The EMBO Journal vol.11 no.2 pp.473-477, 1992

A viral gene confers hypovirulence-associated traits to the chestnut blight fungus

Gil H.Choi and Donald L.Nuss[1]

Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ, 07110 USA

[1]Corresponding author

Communicated by A.van Kammen

A viral double-stranded (ds)RNA associated with reduced virulence (hypovirulence) and the accompanying biological control of the chestnut blight fungus, *Cryphonectria parasitica*, was shown recently to contain two contiguous coding domains designated ORF A and ORF B. We report here that transformation of an isogenic virulent, dsRNA-free *C.parasitica* strain with a cDNA copy of ORF A conferred traits similar to those exhibited by the dsRNA-containing hypovirulent strain: characteristics included reduced pigmentation, reduced laccase accumulation and suppressed conidiation. However virulence was not reduced, indicating an apparent uncoupling of associated traits from hypovirulence. These results establish a direct cause and effect relationship between a viral dsRNA genetic element present in a hypovirulent *C.parasitica* strain and specific phenotypic traits. They demonstrate further that these traits are not the result of a general reaction of the fungus to the presence of the replicating viral RNA, but are caused by a specific viral coding domain.

Key words: chestnut blight/*Cryphonectria parasitica*/hypovirulence-associated virus/transmissible hypovirulence

Introduction

Naturally occurring strains of the chestnut blight fungus, *Cryphonectria parasitica*, that exhibit reduced levels of virulence (hypovirulence) have been shown to control disease progress under certain conditions (Van Alfen *et al.*, 1975; Grente and Berthelay-Sauret, 1978; Anagnostakis, 1982, 1990). In addition to reduced virulence, these strains are distinguished by associated traits, e.g. reduced levels of sporulation and orange pigmentation (Elliston, 1978, 1985; Anagnostakis, 1982, 1984), and by the presence of dsRNA genetic elements (Van Alfen *et al.*, 1975; Day *et al.*, 1977). Conversion of dsRNA-free virulent strains to the hypovirulence phenotype is coincident with transmission of dsRNAs during anastomosis with compatible hypovirulent strains, providing the basis for disease control (Van Alfen *et al.*, 1975; Grente and Berthelay-Sauret, 1978; Anagnostakis and Day, 1979). Although there is considerable correlative evidence to support the proposal that dsRNA genetic elements are responsible for hypovirulence and associated traits, a direct cause and effect relationship has not been demonstrated previously.

Recent studies have provided an emerging view of the structural and functional properties of a hypovirulence-associated dsRNA. The largest dsRNA present in hypovirulent strain EP713, L-dsRNA (12 712 bp), was recently shown to contain two contiguous coding domains, designated ORF A and ORF B, consisting of 622 and 3165 codons, respectively (Shapira *et al.*, 1991b). Both open reading frames encode polyproteins that undergo autoproteolytic processing during translation (Shapira *et al.*, 1991b; Choi *et al.*, 1991b). Both proteolytic activites have been characterized by mutational analysis and were shown to resemble a growing family of virus-encoded papain-like proteases (Choi *et al.*, 1991a; Shapira and Nuss, 1991). Domains that contain putative RNA-dependent RNA polymerase and RNA helicase motifs have also been identified (Shapira *et al.*, 1991b; Koonin *et al.*, 1991). Due to the similarity of the L-dsRNA genetic organization and apparent expression strategy to those of several viral genomes, the term hypovirulence-associated virus (HAV) has been used to denote this class of genetic elements (Shapira *et al.*, 1991b).

Recent progress in elucidating the basic genetic organization of L-dsRNA and the development of a DNA-mediated transformation system for *C.parasticia* (Churchill *et al.*, 1990; Hillman *et al.*, 1989) has allowed us to address the question of whether the phenotypic traits exhibited by a hypovirulent *C.parasitica* strain are the result of a general response of the host to the physical presence of replicating dsRNAs, or whether these traits are dependent upon functions encoded by specific HAV sequences.

