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Infrequent Transmission of Double-stranded RNA Virus Particles but Absence of DNA Proviruses in Single Ascospore Cultures of *Gaeumannomyces graminis*

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SUMMARY

A field isolate, 3b1a, of the wheat take-all fungus *Gaeumannomyces graminis* var. *tritici*, was previously shown to be infected with three serologically unrelated viruses, A, B, and C. It is now shown that virus B can be separated into two distinct strains, designated B1 and B2. All four viruses, A, B1, B2 and C, were faithfully transmitted into conidia. However, six out of eight single ascospore cultures derived from 3b1a single conidial cultures were shown to be virus-free. The remaining two single ascospore cultures each contained only one virus, which appeared to be the same in each culture. This virus was serologically indistinguishable from virus B1, but had dsRNA components of mol. wt. lower than those of virus B1 and had only the smaller of the two capsid polypeptide species of virus B1. No DNA provirus molecules homologous to viruses B1, B2 or C could be detected in two of the virus-free ascospore cultures or in one of the virus-infected ascospore cultures. Very low concentrations of virus particles were detected in hyphal tip isolates of *G. graminis*. After prolonged storage and subculturing of these isolates, the concentration of virus particles had increased to the level of the parent culture from which the hyphal tip isolates were derived.

INTRODUCTION

Isometric virus particles with genomes of double-stranded RNA (dsRNA) occur commonly in field isolates of the wheat take-all fungus, *Gaeumannomyces graminis* var. *tritici* (Rawlinson & Buck, 1981). Viruses, obtained from different fungal isolates, show considerable differences in their serological properties (Frick & Lister, 1978) and in the numbers and mol. wt. of their dsRNA and capsid polypeptide components (Buck *et al.*, 1981 *a*). Such variability could explain conflicting reports on the association of viruses with hypovirulence in the take-all fungus (Lapierre *et al.*, 1970; Lemaire *et al.*, 1970; Rawlinson *et al.*, 1973).

To obtain unequivocal evidence for the effect of a particular virus on the pathogenicity of *G. graminis* it is necessary to obtain isogeneic strains of the fungus with and without virus. Although infection of fungal protoplasts with partially purified virus preparations has been reported (Lhoas, 1971; Pallett, 1976; Ghabrial, 1980), many workers have found this to be an intractable problem (Bozarth, 1975). An alternative strategy would be to free infected cultures from virus particles ('curing'). Rawlinson *et al.* (1973) reported that extracts made from 56 single ascospore cultures derived from four virus-infected field isolates of *G. graminis* were free from virus particles within the limits of detection by electron microscopy. However, Tivoli *et al.* (1979) reported that, whereas no particles could be detected in several ascospore cultures of this fungus shortly after ascospore isolation, virus particles could be obtained from the same cultures after several months' storage. Their results suggested that either ascospores could transmit very low

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levels of virus particles which multiplied over a period of time, or that ascospores could carry virus in an 'eclipsed' state, such as naked dsRNA or DNA provirus.

To assess the potential of ascospore isolation for 'curing' infected *G. graminis* cultures, we analysed several single ascospore isolates derived from two single conidial isolates obtained from a field isolate, 3b1a, which had been shown to contain three serologically unrelated viruses, designated A, B and C (Buck *et al.*, 1981*a*). In the present paper we report that in the majority of ascospore cultures no virus particles, dsRNA or DNA provirus molecules could be detected. We further report the separation of virus B into two strains, designated B1 and B2, and on the transmission of B1 into two single ascospore isolates. The possibility of using hyphal tip cultures for 'curing' virus-infected *G. graminis* is also examined.

METHODS

The following methods were performed as described by Buck *et al.* (1981*a*): polyacrylamide gel electrophoresis (PAGE) of dsRNA; urea–PAGE of ssRNA; SDS–PAGE of polypeptides; electron microscopy; gel immunodiffusion analysis; agarose gel electrophoresis of virus particles.

Buffers. MOPS contained 0·02 M-sodium morpholinopropane sulphonate, 5 mM-sodium acetate, 1 mM-Na_2EDTA, pH 7·4; PBK-1 contained 0·03 M-NaH_2PO_4, 0·15 M-KCl adjusted to pH 7·6 with NaOH; PBK-2 contained 0·03 M-NaH_2PO_4, 0·3 M-KCl adjusted to pH 7·6 with NaOH; SET contained 0·5 M-Tris–HCl, 0·1 M-NaCl, 1 mM-Na_2EDTA, pH 6·9; TE contained 10 mM-Tris–HCl, 2 mM-Na_2EDTA, pH 7·6; TBE contained 0·09 M-Tris, 0·09 M-boric acid, 2·5 mM-Na_2EDTA, pH 8·2; TNE contained 10 mM-Tris–HCl, 0·15 M-NaCl, 2 mM-Na_2EDTA, pH 7·6.

Fungal cultures. G. graminis isolates 3b1a, F6 and F6 SM8 have been described previously (Buck *et al.*, 1981*a*). Single conidial isolates, con D and con E, derived from 3b1a, were kindly supplied by Dr J. W. Deacon. Perithecia were produced, and single ascospores and hyphal tips were isolated, essentially as described by Rawlinson *et al.* (1973). Growth of fungal isolates on solid and liquid media were as described previously (Buck *et al.*, 1981*a*).

