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In vitro and *in vivo* quantification of elicitin expression in *Phytophthora cinnamomi*

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ABSTRACT

The differential expression of four *Phytophthora cinnamomi* elicitin genes was analysed by Real Time RT-PCR. In *in vitro* cultures, the α-cinnamomin gene showed the highest level of expression, the β-cinnamomin gene (*β-cin*) was the most inducible, and the *HAE* transcripts were in low abundance. Transcription of all the elicitins was active during the active growth of the pathogen when infecting cork oak (*Quercus suber*) roots, and as host colonization progressed, the level of *β-cin* expression fell, while that of *α-cin* rose. In an antisense transgenic strain, the silencing of *β-cin* also negatively affected the expression of other elicitin genes in the cluster. The reduced *in planta* growth of the *β-cin* knock-out is related to the altered pattern of elicitin gene expression, supporting the idea that one of the functions of elicitins is related, directly or indirectly, with pathogenesis.

1. Introduction

The class oomycetes includes obligate biotrophs (e.g. downy mildews, white rusts), narrow host range hemibiotrophs (e.g. *Phytophthora infestans*, *Phytophthora sojae*) and wide range host necrotrophs such as *Phytophthora cinnamomi* likely to infect in excess of 3000 species of plants, including *Quercus suber* and *Quercus ilex* subspecies *rotundifolia*, evergreen oaks of major economic and ecological importance in the Iberian Peninsula. *P. cinnamomi* can grow saprophytically in the soil, producing asexual, biflagellate motile zoospores that are attracted, usually to fine roots of susceptible plants where they attach and develop ramifying hyphae.

The elicitins, which belong to a group of conserved holoproteins, are thought to play a key role in the host/*Phytophthora* interaction. These small (typically 98 residue) proteins are abundantly secreted *in vitro* by almost all *Phytophthora* species, as well as by certain species of the related genus *Pythium*. Elicitin gene clusters have been identified in *Phytophthora cryptogea* [1], *P. cinnamomi* [2] (Fig. 1) and *Phytophthora cambivora* (unpublished results). The

structure of these gene clusters is rather conserved, and consists of four open reading frames arranged as two tandem pairs separated by ca. 2 kb. Two of the four genes share homology with the basic and acidic elicitin groups, but the other two encode elicitin isoforms sharing homology with the class II highly acidic elicitins. Elicitin and elicitin-like gene clusters with a different genomic arrangement have been observed in *P. infestans* [3], *P. sojae* and *Phytophthora ramorum* [4]. *P. cryptogea* expresses both the β- and α-cryptogein genes *in vitro*, but not the highly acidic elicitins [1]. Both β- and α-cinnamomins have been detected in *P. cinnamomi* culture filtrates [5], and an RT-PCR analysis of cultured mycelium revealed that all the elicitin genes belonging to the gene cluster were expressed [6].

Although some progress has been made towards an understanding of the function and mechanism of action of the elicitins, their overall role in the biology of *Phytophthora* remains puzzling (for a review see Ref. [7]). In addition to the canonical elicitins, some family members possess different structural domains which suggests a diversity of functions [1,2,4,8–11]. The canonical elicitins are able to load lipids (such as sterols) and effect their transfer between phospholipid membranes [12–17], and as a result, it has been suggested that in *Phytophthora*, they act as sensors to detect both the presence and/or abundance of potential hosts [7]. Antibiotic [18] and structural component [11,19] roles have also been proposed. In addition, some elicitin-like proteins were shown to display phospholipase A2 activity [20].

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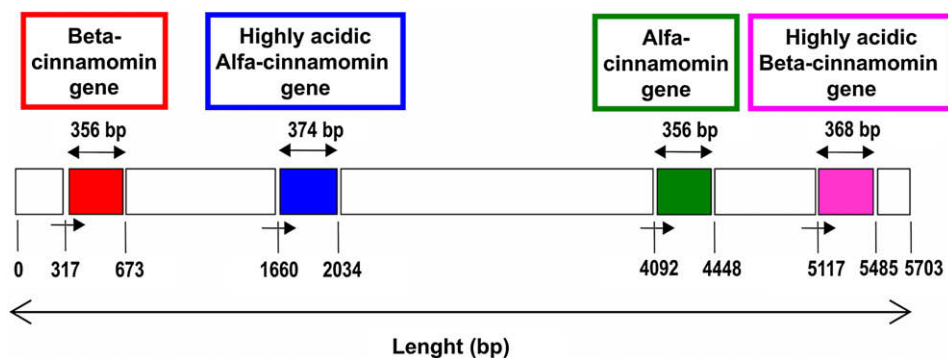*E-mail address:* mhorta@ualg.pt (M. Horta).

Fig. 1. Schematic representation of the elicitin gene cluster in *P. cinnamomi*. Arrows show direction of transcription.

The role of the elicitins in the pathogenicity of *Phytophthora* was until recently controversial, as it has been observed that they can act either as virulence or as avirulence factors. It has been proposed that they are non-specific toxins able to induce necrosis in any plant species [5,21], and act to facilitate pathogen colonization of the host by killing plant tissue via an attack on the membrane lipids [20]. Some authors, however, have shown that the host necrosis associated with the action of elicitin is restricted to certain plant species or even specific cultivars [19,22–25]. In tobacco, the role of the elicitins appears to be as an avirulence factor [26,27]. The elicitin genes are down-regulated during the early stages of pathogen infection involving *P. infestans*-potato [19] and *Phytophthora parasitica*-tobacco [28] compatible interactions, although expression of an elicitin gene in *P. parasitica* was retained throughout the compatible interaction with tomato [28]. Elicitin expression has also been observed during the infection process of *Phytophthora quercina* on *Quercus robur* [29]. This apparent diversity of behaviour in elicitin expression displaying dual and conflicting functions depending on the genotype of the host and other variables is better explained by the concept of effector, a neutral term that does not imply a negative or positive impact on the outcome of the disease interaction [30]. Viewed as effectors, elicitins are molecules that help the oomycete to manipulate biochemical and physiological processes in their host plants. In compatible interactions, effectors promote infection by suppressing defence responses, enhancing susceptibility, or inducing disease symptoms. In incompatible interactions effectors are recognized by the products of plant resistance genes, resulting in host cell death and effective defence responses.

The objective of the present work was to determine the expression level of elicitin genes in *P. cinnamomi*. For this purpose, we have exploited both *in vitro* cultures of the pathogen, and infected plant tissues. In addition, we have sought to evaluate the effect of inhibiting the expression of the β-cinnamomin gene (*β-cin*) on the infection process of *Q. suber*, a species severely affected by a decline disease caused by *P. cinnamomi* [31,32] and that has a major economic importance to the economies of south-western Iberia and north-western Africa.

2. Materials and methods

2.1. Biological material

P. cinnamomi isolates PA45 and PA37 were isolated from soil samples from sites affected by the cork oak decline disease on the Algarve region (southern Portugal), using a plant bait (*Granny Smith* apples in aqueous soil solution). The isolates were identified by their morphology when grown in PARPH selective medium [32] and by a colorimetric molecular assay [33]. They were grown in the dark at 25 °C in clarified V8 (Campbell Soup), either in semi-solid (agar) or liquid medium. For the production of elicitins, the cultures were transferred to ESM (Elicitin Secretion Medium) (0.05% w/v KH_2PO_4, 0.025% w/v $MgSO_4.7H_2O$, 0.1% w/v asparagine, 1 mg/l thiamine, 0.05% w/v yeast extract, 2% w/v glucose). These isolates are preserved at the Mycological Library of the Laboratory of Molecular Biotechnology and Phytopathology of the University of Algarve (UALG).

Q. suber acorns were surface sterilized, germinated in sterile vermiculite and grown in a greenhouse until their root length reached 5–6 cm.

