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*Erwinia amylovora hrp*N mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco

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Abstract

Erwinia amylovora is the bacterium responsible for fire blight, a necrotic disease affecting many rosaceous plants and especially pear tree and apple tree. A protein named harpin, secreted through the Hrp secretion pathway and able to elicit an hypersensitive reaction (HR) on tobacco has recently been isolated. Mutants in *hrp*N, the gene encoding harpin were described as non pathogenic on immature pear fruit and unable to elicit an HR on tobacco [Wei *et al.*, 1992; Wei and Beer, 1993]. In this paper, the phenotype on plant of *hrp*N mutants was carefully determined. *hrp*N mutants expressed a weak but significant virulence on host plants. Furthermore, when infiltrated into tobacco leaf mesophyll, the *hrp*N mutants elicited varied responses that fluctuated from null reaction to full necrosis of the infiltrated area. These results show that harpin is not absolutely required neither for pathogenicity on host plant nor for elicitation of an hypersensitive reaction on tobacco. Furthermore, in all the tests performed, mutant blocked in harpin secretion remained non pathogenic and unable to elicit an HR on tobacco. This suggests that factor(s), different from harpin, involved both in pathogenicity and HR eliciting ability is (are) secreted through the Hrp secretion pathway.

Abbreviations: HR = hypersensitive reaction; NSI = necrosis severity index; CFU = colonie forming units.

Erwinia amylovora is the bacterium responsible for fire blight, a disease affecting many rosaceous plant and especially pear tree and apple tree [Van der Zwet and Keil, 1979]. Genetic studies on this bacterial plant pathogen lead to the identification of two main gene clusters, named *ams* and *hrp*, both necessary for pathogenicity. The *ams* gene cluster is involved in the biosynthesis of an extracellular polysaccharide named amylovoran [Belleman and Geider, 1992; Bernhard *et al.*, 1993]. The *hrp* cluster encodes functions necessary both for pathogenicity on host plant and for the elicitation of the hypersensitive response (HR) on non host plant [Steinberger and Beer, 1988; Barny *et al.*, 1990; Walter *et al.*, 1990; Bauer and Beer, 1991].

Besides *E. amylovora*, *hrp* clusters have been identified and cloned in many phytopathogenic bacteria such *Pseudomonas solanacearum*, different pathovars of *Xanthomonas campestris* and different pathovars of *Pseudomonas syringae* [Lindgren *et al.*, 1986, Boucher *et al.*, 1986, 1987; Arlat *et al.*, 1991; Bonas *et al.*, 1991; Kamoun and Kado 1990; Huang *et al.*, 1991; Mukhopadhyay *et al.*, 1988; Rhame *et al.*, 1991; Xiao *et al.*, 1992]. To date, DNA homology has been detected between several *hrp* genes in strains representative of all these Gram negative bacteria [Laby and Beer, 1990; Arlat *et al.*, 1991; Gough *et al.*, 1992; Fenselau *et al.*, 1992]. Furthermore, it has been shown that several *hrp* gene products share homology with pathogenicity determinants

involved in the specific secretion of virulence related proteins in animal pathogenic bacteria of the genus *Yersinia* and *Shigella* [Gough *et al.*, 1992; Fenselau *et al.*, 1992]. These homologies suggest that the conserved *hrp* gene products are involved in the secretion of protein(s) able to elicit the HR on non host plant and/or important for pathogenicity [for a review see van Gijsegem *et al.*, 1993].

