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Short communication

Structure and expression *in planta* of *Botrytis cinerea* ubiquitin genes*

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Abstract

To identify genes of the necrotrophic pathogenic fungus *Botrytis cinerea* that are expressed during infection of tomato leaves, a differential screening of a genomic library with radioactively labelled cDNA was performed. This resulted in the identification of a *B. cinerea* gene, denominated *Bcubi*4, which encodes a precursor protein consisting of four identical head-to-tail repeats of a 76 aa ubiquitin unit. Subsequently a gene denominated *Bcubi*1CEP79, encoding a single ubiquitin unit joined to a Carboxyl Extension Protein of 79 amino acids, was isolated. The expression of the two ubiquitin genes was studied during pathogenesis of *B. cinerea* on tomato. *Bcubi*1CEP79, but not *Bcubi*4, mRNA was transiently induced at 16 h after inoculation. The increased expression of the *Bcubi*1CEP79 gene at this stage of pathogenesis might be required for enhanced ribosomal biogenesis.

Introduction

The ubiquitous plant pathogen *Botrytis cinerea* Pers.: ex. Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetz.), is a necrotrophic fungus with a broad host range (reviewed by Prins et al., 2000). The infection of a host plant by *B. cinerea* is accompanied by an oxidative burst (Deighton et al., 1999) and results in many different chemical defence responses by the host, such as the synthesis of phytoalexins and pathogenesis-related (PR) proteins, as well as lignification of the plant cell wall. These defence responses presumably impose a stress on the pathogen, resulting in a temporary cessation of fungal growth (Benito et al., 1998), referred to as a quiescent phase (Williamson, 1994; Prusky, 1996). When fungistasis leads to protein misfolding, aberrant proteins may accumulate in the fungus and must be removed. One way of removing non-functional proteins is by proteasomes through a process requiring ubiquination of proteins (Ciechanover, 1994).

Ubiquitins are highly conserved among eukaryotes and are encoded by two classes of genes. Polyubiquitins consist of a 76 aa coding unit in multiple direct repeats. The C-terminal unit is followed by a single amino acid that is not conserved. The second class of genes encodes a single ubiquitin unit fused in frame with a stretch of unrelated amino acids at the C-terminal end, known as the Carboxyl Extension Protein (CEP). Within the CEPs, two types can be identified: those of 52 or 76–80 amino acids (Monia et al., 1990). Once a target protein is ubiquinated by covalent attachment of ubiquitin units, the conjugate is transported to the proteasome were the target protein is proteolytically cleaved (Ciechanover, 1994). Besides ubiquination of cytoplasmic proteins, ubiquitin can also be covalently attached to regulatory proteins, thereby fine-tuning cell processes like cell cycling (von Kampen et al., 1996).

*The nucleotide sequence data are in the EMBL and GenBank databases under accession numbers AF060501 (*Bcubi4) and AF060232 (Bcubi1CEP79).*

Ubiquitin has been studied most extensively in *Saccharomyces cerevisiae*. Polyubiquitin is suggested to be required for resistance of cells to high temperatures, starvation and other stresses (Özkaynak et al., 1987; Finley et al., 1987). Furthermore, polyubiquitin contributes to oxidative stress tolerance (Cheng et al., 1994) and catabolite derepression (Watt and Piper, 1997). Ubiquitin has also been studied in a number of plant pathogens such as *Phytopthora infestans* (Pieterse et al., 1991), *Magnaporthe grisea* (McCafferty and Talbot, 1998) and *Gibberella pulicaris* (Loser and Weltring, 1998). In this study, mono- and polyubiquitin genes of *B. cinerea* were isolated and their expression during pathogenesis was analysed.