2.2. Transformation of *P. cinnamomi*

The pHAMT35H construct (based on pUC19, and kindly provided by Howard Judelson) contains an expression cassette consisting of the hygromycin phosphotransferase gene (*hpt*) (present in a 1253 bp *Sma*I fragment) regulated by the *ham34* promoter (present in a 920 bp *Hind*III-*Sma*I fragment) and by the *ham34* terminator (present in a 550 bp *Sma*I-*Eco*RI fragment of *Bremia lactucae*) (EMBL Genebank accession number: X16984). The pHAMT35-fatss plasmid contains the *β-cin* coding sequence and a 5′ untranslated sequence in antisense orientation fused to the *ham34* promoter. This was obtained by PCR amplification of a 416 bp fragment of *β-cin*, using as primers 5′-AGC TAC CCG GGG CTC CGT CAC ACG CAC GAC ATC and 5′-AGA CTC CCG GGT TAG AGC GAC GCG CAC TTG GAC, digesting this fragment with *Sma*I and finally cloning it into *Sma*I-digested pHAMT35H. Correctly oriented clones were selected by restriction analysis with *Bgl*I. Plasmids were propagated in *Escherichia coli* (TOP 10 F′ Cells, Invitrogen) and plasmid DNA was extracted and purified with the Qiagen Plasmid Midi Kit (Qiagen), following the manufacturer's instructions.

The transformation of *P. cinnamomi* was based on published protocols [26,34–36]. Mycelium was grown for three days and protoplasts were prepared by gentle agitation at 25 °C for 2.5 h in the dark in a solution of 7 mg/ml Driselase (Sigma) and 7 mg/ml Cellulase (Onozuka) in 0.64 M KCl, 0.2 M CaCl₂. The addition of 80 μg Lipofectin (Life Technologies) mediated the co-transformation of 10^7 protoplasts with 20 μg pHAMT35H and 40 μg pHAMT35-fatss in MTC-20 buffer (1 M mannitol, 10 mM Tris–HCl, 20 mM $CaCl_2$; pH 7.5). Protoplasts were diluted $10\times$ in a mixture of clarified V8 liquid medium and 1 M mannitol solution (1:4 v/v). After 18 h at 25 °C in the dark, young mycelia were recovered by centrifugation and dispersed on V8 agar containing 250-μg/ml hygromycin. Transformants emerged within ten days.

2.3. Selection of transformants

Genomic DNA was extracted and purified from the mycelium with the Dneasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. The presence of pHAMT35H in hygromycin resistant clones was verified by a PCR using primers 5′-CGC CGA TGG TTT CTA CAA and 5′-CCA CGC TCC GTC AGG ATC TTC, which amplify an 839 bp fragment of the *hpt* coding sequence. Similarly, the presence of pHAMT35-fatss was verified using primers 5′-CTT TTG CGT CCT ACC ATC CGT TA and 5′-GGG GCT CCG TCACAC GCA CGC CAT C, which amplify a segment of the *ham34* promoter and all the *β-cin* antisense sequence (total length 754 bp). The PCR cycling conditions were 94 °C/3 min, followed by 30 × 94 °C/45 s; 65 °C/ 45 s, 72 °C/45 s, and ending with 72 °C/7 min. Each 50 μl PCR contained 10 mM dNTP, 0.4 μM of each primer, 100 ng genomic DNA or 1 ng plasmid DNA, and 2.5 U *Taq* DNA polymerase in the appropriate buffer. Aliquots of the PCR reactions were separated on 1% (w/v) agarose gels and stained with ethidium bromide, to check for the presence of the expected amplicon.

2.4. Analysis of elicitins production

ESM and V8 culture filtrates were harvested after five and 15 days of mycelial growth. Secreted proteins were concentrated by precipitation with trichloroacetic acid and separated by SDS-PAGE (17.5% PA). The proteins present in the gels were then electro-blotted onto a nitrocellulose membrane using a Trans-blot SD Transfer Cell (Biorad) and immuno-detected with monoclonal mouse antibodies recognising *β*-cinnamomin (kindly provided by Carlos Novo) as follows: the membranes were treated with PBS–T (phosphate buffered saline, pH 7.4 – Tween 20 (0.5% v/v) solution (Sigma) for 30 min, exposed for 16 h to a 1:1000 dilution in PBS–T of the primary antibody, washed 3× for 10 min in PBS–T, exposed for 2 h to a 1:6700 dilution in PBS–T of the secondary antibody (goat anti-mouse IgG) conjugated with alkaline phosphatase), once again washed 3× for 10 min in PBS–T, and finally immersed in BCIP/NBT Liquid Substrate System (Sigma) in the dark for 10 min. The stained membranes were washed in distilled water and dried, before being photographed.

2.5. Zoospore production and bioassay

Zoospores were prepared following the method described in Ref. [37].

Q. suber roots were covered in fully colonized V8 agar and incubated in the dark at 25 °C for 12, 24 and 36 h. Negative controls were provided by roots in contact with non-colonized agar. After the incubation period, the agar was removed, along with all external mycelia growth. The roots were examined for the presence

and extent of necrosis and then frozen to −80 °C. The assays were repeated three times with each pathogen strain.

2.6. Real time PCR assay

Total RNA was isolated from mycelia and root using the Rneasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. The integrity of the RNA was assessed by formaldehyde agarose gel electrophoresis (1.2% agarose). Residual DNA was removed by *DNase* I (Roche) treatment, following the manufacturer's instructions. Four μg of RNA were reverse transcribed using 400 U SuperScript II RNase H-Reverse Transcriptase (Invitrogen) in the presence of 4 μl random hexanucleotides (Roche), following the manufacturer's instructions. *Taqman* primers and probes (Table 1) for the quantification of the expression of elicitin genes were designed using Primer Express Software (Applied Biosystems) and were synthesized by Eurogentec. The fluorogenic hybridization probes were labelled with TAMRA (tetramethylcarboxyrodamin) attached to the 3′ end and FAM (6-carboxyfluorescein) covalently linked to the 5′ end of the oligonucleotide. Real Time PCR was performed using an iCycler iQ Detection System (Biorad). Each 25 μl reaction contained 50 ng RNA equivalent (unknown samples) or 0.005–500 pg plasmid DNA (calibration curves), as well as 125 μl qPCR Master Mix (Eurogentec: dNPTs, Hot Goldstar DNA polymerase, MgCl$_2$, Uracil-N-Glicosilase, stabilizers and passive reference), 300 nM of each primer and 200 nM *Taqman* probe. The reactions were run in triplicate and incubated at 50 °C for 2 min, at 95 °C for 10 min, followed by 45 × 95 °C/15 s, 62 °C/60 s.

The endogenous control used to normalize the amount of sample RNA was the *P. cinnamomi* actin gene. Amplification primers (given in Table 2) were targeted to the coding regions of the *P. infestans* [38] and *Phytophthora megasperma* [39] actin genes. Independent measures of the oomycete actin mRNA levels in *in vitro* and *in planta* conditions were made by Northern Blots using digoxigenin-labelled probes for actin and for LSU-rRNA as a loading control. Procedures followed the instructions supplied by the manufacturer ("DIG Application Manual for Filter Hybridization"; 2003, Roche Diagnostics Corporation).

Taqman primers and probe for the specific quantification of the expression of *P. cinnamomi* actin were designed as described below (Table 1). No *Q. suber* transcripts were detected during real time PCR assays using these primers and probe.

P. cinnamomi cDNA was generated by the reverse transcription of 5 μg total RNA with SuperScript II RNase H-Reverse Transcriptase (Invitrogen) primed with 1 μl of Oligo(dT)$_{12–18}$, and the actin amplicon derived from this template was cloned via the TA Cloning Kit (Invitrogen) and sequenced to define the necessary *Taqman* primers and probe (Table 1). Fragments of each elicitin gene containing the entire coding sequence, were similarly amplified from

Table 1
Sequences of the *Taqman* primer and probes.

