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ORIGINAL PAPER

M. Malnoy · J. S. Venisse · E. Chevreau

Expression of a bacterial effector, harpin N, causes increased resistance to fire blight in *Pyrus communis*

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Abstract The rapid and effective activation of disease resistance responses is essential for plant defense against pathogen attack. These responses are initiated when pathogen-derived molecules (elicitors) are recognized by the host. In order to create novel mechanisms for fire blight resistance in pear, we have generated transgenic pears expressing the elicitor harpin N_{ea} from *Erwinia amylovora* under the control of the constitutive promoter *CaMV35S*. The transient expression of *hrpN*$_{ea}$ in pear cells did not provoke any apparent damage. Therefore, stable constitutive expression of *hrpN*$_{ea}$ was studied in 17 transgenic clones of the very susceptible cultivar "Passe Crassane." Most transgenic clones displayed significant reduction of susceptibility to fire blight in vitro when inoculated by *E. amylovora*, which was positively correlated to their degree of expression of the transgene *hrpN*$_{ea}$. These results indicate that ectopic expression of a bacterial elicitor such as harpin N_{ea} is a promising way to improve pear resistance to fire blight.

Abbreviations cfu: Colony forming unit · *nptII*: Gene encoding neomycin phosphotransferase II · OH: Old Home · PC: Passe Crassane

M. Malnoy (✉)
Department of Plant Pathology,
University of Cornell,
NYSAES,
Geneva, NY 14456, USA
e-mail: mam262@cornell.edu
Tel.: +1-315-7872468
Fax: +1-315-7872389

J. S. Venisse
PIAF, Université Blaise Pascal-Campus des Cézeaux,
63177 Aubiere cedex, France

E. Chevreau
UMR GenHort (INRA/INH/UA),
42 rue Georges Morel,
49071 Beaucouzé cedex, France

Introduction

Fire blight is the major bacteriosis of *Maloideae* (pear, apple, and other members of the *Rosaceae*) caused by the necrogenic bacterium *Erwinia amylovora*. After entering the plant through natural openings or wounds, the bacteria spread quickly along the stems to the main branches, producing the characteristic symptom of this disease: a necrotic shoot blight which can kill a tree in one season [52]. In nonhost plants such as *Arabidopsis thaliana* or tobacco, *E. amylovora* elicits a hypersensitive reaction (HR) characterized by a rapid and localized collapse of tissues.

Transposon mutagenesis, along with *ams* genes required for synthesis of extracellular polysaccharides [7] and *dsp* genes required strictly for pathogenicity [9, 20], led to the identification of a gene cluster named *hrp* that is necessary for pathogenicity on host plants and HR elicitation on nonhost plant [4, 31]. This cluster encodes components of a type III secretion pathway [28] and regulatory and secreted proteins. Today, four proteins of *E. amylovora* are known to be secreted via the Hrp pathway: DspA/E, HrpW, HrpA, and harpin N_{Ea}. Harpin N_{Ea} was the first protein characterized by molecular analysis [57]. It is a glycine-rich protein of 44 kDa, lacking cysteine, heat stable, and able to induce an HR when purified and infiltrated into tobacco leaves. The gene encoding harpin N_{Ea}, named *hrpN*, is located on the bacterial chromosome. *E. amylovora hrpN* mutants, blocked in harpin N_{Ea} synthesis, retain a weak but significant ability to induce disease on host plants [3].

Although harpin N_{Ea} was discovered several years ago, its precise role during the colonization of host and nonhost plants and its site of action are still unclear. A general effect of harpin N_{Ea} after infiltration into nonhost plant is the expression of an HR [2, 22, 35]. In tobacco, Xie and Chen [58] observed a rapid inhibition of ATP synthesis following harpin treatment, suggesting that altered mitochondrial functions play an important part in harpin-induced HR. Defense response induced in *Arabidopsis* suspension cell by the harpin is associated with the accumulation of reactive oxygen species and, specifically, with altered mito-

chondrial functions such as mitochondrial ROS production, membrane depolarization, and cytochrome *c* release [33]. Production of active oxygen species [2, 15] and increase in cytosolic calcium [8] have also been reported. In cell suspension cultures from tobacco and *Arabidopsis*, harpin also induced membrane depolarization, extracellular alkalization, and potassium efflux, which have a pronounced effect on the plasmalemma, affecting H^+-ATPase, ion channels, membrane carriers, and the oxidative status [47, 48, 58]. Recently, modification of plasma membrane ion channels activities was observed in *Arabidopsis* suspension cells treated with harpin N_{Ea} [17].

Harpin N_{Ea} is also a known inducer of systemic acquired resistance (SAR) in plants. In *A. thaliana*, Dong et al. [16] demonstrated that harpin elicits resistance to *Peronospora parasitica* and *Pseudomonas syringae* pv. tomato, accompanied by induction of the SAR genes *PR-1* and *PR-2*. Harpin N_{Ea} acts through the *NPR1*/*NIM1*-mediated SAR signal transduction pathway in a salicylic acid-dependent fashion. The site of action of harpin in the SAR regulatory pathway is upstream of salicylic acid. The practical potential of harpin N_{Ea} as an elicitor of defense reaction has been tested on several plant species challenged with various disease agents [56].