Materials and methods

Screening of genomic library

A genomic library of *B. cinerea* strain SAS56 in λEMBL3 was prepared according to the manufacturer's instructions (Promega). From this library, 15,000 plaque-forming units, which represented five genome equivalents, were plated. Four replica filters (Hybond-N^+, Amersham) were prepared. For a differential screening, poly(A)$^+$ RNA was isolated from *B. cinerea* grown *in vitro* and a tomato–*B. cinerea* interaction (16 h after inoculation, as described by Benito et al., 1996). There was 3% of *B. cinerea* RNA in the total infected plant RNA population that was used for preparing the 'Interaction cDNA', based on densitometric scanning of an autoradiograph of RNA hybridisation experiments with a *B. cinerea* actin probe, (Benito and van Kan, 1998). This RNA was reverse transcribed into cDNA in the presence of [α-^{32}P]dATP and hybridised to duplicate filters of the genomic library of *B. cinerea*. Since the fungal poly(A)$^+$ RNA in the interaction sample represents at maximum 5% of the total (Benito et al., 1996), the *in vitro* probe was supplemented with 19-fold excess poly(A)$^+$ RNA from uninfected tomato leaves. Filters were hybridised overnight at 65 °C in 0.5 M sodium phosphate buffer pH 7.2; 7% SDS (Church and Gilbert, 1984), washed with 0.5 × SSC; 0.5% SDS at 65 °C and exposed to Kodak X-OMAT AR film. Plaques that hybridised differentially on both replica filters were isolated and subjected to a second round of hybridisation. DNA of positive phages was isolated using the Qiagen phage DNA isolation kit (Qiagen Inc., USA) and analysed by Southern blotting. Hybridising fragments were cloned into pBluescript-SK II (−) (Stratagene) as described in Sambrook et al. (1989).

Sequence analysis

DNA sequencing was performed with Applied Biosystems, 377 DNA Sequencer, using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, with Amplitaq DNA Polymerase, and analysed using Lasergene software package (DNAstar Inc., Madison, USA). Nucleotide and amino acid homology analysis was performed using the Blast program (Gish and States, 1993; Altschul et al., 1997).

Analysis of gene expression in planta

Tomato leaves (*Lycopersicon esculentum* cv. Money Maker genotype *Cf4*) were inoculated with conidia of *B. cinerea* according to Benito et al. (1998). Inoculated leaves were incubated at 4 and 20 °C. At 16, 32, 48, 72, 96 and 120 h after inoculation, leaflets were harvested. RNA was extracted and electrophoresed as described by Van der Vlugt-Bergmans et al. (1997). As probes, a 400-bp *Xho*I/*Acc*I polyubiquitin fragment (nt. 741–1141), a fragment of the actin gene (Benito et al., 1998) and a fragment of the 27S rDNA gene (Prins, unpublished) were used.

Results

A differential screening of a *B. cinerea* genomic phage library in λEMBL3 was carried out as described by Pieterse et al. (1991), using two batches of radioactively labeled cDNA: 'Interaction cDNA' synthesised on RNA isolated from a *B. cinerea*-tomato interaction 16 h post inoculation (h.p.i.) and '*In vitro* cDNA' synthesised on RNA from *B. cinerea* grown in liquid culture (Benito et al., 1996; Benito and van Kan, 1998). There was 3% of *B. cinerea* RNA in the total infected plant RNA population that was used for preparing the 'Interaction cDNA'. One of the differentially hybridising phages was purified and a hybridising restriction fragment derived from this phage was cloned and sequenced. Sequence similarity was found with ubiquitin, a 76 amino acid protein that is highly conserved in all eukaryotes.

Southern analysis of *B. cinerea* genomic DNA, digested with four restriction enzymes, indicated that

the gene hybridised strongly to a single band in each digest and weakly to a second band (results not shown). In view of the presence of multiple ubiquitin genes in *S. cerevisiae* and a number of plant pathogenic fungi (Özkaynak et al., 1987; McCafferty and Talbot, 1998; Loser and Weltring, 1998), the library was rescreened for additional *B. cinerea* ubiquitin genes. This resulted in the identification of a second type of phages, which also contained a gene encoding ubiquitin. The gene that was isolated first, denominated *Bcubi*4 (Genbank accession number AF060501), contains an ORF encoding a polyubiquitin precursor protein, consisting of four identical ubiquitin units. As in all polyubiquitin genes, the last unit is followed by an additional amino acid preceding the translation termination codon, in this case a glutamine residue. The second gene, denominated *Bcubi*1CEP79 (Genbank accession number AF060232), contains an ORF encoding a single ubiquitin unit joined to a CEP of 79 aa (hereafter referred to as CEP79). The amino acid sequences of the five ubiquitin units in both *B. cinerea* genes are exactly identical. Figure 1 shows an alignment of the *B. cinerea* ubiquitin units with homologues from other fungi. Figure 2 shows that the extension protein CEP79 has homology to ubiquitin–CEPs from other fungi, especially in the N-terminus. A potential nuclear localisation signal and a putative nucleic acid binding site (Özkaynak et al., 1987) are found in the CEP79 moiety. Both motifs are conserved in all ubiquitin–CEPs shown in the alignment in Figure 2. One of the homologues is the 76 amino acid UBI3 extension protein of *S. cerevisiae* (Özkaynak et al., 1987). This protein is cleaved from the ubiquitin moiety, and participates in ribosomal biogenesis (Finley et al., 1989).

