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Expressed sequence tags from the phytopathogenic fungus *Botrytis cinerea*

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Abstract

A large set of genes was identified in the phytopathogenic fungus *Botrytis cinerea* by using an expressed sequence tag approach. The fungus was grown in axenic culture and a cDNA library was produced. From this library, 6559 ESTs were obtained. The combined sequences represent 3026 unisequences that corresponds to approximately one-quarter of the estimated total number of genes in *B. cinerea*. Approximately 18% of the ESTs showed significant similarities with genes coding for proteins with known functions, ~56% were similar to genes coding for proteins with unknown functions and ~26% were orphans. A substantial *B. cinerea* gene inventory including putative virulence factors was therefore obtained and is now available at the Génoplante-Info Database interface (http://urgi.infobiogen.fr///Projects/GPiDB/Interface/).

Abbreviations: CWDEs – cell wall degrading enzymes; ESTs – expressed sequence tags; GPiDB – Génoplante-Info DataBase.

Introduction

The necrotrophic fungus *Botrytis cinerea* (asexual stage of the teleomorph, *Botryotinia fuckeliana*) is the causal agent of grey mould that is responsible for important diseases of dicotyledonous plants, inflicting serious food and ornamental crop losses (Elad and Evenson, 1995). Chemical control remains the main way to reduce the incidence of grey mould in most crops but *B. cinerea* has a high ability to quickly adapt to new fungicides and to develop resistance (Leroux et al., 2002). Understanding the infection mechanisms may contribute to develop new strategies to control this disease. Genetic transformation and homologous recombination are efficient in *B. cinerea* (Levis et al., 1997) making targeted gene inactivation an available tool. Using this strategy, some cell wall degrading enzymes (CWDEs, ten Have et al., 1998; Valette-Collet et al., 2003) and signal transduction components (Zheng et al., 2000; Schulze Gronover et al., 2001; Klimpel et al., 2002) were shown to be virulence factors. The limitation of such targeted gene inactivation approaches is that it involves potential candidate genes. Genome-wide approaches, recently applied to a number of fungi, should allow the discovery of new pathogenicity factors (Sweigard and Ebbole, 2001;

Yoder and Turgeon, 2001; Lorenz, 2002; Tunlid and Talbot, 2002). Among these approaches, the production of expressed sequence tags (ESTs) has allowed the identification of large sets of genes in the phytopathogens *Phytophthora infestans*, *Mycosphaerella graminicola*, *Magnaporthe grisea*, *Blumeria graminis*, *Fusarium graminearum* and *Verticillium dahliae* (Kamoun et al., 1999; Keon et al., 2000; Kim et al., 2001; Rauyaree et al., 2001; Thomas et al., 2001; Neumann and Dobinson, 2003; Trail et al., 2003). A similar EST approach was used to identify a large set of expressed genes and putative virulence factors in *B. cinerea*. In order to avoid the isolation of plant ESTs, a cDNA library was made from the fungus grown in axenic culture. From this cDNA library, 6559 ESTs have been produced. They were stored and analyzed in the Génoplante-Info DataBase (GPiDB) developed by Samson et al. (2003). This analysis generated a non-redundant set of 3026 unisequences that were compared to known proteins. The resulting *B. cinerea* gene inventory should facilitate the identification of genes coding for putative virulence factors including CWDEs, signal transduction components and secondary metabolism enzymes.

Materials and methods

Strain and culture conditions

The *B. cinerea* T4 strain was isolated from infected tomatoes (*Lycopersicum esculentum*) grown in a glasshouse in Avignon, France. 10^8 conidia from the T4 strain were used to inoculate 100 ml of the following medium: 10 g l^{-1} glucose, 1.52 g l^{-1} KH_2PO_4, 0.52 g l^{-1} KCl, 0.152 g l^{-1} $MgSO_4 \cdot 7\ H_2O$, 2 g l^{-1} peptone, 0.1 g l^{-1} $NaNO_3$. After 65 h rotated culture (70 rpm) at 21 °C, the mycelium was filtered and frozen at −80 °C.

Construction of the cDNA library

Total RNA from *B. cinerea* was isolated from 1 g of mycelium using the guanidine hydrochloride extraction method (Sambrook et al., 1989). Poly(A)+ mRNA was isolated using the mRNA purification kit (Pharmacia Biotech) according to the manufacturer's instructions. The cDNA synthesis was performed with Time Saver Synthesis kit (Pharmacia Biotech) and TimeSaver cDNA Synthesis Kit (Pharmacia Biotech). The *Not I*-(dT)18 primer was used to polymerase the first strand, and the obtained cDNA were cloned directionally in the pBSII SK+ vector (Stratagene) previously digested by *Not* I and *EcoR* I. Ligation products were transformed into *Escherichia coli* DH5α competent cells.