Improvement of fire blight resistance in pear is currently performed through conventional breeding [36], but this method requires a long time and causes recombination in the genome, which hampers the selection of high-quality commercial cultivars. Genetic engineering, which preserves the genetic background of the cultivars, seems to be a promising strategy to introduce resistance against pathogens in pear. Most of the strategies currently tested to enhance pear resistance to fire blight through genetic engineering are based on transgenes integrating with a direct antibacterial activity such as attacin E [49], or acting against *E. amylovora* pathogenicity factors through exopolysaccharide destruction with a depolymerase gene [42], or competing for iron nutrition with a lactoferrin gene [41]. In this work, we have chosen to develop a wide-spectrum strategy based on the elicitation of plant defense.

Under attack by pathogens, plants have evolved a broad array of induced defense responses, including cell-wall reinforcement and de novo synthesis of antimicrobial phytoalexines [44] and of pathogenesis-related (PR) proteins [54]. This rapid induction of defense mechanisms requires that the plant recognizes the pathogens through the interaction of pathogen-derived molecules, called elicitors, with the corresponding putative plant-derived receptors [25]. Elicitors appear to trigger a common network of signaling pathways that coordinate the defense responses of plants. Genetic engineering of induced defense mechanisms through the introduction of an elicitor gene in the plant genome has already been tested to generate resistance to pathogenic microorganisms. One approach is to place an elicitor gene under the control of a strictly pathogen-inducible promoter, in order to create local and limited cell death at the point of infection. This has been successfully achieved to protect tobacco from the oomycete *Phytophtora cryptogea*, by expression of either an oomycete (elicitin, [30]) or bacterial (PopA, [6]) elicitor under the control of the *hsr203J* promoter. A similar strategy, based on the expression of the harpin N_{Ea} from *E. amylovora* under the pathogen-inducible promoter *gst1*, was tested on non-host plants of fire blight, and it decreased the infection of *A. thaliana* by the oomycete *Peronospora parasitica* [5] and of potato by the oomycete *Phythophthora infestans* [37]. Work is also in progress to study the effects of a constitutive or pathogen-inducible expression of harpin N_{Ea} in transgenic apple [1].

The constitutive expression of $hrpN_{Ea}$ gene in pear through genetic engineering could further increase knowledge on the role of harpin in compatible interactions. The present paper reports (1) the development of a transient expression system of harpin N_{Ea} in pear based on agroinfiltration, to assess the harmlessness of intracellular harpin accumulation and (2) the introduction and expression of a harpin N_{Ea} gene in several transgenic lines of pear.

Materials and methods

Plasmid construction and *Agrobacterium* strains

The binary expression vector pBinHrpN_{Ea} was constructed by M. Boccara (INA-PG, Paris) by cloning a $hrpN_{Ea}$ gene without signal sequence [4] into the pBin35S-mGFP4 plasmid [23] at the *Xba*I and *Sac*I sites (Fig. 1). The pBinHrpN_{Ea} binary vector was introduced by electroporation into the supervirulent *Agrobacterium tumefaciens* strain EHA105 [27].

The binary expression vector pFAJ3000 [14] contained an *ntp*II-based expression cassette as selectable marker and *uidA*-intron expression cassette obtained as a 3-kb *Hin*dIII fragment from p35S GUS INT [53] (Fig. 1). It was introduced by electroporation into the supervirulent *A. tumefaciens* strain EHA101 [26].

E. amylovora strains

The wild-type strain CFBP1430 and harpin-N_{Ea}-deficient mutant (PMV 6112: CFBP1430 *hrpN*16::Mu*d*IIPR13, $Path^{+/-}$, $HR^{+/-}$,CM^R; [3]) of *E. amylovora* were grown overnight at 26°C in King's medium B (KB) [32] containing chloramphenicol (20 μg ml^{-1}) for the mutant. Inocula were prepared in sterile distilled water and adjusted at concentration of 10^6 or 10^7 cfu ml^{-1}.

Plant material

The pear cultivar "Passe Crassane" (PC) was chosen for this study because of its high susceptibility to fire blight. The pear cultivar "Old Home" (OH) was used as a resistant control for fire blight resistance evaluation. All clones were propagated in vitro as previously reported [34].