Standard virus extraction. Fungal mycelium (10 g wet wt., approx. 2 g dry wt.) was homogenized by two passages through a Pascall Triple Roll Mill (Pascall Engineering Co., Crawley, West Sussex, U.K.) and then blended for 1 min at full speed in an Atomix (Measuring and Scientific Equipment Co., Crawley, West Sussex, U.K.) with 30 ml ice-cold PBK-1 buffer. After dilution to 100 ml with PBK-1 buffer and stirring at 4°C for 4 h, mycelial debris was removed by low-speed centrifugation. The virus was then partially purified by two cycles of high-speed (27000 rev/min; Beckman type 30 rotor, 16 h) and low-speed centrifugation and finally resuspended in 1 ml PBK-1 buffer. Preparations, in which virus particles could be detected at this stage, were further purified by centrifugation through linear density gradients of sucrose [10 to 40% (w/w) in PBK-1 buffer] at 27000 rev/min (Beckman SW27 rotor) for 3 h. Fractions containing virus particles were dialysed against PBK-1 buffer and stored at 4 °C.

Large-scale virus extraction. For increasing the sensitivity of detecting virus particles in ascospore isolates the procedure of Buck & Girvan (1977) with 1 to 5 kg wet wt. of mycelium was used with the following modifications. (i) Polyethylene glycol precipitates were resuspended in 5 times their wet wt. of PBK-2 buffer. (ii) Virus was pelleted at 30000 rev/min for 16 h in a Beckman Ti45 rotor. (iii) Virus pellets were resuspended in 20 to 30 ml PBK-1 buffer. (iv) Putative virus was not purified by sucrose density gradient centrifugation (SDGC) but was re-pelleted by centrifugation at 25000 rev/min in a Beckman type 65 rotor for 16 h and the pellet was resuspended in 1 ml PBK-1 buffer and examined for virus content by immune electron microscopy.

Binding of virus particles to bentonite. A 0·1 ml amount of bentonite suspension (5 mg/ml), prepared according to Fraenkel-Conrat *et al.* (1961), was mixed with 1 ml of virus in PBK-1 or TNE buffer and gently shaken for 5 min. The bentonite was pelleted (Beckman Microfuge B, full speed, 1 min) and the supernatant was removed. The bentonite was resuspended in 1 ml of buffer and then re-pelleted.

Standard dsRNA extraction. Nucleic acids were extracted from fungal mycelium (about 10 g wet wt.) essentially as described by Moffitt & Lister (1975). dsRNA was separated from DNA and ssRNA by chromatography on columns of CF 11 cellulose (Franklin, 1966). Nucleic acid in fractions eluting with SET buffer was precipitated with 2·5 vol. ethanol and 5 μg yeast tRNA as carrier.

Immune electron microscopy. This was carried out as described by Derrick (1973). Antisera to 3b1a viruses and F6 viruses (Buck *et al.*, 1981*a*) were diluted 1:10 and 1:100 in PBK-1 buffer.

Fungal DNA. This was extracted as described by Timberlake (1978). It consisted of nuclear (n)DNA and mitochondrial (m)DNA in the ratio of 4:1 by weight. Details of DNA characterization and determination of the genome sizes of *G. graminis* nDNA [29×10^6 base pairs (bp)] and mDNA (25×10^3 bp) will be reported elsewhere.

Agarose gel electrophoresis. DNA, or DNA restriction digests, were electrophoresed in 0·1 to 1·8% agarose horizontal slab gels in TBE buffer containing 0·005% ethidium bromide at 100 V for 4 to 18 h. DNA bands were located by fluorescence on an ultraviolet transilluminator. DNA samples to be sized were sometimes

electrophoresed in alkaline agarose gels (McDonell *et al.*, 1977). *Hin*dIII and *Eco*RI digests of phage λ DNA and φX174 DNA were used as standards.

Restriction endonuclease digestions. These were carried out for 1 h at 37 °C in the following buffers. *Eco*RI: 100 mM-Tris–HCl, 50 mM-NaCl, 5 mM-$MgCl_2$, 100 μg/ml bovine serum albumin, pH 7·0; *Hin*dIII: 6 mM-Tris–HCl, 50 mM-NaCl, 6 mM-$MgCl_2$, 100 μg/ml bovine serum albumin, pH 7·4. Reactions were terminated by addition of 0·1 vol. 10% SDS and the mixture was heated to 65 °C for 10 min.

DNA gel transfer hybridizations. DNA was transferred to nitrocellulose filters as described by Southern (1975). Hybridization with labelled ssRNA probe was carried out in a 50% formamide buffer at 42 °C for 18 to 48 h as described by Thomas (1980). ^{32}P-labelled RNA probes were prepared either from *G. graminis* RNA or from ssRNA transcripts, synthesized *in vitro* with 3b1a virion RNA polymerases (Buck *et al.*, 1981*b*). ssRNA was treated with 0·1 M-NaOH at 4 °C for 1 h to cleave it to an average size of 100 nucleotides (as assessed by urea–PAGE with wheat germ rRNAs and tRNA as markers) and then end-labelled with [γ-^{32}P]ATP and polynucleotide kinase (Szekely & Sanger, 1969).

