Characterization of debilitation-associated mycovirus infecting the plant-pathogenic fungus Sclerotinia sclerotiorum

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It was previously reported that three dsRNA segments, designated L, M and S, were isolated from Sclerotinia sclerotiorum strain Ep-1PN and that the M dsRNA segment was coincident with hypovirulence and debilitation of the fungal host. Here, the complete nucleotide sequence of the M dsRNA of 5419 nt, excluding the poly(A) tail, was determined. Sequence analysis revealed the occurrence of a single open reading frame (nt 93–5195) encoding a protein with significant similarity to the replicases of the ‘alphavirus-like’ supergroup of positive-strand RNA viruses. The M dsRNA-encoded putative replicase protein contained the conserved methyl transferase, helicase and RNA-dependent RNA polymerase (RdRp) domains characteristic of the replicases of potex-like plant viruses (flexiviruses) and Botrytis virus F (BVF), a flexuous rod mycovirus infecting the phytopathogenic fungus Botrytis cinerea. Furthermore, convincing evidence is presented showing that ascospore descendents derived from the debilitated strain Ep-1PN were devoid of dsRNA and exhibited normal colony morphology. Moreover, it was demonstrated that the debilitation phenotype was transmitted from the parental debilitated strain to its normal ascospore progeny via hyphal anastomosis. These results suggest that the M dsRNA from strain Ep-1PN is derived from the genomic RNA of a positive-strand RNA virus, which we designated Sclerotinia sclerotiorum debilitation-associated RNA virus (SsDRV).

Although phylogenetic analysis of the conserved RdRp motifs verified that SsDRV is closely related to BVF and to the allexiviruses in the family Flexiviridae, SsDRV is distinct from these viruses, mainly based on the lack of coat protein and movement protein.

INTRODUCTION

Hypovirulent or debilitated strains of plant-pathogenic fungi that carry transmissible viruses have attracted much interest because of their potential exploitation as biological control agents, as well as their use as probes for deciphering mechanisms of fungal pathogenesis. Evidence of a viral aetiology for the debilitating disease of the plant-pathogenic fungus Cochliobolus (Helminthosporium) victoriae, the causal agent of Victoria blight of oats (Lindberg, 1959), was recently presented (Ghabrial, 2001; Ghabrial et al., 2002). Hypovirulent strains of Cryphonectria (Endothia) parasitica, the highly destructive chestnut blight fungus, were first reported in 1965 (Grente, 1965) and were later utilized successfully to control chestnut blight in Europe (Anagnostakis, 1982). The development of an infectious cDNA-based reverse genetics system for hypoviruses has significantly facilitated the undertaking of fundamental studies on various aspects of hypovirus–Cryphonectria parasitica interactions (Dawe & Nuss, 2001). There are several other examples of mycovirus-associated hypovirulence/debilitation including the mitoviruses in Ophiostoma novo-ulmi (Hong et al., 1999) and Sclerotinia homoeocarpa (Deng & Boland, 2004; Deng et al., 2003) and the unclassified mycoviruses infecting Diaportha ambigua (Preisig et al., 2000), Fusarium graminearum (Chu et al., 2002) and Botrytis cinerea (Castro et al., 2003). Furthermore, a mycoreovirus was recently isolated...
and identified from a hypovirulent strain of *Cryphonectria parasitica* (Suzuki *et al.*, 2004). Among these mycoviruses, the interactions between hypoviruses and the chestnut blight fungal host have been best studied and the potential utilization of hypoviruses in biological control has been well documented (Allen *et al.*, 2003; Anagnostakis *et al.*, 1998; Hoegger *et al.*, 2003; Nuss, 1992, 1996; Suzuki & Nuss, 2002).