Results and discussion

The potential phenotypic contribution of the first open reading frame present in L-dsRNA, ORF A, was tested by transforming the virulent *C.parasitica* strain EP155 (which is isogenic to the L-dsRNA-containing hypovirulent strain EP713) with vector pAXHY2. This vector contains the ORF A coding domain fused upstream to the *C.parasitica* glyceraldehyde-3-phosphate dehydrogenase (*gpd-1*) promoter and fused downstream to the *gpd-1* terminator (Figure 1A). pAXHY2 also contains the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker flanked by the *trp*C promoter and terminator domains from *Aspergillus nidulans* (Cullen *et al.*, 1987). Plasmid pAXHY5, which contains ORF A in the reverse orientation, served as a control vector. Selected transformants were subjected to Southern hybridization analysis in order to confirm integration of the appropriate transformation vector. Two different sets of ORF A-containing fragments were liberated from each vector by *Pst*I digestion: fragments of 2.7 kb and 1.1 kb from pAXHY2 and two 1.9 kb fragments from pAXHY5 (illustrated in Figure 1A). As shown in Figure 1B, fragments of the expected size were observed when a blot containing *Pst*I-digested genomic DNA of four independent pAXHY2 transformants [designated (+) ORF A transformants A1, A5, A6 and A10] and two independent pAXHY5

