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Involvement of three pathogenicity factors of *Erwinia amylovora* in the oxidative stress associated with compatible interaction in pear

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Abstract ***Erwinia amylovora*, the causal agent of fire blight of *Maloideae*, induces in its susceptible host plants an oxidative burst as does an incompatible pathogen. In this paper we present evidence that the elicitation of this phenomenon is the result of the combined action of two Hrp effectors of the bacteria, HrpN and DspA. We also confirmed that desferrioxamine, the siderophore of *E. amylovora*, is necessary for the bacteria to tolerate high levels of hydrogen peroxide. Two other pathogenicity factors of the bacteria, the HrpW effector and the capsule, do not seem to play any role in the elicitation of the oxidative burst nor in the protection of the bacteria.**

Key words: Oxidative burst; Hrp effector; Desferrioxamine; Capsule; *Erwinia amylovora*; *Pyrus communis*

1. Introduction

Erwinia amylovora is the bacterium responsible for fire blight, a necrotic disease of *Maloideae* (apple, pear and some ornamental trees). Three main genetic determinants are involved in the pathogenicity of this bacterium. As other necrogenic bacteria, *E. amylovora* possesses *hrp* genes which are involved in the elicitation of the hypersensitive reaction (HR) in non-host plants and/or in the pathogenesis in susceptible host plants [1,2]. These genes, clustered within a 40 kb genomic region, encode three different types of proteins based on their functions: regulatory, secretory and secreted (for a review see [3]). Regulatory proteins control the expression of the other *hrp* genes, and secretory proteins are structural components of a type III secretion (TTS) apparatus delivering proteins outside the bacterial cell. Four secreted proteins have been characterized so far: (i) the major HR elicitor HrpN (harpin), also involved in pathogenicity [4,5]; (ii) an essential pathogenicity determinant, DspA [6], also designated DspE [7] for its homology with AvrE of *Pseudomonas syringae* pv. *tomato*; (iii) the protein HrpW [8,9], which shares structural similarities with HrpN of *E. amylovora* and PopA of *Ralstonia solanacearum* and is partly homologous to class III pectate lyases; and (iv) the HrpA pilus structural protein, which plays a key role in the secretion of Hrp proteins [10]. Whether the Hrp-secreted proteins (except HrpA) are injected into the plant cell or not remains to be established, as well as their biological functions. In addition to the TTS system and secreted protein effectors, the extracellular polysaccharides (EPS), which form a capsule around the bacterial cell, play a crucial role in pathogenicity by protecting the bacteria against host defense reactions (for a review see [11]). Finally, *E. amylovora* produces hydroxamate-type siderophores belonging to the class of desferrioxamines (DFOs), mainly DFO E [12,13]. These siderophores are involved in the virulence of the bacterium and their role in its protection in oxidative conditions has been proposed [14].

Oxidative burst, i.e. the massive production of reactive oxygen species (ROS) including $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$, is generally associated with incompatible plant/pathogen interactions (for a review see [15]). However, we recently showed that *E. amylovora* induces in its susceptible hosts such a stress response as well as related consequences (lipid peroxidation, electrolyte leakage, modulation in the antioxidant status) with intensity and kinetics similar to those induced by an incompatible bacterium [16]. This ability is linked to a functional TTS system in the bacterium, as a *hrp* secretory mutant does not induce such a plant response. These data suggest that (i) *E. amylovora* is first recognized as an incompatible pathogen by its susceptible host, (ii) this bacterium copes with – and even takes advantage of – the lethal action of ROS on plant cells for a successful pathogenesis, and (iii) Hrp effectors are responsible for the triggering of the oxidative burst.

In this paper, we investigated which of the Hrp effectors (amongst HrpN, DspA and HrpW) is responsible for the elicitation of the oxidative burst during the compatible interaction between *E. amylovora* and pear. In addition, the possible role of the capsule and of the siderophore in the protection of the bacteria in oxidative conditions was examined.

2. Materials and methods

2.1. Bacterial strains and construction

The relevant characteristics of strains used in this study are given in Table 1. To construct the M64 *hrpN–dspA* mutant (*Ea hrpN–dspA*), plasmid pSG1, containing a *uidA*–Km cassette inserted into the *Sma*I site of *dspA* [6], was electroporated into the *hrpN*::Mu*d*IIPR13 mutant PMV6112 (*Ea hrpN*). Marker exchange recombination was performed as previously described [6] and recombination event was checked by Southern analysis. A secretion test confirmed that *Ea hrpN–dspA* was unable to secrete HrpN and DspA, but remained able to secrete HrpA and HrpW (data not shown).