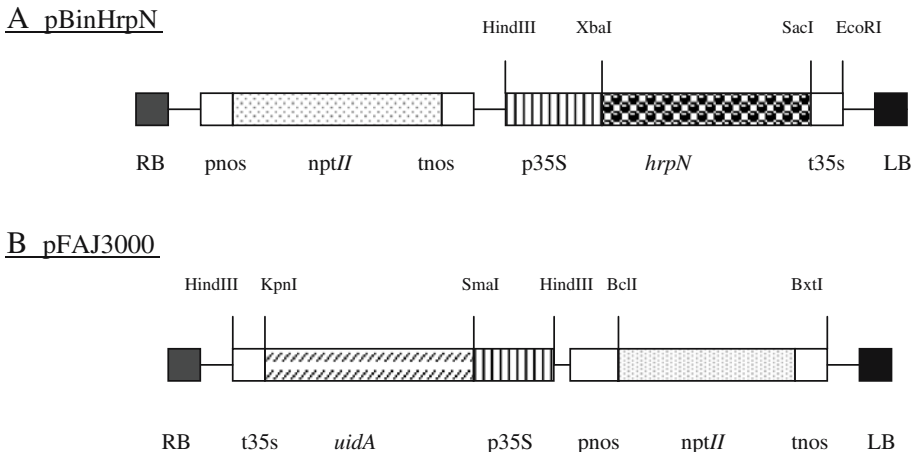

Fig. 1 Schematic diagram of the T-DNA region from the binary vectors pBinHrpN$_{Ea}$ (**a**) and pFAJ3000 (**b**). *RB* and *LB*, T-DNA right and left border sequences, respectively; *pnos* and *tnos*, nopaline synthase gene promoter and terminator, respectively; *p35S* and *t35S*, cauliflower mosaic virus 35S promoter and terminator, respectively; *nptII*, neomycin phosphotransferase II; *hrpN*, 2-kb sequence of *hrpN$_{Ea}$* cDNA; *uidA*, intron-containing β-glucuronidase gene

Transient gene expression and infiltration procedures

A. tumefaciens EHA101 pFAJ3000 or EHA105 pBinHrpN$_{Ea}$ were grown in KB, supplemented with antibiotics (kanamycin at 100 mg l^{-1}, rifampicin 50 mg l^{-1}), overnight at 26°C. Inocula were prepared in distilled water at concentration of 10^8 cfu ml^{-1}. The youngest leaves of in vitro pear microshoots were inoculated by vacuum infiltration in the inoculum for 5 min. During vacuum application, the leaves were gently swirled to avoid trapping of air bubbles. The vacuum was broken rapidly, and the leaves were briefly dried on sterile Whatmann paper. The treated leaves were placed with the adaxial side on the regeneration medium [43]. After 4 days in the dark at 24°C, the leaves were transferred to the same medium supplemented with cefotaxim (200 mg l^{-1}), timentin (100 mg l^{-1}), and kanamycin (100 mg l^{-1}). In preliminary experiments, an agroinfiltration technique was developed with an *uidA* marker gene in two pear genotypes (Conference and PC) by testing different inoculation techniques and concentrations of *A. tumefaciens* (data not shown). Depending on the genotype, the level of GUS expression reached a maximum 6 or 8 days after infiltration, and the percentage of leaf transformation varied from 60 to 90%.

Detached leaves from in vitro plants were vacuum infiltrated with bacterial suspensions (*E. amylovora* CFBP 1430 or PMV6112 at 10^7 cfu ml^{-1}), harpin N$_{Ea}$ (500 ng ml^{-1}), or water (control) as described by Faize et al. [18]. Harpin N$_{Ea}$ was produced from *E. coli* K38 pGp1-2 as described by Gaudriault et al. [21]. The protein concentration was measured according to the method of Bradford [11].

Immunodetection of harpin NEa protein

Harpin N$_{Ea}$ was used for rabbit subcutaneous immunization (three injections with 50 μg of protein each at intervals of 2 weeks). The antiserum was collected 6 days after the third injection.

Leaves agroinfiltrated with EHA105 pBinHrpN$_{Ea}$ or infiltrated with harpin N$_{Ea}$ were transferred onto a nitrocellulose membrane by immunoprinting (Hybond ECL, Amersham, UK). The membrane was incubated in PBS buffer (containing 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) added with 0.1% Tween 20 (v/v) and 10% powdered milk (w/v) for 1 h at 37°C, rinsed in PBS buffer three times for 5 min, and incubated in PBS buffer supplemented with the anti-harpin N$_{Ea}$ antibody diluted 1/1,000 for 2 h at 37°C. The membrane was then rinsed and incubated for 1 h at 37°C in PBS buffer containing the second antibody coupled with alkaline phosphatase enzyme (diluted 1/1,000). The membrane was placed in the staining solution [66 μl of Tetrazolium blue (75 mg ml^{-1}), 50 μl of 5-bromo 4-chloro indolylphosphatase (50 mg ml^{-1}) in 15 ml of staining buffer (containing 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 M Tris)]. The staining was stopped with HCl 0.2 N.

Stable transformation experiments and evidence of transformation

Plant transformation experiments were carried out as previously described [43] on 1,200 fully expanded leaves from in vitro growing shoots of PC, excised 2 weeks after subculture.

DNA isolation from leaves of in vitro shoots and polymerase chain reaction (PCR) experiments were carried out as previously described [43]. Specific primers (forward 5′-GCGCTGAACGATATGTTAGG-3′ and reverse 5′-AAG GAGCTGCAGACCAT-3′, annealing, 54°C) were used to amplify a 500-bp fragment of the harpin N$_{Ea}$ gene. Similarly specific primers (forward 5′-GAGGCTATTCGGC TATGACTG-3′ and reverse 5′-ATCGGGAGCGGCGA TACCGTA-3′, annealing, 60°C) were used to amplify a 700-bp fragment of the *nptII* gene.

