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First evidence for improved resistance to fire blight in transgenic pear expressing the *attacin E* gene from *Hyalophora cecropia*

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Abstract

Fire blight, caused by *Erwinia amylovora*, is the most important bacterial disease of pear (*Pyrus communis* L.). Attacin E, a lytic protein originating from *Hyalophora cecropia*, has been previously reported to be active against *E. amylovora* in transgenic apple. Integration of the *attacin E* gene under control of a derivative of the constitutive promoter *CaMV35S* was accomplished using a transformation protocol developed for several pear cultivars. The integration was checked by polymerase chain reaction (PCR) for 11 lines of the cultivar Passe Crassane. Expression was determined in in vitro plantlets. The differences in transcription levels among lines revealed by comparative reverse transcription PCR correlated very well with the differences in attacin E accumulation observed by Western blot analysis. Fire blight inoculations were performed in vitro on all transgenic lines. A significant reduction of symptoms was observed for six lines, in comparison with the susceptible control Passe Crassane.

Keywords: Pear; Fire blight; Resistance; Genetic transformation; Attacin

1. Introduction

Fire blight is a major disease of the European pear (*Pyrus communis* L.) and other members of the *Rosaceae* family, caused by the necrotic bacterium *Erwinia amylovora*. It is responsible for serious production losses in Europe and North America. Conventional breeding requires a long time and causes recombination in the genome, which hampers the selection of high-quality commercial cultivars. Genetic transformation, which preserves the genetic background of the transformed cultivar, offers an attractive alternative method for the introduction of genes conferring resistance into specific varieties of vegetatively propagated species. An efficient regeneration system for pear is available [1] and transgenic plants have recently been obtained from pear cultivars [2].

Many non-plant lytic proteins with antibacterial effects have been identified [3]. Among these, peptides secreted into the haemolymph of pupae of the Cecropia moth (*Hyalophora cecropia*) in response to bacterial infection have been extensively studied [4]. Besides the cecropins, a family of small basic proteins [5], and lysozymes, a group of ubiquitous enzymes [6], the attacins represent the largest antibacterial proteins known in haemolymph (MW ≅ 20 kDa). Six attacins were isolated [7] and displayed antibacterial activity directed against the outer membrane of *Escherichia coli*, affecting permeability [8]. Synthesis of the outer membrane proteins has also been reported to be inhibited [9,10]. A possible synergy

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between attacin, cecropin and lysozyme has been suggested [8] because of the complementary mode of action of these three proteins against bacterial membranes. Minimum inhibitory concentration (MIC) of attacin E against different strains of *E. coli* was 8 μM [8], whereas only 1 μM or less of cecropins was lethal [4]. While MICs of cecropins have been determined against a variety of phytopathogenic bacteria [11], to our knowledge, no information is available about the activity of attacin E against plant pathogens.

Introduction of antibacterial protein genes from insects to enhance plant resistance was previously suggested by Jaynes et al. [12] and Casteels et al. [13]. In particular, expression of *cecropin B* gene and synthetic analogues has been widely analyzed. Although enhanced resistance to bacteria was reported in some cases [11,14], susceptibility was not modified in others [15,16]. Degradation of cecropins by intercellular fluids (IF) [16–18] could explain the lack of resistance. In comparison with cecropins, nothing is known about activity of attacins and their stability in planta or in IF. The first transgenic plants expressing the *attacin E* gene, driven by the potato proteinase inhibitor II [14] or by the *CaMV35S* promoter [19], were obtained from apple rootstock and displayed enhanced resistance to fire blight [20]. In this report, we describe for the first time the successful transformation of pear with the *attacin E* gene and its expression in transgenic lines.

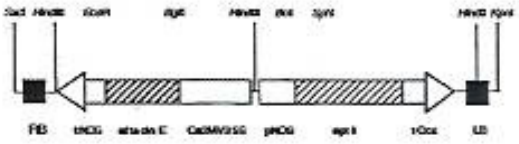

Fig. 1. Schematic representation of *attacin E* construct used in transformation experiments with pFM3002. The external region to the T-DNA borders is identical to that of pFAJ3000. Components are represented by boxes. RB and LB, right and left borders; *Ca2MV35S*, derivative of the cauliflower mosaic virus promoter containing a tandem duplication of 250 bp of upstream sequences; pNos and tNos, promoter and termination signal respectively of the nopaline synthase gene; *nptII*, coding region of the neomycinphosphotransferase gene (kanamycin resistance); tOcs, termination signal of the octopine synthase gene.

