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

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**A B S T R A C T**

The differential expression of four Phytophthora cinnamomomi 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 reduction 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.

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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gene clusters have been identified in Phytophthora cryptogae [1], P. cinnamomi [2] (Fig. 1) and Phytophthora cambivora (unpublished results).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. cryptogae expresses both the β- and α-cryptogenes 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]. Antibiocide [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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The role of the elicitors 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 elicitor is restricted to certain plant species or even specific cultivars [19, 22–25]. In tobacco, the role of the elicitors appears to be an avirulence factor [26, 27]. The elicitor 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 elicitor 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 elicitor expression displaying dual and conflicting functions depending on the genotype of the host and other variables are 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, elicitors 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 elicitor 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 PA46 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 elicitors, the cultures were transferred to ESM (Elicitin Secretion Medium) (0.05% w/v KH2PO4, 0.025% w/v MgSO4•7H2O, 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 Smal fragment) regulated by the ham34 promoter (present in a 920 bp HindIII-Smal fragment) and by the ham34 terminator present in a 550 bp Smal- EcoRI fragment of Bremia lactucae (EMBL Genebank accession number: X16984). The pHAMT35-fats 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 AGC GAC CAC GAC ATC and 5’-AGA CTC CCG GGT TAG AGC GAC CCC TTG GAC, digesting this fragment with BglI and SmaI and cloning it into Smal-digested pHAMT35H. Correctly oriented clones were selected by restriction analysis with BgII. 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 105 protoplasts with 20 μg pHAMT35H and 40 μg PHAMT35-fats in MTC-20 buffer (1 M mannitol, 10 mM Tris-HCl, 20 mM CaCl2; pH 7.5). Protoplasts were diluted 10× 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 GAAG
TTT TTT CTA CAA and 5'-CGC GCC CCC ACC AGA ATC TTC, which amplify an 839 bp fragment of the hpt coding sequence. Similarly, the presence of pHAMT35-fatts was verified using primers 5'-CCT
TTG GGT ACC ATG TCA CCA TGA TGG CTG GCT CCC ACC GCA GCC 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 amplicons.

2.4. Analysis of elicitors 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-botted onto a nitrocellulose membrane using a Trans-blot SD Blotting apparatus (BioRad). The proteins present in the gels were then electro-botted onto a nitrocellulose membrane using a Trans-blot SD Blotting apparatus (BioRad). The proteins present in the gels were then electro-botted onto a nitrocellulose membrane using a Trans-blot SD Blotting apparatus (BioRad). The proteins present in the gels were then electro-botted onto a nitrocellulose membrane using a Trans-blot SD Blotting apparatus (BioRad). The proteins present in the gels were then electro-botted onto a nitrocellulose membrane using a Trans-blot SD Blotting apparatus (BioRad). The proteins present in the gels were then electro-botted onto a nitrocellulose membrane using a Trans-blot SD Blotting apparatus (BioRad).

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) were designed using Primer Express Software (Applied Biosystems) and 5′-fluorescently labelled probes were validated using the 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 (Invitrogen) and 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 a fragment of 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).

Table 1

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<th>Forward primer sequence (5′ → 3′)</th>
<th>Taqman probe sequence (FAM-5′ → 3′-TAMRA)</th>
<th>Reverse primer sequence (5′ → 3′)</th>
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<td>Beta cin 67 bp</td>
<td>CAGACCCGCCCCTACCAA</td>
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<tr>
<td>HAF Alfa cin 75 bp</td>
<td>ACAGCAGCGCCAGGATGCTGGTCTGGTCTG</td>
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<td>CCCCTTGATGTTGCTGAGG</td>
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cDNA (using the primers shown in Table 2), cloned using the TA Cloning Kit (Invitrogen) and sequenced. The resulting plasmids were linearized with NotI (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 $10^3$–$10^7$ ss DNA/μl.

Sample normalisation was effected using the relationship: (normalised number of elicitor 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 elicitor transcripts. The expression level of the cin gene was expressed relative to their level measured in 3-day-old cultures of PA45 in V8 medium (3d_PA45_V8).