Control of the ploidy level

Ploidy level of the transgenic lines and control was estimated by flow cytometry. Nuclei were isolated from in vitro leaves by manual chopping with a razor blade directly into the Brown et al. [13] buffer. After addition of 4, 6-diamino-2-phenylindole dihydrochloride (2% v/v) and filtration through a 20-μm nylon mesh, the mixture

was analyzed with a cytometer (Cell analyser II; Partec, Germany).

Semiquantitative reverse transcription PCR analysis

Reverse transcription (RT) was conducted as described by Promega (Madison, WI) with 1 μg of total RNA, extracted from 0.5 g of young leaves excised from in vitro shoot according to Malnoy et al. [39]. In order to evaluate relative differences in cDNAs between transgenic clones, comparative kinetic analysis was conducted by PCR using a procedure described by Malnoy et al. [40].

The intensity of signals was quantified by the software Gel Analyst-Clara Vision, and the ratio of $HrpN_{Ea}$ to EF1-α was calculated. This experiment was performed at least twice, and one representative result is presented. The results were similar between the two experiments.

Protein extraction and Western blot

Protein extraction was carried out from 200 mg of leaves from in vitro shoots for each transgenic clone according to Schuster and Davies [51], with the modification described by Reynoird et al. [49]. Protein extracts were quantified against a bovine serum albumine (BSA) standard using a colorimetric assay according to Bradford [11]. For Western analysis, 2.5-μg aliquots of protein extract from control and transgenic clones in Laemmli buffer were separated on 16% SDS-tricine polyacrylamide (w/v) gel according to the discontinuous procedure of Schägger and Von Jagow [50]. After electrophoresis, proteins were blotted onto Hybond C nitrocellulose membrane (Amersham) by passive transfer. Polyclonal rabbit anti-harpin N_{Ea} antiserum (raised with the help of Dr. Brisset) was used. Harpin N_{Ea} was detected with the enhanced chemiluminescence Western blotting detection system (ECL, Amersham) using horseradish peroxydase-labeled secondary antibody, according to the manufacturer's instruction.

Quantification of harpin accumulation by ELISA

Soluble proteins were extracted from 200 mg of leaves from in vitro shoots for each transgenic line according to Schuster and Davies [51], with the modification described by Reynoird et al. [49].

Enzyme-linked immunosorbent assay (ELISA) microtiter plates were coated with 100 μl of crude extract (10 μg of proteins applied in triplicate) and three dilution series of harpin N_{Ea} for 24 h at 4°C. The uncoated sites were blocked with 1% (w/v) BSA prepared in phosphate buffer (PBS) after washing three times with PBS containing 0.05% (v/v) Tween 20 (PBSt). Primary harpin N_{Ea} antibodies were diluted 1,000-fold in PBS, and 100 μl was applied to the wells. The plates were incubated for 2 h at 37°C. After three washes with PBSt, the plates were filled with 100 μl of secondary antibodies (goat anti-rabbit antibodies coupled to alkaline phosphate, Sigma) diluted 800-fold in PBSt. The plates were incubated for 1 h at 37°C, and alkaline phosphate activity was measured at 405 nm after 30 min of incubation at 37°C by using *p*-nitrophenylphosphate (50 μl) and stopping reaction with 5 N NaOH.

Determination of fire blight resistance

The level of resistance of transgenic clones was evaluated in vitro according to Brisset et al. [12]. Before inoculation, shoots were micropropagated on basal multiplication medium without kanamycin during at least three subcultures. Shoots (2–3 cm high) were subcultured in baby food jars (five per jar) 2 weeks before inoculation. For each clone, five jars were used and experiments were repeated three to four times. The youngest expanded leaf was wounded with teeth-nosed dissecting forceps dipped into the bacterial suspension. Inoculated shoots were incubated in the dark at 24±1°C, and symptom development was observed after 10 days. Disease severity was assessed by estimating the necrosis extent from the inoculated leaf and apex to the base using a scale of 0 to 3: 0=no necrosis; 1=necrotic apex; 2=less than half of the shoot necrosed; 3=more than half of the shoot necrosed. Shoots of nontransformed lines of PC and OH were used as susceptible and resistant controls, respectively.

Data were compared pair-wise between transgenic clones and nontransformed PC using the nonparametric *H* test of Kruskall and Wallis. Data analysis was carried out using the SAS/STAT 6.06 software, NPAR1WAY procedure.

Results

Transient gene expression of the harpin NEa in pear

When harpin N_{Ea} was infiltrated into host plant cells, no macroscopic necrotic reaction was observed [57]. Therefore, harpin in the apoplast of pear does not trigger deleterious effects. However, the effects of intracellular accumulation of harpin N_{Ea} in host plant cells are not described. Therefore, before attempting the stable transformation of pear with the harpin N_{Ea} gene, we performed transient gene expression to assess the harmlessness of intracellular harpin accumulation. Infiltration technique was therefore chosen as the transient expression technique, which allowed a regular expression and prevented necrosis.

