Mol Gen Genet (1994) 245:265–271

ORIGINAL PAPER

Masashi Hongo · Atsushi Miyasaka
Fumihiko Suzuki · Teruyoshi Hashiba

Expression of the linear DNA plasmid pRS64 in the plant pathogenic fungus *Rhizoctonia solani*

Received: 22 November 1993 / Accepted: 28 April 1994

Abstract The plant pathogenic isolate RI-64 of anastomosis group 4 of *Rhizoctonia solani* possesses three linear DNA plasmids (pRS64-1, −2, and −3). Unique poly(A)$^-$ RNA, 0.5 kb in length and hybridizable with the pRS64 DNAs was found in mycelial cells of the isolate RI-64. The overall homology at the nucleotide level between pRS64-1, −2, and −3, and the cDNA prepared from the poly(A)$^-$ RNA was 100%, 73%, and 84%, respectively. The open reading frames found in pRS64-1, −2, and −3 (ORF1-1, ORF2-1, and ORF3-1) are 68 amino acids long. The amino acids sequence showed no significant homology with known proteins. Extracts from *Escherichia coli* cells expressing ORF1-1 contain a specific protein of 7 kDa. Antisera raised against the ORF1-1 product obtained from *E. coli* cells cross-reacted with the specific proteins found in the mycelia. The results indicate that the DNA plasmids found in *R. solani* contain a sequence that encodes a specific protein which may be involved in determination of plant pathogenicity.

Key words Fungus · *Rhizoctonia solani*
Linear plasmid · Expression · Specific protein

Introduction

Rhizoctonia solani is a plant pathogenic fungus that has a wide host range. Vegetative incompatibility in this fungus is determined by anastomosis groups (AGs) and intraspecific groups (ISGs). The *R. solani* isolates obtained in Japan have been divided into nine AGs and ISGs (Ogoshi 1976). Plasmid DNAs designated pRS64-1, −2, and −3 have been identified in the isolate RI-64 of *R. solani* AG4. These are linear DNA plasmids of similar size (2.7 kb) (Hashiba 1988; Miyashita et al. 1990).

Linear DNA plasmids have been detected in a wide variety of higher plants and fungi, including plant pathogenic fungi (reviewed in Esser et al. 1986; Meinhardt et al. 1990; Sakaguchi 1990). Most of the linear plasmid DNAs found in plant pathogenic fungi are associated with terminal proteins covalently attached to their 5′-termini (Kistler and Leong 1986). However, the pRS64 DNAs of *R. solani* have hairpin loops at both termini (Miyashita et al. 1990).

We examined the sequence homology among plasmid DNAs found in representative isolates by Southern blot analysis, using nick-translated plasmid DNAs as probes. Considerable sequence homology was observed among plasmid DNAs obtained from isolates within the same AG and ISG. This suggested that the distribution of plasmid DNAs correlates with the distinct host range of AGs and ISGs of *R. solani* (Miyasaka et al. 1990). Recently, we showed the existence of chromosomal DNA sequences homologous to pRS64 from isolates of AG4 (Wako et al. 1991).

Little is known about the function of the linear plasmid DNAs in plant pathogenic fungi. The linear mitochondrial plasmids, which are similar in structure (linearity, terminal inverted repeats), most probably code for a DNA polymerase and RNA polymerase (Düvell et al. 1988; Oeser and Tudzynski 1989; Gessner-Ulrich and Tudzynski 1992; Hermanns and Osiewacz 1992).

To understand the function and role of the plasmid DNA in *R. solani*, it is essential to identify plasmid-encoded transcripts and proteins. In this paper, we describe the isolation and sequence determination of the linear DNA plasmid pRS64 transcript expressed in *Escherichia coli*.

