Research Note

Starvation-Induced Genes of the Tomato Pathogen *Cladosporium fulvum* Are Also Induced During Growth In Planta

Mark Coleman, Beatrice Henricot, Jose Arnau, and Richard P. Oliver

Norwich Molecular Plant Pathology Group, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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The pathogenicity of fungal pathogens is presumably dependent on genes that are expressed during infection. In order to isolate such genes from the tomato pathogen *Cladosporium fulvum*, and to test the hypothesis that starvation-induced genes are also plant induced, a cDNA library was prepared from mycelia grown in a defined medium and then transferred to a starvation medium. The library was then screened with cDNA probes prepared from starved and replete fungal mycelium. Five unique, differentially expressed cDNAs were isolated from 1,000 clones screened. Northern (RNA) hybridization confirmed that all five were starvation induced. Interestingly, all five were also found to be plant induced. The identity of two of the clones was indicated by partial DNA sequencing as alcohol and aldehyde dehydrogenase. The observed correlation between starvation induction and plant induction is discussed.

Fungal phytopathogenicity is presumed to be dependent upon pathogen factors required for penetration, survival, symptom development, and growth within the plant host. Progress in isolating and characterizing genes required for pathogenicity has been reviewed (Oliver and Osbourn 1995; VanEtten et al. 1995; Bhattacharyya et al. 1997). Many groups have taken a direct approach to clone genes whose products were suspected to be required for disease development. Such genes include those encoding cell wall-degrading enzymes, enzymes degrading preformed or induced anti-fungal toxins and phytotoxin synthesis. In order to identify genes involved in novel or unsuspected steps in the infection process, other groups have used “black box” approaches such as the analysis of differential gene or protein expression and random mutagenesis. In the former case, genes induced by growth in

the plant (Pieterse et al. 1993a, 1993b; Talbot et al. 1993; Benito et al. 1996), specific to developmental stages (Hwang and Kolattukudy 1995; Justesen et al. 1996) or proteins only found in infected tissue (Honee et al. 1994; Marmeisse et al. 1994) have been isolated. In the latter case, mutants deficient in pathogenicity were isolated by, for example, a restriction enzyme-mediated integration method (Bölker et al. 1995). Direct proof of involvement in pathogenicity of the genes identified above has only been obtained in a handful of cases.

This paucity of direct evidence about the nature of pathogenicity determinants demands that new approaches be developed and tested. The isolation of pathogen genes expressed specifically during infection of the host plant has provided an alternative to predictive approaches for the identification of pathogenicity determinants. Differential screening of a *Phytophthora infestans* genomic DNA library with cDNA from infected plants and from in vitro-grown fungus identified nine genes that exhibited in planta-induced expression (Pieterse et al. 1991, 1993a, 1993b, 1994a, 1994b). The identity of many of these genes and their requirement for pathogenicity are at present unclear. The *MPGI* gene of *Magnaporthe grisea* was isolated by the differential screening of a cDNA library constructed from *M. grisea*-infected rice leaves. Presumed to encode a hydrophobin, its disruption resulted in a significant reduction in virulence (Talbot et al. 1993). With a differential display reverse transcriptase-polymerase chain reaction method, five *Botrytis cinerea* cDNAs were isolated but no homology was found with any known genes (Benito et al. 1996).

We are interested in identifying pathogenicity determinants of *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* (Cooke) Cif.) a non-obligate biotrophic pathogen of tomato (*Lycopersicon esculentum* Mill.). We noticed that a number of fungal pathogen genes that exhibit in planta-induced expression have been shown to be also expressed in vitro under conditions of nutrient deprivation. Thus, the *avr9* gene of *C. fulvum*, which encodes an elicitor of host defense, exhibits in vitro expression only in response to acute nitrogen deprivation (van den Ackerveken et al. 1994). The in planta-induced genes *ipiB* and *ipiO* from *P. infestans* also exhibit greatly enhanced expression in vitro in response to nutrient deprivation; for *ipiB*, carbon deprivation is sufficient to cause induction

Corresponding author: Richard P. Oliver; Fax: +45 3327 4766; E-mail: rpo@crc.dk

Present address of Beatrice Henricot and Richard P. Oliver: Physiology Department, Carlsberg Laboratory, Gamle Carlsberg Vej 10, Copenhagen, DK-2500, Denmark.

Present address of Jose Arnau: Biotechnology Institute, Kogle Alle 2, Horsholm, DK-2970, Denmark.

(Pieterse et al. 1994a). Similarly, *M. grisea MPG1* expression is induced in vitro in response to nutrient deprivation (Talbot et al. 1993). More recently, a search for genes that would impair nitrogen metabolism identified two genes, *NPR1* and *NPR2*, that are essential for *MPG1* expression and hence pathogenicity (Lau and Hamer 1996).

We set out to test the hypothesis that a significant proportion of in vitro starvation-induced genes of *C. fulvum* would also be plant induced. In the current study, we have isolated five cDNAs of *C. fulvum* genes that exhibit expression induced under conditions of carbon and nitrogen deprivation. Analysis of the expression of these genes during tomato infection showed that all five also exhibited induced expression in planta. Starvation may thus represent at least a component of the signal for the expression of genes required for growth on the host and the isolation of starvation-induced genes may provide a rapid means of identifying putative fungal pathogenicity determinants.