Target mRNA amplicon size	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
	Taqman probe sequence (FAM-5′ →3′-TAMRA)	
Beta cin 67 bp	CAGACCGCCGCGTACAA	CCTTGGAGCACTGCGAGAAGG
	ACGCTCGTGAGTATCCTGTCCGAGTCG	
HAE Alfa cin 75 bp	GTTTGGTGATCAACGTGTACGA	ACGACGCCGAGGACGA
	CACGTCGACACAAAGTCGTTCGAG	
Alfa cin 67 bp	CGGCGCAGTACAAGCTCAT	AGCGTCACGATCTTCTTGATCA
	CGTCGACGGCGTGCAACACC	
HAE Beta cin 68 bp	GGCCCAGTACAAGCTCATGTG	TCAACGAGACGATCTCAGTGATC
	TCGACGGCGTGCAACACCA	
Actin 65 pb	CGTGGTGTGCTTACGCTGAA	TCCATGTCGTCCCAGTTGGT
	CCCCATCGAGCACGGCATGC	

Table 2
Sequences of primers used to clone actin and elicitin genes.

Target gene amplicon size	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
Actin 527 bp	GCCGGTGACGACGCCCCGCG	CCACGCTCCGTCAGGATCTTC
Beta Cin 490 bp	ACATCCACTCGCCACCTCC	TGCTCCTCCATCCTCAGTAA
Hae Alfa Cin 564 bp	CACCACTCAAGCCCCACTTCCG	GCTCGCCCTCATAACCAAGC
Alfa Cin 537 bp	GCACCCTTCCTCCCCACCACC	GCTTTACAAACGCTCGAGCG
Hae Beta Cin 523 bp	GCACAAACCTCTTCACCCACCC	CGGGTAAGTCTTTTGCAATG

the relevant cDNA (using the primers shown in Table 2), cloned using the TA Cloning Kit (Invitrogen) and sequenced. The resulting plasmids were linearized with *Not*I (New England Biolabs), and the optical density at 260 nm of various dilutions was measured for quantification (five replicated optical measurements per sample). These quantifications were used, along with an estimate for the molecular weight of single-stranded (ss) recombinant DNA (Vector NTI 8® program (Informax)), to create a set of calibration standards in the concentration range 10^9–10^1 ss DNA/μl.

Sample normalisation was effected using the relationship: (normalized number of elicitin transcripts) = [(number of actin transcripts produced by PA45 after 3 days of culture in V8 medium)/(number of actin transcripts in sample)] × number of the elicitin transcripts. The expression level of the *cin* genes was expressed relative to their level measured in 3-day-old cultures of PA45 in V8 medium (3d_PA45_V8).

3. Results

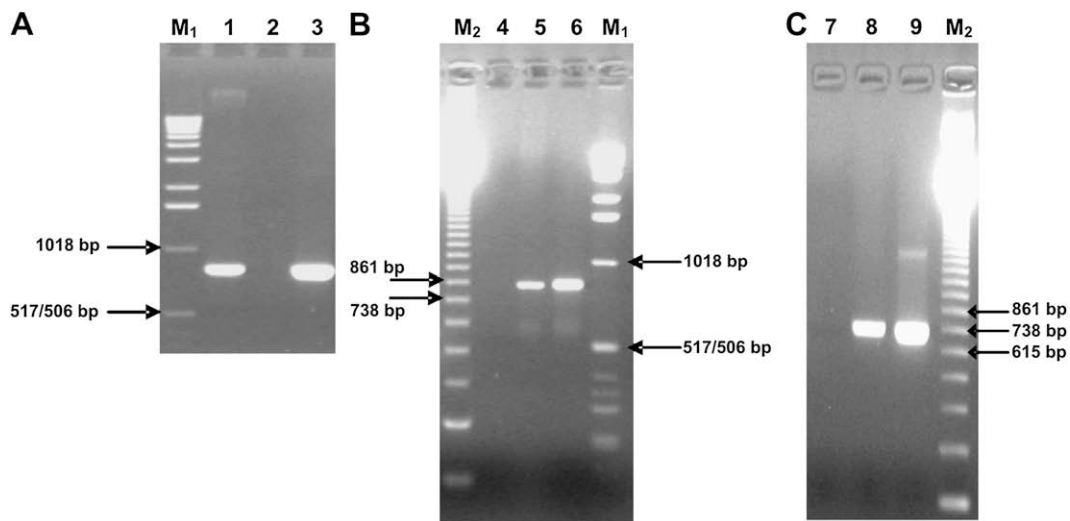### 3.1. Genetic transformation of P. cinnamomi

P. cinnamomi was particularly recalcitrant to transformation. Several transformants were obtained, but lost their ability to grow in the presence of hygromycin, except two which grew in a stable manner; one of these (FATSS) was derived from isolate PA45, and the other (13C) from isolate PA37. The FATSS strain carries both pHAMT35H and pHAMT35-fatss (Fig. 2A and C). The 13C strain only carries pHAMT35H (Fig. 2B). PCR amplification targeted to either *hpt* or *ham34+fatss* on genomic DNA extracted from the transformed strains after successive passaging on hygromycin-

containing and non-containing media showed that the transgenes were stably incorporated. When grown *in vitro* in the absence of antibiotic selection, no visible difference in growth rate between wild type and the transgenic strains was observed (data not shown).

The expression of the endogenous *β-cin* in response to the presence of the antisense transgenic sequence in the FATSS strain was firstly assessed in culture filtrates following growth on ESM and V8 liquid media. A western blot analysis revealed that no *β*-cinnamomin was produced by the FATSS strain (at least at levels detectable by the antibody hybridization assay) (Fig. 3), whereas, in contrast, it was produced by PA45, PA37 and strain 13C. Zoospores were not freely produced by either of the transgenic strains.

3.2. Infection of Q. suber roots

As transgenic zoospores were not available, infection of the host roots was effected using mycelium. The earliest necrotic lesions on the roots of PA45, PA37 and 13C appeared after about 12 h, in those areas in direct contact with the inoculum; at this time, the FATSS strain gave no visible symptoms of infection on the roots. By 24 h, the original lesions caused by the wild type isolates and the 13C transformant had extended along the root, while roots infected with the FATSS strain showed the first sign of necrosis, similar in size to those which had developed within 12 h on the wild type and 13C strains (Fig. 4). By 36 h post-inoculation, the root necrosis caused by the FATSS strain had spread, while in the roots infected with the other strains, the lesions remained of similar size, were localised in the non-suberized region of the root, and showed early

Fig. 2. Screening for the presence of transgenes in wild type and transformed strains of *P. cinnamomi* by separation of PCR products in agarose gel electrophoresis. (A) and (B) refer to detection of *hpt* (as shown by an 839 bp amplicon); (C) refers to detection of *ham34* + *fatss* (as shown by a 754 bp amplicon). Templates for the PCRs were: (1) and (8) FATSS genomic DNA, (2) and (7) PA45 (wild type) genomic DNA, (3) and (6) pHAMT35H plasmid, (4) PA37 (wild type) genomic DNA, (5) 13C genomic DNA, (9) pHAMT35H-*fatss*, plasmid M₁: 1 kb DNA ladder (Invitrogen), M₂: 123 bp DNA ladder (Invitrogen).
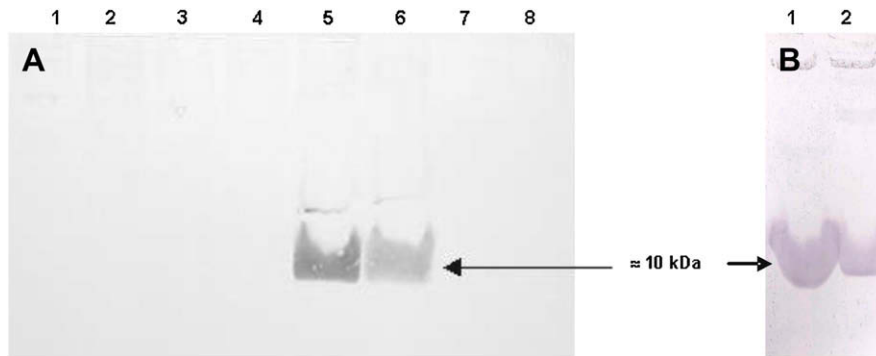
Fig. 3. Western blotting detection of β-cinnamomin (*arrowed*) in culture filtrates. (A) FATSS (tracks 1–4), PA45 (tracks 5–8). (1) 5-day ESM, (2) 15-day ESM, (3) 5-day V8, (4) 15-day V8, (5) 15-day ESM, (6) 5-day ESM, (7) 15-day V8, (8) 5-day V8. (B) (1) 15-day ESM culture filtrate of PA37, (2) 15-day ESM culture filtrate of 13C.

