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Protection of apple against fire blight induced by an *hrp*L mutant of *Erwinia amylovora*

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Abstract

A regulatory *hrp*L non-virulent mutant of *Erwinia amylovora* is effective in controlling fire blight disease when inoculated on apple seedlings simultaneously with the pathogenic parental strain. Mechanisms involved in this protective effect were investigated. The use of two marker genes, *uid*A and *lac*Z, expressed in the *hrp*L mutant and the pathogenic strain, respectively, allowed to localize simultaneously the two inoculated strains in plant tissue. An anti-β-glucuronidase antibody was also used to detect the *hrp*L mutant. Both techniques indicated that the two strains localized mainly in separate areas of the leaf tissue. In addition, leaves infiltrated with the *hrp*L mutant exhibited a significant increase in peroxidase activity in contrast to a *hrp* secretion mutant known to be less effective in the protection. It is suggested that protection obtained with the *hrp*L mutant relies on the physical separation between the mutant and the parental strain after co-inoculation and the rapid and sustained activation of plant defense mechanisms in reactive tissue, *i.e.* not invaded by the virulent strain.

Additional key words: apple defense response, histochemical assays, *Malus* × *domestica.*

Introduction

Erwinia amylovora causes fire blight, a necrotic disease occurring on apple and pear trees as well as on woody ornamentals, including *Crataegus*, *Cotoneaster* and *Pyracantha*. Like most Gram negative phytopathogenic bacteria, *E. amylovora* chromosome includes a cluster of genes named *hrp* which is required for both the elicitation of the hypersensitive reaction (HR) in incompatible interactions and the disease in compatible ones (Barny *et al.* 1990, Bauer and Beer 1991). The *hrp* cluster of *E. amylovora* encodes a type III secretion apparatus which is common to several phytopathogenic bacteria (Lindgren *et al.* 1986, Huang *et al.* 1988, Fenselau and Bonas 1995, Bogdanove *et al.* 1996) and travelled by several proteins involved in pathogenicity, such as harpins N and W (Wei *et al.* 1992, Gaudriault *et al.* 1998, Kim and Beer 1998), DspA (Gaudriault *et al.* 1997) and HrpA (Hu *et al.* 1999). This cluster is under the control of a complex regulatory network involving *hrp*X, *hrp*Y and *hrp*S leading to the activation of *hrp*L, which seems to be the master switch of the *hrp* system of *E. amylovora*. The *hrp*L is under the partial control of *hrp*S gene; it is a ECF sigma factor and positively regulates the *hrp* gene cluster (Wei and Beer 1995, Wei *et al.* 2000).

The *hrp* mutants of *E. amylovora* have been shown to protect apple seedlings and apple flowers from developing fire blight symptoms caused by the virulent parental strain (Tharaud *et al.* 1997, Faize *et al.* 1999). When inoculated either in advance of – or simultaneously with – the virulent strain, *hrp*S and *hrp*L mutants were significantly more protective than *hrp* secretory mutants (*hrc*V) (Tharaud *et al.* 1997). Such a protective effect was associated, in the case of *hrp*L or *hrp*S mutants only, with a decrease in population of the virulent strain.

In a first approach to elucidate the mechanisms leading to protection we showed (Faize *et al.* 1999) that the protective ability of a *hrp*S mutant was correlated with the induction in the host plant of defense reactions including increased activities of phenylalanine-ammonia lyase (PAL; EC 4.3.1.5) and of peroxidase (POX; EC 1.11.1.7). Because of the partial control exerted on

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Abbreviations: β-Gal - β-galactosidase activity; CFU - colony-forming units; Gus - β-glucuronidase activity; POX - peroxidase.
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the *hrp* cluster by the *hrp*S gene these results suggested that in this mutant some pathogenicity factors were released at a level sufficient to induce plant defense response but not high enough to cause disease.