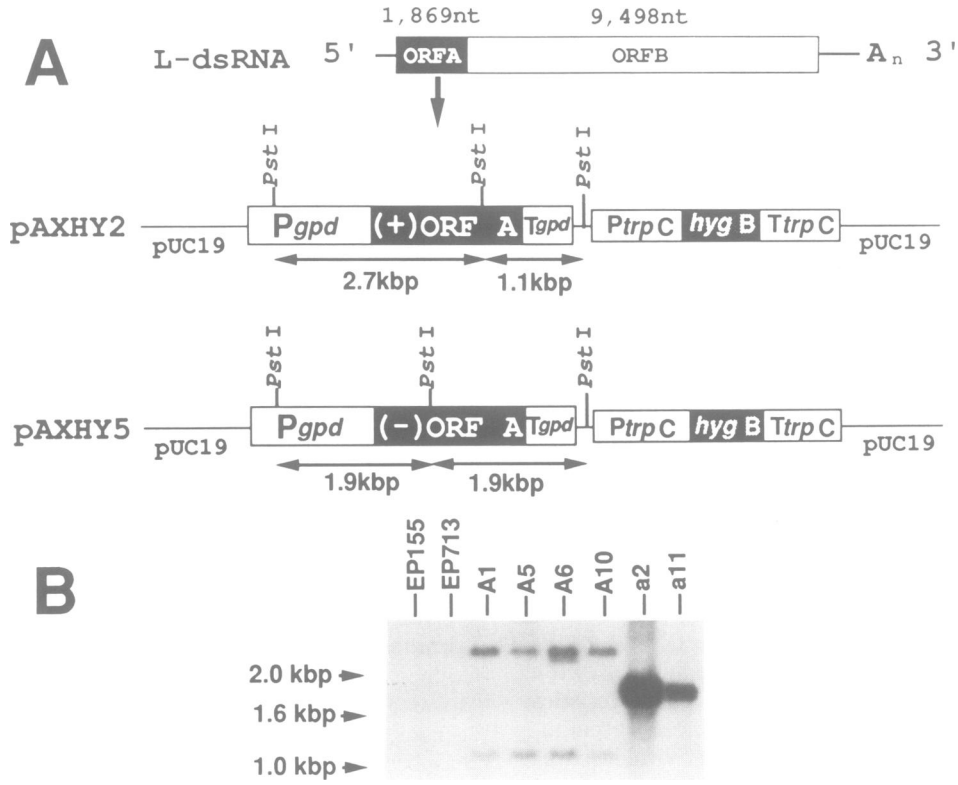

Fig. 1. Description of transformation vectors pAXHY2 and pAXHY5 and Southern analysis of *C.parasitica* transformants. **A.** Plasmid pAXHY2 contains the first coding domain of the HAV L-dsRNA, ORF A, flanked by the *C.parasitica* glyceraldehyde-3-phosphate dehydrogenase gene promoter (P*gpd*) and terminator (T*gpd*). It was shown that ORF A encodes a polyprotein that is autocatalytically processed into two polypeptides, designated p29 and p40 (Choi *et al.*, 1991b). The *gpd-1* promoter region extended 1696 nt upstream of map position 376 of the published *gpd-1* sequence, while the terminator extended from map position 1571 to position 2172 (Choi and Nuss, 1990). This plasmid also contains the *E.coli* hygromycin B phosphotransferase gene (*hyg*B) preceded by the *A.nidulans trp*C promoter (P*trp*C) (Cullen *et al.*, 1987), all in a pUC19 background. Plasmid pAXHY5 is the same as pAXHY2 except that the ORF A coding domain is in the antisense orientation. The location of *Pst*I sites within and flanking the ORF A coding region are also indicated. The arrows located under the ORF A region in each plasmid indicate the sizes of ORF A containing fragments that are expected to be liberated by *Pst*I digestion. **B.** Southern analysis of *Pst*I-digested genomic DNA prepared from four (+) ORF A transformants (A1, A5, A6 and A10), two (−) ORF A transformants (a2 and a11), untransformed strain EP155 and hypovirulent strain EP713, all probed with the ORF A coding sequence. The positions of marker fragments are indicated at the left.

transformants [designated (−) ORF A transformants a2 and a11] were probed with the ORF A sequence. Based on the relative intensity of the hybridized bands, it appeared that each of the four (+) ORF A transformants contained the same approximate number of copies of integrated pAXHY2 DNA, while the copy numbers for the two (−) ORF A transformants were several fold higher. No homology to ORF A sequences was observed for genomic DNA prepared from either strain EP155 or EP713.

The isogenic virulent and hypovirulent strains EP155 and EP713 clearly exhibit observable phenotypic differences that provide convenient markers for transformation studies aimed at examining the consequence of introducing HAV genetic information. When grown under standard laboratory conditions, strain EP155 is orange in color, conidiates abundantly and produces consistent levels of phenol oxidase activity of the laccase type. In contrast, strain EP713 appears white, is severely suppressed in conidiation and produces significantly reduced levels of laccase (Hillman *et al.*, 1990).

As indicated in Figure 2, transformation with the (−) ORF A vector pAXHY5 resulted in no alteration in colony morphology or reduction in orange pigmentation (compare transformants a2 and a11 with EP155). This result indicates that the introduction of vector sequences, including the hygromycin B phosphotransferase gene, does not significantly alter fungal phenotype. Remarkably, the four independent (+) ORF A transformants, A1, A5, A6 and A10, all resembled strain EP713. Orange pigment production was severely reduced throughout the culture period in which the colonies were actively expanding. However, unlike strain EP713, mature (+) ORF A transformant colonies developed a very low level of orange pigmentation. It is noteworthy that no alterations in pigmentation were previously observed among any hygromycin resistant isolates generated from numerous unrelated transformation experiments. In contrast, all (+) ORF A transformants, including the four presented here, showed reduced pigment production.

There have been several reports that hypovirulent *C.parasitica* strains are deficient in phenol oxidase activity of the laccase type (Rigling *et al.*, 1989; Hillman *et al.*, 1990) and that the difference in relative laccase levels can readily be detected by a color reaction on Bavendamm's medium (Rigling *et al.*, 1989). As indicated in Figure 3, strain EP155 produces a dark brown color when grown on this medium, while strain EP713 exhibits a very weak color reaction. Similar to the results observed for pigmentation, transformants a2 and a11 resembled EP155 in terms of color reaction while laccase accumuation was reduced in all four