We compared the transient expression of $hrpN_{Ea}$ and *uidA* in PC to distinguish the macroscopic symptoms due to the *Agrobacterium* infiltration from those due to the effect of the harpin N_{Ea} protein in the tissues.

Six days after infiltration with the *Agrobacterium* strains EHA105 pFAJ3000 or $pBinHrpN_{Ea}$ (Fig. 1) or with purified harpin N_{Ea} (0.5 mg ml^{-1}), no necrosis was detected in the leaves. After infiltration with the wild *E. amylovora* strain (CFBP1430) and the harpin-N_{Ea}-deficient mutant (PMV6112), necrosis appeared between 2 and 3 days. How-

ever, after 6 days, the mutant harpin N_{Ea} had induced less necrosis than the wild type. This result confirms that harpin N_{Ea} is not the only pathogenicity factor expressed by *E. amylovora*. Immunodetection revealed the presence of harpin N_{Ea} in leaves infiltrated with EHA105 pBinHrpN$_{Ea}$ or the purified harpin N_{Ea} and not in leaves infiltrated with EHA101 pFAJ3000 (Fig. 2). However, in leaves infiltrated with the harpin N_{Ea}, the amount of harpin N_{Ea} signal was lower than in leaves infiltrated with EHA105 pBinHrpN$_{Ea}$. This difference could be explained by a partial degradation of the harpin N_{Ea} in the apoplast 6 days after infiltration.

This experiment demonstrated that intracellular accumulation of harpin N_{Ea} did not cause visible deleterious effects on pear leaves. Therefore, stable transformation was attempted.

Transformation rates and ploidy level of transgenic clones

One transformation experiment with the binary vector pBinHrpN$_{Ea}$ harboring the harpin N_{Ea} gene driven by the 35S promoter was performed on 1,200 leaves from in vitro shoots of PC and produced 20 independent transgenic buds. This rate of transformation (1.6%) was similar to those previously reported with the pear cv. PC [41]. Presence of the *hrpN$_{Ea}$* gene was tested by PCR analysis on the 20 clones growing on kanamycin selective medium. All clones amplified the expected fragment from the *hrpN$_{Ea}$* gene. Ploidy level of all transgenic clones determined by flow cytometry proved to be diploid, like that of the nontransformed PC, with the exception of three tetraploid clones. Such a high frequency of chromosome doubling (about 10%) has already been observed among PC transgenic clones [38]. In total, 17 diploid transgenic clones were further analyzed.

Resistance to *E. amylovora*

In order to characterize precisely the behavior of the transgenic clones, 80 to 100 in vitro shoots per clone were inoculated with *E. amylovora* (CFBP 1430, at 10^7 and 10^6 cfu ml^{-1}) in three independent experiments. Trends were similar in the replicated experiments, as indicated by the low confidence intervals of the means (Fig. 3). Ten days after inoculation with an inoculum at 10^7 cfu ml^{-1}, 90% of nontransformed PC shoots showed necrosis, whereas fewer than 15% of OH shoots (resistant control) were infected with low severity (Fig. 3a). Most of the *uidA* clones showed no reduction of susceptibility; only three of the 13 clones showed intermediate levels of susceptibility ($p<0.01$) (data not shown). Instead, almost all the transgenic clones expressing the *hrpN$_{Ea}$* gene displayed intermediate levels of susceptibility to *E. amylovora*. Ten clones had a significant ($p<0.001$) reduction of fire blight symptoms in comparison to untransformed PC. When challenged with a lower inoculum concentration (10^6 cfu ml^{-1}), all the transgenic clones were less susceptible ($p<0.001$) to *E. amylovora* than the control PC (Fig. 3b), although they did not express the same level of resistance as the resistant genotype OH. Results from inoculations with the two different inoculum concentrations were highly positively correlated among the 17 transgenic clones ($r=0.89$, $p<0.01$). The two transgenic clones, 11Q and 11T, had the lowest level of susceptibility at each inoculum concentration.

Harpin N_{Ea} expression

To address the molecular basis for the range of disease susceptibility observed in the harpin N_{Ea} transgenic clones, harpin N_{Ea} mRNA and protein levels were determined from the transgenic clones in vitro and compared to nontransformed PC plants under the same conditions (Fig. 4). Transgenic clones displayed different levels of

