora*. BMC Plant Biol. 6:23.
- Boureau, T., Siemer, S., Perino, C., Gaubert, S., Patrit, O., Degrave, A., Fagard, M., Chevreau, E., and Barny, M.-A. 2011. The HrpN effector of *Erwinia amylovora*, which is involved in type III translocation, contributes, directly or indirectly, to callose elicitation on apple leaves. Mol. Plant-Microbe Interact. 24:577-584.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
- Brisset, M. N., and Dugé de Bernonville, T. 2011. Device for determining or analyzing the state of stimulation of the natural defenses of plants or portions of plants. International application published under patent cooperation treaty PTC/FR2011/051470. WO/2011/161388.
- Chevreau, E., Dupuis, F., Taglioni, J. P., Sourice, S., Cournol, R., Deswartes, C., Bersegeay, A., Descombin, J., Siegwart, M., and Lloridon, K. 2011. Effect of ectopic expression of the eutypine detoxifying gene *Vr-ERE* in transgenic apple plants. Plant Cell Tiss. Org. Cult. 106:161-168.
- Conway, J. F., and Parry, D. A. D. 1990. Structural features in the heptad sub structure and longer range repeats of two-stranded α -fibrous proteins. Int. J. Biol. Macromol. 12:328-334.
- Cowan, M. M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12:564-582.
- Denecke, J., Botterman, J., and Deblaere, R. 1990. Protein secretion in plant cells can occur via a default pathway. Plant Cell 2:51-59.
- Dong, H., Delaney, T. P., Bauer, D. W., and Beer, S. V. 1999. Harpin induces resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *Nim1* gene. Plant J. 20:207-215.
- Dugé de Bernonville, T., Gaucher, M., Flors, V., Gaillard, S., Paulin, J. P., Dat, J. F., and Brisset, M. N. 2012. T3SS-dependent differential modulations of the jasmonic acid pathway in susceptible and resistant genotypes of *Malus* spp. challenged with *Erwinia amylovora*. Plant Sci. 188:189-199.
- 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 (Fed. Eur. Biochem. Soc.) Lett. 497:82-84.
- Faize, M., Malnoy, M., Dupuis, F., Chevalier, M., Parisi, L., and Chevreau, E. 2003. Chitinases of *Trichoderma atroviridae* induce scab resistance and some metabolic changes in two cultivars of apple. Phytopathology 93:1496-1504.
- Faize, M., Sourice, S., Dupuis, F., Parisi, L., Gautier, M. F., and Chevreau, E. 2004. Expression of wheat puroindoline-b reduces scab susceptibility in transgenic apple (*Malus × domestica* Borkh.). Plant Sci. 167:347-354.
- Galan, J. E., and Collmer, A. 1999. Type III secretion machines: Bacterial devices for protein delivery into host cells. Science 284:1322-1328.
- Gonzalez, L., Plecs, J. J., Jr., and Alber, T. 1996. An engineered allosteric switch in leucine-zipper oligomerization. Nature Struct. Biol. 3:510-515.
- Gopalan, S., Wei, W., and He, S. Y. 1996. Hrp gene-dependent induction of *hml1*: A plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. Plant J. 10:591-600.
- Hao, F., and Wei, Z. M. 2001. Hypersensitive response eliciting domains and use thereof. International application published under patent cooperation treaty PCT/US101/18820.
- Harris, J. C., Cottrell, S. L., Plummer, S., and Lloyd, D. 2001. Antimicrobial properties of *Allium sativum* (garlic). Appl. Microbiol. Biotechnol. 57:282-286.
- He, S. Y., Huang, H. C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpin_{PSG}: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255-66.
- Hood, E. E., Gelvin, S. B., Melchers, L., and Hoekema, A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208-218.
- Hückelhoven, R. 2007. Cell wall-associated mechanisms of disease resistance and susceptibility. Annu. Rev. Phytopathol. 45:101-127.
- Ishaq, M., Wolf, B., and Ritter, C. 1990. Large-scale isolation of plasmid DNA using cetyltrimethylammonium bromide. Biotechniques 9:19-20.
- Ji, Z., Song, C., Lu, X., and Wang, J. 2011. Two coiled-coil regions of *Xanthomonas oryzae* pv. *oryzae* harpin differ in oligomerization and hypersensitive response induction. Amino Acids 40:381-392.
- Jin, Q., Hu, W., Brown, I., McGhee, G., Hart, P., Jones, A. L., and He, S. Y. 2001. Visualization of secreted Hrp and Avr proteins along the Hrp pilus during type III secretion in *Erwinia amylovora* and *Pseudomonas syringae*. Mol. Microbiol. 40:1129-1139.
- Johnson, A. E., and van Waas, M. A. 1999. The translocon: A dynamic gateway at the ER membrane. Annu. Rev. Cell. Dev. Biol. 15:799-842.
- 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, J. G., Jeon, E., Oh, J., Moon, J. S., and Hwang, I. 2004. Mutational analysis of *Xanthomonas* harpin HpaG identifies a key functional region that elicits the hypersensitive response in nonhost plants. J. Bacteriol. 186:6239-6247.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
- Laemmli, D. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-683.