Sequencing

DNA sequencing was made at the Génoscope (Evry, France). Plasmid DNAs were purified with the standard alkaline lyses protocol (Sambrook et al., 1989) using the Qiagen kit, and sequenced with the T7 universal forward Primer using the ABIPRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq (Perkin-Elmer) and an ABI 377 DNA sequencer. 6559 ESTs were obtained and deposited at GenBank database (numbers ranging from AL110508 to AL117185).

EST clustering pipeline and functional annotation

Clustering pipeline, database and tools used in this analysis were previously described by Samson et al. (2003). *B. cinerea* ESTs were analyzed in four successive steps: cleaning, masking, clustering and alignment. The cleaning step was done by using *cross_match* (http://www.phrap.org) that allows the removal of contaminated sequences. Repeats and low complexity sequences were masked using *RepeatMasker* (http://repeatmasker.genome.washington.edu) and the associated Repbase database (Jurka, 2000). Clustering was performed with a multi-step procedure, starting with the transitive regrouping of repeat-masked sequences that shared at least a subsequence of 80 bp with 96% similarity (Glémet and Codani, 1997). Eventually, sequences from each cluster were assembled using CAP3 (Huang and Madan, 1999) to form contigs.

For functional classification, unisequence were compared with the NCBI non-redundant protein database using the BLASTX program (Altschul et al., 1997). A match was considered to be significant if the expected (E) value was less than $1e^{-5}$. Unisequences with significant BLASTX matches were further divided on the basis of their likely cellular function following the general categories outlined at the Gene Ontology Consortium (http://www.geneontology.org).

Data availability and tools

For the most efficient use of the EST data, Génoplante-Info offers web tools available at http://urgi.infobiogen.fr/Projects/GPiDB/Interface/. It allows users to query and/or get information on each contig (consensus, origin of its members, alignments, translation, putative ORF etc.), library (type, tissue, development stage etc.), or annotations (description of hits, *E*-value and positions). Moreover, one can execute some annotation tasks (ClustalW, BLAST, primer3 etc.) and access graphical visualization of contigs. To initiate the user to this interface, a tutorial is supplied.

Results and discussion

Construction of a cDNA library and single-pass sequencing of 6559 cDNAs

A cDNA library was constructed with poly(A)+ mRNA isolated from *B. cinerea* mycelium grown under axenic liquid culture conditions. cDNAs were cloned directionally into the pBSII SK+ vector and 6900 clones were obtained. This library was named *bcns* and was evaluated by PCR analysis conducted on 600 clones. PCR with universal primers situated on each side of the insert, showed that 96% of the clones had inserts longer than 400 bp. The size of the inserts ranged from 200 to 3900 bp with an average size of 980 bp (Data not shown). All 6900 clones were sequenced from the 5′ end using the T7 universal primer and 6559 ESTs were obtained from single-pass sequencing. Their average readable sequence length was 501 nucleotides. In less than 1% of cases, sequencing revealed a poly(A) tract on the 5′ end suggesting that the cDNA had been inserted in the reverse orientation.

Distribution of ESTs into 891 contigs and 2135 singletons

ESTs were stored in the GPiDB database (Samson et al., 2003) in which they have been organized into contigs. Before clustering, ribosomal RNA (16 sequences), vector sequences (36 sequences) and sequences shorter than 60 bp (55 sequences) were removed. The remaining 6452 sequences were distributed into 891 contigs (containing two or more ESTs) and 2135 singleton sequences. Therefore, the estimated number of unisequences identified was 3026. Thirty-three percent (2135 out of 6452) of the ESTs appeared only once. The remaining 67% correspond to redundant ESTs and form contigs ranging from 2 to 61 sequences. This redundancy rate is similar to the rates obtained from other EST programs of similar scale (Thomas et al., 2001; Trail et al., 2003). The unisequences identified represent 3026 putative genes expressed during *B. cinerea* mycelial growth in liquid culture. Genome size and number of genes are only available in several ascomycetes including *Neurospora crassa*, *M. grisea* and *F. graminearum* (Galagan et al., 2003; http://www.broad.mit.edu/annotation/). The genome sizes of these three species are about 40, 39 and 36 Mb respectively, and the corresponding number of genes are around 10,000, 11,000 and 12,000. *Botrytis cinerea*'s genome size was estimated to vary between 34 and 40 Mb depending on the strains (van Kan et al., 1993). Assuming that *B. cinerea* has a gene density similar to *N. crassa*, *M. grisea* and *F. graminearum*, its number of genes could be between 10,000 and 12,000. Therefore, the genes identified in our study represent approximately one-quarter of *B. cinerea* genes.