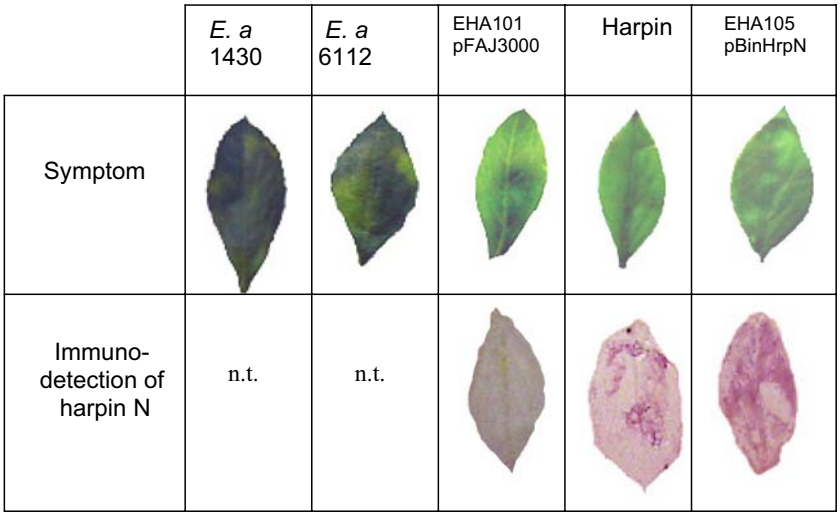

Fig. 2 Effect of infiltration of *Erwinia amylovora* (*E.a.*) 1430, *E.a.* 6112, *Agrobacterium tumefaciens* (*A.t.*) EHA101 pFAJ300, harpin N_{Ea}, and *A.t.* EHA105 pBinHrpN$_{Ea}$ in pear in vitro leaves, 6 days after inoculation. Bacterial suspensions were adjusted to 10^7 cfu ml^{-1} for *E. amylovora* and 10^8 cfu ml^{-1} for *Agrobacterium tumefaciens*. The *red staining* indicates the presence of harpin N_{Ea} after immunodetection. *NT* Not tested because of leaf necrosis