dsRNA-associated hypovirulence in *Sclerotinia sclerotiorum* was first reported with strain 91 (Boland, 1992) and later with strain Ep-1PN (Li *et al.*, 1999b). Evidence for dsRNA-associated hypovirulence was also presented with other *Sclerotinia* spp. including *Sclerotinia minor* and *Sclerotinia homoeocarpa* (Melzer & Boland, 1996; Zhou & Boland, 1997). As indicated above, the dsRNA in the case of *Sclerotinia homoeocarpa* was confirmed to be of mitovirus origin (Deng & Boland, 2004). Strain Ep-1PN, which was originally isolated from a sclerotium on diseased eggplant (*Solanum melongena*), is hypovirulent to its hosts and exhibits a debilitation phenotype in culture characterized by reduced growth rate and abnormal colony morphology (Jiang *et al.*, 1998; Li *et al.*, 1996). Three dsRNA segments, designated L, M and S dsRNA with estimated sizes of 7.4, 6.4 and 1.0 kb, respectively, were reported in association with this debilitated strain (Li *et al.*, 1999b). Of these three dsRNA segments, only the M dsRNA was consistently detected in association with the debilitation phenotype (Li *et al.*, 1999b). It is not known, however, whether the M dsRNA in *Sclerotinia sclerotiorum* strain Ep-1PN is of viral origin. In the present study, molecular cloning and sequencing of the M dsRNA segment was carried out and the sequences generated were used for sequence and phylogenetic analyses to determine whether the M dsRNA is related to previously characterized mycoviruses and plant viruses.

**METHODS**

**Fungal strains.** *Sclerotinia sclerotiorum* strain Ep-1PN was originally isolated from a sclerotium collected from a diseased eggplant and its hypovirulence on eggplant and on oilseed rape was demonstrated (Li *et al.*, 1996). Strain Sunf-M, a normal wild-type strain, was originally isolated from a sclerotium on a diseased sunflower plant (*Helianthus annuus*). All fungal strains were grown at 18–22 °C on potato dextrose agar (PDA) medium and stored on PDA slants at 4–8 °C.

**Transmission of the debilitation phenotype.** Sclerotia of Ep-1PN were treated for carpogenic germination to obtain ascospores (Li *et al.*, 1999a). Single ascospores were picked with the aid of a stereomicroscope and transferred to fresh medium. Colony growth rate and the morphology of the sexual offspring cultures were observed and compared with those of the debilitated and normal strains Ep-1PN and Sunf-M, respectively. Thirty-two ascospore progeny that showed normal colony growth and morphology were selected to evaluate their virulence to oilseed rape using standard methods (Li *et al.*, 1996). A single ascospore isolate, Ep-1PNA1, and the normal wild-type isolate Sunf-M were used in the hyphal anastomosis transmission assays along with the debilitated strain Ep-1PN. Mycelial plugs from the colony edge of the debilitated (Ep-1PN) and normal (Ep-1PNA1 or Sunf-M) strains were placed side by side, 1.5–2.0 cm apart, on the surface of a PDA plate and the plates were incubated at 20–22 °C. After the hyphae from the two colonies had intermingled, the hyphal tips at the colony margin of strain Ep-1PNA1 or Sunf-M were observed with a stereo-microscope and the tips were cut and transferred onto fresh PDA plates. The growth rate and morphology of the newly developed colonies were compared with those of the debilitated and normal strains.

**Extraction of dsRNA.** The procedure for dsRNA extraction described by de Paulo & Powell (1995) was used with minor modifications. Mycelium for dsRNA isolation was grown for 7–10 days on cellophane membranes placed on top of the PDA medium in Petri plates. Following harvesting, the mycelium was stored at −80 °C before use.

**cDNA synthesis, molecular cloning and sequencing.** A sample of approximately 0–50 μg dsRNA was mixed with 0–74 μg random hexamers and 2 μl 100 % DMSO, and DEPC-treated double-deionized water was added to a final volume of 10 μl. The mixture was heated at 95 °C for 15 min and chilled on ice for 3 min. First- and second-strand cDNAs were synthesized as described by Sambrook *et al.* (1989). The resulting cDNA was purified by filtration through a Sephadex G-50 column and A-tailed with *Tag* DNA polymerase and dATP at 72 °C for 30 min. The A-tailed double-stranded cDNA was ligated into the pGEM-T Easy vector according to the manufacturer’s instructions (Promega). The recombinant vector was transformed into competent cells of *Escherichia coli* strain JM109.