- Landschulz, W. H., Johnson, P. F., and McKnight, S. L. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA-binding proteins. Science 240:1759-1764.
- Larsson, C., Sommarin, M., and Widell, S. 1994. Isolation of a highly purified plant plasma membranes and separation of inside-out and right-side-out vesicles. Methods Enzymol. 228:451-469.
- Lee, J., Klusener, B., Tsiamis, G., Stevens, C., Neyt, C., Tampakaki, A. P., Panopoulos, N. J., Noller, J., Weiler, E. W., Cornelis, G. R., Mansfield, J. W., and Nurnberger, T. 2001a. HrpZ_{PSph} from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms ion-conducting pore in vitro. Proc. Natl. Acad. Sci. U.S.A. 98:289-294.
- Lee, J., Klessig, D. F., and Nurnberger, T. 2001b. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene *HIN1* independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. Plant Cell 13:1079-1093.

- Lupas, A. N., and Gruber, M. 2005. The structure of alpha-helical coiled coils. *Adv. Protein Chem.* 70:37-78.
- Lupas, A. N., Van Dyke, M., and Stock, J. 1991. Predicting coiled coils from protein sequences. *Science* 252:1162-1164.
- Malkus, P., Jiang, F., Schekman, R. 2002. Concentrative sorting of secretory cargo proteins into COPII-coated vesicles. *J. Cell Biol.* 159:915-21.
- Malnoy, M., Venisse, J. S., and Chevreau, E. 2005. Expression of a bacterial effector, HrpN, causes increased resistance to fire blight in *Pyrus communis*. *Tree Genet. Genomes* 1:41-49.
- Malnoy, M., Borejsza-Wysocka, E. E., Pascal-Omenaca, L., Aldwinckle, H. S., Oh, C. S., and Beer, S. V. 2008. Silencing of HIPM, the apple protein that interacts with HrpN of *Erwinia amylovora*. *Acta Hort.* 793:261-264.
- Miao, W. G., Song, C. F., Wang, Y., and Wang, J. S. 2010. Hpa_{xm} from *Xanthomonas citri* subsp. *malvacearum* is a novel harpin with two heptads for hypersensitive response. *J. Microbiol. Biotechnol.* 20:54-62.
- Milčevićová, R., Gosch, C., Halbwirth, H., Stich, K., Hanke, M. V., Peil, A., Flachowsky, H., Rozhon, W., Jonak, C., Oufir, M., Hausman, J. F., Matušiková, I., Fluch, S., and Wilhelm, E. 2010. *Erwinia amylovora*-induced defense mechanisms of two apple species that differ in susceptibility to fire blight. *Plant Sci.* 179:60-67.
- Norelli, J., Mills, J. A., and Aldwinckle, H. S. 1996. Leaf wounding increases efficiency of *Agrobacterium*-mediated transformation of apple. *HortSci.* 31:1026-1027.
- Norelli, J. L., Farrell, R. E., Jr., Bassett, C. L., Baldo, A. M., Lalli, D. A., Aldwinckle, H. S., and Wisniewski, M. E. 2009. Rapid transcriptional response of apple to fire blight disease revealed by cDNA suppression subtractive hybridization analysis. *Tree Genet. Genomes* 5:27-40.
- Oh, C. S., and Beer, S. V. 2007. AtHIPM, an ortholog of the apple HrpN-interacting protein, is a negative regulator of plant growth and mediates the growth-enhancing effect of HrpN in *Arabidopsis*. *Plant Physiol.* 145:426-436.
- Oh, J., Kim, J. G., Jeon, E., Yoo, C. H., Moon, J. S., Rhee, S., and Hwang, I. 2007. Amyloidogenesis of type III-dependent harpins from plant pathogenic bacteria. *J. Biol. Chem.* 282:13601-13609.
- O'Shea, E. K., Rutkowski, R., and Kim, P. S. 1989. Evidence that the leucine zipper is a coiled coil. *Science* 243:538-542.
- Paetzel, M., Karla, A., Strynadka, N. C., and Dalbey, R. E. 2002. Signal peptidases. *Chem. Rev.* 102:4549-4580.
- Paulin, J. P., and Samson, R. 1973. Le feu bactérien en France. II. Caractères des souches d'*Erwinia amylovora* (Burrill). Winslow et al., 1920, isolées du foyer franco-belge. *Ann. Phytopathol.* 5:389-397.
- 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.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:2002-2007.
- Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. 2009. Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5:308-316.
- Pike, S. M., Adam, 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 *Erwinia amylovora*. *Physiol. Mol. Plant Pathol.* 53:39-60.
- Pontais, I., Treutter, D., Paulin, J. P., and Brisset, M. N. 2008. *Erwinia amylovora* modifies phenolic profiles of susceptible and resistant apple through its type III secretion system. *Physiol. Plantarum* 132:262-271.