signs of tissue dehydration. For one set of roots, the infection was allowed to proceed for a further 12 h (48 h post-inoculation), by which time there was little visible difference between the appearance of roots infected with any of the strains, except that tissue dehydration was less advanced in the FATSS infected roots. Non-inoculated control roots displayed only a faint browning, an oxidation reaction caused by their exposure to air. The three independent assays produced comparable outcomes.

3.3. Quantification of elicitin transcripts

The choice of actin mRNA as a stable endogenous control to normalize the amount of sample RNA was validated by evaluation of the oomycete actin mRNA levels in *in vitro* and *in planta* conditions. Hybridization of Northern Blots at 60 °C confirmed the full specificity of DIG labelled probes towards *Phytophthora* mRNA. The signal obtained with the LSU probe was proportional to that
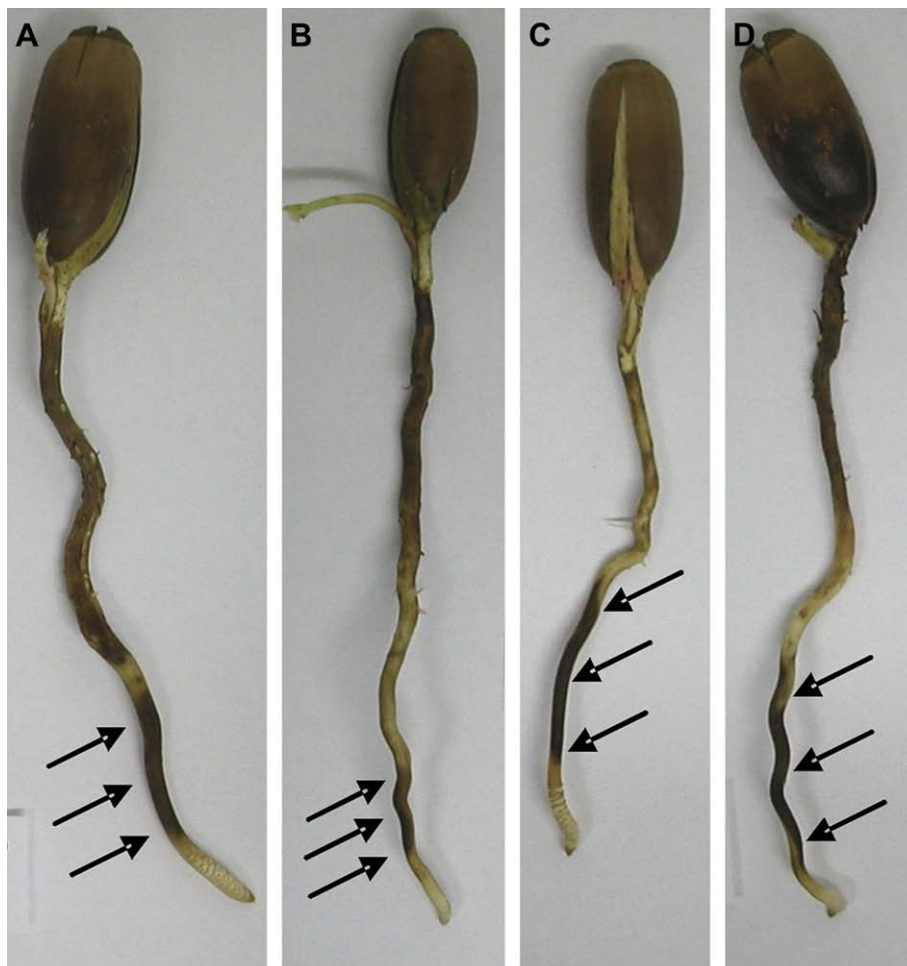

Fig. 4. Infected roots, 24 h post-inoculation. Cork oak roots were covered in fully colonized V8 agar and incubated in the dark at 25 °C. Necrotic tissue is indicated by an *arrow*. (A) PA45, (B) FATSS, (C) PA37, (D) 13C.