RT-PCR amplification using sequence-specific primers was used to produce cDNA clones for dsRNA regions not covered by the cDNA clones generated with random primers. Denatured dsRNA samples were reverse transcribed using Superscript reverse transcriptase (Gibco-BRL) and a sequence-specific reverse primer and incubated for 60 min at 45–48 °C. After reverse transcription, the mixture was treated with RNase H (1 U at 37 °C for 30 min; TaKaRa) and 5 % of the reaction volume (1 μl) was used for PCR amplification with the pertinent forward and reverse primers and Platinum R *Pfu* DNA polymerase (Gibco-BRL). The resulting PCR product was fractionated by electrophoresis on an agarose gel and purified using a gel extraction kit (DingGuo). The PCR product was then A-tailed with *Tag* DNA polymerase at 72 °C for 30 min and cloned into the pGEM-T Easy cloning vector.

Clones for the terminal sequences of the dsRNA were generated by T4 RNA ligase oligonucleotide-mediated amplification as described by Lambden *et al.* (1992). The 3’ terminus of each strand of dsRNA was ligated at 4–8 °C for 18 h with the 5’-end phosphorylated oligonucleotide 5’-GCAATGGCACATGCGTGATTAAGGTTAATTGCGC(-NH2)3-3’. The reaction product was treated with RNase H as described above and the cDNA was amplified with another primer complementary to the RNA ligated oligonucleotide (5’-TCTAATCAAGAATTGCGATGATC-3’) and the sequence-specific primer corresponding to the 5’- and 3’-terminal sequences of the dsRNA, respectively. The expected PCR products were recovered and purified with a gel extraction kit (DingGuo), A-tailed and cloned into the pMD18-T cloning vector (TaKaRa).

Sequencing was carried out by the dideoxynucleotide termination method using a Big Dye Terminator Sequencing kit (BigDye terminator v. 2.0; ABI) and an ABI PRISM 377-96 automated sequencer (Shanghai Sangon). M13 universal primers or sequence-specific
primers were used for sequencing and each base was sequenced at least twice. Sequence analysis, alignments and phylogenetic analysis were carried out by using the DNAMAN, CLUSTAL_W and GENETREE programs.

**Northern hybridization.** Northern hybridization analysis was performed as previously described (Jiang & Ghabrial, 2004). To verify the authenticity of the cDNA clones generated with the purified dsRNA, the cDNA clones were labelled with [³²P]dCTP using a radiolabelling kit (TaKaRa) and used to probe the RNA blot.

**RT-PCR.** RT-PCR was used to verify the absence of dsRNA in the ascospore progeny derived from the debilitated strain Ep-1PN. Total RNA samples from the debilitated strain Ep-1PN and its ascospore descendants were isolated according to Sambrook et al. (1989). First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega) and the reverse primer SsDRV-PCRpREV (5'-CAGTCCCTAGTTTCATCTCGTTCC-3'). The first-strand cDNA was then subjected to PCR using the SsDRV-PCRpREV primer and the forward primer SsDRV-PCRpFOR (5'-TGGCAGGAAACAGTCATGGCAAC-3'). The conditions for cDNA amplification included an initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 62.5°C and 1 min at 72°C, with a final elongation step of 10 min at 72°C. PCR products, with a predicted size of 870 bp, were fractionated by gel electrophoresis on 1% agarose gels and stained with ethidium bromide.

**RESULTS**

**cDNA cloning and sequencing of debilitation-associated dsRNA**

Agarose gel electrophoresis of dsRNA isolated from mycelial extracts of strain Ep-1PN revealed the presence of a single dsRNA segment that was slightly larger than the genomic dsRNA of Helminthosporium victoriae virus 190S (5178 bp; Fig. 1). This dsRNA segment corresponded to the M dsRNA detected in an earlier report in strain Ep-1PN, the size of which was estimated as 6-9 kbp using DNA size markers (Li et al., 1999b). Occasionally, two additional dsRNA segments of 7-4 and 1-0 kbp were detected, as previously reported by Li et al. (1999b).

The M dsRNA segment was purified from low melting point agarose and subjected to cDNA synthesis and cloning. Two non-overlapping cDNA clones, SS7 and SS15, with inserts of 232 and 473 bp, respectively, were originally obtained using random primers. Northern blot hybridization analysis of the dsRNA, using radiolabelled SS7 and SS15 cDNA clones as probes, showed that both cDNA clones contained sequences derived from the M dsRNA segment (data not shown). Other clones were derived from RT-PCR with specific primers, oligo(dT) primers or random primers. Computer-assisted sequence assembly indicated that a full-length cDNA of 5419 bp, excluding the poly(A) tail, had been cloned that was 3' co-terminal with the M dsRNA. The cloning strategy for dsRNA is outlined in Fig. 2(a).