Communicated by M. Sekiguchi

M. Hongo · A. Miyasaka · F. Suzuki · T. Hashiba (✉)
Laboratory of Plant Pathology, Faculty of Agriculture,
Tohoku University, Sendai, 981 Japan

Materials and methods

Fungal isolates

R. solani isolates C-527 (AG1), H-16 and C-428 (AG2), ST-9 (AG3), RI-64, GM-11 and R101 (AG4), ST-8 (AG5), and KNB2-2 (AG6) were used in this study. Cultures were grown and maintained on potato/sucrose agar medium (200 g/l potato, 20 g/l sucrose, 20 g/l agar).

Preparation of total RNA

Mycelia were grown without shaking in polypeptone/potato/sucrose liquid medium. After 5–7 days mycelia were harvested on a paper filter. Total cellular RNAs were prepared using the procedure of Chomczynski and Sacchi (1987), with slight modifications. Fresh mycelia were chilled in liquid nitrogen and pulverized using a motor driven pestle. The mycelia were added to 10 volumes of a denaturing solution consisting of 4 M guanidium thiocyanate, 50 mM TRIS-HCl pH 7.5, 25 mM EDTA, 25 mM sodium citrate pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% sarcosyl. The mixture was homogenized and transferred to a new tube. Then, 1/10 volume of 2 M sodium acetate pH 4.0, 1 volume of water-saturated phenol and 1/5 volume of chloroform-isoamylalcohol (49:1) were added to the homogenate. The suspension was shaken vigorously and cooled on ice for 15 min. The sample was centrifuged at $10000 \times g$ for 20 min at 4° C. The aqueous phase was transferred to a new tube, followed by mixing with an equal volume of isopropanol, and then placed at −20° C for 1 h. The tube was centrifuged at $10000 \times g$ for 20 min at 4° C. The RNA pellet was suspended in 4 ml of 4 M LiCl, transferred to a new Corex tube, and placed on ice for 2 h. After centrifugation at $5000 \times g$ for 20 min at 4° C, the RNA pellet was dissolved in 3 ml of the denaturing solution and precipitated with an equal volume of isopropanol at −20° C for 45 min. Following the precipitation, the RNA sample was rinsed with 75% ethanol, sedimented and dissolved in water.

Isolation of poly(A)$^-$ RNA and poly(A)$^+$ RNA from total RNA

Poly(A)$^+$ RNA was obtained from total RNA using an Oligotex-dT 30 column (Rochel). Poly(A)$^-$ RNA was obtained from the flowthrough solution not bound to oligo(dT)-latex particles.

Northern hybridization

The RNA samples were denatured in a solution of 12 mM TRIS, 6 mM sodium acetate, 0.3 mM EDTA pH 7.0 (TAE) containing 1 mM glyoxal and 50% DMSO, and then separated by electrophoresis in a 1.2% TAE agarose gel. RNA was transferred from agarose gels to Gene Screen Plus membranes (NEN Research products, Dupont) by the capillary procedure. RNA was hybridized to DNA probes prepared with a labeling kit (Amersham) in 50% formamide, 1% SDS, 5 × SSPE, 5 × Denhardt's solution and 100 µg denatured salmon sperm DNA/ml (1 × SSPE contains 0.18 M NaCl, 0.01 M $NaH_2PO_4 \cdot 2H_2O$, and 20 mM EDTA pH 7.7; 1 × Denhardt's solution contains 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrollidone) at 42° C for 16–24 h. After hybridization, membranes were washed twice in 2 × SSC at room temperature for 5 min, twice in 2 × SSC, 1% SDS at 42° C for 30 min and once in 0.1 × SSC at 42° C for 30 min, and autoradiographed with intensifying screens at −70° C. All procedures were carried out as described by Sambrook et al. (1989).