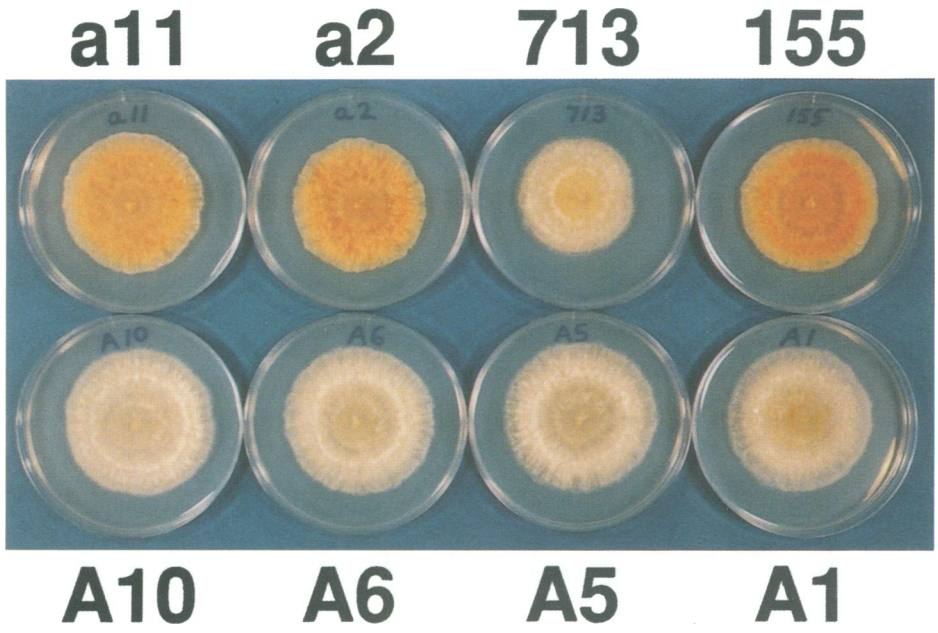

Fig. 2. Effect of ORF A transformation on pigmentation and colony morphology. Transformation of EP155 spheroplasts was performed essentially as described (Churchill *et al.*, 1990), followed by selection on hygromycin B-containing medium (40 μg/ml). Transformants used in this study were further selected on a higher level of hygromycin B (200 μg/ml). Transformants containing either vector pAXHY2 [(+) ORF A transformants, A1, A5, A6 and A10] or vector pAXHY5 [(−) ORF A transformants, a2 and a11] were grown in parallel with untransformed EP155 and the isogenic hypovirulent strain EP713 for five days on potato dextrose agar (Difco) on the laboratory bench (light <2000 lux, temperature 22−24°C as described (Hillman *et al.*, 1990).