**Sequence analysis of the M dsRNA**

Sequence analysis revealed the occurrence of a single open reading frame (ORF) (nt 93–5195) encoding a putative protein of 1700 aa containing the conserved methyl transferase, viral RNA helicase and RNA-dependent RNA polymerase (RdRp) domains characteristic of the replicase genes of many of the positive-strand RNA viruses (Fig. 2b). Homology searches of the methyl transferase, helicase and RdRp conserved motifs of the M dsRNA-encoded replicase indicated that it was closely related to the potex-like plant viruses in the family Flexiviridae and to Botrytis virus F (BVF), a flexuous rod mycovirus that infects the plant-pathogenic fungus B. cinerea (Table 1, Figs 3 and 4). Taken together these results suggested that the M dsRNA probably represents the replicative form, or a replicative intermediate, of the genomic RNA of a positive-strand mycovirus infecting the debilitated *Sclerotinia sclerotiorum* strain Ep-1PN. We designated this virus *Sclerotinia sclerotiorum* debilitation-associated RNA virus (SsDRV).

**Phylogenetic analysis**

A dendrogram based on multiple alignments of RdRp conserved motifs of SsDRV and viruses in the family Flexiviridae...
Fig. 2. Schematic representation of the strategy used in cDNA cloning of SsDRV dsRNA and predicted genome organization. (a) Non-overlapping cDNA clones SS15 and SS7 were synthesized using random hexamer primers and denatured dsRNA as a template. Sequences of the region of the dsRNA that was not covered by these two cDNA clones were obtained from cloned RT-PCR products using sequence-specific primers (designed based on the sequences of SS15 and SS7). A series of overlapping cDNA clones was generated by RT-PCR using sequence-specific forward primers and oligo(dT) primers; alternatively, sequence-specific reverse primers and random forward primers were used (clones A–N) and clones corresponding to 5' and 3' termini were amplified by using the method of Lambden et al. (1992) (clones O–S). (b) Diagrammatic representation of the genomic organization of SsDRV dsRNA showing the presence of one ORF and its putative encoded protein. The ORF encodes a putative protein containing a methyl transferase domain, a helicase domain typical of the superfamily 1 of viral RNA helicases and eight conserved motifs characteristic of the RdRp of plant positive-strand RNA viruses.

Table 1. Percentage amino acid sequence identity and similarity of the helicase of SsDRV and those of selected viruses in the family Flexiviridae

The number of identical or similar amino acid residues/total number of residues included in the sequence comparison analysis is shown. Values in parentheses are the identity or similarity scores as calculated by the GAP program in the UWGCG package.