RNA gel transfer hybridizations. These were carried out by a modification of the method of Thomas (1980). dsRNA in 0·1 × TNE buffer (17μl) was mixed with 30 μl formamide, 10 μl 37% formaldehyde, 3 μl 10 × MOPS buffer and incubated at 60 °C for 30 min. After cooling in ice the denatured RNA was electrophoresed in an agarose gel (0·6 to 1·5%) in MOPS buffer, containing 2·2 M-formaldehyde, at 100 V for 3 to 8 h. RNA transfer to nitrocellulose and hybridization to ^{32}P-labelled ssRNA probe was as described above, in the 'DNA gel transfer hybridizations' section.

RESULTS

Separation of virus B into two distinct viruses, B1 and B2

Bentonite was added to a mixture of viruses, A, B, and C, from *G. graminis* isolate 3b1a and then removed by centrifugation. Analysis of the dsRNA and polypeptide components of the bentonite and supernatant fractions by PAGE and SDS–PAGE, after disruption of the virus particles, showed that virus A became bound to the bentonite, while virus C did not. Virus B, however, was separated into two components. Of the four dsRNA components associated with this virus the bentonite-bound fraction contained only the two smaller dsRNAs and two polypeptide components (mol. wt. 73000 and 68000) in the ratio of about 10 :1. The supernatant contained the two larger dsRNAs and the two polypeptide components in the ratio of about 2 :1. [The mol. wt. of these polypeptides were reported erroneously by Buck *et al.* (1981*a*) as 68000 and 66000.] These results indicate that virus B is heterogeneous, being composed of two distinct types of particles designated B1 (bentonite-supernatant) and B2 (bound to bentonite).

Virus B had been shown previously to consist of particles of uniform size (35 nm diam.), to give rise to a single precipitin line in gel double-diffusion tests against an antiserum to 3b1a viruses, to migrate as a single band in agarose gel electrophoresis and to elute as a single peak from a column of DEAE-cellulose (Buck *et al.*, 1981*a*). It is likely, therefore, that B1 and B2 are related strains of a single virus. To determine this, unseparated B1 + B2 and a bentonite-supernatant fraction containing B1 were placed in adjacent wells in a gel and allowed to diffuse against the 3b1a antiserum. The single precipitin line formed by the mixture of B1 and B2 gave rise to a spur with the precipitin line formed by B1 alone, indicating that B1 and B2 are not serologically identical. We have been unable to find conditions for elution of B2 from bentonite that did not result in disruption of the virus particles. Therefore, no serological tests with separated B2 have been carried out.

3b1a virus B is serologically related to virus F6-B, one of three viruses isolated from *G. graminis* isolate F6 SM8 (Buck *et al.*, 1981*a*). Since 3b1a virus B has now been shown to be a mixture it was of interest to determine whether both B1 and B2 were related to F6-B. (There was no evidence for heterogeneity in F6-B, which contained only two dsRNA components.) In gel diffusion tests against an antiserum to F6 viruses (Buck *et al.*, 1981*a*) a mixture of B1 and B2 gave rise to a single precipitin line which formed a spur with the precipitin line formed by virus F6-B. When the precipitin line formed by B1 and B2 was cut out, incubated with SDS to disrupt the virus particles and analysed by PAGE, it was found that the dsRNA components of both B1 and B2 were present, i.e. B1 and B2 are both related to virus F6-B. This strengthens the view that B1 and B2 are related strains of the same virus.

Viruses in single conidial isolates

Since 3b1a is a field isolate of *G. graminis* it could be a mixture of individual fungal strains, some of which might lack one or more of the four viruses. To determine whether viruses can be transmitted into ascospores (sexual spores) it is necessary to start with genetically homogeneous infected fungal strains. Cultures derived from single conidia (asexual spores) are suitable for this since the conidia are single cells and are predominantly if not exclusively uninucleate (Deacon, 1976; Deacon & Henry, 1978). Two single conidial cultures from 3b1a, designated con D and con E, have been shown to contain virus particles (Rawlinson *et al.*, 1977). All of the nine dsRNA components found in 3b1a (Buck *et al.*, 1981*a*) were also found in each of the single conidial isolates. The cultures con D and con E therefore contained all the four viruses (A, B1, B2 and C) of 3b1a.