In *S. cerevisiae*, ubiquitin gene expression is regulated by carbon and nitrogen sources in the medium (Watt and Piper, 1997). In *B. cinerea*, no obvious

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Botrytis cinerea              MQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG(Q)
Neurospora crassa             ........................Q.......................................................(F)
Magnaporthe grisea            ........................S.......................................................(.)
Gibberella pulicaris          ........................S.......................................................(.)
Saccharomyces cerevisiae      ........................S.......................................................(N)
Candida albicans              ........................S.......................................... ....(F)
Phanerochaete chrysosporium   ..........................................................................(L)
Phytophthora infestans        .............D..P..S.....Q.......................................................(N)
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Figure 1. Alignment of *B. cinerea* ubiquitin units with homologues from other fungi. The amino acid sequences of BcUBI4 (four identical units, accession AF060501) and BcUbi1CEP79 (one unit, accession AF060232) were aligned with polyubiquitins from *Neurospora crassa* (X13140, four identical units; Taccioli et al., 1989), *Magnaporthe grisea* (AF056625, five identical units; McCafferty and Talbot, 1998), *Gibberella pulicaris* (AJ007936, four identical units; Loser and Weltring, 1998), *Saccharomyces cerevisiae* (X05731, five identical units; Özkaynak et al., 1987), *Candida albicans* (Z54197; unpublished), *Phanerochaete chrysosporium* (Z24723, five identical units; Escher, 1995), and *Phytophthora infestans* (X55717, three identical units; Pieterse et al., 1991). The additional C-terminal amino acid following the ultimate ubiquitin unit is indicated between brackets. A dot represents an amino acid that is identical to the *B. cinerea* ubiquitin moiety.

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Botrytis cinerea              GKKRKKKVYTTPKKIKHKRKKTKLAVLKYYKVDGDGKIERLRRECPTPDCGAGVFMAAMHDRQYCGRCHLTYIFDDANK 79
Aspergillus nidulans          ..............................................S.E....I.....QN.....K.....V..ESK  78
Neurospora crassa             ................................S.............NET..........Q.............V.EKSS  78
Magnaporthe grisea            ..................H..V.....STTRSTVTERLSASAAS..NET....I.....Q.............V..KKD  78
Saccharomyces cerevisiae      ..................H..V.....S.....AE..VTK.....SN.T......L.NHK..L...K..SV.KVNA    76
Candida albicans              ..................HR.......T.....NE.NV.........A.T....I...N.K......K....LKAN     75
Schizosaccharomyces pombe     .......T..........H..VE.........ED..SVK.......N-..-...ST...NHK..L........LKLEN    74
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Figure 2. Alignment of the *B. cinerea* ubiquitin–CEP79 moiety with homologues from other fungi: *Aspergillus nidulans* (accession AF175521, Noventa-Jordão et al., 2000), *Neurospora crassa* (X15338, Taccioli et al., 1991), *Magnaporthe grisea* (AF056624; McCafferty and Talbot, 1998), *Saccharomyces cerevisiae* (X05730, Özkaynak et al., 1987), *Candida albicans* (Y15608; unpublished) and *Schizosaccharomyces pombe* (CAB11297, unpublished). A dot represents an amino acid that is identical to the *B. cinerea* CEP79 protein.The nuclear localisation signal (positions 2–7) is underlined. The four cysteine residues that form part of the metal binding site are indicated in bold and double underlined. The number behind the sequence indicates the length of the CEP.