In this paper we investigated the mechanisms involved in the protective effect obtained with a *hrp*L mutant since the *hrp*L gene seems to be the key component for the regulation of the *hrp* system of *E. amylovora*. In this context, we engineered from the virulent strain CFBP1430 an *hrp*L mutant (M73) and a pathogenic strain (FB2224) harbouring gene fusions that constitutively expressed β-glucuronidase (Gus) and β-galactosidase (β-Gal) activities, respectively. This allowed us to observe the location of both strains in tissues of the host leaf when inoculated either alone or simultaneously (co-localization). We also analyzed the ability of the engineered *hrp*L mutant to elicit apple defense mechanisms, using POX as a defense marker.

Materials and methods

Plants and growth conditions: Apple (*Malus* × *domestica*) seedlings from open-pollinated cv. Golden Delicious were grown in pots containing commercial soil mix in the greenhouse (20 °C, 80 % relative humidity, natural photoperiod). For flower studies, branches of apple trees of the same cultivar were collected in the orchard in early spring after cold requirements were fulfilled and placed in buckets filled with gravel and water in the greenhouse. The flowers were cross-pollinated and inoculated 24 h later.

Bacterial strains, plasmids, culture media and growth assays: The relevant characteristics of bacterial strains and plasmids are listed in Table 1. *E. amylovora* strains were grown at 27 °C for 24 h on KB medium (King *et al.* 1954) supplemented with the appropriate antibiotic (20 µg cm^{-3}). *Escherichia coli* was cultivated on LB medium (Miller 1972) at 37 °C for 24 h. Growth curves of *E. amylovora* strains were determined by using microplate photometer (*Bioscreen, Labsystems*, Helsinki Finland). 0.32 cm^3 of liquid M9 minimal medium (Sambrook *et al.* 1989) supplemented with galactose (0.2 %) and nicotinic acid (0.02 %) were added to 0.03 cm^3 of bacterial suspension (3×10^8 CFU cm^{-3}). Absorbance (492 nm) was read automatically every 15 min during 60 h.

Screening for the virulent strain expressing a LacZ fusion: In order to use a virulent strain of *E. amylovora* that expressed a β-Gal activity, a bank of mutants obtained after insertion of MudIIPR13 phage (carrying the lacZ gene of *E. coli* for β-Gal activity) in the virulent CFBP1430 (Barny *et al.* 1990) was successively screened on McConkey medium (Sambrook *et al.* 1989) supplemented with lactose (1 %), then on M9 minimal medium supplemented with galactose (0.2 %), nicotinic acid (0.02 %) and 5-bromo-4-chloro-3-indolyl-ß-D-galacto-pyranoside (X-Gal) (40 µg cm^{-3}). Strains which appeared pink then blue on these media respectively, were tested for pathogenicity on apple seedlings and for elicitation of an HR on tobacco, as described by Tharaud *et al.* (1994). One strain expressing a LacZ fusion, eliciting an HR on tobacco and exhibiting both a pathogenicity level and a growth curve identical to the parental strain CFBP1430 (data not shown) was selected and named FB2224.

Construction of hrpL mutant expressing Gus fusion: Plasmid DNA extraction, restriction enzyme digestion, DNA ligation, transformation of competent $DH_{5\alpha}$ *E. coli* strain, Southern blotting and hybridization were performed as described by Sambrook *et al.* (1989).

In order to obtain an *hrp*L mutant that expressed a Gus activity, an *hrp*L::*uid*A transcriptionnal fusion was constructed by cloning the *uid*A-Km cassette (carrying a gene for Gus activity) into the plasmid pMAB71 (Gaudriault *et al.* 1997) carrying the *hrp*L gene. The plasmid pMAB71 which harbors the 3.8-kb EcoRI fragment carrying *hrp*L was linearized with *Bgl*II within *hrp*L, 151 nucleotides after the start codon. The 3.6-kb *uid*A-Km cassette was liberated from pUIDK1 following restriction with *Sma*I enzyme. After filling in with Klenow the linearized plasmid, pMAB71 was ligated with the border fragment of the *uid*A-Km cassette. The orientation of the cassette in the resulting plasmid was determined by restriction enzyme analysis. The plasmid carrying the cassette in the correct orientation was

Table 1. Bacterial strains, plasmids and relevant characteristics.