Construction of the cDNA library

The total RNA was fractionated by sucrose density gradient (5–30% w/v) centrifugation. The RNA obtained from fractions containing the poly(A)$^-$ RNA (5 µg) was 3′-polyadenylated in 100 µl of 100 mM TRIS-HCl pH 8.2, 20 mM $MgCl_2$, 500 mM NaCl, 5 mM $MnCl_2$, 0.5 mM AMP and 250 µg/ml bovine serum albumin, using 100 U of RNase H and 2 U of poly(A) polymerase at 37° C for 30 min. The RNA was then phenol-extracted and precipitated with 10 µg glycogen and ethanol. Double-stranded cDNA (dscDNA) was synthesized as described by Gubler and Hoffman (1985). The dscDNA was repaired using T4 DNA polymerase, methylated and ligated with *Eco*RV linkers at both ends. After digestion with *Eco*RV, the dscDNA was inserted into the *Eco*RV site of phagemid pBluescript SK$^+$ (Stratagene). The cDNA was cloned in *E. coli* XL-1 Blue. The RNA species from which the cloned cDNA had been transcribed was detected by colony hybridization using a labeled cloned fragment of pRS64-2 as a probe.

Preparation of cloned fragments of pRS64

pRS64 was digested with restriction endonucleases and ligated with pUC19. The ligation mixture was used to transform *E. coli* JM109. Plasmid DNAs of several Amp^{r}Lac$^-$ transformants were analyzed by the alkaline lysis minipreparation method (Birnboim and Doly 1979). DNA probes (Fig. 1) were prepared using a labeling kit (Amersham).

Cloning procedure and DNA sequencing

All DNA manipulations were conducted as described by Sambrook et al. (1989). DNA sequences were determined by the dideoxynucleotide chain-termination method (Sanger et al. 1977) except that Sequenase 2.0 (USB) was used instead of the Klenow fragment of DNA polymerase. Computer analysis of nucleotide sequences was performed with DNASIS (Hitachi Software Engineering).

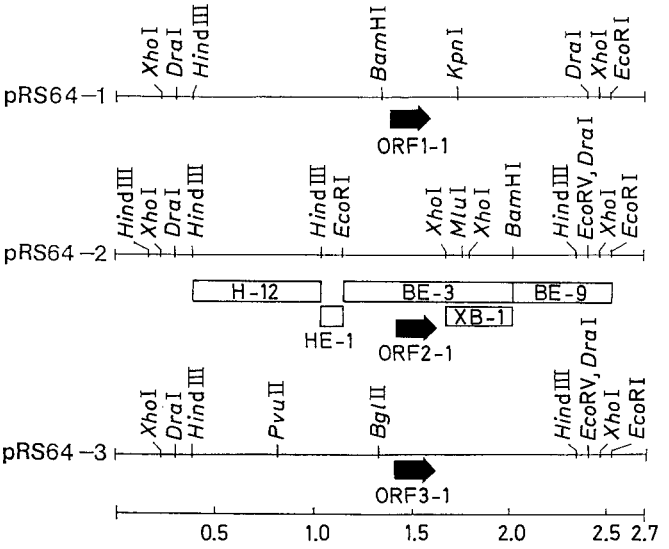

Fig. 1 Partial restriction maps of pRS64 plasmids of *Rhizoctonia solani* anastomosis group 4 (AG4). Restriction maps of pRS64-1, −2, and −3 are shown as described by Miyashita et al. (1990). Restriction fragments that were used as hybridization probes in Northern blot analysis are indicated as *open boxes*. *Arrows* indicate position and orientation of open reading frames (ORFs) of plasmid pRS64

Construction of pGEM-ORF1-1

The DNA fragment of ORF1-1 was ligated in frame with the leader peptide of the T7 gene 10 at one end and with the T7 terminator at the other end. Plasmid pGEMEX1 was digested with *Bam*HI and *Hin*dIII and the excised fragment was replaced with the coding sequence of pRS64-1, ORF1-1. The sequences cloned into the pGEMEX1 vector were specifically expressed as T7 gene 10 fusion proteins in JM109 (DE3). The resulting construct, pGEM-ORF1-1, was then transformed into *E. coli* cells. The nucleotide sequence surrounding the junction between ORF1-1 and the T7 gene 10 was determined by the dideoxy method (Sanger et al. 1977).