Several *hrp* gene products have recently been shown to be involved in a specific secretion pathway [He *et al.*, 1993; Wei and Beer 1993; Arlat *et al.*, 1994]. Protein elicitors, secreted through this pathway and able to induce the HR on tobacco, have been isolated from *E. amylovora* E321, *P. syringae* pv. *syringae* and *P. solanacearum* [Wei *et al.*, 1992; He *et al.*, 1993; Arlat *et al.*, 1994]. These elicitor proteins, named harpin in *E. amylovora* and *P. syringae* pv. *syringae* and PopA in *P. solanacearum*, are heat stable, hydrophilic, glycin rich proteins but the genes encoding them show little or no homology [He *et al.*, 1993; Arlat *et al.*, 1994]. Furthermore, no homology has been detected with any known protein and the function of these elicitor proteins remains an open question. *P. solanacearum* PopA mutants are still pathogenic and able to elicit an HR on tobacco [Arlat *et al.*, 1994]. This constrasts with *E. amylovora* strain mutated in *hrp*N, the gene encoding harpin, which has been described as non pathogenic on immature pear fruit and unable to elicit an HR on non host plant, suggesting a crucial role for this protein in both processes [Wei *et al.*, 1992]. However, the reaction induced on an isolated organ like an immature pear fruit might not reflect pathogenicity properly. To assess *hrp*N mutants pathogenicity further, several *hrp*N mutants were constructed and their pathogenicity on host plant and HR inducing ability on tobacco were examined.

To generate mutations in *hrp*N, the 1.3 kb *Hin*dIII fragment carrying *hrp*N was first subcloned from pPV133 into the *Hin*dIII site of pRK767 (see Table 1 for plasmids and strains description). The resulting plasmid, named pMAB17, was mutagenized following the procedure described by Stachel *et al.* [1985]: pMAB17 was introduced by transformation into strain S17-1 (pSShe, pTn3-gus), selecting simultaneously for tetracycline (pMAB17), chloramphenicol (pSShe) and kanamycin (pTn3-gus). The presence of the three plasmids was checked on one transformant and this transformant was mated with the *pol*A recipient strain C2110 (Nal^r). Neither pSShe nor pTn3-gus are maintained after mobilization into C2110 since both plasmids are *pol*A dependant for their replication. Thus, plating the mating mixture on LB supplemented with Nal, Tet and Kan allowed the selection of C2110 (pMAB17::Tn3-gus) transconjugants. Plasmid DNA was isolated from 10 tranconjugants and the position and the orientation of the Tn3-gus transposon inside of pMAB17 were mapped with *Eco*R1 and *Hin*dIII. On two plasmids, pMAB18 and pMAB19, Tn3-gus inserted respectively 0.5 kb and 0.9kb inside *hrp*N in opposite orientation to *hrp*N transcription. To marker exchanged both insertions into insertions into the chromosome of the wild-type strain CFBP1430, pMAB18 and pMAB19 were first mobilized into CFBP1430 [Barny *et al.*, 1990]. The resulting strains, CFBP1430(pMAB18) and CFBP1430(pMAB19) were cycled more than 10 times on low phosphate medium (0.1 M Tris, 500 mM magnesium sulfate, 7.5 mM ammonium sulfate, 0.2% galactose, pH7. After sterilization, potassium phosphate buffer was added to 250 mM final) supplemented with Km to maintain the selection for the presence of the transposon. The bacteria were then plated on L medium supplemented with Km and on L medium supplemented with Km and Tet. Km resistant, Tet sensitive colonies recovered respectively from CFBP1430(pMAB18) and CFBP1430(pMAB19) were named M30 and M29. To verify that M30 and M29 had acquired Tn3-gus *via* a double recombination event, total genomic DNA from these two mutants and the wild type strain was extracted [Ausubel *et al.*, 1987], digested with *Hin*dIII and, after electrophoretic separation transferred to hybond N membrane (Amersham) for hybridization experiments. The membrane was probed with pPV133 DNA previously labelled with digoxigenin and chemoluminescent detection was performed using anti-digoxygenin-alkaline-phosphatase-conjugate and AMPPD (3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane) as chemoluminescent substrate as described by the supplier (Boehringer Mannheim). With the wild type strain DNA, this probe detected the 1.3 kb *Hin*dIII fragment