obtained with the actin probe in the same sample. In RNA extracted from various *in vitro* cultures, all LSU bands had identical intensity, showing that an identical quantity of *Phytophthora* RNA was present in all lanes; the intensity of actin mRNA bands was similar for all samples, showing that actin mRNA levels were also identical. In RNA extracted from infected roots, samples with increasing times of infection showed a corresponding increase of the RNA LSU band signal intensity. This was expected, as it reflects a rise in *Phytophthora* RNA quantities with the time course of root infection. The signal of actin mRNA followed a similar pattern, increasing proportionally to the LSU signal.
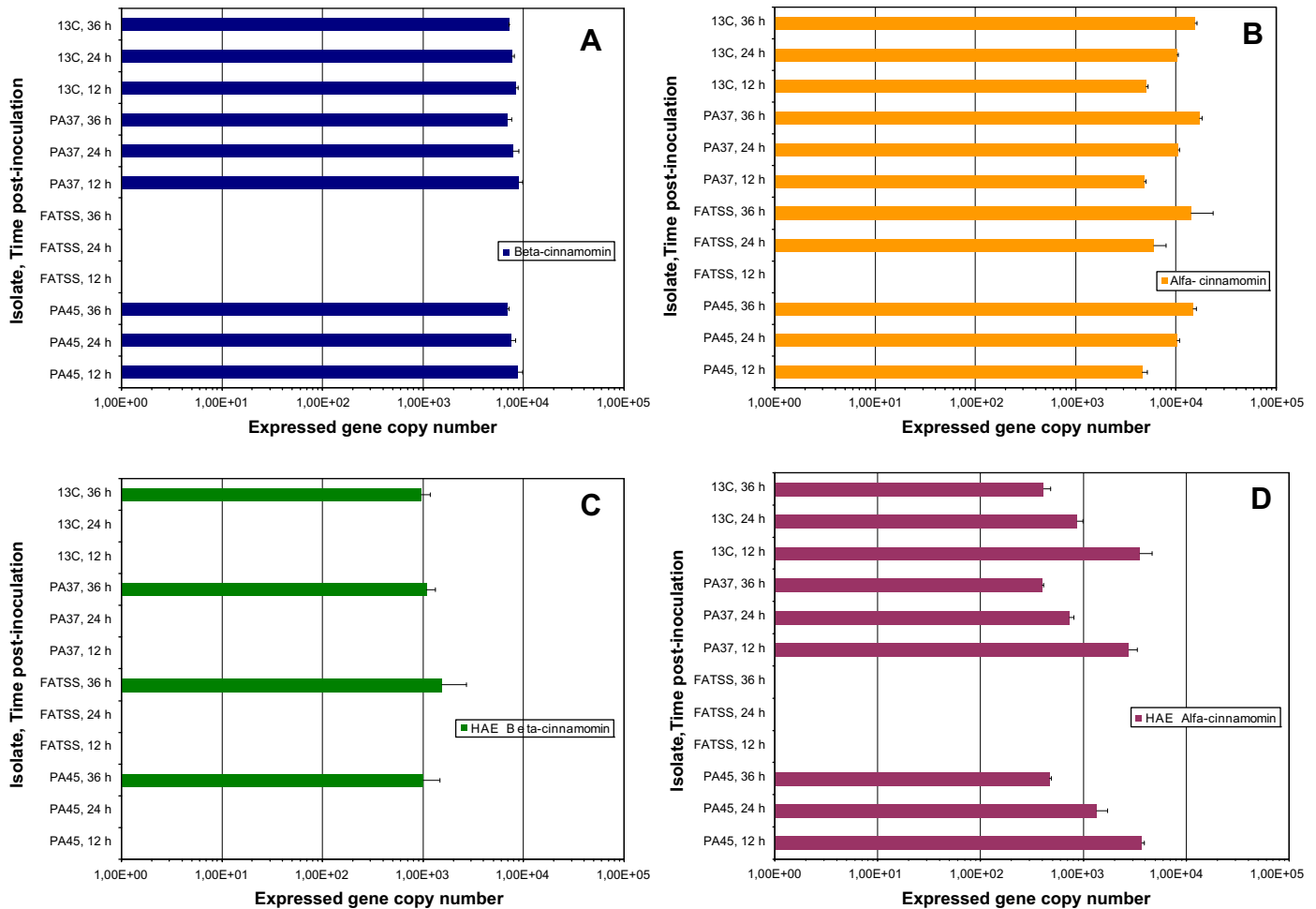
The quantification of elicitin gene expression is summarized in Figs. 5 and 6. The two wild type PA45 and PA37 isolates expressed the four elicitin genes present in the genomic cluster in both culture media. The general level of expression was substantially higher in ESM than in V8, and decreased in both media as the culture aged. In 3-day V8 cultures, α-cin was expressed about 15× more abundantly than β-cin, 23× more than HAEβ-cin and 142× more than the HAEα-cin; in 3-day ESM cultures, α-cin was expressed about 1.5× more abundantly than β-cin, 18× more than HAEβ-cin and 231× more than the HAEα-cin. The type of medium (V8 or ESM) had a large effect on the level of expression – for β-cin, this was 134 fold higher in ESM than in V8, while the equivalent induction for α-cin was only 14 fold, for HAEβ-cin 17 fold and for HAEα-cin eight fold. The 13C transformant showed a pattern of elicitin gene expression similar to that of its wild type progenitor, PA37. However, in contrast, the FATSS strain lacked any transcript of either β-cin and HAEα-cin. The number of α-cin and HAEβ-cin transcripts in 3-day V8 cultures of FATSS was, respectively, 1.8 and 1.6× less than 3d_PA45_V8. The extent of the induction of expression by the ESM medium was also less substantial, at only 2.8× (α-cin) and 2.5× (HAEβ-cin) 3d_PA45_V8.

In planta, the PA45, PA37 and 13C strains behaved identically – β-cin and HAEα-cin transcripts were detectable at the time of the first post-inoculation assay (12 h), and their expression level was, respectively, 154 and 496 fold more abundant than 3d_PA45_V8. The level of expression decreased with time (especially noticeable for HAEα-cin), as the root tissues became fully colonized. The expression of β-cin decreased by 11% (12–24 h) and 19.5% (12–36 h). HAEα-cin expression fell by 70.8% (12–24 h) and 86.5% (12–36 h). The α-cin transcript was readily detectable by 12 h post infection, and its expression increased with time, as the root tissue became fully colonized, but HAEβ-cin was only detectable when the root tissue was fully colonized. After 12 h, α-cin was expressed 6× more abundantly than 3d_PA45_V8; after 24 h the level had increased to 13× and by 36 h to 20×. The FATSS strain produced a quite different pattern of elicitin expression, since neither any β-cin nor any HAEα-cin transcripts were detectable. The α-cin transcript was not detectable 12 h post-inoculation, although pathogen biomass was present, as indicated by actin detection at this point (not shown). At
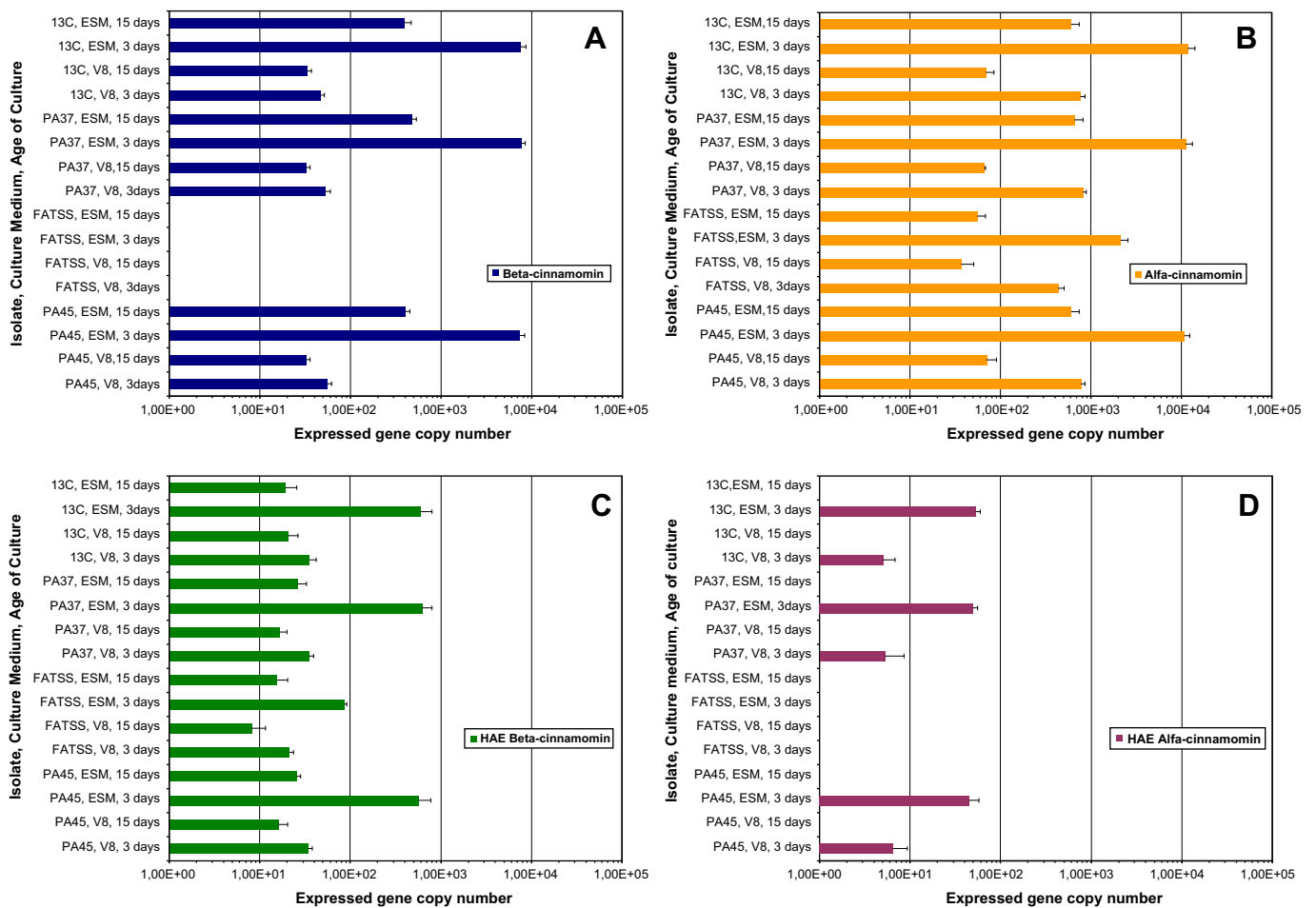
Fig. 5. Quantification of the genetic expression of elicitin genes *in vitro* by Quantitative real time PCR. RNA was extracted from mycelia of the wild type isolates (PA45 and PA37) and from genetically transformed strains (FATSS and 13C) grown in V8 or ESM media during 3 or 15 days. Mean values obtained over three independent assays are expressed as the number of gene copies present in 50 ng of *P. cinnamomi* cDNA. Gene copy number was calculated using the absolute standard curve method and normalized against actin gene expression in PA45 mycelium grown in V8 for three days. A: β-cinnamomin; B: α-cinnamomin; C: HAE β-cinnamomin; D: HAE α-cinnamomin.