An estimate for the average number of dsRNA molecules per hyphal cell was obtained from the expression:

$$\frac{\text{Wt. dsRNA/unit wt. of mycelium}}{\text{Wt. nDNA/unit wt. of mycelium}} = \frac{\text{Mol. wt. dsRNA} \times \text{no. copies/cell}}{\text{Mol. wt. nDNA} \times \text{no. copies/cell}}$$

The wt. of nuclear DNA per g dry wt. of mycelium was 450 μg and the nuclear genome size was $2{\cdot}9 \times 10^6$ bp corresponding to a mol. wt. of $1{\cdot}8 \times 10^{10}$ (J. J. P. McFadden & K. W. Buck, unpublished results). *G. graminis* is haploid throughout the vegetative phase of its life cycle (Asher, 1981) and most hyphal cells are uninucleate (Chambers, 1970). The amounts of dsRNA obtained from isolate 3b1a con D and con E were similar (10 μg per g dry wt. of mycelium). Cell breakage (estimated microscopically) was 80% and the recovery (estimated by analysis of debris fractions and supernatants by PAGE) was about 70%. The amount of dsRNA per g dry wt. of mycelium was therefore 18 μg. The average mol. wt. of a 3b1a dsRNA component is $1{\cdot}8 \times 10^6$. Using these values the average number of dsRNA molecules per hyphal cell was calculated to be about 400. As each virus particle contains only one molecule of dsRNA (Buck *et al.*, 1981*a*), the numbers of virus particles per cell should also be approx. 400 if all the dsRNA is encapsidated.

In large-scale extractions the amount of virus obtained from 3b1a cultures was 70 μg per g dry wt. The cell breakage was 90% and the recovery (estimated as for dsRNA after SDS disruption of virus particles) was 80%. Therefore, the wt. of virus per g dry wt. of mycelium is approx. 100 μg. Taking the average value of 20% dsRNA for 3b1a viruses (Rawlinson *et al.*, 1973), this corresponds to 20 μg of dsRNA per g mycelium. This is in good agreement with the value obtained in direct dsRNA extraction and indicates that most if not all of the dsRNA is encapsidated in virus particles.

Production and analysis of single ascospore cultures

G. graminis is a homothallic ascomycete fungus and ascospores can be produced from a single homokaryotic strain. Cultures of both con D con E, under appropriate conditions, readily produced perithecia. A single perithecium was isolated from a con D culture and the asci and ascospores were allowed to extrude. Four single ascospores were selected at random and cultured. These cultures were designated con D/A1, con D/A2, con D/A3 and con D/A4. Similarly, four single ascospore cultures were obtained from con E and designated con E/A1, con E/A2, con E/A3 and con E/A4. Standard dsRNA extractions were carried out on all eight isolates, but dsRNA could be detected in only two of them, con D/A1 and con E/A2.

Isolation and characterization of virus particles from single ascospore isolates con D/A1 and con E/A2

Isometric virus particles, 34 to 36 nm in diameter, were isolated from cultures of both con D/A1 and con E/A2 and are designated viruses Asc 1 and Asc 2 respectively. In agarose gel electrophoresis, Asc 1 and Asc 2 comigrated with each other and also with B1 and B2. In gel immunodiffusion tests against antiserum to 3b1a viruses, Asc 1 and Asc 2 each gave single precipitin lines which fused when the viruses were placed in adjacent wells. Fusing precipitin lines were also obtained between strain B1 and either Asc 1 or Asc 2 virus. However, a spur was

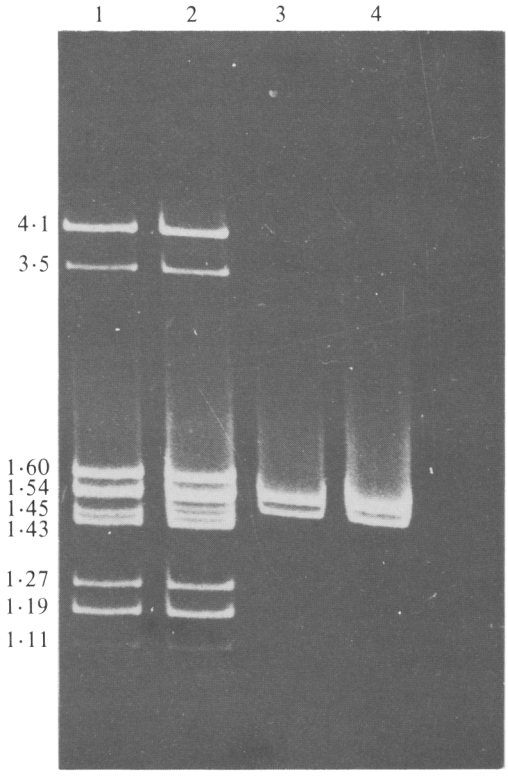

Fig. 1. PAGE of virus dsRNA samples. Lane 1, dsRNA from a mixture of 3b1a viruses A, B1, B2 and C; lane 2, dsRNA from a mixture of 3b1a viruses A, B1, B2 and C plus Asc 2 virus dsRNA; lane 3, Asc 2 virus dsRNA; lane 4, Asc 1 virus dsRNA. Electrophoresis, from top to bottom, was at 60 V for 24 h. The gel was stained with ethidium bromide and photographed on an ultraviolet transilluminator. Numbers at the side of the gel are mol. wt. $\times 10^{-6}$.