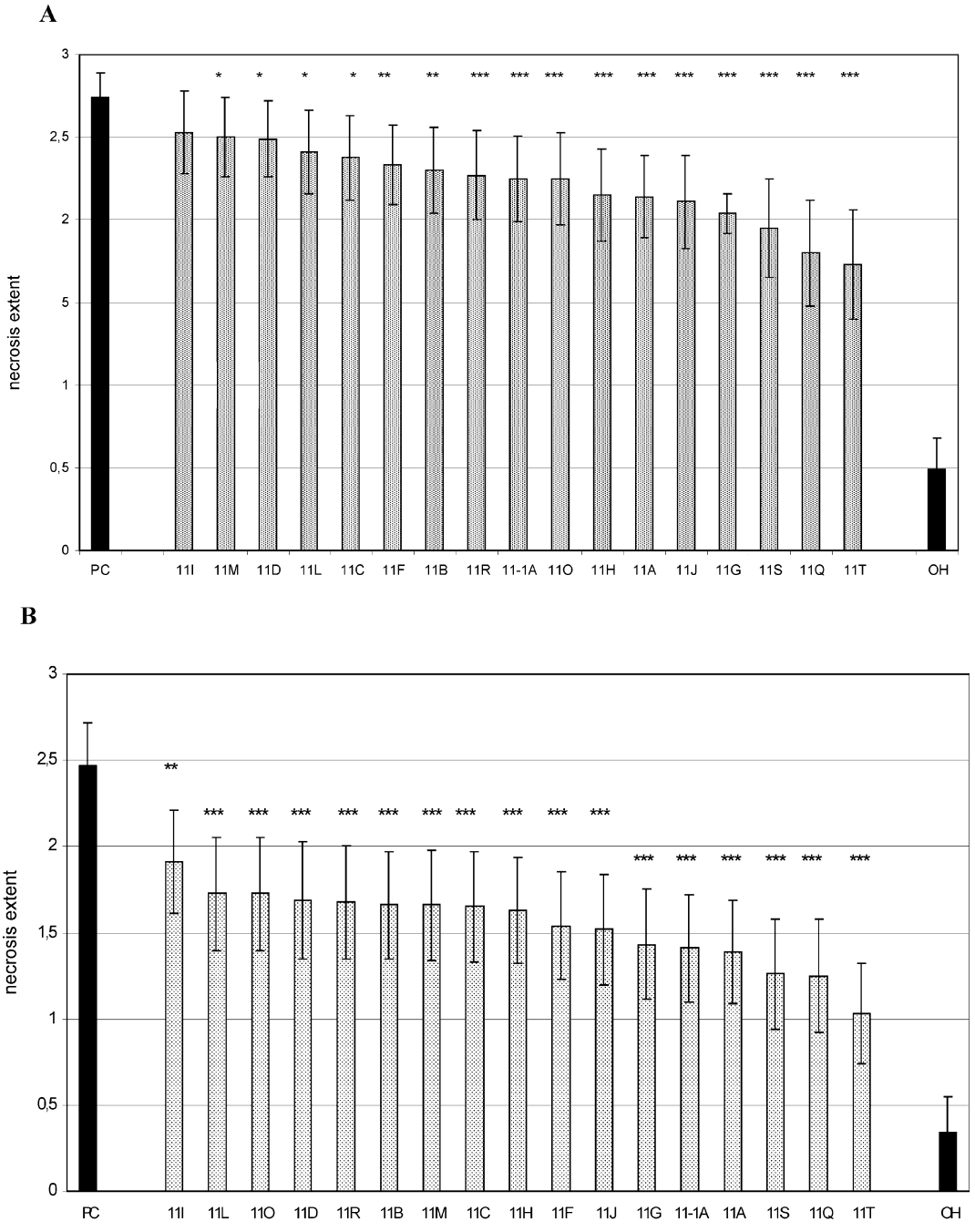

Fig. 3 Fire blight susceptibility of in vitro transgenic clones harpin N_{Ea} (11-1A to 11T) compared to untransformed PC and OH, inoculated with *Erwinia amylovora* (CFBP 1430) at 10^7 cfu ml^{-1} (**a**) or at 10^6 cfu ml^{-1} (**b**). Data correspond to the mean of 90 microshoots from 3–4 separate experiments. *Bars* represent the confidence interval at α=0.05. Transgenic clone susceptibility is significantly different from PC at *p<0.05, **p<0.01, and ***p<0.001 according to Kruskall and Wallis test

mRNA, as revealed by RT-PCR (Fig. 4a), while no *hrpN*$_{Ea}$ mRNA was detected in the nontransformed control. The accumulation of harpin N_{Ea} protein was examined by Western blot analysis with antiserum raised against harpin N_{Ea} (data not shown). One specific band of 45 kDa, matching with the molecular mass of the full-length harpin N_{Ea} protein, was detected in all transgenic clones, with various signal intensities. This 45-kDa signal was not observed in the extracts of the nontransformed control (data not shown).

Quantification by ELISA confirmed that the transgenic clones expressed the harpin N_{Ea} protein at different levels (1–8 ng harpin N_{Ea} μg^{-1} of soluble proteins) (Fig. 4b). The abundance of harpin N_{Ea} protein was positively correlated (r=0.84, p<0.01) with the abundance of *hrpN*$_{Ea}$ transcripts evaluated by the ratio of RT-PCR signal intensity between *hrpN*$_{Ea}$ and the control EF1-α (Fig. 4c). The three highest producers of harpin N_{Ea}, both at the transcriptional and translational levels, were the clones 11T, 11O, and 11Q.