<table>
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<tr>
<th>Genus</th>
<th>Virus</th>
<th>Abbreviation</th>
<th>GenBank accession no.</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
<th>Reference</th>
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<tr>
<td>Unclassified</td>
<td>Botrytis virus F</td>
<td>BVF</td>
<td>NP_068549-1</td>
<td>85/242 (35%)</td>
<td>129/242 (53%)</td>
<td>Howitt et al. (2001)</td>
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<td>Potexvirus</td>
<td>Clover yellow mosaic virus</td>
<td>CIYMV</td>
<td>NP_077079-1</td>
<td>79/235 (33%)</td>
<td>129/235 (54%)</td>
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<td>Pepino mosaic virus</td>
<td>PepMV</td>
<td>CAD27508-1</td>
<td>83/239 (34%)</td>
<td>129/239 (53%)</td>
<td>Cotillon et al. (2002)</td>
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<td>Scallion virus X</td>
<td>ScaVX</td>
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<td>82/241 (34%)</td>
<td>130/241 (53%)</td>
<td>Chen et al. (2002)</td>
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<td>White clover mosaic virus (strain O)</td>
<td>WCMV</td>
<td>P15402</td>
<td>78/241 (32%)</td>
<td>126/241 (52%)</td>
<td>Beck et al. (1990)</td>
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<td></td>
<td>Potato aucuba mosaic virus</td>
<td>PAMV</td>
<td>NP_619745-1</td>
<td>82/244 (33%)</td>
<td>135/244 (55%)</td>
<td>Xu et al. (1994)</td>
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<td>Strawberry mild yellow edge virus</td>
<td>SMYEV</td>
<td>NP_620642-1</td>
<td>85/240 (35%)</td>
<td>126/240 (52%)</td>
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<td>Cactus virus X</td>
<td>CVX</td>
<td>NP_148778-1</td>
<td>78/235 (33%)</td>
<td>130/235 (55%)</td>
<td>Liou et al. (2004)</td>
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<td>Potato virus X</td>
<td>PVX</td>
<td>NP_056753-1</td>
<td>81/238 (34%)</td>
<td>127/238 (53%)</td>
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<td>Bamboo mosaic virus</td>
<td>BaMV</td>
<td>NP_042582-1</td>
<td>72/233 (30%)</td>
<td>124/233 (53%)</td>
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<td>Papaya mosaic virus</td>
<td>PapMV</td>
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<td>121/235 (51%)</td>
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<td>Tulip virus X</td>
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<td>NP_702988-1</td>
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<td>114/245 (46%)</td>
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<td>NP_040778-1</td>
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<td>133/242 (54%)</td>
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<td>Cymbidium mosaic virus</td>
<td>CymMV</td>
<td>AAB71853-1</td>
<td>77/242 (31%)</td>
<td>122/242 (50%)</td>
<td>Kim et al. (1998)</td>
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<td>Allexivirus</td>
<td>Shallot virus X</td>
<td>ShVX</td>
<td>NP_620648-1</td>
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<td>125/240 (52%)</td>
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<td>Garlic virus A</td>
<td>GarV-A</td>
<td>NP_569126-1</td>
<td>68/240 (28%)</td>
<td>125/240 (52%)</td>
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<td>Garlic virus X</td>
<td>GarV-X</td>
<td>NP_044571-1</td>
<td>73/241 (30%)</td>
<td>124/241 (51%)</td>
<td>Song et al. (1998)</td>
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<td>Mandarivirus</td>
<td>Indian citrus ringspot virus</td>
<td>ICRSV</td>
<td>NP_203553-1</td>
<td>76/234 (32%)</td>
<td>123/234 (52%)</td>
<td>Rustici et al. (2002)</td>
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</table>
is shown in Fig. 5. The resulting tree, which is supported by robust bootstrapping values (1000 replicates), showed that SsDRV is closely related to BVF and that these two mycoviruses cluster with several allexiviruses (family Flexiviridae) including Garlic virus E, Garlic virus A, Garlic virus X and Shallot virus X (Fig. 5).

The debilitation phenotype is not transmissible via sexual spores

Ascospore progeny derived from the debilitated strain Ep-1PN (Fig. 6a) showed a normal growth rate and the typical colony morphology of Sclerotinia sclerotiorum (Fig. 6b, isolate Ep-1PN1). A total of 3120 single ascospore isolates, which were obtained from seven apothecia, were included in this study. Thirty-two of these ascospore descendents, designated Ep-1PN1 to Ep-1PN32, were randomly selected and subjected to RT-PCR analysis (see below). The growth rate of the ascospore progeny examined was about 20 mm day$^{-1}$ on PDA plates maintained at 20–22 °C. Colonies of the parental strain Ep-1PN, on the other hand, developed sectors and expanded at a rate of 1–5 mm day$^{-1}$. The ascospore progeny was similar in virulence to the wild-type strain Sunf-M when allowed to colonize detached leaves of oilseed rape. The resultant lesions had a diameter of about 4–0 cm when the leaves were incubated at 20–22 °C with 100% relative humidity for 2 days. Under the same conditions, strain Ep-1PN produced no lesions or small
lesions (<1.0 cm in diameter). The colony morphology of all ascospore progeny examined was significantly different from the parental hypovirulent strain, but similar to the normal wild-type strain Sunf-M (Fig. 6d).