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli[a]		
DH5-a	High efficiency transformation derivative of DH5	Sambrook *et al.*, 1989
S17-1	Δ*rec*A, ::RP4-2Tc::MuKm::Tn7, *hsd*R, *thi*, *pro*	Simon *et al.*, 1983
C2110	*pol*A1, Nal^r, Rif^r	Leong *et al.*, 1982
E. amylovora[a]		
CFBP1430	Wild type, isolated from hawthorn	Paulin and Samson, 1973
PMV6023	*hrp*::Mud*II*PR13, Cm^r	Barny *et al.*, 1990
PMV6076	Δ*hrp*, ::Mud*II*PR13, Cm^r	Barny *et al.*, 1990
M29	*hrp*N::Tn3-gus derivative of CFBP1430	This work
M30	*hrp*N::Tn3-gus derivative of CFBP1430	This work
Plasmid		
pRK767	10.7 kb mobilizable, *pol*A independant vector, Tet^r	Gill and Warren, 1988
pTn3-gus	*tnp*A, Tn3HoHo derivative carrying a promoter-less β-glucuronidase gene, *pol*A dependant vector	B. Staskawicz[b]
pSShe	supply TnpA activity *in trans*, *pol*A dependant vector	Stachel *et al.*, 1985
pMAB17	pRK767 with the 1.3 kb *Hin*dIII fragment carrying *hrp*N	This work
pMAB18	pMAB17::Tn3-gus	This work
pMAB19	pMAB17::Tn3-gus	This work
pPV133	pLA2917 derivative carrying part of the *hrp* cluster	Barny *et al.*, 1990

[a] *E. amylovora* and *Escherichia coli* strains were respectively grown at 27 °C and 37 °C in either Luria Broth (L, Sambrook *et al.*, 1989) or King's medium B [KB, King *et al.*, 1954]. Media were supplemented with antibiotics, when indicated, at the following concentrations: chloramphenicol (Cm) 10 mg ml^{-1}, kanamycin (Km) 20 mg ml^{-1}, tetracycline (Tet) 5 mg ml^{-1}, and nalidixic acid (Nal) 25 mg ml^{-1}.

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carrying *hrp*N. With the mutants DNA, this probe failed to detect the 1.3 kb *Hin*dIII fragment carrying *hrp*N while two new fragments of different sizes were detected. This indicated that in M29 and M30, Tn3-gus inserted into the 1.3 kb *Hin*dIII fragment carrying *hrp*N.

The pathogenicity of both mutants was tested on host plant. Since similar results were obtained for both mutants, only the results obtained with M29 will be presented.

To determine pathogenicity of *hrp*N mutant, M29 was inoculated on 20 Pyrus seedlings and 20 Malus seedlings, in the greenhouse. As control, the wild type strain was also inoculated on the same number of plants. Nine days after inoculation, systemic necrosis reaching the stem were observed on most of the plant inoculated with the wild type strain (Table 2). After this period, no symptoms were detected on the apple seedlings inoculated with M29 while symptoms restricted to the inoculated leaf were detected on the pear seedlings. Twenty days after inoculation, a few of the pear seedlings symptoms had evolved to stem necrosis while the apple seedlings remained symptomless (Table 2). The experiment was repeated three times with similar results. The phenotype on plant seedlings *hrp*N mutants was similar to the phenotype described by Tharaud *et al.* [1994] for the mutant PMV6112: non pathogenic on apple seedlings and expressing a reduced virulence on pear seedlings. Sequencing of the border of the transposon insertion responsible for

Table 2. Comparative pathogenicity ratings for the wild type strain CFBP1430 and the harpin-deficient mutant M29 on pear and apple seedlings

	Number of plants with systemic symptoms			
	After 9 days		After 20 days	
	Pear	Apple	Pear	Apple
CFBP1430	16/20	18/20	17/20	18/20
M29	0/20	0/20	3/20	0/20

Pathogenicity tests were performed in the green house on seedlings at the 6 to 8 leaves stage. Pear seedlings (Kirchensallers) and apple seedlings (open pollinated Golden delicious) were inoculated as previously described [Laurent *et al.*, 1987].

PMV6112 phenotype proved that PMV6112 was an *hrp*N mutant with the transposon inserted out of frame 16 nucleotides after *hrp*N start codon.

To further assess *hrp*N mutants virulence, axenically grown unrooted microcuttings were used. This type of plant material is very susceptible to fire blight. Furthermore, the pear cultivar and the apple cultivar micropropagated for this study are both very susceptible to fire blight. Thus, the use of this plant material should allow to detect any residual pathogenicity on both hosts.