A cDNA library prepared in λ ZAPII (Stratagene, La Jolla, CA) from *C. fulvum* transferred to B5-NC was screened to select clones representing starvation-induced genes. The screening of approximately 1,000 plaques identified 10 clones that appeared to hybridize more strongly to the cDNA probe prepared from starved *C. fulvum*. These 10 cDNA clones were excised from λ ZAPII to yield plasmid clones, designated pSI-1 to pSI-10. DNA preparations of each were examined by a combination of restriction digestion, cross-hybridization, and partial sequencing to reveal which clones were unique. The clone *pSI-4* was found to be a short version of pSI-5 and pSI-10 was identical to pSI-2, 3, 6, and 8. This left five unique, putatively differentially expressed clones.

The differentially hybridizing cDNA clones pSI-1, pSI-5, pSI-7, pSI-9, and pSI-10 were used to probe Northern (RNA) blots containing (i) approximately equal quantities of poly A⁺ RNA isolated from *C. fulvum* grown under starvation and control conditions, and (ii) approximately equal quantities of total RNA isolated from tomato leaves harvested 8 or 14 days post *C. fulvum* inoculation and harvested from uninfected plants (Fig. 1). *C. fulvum* actin, which is presumed to be constitutively expressed, was used as a control. The hybridization patterns obtained confirmed that all five cDNA clones represented genes that exhibited starvation-induced expression (Fig. 1, lanes 1 vs. 2). Importantly, all five of the genes also appeared to be induced during pathogenesis, in that the hybridization to infected plant RNA (Fig. 1, lane 4) exceeded the hybridization to RNA from control, in vitro-grown mycelium (Fig. 1, lane 1). RNA from uninfected tomato plants hybridized to none of the probes (Fig. 1, lane 5).

To assess quantitatively the degree of induction of gene expression during starvation and growth in planta, phosphoimage analysis (Fuji BAS1000 IP) was used to quantitate the intensity of Northern blot hybridization signals. The fold induction under different conditions (relative to the fully fed mycelium) is shown in Table 1. These data clearly demonstrate that all of the identified starvation-induced cDNA clones represent genes expressed in planta and the level of in planta gene expression is very much greater than that exhibited during growth in full minimal medium. The genes fall broadly into two classes: one class whose expression in planta is relatively less than in vitro starvation conditions (pSI-9, pSI-10), and one class represented by pSI-1, pSI-5, and pSI-7,

for which there is a striking similarity between the degree of induction upon starvation and during growth upon tomato. Four of the clones (pSI-1, 7, 9, and 10) were essentially full length. There was a 300-fold range in the absolute signal intensities of the different clones following hybridization to RNA of starved mycelium, indicating the technique can identify weakly expressed genes.

Terminal DNA sequences obtained with vector primers were compared with the Owl data bases, using BLASTX (Altschul et al. 1990). Such analysis revealed a clear homology between the sequences of two of the clones and known

Fig. 1. Hybridization of pSI clones to polyA⁺ RNA from fully fed *Cladosporium fulvum* (lane 1), starved *C. fulvum* (lane 2), or total RNA 8 (lane 3) or 14 (lane 4) days tomato post-infection and (lane 5) uninfected plants. *C. fulvum* race 4 was grown in liquid B5 medium (Gamborg et al. 1968) with shaking at 100 rpm (Innova 2300, New Brunswick Scientific, Basingstoke, Hampshire, UK). Spores (1×10^5) prepared from 12-day-old, V8-grown *C. fulvum* were used to inoculate 250-ml flasks containing 50 ml of B5 medium. After 96 h, the mycelium was filtered and washed with B5 medium containing no carbon or nitrogen source (B5-NC); the washed mycelium was immediately transferred to a 250-ml flask containing 50 ml of B5-NC and shaken as before. The mycelium was collected by filtration 16 h after transfer, frozen in liquid nitrogen, and lyophilized prior to RNA isolation. The above constituted the "starvation conditions." Fully fed *C. fulvum* race 4 was grown and treated in an identical manner except that the mycelium was washed and resuspended in fresh B5 medium. Total RNA was isolated from uninfected or infected tomato leaves (Dickinson et al. 1993). RNA was prepared as in Logemann et al. (1987). PolyA⁺ RNA was prepared with Poly(A) Quick columns (Stratagene, La Jolla, CA).

genes (Table 1 and Fig. 2). pSI-9 and pSI-10 show most homology to aldehyde dehydrogenase (EC 1.2.1.3) of *C. herbarum* and alcohol dehydrogenase (EC 1.1.1.1) of *Aspergillus nidulans*, respectively. Complete sequencing of pSI-1 and pSI-5 has so far not revealed a convincing homology to any sequence in the data base. pSI-7 shows a strong homology to a yeast cDNA (YCR 68W; BLAST score 431) whose function is still unknown. However, hydrophobic domains present in the amino acid sequence suggest that it might be a membrane protein.