**Transmission of debilitation via hyphal anastomosis**

The debilitation phenotype of strain Ep-1PN was transmitted to strain Ep-1PNA1, a derivative single-ascospore isolate, following hyphal anastomosis (Fig. 7a). At first, the hyphae at the margin of the Ep-1PNA1 colony closest to Ep-1PN exhibited excessive branching and the abnormal branching subsequently spread throughout the entire colony edge (Fig. 7a, lower left plate). When hyphal tips were cut from the abnormal branching hyphae at the margin of the colony and transferred to fresh PDA plates, the newly developed colonies were similar in morphology and virulence to Ep-1PN (Fig. 6c). The debilitation phenotype, however, was seldom transmitted to the vegetatively incompatible strain Sunf-M (Fig. 7a, upper right plate). The mycelial plug from strain Ep-1PN was placed on the plate 1 day prior to transferring mycelial plugs of Ep-1PNA1 or Sunf-M. The plates were incubated at 22 °C for 4 days. The control plate (lower right plate) was only inoculated with Ep-1PNA1. (b). RT-PCR analysis of SsDRV RNA. Total RNA samples from the parental debilitated Ep-1PN strain and its ascospore progeny were subjected to RT-PCR analysis using SsDRV-specific primers. The generated RT-PCR products were fractionated on 1.0% agarose gel and stained with ethidium bromide. Lanes 1–4, RT-PCR products of the debilitated strain and three of its ascospore descendents with normal colony morphology, Ep-1PN, Ep-1PNA1, Ep-1PNA2 and Ep-1PNA3, respectively; lanes 5–7, RT-PCR products from three strains that were recipients of SsDRV via hyphal fusion of the virus-free ascospore progeny with the parental debilitated strain.

RT-PCR analysis was performed using total RNA samples from mycelial extracts of the debilitated strain Ep-1PN, three ascospore progeny derivatives (Ep-PNA1, Ep-PNA2 and Ep-PNA3) and the corresponding debilitated colonies produced subsequent to hyphal anastomosis with the parental strain EP-PN1. Whereas no PCR products were generated with RNA samples from the ascospore progeny, a cDNA fragment of the predicted size (870 bp) was amplified with RNA samples from strain Ep-1PN and recipient debilitated strains generated by hyphal fusion of the virus-free ascospore progeny with the parental debilitated strain (Fig. 7b).
DISCUSSION

This study is the first report on the molecular characterization of a mycovirus infecting a hypovirulent strain of Sclerotinia sclerotiorum that exhibits a debilitation phenotype. Sequence analysis of the full-length cDNA clone containing the coding sequence of a putative viral replicase revealed the presence of conserved methyl transferase, helicase and RdRp domains characteristic of the replicase proteins of the 'alphavirus-like' supergroup of positive-strand RNA viruses and with significant similarity to potex-like positive-strand plant viruses (Koonin & Dolja, 1993). These findings support the conclusion that SsDRV is closely related to the positive-strand ssRNA potex-like plant viruses belonging to the family Flexiviridae and to BVF, a flexuous rod mycovirus with positive-strand RNA infecting the plant-pathogenic fungus B. cinerea (Howitt et al., 2001). The International Committee on Taxonomy of Viruses has recently grouped the plant viruses with flexuous rod morphology belonging to the genera Potexvirus, Mandarivirus, Allexivirus, Carlavirus, Foveavirus, Capillovirus, Vitivirus and Trichovirus under the newly created family Flexiviridae (Adams et al., 2005).

Unlike the genomes of flexiviruses, which contain three to six ORFs, SsDRV genomic RNA contains only a single ORF with significant sequence similarity to ORF1 of flexiviruses and BVF. The product of the SsDRV single ORF (193 kDa) is of comparable size to those encoded by ORF1 of flexiviruses and BVF (150–250 kDa). Also similar to flexiviruses and BVF, SsDRV ORF follows a short 5′-untranslated leader sequence (Adams et al., 2005; Howitt et al., 2001). Although the mycovirus BVF encodes a coat protein (CP), it lacks a coding sequence for a movement protein (MP). Likewise, SsDRV does not code for a MP.

