Complex of Virus-Like Particles Containing Double-Stranded RNA from *Thielaviopsis basicola*

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Six randomly selected isolates of *Thielaviopsis basicola* were found to contain spherical virus-like particles (VLPs) approximately 40 nm in diameter. One isolate, ATCC 34114, selected for further analysis contained a complex of VLPs that sedimented as eight or more bands in sucrose density gradients and contained five size classes of double-stranded RNA. Five discrete precipitation lines were obtained in immunoelectrophoresis, which indicated that this isolate of *T. basicola* contains five distinct species of VLPs.

Virus-like particles (VLPs) of fungi are of interest to mycologists because they potentially affect the growth habits or production of secondary metabolites of fungi and to virologists because they contain double-stranded RNA (ds-RNA) as their genome (3, 14, 18). In an effort to extend our knowledge of VLPs among the fungi that are important root pathogens of crop plants, we tested six isolates of *Thielaviopsis basicola* from the collection of G. Papavizas (U.S. Department of Agriculture, Beltsville, Md.) for their presence. All isolates tested were found to contain spherical VLPs approximately 40 nm in diameter. One isolate that was especially slow growing was selected for further study of its VLPs.

MATERIALS AND METHODS

T. basicola ATCC 34114 was grown at 25°C in a complex corn steep medium on still culture (W. M. Stark, W. J. Kleinschmidt, and G. W. Probst, U.S. Patent 3,108,047, Oct. 1963), harvested after 7 days, and purified by differential centrifugation (5). Final purification by ultracentrifugation in a zonal rotor (5) resulted in a complex of centrifugal components. Fractions were examined by electron microscopy and UV spectroscopy. Portions of fractions containing VLPs were pooled to form a reconstituted sample. One OD₂₆₀ (optical density at 260 nm) unit of the reconstituted sample, the original sample, and each of the tubes containing VLPs were centrifuged on a 10 to 40% linear sucrose density gradient and fractionated on an ISCO (ISCO, Lincoln, Neb.) density gradient fractionator (13).

Antiserum to the reconstituted sample was prepared by intramuscular injections of a rabbit with the VLP preparation mixed with Freund complete adjuvant (Difco, Detroit, Mich.).

Established methods or previously described tech-

niques were used for serology (2, 13), immunoelectrophoresis (7), orcinol tests (11), diphenylamine tests (11), extraction of nucleic acid (10, 17), thermomelting (T_m) (5), Cs₂SO₄ density gradient centrifugation (4), and polyacrylamide gel electrophoresis of ds-RNA (6, 15).

RESULTS

The VLP preparation purified by zonal centrifugation contained overlapping bands that sedimented at the rate of approximately 100 to 150S.

The bands were fractionated into eleven fractions (7 through 17). Each fraction was analyzed by electron microscopy, UV absorbancy, and sucrose density gradient centrifugation (Fig. 1). Fraction 7 contained particles that were penetrated by the uranyl acetate, and the ratio of UV absorbancy (260/280 nm) was 0.71. In fractions 15 through 17 most particles were not penetrated by the uranyl acetate, and the 260/280 ratio was 1.47. Intermediate fractions contained both penetrated and unpenetrated particles, and the 260/280 ratios were intermediate.

When portions containing approximately 1 OD₂₆₀ unit from each of the fractions were centrifuged on sucrose density gradients, each fraction contained several overlapping bands. (Figure 1 shows only the odd-numbered fractions.) Eight bands could be distinguished, which corresponded in sedimentation velocity to eight bands in the unfractionated sample (prezonal sample, Fig. 1). Pooling portions from each fraction resulted in a reconstituted sample that had the same pattern in sucrose density gradient analysis as the prezonal sample in the region of the VLPs but was free of the contaminating substances that banded above and below the VLP region of the gradient. This sample contained only VLPs in sucrose density gradient

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and electron microscopic analysis. Figure 2 is a negatively stained electron micrograph of the reconstituted sample. It contains particles which

FIG. 1. Sucrose density gradient analysis of VLP extracts from *T. basicola* fractionated by zonal ultracentrifugation. The lower six traces are samples from the indicated tube numbers that contained VLPs. The reconstituted sample is a composite containing portions of all fractions containing VLPs. The prezonal sample is a portion of the unfractionated sample.

were penetrated and not penetrated by the stain and which measured 40 nm in diameter.

The antiserum to the complex was analyzed by Ouchterlony double diffusion and immunoelectrophoresis. It reacted with its homologous antigen at a titer of 1:512, but did not react with the VLPs of *Penicillium stoloniferum* (7), *P. chrysogenum* (19), *P. brevicompactum* (22), *Aspergillus flavus* (21), or *Helminthosporium maydis* (5). Immunoelectrophoresis resulted in five distinct serological reactions (Fig. 3).