Strain/plasmid			Relevant characteristics	Source/reference
Strains	*E. amylovora*	CFBP1430	Wild type, isolated from *Crataegus*	Paulin and Samson 1973
		FB2224	CFBP1430 ::Mu*d*IIPR13, LacZ, Cmr, HR+, Path+	this work
		PMV6023	CFBP1430 *hrc*V::Mu*d*IIPR13, LacZ, Cmr, HR-, Path-	Barny 1995
		M73	CFBP1430 *hrp*L::*uid*A, Gus, Kmr, HR-, Path-	this work
	Escherichia coli	$DH_{5\alpha}$	Strain highly transformable	Invitrogen BRL
Plasmids		pMAB71	pUC18 with the 3.8 Kb *Eco*RI fragment carrying *hrp*L	Gaudriault *et al.* 1997
		pMAB108	pMAB71 carrying a *uid*A-Km cassette inside *hrp*L	this work

named pMAB108. Plasmid pMAB108 containing the *hrp*L::*uid*A transcritionnal fusion was introduced into the virulent CFBP1430 by electroporation as described by Franza and Expert (1991). Transformants were grown on LB medium containing kanamycin as selective marker for the cassette and colonies were screened for kanamycin resistance (20 µg cm^{-3}) and ampicillin sensitivity (100 µg cm^{-3}) to identify marker exchange recombinants.

The marker exchange mutant was named M73. It expressed a high level of Gus activity (Jefferson *et al.* 1986) when grown on M9 minimal medium supplemented with galactose, exhibited the typical Hrp minus phenotype and was unable to induce an HR on tobacco or disease on apple seedlings.

Assays for protective ability: The protective ability of the engineered *hrp*L mutant M73 was tested against the virulent strains CFBP1430 and FB2224 on young apple seedlings and apple flowers as described by Tharaud *et al.* (1997) and Faize *et al.* (1999), respectively. This ability was compared to that of the *hrc*V mutant PMV6023, previously shown to have a low protective ability (Tharaud *et al.* 1997). Briefly, suspensions of the nonvirulent (3×10^9 CFU cm^{-3}) and of the virulent (3×10^8 CFU cm^{-3}) strains were mixed, then immediately inoculated (co-inoculation). Apple flowers were inoculated with a drop (0.01 cm^3) of bacterial suspension inserted onto the hypanthium. For apple seedlings, one drop (0.01 cm^3) of bacterial suspension was deposited on the youngest expanded leaf wounded by an incision perpendicular to the midrib. Percentages of infected fruitlets or infected seedlings respectively were compared to water controls.

Histochemical Gus and β-Gal activity assays: The localization of the virulent strain FB2224 (by the blue staining of its β-Gal activity) and of the *hrp*L mutant M73 (by the Magenta red coloration of its Gus activity) was studied in leaves of apple seedlings inoculated as described above. Strains were either inoculated alone (single inoculation) or simultaneously (co-inoculation). As FB2224 inoculated alone induced typical symptoms up to 0.5 cm from the wound within three days, we chose to investigate bacterial localization in invaded tissues three days after inoculation**.**

Histochemical Gus and β-Gal activities were assayed as described by Etchebar *et al.* (1998) with slight modifications. For Gus activity, leaf pieces (0.5 × 0.5 cm) were excised around the inoculation site (0.5 cm far from the injury). They were rinsed in sodium phosphate buffer (50 mM, pH 7), prefixed for 1 h at 4 °C (1 % *p*-formaldehyde, 50 mM phosphate buffer), rinsed 4 times in the phosphate buffer then incubated in the reaction medium (50 mM phosphate buffer, pH 7, 5 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1 mg cm^{-3} of *Magenta-Gluc*) for 2 h at 37 °C, and finally rinsed in phosphate buffer. For β-Gal activity, the leaf pieces were fixed 1 h with 1.25 % (v/v) glutaraldehyde prepared in cacodylate buffer (0.1 M, pH 7.2) to eliminate the endogenous plant β-Gal activity. They were rinsed twice in the same buffer, then incubated overnight at room temperature in the reaction medium (50 mM cacodylate buffer, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.08 % X-Gal) and finally rinsed in cacodylate buffer. Samples were lighted by 70 % hot ethanol and observed directly with light microscopy or sliced into semi-thin (1 µm) sections and colored with toluidine blue (1 %).