Functional classification

To make a functional classification of *B. cinerea* ESTs, each unisequence was compared with the NCBI non-redundant protein database using the BLASTX program (Altschul et al., 1997). This classification was established based on the database entry giving the best *E*-value and the Gene Ontology Consortium (www.geneontology.org). About 18% of the ESTs showed significant matches to genes coding for proteins with known functions (proteins classified in Gene Ontology), ~56% were similar to genes coding for proteins with unknown functions (not classified in Gene Ontology) and ~26% did not shown any similarity to any sequences in public databases. This high frequency of orphan sequences is similar to that observed in previous fungal EST projects (Yoder and Turgeon, 2001). In some cases, this lack of similarity to protein database entries could be due to the sequence being derived from the 5′ non-translated region of the cDNA (Skinner et al.,

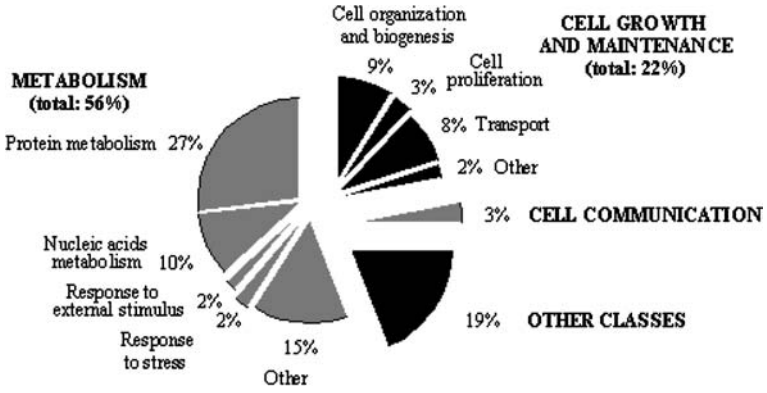

Figure 1. Distribution of 118 ESTs into functional classes according to the Gene Ontology classification. Although initial comparisons were made using consensus sequences from contigs and singletons, percentages are calculated from the initial number of ESTs to represent the relative levels of expression of genes in each category.

2001). Figure 1 presents the assignment of the 1181 ESTs having significant matches to proteins with known functions to functional categories as well as their relative abundances. As in other fungal EST projects, all major functional categories were represented with a majority of the identified ESTs related to metabolism (56%) and to cell growth and maintenance (22%). The most expressed gene (CTG_BotF_1.1-AL110794, 61 ESTs) did not show any similarity to proteins of known function. This gene, which seems to encode a very small protein (81 amino acids), was investigated by gene knock-out. The corresponding mutant did not show any particular phenotype neither *in vitro* nor *in planta*. We could not therefore draw any conclusions about its function (C. Levis, unpublished data).

Genes homologous to known fungal virulence factors

The sequence similarities highlighted by the BLASTX results enabled the identification of genes homologous to known fungal virulence factors or genes already described as required for full pathogenicity in *B. cinerea* (Table 1). Many fungal phytopathogens produce CWDEs that are known to degrade the plant cell wall, thereby facilitating *in planta* growth and providing nutrients to the invader (ten Have et al., 2002). Among the six endopolygalacturonases that have been

Table 1. Genes homologous to known fungal virulence factors or described as required for full pathogenicity in *Botrytis cinerea*