Fig. 6. Quantification of the genetic expression of elicitin genes *in planta* by Quantitative real time PCR. RNA was extracted from cork oak root tissues after 12, 24 or 36 h post-inoculation with the wild type isolates (PA45 and PA37) and genetically transformed strains (FATSS and 13C). Mean values obtained over three independent assays are expressed as number of gene copies present in 50 ng of *P. cinnamomi* cDNA. Gene copy number was calculated using the absolute standard curve method and normalized against actin gene expression in PA45 mycelium grown in V8 for 3 days. A: β-cinnamomin; B: α-cinnamomin; C: HAE β-cinnamomin; D: HAE α-cinnamomin.

24 h was 8× more abundant than 3d_PA45_V8, and after 36 h expression had increased a further 2.25×. The *HAEβ-cin* transcript was only detectable in the most advanced stage of infection (36 h), when its expression level was 42× higher than 3d_PA45_V8.

4. Discussion

4.1. In vitro studies

Reference genes classically used as control in gene expression research have been subjected to apparently contradictory results. For example, Yan and Liou (2006) [40] showed that actin and elongation factors may not be suitable controls in *P. parasitica* studies and indicated the ubiquitin-conjugating enzyme encoding gene (*Ubc*) as one of the best. Moreover, it was shown that the *Ubc* gene can be a bad choice for *P. cinnamomi* and that the elongation factor 1α is more appropriate [41]. The more suitable standard may vary with the specific conditions of each study, therefore it is very important to test the stability of the standards according to the type of biological material and the particular conditions of a given experiment. In our experimental conditions, northern blots proved that actin is acceptable as constitutive gene control.

Both the P45 and P37 isolates expressed β-cin at a lower level than α-cin, which contrasts with the experience in *P. cryptogea*, where the expression of the basic elicitin gene dominated in ESM medium [1] and a greater amount of the *β-cryptogein* than the *α-cryptogein* was present in culture filtrates [42]. Note, however, that probes for the Northern analysis on *P. cryptogea* were based on 3′ UTR sequences, which present more variability than coding regions. Furthermore, a probe directed to the coding region of the basic cryptogein detected a number of genes in addition to the elicitin genes in the cluster [1]. As a result, the Northern signal is likely to have been restricted to transcript originating from the target genomic cluster. Rather than Northern analysis, we used a real time RT-PCR assay with primers and probes targeted to the four specific elicitin coding regions. These sequences are less locus-specific than are 3′ UTR ones, so the possibility cannot be excluded that a part of the signal derived from transcripts of gene copies located outside the cluster. However, data on quantification of canonical cinnamomins in culture filtrates is missing and as yet the correlation between transcript number and protein abundance has not been properly established.

An RT-PCR approach has been successfully used to detect highly acidic elicitin mRNAs in both *P. cinnamomi* [6] and *Phytophthora insolita* [7]. Our real time PCR experiments confirmed that *HAE* transcripts are present in *P. cinnamomi*, although their abundance is much lower than that of the canonical elicitins. This low expression level probably explains why *HAE* transcripts could not be detected

in *P. cryptogea* [1], since the sensitivity of the Northern blot is some orders of magnitude less than that of real time RT-PCR [43]. However, whether these class II elicitin transcripts are effectively translated *in vivo* has yet to be demonstrated.

The marked diminution in the expression of the elicitin genes in 15-day cultures suggests that the major demand for the elicitins occurs during the active growth phase of the mycelium. As cultures age and nutrient availability increasingly becomes limiting, active hyphal growth occurs only locally and chlamydospore formation is initiated. The large quantities of elicitin present in filtrates of aged ESM cultures therefore represent their accumulation during the stage of actively growing mycelium, rather than the product of active transcription and translation.

Abundant β-cinnamomin was present in ESM cultures of P45, PA37 and 13C strains, but was lacking in the FATSS transformant. Thus, the absence of β-cinnamomin in culture filtrates of this strain must have been effected by the presence of the antisense *β-cin* sequence, rather than by any transgene sequence(s) shared with strain 13C. The antisense-driven inhibition of *β-cin* gene in FATSS acted at the transcription level, in the same way that other genes have been silenced in various *Phytophthora* spp [26,44–47]. This form of genetic silencing requires a level of sequence homology between the transgene and the endogenous target of at least 80% [48]. The antisense sequence of *β-cin* is highly homologous with the other three members of the elicitin cluster. In addition to the total suppression of *β-cin*, in the FATSS transformant, no *HAEα-cin* (77% homology with *β-cin*) and a reduction in the abundance of *α-cin* and *HAEβ-cin* (83.5% and 81.2% homology, respectively) transcripts were observed. As the number of *HAEα-cin* transcripts is low in the wild type pathogen, its effective silencing is probably rather easily achieved. Alternatively, a failure in transcription resulting from transgenesis has been attributed to an induction of heterochromatin changes in the region of the silenced gene [48,49]. Of the four elicitin cluster members, *HAEα-cin* lies the closest to *β-cin*, and thus may be more susceptible than both *α-cin* and *HAEβ-cin* to disruption of transcription caused by localised chromatin alterations.

In the present study it is shown that the impaired capacity to produce zoospores is not related to cinnamomin production because the hygromycin expressing resistance 13C transformant produces cinnamomins while being similarly affected in zoosporogenesis as cinnamomin-silent FATSS strain. The fact that the transgenic strains did not freely produced zoospores was already reported [45] and was attributed to the expression of the resistance cassette.

4.2. In planta studies

The four elicitin genes known in *P. cinnamomi* presented different levels of expression, both *in vitro* and *in planta*. Moreover, the two pairs (*β-cin* and *HAEα-cin*; *α-cin* and *HAEβ-cin*) showed apparently concerted patterns of induction/repression. In spite of the synthesis of each elicitin being directed by its own promoter *HAE* gene expression appears to be regulated by the same transcription factors that control the expression of the canonical elicitin gene located upstream in the cluster. Thus, either the highly acidic elicitins are functional and act in concert with the canonical elicitins that are expressed at the same time, or the *HAE* transcripts are only the product of pseudogenes expressed solely under the influence of factors that act preferably on canonical elicitin genes, inducing the expression of the closest *HAE* gene. The *in planta* differential expression pattern of the elicitin genes shows that the basic and acidic elicitins have different, although possibly correlated biological roles, with the former involved in the initial invasion of the host, and the latter in propagation and survival after colonization. More generally, the differential expression of each

elicitin may reflect the response of the oomycete to its surrounding environment, adjusting the balance of elicitins present according to the prevailing pH conditions. In fact calculated pI varies from 3.38 for the most acidic one to 7.81 (the basic elicitin) and this difference could favour interactions with biological membranes and ease the load of lipid molecules by different elicitins under various pH conditions.