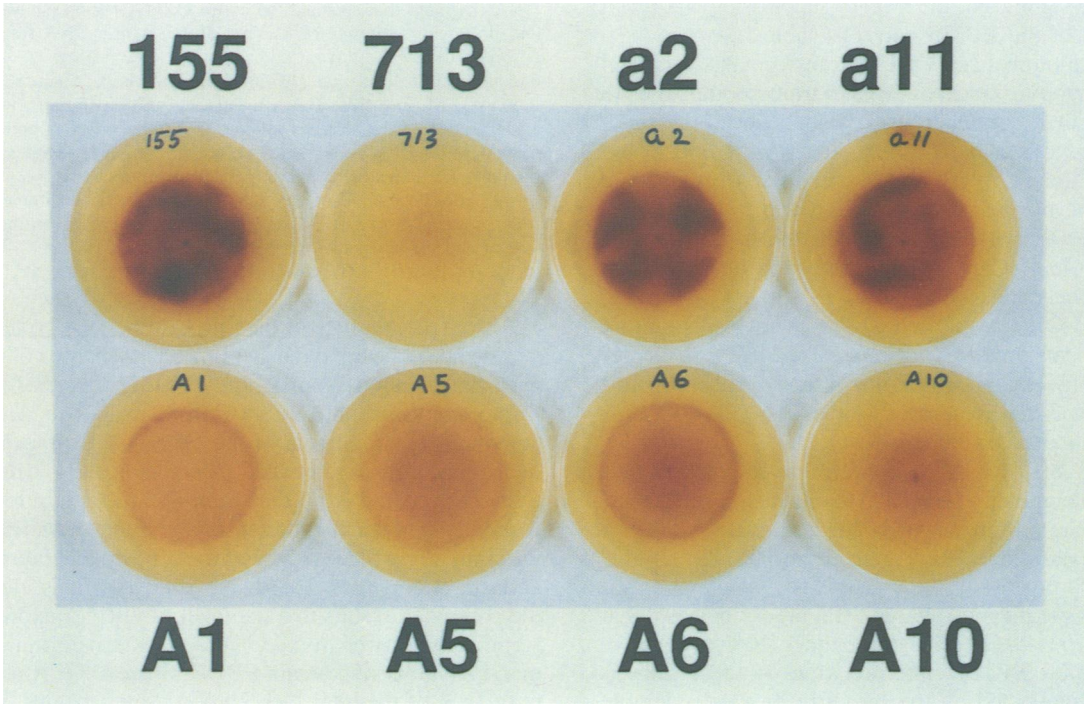

Fig. 3. Effect of ORF A transformation on accumulation of laccase activity. Strains EP155, EP713 and the (+) ORF A and (−) ORF A transformants were grown for five days in the dark at 22−24°C on Cellophane covering the surface of agar plates containing Bavendamm's medium (Bavendamm, 1928).

Table I. Effect of ORF A transformation on conidiation

Strain	Conidia/ml
EP155	1.6×10^7
EP713	$<1.0 \times 10^4$
a2	1.4×10^7
a11	1.7×10^7
A1	N.D.
A5	6.2×10^5
A6	9.5×10^5
A10	3.7×10^5

Colonies were grown on potato dextrose agar plates as described in Figure 2 for 10 days. Conidia were liberated with a glass rod after flooding plates with 10 ml of 0.15% Tween 80. The number of conidia was counted using a hemacytometer. Results are presented as average number of conidia per ml of the original 10 ml suspension. Strain designations are in Figure 2. N.D. = not determined.

(+) ORF A transformants. However, there was some apparent variation in the level of laccase reduction. Transformant A6 exhibited a slightly darker color reaction than did transformant A10, and laccase activity appeared to be reduced less in all four (+) ORF A transformants than in strain EP713.

Suppressed conidiation is another common hypovirulence-associated characteristic. The level of suppression in the case of strain EP713 is consistent and nearly complete under standard laboratory conditions (Hillman *et al.*, 1990, Table I). As indicated in Table I, the (−) ORF A transformants produced conidia at a level comparable to that of the untransformed EP155 control. In contrast, (+) ORF A transformants produced conidia at a level between 16−40-fold less than the level produced by the EP155 control. While significant, the level of suppression was much less than that of the EP713 control (Table I).

The observation that (+) ORF A transformants exhibited a number of hypovirulence-associated traits prompted us to examine whether virulence was also reduced in these transformants. Dormant chestnut stems were inoculated (Jaynes and Elliston, 1980) and the relative virulence, as a function of the mean canker radial growth, was analyzed (SAS system for personal computer release 6.04 from SAS Institute Inc., Cary, NC). There was no significant difference between the canker sizes for (+) ORF A (A6 and A10) and (−) ORF A (a2 and a11) transformants and all four transformants produced cankers at least five times larger than the EP713 cankers (data not shown). Based on this assay, there was no evidence that the (+) ORF A transformants were reduced in virulence.

The failure of ORF A to confer the complete hypovirulence phenotype may be related to one of several factors, including the requirement for a higher level of ORF A expression or additional HAV genetic information. In this regard, measurement of ORF A-specific single-stranded RNA by slot hybridization analysis (Figure 4) indicated that the ORF A (+) transformants contained < 10% of the level present in strain EP713. Interpretation of these data is potentially complicated by the presence of a considerable amount of internally deleted forms of the L-dsRNA species in strain EP713 (Shapira *et al.*, 1991a). However, PCR mapping analysis of the major defective RNAs have indicated that the deletion breakpoints lie within the internal portion of the L-dsRNA sequence, i.e. the 5′ terminal region containing ORF A appears to be intact (Shapira *et al.*,