2. Materials and methods

2.1. Plant material

The Passe Crassane (PC) cultivar was chosen for transformation experiments because of its high susceptibility to fire blight. For the in vitro fire blight resistance test, the resistant cv. Old Home (OH) and non-transformed PC were used as controls. Shoots were propagated in vitro as previously reported [1].

2.2. Plasmid constructions

Two binary expression vectors, pFAJ3000 and pFM3002, were used: pFAJ3000 [21] contained an *npt*II-based expression cassette as selectable marker and a *uid*A-intron expression cassette obtained as a 3 kbp *Hin*dIII fragment from p35S GUS INT [22], adjacent to the right border; pFM3002 derived from pFAJ3000 by replacement of the *uidA*-intron cassette by an *attacin E* expression cassette excised from pLDB11[23] as a *Hin*dIII fragment, containing the *attacin E* gene [12] driven by a derivative of the cauliflower mosaic virus promoter (*Ca2MV35S*) containing a tandem duplication of 250 bp of upstream sequence [24] and the nopaline synthase transcriptional terminator sequence (Fig. 1). The binary vectors were transferred by electroporation to supervirulent *Agrobacterium tumefaciens* strain EHA 101 [25].

2.3. Transformation experiments and propagation of transgenic lines

Five hundred fully expanded leaves were excised from shoots 2 weeks after subculture and transformation experiments were carried out as previously reported [2], using *A. tumefaciens* strain EHA101 containing either pFM3002 or pFAJ3000. Transgenic shoots were subcultured on basal medium with kanamycin (100 mg/l) as selective agent, then rooted as described in [2].

2.4. Evidence of transformation

DNA isolation from leaves of in vitro shoots and polymerase chain reaction (PCR) experiments were carried out as previously described [2]. In order to check the presence of *attacin E* or *uidA*

gene, specific primers were designed: 5′-CTTACGCTCAACTCCGATG-3′ (forward) and 5′-AATCCGAAGTTAGGCTCCC-3′ (reverse) for amplification of a 530 bp fragment in *attacin E* gene, 5′-GGTGGGAAAGCGCGTTACAAG-3′ (forward) and 5′-GTTTACGCGTTGCTTCCGCCA-3′ (reverse) for amplification of a 1.2 kb fragment in *uidA* gene. Annealing was performed at 58°C.

2.5. Control of the ploidy level

Ploidy level in the transgenic lines and control was estimated by flow cytometry. Nuclei were isolated from leaves by manual chopping with a razor blade directly into Brown et al. buffer [26] with 2 % 4,6 diamino 2 phenyl indole dihydrochlorode (Cheminex), then filtered through a 4 μm nylon mesh and analysed with a cytometer (Cell analyzer II; Partec, Germany). Pea leaf nuclei were used as internal reference.

2.6. Determination of expression levels

2.6.1. RNA extraction, Northern analysis and semi-quantitative reverse transcription PCR analysis

Total RNA (50–100 μg) was extracted as described by Verwoerd et al. [27] from 0.5 g of young leaves excised from in vitro shoots. Purification was achieved by addition of 2-butoxyethanol in order to precipitate polysaccharides [28].

For Northern blot analysis, 10 μg of total RNA were denatured and separated by electrophoresis on a 1.2% agarose/formaldehyde gel in MOPS buffer, then transferred to Hybond™-N nylon membranes (Amersham, UK) as described by Sambrook et al. [29]. Filter was prehybridized for 2 h then hybridized overnight at 50°C in sodium dodecyl sulfate (SDS)-formamide buffer [30], with 50 ng of an *attacin E* DNA probe. *Attacin E* DNA was obtained by digestion of pFM3002 with *Hin*dIII, then ^{32}P-labelled with the 'Ready to go' kit (Pharmacia), purified on TE MIDI Select-D G-50 columns (5′-3′ Inc., USA). After hybridization, the filter was washed according to manufacturer's recommendations and finally exposed for 4 days on X-OMAT™ film with one intensifying screen at −80°C.