For immunodetection of bacteria expressing Gus activity, 1-µm-thin sections were labelled with anti β-glucuronidase rabbit antibody (*Sigma*) as follows: sections were incubated for 5 min in phosphate saline buffer, pH 7.4, containing 0.1 % BSA and 0.01 % *Tween 20*, incubated for 20 min in goat antibody diluted at 1/30 in phosphate saline buffer then incubated overnight at 4 °C in anti-β-glucuronidase rabbit antibody diluted at 1/500. After several washes in the same buffer without *Tween*, sections were incubated (1 h in the darkness) in a goat antibody raised against rabbit IgG and labelled with fluorescein isothiocyanate freshly diluted at 1/400. Sections were then washed several times in buffer and water and finally mounted in antifading medium (*Vector H 1000*) and observed under UV microscopy.

Assays for plant POX activities: To test the ability of the engineered *hrp*L mutant M73 to elicit plant defense, POX activities were assessed in leaves of apple seedlings previously infiltrated with bacterial suspension as described by Faize *et al.* (1999), using water, the *hrc*V mutant PMV6023 and the virulent CFBP1430 as controls. Leaf tissues were ground in 50 mM acetate buffer pH 5.8 containing 0.01 % Triton X-100, 1 mM β-mercaptoethanol, 1 mM PMSF and 8 % (m/v) of polyvinylpolypyrrolidone. The homogenate was centrifuged at 16 000 *g* for 20 min and the supernatant assayed for POX activity according to Moerschbacher *et al.* (1986). The protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

Results

Protective ability of the engineered hrpL against fire blight: The percentage of apple seedlings showing fire blight symptoms was very high when the standard virulent CFBP1430 was inoculated alone (98 %) and though significantly reduced, remained higher than 50 % when co-inoculated with the *hrc*V mutant (Fig. 1*A*). When CFBP1430 was co-inoculated with the *hrp*L mutant the percentage of infected seedlings (10 %) was

drastically reduced. Similarly the percentage of infected flowers was significantly reduced when CFBP1430 was inoculated with the *hrp*L mutant (Fig. 1*B*). The protective effect of the *hrp*L mutant was also assessed against the virulent strain FB2224 which carried a LacZ fusion and exhibited a level of pathogenicity identical to that of the parental strain. The same levels of protection were obtained against this strain in the two bioassays (data not shown).