Table 1 *Properties of viruses in* G. graminis *isolate 3b1a and in two derived ascospore isolates*

Virus	Particle diam. (nm)	dsRNA components (mol. wt. $\times 10^{-6}$)	Polypeptide components (mol. wt. $\times 10^{-3}$)	Electrophoretic mobility (mm/h) towards anode
3b1a-A*	40	4·1, 3·5	87, 83, 78	2·1
3b1a-B1†	35	1·60, 1·54	73, 68	1·4
3b1a-B2	35	1·45, 1·43	73, 68	1·4
3b1a-C*	35	1·27, 1·19, 1·11	55	1·1
3b1a con D/A1-Asc 1†	35	1·52, 1·47	68	1·4
3b1a con E/A2-Asc 2†	35	1·52, 1·47	68	1·4

* Data from Buck *et al.* (1981*a*).
† These viruses were serologically indistinguishable.

formed when either Asc 1 or Asc 2 virus and a mixture of strains B1 and B2 were placed in adjacent wells. It was concluded that Asc 1, Asc 2 and B1 are serologically indistinguishable; however, they are all serologically distinct from B2.

The dsRNA components of Asc 1 and Asc 2 viruses were examined by PAGE. In both cases only two dsRNA components were detected (Fig. 1) and in mixtures the dsRNA components of Asc 1 and Asc 2 viruses comigrated. On co-electrophoresis of Asc 1 dsRNAs and 3b1a dsRNAs in slab gels (Fig. 1) it is seen that the higher mol. wt. Asc 1 dsRNA band comigrated with the B1 dsRNA component of mol. wt. $1{\cdot}54 \times 10^6$, but that the lower mol. wt. Asc 1 dsRNA band migrated between the latter component and the B2 dsRNA component of mol. wt. $1{\cdot}45 \times 10^6$.

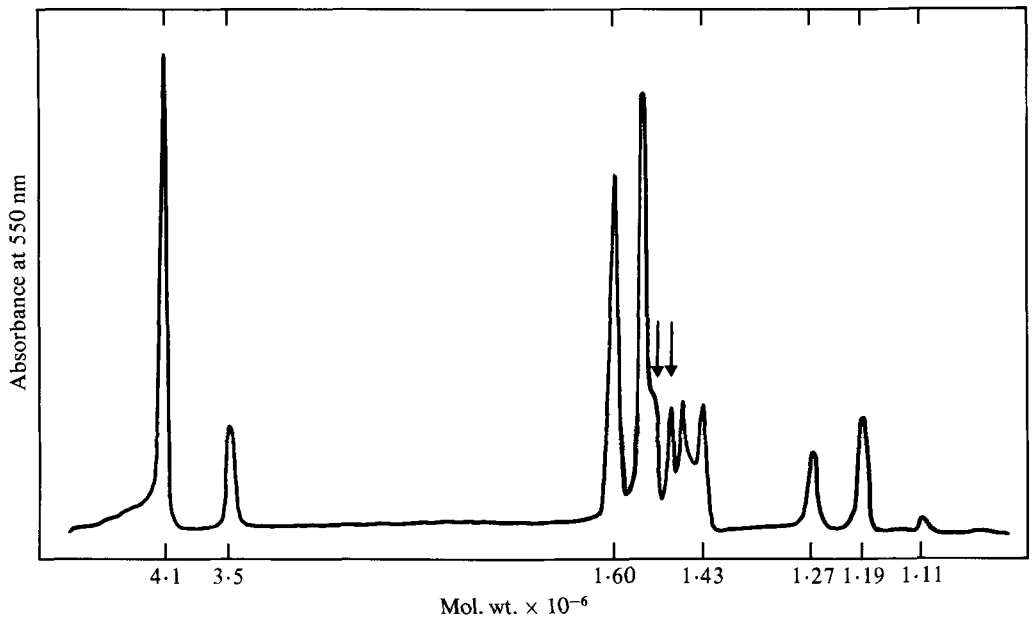

Fig. 2. Gel scan of a mixture of dsRNAs from 3b1a viruses A, B1, B2 and C, and Asc 2 virus, after PAGE in a cylindrical gel at 6 V/cm for 24 h. The gel was stained with toluidine blue and scanned at 550 nm. Asc 2 virus dsRNA components are indicated by arrows.

However, when the co-electrophoresis was carried out in cylindrical gels, which gave increased resolution, it was found that both of the Asc 1 virus dsRNAs had mobilities distinct from all of the nine dsRNAs of 3b1a viruses (Fig. 2). The mol. wt. of Asc 1 (and hence also Asc 2) dsRNAs were estimated to be $1{\cdot}52 \times 10^6$ (2360 bp) and $1{\cdot}47 \times 10^6$ (2290 bp). On analysis by SDS–PAGE both Asc 1 and Asc 2 viruses were found to contain a single polypeptide species of mol. wt. 68000. The average number of virus particules per hyphal cell was estimated to be 20 for Asc 1 virus and 50 for Asc 2 virus.

The properties of Asc 1 and Asc 2 viruses are compared with those of the four viruses of 3b1a in Table 1.

Search for virus particles and dsRNA molecules in single ascospore isolates con D/A2, con D/A3, con D/A4, con E/A1, con E/A3 and con E/A4

Detection of dsRNA

dsRNA from standard extractions of approx. 2 g dry wt. of fungal mycelium derived from each of the above six ascospore isolates was analysed by PAGE. No dsRNA could be detected in any of the isolates. Because the minimum amount of dsRNA detectable in one band with ethidium bromide staining was 100 ng, and, by using the calculation described above, the minimum number of molecules of each of the nine 3b1a dsRNA species per cell which could be detected is two, then the sensitivity of the method is not quite great enough to be sure that the above ascospore isolates are completely free from dsRNA.