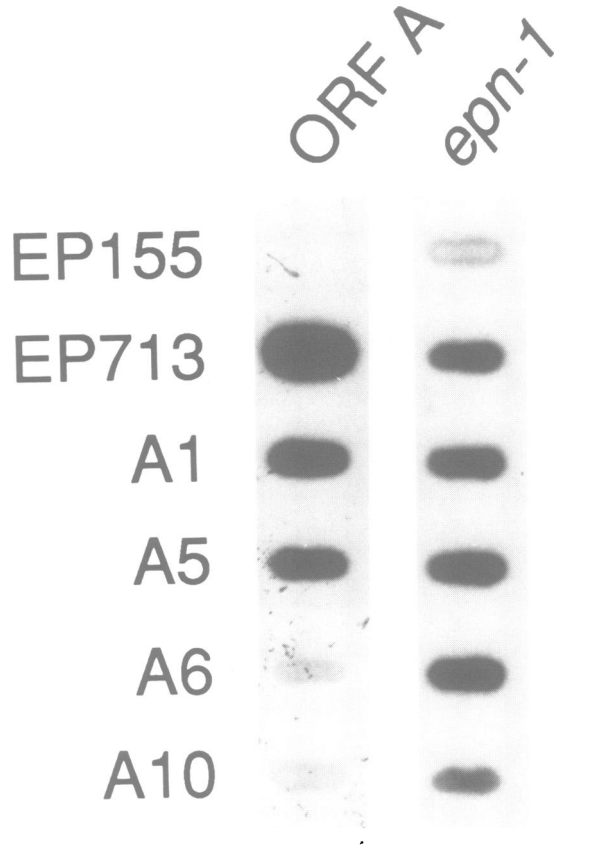

Fig. 4. Slot blot analysis of ORF A specific transcripts present in strain EP713 and in ORF A (+) transformants. As indicated in Materials and methods, each well was loaded with ~15 μg of denatured ssRNA. The same set of samples were probed first with a DNA fragment that spanned the entire ORF A region (shown at left) and, following stripping of the first probe, with a DNA fragment that corresponded to the coding domain of the *C.parasitica* endothiapepsin gene (*epn-1*, Choi,G.H., Rae,B.P., Shapira,R., Pawlyk,D.M. and Nuss,D.L., manuscript in preparation), to provide an indication of the relative amount of cellular mRNA applied to each well. The fungal strains from which the RNA samples were prepared are indicated at the left. Hybridization signals were quantified with the aid of an LKB Ultrascan laser densitometer. The relative amounts of ORF A-specific transcripts present in the fungal strains and transformants were 0%, 100%, 8%, 4%, <1% and <1% for EP155, EP713, A1, A5, A6 and A10, respectively.

1991a). The presence of the ORF A-encoded protein, p29, was demonstrated previously by Western analysis of mycelial extracts prepared from strain EP713 (Rae *et al.*, 1989). Using the same antisera, we were unable to detect p29 convincingly in extracts of the ORF A (+) transformants under conditions in which p29 was readily detected in extracts prepared from the strain EP713 (data not shown). It should be noted that the sensitivity of the Western analysis was limited by the low titer of the affinity-purified anti-p29 antisera (Rae *et al.*, 1989). Nevertheless, it is clear from the combined results that the level of ORF A expression is significantly lower in the ORF A (+) transformant strains than in hypovirulent strain EP713. Since ORF A represents only 16% of the total L-dsRNA coding capacity, it is also possible that additional L-dsRNA genetic information is required to confer the entire spectrum of the EP713 phenotype. Virulence and different associated characteristics such as pigmentation or laccase production could be affected by independent, or a combination of different L-dsRNA coding domains. The phenotypic consequences of intro-

ducing combinations of L-dsRNA coding domains and methods for increasing the level of expression of individual domains are currently being examined.

Irrespective of the mechanism involved, the apparent uncoupling of hypovirulence from associated traits is highly significant and raises the possibility that hypovirulent strains could be engineered to exhibit specific traits. It may be possible to delete portions of the HAV sequence that are, for example, responsible for reduced levels of sporulation while modifying other portions of the molecule to enhance hypovirulence. Introduction of these engineered molecules into virulent fungal strains by various means could then result in the generation of effective hypovirulent strains that can be disseminated more efficiently through abundant sporulation.