Reverse transcription (RT) was carried out from 1 μg total RNA according to Rosati et al. [31]. In order to evaluate relative differences in cDNAs between transgenic lines, comparative kinetic analysis was conducted by PCR as suggested by Horikoshi et al. [32]. Initial amounts of PCR substrates were adjusted for each line on the basis of an equivalent amplification of a cDNA from a member of the constitutively expressed gene family encoding the alpha subunit of translation elongation factor 1 (*EF1*-α) [33]. In order to evaluate differences among lines proportional to differences in initial amounts, we limited the amplification to 20 cycles. *EF1*-α PCR reactions were carried out on 2 μl reverse transcription products. Degenerated *EF1*-α primers (forward, 5′-ATTGTGGTCATTGGYCAYGT-3′; and reverse, 5′-CCAATCTTGTAVACATCCTG-3′), custom designed from multiple sequence alignments [31], were used to amplify a 702 bp fragment. In a second step, comparative amplification was carried out with *attacin E* primers on equivalent total cDNA amounts, adjusted in a volume of 2 μl, using the minimal number of cycles required to distinguish differences. The PCR reactions were run with products from Eurogentec (Seraing, Belgium) and 1 μM primers in a Minicycler (MJ Research, USA). The thermocycler program was: 94°C, 5 min, (20 cycles of: 94°C, 30 s; 58°C, 1 min; 72°C, 1 min); 72°C, 15 min for specific *attacin E* and *EF1*-α amplifications. After gel electrophoresis on 0.8% (w/v) agarose-ethidium bromide gel and detection by ultraviolet light fluorescence, amplified products were blotted onto a Hybond™-N nylon membrane following standard procedures of the manufacturer (Amersham, UK). The filter was prehybridized for 2 h at 42°C in SDS-formamide buffer [30], then hybridized overnight under the same conditions, with *attacin E* and constitutively expressed *EF1*-α probes prepared as already described. After hybridization and washing, membranes were exposed for 5–24 h to X-OMAT™ film with one intensifying screen at −80°C.

2.6.2. Protein extraction and Western analysis

Protein extraction was carried out from 100 mg of leaves excised from in vitro shoots for each transgenic line according to Schuster and Davies [34], with the following modifications: 1 mM phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases, was added to the extraction buffer (0.7 M sucrose, 0.5 M Tris, 50 mM EDTA, 0.1 M KCl, 2% (v/v) β-mercaptoethanol).

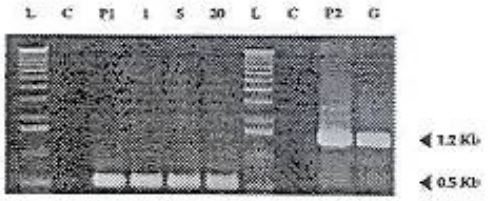

Fig. 2. PCR analysis of transgenic pear plants. Gel electrophoresis of *attacin E* and *uidA* gene PCR products; lane C, non-transformed plant; lanes P1 and P2, plasmids pFM3002 and pFAJ3000; lanes 1, 5 and 20, *attacin E* transgenic lines 1, 5 and 20; lane G, GUS transgenic line; lane L, molecular weight marker. Fragment sizes are indicated in kb.

Protein extracts were resuspended in 50 μl 0.1 M Tris–HCl (pH 6.8), 7 mM dithiothreitol, and quantified against a bovine serum albumin standard using a colorimetric assay according to Bradford [35]. For Western analysis, 10 μg aliquots of protein extract from control and transgenic lines in Laemmli buffer were separated on 16.5% SDS-tricine polyacrylamide gel according to the discontinuous procedure of Schägger and Von Jagow [36]. After electrophoresis, proteins were blotted onto Hybond C nitrocellulose membrane (Amersham, UK) by passive transfer. Polyclonal rabbit anti-attacin antiserum [7] was used and attacin E was detected with the enhanced chemoluminescence Western blotting detection system (ECL, Amersham, UK) using horseradish peroxidase-labelled secondary antibody, according to the manufacturer's instructions.

2.6.3. Determination of in vitro resistance

Before inoculation, shoots were micropropagated on basal multiplication medium [2] 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 line, five replicate jars were used and experiments were repeated three times. *E. amylovora* strain CFBP1430 was grown overnight on King's medium B [37] at 26°C and resuspended in water at a concentration of 5×10^7 colony forming units (CFU)/ml. The youngest expanded leaf was wounded with teeth-nosed dissecting forceps dipped into the bacterial suspension as already described [38]. 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 progression of necrosis from the inoculated leaf and apex to the base using a scale of 0–3: 0, no damage; 1, necrotic apex; 2, less than half of the shoot necrosed; 3, more than half of the shoot necrosed. For each line, susceptibility was calculated as the average of all replicates (about 75 replicates per line). Shoots of non-transformed lines of PC and OH were used as controls, and a transgenic PC line expressing the β-glucuronidase and the NPTII enzymes was also inoculated in order to estimate the effect of the expression of the transgenes that were assumed to lack antibacterial activity.

Resistance levels were compared pairwise between transgenic lines and non-transformed PC using the non-parametric H test of Kruskall and Wallis. Data analysis was carried out using the SAS/STAT 6.06 software, NPAR1WAY procedure.