*hrp*N mutant M29 was inoculated on 8 apple and 8 pear microcuttings. As positive control, the wild-type strain CFBP1430 was also inoculated on the same number of plantlets. Two *hrp* mutants previously isolated, PMV6076 and PMV6023 [Barny *et al.*, 1990] were also inoculated as control. The first one PMV6076, showed a large deletion spanning the whole *hrp* region. The second one, PMV6023, carried a Mu*d*IIPR13 insertion that could be easily localized into the *hrp* transcriptional unit V1 described in Ea 321 [Wei

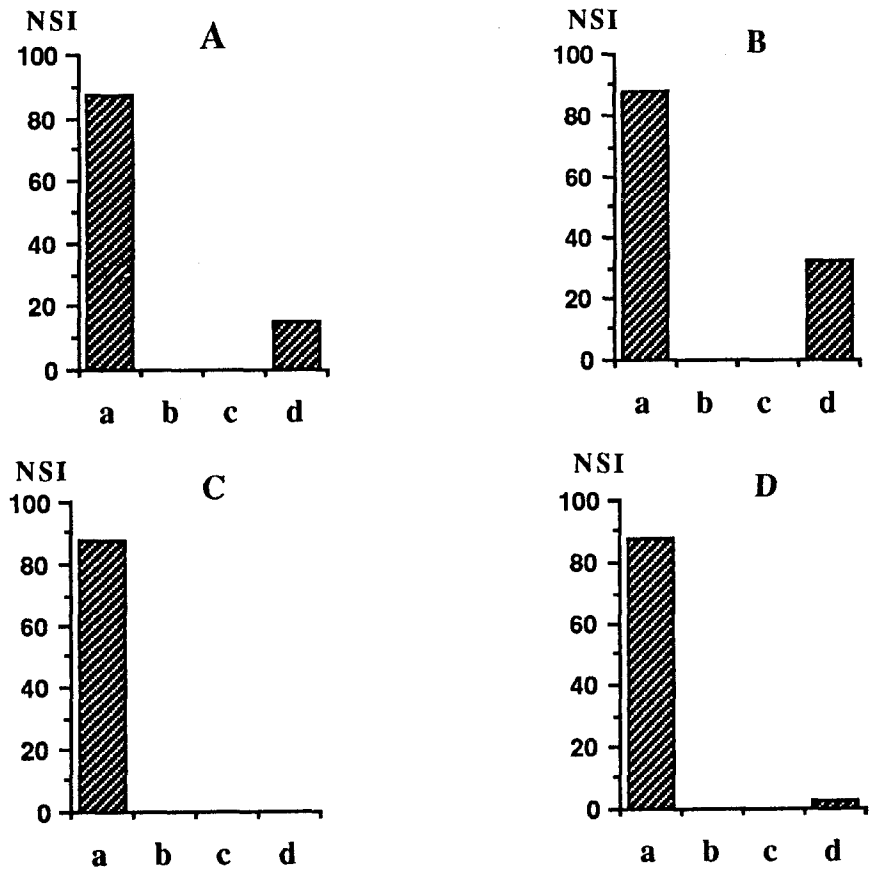

Fig. 1. Comparative pathogenicity of the wild type strain CFBP1430, the harpin-deficient mutant M29 and the *hrp* mutants PMV6023 and PMV6076 on pear and apple microcuttings. The pear microcuttings (cv. Comice) were propagated on modified Lepoivre medium (Leblay *et al.*, 1991). The apple microcuttings (cv. Gala) were propagated on MS medium [Murashige and Skoog, 1962] supplemented with myoinositol (100 mg ml^{-1}), thiamine HCl (0.4 mg ml^{-1}), benzylaminopurine (1 mg ml^{-1}) and indole-3-butyric acid (0.1 mg ml^{-1}). Microcuttings were maintained in the growth chamber and were inoculated with surgical pliers previously dipped into a 10^8 cfu ml^{-1} suspension. To quantitatively assess the results obtained on microcuttings, a necrosis severity index (NSI), representing the mean necrosis for all the inoculated plantlets, was calculated as described by Duron *et al.* [1987]. Briefly, NSI is calculated as follows: $NSI = (1n_1(Nm)+2n_2(Np)+3n_3(Ns)+4n_4(Nwp)/n_1+n_2+n_3+n_4)\ 100$, where Nm = necrosis reaching the midrib of the inoculated leaf; Np = necrosis reaching the petiole of the inoculated leaf; Ns = Necrosis reaching the stem; Nwp = necrosis of the whole plantlet; and $n_{1,2,3,4}$ = number of plantlets showing respectively the degree of severity Nm, Np, Ns, Nwp. The NSI was calculated on eight pear microcuttings (A&B) and 8 apple microcuttings (C&D) seven days (A&C) and ten days (B&D) after inoculation. The NSI obtained following inoculation with CFBP1430 (lane a), PMV6076 (lane b), PMV6023 (lane c) and M29 (lane d) are represented.