Interestingly, several mycoviruses that are associated with debilitation/hypovirulence of their host fungi have been shown to be highly similar to positive-strand RNA plant viruses and phylogenetically distant from the typically avirulent fungal partitiviruses, chrysosoviros and totiviruses with dsRNA genomes (Chu et al., 2002; Jiang & Ghabrial, 2004; Nuss, 1992; Preisig et al., 2000; Revill et al., 1999; Yu et al., 2003). This is also true for SsDRV, which is associated with hypovirulence and debilitation and has significant similarity to plant viruses in the family Flexiviridae. Single-stranded RNA mycoviruses with typical viral capsids have been isolated from Agaricus bisporus (Revill et al., 1999), B. cinerea (Howitt et al., 2001) and Pleurotus ostreatus (Yu et al., 2003), but mycoviruses infecting Cryphonectria parasitica and D. ambigua do not encode capsid proteins (Choi & Nuss, 1992; Preisig et al., 2000). The finding that SsDVR genomic RNA does not code for a CP is consistent with our inability to purify or detect viral particles in strain Ep-1PN. Because of their intracellular mode of transmission, mycoviruses can dispense with an extracellular route of infection and the required packaging function (Ghabrial, 1998).

Hypovirulent strains of Cryphonectria parasitica have been used successfully to control chestnut blight (Anagnostakis, 1982). The development of an RNA transfection system, which involves the introduction of a synthetic copy of the hypovirus positive-strand RNA directly into fungal spheroplasts, overcame the limitation caused by the complicated vegetative compatibility system and expanded the host range of hypoviruses to fungal species closely related to Crypho-nectria parasitica (Anagnostakis et al., 1998; Chen et al., 1994). We determined recently that the debilitated strain Ep-1PN could survive on a leaf of oilseed rape for more than 1 week and it protected leaves from attack by Ep-1PNA1, a virulent ascospore progeny of Ep-1PN with normal colony morphology. The debilitation phenotype of Ep-1PN also could be transmitted to Ep-1PNA1 in the soil and subsequently protected seedlings against invasion by normal virulent strains (data not shown). Melzer et al. (2002) demonstrated interspecific transmission of dsRNA from the hypovirus isolate Ss275 of Sclerotinia sclerotiorum to virulent isolates of Sclerotinia minor through hyphal interaction. It may, however, be very difficult from a practical viewpoint to control diseases caused by Sclerotinia sclerotiorum with SsDRV. The vegetative compatibility system of Sclerotinia sclerotiorum is highly complicated and strains belonging to several compatibility groups may occur in the same field (Kohn et al., 1991). Moreover, the homothallic nature of sexual reproduction of Sclerotinia sclerotiorum would prevent the use of an infectious cDNA transformation strategy, which offers promising opportunities for control of chestnut blight.

SsDRV could be exploited as a tool to probe the mechanism of pathogenicity of Sclerotinia sclerotiorum in a manner comparable to that demonstrated with the hypovirus/chestnut blight fungus system. In the hypovirus/Cryphonectria parasitica system, the expression of many host genes, including those involved in signal transduction, were suppressed by hypovirus infection (Allen et al., 2003). Furthermore, the hypovirus-encoded proteins play important roles as determinants of phenotypic traits associated with hypovirulence; for example, the p29 protease modulates the levels of pigmentation and sporulation of the hypovirulent fungal host (Craven et al., 1993). The mechanism underlying SsDRV-mediated debilitation and hypovirulence of Sclerotinia sclerotiorum is most likely different from that determined for the hypovirus/Cryphonectria parasitica system based on the current information on the genomic organization of the two viruses. Based on the broad differences in genomic organization and expression strategy of the mycoviruses that are associated with hypovirulence/debilitation phenotypes, it is most likely that multiple mechanisms for these phenotypes exist in nature.

In summary, we have characterized the potex-like mycovirus SsDRV at the molecular level and presented several lines of evidence supporting its association with the debilitation phenotype in Sclerotinia sclerotiorum strain Ep-1PN. Unequivocal evidence that SsDRV is the cause of
debilitation would require the development of infectivity assays using infectious cDNA clones, which are unavailable at present. We showed that SsDRV replicase has features typical of those of viruses in the family Flexiviridae and the mycovirus BVF. The major difference between SsDRV and flexiviruses is the lack of the CP and MP. Whereas both SsDRV and BVF lack the MP, BVF, but not SsDRV, encodes the CP and packages its ssRNA genome in flexuous rod-shaped particles. Furthermore, BVF has the unusual feature of the presence of a readthrough opal codon in its replicase-coding region (Howitt et al., 2001). Thus, these two mycoviruses are distinct enough from each other and from the previously established flexiviruses to warrant placing them in two separate new genera, as yet unassigned, in the family Flexiviridae.

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