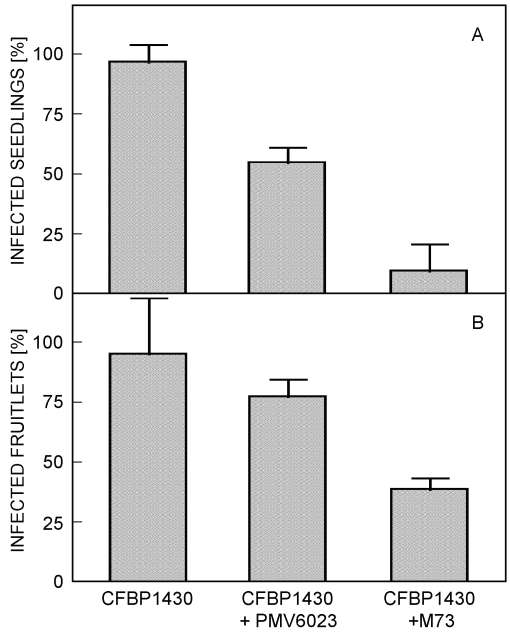

Fig. 1. Comparison of the protective effect of the regulatory (*hrp*L) mutant M73 and of the secretion deficient (*hrc*V) mutant PMV6023 on wounded apple seedlings (*A*) and apple flowers (*B*). For each repetition, twenty seedlings were inoculated, after wounding through the midrib of the youngest expanded leaf and depositing 0.01 cm^3 of bacterial suspension (pathogenic strain 3×10^8 CFU cm^{-3}, non virulent mutant 3×10^9 CFU cm^{-3}); thirty flowers of a branch were inoculated by depositing 0.01 cm^3 of bacterial suspension onto the hypanthium. Percentages of infected seedlings were assessed three weeks after inoculation; values are means from three repetitions and *vertical bars* represent standard errors ($P = 0.05$). Percentages of infected fruitlets were assessed two weeks after inoculation; results are means from six replicate branches and *vertical bars* represent standard errors ($P = 0.05$).

Localization of the hrpL mutant and of the virulent strain after single- or co-inoculation: The virulent strain FB2224 inoculated alone was detected in the midrib and in the mesophyll (Fig. 2*A*). A blue staining was never detected in the control leaf (not shown). Semi-thin transverse sections through the primary leaf vein colored with toluidine blue, showed an abundance of bacterial cells in distorted xylem elements and in lysigenous cavities derived from enlarged and damaged interfascicular parenchyma cells of the midrib (Fig. 2*B*). Bacteria were also detected in the intercellular spaces of perifascicular parenchyma and of dead cells of the mesophyll (not shown).

The *hrp*L mutant M73 inoculated alone was only detected in the midrib near the injury (Fig. 2*C*). Semi-thin transverse sections of the leaf showed bacteria in younger cells that will form xylem vessel (protoxylem) as well as in differentiated vessels (metaxylem) (Fig. 2*D*). Conversely to the tissues inoculated with the virulent strain, no lysigenous cavities were observed with the *hrp*L mutant.

Double staining of leaves simultaneously inoculated with the *hrp*L mutant M73 and the virulent strain FB2224 revealed that the blue stained areas were distinct from the *Magenta* red areas (Fig. 3*A*). These two strains invaded separate xylem elements (Fig. 3*B*). Such results were noticed in each of three different experiments.

In order to reinforce this observation we used an anti-β-glucuronidase antibody allowing the detection of the *hrp*L mutant M73 in semi-thin transverse sections. After single inoculation, we observed an immunostaining (fluorescence) of the *hrp*L mutant in xylem vessels. These semi-thin transverse sections were not fluorescent when untreated with the antiserum. Semi-thin sections from leaf inoculated with the virulent strain FB2224 alone (which did not harbour the Gus fusion) did not exhibit any immunostaining (data not shown).

When the *hrp*L mutant and the virulent strain were inoculated simultaneously we observed, in the midrib of the leaf, xylem vessels with immuno-marked (fluorescent) bacteria and xylem vessels containing non fluorescent bacteria (Fig. 3*C*). These non fluorescent bacteria were also detected in distorted xylem elements as noticed with the virulent strain inoculated alone. This confirmed that the mutant and the virulent strain were located mainly in separate areas of the leaf tissue.

POX activities: Leaves of apple seedlings infiltrated with the hrpL mutant M73 exhibited a significant increase in POX activity as compared to water as soon as 7 h after inoculation (Table 2). This activity remained high during the whole duration of the experiment (120 h). In leaves infiltrated with the hrcV mutant an increase in POX activity occurred which however was usually non significantly higher than that of leaves infiltrated with water. When leaves were infiltrated with the virulent CFBP1430 an increase in POX activity was observed up to 15 h after infiltration. Then a complete necrosis of the infiltrated leaves occurred preventing any further analysis.

Discussion

It has been shown previously that *hrp* mutants of *E. amylovora* altered in their regulatory functions were the most effective in protecting against fire blight (Tharaud *et al.* 1997, Faize *et al.* 1999). In this paper, we

investigated the possible basis of the protective ability of a regulatory *hrp*L mutant.