For the preparation of inocula, bacteria were subcultured at 26°C for 24 h on solid King's medium B [19] supplemented with appropri-

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Table 1
Strains used in this work

Designation[a] (abbreviation[b])	Relevant characteristics[c]	Reference or source
Erwinia amylovora		
CFBP1430 (*Ea wt*)	Wild-type strain, isolated from *Crataegus*	[17]
PMV6023 (*Ea hrp sec*)	*hrcV*::Mud*II*PR13, Cm^r	[5]
PMV6112 (*Ea hrpN*)	*hrpN*::Mud*II*PR13, Cm^r	[18]
M52 (*Ea dspA*)	*dspA*::*uidA-Km*, Km^r	[6]
M56 (*Ea hrpW*)	*hrpW*::Mud1734, Km^r	[8]
M64 (*Ea hrpN–dspA*)	*hrpN*::Mud*II*PR13,*dspA*::*uidA-Km*, Cm^r, Km^r	This work
PMV6089 (*Ea ams*)	*ams*::Mud*II*PR13, EPS$^-$, Cm^r	[18]
VD61 (*Ea dfo*)	*dfo-61*::Mud*II*PR13, Cm^r	[14]
Pseudomonas syringae pv. *tabaci*		
CFBP2106 (*Pst wt*)	Wild-type strain, isolated from *Nicotiana tabacum*	CFBP

[a]CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; PMV, Pathologie Moléculaire et Végétale, INRA–INAPG, Paris, France.
[b]Abbreviations used in this study.
[c]*hrc*, hypersensitive response and conserved (*hrp* secretion mutant); Cm^r, chloramphenicol resistance; Km^r, kanamycine resistance; *ams*, amylovoran synthase; EPS, exopolysaccharides; *dfo*, desferrioxamine.

ate antibiotics (20 μg/ml) for the transposon mutants. All experiments were carried out with inocula prepared in sterile distilled water to yield the concentration of 10^7 cells/ml.

2.2. Plant material and inoculation procedures

Experiments were performed on unrooted microcuttings of *Pyrus communis* cv. Passe Crassane chosen for its high susceptibility to fire blight. Plants were propagated in vitro as previously reported by [20], and grown in an environmentally controlled growth chamber at 22°C with a 14/10 h (light/dark) period. For each experiment, actively growing shoots were transferred onto a fresh medium 1 week before inoculation. Inoculations were performed either by wounding the youngest expanded leaf of each plant with a teeth-nosed dissecting forceps previously dipped into bacterial suspensions (wounding procedure [21]), or by vacuum-infiltration of the whole plants immersed in bacterial suspensions (infiltration procedure). After vacuum-infiltration, plants were blotted dry, and replaced onto their medium. Inoculated plants were incubated under constant light and temperature (22°C).

2.3. Assays for activities of antioxidative enzymes

Enzymatic extracts were obtained from whole microcuttings. Enzyme extractions and measurements of ascorbate peroxydase (AsPOX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2) and glutathione-*S*-transferase (GST; EC 2.5.1.18) activities and of protein contents were assayed as previously described [16]. Catalase activity (KAT; EC 1.11.1.6) was assayed according to Aebi [22]. In each experiment, three replicates of two plants per treatment and per time point were homogenized, and experiments were repeated at least three times.

2.4. In vitro bacterial growth in presence of H_2O_2

Minimal inhibitory concentration (MIC) of H_2O_2 on bacterial growth was determined in liquid M9 minimal medium [23] supplemented with galactose (0.2%) (w/v) and nicotinic acid (0.01%) (w/v) (*hrp*-inducing medium [6]). Bacteria at initial concentration of 10^6 cells/ml were exposed to concentrations of H_2O_2 ranging from 0.062 to 2 mM. Bacterial growth was assessed automatically every 2 h by optical density measurements at 600 nm (Microbiology Analyser Bioscreen C, Labsystem). Each concentration of H_2O_2 was tested in triplicate and experiments were performed twice.