Unisequence[a] (number of ESTs)	BlastX[b] first hit: organism, protein, *E*-value	Reference
CTG_BotF_3.1-AL110663 (56)	*Blumeria graminis* GEGH 16 protein, 3e-25	Justesen et al. (1996)
CTG_BotF_4.1-AL111402 (37)	*B. cinerea* endopolygalacturonase BCPG1, e-148	ten Have et al. (1998)
CTG_BotF_21.1-AL111165 (22)	*B. cinerea* cyclophilin BCP1, 0	Viaud et al. (2003)
CTG_BotF_140.1-AL112170 (8)	*Paracoccidioides brasiliensis* calmodulin, 7e-78	de Carvalho et al. (2003)
CTG_BotF_471.1-AL112730 (3)	*Fusarium sporotrichioides* trichodiene oxygenase, 2e-41	Hohn et al. (1995)
CTG_BotF_180.1-AL111095 (6)	*Colletotrichum gloesporioides* CAP20 protein, 5e-32	Hwang et al. (1995)
CTG_BotF_240.1-AL113203 (4)	*Metarhizium anisopliae* Calcium/calmodulin dependant kinase, 1e-121	KCC1_METAN, O1440[c]
CTG_BotF_249.1-AL114358 (5)	*Sclerotinia sclerotiorum* zinc finger protein PAC1, 0	Rollins and Dickman (2001)
CTG_BotF_623.1-AL113969 (2)	*B. cinerea* tetraspanin BcPLS1, e-98	Gourgues et al. (2004)
CTG_BotF_686.1-AL110870 (2)	*Neurospora crassa* calcineurin B, 7e-87	Prokisch et al. (1997)
CTG_BotF_899.1-AJ309701 (1)	*B. cinerea* pectin methyl esterase, 0	Valette-Collet et al. (2003)
CL_BotF_2829.1-AL113673 (1)	*Neurospora crassa* calcineurin A, 6e-51	Higuchi et al. (1999)

[a] GenBank accession number corresponding to the first EST put into the contig.
[b] For BLASTX hit, EST sequences were BLASTX-searched aainst the GenBank number protein database.
[c] GenBank accession number was indicated when the work has not been published.

characterized in *B. cinerea*, only BCPG1 was present in our ESTs. Moreover, the *BCPGI* gene appeared highly expressed (37 ESTs). This result is consistent with the expression studies of ten Have et al. (2001, 2002) which conclude that *BCPG1* is the major transcript with a high basal expression in different media and host plants while other *BCPG* genes have a more complex expression regulation with inductions by galacturonic acid or low ambient pH. BCPG1 was shown to be required for full virulence (ten Have et al., 1998) and to activate grapevine defense reactions unrelated to its enzymatic activity (Poinssot et al., 2003). The PME1 gene coding for pectin methylesterase, another CWDE characterized as a virulence factor (Valette-Collet et al., 2003), was also found among the ESTs. Other putative CWDEs (glucanases, cellobiohydrolases, xylanases etc.) have been identified among the ESTs, but they are not mentioned in Table 1 as their role in pathogenicity remains to be established. Moreover, it is likely that many substrate-induced genes are not present because of the chosen medium. Supplementary cDNA libraries made from the fungus grown in conditions closer to those occurring during infection could increase the representation of CWDEs. Freimoser et al. (2003) have shown that growing the entomopathogen *Metarhizium anisopliae* in a medium containing chitin and cockroach cuticle allowed the isolation of a cDNA library with a high representation of secreted depolymerases.

Studies on signal transduction cascades revealed that similar transduction components including the cAMP-dependant protein kinase (PKA; Kronstad et al., 1998) and MAP kinases (Xu, 2000) are involved in the regulation of the infectious process in distantly related fungi. In *B. cinerea*, both a MAP kinase pathway (Zheng et al., 2000) and the PKA pathway (Schulze Gronover et al., 2001; Klimpel et al., 2002) were involved in pathogenicity. Another signalling pathway, depending on calcium and calcineurin, was identified in our EST set. This pathway was shown to be involved in fungal morphology and virulence (Rasmussen et al., 1994; Prokish et al., 1997; Kim et al., 1998; Kothe and Free, 1998; Lee and Lee, 1998; Fox and Heitman, 2002). Genes involved in this pathway, including those coding for calcineurin A, calcineurin B, calmodulin, and calcium/calmodulin-dependant kinase, were found among the ESTs (Figure 1). Moreover, the gene coding for cyclophilin A, which is known to interact with calcineurin and to be a virulence determinant in *M. grisea* and *B. cinerea* (Viaud et al., 2002, 2003), was also present. Expression of these genes suggests that the calcineurin pathway was activated during the culture conditions used. Other expression studies using macroarrays have shown that this pathway controls the expression of several genes including putative and known virulence factors like *bcpg1* (Viaud et al., 2003). Another interesting transduction component among the ESTs is the gene coding for the transcription factor PACC involved in pH sensing. Ambiant pH may be a regulatory cue for pathogenicity (Prusky and Yakoby, 2003) and the involvement of PACC in virulence was demonstrated in *F. oxysporum* (Caracuel et al., 2003) and in *Sclerotinia sclerotiorum*, a species closely related to *B. cinerea* (Rollins and Dickman, 2001).