We therefore developed a gel transfer hybridization (blotting) technique which increased the sensitivity 1000-fold (see Methods). After agarose gel electrophoresis of denatured dsRNA, bands corresponding in mobility to both ssRNA components and dsRNA components could be detected if gels were stained with toluidine blue. Surprisingly, after transfer to nitrocellulose, both 'dsRNA' and ssRNA bands hybridized with the labelled probe (Fig. 3*a*). It is likely that the 'dsRNA' resulted from partial re-annealing of denatured RNA (or partial denaturation of dsRNA), leaving sufficient unpaired bases for hybridization with the probe. By this method

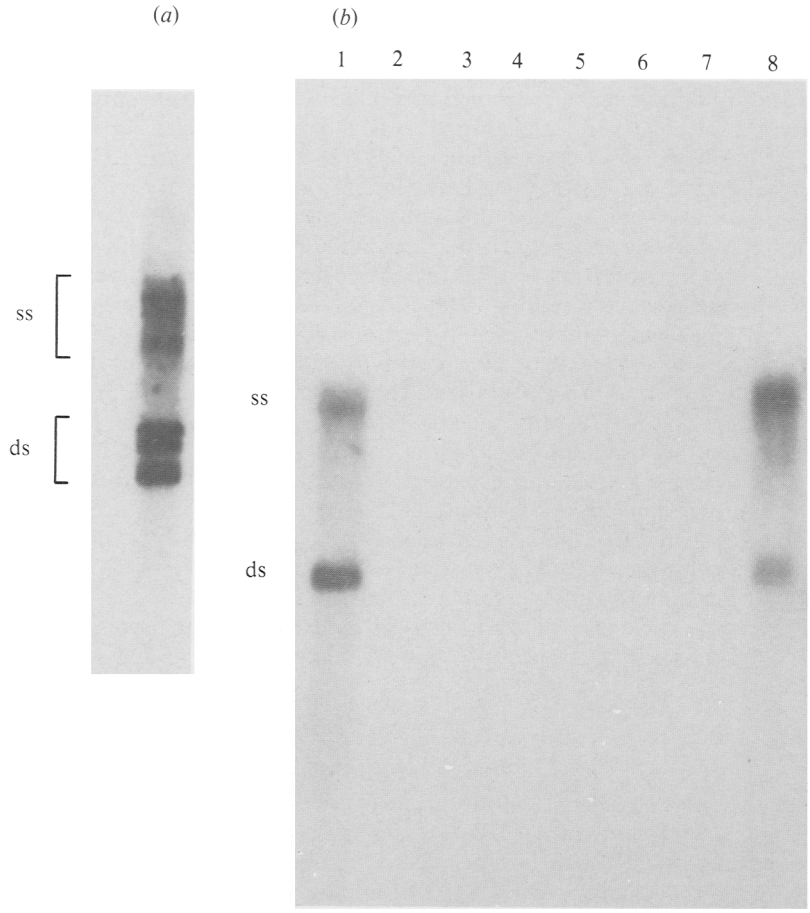

Fig. 3. Gel transfer hybridization of dsRNA from 3b1a viruses. dsRNA was denatured, electrophoresed at 10 V for 6 h in a 1% agarose/formaldehyde gel, transferred to nitrocellulose and hybridized with a ^{32}P-labelled probe ($0{\cdot}5 \times 10^7$ ct/min/μg), prepared from ssRNA transcripts of 3b1a virus B1, B2 and C dsRNAs. The 'blots' were autoradiographed for 24 h. Bands migrating in the positions of dsRNA and ssRNA for each virus are indicated. Electrophoresis was from top to bottom. (*a*) dsRNA from a mixture of 3b1a viruses A, B1, B2 and C. (*b*) Lanes 1 and 8, RNA extracts from 20 mg dry wt. of mycelium of con E/A2 and con D/A1 respectively. Lanes 2 to 7, RNA extracts from 2 g dry wt. of mycelium of con E/A1, con E/A3, con E/A4, con D/A2, con D/A3 and con D/A4 respectively.

0·1 ng RNA in a band could be detected. However, resolution in agarose was not as good as in polyacrylamide, and bands closely spaced in PAGE were unresolved in the agarose gels. Furthermore, since virus A has no transcriptase activity (Buck *et al.*, 1981*b*), ssRNA probes of this virus were not available, and virus A dsRNA molecules could not be detected by this method.

When the method was applied to the eight ascospore isolates derived from con D and con E, dsRNA from 20 mg dry wt. of con D/A1 and of con E/A2 was readily detected (Fig. 3*b*, lanes 1 and 8). However, no bands could be detected when RNA from 2 g dry wt. of the other six ascospore isolates was analysed (Fig. 3*b*, lanes 2 to 7). It is concluded that these isolates could not contain more than one copy of any of the dsRNA components of viruses B1, B2 or C per 500 hyphal cells.