3. Results

3.1. Pathogenicity of strains

Pathogenicity of strains was tested on pear microcuttings inoculated either by wounding or by vacuum-infiltration in order to analyze (i) the ability of bacteria to invade the plants from the site of inoculation (wounding procedure) or (ii) the local reaction of plant cells challenged by bacteria (infiltration procedure).

With the wounding procedure, *E. amylovora* wild-type strain (*Ea wt*) induced a progressive necrosis from the inoculation site, becoming systemic within 10 days, while *P. syringae* pv. *tabaci* wild-type strain (*Pst wt*) did not induce any visible symptoms. Results obtained with single mutants were in accordance with previous works mainly performed on apple or pear seedlings [1,5,6,8,14,24]. (i) The *hrpW* mutant (*Ea hrpW*) showed similar virulence as *Ea wt*. (ii) The *hrp* secretion mutant (*Ea hrp sec*), the *dspA* mutant (*Ea dspA*), and the *ams* mutant (*Ea ams*) were non-pathogenic. (iii) Most plants inoculated with *Ea hrpN* showed but limited necrosis of the inoculated leaves; 25% of plants only showed progressive necrosis becoming systemic. (iv) The *dfo* mutant (*Ea dfo*) induced delayed systemic necrosis when compared to *Ea wt*. Finally, plants inoculated with the double mutant *Ea hrpN–dspA* remained symptomless.

With the infiltration procedure, the four strains *Ea wt*, *Ea hrpW*, *Ea ams* and *Pst wt* induced a complete necrosis of the plant within 36 h. *Ea hrp sec*, *Ea dspA* and *Ea hrpN–dspA* did not induce any symptoms. Plants infiltrated with *Ea hrpN* exhibited a delayed generalized necrosis when compared to *Ea wt* (60 vs. 36 h). On the contrary, *Ea dfo* induced a complete necrosis earlier than *Ea wt* (24 vs. 36 h).

3.2. Oxidative stress events during infection

Occurrence of an oxidative stress (i.e. activation of AsPOX, GR, KAT and GST) was analyzed in pear microcuttings inoculated by the various strains with the infiltration procedure. Results showed that *Ea wt* and the two mutants, *Ea hrpW* and *Ea ams*, activated the diverse antioxidative enzymes with kinetics similar to those induced by *Pst wt* (Fig. 1). Activation generally began 12 h after infiltration of strains and reached a maximum 24 h later. A decrease was observed thereafter which coincided with generalized necrosis of the whole plants. On the other hand, no significant increase of enzymatic activities was recorded after infiltration of *Ea hrp sec* or *Ea hrpN–dspA* when compared to the water control. *Ea hrpN* and *Ea dspA* were able to activate the four families of enzymes with delayed and progressive kinetics when compared to *Ea wt*. This residual ability was higher for *Ea hrpN* than for *Ea dspA*. As far as *Ea dfo* was concerned, the maximum of activation of the various families of enzymes was usually higher than with *Ea wt* during early time points (16 and 12 h), and activities rapidly decline after 18 h, coinciding with the early generalized necrosis of the microcuttings.