The most significant observation resulting from this study is that a specific L-dsRNA coding domain, in the absence of replicating HAV RNA, is sufficient to confer certain traits that are exhibited by the corresponding untransformed hypovirulent strain. This result establishes a direct cause and effect relationship between the viral dsRNA present in a hypovirulent *C.parasitica* strain and specific traits associated with that strain. Additionally, it demonstrates clearly that these phenotypic traits are not the result of some general reactions of the fungus to the physical presence of replicating viral RNA but are caused by specific viral coding domains. Finally, the combined results suggest that means are now available for elucidating the molecular basis of transmissible hypovirulence and for determining the feasibility of extending this form of biological control to other pathogenic fungi.

Materials and methods

Fungal strains and growth conditions

C.parasitica strains EP155 (virulent, dsRNA-free) and EP713 (isogenic to EP155, hypovirulent, contains L-dsRNA) were maintained as described previously (Hillman *et al.*, 1990). Transformed strains were grown on potato dextrose agar (Difco) on the laboratory bench (light <2000 lux, temperature 22–24°C) as described by Hillman *et al.* (1990). To test for laccase activity, colonies were grown in the dark at 22–24°C on Cellophane covering the surface of agar plates containing Bavendamm's medium (Bavendamm, 1928).

Transformation

Transformation vector pAXHY2 contained the entire first open reading frame, ORF A, of L-dsRNA flanked by the *C.parasitica* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd-1*) promoter and terminator and the *E.coli* hygromycin B phosphotransferase gene preceded by the *A.nidulans trpC* promoter (Cullen *et al.*, 1987) all in a pUC19 background (see Figure 1A). The *gpd-1* promoter region extended 1 696 nt upstream of map position 376 of the published *gpd-1* sequence, while the terminator extended from map position 1571 through position 2172 (Choi and Nuss, 1990). Transformation of EP155 spheroplasts was performed essentially as described by Churchill *et al.* (1990), followed by selection on hygromycin B-containing medium (40 μg/ml).

Nucleic acid preparation and analysis

Total nucleic acids were prepared from *C.parasitica* cultures by the method of Borges *et al.* (1990) with minor modifications. Mycelium (1–2g) was harvested, blotted dry and pulverised in liquid nitrogen with a mortar and pestle. The mycelial powder was transferred to a 50 ml polypropylene Corning centrifuge tube containing 15 ml of cold buffer (4 mM spermidine, 10 mM EDTA, 0.1 M NaCl, 0.5% SDS, 10 mM freshly added β-mercaptoethanol and 40 mM Tris–HCl, pH 8.0) and shaken vigorously. The mixture was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. To the aqueous phase was added 0.1 vol of 3 M sodium acetate (pH 5.5) and nucleic acids were precipitated by incubating on ice for 10 min after addition of 2 vol of cold ethanol. The precipitate was collected by centrifugation at 10 000 r.p.m. in a Beckman JS-13 rotor for 10 min, rinsed with 75% ethanol and dissolved in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). These preparations were used for Southern hybridization analysis after digestion with appropriate restriction endonucleases (Sambrook *et al.*, 1989).

For slot hybridization analysis, single-stranded (ss) RNA was selected from total nucleic acid preparations by precipitation with LiCl (final concentration of 2 M) for 4 h in ice followed by centrifugation at 10 000 *g* for 10 min at 4°C. Approximately 15 μg of ssRNA was denatured with deionized glyoxal (Sambrook *et al.*, 1989) and applied to a GeneScreen Plus membrane (DuPont) with the aid of a slot blot manifold (BRL). Hybridization was performed with ^{32}P-labeled DNA probes at 60°C in a solution containing 1% SDS, 1 M NaCl, 10% dextran sulfate and 100 μg salmon sperm DNA/ml. Following hybridization, blots were washed sequentially with 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min at room temperature, with 2 × SSC, 1% SDS at 60°C for 30 min and with 0.1 × SSC at room temperature for 30 min.