### Table 2

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<th>Reverse primer sequence (5’→3’)</th>
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<td>Actin 527 bp</td>
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<td>Beta Cin 490 bp</td>
<td>ACATCCCTTGGCCACCTCC</td>
<td>TGTTCCCCCTCATCTCAAGGAA</td>
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<tr>
<td>Hae Alfa Cin 564 bp</td>
<td>CACCACTGAGGCGCCCTCC</td>
<td>GCTTACAAAGGCTCCAGG</td>
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<tr>
<td>Alfa Cin 537 bp</td>
<td>GCACCTCTCTCCCTCCACCACC</td>
<td>GCCTTTAAGCTTCCTCC</td>
</tr>
<tr>
<td>Hae Beta Cin 523 bp</td>
<td>GCACAAACCTCTCCCTTACACC</td>
<td>CCCTTTAAAGCTTCCTCC</td>
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### 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 carried the other (13C) from isolate PA37. The FATSS strain carries both pHAMT35H and pHAMT35-fatss (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-cin 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 root necrosis.
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. 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.

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, a-cin was expressed about 15× more abundantly than β-cin, 23× more than HAEβ-cin and 142× more than the HAEα-cin; in 2-day ESM cultures, a-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 to 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 HAEα-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

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**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-cinamomin; B: α-cinamomin; C: HAE β-cinamomin; D: HAE α-cinamomin.
24 h was 8× more abundant than 3d_PA45_V8, and after 36 h expression had increased a further 2.25×. The HAE-β-cinnamomin 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. cryptogaea, where the expression of the basic elicitin gene dominated in ESM medium [1] and a greater amount of the β-cryptogcin than the α-cryptogcin was present in culture filtrates [42]. Note, however, that probes for the Northern analysis on P. cryptogaea were based on 3′ UTR sequences, which present more variability than coding regions. Furthermore, a probe directed to the coding region of the basic cryptogcin 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 cinnamomin 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...
colonization. More generally, the differential expression of each elicitin across the host, and the latter in propagation and survival after colonization 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 induced by the presence of the antisense β-elicitin sequence, rather than by any transgene sequence(s) shared with strain 13C. The antisense-driven inhibition of β-elicitin 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 transgenic and the target genomic DNA homologues, and potentially target of at least 80% [48]. The antisense sequence of β-elicin is highly homologous with the other three members of the elicitin cluster. In addition to the total suppression of β-elicitin in the FATSS transformant, no HAEa-elicitin (77% homology with β-elicitin) and a reduction in the abundance of α-elicitin and HAEβ-elicitin (83.5% and 81.2% homology, respectively) transcripts were observed. As the number of HAEa-elicitin 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, HAEa-elicitin lies closest to β-elicitin, and thus may be more susceptible than both α-elicitin and HAEβ-elicitin to disruption of transcription caused by localised chromatin alterations.

In the present study it was 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 produce zoospores were 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-elicitin and HAEa-elicitin; α-elicitin and HAEβ-elicitin) 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 elicities 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 elicities 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 elicities 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 elicities under various pH conditions.

The infection process on Q. robur roots was characterised by an increase in the expression of α-cinin 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 cinnamomi, the α-citriconin 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 elicitor expression were slighter than those in the P. parasitica experiment. 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 introduction of Q. robur roots by P. quercina was characterised by the ubiquitous presence of β-querocin, 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-elicitin gene expression which does not have a direct relation with the totality of the pathogen biomass. The in planta expression of β-elicitin 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 synthetized by a given strain is constant, there may well be a common role for the elicits across all the Phytophthora spp. and, that the class IA and IB elicits are in effect interchangeable, with similar functions. However, the level of α-infestin did not fall below that present in the wild type form, 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 α-cinnamomin by an over-expression of α-cinin. However, the experimental observation was that, on the contrary, the activity of α-cinin was, if anything, partially down-regulated as silencing of β-elicitin 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 (htp) 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.

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