Secondary metabolism is commonly associated with fungal morphological differentiation processes occurring during the stationary phase of growth (Calvo et al., 2002). Many genes related to the secondary metabolism were found in *B. cinerea* ESTs suggesting that 65 h-old mycelium contains cells that are already producing secondary metabolites. Several of these genes may play a role in pathogenicity. Nine unisequences were genes coding for putative P450 monooxygenases. Among them, was one very similar to the *TRI4* trichodiene oxygenase gene of *Fusarium sporotrichioides* which is involved in the biosynthesis of trichothecenes (Hohn et al., 1995). Trichothecenes constitute a large family of phytotoxic sesquiterpenes that inhibit eukaryotic protein synthesis. In phytopathogenic fungi, such toxin encoding genes are frequently clustered and coordinately regulated (Desjardins and Hohn, 1997; Walton, 2000). In common with these genes, the *B. cinerea* gene homologous to *TRI4* is closely linked to two other genes possibly involved in sesquiterpenes synthesis. In addition, the three genes are co-regulated through the calcineurin signaling pathway (Viaud et al., 2003).

Three putative peptide synthase genes and five putative polyketide synthase genes were identified among the ESTs. These genes are members of protein families more abundant in plant-pathogenic fungi than in saprophytic fungi (Yoder and Turgeon, 2001). Moreover, some of their members are known to be involved in fungal pathogenicity

(Walton, 1996; Yang et al., 1996; Kroken et al., 2003).

Ten ABC transporters genes have been identified among the ESTs (Vermeulen et al., 2001). In phytopathogenic fungi, the functions of these transporters comprise secretion of toxins that acts as virulence factors and protection against plant defense compounds and synthetic fungicides (Schoonbeek et al., 2002).

Finally, the EST set also provided the *BcPLS1* gene, the homologue to *MgPLS1* that is required for appressorium-mediated penetration of *M. grisea* into host plants (Gourgues et al., 2002). These two genes are coding for membrane proteins called tetraspanins. Recently, Gourgues et al. (2004) have demonstrated that *BcPLS1* and *MgPLS1* null mutants have similar penetration defects suggesting that fungal tetraspanins control an appressorial function conserved among fungi independent of their taxonomic relatedness or infection strategies.

Some other unisequences from the *B. cinerea* ESTs showed high similarity with proteins that have been described as fungal virulence factors but whose biochemical functions remain unknown. For example, one highly expressed unisequence (56 ESTs) appeared to encode a protein similar to GEGH16 of *Blumeria graminis* and to GAS1 and GAS2 virulence factors described in *M. grisea*. *GEGH16* gene belongs to a multigene family and is expressed *in planta* (Justesen et al., 1996). *GAS1* and *GAS2* genes are homologous to *GEGH16* and are expressed specifically during appressorium formation (Xue et al., 2002). Moreover, mutants deleted in *GAS1* and *GAS2* were reduced in plant penetration and lesion development. Another example is one unisequence that showed similarities with CAP20 protein characterized in *Colletotrichum gloesporioides*. The *CAP20* gene is expressed solely during appressorium formation and gene-disrupted mutants incapable of expressing *CAP20* showed a drastically decreased virulence (Hwang et al., 1995).

All together the pathogenicity genes mentioned above show that *B. cinerea* homologues of genes expressed during infection are present in our EST collection from axenic culture derived mycelium. The genes identified by our EST approach were all spotted on high-density filters to make cDNA arrays to investigate gene expression in different physiological states or genetic backgrounds (Viaud et al., 2003). This approach is now being used to identify gene expressed during plant infection (Gioti et al., unpublished data). Identification of new virulence factors will rely on such large-scale expression studies and from phenotypic analysis of gene inactivations.

The increasing number of EST data derived from fungal pathogens and related species in public databases provides the opportunity to compare sets of genes from different fungal pathogens. Unsurprisingly, 17% of *B. cinerea* unisequences had their most significant match with ESTs from the closely related species *B. graminis*. 28% had a significant match with ESTs from *M. grisea,* which is probably due to the high number of ESTs (>8000) generated in this species. Moreover, many of the putative pathogenicity related genes mentioned in our study were also present in other EST collections from fungal pathogens. In order to group and to investigate these EST data, bioinformatics resources have been developed (Soanes et al., 2002). The GPiDB database presented here allows to search 3026 unisequences from *B. cinerea* and to retrieve the corresponding ESTs by using text queries or homology searches.

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