The infection process on *Q. suber* roots was characterised by an increase in the expression of *α-cin* over time up to 36 h post-inoculation, broadly consistent with the expression patterns of equivalent pathogen elicitors in other host/pathogen systems. In *Phytophthora citricola*, the α-citricolinin transcript was only detectable in the root of *Fagus sylvatica* some 6 h post-inoculation, and a significant rise in the level of expression occurred after three days and reached its maximum on the fifth day [50]. Similarly, α-infestin, produced by *P. infestans* was first detectable one day after the inoculation of tomato leaves, and its peak of expression occurred after four days [51]. Almost identical patterns of elicitin expression have been observed in potato [19] and tomato [28] infected with either *P. infestans* or *P. parasitica*. A more rapid host colonization was achieved in the present experiments, because the initial pathogen load was higher. As a result, the conditions necessary to induce *α-cin* expression were reached more rapidly. The peak expression of the α-elicitins is associated with the first appearance of host tissue necrosis, which represents the stage when sporulation begins. Thus the role of the α-elicitins may be connected with the process of sporulation and/or pathogen survival under saprophytic conditions; however, direct evidence for this role is lacking, since *P. infestans* not expressing α-infestin was unchanged with respect to both its virulence and sporulation phenotype [26].

It was shown that the invasion of *Q. robur* roots by *P. quercina* was characterised by the ubiquitous presence of β-quercinin, with its production increasing in concert with the increase in pathogen biomass over the first five days post infection [29]. The present observations concern the *β-cin* gene expression which does not have a direct relation with the totality of the pathogen biomass. The *in planta* expression of *β-cin* decreased during the infection process of *Q. suber* roots by wild type strains of *P. cinnamomi*. Probably, gene expression only occurs in the hyphae during first contact with the host, ceasing soon thereafter. This initial message is likely to be rapidly degraded but the translated elicitin will be retained. Thus, the transcripts detected reflect the early expression of the β-elicitin in hyphae present in the "battle front" with the host; its expression in the initial phase of tissue invasion indicates a role of the protein in pathogenesis.

When *P. infestans* was transformed with the gene encoding β-cryptogein, the quantity of heterologous protein was similar to that produced in *P. cryptogea* itself, and the level of endogenous α-infestin was reduced, leaving the total amount of elicitin present largely unchanged from that in a control transgenic strain expressing resistance to hygromycin B [52]. As a result, it has been suggested that, since the global amount of elicitin synthesized by a given strain is constant, there may well be a common role for the elicitins across all the *Phytophthora* spp. and, that the class IA and class IB elicitins are in effect interchangeable, with similar functions. However, the level of α-infestin did not fall below that present in the wild type, so there is most likely a residual specific function for α-infestin in *P. infestans*. Assuming that the quantity of canonical elicitin is conserved and that the biological roles of class IA and IB elicitin are identical, the prediction is that the FATSS transformant should have compensated for the absence of β-cinnamomin by an over-expression of *α-cin*. However, the experimental observation was that, on the contrary, the activity of *α-cin* was, if anything, partially down-regulated as silencing of *β-cin* also

affected α-*cin* expression. Thus, one (or both) of the prior assumptions above cannot be justified. Evidence that each elicitin group has its particular and distinct function has recently been provided by Jiang et al. (2006), who used a phylogenetic comparison to show that the elicitin family diversified prior to speciation, and that in various *Phytophthora* spp., variation is maintained by selective purification.

The lack of any growth rate difference between the wild type and the transgenic strains in the absence of antibiotic selection showed that the slower infectivity of the FATSS transformant was not due to any per se defect in growth, nor to the presence of the selective marker (*hpt*) gene, which was also present in the 13C strain. Thus we have concluded that the depression of virulence must be a direct consequence of the silencing of β-*cin* unless the FATSS strain suffered from other (unknown) genetic changes as a result of the transformation process (induced, for example by the random insertion of transformation vectors on the genome). This could best be resolved by the isolation of further transgenic lines of *P. cinnamomi*, in which β-*cin* has been silenced. It is however, a hard task at this stage, as this species is highly recalcitrant to transformation.

The FATSS transformant, with its altered patterns of elicitin expression, suffered a loss in virulence compared to its wild type progenitor. Its ability to degrade host roots appears, from preliminary evidence, to also be considerably impaired. We are presently investigating comparative expression profiling platforms such as cDNA-AFLP to reveal the genes related to the action of elicitins or responsible for the determination of virulence.

Acknowledgements

References

[1] Panabiéres F, Marais A, Le Berre J-Y, Penot I, Fournier D, Ricci P. Characterization of a gene cluster of *Phytophthora cryptogea* which codes for elicitins, proteins inducing a hypersensitive-like response in tobacco. Molecular Plant-Microbe Interactions 1995;8:996–1003.

[2] Duclos J, Fauconnier A, Coelho AC, Bollen A, Cravador A, Godfroid E. Identification of an elicitin gene cluster in *Phytophthora cinnamomi*. DNA Sequence. Journal of Sequencing and Mapping 1998;a;9:231–7.

[3] Jiang RHY, Dawe AL, Weide R, van Staveren M, Peters S, Nuss D, et al. Elicitin genes in *Phytophthora infestans* are clustered and interspersed with various transposon-like elements. Molecular Genetics and Genomics 2005;273:20–32.

[4] Jiang RHY, Tyler BM, Whisson SC, Hardham AR, Govers F. Ancient origin of elicitin gene clusters in *Phytophthora* genomes. Molecular Biology and Evolution 2006;23:338–51.

[5] Pernollet JC, Sallatin M, Sallé-Tourne M, Huet JC. Elicitin isoforms from seven *Phytophthora* species: comparison of their physico-chemical properties and toxicity to tobacco and other plant species. Physiological and Molecular Plant Pathology 1993;42:53–67.

[6] Duclos J, Trincão Aurélio M, Graça J, Coelho AC, Fauconnier A, Jacquet A, et al. Identification of an elicitin gene cluster in *Phytophthora cinnamomi* and analysis of the necrotic activity of a purified recombinant β-cinnamomin. In: Proceedings of the twelfth forum for applied biotechnology. Belgium: University of Gent; 1998. p. 1695–8.

[7] Ponchet M, Panabières F, Milat M-L, Mikes V, Montillet J-L, Suty L, et al. Are elicitins cryptograms in the plant – oomycete communications? Cellular and Molecular Life Sciences 1999;56:1020–47.

[8] Baillieul F, Fritig B, Kauffmann S. Occurrence among *Phytophthora* species of a glycoprotein eliciting a hypersensitive response in tobacco and its relationships with elicitins. Molecular Plant-Microbe Interactions 1996;9:214–6.

[9] Kamoun S, Lindqvist H, Govers F. A novel class of elicitin-like genes from *Phytophthora infestans*. Molecular Plant-Microbe Interactions 1997;10:1028–30.

[10] Panabières F, Amselemb J, Galiana E, Berre J. Gene identification in the oomycete pathogen *Phytophthora parasitica* during *in vitro* vegetative growth through expressed sequence tags. Fungal Genetics and Biology 2005;42:611–23.

[11] Qutob D, Huitema E, Gijzen M, Kamoun S. Variation in structure and activity among elicitins from *Phytophthora sojae*. Molecular Plant Pathology 2003;4:119–24.

[12] Boissy G, O'Donohue M, Gaudemer O, Perez V, Pernollet JC, Brunie S. The 2.1 Å structure of an elicitin-ergosterol complex: a recent addition to the sterol carrier protein family. Protein Science 1999;8:1191–9.

[13] Mikes V, Milat ML, Ponchet M, Ricci P, Blein JP. The fungal elicitor cryptogein is a sterol carrier protein. FEBS Letters 1997;416:190–2.

[14] Mikes V, Milat ML, Ponchet M, Panabières F, Ricci P, Blein J-P. Elicitins, proteinaceous elicitors of plant defense, are a new class of sterol carrier proteins. Biochemical and Biophysical Research Communications 1998;245:133–9.