When inoculated alone, the virulent strain invaded mainly xylem vessels and formed lysigenous cavities as already reported (Bogs *et al.* 1998, Dellagi *et al.* 1999). Cells of the *hrp*L mutant could be detected in xylem vessels as well, but lysigenous cavities were never observed. By using a two-step staining technique we observed, after co-inoculating the *hrp*L mutant and the virulent strain, that the virulent and the mutant strains were mostly detected in different xylem vessels. This was confirmed by an additional technique involving immunostaining for GUS. Thus, through two distinct approaches it appeared that though mixed-inoculated, cells of the *hrp*L mutant and of the virulent strain did not coexist at the same site of the leaf tissues. These observations, which were consistently made, supported our previously proposed hypothesis of a competition between the two strains for space in leaf tissues (Tharaud *et al.* 1997). Etchebar *et al.* (1998) showed, using such a two-step staining method, that an *hrp* mutant of *Ralstonia solanacearum*, able to prevent tomato wilt caused by the virulent parental strain, localized in xylem vessels of roots distinct from those invaded by the virulent strain. This successful colonization played a key role in the protective efficacy of the mutant. In their experimental procedure however, the mutant strain was root-inoculated one week before the virulent strain, thus allowing the

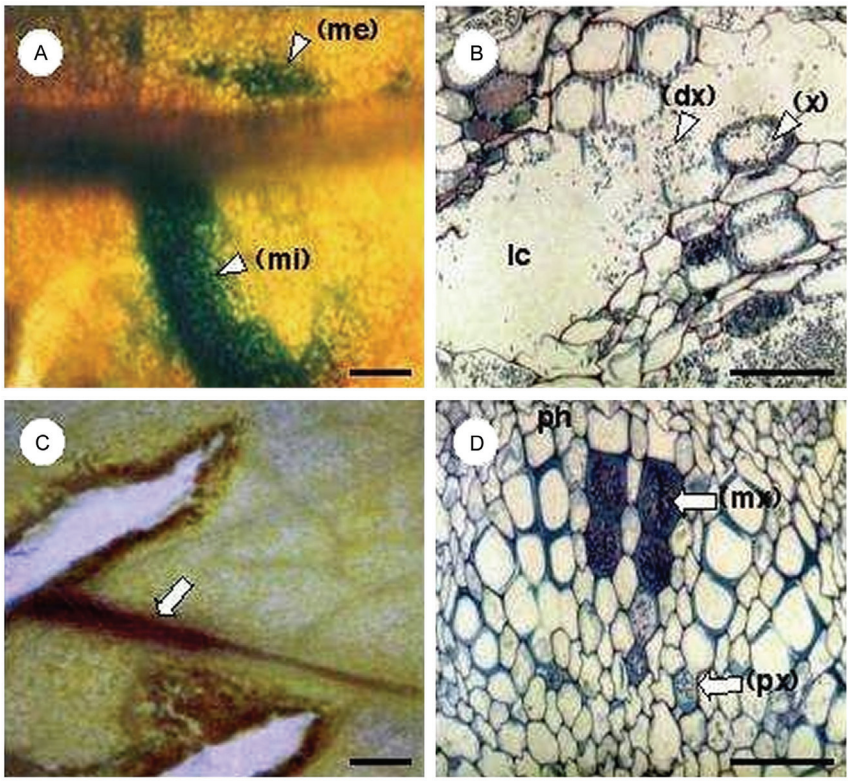

Fig. 2. Localization of bacteria in leaves of apple seedlings 3 d after single inoculation with *E. amylovora* virulent strain FB2224 (*heads of arrow* in *A* and *B*) or the *hrp*L mutant M73 (*arrows* in *C* and *D*). *A* - Portion of the leaf showing localization of the virulent strain FB2224 by histochemical staining of its β-galactosidase activity (blue color) in the midrib (mi) and in the mesophyll (me). *B* - Transverse section through the midrib stained with toluidine blue showing localization of the virulent strain in xylem element (x), in distorted xylem element (dx), and in lysigenous cavity (lc). *C* - Portion of the leaf showing localization of the *hrp*L mutant M73 by histochemical staining of its β-glucuronidase activity (*Magenta* red color) in the midrib. The two diagonal violet areas are tears in the tissue section. *D* - Transverse section through the midrib stained with toluidine blue showing the *hrp*L mutant in metaxylem (mx) as well as in protoxylem vessels (px). ph - phloem. *Scale bars* = 100 (*A*, *C*) or 20 µm (*B*, *D*).