Acknowledgements

We thank D.M.Pawlyk for expert technical assistance.

References

Anagnostakis,S.L. (1982) *Science*, **215**, 466–471.
Anagnostakis,S.L. (1984) In Jennings,D.H. and Rayner,A.D.M. (eds), *The Ecology and Physiology of the Fungal Mycelium*, Cambridge University Press, Cambridge, UK, pp. 353–366.
Anagnostakis,S.L. (1990) *Forest Science*, **36**, 113–124.
Anagnostakis,S.L. and Day,P.R. (1979) *Phytopathology*, **69**, 1226–1229.
Bavendamm,W. (1928) *Z. Pflanzenkrank. Pflanzenschutz*, **38**, 257–276.
Borges,M.I., Azeveto,M.O., Bonatelli,R., Jr, Felipe,M.S.S. and Astolfi-Filho,S. (1990) *Fungal Genetics Newsletter*, **37**, 10.
Choi,G.H. and Nuss,D.L. (1990) *Nucleic Acids Res.*, **18**, 5566.
Choi,G.H., Pawlyk,D.M. and Nuss,D.L. (1991a) *Virology*, **183**, 747–752.
Choi,G.H., Shapira,R. and Nuss,D.L. (1991b) *Proc. Natl. Acad. Sci. USA*, **88**, 1167–1171.
Churchill,A.C.L., Ciufetti,L.M., Hansen,D.R., Van Etten,H.D. and Van Alfen,N.K. (1990) *Curr. Genet.*, **17**, 25–31.
Cullen,D., Leong,S.A., Wilson,L.J. and Henner,D.J. (1987) *Gene*, **57**, 21–26.
Day,P.R., Dodds,J.A., Elliston,J.E., Jaynes,R.A. and Anagnostakis,S.L. (1977) *Phytopathology*, **67**, 1393–1396.
Elliston,J.E. (1978) In MacDonald,W.L., Cech,F.C., Luchok,J. and Smith,H.C. (eds), *Proceedings of the American Chestnut Symposium*. West Virginia University Press, Morgantown, WV, USA, pp. 95–100.
Elliston,J.E. (1985) *Phytopathology*, **74**, 151–158.
Grente,J. and Berthelay-Sauret,S. (1978) In MacDonald,W.B., Cech,F.C., Luchok,J. and Smith,H.C. (eds), *Proceedings of the American Chestnut Symposium*. West Virginia University Press, Morgantown, WV, USA, pp. 30–34.
Hillman,B.I., Rae,B., Tartaglia,J. and Nuss,D.L. (1989) *Molecular Biology of Plant Pathogen Interactions*, Proc. UCLA Symposium, Vol. 101, pp. 59–70.
Hillman,B.I., Shapira,R. and Nuss,D.L. (1990) *Phytopathology*, **80**, 950–956.
Jaynes,R.A. and Elliston,J.F. (1980) *Phytopathology*, **70**, 453–456.
Koonin,E.V., Choi,G.H., Nuss,D.L., Shapira,R. and Carrington,J.C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10647–10651.
Rae,B.P., Hillman,B.I., Tartaglia,J. and Nuss,D.L. (1989) *EMBO J.*, **8**, 657–663.
Rigling,D., Heiniger,U. and Hohl,H.R. (1989) *Phytopathology*, **79**, 219–223.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
Shapira,R. and Nuss,D.L. (1991) J. Biol. Chem., **266**, 19419–19425.
Shapira,R., Choi,G.H., Hillman,B.I. and Nuss,D.L. (1991a) *EMBO J.*, **10**, 741–746.
Shapira,R., Choi,G.H. and Nuss,D.L. (1991b) *EMBO J.*, **10**, 731–739.
Van Alfen,N.K., Jaynes,R.A., Anagnostakis,S.L. and Day,P.R. (1975) *Science*, **189**, 890–891.

Received on September 10, 1991; revised on October 29, 1991