[15] Osman H, Mikes V, Milat ML, Ponchet M, Marion D, Prange T, et al. Fatty acids bind to the fungal elicitor cryptogein and compete with sterols. FEBS Letters 2001;489:55–8.

[16] Rodrigues ML, Archer M, Martel P, Miranda S, Thomaz M, Enguita FJ, et al. Crystal structures of the free and sterol-bound forms of β-cinnamomin. Biochimica et Biophysica Acta 2006;1764:110–21.

[17] Vauthrin S, Mikes V, Milat ML, Ponchet M, Maume B, Osman H, et al. Elicitins trap and transfer sterols from micelles, lipossomes and plant plasma membranes. Biochimica et Biophysica Acta 1999;1419:335–42.

[18] Grant BR, Ebert D, Gayler KR. Elicitins: proteins in search of a role? Australasian Plant Pathology 1996;25:148–57.

[19] Kamoun S, van West P, de Jong AJ, de Groot KE, Vleeshouwers VGAA, Govers F. A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. Molecular Plant-Microbe Interactions 1997;10:13–20.

[20] Nespoulos C, Gaudemer O, Huet JC, Pernollet JC. Characterization of elicitin-like phospholipases isolated from *Phytophthora capsici* culture filtrate. FEBS Letters 1999;452:400–6.

[21] Huet J-C, Sallé-Tourne M, Pernollet J-C. Amino acid sequence and toxicity of the α-elicitin secreted with ubiquitin by *Phytophthora infestans*. Molecular Plant-Microbe Interactions 1994;7:302–4.

[22] Bonnet P, Bourdon E, Ponchet M, Blein JP, Ricci P. Acquired resistance triggered by elicitins in tobacco and other plants. European Journal of Plant Pathology 1996;102:181–92.

[23] Devergne JC, Fort MA, Bonnet P, Ricci P, Vergnet C, Delaunay T, et al. Immunodetection of elicitins from *Phytophthora* spp. using monoclonal antibodies. Plant Pathology 1994;43:885–96.

[24] Kamoun S, Young M, Glascock C, Tyler B. Extracellular protein elicitors from *Phytophthora*: host-specificity and induction of resistance to bacterial and fungal phytopathogens. Molecular Plant-Microbe Interactions 1993;6:15–25.

[25] Yu LM. Elicitins from *Phytophthora* and basic resistance in tobacco. Proceedings of the National Academy of Sciences of USA 1995;92:4088–94.

[26] Kamoun S, van West P, Vleeshouwers VG, de Groot KE, Govers F. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell 1998;10:1413–25.

[27] Kamoun S, Huitema E, Vleeshouwers VGAA. Resistance to oomycetes: a general role for the hypersensitive response? Trends in Plant Science 1999;4:196–200.

[28] Colas V, Conrod S, Venard P, Keller H, Ricci P, Panabières F. Elicitin genes expressed *in vitro* by certain tobacco isolates of *Phytophthora parasitica* are down regulated during compatible interactions. Molecular Plant-Microbe Interactions 2001;14:326–35.

[29] Brummer M, Arend M, Fromm J, Schlenzig A, Oßwald WF. Ultrastructural changes and immunocytochemical localization of the elicitin quercinin in *Quercus robur* L. roots infected with *Phytophthora quercina*. Physiological and Molecular Plant Pathology 2002;61:109–20.

[30] Kamoun S. Groovy times: filamentous pathogen effectors revealed. Current Opinion in Plant Biology 2007;10:358–65.

[31] Brasier CM, Robredo F, Ferraz JFP. Evidence for *Phytophthora cinnamomi* involvement in Iberian oak decline. Plant Pathology 1993;42:140–5.

[32] Moreira-Marcelino ACM. Aspectos da interacção entre *Phytophthora cinnamomi* e a doença do declínio em *Q. suber* e *Q. rotundifolia*. PhD Thesis, Universidade do Algarve, Faro, Portugal; 2001.

[33] Coelho AC, Cravador A, Bollen A, Ferraz JFP, Moreira AC, Fauconnier A, et al. Highly specific and sensitive non-radioactive identification of *Phytophthora cinnamomi*. Mycological Research 1997;101:1499–507.

[34] Bottin A, Larche L, Villalba F, Gaulin E, Esquerré-Tugayé M-T, Rickauer M. Green fluorescent protein (GFP) as gene expression reporter and vital marker for studying development and microbe-plant interaction in the tobacco pathogen *Phytophthora parasitica* var. *nicotianae*. FEMS Microbiology Letters 1999;176:51–6.

[35] Judelson HS, Tyler BM, Michelmore RW. Transformation of the oomycete pathogen *Phytophthora infestans*. Molecular Plant-Microbe Interactions 1991;4:602–7.

[36] Judelson HS, Coffey MD, Arredondo FR, Tyler BM. Transformation of the oomycete pathogen *Phytophthora megasperma* f. sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. Current Genetics 1993;23:211–8.

[37] Byrt P, Grant BR. Some conditions governing zoospore production in axenic cultures of *Phytophthora cinnamomi* Rands. Australian Journal of Botany 1979;27:103–15.

[38] Unkles SE, Moon RP, Hawkins AR, Duncan JM, Kinghorn JR. Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. Gene 1991;100:105–12.

[39] Dudler R. The single-copy actin gene of *Phytophthora megasperma* encodes a protein considerably diverged from any other known actin. Plant Molecular Biology 1990;14:415–22.

[40] Yan HZ, Liou RF. Selection of internal control genes for real time quantitative RT-PCR assay in the oomycete plant pathogen *Phytophthora parasitica*. Fungal Genetics and Biology 2006;43:430–8.

[41] King M. PhD Thesis, Murdoch University, Australia; 2007.

[42] Le Berre JY, Panabières F, Ponchet F, Bonnet P, Marais A, Ricci P. Occurrence of multiple forms of elicitins in *Phytophthora cryptogea*. Plant Physiology and Biochemistry 1994;32:251–8.

[43] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 2000;25:169–93.

[44] Blanco F, Judelson H. A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. Molecular Microbiology 2005;56:638–48.

[45] Gaulin E, Jauneau A, Villalba F, Rickauer M, Esquerré-Tugayé M-T, Bottin A. The CBEL glycoprotein of *Phytophthora parasitica* var. *nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates. Journal of Cell Science 2002;115:4565–75.

[46] Latijnhouwers M, Govers F. A *Phytophthora infestans* G-protein β subunit is involved in sporangium formation. Eukaryotic Cell 2003;2:971–7.

[47] Latijnhouwers M, Ligterink W, Vleeshouwers VG, van West P, Govers F. A G alpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. Molecular Microbiology 2004;51:925–36.

[48] Kamoun S. Molecular genetics of pathogenic oomycetes. Eukaryotic Cell 2003;2:191–9.

[49] Judelson HS, Tani S. Transgene-induced silencing of the zoosporogenesis-specific NIFC gene cluster of *Phytophthora infestans* involves chromatin alterations. Eukaryotic Cell 2007;6:1200–9.

[50] Fleischmann F, Koehl J, Portz R, Beltrame AB, Oßwald W. Physiological changes of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitin "Citricolin" to pathogenesis. Plant Biology 2005;7:650–8.

[51] Huitema E, Vleeshouwers VGAA, Cakir C, Kamoun S, Govers F. Differences in intensity and specificity of hypersensitive response induction in *Nicotiana* spp by INF1, INF2A and INF2B of *Phytophthora infestans*. Molecular Plant-Microbe Interactions 2005;18:183–93.

[52] Panabières F, Birch PRJ, Unkles SE, Ponchet M, Lacourt I, Venard P, et al. Heterologous expression of a basic elicitin from *Phytophthora cryptogea* in *Phytophthora infestans* increases its ability to cause leaf necrosis in tobacco. Microbiology 1998;144:3343–9.