Table 2. Time course of POX activity [µmol(tetraguaiacol) g^{-1}(protein) min^{-1}] in non excised apple leaves after vacuum infiltration with water or bacterial suspensions PMV6023 (secretory deficient *hrc*V mutant) or M73 (regulatory *hrp*L mutant) at 10^9 CFU cm^{-3}, CFBP1430 (virulent strain) at 10^8 CFU cm^{-3}. Data are medians of nine repetitions from three independent experiments. In each column, values with the same letters are not significantly different (Kruskal-Wallis test, $P = 0.05$).

	7 h	15 h	72 h	120 h
water	492 a	426 a	541 a	766 a
PMV6023 (hrcV)	1128 ab	1088 ab	1975 ab	2790 b
M73 (hrpL)	1487 b	2520 b	2491 b	4012 c
CFBP1430 (virulent)	911 ab	2175 b	necrosis	necrosis

mutant to colonize some vessels before application of the virulent strain. In our system, the mutant and the virulent strains were simultaneously inoculated as a mixture thus suggesting that events leading to the separation of the two populations take place shortly after co-inoculation. A mutual exclusion between the two strains based on antagonism was investigated. However such an antagonism has not been clearly evidenced *in vitro* in our experiments (data not shown). The basis of the physical separation between the two strains therefore remains to be established.