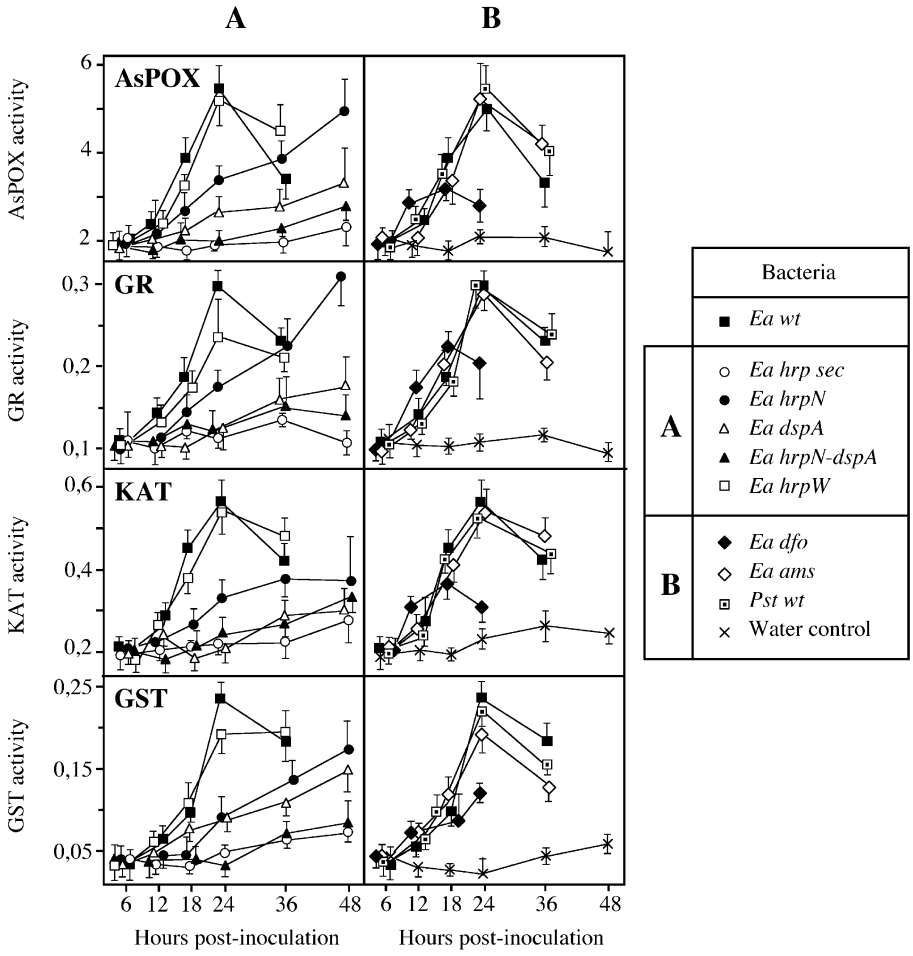

Fig. 1. Changes in the activities of AsPOX, GR, KAT and GST in pear microcuttings vacuum-infiltrated with *E. amylovora* (wild-type strain or pathogenicity mutants), *P. syringae* pv. *tabaci* (wild-type strain) or sterile water. Bacterial suspensions were adjusted at 10^7 cells/ml. Enzyme activities are expressed in μmol of ascorbic acid per mg of proteins and per min (APOX), in μmol of NADPH per mg of proteins and per min (GR), in mmol of H_2O_2 reduced per mg of proteins and per min (KAT) and in μmol of conjugate per mg of proteins and per min (GST). Data are means ± S.E.M. of at least nine repetitions from three independent experiments.

3.3. *In vitro* bacterial resistance to H_2O_2

Growth curves of bacteria exposed to increasing concentrations of H_2O_2 showed that *Ea wt* was much more resistant to H_2O_2 than *Pst wt* with a MIC of 2 mM for the former and 0.125 mM for the latter (Fig. 2). *Ea ams* (as well as the various *hrp* or *dsp* mutants, data not shown) behaved as *Ea wt*, whereas *Ea dfo* was significantly more sensitive to H_2O_2 with a MIC of 0.250 mM.

4. Discussion

The ability of the HrpN protein to elicit an oxidative burst has already been demonstrated in non-host plants of *E. amylovora* such as tobacco and soybean [25,26]. Here we show for the first time that HrpN and DspA both participate in the induction of an oxidative burst in host plants of *E. amylovora* during the compatible interaction. In addition, the unability of the *hrpN–dspA* double mutant to induce any plant response (similarly to a *hrp* secretion mutant) definitely established the preponderant involvement of these two Hrp effectors in the initiation of this phenomenon in compatible interaction.

It is not known whether HrpN and DspA trigger different targets in planta. It has been shown that in apple HrpN is released into the apoplast [27]. In *Arabidopsis thaliana* it regulates several ion channel activities probably after its insertion into the plasma membrane [28]. Less is known about DspA. Whether this protein is released into the apoplast or translocated into the plant cell as suggested for Avr proteins of *Pseudomonas* or *Xanthomonas* species [7] has not yet been determined. However, DspA seems to have a higher potential to elicit the oxidative stress than HrpN. The *dsp*A mutant (secreting HrpN) was generally more affected in its ability to activate antioxidative enzymes than the *hrp*N mutant (secreting DspA). This is in accordance with the pathogenicity of these mutants. The *dsp*A mutant does not produce any symptom, while the *hrp*N mutant is still able to cause some fire blight symptoms, although restricted (or delayed according to the inoculation procedure) when compared to the wild-type strain.

The survival of bacteria under increasing oxidative stress in vitro showed that *E. amylovora* tolerates more than 10 times higher concentrations of H_2O_2 than *P. syringae* pv. *tabaci*. This is in accordance with the fact that the former induces, tolerates and even takes advantage of an oxidative burst during compatible interactions, conversely to the latter, which does not induce a burst in its host plant [16].