3. Results

3.1. Transformation rates

Two transformation experiments with the binary vector pFM3002 harbouring the *attacin E* gene were performed on leaves from in vitro shoots and gave transformation rates of 1.0 and 1.3%, respectively, whereas a rate of 3.4% was obtained in transformation experiment with the binary vector pFAJ3000 harbouring the *uidA* gene. These rates of transformation were similar to those obtained with other *Rosaceae* woody plants [39–41] and one order of magnitude lower than those previously reported on pear cultivar Conference [2]. Transformation with the *attacin E* gene, as well as the *uidA* gene, was confirmed by PCR analysis for all the lines growing on kanamycin selective medium (Fig. 2). Eleven attacin E lines were subcultured on selective medium in the presence of 100 mg/l kanamycin without cefotaxime, and absence of residual *A. tumefaciens* was checked by PCR before acclimatization of the plants (data not shown).

3.2. Ploidy levels of transgenic lines

Ploidy levels were determined by flow cytometry. Leaves from in vitro shoots or from acclimatized plants were composed exclusively of 2C cells. All the transgenic lines including line 5, which had

strongly reduced growth in vitro and in the greenhouse, showed the 2C value of the non-transformed diploid PC.

3.3. Attacin E gene transcription

We analyzed the transcription of foreign DNA in the transgenic lines. Precipitation with 2-butoxyethanol eliminated most of the contaminants from RNA extracts, as confirmed by the high absorbance ratios A_{260}/A_{230} (data not shown). Preliminary Northern blot experiments were unsuccessful and we were not able to detect clearly the *attacin E* transcript (data not shown). Since levels of mRNA could be limiting, we used RT-PCR to compare the levels of transcription. After retrotranscription, the amounts of cDNA were firstly adjusted among samples based on equivalent amplification of an *EF1-α* fragment, then used as templates for *attacin E* PCR. Amplification products showed differences among transgenic lines after gel electrophoresis, blotting on nylon membrane and hybridization with a ^{32}P-labelled *attacin E* fragment (Fig. 3). No amplification occurred in the non-transformed control, whereas transgenic lines showed variable but always detectable expression. In particular, lines 9, 13, 5, 10 and 21 displayed high transcription levels. By contrast, lines 4, 20 and 22 displayed very low transcription.

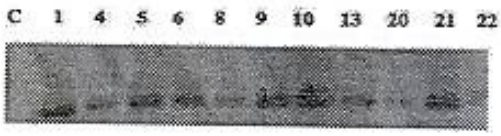

Fig. 3. Transcription of *attacin E* gene in leaves of *in vitro* shoots from control (lane C) and the transgenic lines (lanes 1–22) studied by comparative RT-PCR. Differences among transcription levels of transgenic clones were estimated after specific PCR (20 cycles) on equivalent total cDNA amounts. After blotting, nylon membranes were hybridized using as a probe the ^{32}P-labeled 1.6 kb *Hin*dIII fragment from pFM3002.

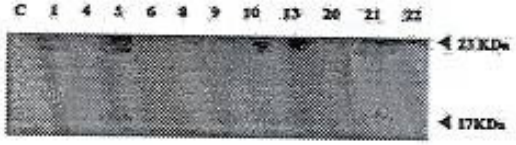

Fig. 4. Western blot analysis of control (lane C) and transgenic pear lines expressing the *attacin E* gene (lanes 1–22). The sizes of the full-length (23 kDa) attacin E protein and possible degradation product are indicated on the left.

3.4. Transgenic protein detection

Transgenic lines were assayed for attacin E. Proteins extracted from leaves of in vitro shoots amounted to 4 ‰ of the fresh weight. Western analysis detected expression in all the transgenic lines (Fig. 4). The non-transformed control showed no cross-reactivity with attacin E antibody. Another protein with a lower molecular weight (MW ≅ 17 kDa) was also detected ahead of the 23 kDa signal corresponding to attacin E. Since this protein was only present in the extracts from transgenic lines and proportional to the 23 kDa signal, it may have resulted from partial breakdown of attacin E or one of its precursors. Cross-hybridization with the putative breakdown product was not suppressed by adding or substituting another protease inhibitor (E-64, trans-epoxysuccinyl-L-[4-guanidino]butane) for PMSF (data not shown). Levels of amplified cDNA and protein amounts were in good agreement for all lines, except for line 9 which exhibited a moderate amount of attacin E. Since this line had a high level of transcription, its translation may have been reduced.