changes in ubiquitin mRNA levels were observed under different growing conditions (results not shown). The expression of the *B. cinerea* ubiquitin genes *in planta* was investigated. Detached tomato leaves were inoculated with a conidial suspension of *B. cinerea* strain SAS56 as described by Benito et al. (1998) and harvested at different time points for RNA extraction. A blot containing total RNA isolated from infected tomato leaflets, as well as from *in vitro* cultures of *B. cinerea*, was hybridised with a ubiquitin probe, a *B. cinerea* actin probe (Benito et al., 1998) and a 27S rDNA probe (Prins, unpublished data). The results of the hybridisation are shown in Figure 3. The rDNA probe detects both plant and fungal rRNAs and was used to visualise RNA loading in each lane. The actin mRNA level increased over time, especially at 96 and 120 h.p.i. This increase reflects the increase of fungal biomass in the infected tissue, which occurred in two steps. In the first 16 h.p.i. primary necrotic lesions were formed by germinating conidia that penetrate the host tissue. The primary lesions remained quiescent for 2–3 days and only started to expand after 72 h.p.i. under the conditions used in this inoculation (Benito et al., 1998). The ubiquitin probe recognised

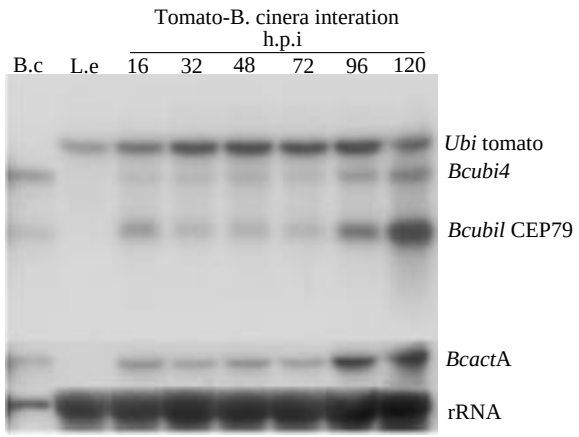

Figure 3. Expression analysis of *B. cinerea* ubiquitin genes *in planta*. Total RNA was isolated from a *B. cinerea* liquid culture (lane B.c.), from uninoculated tomato leaf (lane L.e.) and from *B. cinerea*-infected tomato leaves at different time points (numbers indicate h.p.i.), electrophoresed and blotted. In the left lane (B.c.) only 1/20th of the amount of RNA was loaded as compared to the other lanes, to approximate the amount of fungal RNA in the infected leaves at 16 h.p.i. (Benito et al., 1996). The blot was hybridised with the *Bcubi4* probe (upper panel), the *B. cinerea* actin gene *Bcact*A (Benito et al., 1998; middle panel) and a *B. cinerea* 27S rDNA probe (Prins, unpublished data; lower panel).

the two types of *B. cinerea* ubiquitin mRNAs, as well as the tomato heptaubiquitin (Rollfinke et al., 1998). The level of *Bcubi*4 mRNA increased during the infection, and it follows the pattern of the actin mRNA. Thus, the *Bcubi*4 expression level remained proportional to the fungal biomass during infection. *Bcubi*1CEP79 mRNA, however, was transiently induced at 16 h.p.i. as compared to the actin signal. At this timepoint the proportion of fungal RNA in the total infected leaf RNA was estimated to be 1–5% (Benito et al., 1996; Benito and van Kan, 1998). Twenty-fold less RNA was loaded in the *B. cinerea in vitro* control lane than in the other lanes, in order to obtain a hybridisation intensity of this sample, that would be equivalent to a proportion of 5% *B. cinerea* RNA in the total infected leaf RNA. The *Bcubi*1CEP79 mRNA hybridisation signal in the interaction at 16 h.p.i. is clearly higher than the control sample, while the actin hybridisation signals in both samples are similar. After this timepoint the *Bcubi*1CEP79 mRNA level first decreased and then increased again, following the pattern of the actin signal. The tomato heptaubiquitin mRNA level gradually increased during the infection by *B. cinerea*, possibly as a stress response to the infection. The transient induction pattern of the *Bcubi*1CEP79 mRNA in early stages of the infection was reproducible in a second experiment, not only in an infection at 20 °C, but also at 4 °C (results not shown). In the second experiment, the tomato heptaubiquitin mRNA level remained constant, albeit at a high level (results not shown).