and Beer, 1993] since the restriction map of strain CFBP1430 and Ea321 are identical over the whole length of the *hrp* region [Bauer and Beer, 1991; Laby and Beer, 1992]. Mutations in this transcriptional unit abolishe harpin secretion but not harpin synthesis [Wei and Beer; 1993]. The symptoms were scored seven and ten days after inoculation (Fig. 1).

Most of the apple microcuttings inoculated with the wild-type strain CFBP1430 developed typical symptoms of fire blight seven days after inoculation while no symptoms were detected on the plantlets inoculated with the *hrp*N mutant M29 or with PMV6023 and PMV6076. (Fig. 1C). Ten days after inoculation the plantlets inoculated with PMV6076 or PMV6023 remained symptomless while residual symptoms, typical of the fire blight disease, were observed on a few microcuttings inoculated with the *hrp*N mutant M29 (Fig. 1D and Fig. 2). These residual symptoms never evolved to systemic symptoms even 3 weeks post-inoculation and in most cases, the inoculated leaf fell down.

Seven days after inoculation, most of the pear microcuttings inoculated with the wild-type strain CFBP1430 showed systemic symptoms and none of those inoculated with PMV6076 and PMV6023 developed symptoms (Fig. 1A). At the same time, fire blight symptoms began to appear on most of the plantlets inoculated with the *hrp*N mutant M29 (Fig. 1A). These symptoms evolved and necrosis of the whole plantlet could be observed 10 days after inoculation (Fig. 1B and Fig. 2). Since significant symptoms were observed with *hrp*N mutant M29 on pear microcuttings, the bacterial growth was measured on this plant material 3 days and 10 days after inoculation (Fig. 3). As expected, the wild type strain CFBP1430 grew rapidly on plant tissue. Mutant M29 also grew significantly after inoculation although at a slower rate than the growth observed with the wild type strain. This contrasts with mutant PMV6023 for which no significant growth was detected even 10 days after inoculation.

These results show that, on the *in vitro* plant material, more susceptible to fire blight than the seedlings, residual symptoms are observed on apple but a clear difference is still observed between pear and apple. This shows that the outcome of the infection for *hrp*N mutants

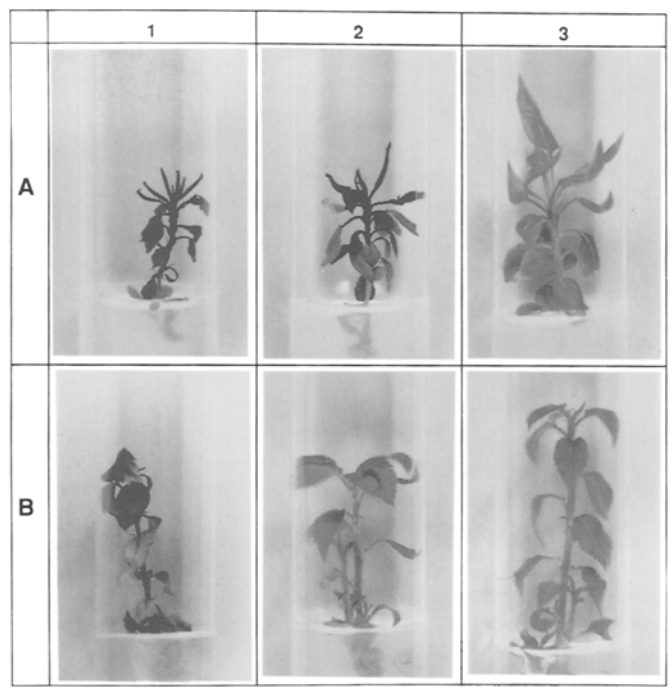

Fig 2. Representative symptoms detected on pear microcuttings (A) and apple microcuttings (B) ten days after inoculation with the wild type strain (lane 1), with the *hrp*N mutant M29 (lane 2) and with the mutant PMV6023 (lane 3).