3.5. In vitro resistance tests against E. amylovora

Preliminary experiments with different concentrations of inoculum varying from 10^5 to 10^8 CFU/ml showed that 5×10^7 CFU/ml were necessary to obtain complete infection of the susceptible control PC after 10 days (data not shown). With these conditions, shoots exhibited first symptoms on veins and petioles after only 3 days. After 10 days, less than 10% of OH shoots (resistant control) were infected with low severity, whereas 90% of untransformed PC shoots showed maximum necrosis (Fig. 5). The transgenic control expressing the *uidA* gene showed the same symptoms as the susceptible control. Transgenic lines displayed intermediate levels of susceptibility (Fig. 6). Six lines were significantly ($P < 0.05$) more resistant than PC in rank comparison, whereas five lines showed little or no difference with the control. Trends were similar in the three sets of experiments, as indicated by the low standard errors. Data on in vitro disease susceptibility and attacin E levels

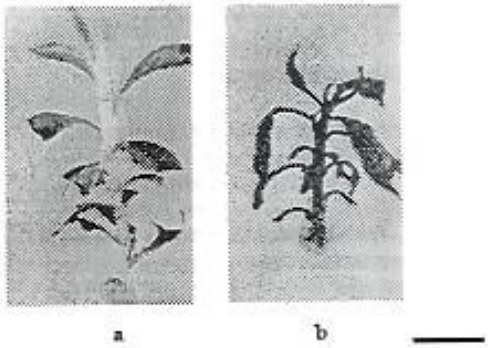

Fig. 5. Behaviour of resistant Old Home (a) and susceptible Passe Crassane (b) in vitro shoots 10 days after inoculation with *E. amylovora* CFPB 1430. Bar = 1 cm.

were in good agreement for most of the lines, especially for the resistant lines 5, 10, 13 and 21, and for the susceptible lines 4 and 20. However, the ranks according to disease susceptibility and attacin E levels were quite different in two cases. Line 22 exhibited an intermediate to high level of resistance despite a small amount of attacin, in agreement with a low transcription level. No increase of resistance was detected for line 9 despite a high level of transcription and a moderate level of attacin E.

4. Discussion

This study is the first report of successful transformation of pear with a gene encoding a lytic protein. The *attacin E* gene was introduced into the susceptible cultivar Passe Crassane. Eleven transgenic lines were obtained for which expression was analyzed in in vitro plantlets. The amounts of *attacin E* transcripts, attacin E and levels of resistance to *E. amylovora* were compared among the transgenic lines.

After retrotranscription of total RNA extracted from leaf samples, we used non-competitive PCR in order to compare the quantity of *attacin E* cDNA among RT products. This procedure relies on the well-established observation of linear relationship between the levels of cDNA before and after amplification prior to the onset of the plateau effect [42]. Amplification of *EF1-α*, an internal housekeeping gene uniformly expressed [43] as an internal standard, allowed adjustment of the amount of total cDNA among samples to yield comparable amplification products. We then amplified a fragment of *attacin E* in the samples. After 20 cycles, detection required blotting of the electrophoresis gel and labelling with a specific probe. By this procedure, we detected differences in transcription levels among the lines.

The expression of attacin E was detected by Western blotting. Leaves of all transgenic lines expressed detectable levels of the protein. The modified promoter *Ca2MV35S* was previously

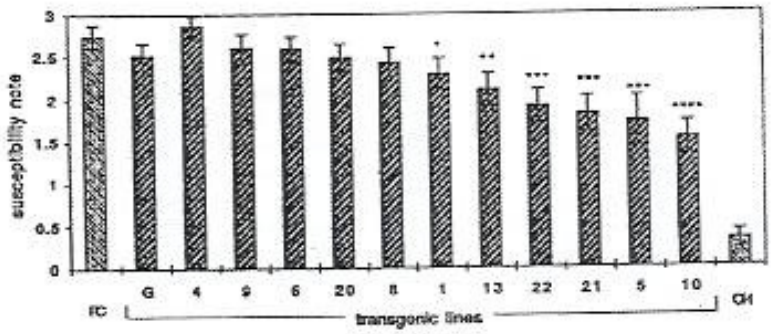

Fig. 6. Disease susceptibility of non-transformed PC and OH cv. and attacin E lines, 10 days after in vitro inoculation with *E. amylovora* CFPB 1430. A GUS transgenic line of PC (G) was also used as control. Each bar is the mean susceptibility score of three sets of experiments on 25 replicate shoots per clone. Lines on top of bars represent standard errors of the means. Susceptibility scores of transgenic plants are significantly different from those of control plants at * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$, according to the Kruskall–Wallis test.

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