Discussion

The induction of ubiquitin gene expression during the infection of host plants has previously been described for other plant pathogens. Ubiquitin gene expression was induced in the oomycete *Phytophthora infestans* during colonisation of potato leaves, suggesting an important role of ubiquitin in pathogenesis (Pieterse et al., 1991). The rice blast fungus *M. grisea* contains two genes, *UEP1* and *UEP3*, encoding monoubiquitin joined to CEPs of 52 and 76 amino acids, respectively (McCafferty and Talbot, 1998). Both genes are highly expressed during active growth and down-regulated during starvation stress. *M. grisea* also contains a polyubiquitin gene *PUB4*, which is predominantly induced by environmental stress (McCafferty and Talbot, 1998). *UEP*1 is the only member of the *M. grisea* ubiquitin gene family that is expressed to detectable

levels during plant colonisation (McCafferty and Talbot, 1998). A polyubiquitin gene was also cloned from the pathogenic fungus *Gibberella pulicaris* (Loser and Weltring, 1998). Increased mRNA levels were detected at elevated temperatures and when the potato phytoalexin rishitin was applied to a liquid culture. Southern analysis suggested the presence of additional ubiquitin genes (Loser and Weltring, 1998). Expression of *G. pulicaris* ubiquitin genes was not studied *in planta*.

Ubiquitin is highly conserved among eukaryotes and is involved in a number of cellular processes like oxidative stress resistance (Cheng et al., 1994), selective protein degradation (Ciechanover, 1994), cell cycle regulation (King et al., 1996), p53-mediated apoptosis (Scheffner et al., 1993), NF-κB-induced apoptosis (Krappmann et al., 1996) and regulation of receptors (Strous et al., 1996). The function of ubiquitin genes in fungi has best been studied in *S. cerevisiae*. Yeast contains three genes denominated *UBI1*, *UBI2* and *UBI3*, that encode monoubiquitins with CEPs of 52, 52 and 76 amino acids, respectively. In addition, the gene *UBI4* encodes a pentaubiquitin that is strongly inducible by starvation, heat shock and other stresses (Özkaynak et al., 1987). The *UBI*4 gene contributes to oxidative stress resistance (Cheng et al., 1994) and is subject to catabolite derepression control (Watt and Piper, 1997).

The CEP moieties of the three *S. cerevisiae* monoubiquitin gene products each have a nuclear localisation signal and a putative metal binding site that can assemble into a nucleic acid binding motif, suggesting that the CEP moieties bind to nucleic acids, either before or after cleavage from the ubiquitin moiety (Özkaynak et al., 1987). Targeted deletion of each of the three monoubiquitin–CEP genes resulted in mutants with severely slower growth rates. The mutants displayed a distortion of the stoichiometry of the ribosomal subunits (Finley et al., 1989). The growth defect caused by deletion of the *UBI*1, *UBI*2, or *UBI*3 gene could be relieved by complementation with gene constructs consisting of the CEP52 or CEP76 moiety alone, lacking the ubiquitin moiety (Finley et al., 1989). Tagging the CEP moiety with a 12 amino acid c-myc epitope revealed that the CEP52 and the CEP76 are incorporated in ribosomes. The CEP52 encoded by *UBI*1 and *UBI*2 is part of the large ribosomal subunit, whereas the *UBI*3-encoded CEP76 tail is part of the small ribosomal subunit (Finley et al., 1989). Incorporation of these CEP moieties into ribosomal subunits is thus essential for proper ribosome biogenesis.

The observation that the *B. cinerea* monoubiquitin gene, but not the polyubiquitin gene, is induced *in planta* at an early stage of infection, suggests that the need for increased production of the CEP79 moiety determines the induction of the *Bcubi*1CEP79 gene, rather than the need for ubiquitin. Since the *S. cerevisiae* CEP76 (homologue of the *B. cinerea* CEP79 moiety) is required for ribosomal biogenesis (Finley et al., 1989), the expression pattern probably reflects a need for a relatively high rate of ribosomal biogenesis in *B. cinerea* at the early stages of the infection process. A similar conclusion was drawn for the rice blast fungus, *M. grisea* (McCafferty and Talbot, 1998). Such a conclusion is in agreement with our observation that a differential display RT-PCR screening for *B. cinerea* genes that are differentially expressed *in planta* (Benito et al., 1996; Benito and van Kan, 1998), resulted in the isolation of a small number of cDNA fragments derived from ribosomal protein encoding genes (Prins, unpublished data). Initially this was considered to be an artefact of the DDRT-PCR procedure. In retrospect, this finding may be the consequence of enhanced ribosomal biogenesis in *B. cinerea* in early stages of pathogenesis, involving the induction of the *Bcubi*1CEP79 gene and other ribosomal protein encoding genes.

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