E. amylovora possesses enzymes such as catalases and peroxidases which can directly break down H_2O_2 [29]. A protec-

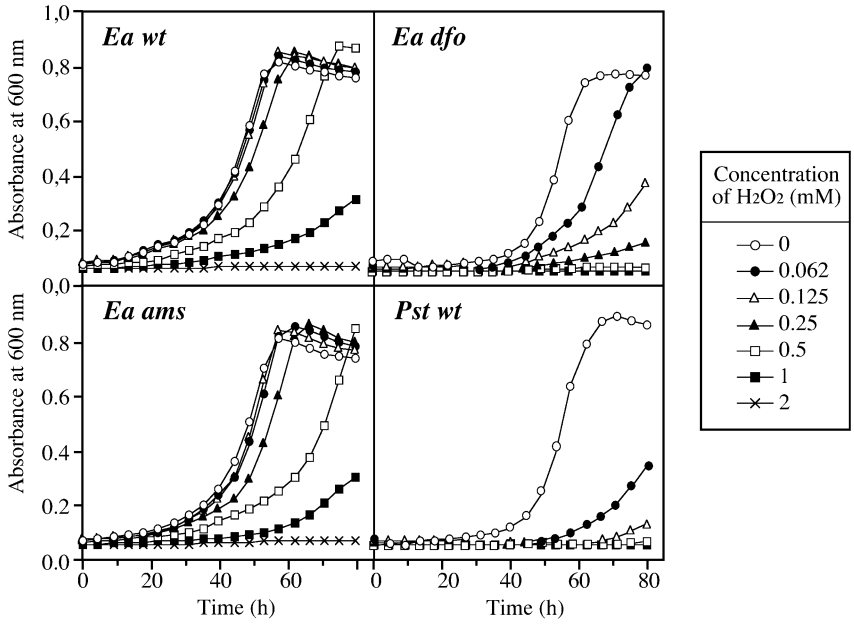

Fig. 2. Growth curves of *E. amylovora* (wild-type strain or pathogenicity mutants) and *P. syringae* pv. *tabaci* (wild-type strain) exposed to increasing concentrations of H_2O_2. Initial bacterial concentrations in liquid M9+ galactose were 10^6 cells/ml. Data, representative of a typical experiment, are means of three replicates.

tive role of the extracellular capsule of phytopathogenic bacteria against oxidative stress has also been proposed by Kiraly et al. [30]. This has not been evidenced in our experiments, at least against H_2O_2. However, capsule undoubtedly protects *E. amylovora* against some other plant defense mechanisms as the *ams* mutant behaves locally like the wild-type strain but is unable to invade the plant from the inoculation site.

On the other hand, a major protection of bacterial cells against oxidative conditions seems to be played by DFO E, the siderophore of *E. amylovora*. Absence of DFO E drastically reduces the ability of the bacterium to survive in vitro in an oxidative environment. Amongst hydroxamate-type siderophores, DFO B produced by various actinomycetes or bacteria is well-known to protect animal tissues against oxidative damage [31]. It prevents the generation of hydroxyl radicals via the Fenton reaction by sequestration of iron and also reacts directly with $O_2^{\bullet-}$ and H_2O_2 to generate nitroxide radicals. The same mode of action may be suggested for DFO E, as proposed by Dellagi et al. [14]. This hypothesis is consistent with our results obtained in planta. The swift kinetics of activation of antioxidative enzymes as well as the quick local necrosis observed in plants infiltrated with the *dfo* mutant suggest that the oxidative burst is more intense than with the wild-type strain. The lack of resistance of the *dfo* mutant to ROS could in turn explain its slower progression in planta when inoculated with the wounding procedure.

On the whole, the close link between the intensity of the oxidative burst and the severity of symptoms observed in pear after inoculation of *E. amylovora* suggests that the production of an optimal level of ROS is needed by this bacterium as a first step for an optimal multiplication in planta resulting in a successful pathogenesis. This level seems to be never or tardily reached when *E. amylovora* lacks DspA or HrpN, respectively, preventing a proper supply of nutrients to the bacteria. By contrast, this level appears overstepped, thus creating excessive oxidative conditions for a non-protected bacterium when *E. amylovora* lacks DFO E.

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