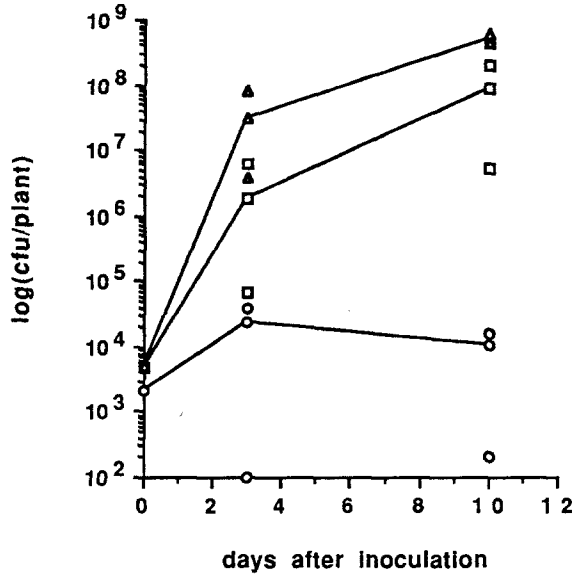

Fig 3. Comparative growth on pear microcuttings of CFBP1430 (triangle), M29 (square) and PMV6023 (circle). The number of bacterial cells (cfu) present on 3 plantlets was measured 3 days and 10 days after inoculation. Each plantlet was dipped in 10 ml of sterile water and vortexed to recover the bacteria present on the surface. The intercellular fluid from the inoculated plantlet was prepared as described by Neema *et al.* [1993] and bacteria were harvested after centrifugation. All recovered bacteria (surface and intercellular fluid) were pooled and serial dilutions were plated on L medium. The mean of recovered bacteria in each of the 3 plantlets is represented as well as the minimal and maximal observed value.

depends on the plant material used for the experiment. This could explain why Wei *et al.* [1992] described *hrp*N mutants as non pathogenic. Actually, they tested *hrp*N mutant pathogenicity on immature pear fruit. This test, on a isolated organ, gives probably partial information on pathogenicity and could therefore be less discriminative than a test on a whole plant. The *in vitro* pear microcuttings are probably a more sensitive material to detect fire blight disease.

Since residual pathogenicity could be detected on host plant, the potentiality of *hrp*N mutants to induce an HR on tobacco was examined. The *hrp*N mutants M30 and M29 were infiltrated into the leaf mesophyll of several tobacco plants. As control each tobacco plant was also infiltrated with the wild-type strain CFBP1430 and with mutants PMV6076 and PMV6023.

In each experiment, the area infiltrated with the wild-type strain developed a typical HR while no reaction was detected on the area infiltrated with PMV6076 or PMV6023 (Table 3). Variable responses were observed in the area infiltrated with the *hrp*N mutants M29 and M30 depending on the experiment. In some experiments no visible HR was observed while in other experiments a full HR or an intermediate reaction, characterized by spotty necrosis of the infiltrated area, occurred (Table 3). The intensity of the HR reaction was not dependent on the concentration of the inoculum. On one tobacco infiltrated with a very high inoculum (10^9 cfu ml^{-1}) no visible HR was observed in the area infiltrated with the *hrp*N mutants M29 and M30 while a typical HR could be observed on another tobacco plant infiltrated with a smaller inoculum of the same strain (10^7 cfu ml^{-1}). Moreover, the two *hrp*N mutants tested always reacted in the same way on the same tobacco plant. Typical necrosis observed on a reacting tobacco plant are shown in Fig. 4.