Detection of virus particles

In small scale extractions virus particles from approx. 2 g dry wt. of mycelium were resuspended in 1 ml of buffer (without purification by SDGC) and examined by electron

microscopy. Serial dilutions of 3b1a virus preparations showed that the limit of detection (an average of one particle per field) corresponded to approx. 1 μg/ml, equivalent to two virus particles per hyphal cell. In partially purified virus preparations, one virus particle in an electron microscope field containing much cell debris was difficult to identify unequivocally; the lower limit of detection for crude virus preparations was about ten particles per hyphal cell.

To be sure that ascospore isolates were completely free from virus particles, the sensitivity of detection was increased in two ways. (1) Between 200 g and 1000 g dry wt. of mycelium was used. Virus particles were isolated by polyethylene glycol (PEG) precipitation and ultracentrifugation and were resuspended in 1 ml of buffer. A trial of the method with con D/A1, which has only 5% of the virus particles of 3b1a, showed that virus particles were recovered with high efficiency (overall recovery approx. 70%). This is because the virus co-precipitates at the PEG stage, and co-pellets at the ultracentrifugation stage, with a considerable amount of cellular material. This method increased the sensitivity of detection between 10- and 50-fold. (2) Grids coated with antiserum to 3b1a viruses were used to examine virus preparations in the electron microscope. This had two advantages. First, virus particles in crude preparations were selected and concentrations as low as 40 ng/ml could be detected. Secondly virus A (diam. 40 nm) could be detected and distinguished from viruses B1, B2 and C (diam. 35 nm). This method gave a further increase in sensitivity of 25-fold.

When methods (1) and (2) were applied to con D/A1 and con E/A2, virus particles were readily detected in crude preparations even after 1000-fold dilution. However, no virus particles could be found in any of the other six ascospore isolates. It was concluded that there could be no more than one virus particle in 1000 hyphal cells in these isolates. The results are in agreement with the dsRNA analysis in the previous section and moreover extend that analysis to include virus A.

Search for DNA proviruses in single ascospore isolates con D/A2, con E/A1 and con E/A2 by gel transfer hybridization

DNA from the three ascospore cultures was digested with *Eco*RI and *Hin*dIII and the fragments were electrophoresed in agarose alongside undigested DNA, transferred to nitrocellulose and hybridized with 1 μg of ssRNA transcripts of viruses B1, B2 and C, end-labelled (^{32}P) to a specific activity of $4{\cdot}7 \times 10^7$ ct/min/μg. The blot was incubated at 42 °C for 72 h, washed and autoradiographed. No bands were visible. To ensure that hybridization conditions were not too stringent for formation of hybrids, the experiment was repeated with the hybridization reaction taking place at 30 °C. Again no bands were detected. To confirm that the DNA on the filter was available for hybridization, the filters were hybridized with a probe consisting principally of *G. graminis* ribosomal RNA. Ribosomal DNA fragments of the expected sizes (J. J. P. McFadden & K. W. Buck, unpublished results) were seen in each of the DNA tracks on the filter. Hence, the DNA was available for hybridization with homologous probe.

To conclude that no DNA provirus copies of viruses B1, B2 and C dsRNAs exist in *G. graminis* DNA it is necessary to consider the sensitivity of the gel transfer hybridization experiment. If the smallest dsRNA component of 3b1a viruses (mol. wt. $1{\cdot}11 \times 10^6$, 1730 bp) is represented as a single DNA copy it would represent 0·006% of the *G. graminis* genome ($2{\cdot}9 \times 10^7$ bp). Hence, the 10 μg of DNA used for gel transfer would contain 3×10^{-4} μg of viral cDNA (for one strand). The ssRNA probe had a specific activity of $4{\cdot}7 \times 10^7$ ct/min/μg. Assuming 90% hybridization (from estimations of the C_0 t values attained), 12000 ct/min would be hybridized in a single cDNA band or 6000 ct/min if the cDNA were cut once by the restriction endonuclease. Since 100 ct/min of ^{32}P in a single band could be detected by autoradiography, the results suggest not more than one DNA provirus copy per 120 *G. graminis* nuclear genomes (or 60 if the cDNA were cut once).

The sensitivity of the method was also estimated experimentally. Known amounts of dsRNA from a mixture of 3b1a viruses were added to samples of 10 μg of *G. graminis* DNA (from con E/A1) that had been digested with *Eco*RI. The mixtures were denatured, electrophoresed, transferred to nitrocellulose and hybridized with the same ssRNA probe used for detection of

DNA provirus. The minimum amount of dsRNA detectable was 10^{-5} μg per band (equivalent to $0{\cdot}5 \times 10^{-5}$ μg of single-stranded cDNA). This indicates a lower degree of sensitivity, i.e. not more than one DNA provirus copy per 60 nuclear genomes (or 30 if the cDNA were cut once), probably because the efficiency of transfer and hybridization of dsRNA is less than that of DNA.