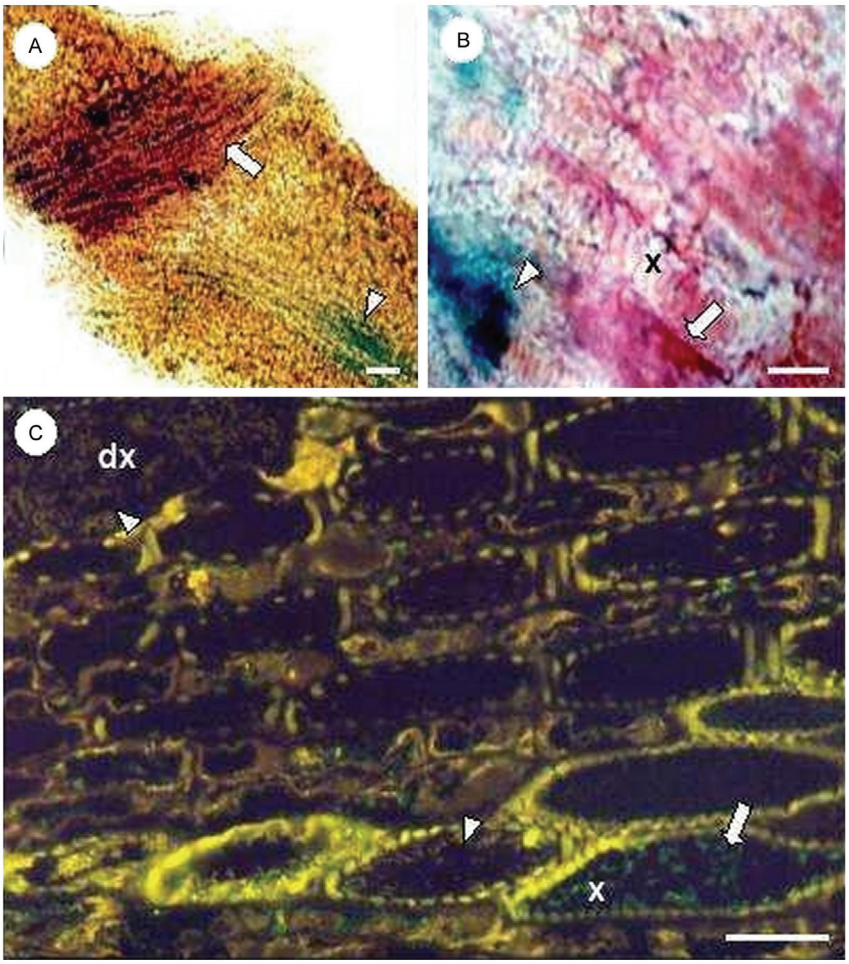

Fig. 3. Localization of bacteria in leaves of apple seedlings three days after co-inoculation with *E. amylovora* virulent strain FB2224 (*heads of arrow*) and the *hrp*L mutant M73 (*arrows*) by histochemical staining of their β-galactosidase (blue color) and β-glucuronidase (*Magenta* red color) activities respectively (*A*, *B*) and by immunostaining of M73 cells harbouring the Gus fusion with an anti-β-glucuronidase rabbit antibody and a fluorescein isothiocyanate coupled anti-rabbit IgG conjugate (green color) (*C*). *A* - Portion of the leaf showing localization of the *hrp*L mutant in the midrib and of the virulent strain in the secondary vein. *B* - Crushing of vessel elements of the midrib showing separate xylem localization of the *hrp*L mutant and of the virulent strain. *C* - Immunomarked transverse section through the midrib showing immunofluorescence of the *hrp*L mutant in xylem element (x) and nonfluorescent bacteria (virulent strain) in xylem element and in distorted xylem element (dx). Presence here and there of dispersed bacteria eventually stained is due to the vessel lysis. *Scale bars* = 100 µm (*A*) or 20 µm (*B*, *C*).

We also investigated the hypothesis of elicitation of plant defense by the *hrp*L mutant using POX activation as an indicator of overall resistance of plant tissues. In a previous work (Venisse *et al.* 2002), we showed that a low concentration (10^7 CFU cm^{-3}) of a virulent strain of *E. amylovora* was able to activate several plant defense responses (including POX), conversely to a *hrc*V mutant. This suggested that Hrp effectors were responsible of the elicitation. In the present work we showed that, at higher concentration (10^9 CFU cm^{-3}) the *hrp*L mutant (and to a lesser extent the *hrc*V mutant) was also capable of a rapid and sustained induction of POX. This suggests the existence of Hrp-independent elicitors in *E. amylovora*. Induction of non-specific responses that are *hrp*-independent has already been described in non-host as well as in host interactions. Jacobek and Lindgren (1993) showed that an *hrp* mutant of *Pseudomonas syringae* pv. *tabaci* induced PAL, chitinase, chalcone synthase and

chalcone isomerase transcripts in bean. Brown *et al.* (1995) reported the formation of large papillae and cell wall changes in pepper mesophyll challenged with *hrp* mutants of *Xanthomonas campestris* pv. *vesicatoria*. More recently, Newman *et al.* (2001) recorded rapid accumulation of phenolic conjugates in pepper in response to a mutant of *Xanthomonas campestris* pv. *campestris* carrying a deletion of the *hrp* cluster. These authors suggested the existence of a general non-specific induction of defense genes by external, *hrp* gene-independent bacterial components such as lipopoly-saccharides, cell surface polysaccharides or bacterial flagellins.

However our work showed a consistent distinction between the two *hrp* mutants (regulatory and secretion deficient) in their ability to activate plant POX. One possibility is that in the plant *hrp*-independent putative elicitors are overproduced by the regulation mutant compared to the secretion mutant. This is in accordance with an hypothesis proposed by Newman *et al.* (2001) that *hrp* genes could regulate processes in the bacteria that modify the levels of *hrp*-independent inducers. The higher protective ability of the *hrp*L mutant could then be explained by the rapid and sustained induction of defenses as indicated by the level of POX activity.

On the whole, our results suggest that *1*) the physical separation between cells of the virulent strain and cells of the regulatory *hrp*L mutant when co-inoculated, and *2*) the sustained induction of *hrp*L-independent plant defenses in tissues that are still reactive because they had not yet been invaded by the virulent strain, contribute together to the observed protection. Research on the basis of the physical separation as well as on the origin of the elicitation is currently in progress.

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