The variability of the HR induced by *hrp*N mutants seems to rely on the tobacco plant used for the experiment. Since no obvious differences in the phenotype were observed between the different tobacco plants, it is likely that subtle physiological differences accounted for the differential HR responses. This variability could explain why Wei *et al.* [1992] described *hrp*N mutants as unable to elicit an HR on tobacco while Tharaud *et al.* [1994] described the *hrp*N mutant PMV6112 as fully able to elicit an HR on tobacco. Testing PMV6112 on several tobacco plants revealed that

Table 3. Comparison of the HR-inducing ability of the wild type strain CFPB1430, harpin-deficient mutants M29 and M30 and Hrp mutants PMV6076 and PMV6023

	Full HR	Intermediate HR	No visible HR
CFBP1430	8	0	0
PMV6076	0	0	8
PMV6023	0	0	8
M29	3	2	3
M30	3	2	3

Hypersensitive reaction was tested on eight different tobacco plants (*Nicotiana tabacum* L cv. Xanthi) grown to a height of 100 cm to 120 cm in the green house. Tobacco leaves were infiltrated with a 10^8 cfu ml^{-1} Appearance of the HR was scored after incubation at room temperature for 24 h.

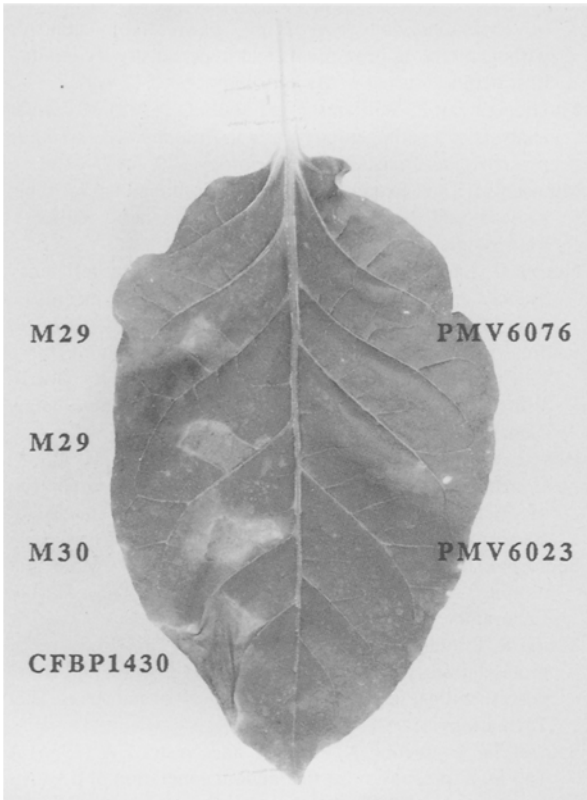

Fig 4. Hypersensitive reaction on tobacco, The left part of the leaf was infiltrated from top to bottom with M29 (10^7 bacteria ml^{-1}), M29 (10^8 bacteria ml^{-1}), M30 (10^8 bacteria ml^{-1}) and CFBP1430 (10^8 bacteria ml^{-1}). The right part of the leaf was infiltrated from top to bottom with PMV6076 (10^8 bacteria ml^{-1}) and PMV6023 (10^8 bacteria ml^{-1}).

its HR inducing ability was as variable as the other *hrp*N insertion mutants presented in this paper. The results presented here showed that *hrp*N mutants, blocked in harpin synthesis, have a weak but significant ability to induce both disease on host plants and HR on non host plants. This implies that pathogenicity/HR inducing factor(s), different from harpin, is (are) still expressed in *hrp*N mutants.

On host plant, the virulence of *hrp*N mutants is severely reduced: the symptoms are delayed and the number of plant showing symptoms is reduced, as compared with the wild type strain. This confirms that harpin is a major virulence determinant in *E. amylovora.*

In all the tests performed, mutant PMV6023, which is blocked in harpin secretion but not in harpin synthesis remained totally non pathogenic and unable to elicit an HR on tobacco, even on the most susceptible plant material tested. This suggests that the pathogenicity/HR inducing determinants responsible for the reduced virulence/HR inducing ability of *hrp*N mutants are secreted through the Hrp secretion pathway. Assessing the importance of these pathogenicity/HR inducing determinants in the disease/HR inducing process awaits the isolation of mutants blocked in the synthesis of these factors.

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