Since total cellular DNA, in which there were 100 copies of mDNA per nDNA genome, was used in these experiments, the sensitivity for detecting a provirus in mDNA is 100 times as great as that for nuclear DNA. It is noteworthy that DNA proviruses to viruses B1, B2 and C could not be detected either in an ascospore culture containing dsRNA (con E/A2) or in two ascospore cultures free from dsRNA (con D/A2 and con E/A1). ssRNA probes to virus A dsRNAs were not available and no tests on possible cDNA copies of virus A dsRNAs were carried out.

Virus particles in hyphal tip cultures of G. graminis

Apical hyphal cells of some virus-infected fungi are free from virus-like particles visible in thin sections (Border *et al.*, 1972). Culture of excised apical hyphal cells, sometimes after thermotherapy, has been used to obtain cultures which were apparently virus-free (Rawlinson *et al.*, 1973) or contained reduced amounts of dsRNA (Castanho & Butler, 1978). We therefore compared hyphal tips of *G. graminis* isolates F6 SM8 with ascospores as sources of virus-free cultures. F6 SM8 contains three serologically unrelated viruses, F6-A, F6-B and F6-C (Buck *et al.*, 1981*a*). These are related to 3b1a viruses A, B1 and B2, and C respectively. Culture F6 SM8 contained an average of 450 virus particles per cell by the methods described for 3b1a condial cultures.

A hyphal tip isolate of F6 SM8 was taken through ten successive hyphal tip subcultures, and two of the final cultures, designated HT1 and HT2, were retained for study. No virus particles or dsRNA components were detectable in standard extractions of HT1 or HT2. However, virus particles were readily detected in crude preparations by immune electron microscopy with F6 antiserum. Comparisons with standard dilutions of F6 viruses indicated 250 ng of virus particles per g dry wt. of HT1 or HT2 mycelium, corresponding to one or two virus particles per cell. But, 18 months and several subculturings after isolation, standard virus and dsRNA extractions revealed all of the original dsRNA components, and also virus particles, in amounts similar to those of the parent culture F6 SM8.

Thus, hyphal tip cultures may retain very low levels of virus particles, which subsequently multiply, and are therefore not suitable for producing isogeneic lines of *G. graminis* with and without virus. We did not wish to use thermotherapy in the present studies since heat can act as a mutagen or as a selective agent for mutants (Burnett, 1975) and the fungal cultures were ultimately required for pathogenicity testing.

DISCUSSION

A virus exclusion mechanism operates during ascospore formation in *G. graminis*. Virus transmission occurred in only two out of eight ascospores examined and even in these two cases only one out of four viruses was transmitted. The remaining six ascospore cultures contained no detectable dsRNA or virus particles. No DNA provirus molecules, homologous with virus B1, B2 or C dsRNAs, could be detected in two ascospore cultures devoid of dsRNA and virus particles, or in one ascospore culture that contained dsRNA virus particles. Production of ascospores from a homokaryotic conidial strain is therefore a satisfactory method of generating isogeneic strains of *G. graminis* with and without virus. It is emphasized, however, that since virus transmission into ascospores can occur, very sensitive methods of detection are needed to ensure that ascospore cultures are completely virus-free.

The appearance of virus particles after storage of apparently virus-free ascospore cultures of *G. graminis* for several months, reported by Tivoli *et al.* (1979), is probably due to transmission of low levels of virus particles into some ascospores. We could find no evidence to support the hypothesis of transmission of DNA proviruses or of naked dsRNA. Our calculations show that very low levels of dsRNA and virus particles could go undetected, unless very sensitive methods of detection are used. We have obtained apparently analogous results with hyphal tip cultures of

G. graminis which appeared to be virus- and dsRNA-free by standard extraction procedures, but immune electron microscopy revealed a low level of virus particles. After storage of the hyphal tip cultures for 18 months, virus particles and dsRNA had increased to the levels of the parental fungal isolate.

In the two cases in which virus transmission into ascospore cultures occurred it appears that the transmitted viruses, Asc 1 and Asc 2, are identical, even though the two ascospore cultures were derived from different conidial isolates. This could indicate a greater resistance of these viruses to the exclusion mechanisms or could be fortuitous. Both Asc 1 and Asc 2 viruses are serologically indistinguishable from B1 virus, but are not identical to it (Table 1). Their dsRNA components are smaller than those of B1 virus and therefore it appears that small deletions in B1 dsRNAs occurred during transmission.

Rawlinson *et al.* (1973) found that most isolates of *G. graminis* from cereal crops after a break of fallow or non-susceptible crops were free from virus particles as judged by standard extraction and detection techniques. However, after three consecutive cereal crops about 80% of isolates contained virus particles readily detected by the standard methods. These and the present results suggest that ascospores may be a more important propagule for the dissemination of *G. graminis* into areas previously free from the fungus than previously thought (Hornby, 1981). If a small proportion of an ascospore population carried low levels of virus particles, these low levels could gradually increase within an individual fungal strain over a period of time (as we have observed in hyphal tip isolates) and the viruses could be transmitted through the fungal population by hyphal anastomoses between different fungal strains. Furthermore, alterations in dsRNA or polypeptide components of a virus following ascospore transmission could help to explain some of the variability found in virus particles from different isolates of *G. graminis*, including those taken from a single field (Frick & Lister, 1978; Buck *et al.*, 1981*a*).

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