The nucleic acid extracted from the purified VLP preparation had a 260/280 ratio of 2.0 and was ds-RNA as evidenced by a positive orcinol test, negative diphenylamine test, sharp thermomelting profile in 0.01× SSC buffer (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) with $T_m = 79.0^\circ\text{C}$ (Fig. 4), density in Cs_2SO_4 , $\rho = 1.6048$, and intercalation of ethidium bromide. Electrophoresis of the RNA on polyacrylamide

FIG. 3. Immunoelectrophoresis of VLP complex from *T. basicola* (Tb-1), using its homologous antiserum (Tb-1 As). Electrophoresis was for 1 h at 5 mA in 0.1 M potassium phosphate buffer, pH 7.0. The serological reactions developed 24 to 36 h before the picture was taken.

FIG. 2. Electron micrograph of VLPs negatively stained with 1% uranyl acetate.

gels and staining with ethidium bromide (5 $\mu\text{g}/\text{ml}$) resulted in five bands visible under UV light. The molecular weights relative to known ds-RNAs were determined by co-electrophoresis on 2.4% polyacrylamide gels. Five peaks with molecular weights of 2.7×10^6 , 3.8×10^6 , 3.9×10^6 , 4.2×10^6 , and 4.5×10^6 were obtained (Fig. 5). This corresponded to the five peaks revealed by ethidium bromide staining.

DISCUSSION

Although VLPs have been reported from some 73 genera of fungi, characterization of the particles by other than electron microscopic observations has been carried out on VLPs from only

10 fungal species (5, 8, 14). This is the first report of VLPs in *T. basicola*, and we have made no attempt to characterize those we found in five other strains.

These VLPs measured 40 nm in diameter, which is the same diameter reported for the VLPs of *P. chrysogenum* (19) and *P. brevicompactum* (22) and close to the size reported for those of *Ustilago maydis* (20). The absence of a serological cross-reaction and the distinctive molecular weights of the ds-RNA indicate that these VLPs are unrelated to any other VLPs thus far described (1, 5, 8, 14).

Preliminary experiments to fractionate the five components distinguished by immunoelectrophoresis for further analysis were only partially successful, and the estimate of time and manpower required to perform the exercise indicated that these experiments were unrealistic. Therefore, further descriptive parameters were not measured.

Whether the VLPs of *T. basicola* represent a complex virus with a segmented genome and five serologically distinct capsid particles or a complex of distinct VLPs, some of which have "full" and "empty" capsids, is uncertain, but the balance of evidence supports the latter hypothesis. Although some viruses are known to have more than one surface antigen, there is no evidence for complex antigenic structure on the surface of any fungal VLP. All of the reaction

FIG. 4. Thermomelting profile of ds-RNA extracted from the complex of VLPs from *T. basicola*.

FIG. 5. Polyacrylamide gel electrophoresis of the ds-RNA of the VLPs isolated from *T. basicola* (Tb-1) and Tb-1 ds-RNA together with marker ds-RNAs of *H. maydis* VLPs (Hm-9), $\phi 6$ bacteriophage ($\phi 6$), and *P. chrysogenum* VLPs (PcV). Electrophoresis was for 4 h at 5 mA/gel on 2.4% polyacrylamide gels.

lines shown in Fig. 3 cross, with no evidence of common antigens among the different electrophoretic species. Therefore they are electrophoretically and serologically distinct.

On the other hand, multiple infections are common in fungal viruses and most, if not all, have "full" and "empty" capsids. Specific strains of *P. stoloniferum* (7), *Aspergillus niger* (9), and *A. foetidus* (16) contain two distinct types of VLPs that are separable by electrophoresis. Strains of *U. maydis* (20) and *Saccharomyces cerevisiae* (1) also appear to contain more than one type of VLP. With some fungal VLPs, mainly those of *P. chrysogenum* (19), *H. maydis* (5), and *A. foetidus* (16), each "full" capsid was found to contain a single segment of ds-RNA. Therefore the serological data and the number of ds-RNA segments suggest, but do not prove, that there are five distinct VLPs.

The significance of fungal viruses (VLPs) in *T. basicola* and most other species is yet to be determined. VLPs may explain a curious relationship between *T. basicola* and certain plant viruses. Hecht and Bateman (12) reported that tobacco plants hypersensitive to tobacco mosaic virus and tobacco necrosis virus were even more resistant if the leaves of the plant were first inoculated with *T. basicola*. They were unable to detect the presence of a virus in *T. basicola* with the techniques available at the time. On the basis of these results, it is considered likely that the *T. basicola* used by them contained fungal VLPs. Whether fungal VLPs can induce resistance to plant viruses in inoculated plants is a matter for future research.

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