- R Core Team 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Racape, J., Belbahri, L., Engelhardt, S., Lacombe, B., Lee, J., Lochman, J., Marais, A., Nicole, M., Nurnberger, T., Parlange, F., Puverel, S., and Keller, H. 2005. Ca²⁺-dependent lipid binding and membrane integration of PopA, a harpin-like elicitor of the hypersensitive response in tobacco. *Mol. Microbiol.* 58:1406-1420.
- Rapoport, T. A. 2007. Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature*. 450:663-9.
- 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.
- Sambrook, J., Fritsch, E. F., and Maniatis T. E. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Sarowar, S., Zhao, Y., Soria-Guerra, R. E., Ali, S., Zheng, D., Wang, D., and Korban, S. S. 2011. Expression profiles of differentially regulated genes during the early stages of apple flower infection with *Erwinia amylovora*. *J. Exp. Bot.* 62:4851-4861.
- Shrestha, R., Park, D. H., Cho, J. M., Cho, S., Wilson, C., Hwang, I., Hur, J. H., and Lim, C. K. 2008. Genetic organization of the *hrp* genes cluster in *Erwinia pyrifoliae* and characterization of HR active domains in HrpN_{EP} protein by mutational analysis. *Mol. Cells* 25:30-42.
- Sinn, J. P., Oh, C.-S., Jensen, P. J., Carpenter, S. C. D., Beer, S. V., and McNellis, T. W. 2008. The C-terminal half of the HrpN virulence protein of the fire blight pathogen *Erwinia amylovora* is essential for its secretion and for its virulence and avirulence activities. *Mol. Plant-Microbe Interact.* 21:1387-1397.
- Strobel, R. N., Gopalan, J. S., Kuc, J. A., and He, S. Y. 1996. Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZ_{PSS} protein. *Plant J.* 9:431-439.
- Suzuki, R., and Shimodaira, H. 2011. Pvcust: Hierarchical clustering with p-values via multiscale bootstrap resampling. R package version 1.2-2. Published online. <http://CRAN.R-project.org/package=pvcust>
- Tampakaki, A. P., and Panopoulos, N. J. 2000. Elicitation of hypersensitive cell death by extracellularly targeted HrpZ_{PSPH} produced in planta. *Mol. Plant-Microbe Interact.* 13:1366-1374.
- Tarafdar, P. K., Vedantam, L. V., Kondreddy, A., Podile, A. R., and Swamy, M. J. 2009. Biophysical investigations on the aggregation and thermal unfolding of harpin (Pss) and identification of leucine-zipper-like motifs in harpins. *Biochem. Biophys. Acta* 1794:1684-1692.
- Treutter, D. 2005. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biol. (Stuttg.)* 7:581-591.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:0034.1-0034.11.
- van Loon, L. C., Rep, M., and Pieterse, C. M. 2006. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44:135-162.
- Venisse, J. S., Gullner, G., and Brisset, M. N. 2001. Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol.* 125:2164-2172.
- Venisse, J. S., Malnoy, M., Faize, M., Paulin, J. P., and Brisset, M. N. 2002. Modulation of defense responses of *Malus* spp. during compatible and incompatible interactions with *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 15:1204-1212.
- Venisse, J. S., Barny, M. A., Paulin, J. P., and Brisset, M. N. 2003. Involvement of three pathogenicity factors of *Erwinia amylovora* in the oxidative stress associated with compatible interaction in pear. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 537:198-202.
- Wang, X. Y., Song, C. F., Miao, W. G., Ji, Z. L., Wang, X., Zhang, Y., Zhang, J. H., Hu, J. S., Borth, W., and Wang, J. S. 2008. Mutations in the N-terminal coding region of the harpin protein Hpa1 from *Xanthomonas oryzae* cause loss of hypersensitive reaction induction in tobacco. *Appl. Microbiol. Biotechnol.* 81:359-369.
- Warnes, G., R. 2012. gplots: Various R programming tools for plotting data. R package version 2.11.0. Published online. <http://CRAN.R-project.org/package=gplots>
- Wei, Z. M., Laby, R. J., Zumoff, C. H., Baeur, 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., Binet, M. N., Blein, J. P., Ricci, P., and Pugin, A. 1995. Evidence for specific, high-affinity binding sites for a proteinaceous elicitor in tobacco plasma membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 374:203-207.
- 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.

AUTHOR-RECOMMENDED INTERNET RESOURCES

- DAS Transmembrane Prediction server server:
www.sbc.su.se/~miklos/DAS
- I-TASSER server: zhanglab.ccmb.med.umich.edu/I-TASSER
- MEMSAT server: bioinf.cs.ucl.ac.uk/psipred
- PRED-TMBB server: bioinformatics.biol.uoa.gr/PRED-TMBB/input.jsp
- PRED-TMR Prediction of Transmembrane regions in proteins server:
athina.biol.uoa.gr/PRED-TMR/input.html
- pvcust, an R package for hierarchical clustering:
www.is.titech.ac.jp/~shimo/prog/pvcust