A significant proportion of the small group of fungal genes shown to be involved in pathogenicity is also induced during in vitro growth under starvation conditions (van den Ackerveken et al. 1994; Talbot et al. 1993). This observation invited the approach used in the current project; i.e., to use mRNA from starved, axenically grown mycelium as the source of the probe. The use of a cDNA library prepared from the same material obviates the need to subclone from a genomic library, makes it straightforward to establish the uniqueness of each clone by DNA sequencing, and gives direct access to the amino acid sequence of the putatively induced protein. It has the disadvantage that 5' untranslated regions are not readily identified.

The coincidence of starvation-induced clones with plant-induced clones is so far five out of five. This suggests that there is more than a coincidental link between the two induction conditions. It has long been noted that many, but not all, cell wall-degrading enzymes are catabolite repressed in plant-pathogenic fungi (Cooper et al. 1988). As such, these genes would be starvation induced. The transcription of the pisatin demethylase gene in *Nectria haematococca* is repressed in rich media (Straney and VanEtten 1994). Also, there are a few reports that auxotrophic mutants of plant pathogens are non-pathogenic, implying that pathways for the biosynthesis of nucleotides and amino acids are induced during growth in the plant (Keitt and Boone 1954; Puhalla and Anagnostakis 1971; Marmeisse et al. 1993). A more generalized relationship between the nutritional environment of a host organism and the pathogenicity of parasites has been hypothesized (Garber 1956; Lewis 1953). The particular role of starvation in inducing gene expression required for pathogenicity is supported by recent evidence on the hypovirulence-inducing mycovirus of *Cryphonectria parasitica* (Choi et al. 1995; Larson et al. 1992; Larson and Nuss 1994). It appears that the hypovirulence-inducing mycovirus attenuates the starvation-induced transcription of genes correlated with pathogenicity, a process that appears to be G-protein mediated (Gao and Nuss 1996).

Evidence of a link between nitrogen metabolism and pathogenicity has been recently demonstrated in *Magnaporthe grisea* by Lau and Hamer (1996). They showed that *npr1* and *npr2* mutants, deficient in various nitrogen source utilization, express low *MPG1* mRNA levels during nitrogen and carbon starvation and consequently are defective in pathogenicity. These findings, added to those reported herein, suggest that a direct study of the starvation response will throw light on the pathogenic process (see also Marzluf 1997).

An advantage of screening a cDNA library is that the amino acid sequences of the putative protein products are immediately apparent from the DNA sequence. Thus, partial DNA sequencing of these clones has provided putative identities of two out of the five clones. The identification of alcohol and aldehyde dehydrogenase as plant-induced genes was unexpected. The significance of the connection between starvation, alcohol metabolism, and pathogenicity, if any, will require more information before it can be fully assessed. Most importantly, it remains to be seen whether disruption of any of

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CHCLAH3      M-TSVQLETPHSGKYEQPTGLFINNEF
pSI-9        MSLSVQLETPSAGKYEQPTGLFINNEW

VKGQEGKTFDVINPSDESIVITQVHEATEKDVDIAVAAAR
VKPVSGKFFDVINPTDESVICQVAEAEKDVDIAVAAAR

QAFEGSWRLETPENRGKLLNNLANL
KRFEGPWRQETPENRGKLLVKLAEL

ADH1_EMENI   M--CIPTMQWAQVAEKVGGPLVYKQIP
pSI-10       MAGQIPQQQWAMVIEKTGGPVEYKQIP

VPKPGPDQILVKIRYSGVCHTDLHAMMGHWPIPVKMPLV
VQQPGPDEVLVNIKFSGVCHTDLHAVNGDWPLPTKLPLV

GGHEGAGIVVAKGELVHEFEIGDQAGIKWLNGSCGECEF
GGHEGAGVIVAKGNLVHDIEIGDHAGVKWINGSCLQCDF

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Fig. 2. Alignment of the pSI-9 open reading frame with the *Cladosporium herbarum* aldehyde dehydrogenase sequence (CHCLAH3) and of pSI-10 with the *Aspergillus nidulans* alcohol dehydrogenase gene (ADH1_EMENI).

Table 1. Characteristics of transcripts of starvation-induced clones

Clone name	Transcript		Infection (days)		Transcript size (bp)	Clone size (bp)	Accession no.
	Starvation abundance ^a	Starvation induction ^b	8 ^c	14 ^c			
pSI-1	25	26	6	25	860	834	Y14552
pSI-5	300	18	3	15	500	326	Y14553
pSI-7	1	5	ND ^d	3.6	2,240	2,166	Y14554
pSI-9	24	26	4	6	2,140	2,000	Y14555
pSI-10	82	92	16	4	1,250	1,250	Y14556

^a Abundance of transcript in starved mycelium relative to pSI-7.

^b Abundance of transcript in starved mycelium relative to full mycelium.

^c Relative abundance of transcript in 8 or 14 days infected plants relative to expression in full mycelium when corrected for fungal biomass as determined by actin expression.

^d Not determined reliably.

the genes isolated above will have any significant effect on pathogenicity.

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