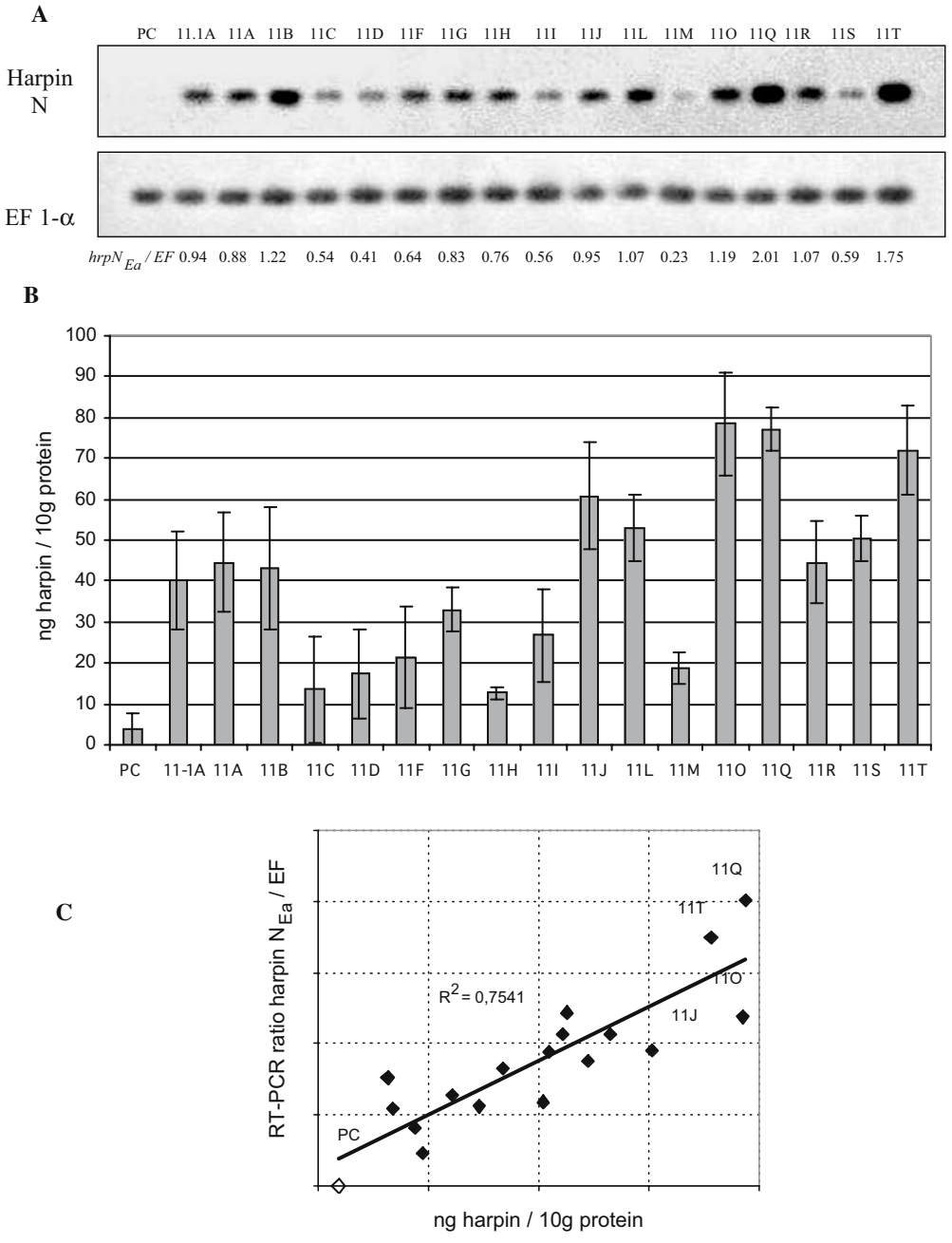

Fig. 4 Characterization of harpin N_{Ea} expression in in vitro leaves of transgenic clones (11-1A to 11T) and in untransformed clone (PC). **a** Comparative RT-PCR analysis of *hrpN*$_{Ea}$ and *EF 1-α* genes. Differences among transcription level of transgenic plants were estimated after 20 cycles. The EF 1-α was an internal control of transcript expression. **b** ELISA determination of harpin N_{Ea} content in 10 μg of crude extract. **c** Correlation between RT-PCR data and ELISA

Discussion

A rapid and effective activation of disease responses is essential for plant defense against pathogen attack. These responses are initiated when pathogen-derived molecules (elicitors) are recognized by the host. Harpin N_{Ea} is one of the key pathogenicity factors delivered outside of *E. amylovora* cell by a type-III secretion system. This protein induces disease-resistance-associated responses such as hypersensitive reaction and accumulation of PR proteins in nonhost plants. However, in host plants, the role of this protein is still unspecified. Recently, Venisse et al. [55] have shown the combined action of harpin N_{Ea} with DspA/E in the elicitation of the oxidative burst during the interaction between *E. amylovora* and pear.

The precise site of action of this protein in host plant has not yet been clearly demonstrated. Perino et al. [46] showed that harpin N_{Ea} is released into the apoplast of apple tissue after *E. amylovora* infection. Jin et al. [29] visualized harpin N_{Ea} secretion along the hrpA pilus. In this study, we demonstrated that harpin N_{Ea} intracellular production (through transient gene expression) or extracellular application (through infiltration of purified harpin N) does not induce any detectable necrosis in host plant, contrary to the effects observed in nonhost plants [19, 45, 57].

Stable integration of harpin N_{Ea} under the control of the constitutive *CaMV35S* promoter did not cause any detectable damage in the transgenic pear clones. These results confirmed preliminary data obtained in transgenic apple expressing this gene under the control of the inducible pro-

moter Gst1 [1]. In a nonhost plant, the expression of harpin N_{Ea} also led to normal growth in the case of potato [37], explained by a harpin N_{Ea} expression lower than the necessary threshold for necrosis. However, in *A. thaliana*, the harpin N_{Ea} expression under the control of *nos* promoter induced dwarfism and macroscopic necrosis in the plants [5]. Thus, we can hypothesize that there are different mechanism of action of transgenic harpin N_{Ea} expressed in host or nonhost plants, or that the mechanism of harpin N_{Ea} could be the same, but host and nonhost plant could differ in harpin N_{Ea} receptors.

Integration of harpin N_{Ea} in the pear genome significantly decreased the in vitro fire blight susceptibility in 10 of 17 clones. Compared to the nontransformed control, the reduction of symptoms reached 36 and 58% with inoculum concentration of 10^7 and 10^6 cfu ml^{-1}, respectively. The fact that the transgenic *uidA* control plants did not show a significant increase in resistance to fire blight compared to the harpin N_{Ea} clones provides the proof that the partial increase in resistance observed in these clones can be attributed to the expression of the harpin N_{Ea} gene in PC. This partial resistance may be attributed to the fact that *E. amylovora* requires other pathogenicity factors (DspE/A) to induce the disease. Expression analysis of harpin N_{Ea} at the transcriptional and translational levels showed a correlation among the relative quantities of mRNA, the amount of protein, and the reduction of symptoms in vitro. These results are encouraging because PC is one of the pear genotypes most susceptible to fire blight. Our results are similar to the preliminary results reported on transgenic apple rootstock 'M.26' expressing this gene under the control of the inducible promoter Gst1 [10].

Our results indicate that the harpin N_{Ea} gene induced partial fire blight resistance in transgenic pear clones. However, assessment of fire blight resistance in field trials will be necessary to confirm these results and evaluate the stability of transgene expression. Indeed, the permanent synthesis of the harpin N_{Ea} might change yield and important fruit quality traits. Furthermore, the constitutive overexpression of this gene which stimulates defense mechanisms might lead to a substantial accumulation of defense-related proteins, several of which are now considered to be potential food allergens [24]. In order to avoid the potential negative effect of this approach, a pathogen-inducible promoter, such as the str246C promoter [40], could be utilized. This genetic engineering approach of pathogen-induced harpin N_{Ea} production in transgenic plants is a powerful tool for generating a large spectrum of bacterial resistance.

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