Purification of the fusion protein

E. coli JM109 (DE3) containing pGEM-ORF1-1 was grown to late log phase and then isopropyl β-D-thiogalactoside (IPTG) was added to the culture medium to a final concentration of 1 mM. The cells were collected by centrifugation, suspended in 20 ml of 1 M TRIS-HCl, pH 7.5 and then disrupted by ultrasonic treatment. Insoluble fractions rich in the fusion protein were collected by centrifugation and solubilized by boiling in 20 ml of the sample buffer (100 mM TRIS-HCl, 4% SDS, 2% 2-mercaptoethanol, 30% sucrose, 0.1% bromophenol blue). After SDS-PAGE, the fusion protein in the gels were stained with 0.1% Coomassie blue, eluted electrophoretically in 2.5 mM TRIS-HCl containing 12.9 mM glycine, and dialyzed against phosphate-buffered saline.

Preparation of antisera

The eluted fusion proteins were used as immunogens. The immunogen was mixed with an equal volume of complete Freund's adjuvant, then injected subcutaneously into white rabbits. About 200 μg of the fusion proteins were used per rabbit. The animals were reimmunized with about 200 μg of the fusion proteins every week. Seven days after the last injection, the rabbits were bled, and the sera were collected and stored at −20° C.

Western blots and antibody reaction

Total fungal proteins were separated and visualized in a 10% SDS-PAGE gel. Electrophoretically separated proteins were transferred to a microcellulose membrane according to the method of Towbin et al. (1979). An anti-rabbit IgG conjugated to alkaline phosphatase was applied following the supplier's protocol; for color detection, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used.

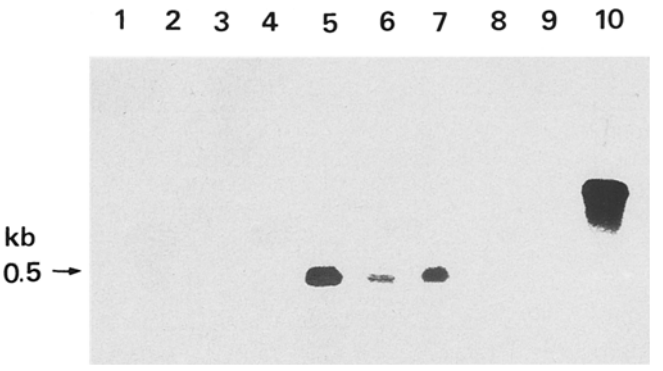

Fig. 2 Northern hybridization analysis of total RNA from *R. solani* AG1–AG6 isolates with the cloned fragment of pRS64 DNA. Total RNA (15 μg) from different *R. solani* isolates was separated by electrophoresis. Total RNA from the gel was blotted onto nitrocellulose and hybridized with the cloned BE-3 of plasmid pRS64-2 (Fig. 1). Lane 1, total RNA prepared from the isolate C-527 (AG1); lane 2, isolate H-16 (AG2); lane 3, isolate C-148 (AG2); lane 4, isolate ST-9 (AG3); lane 5, isolate RI-64 (AG4); lane 6, GM-11 (AG4); lane 7, R101 (AG4); lane 8, ST-8 (AG5); lane 9, KNB2-2 (AG6); and lane 10, cloned fragment (0.05 ng) of plasmid pRS64 (BE-3)

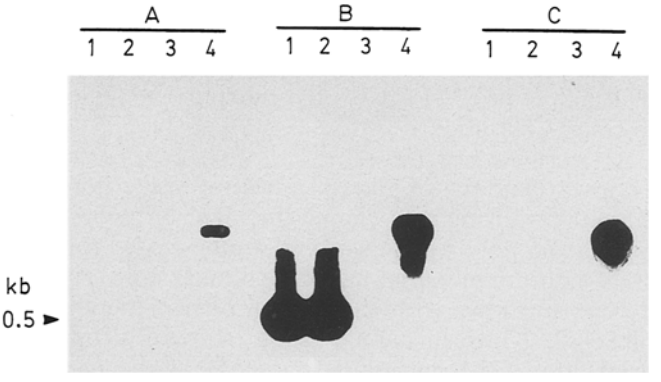

Fig. 3A–C Northern blot analysis of total RNA, poly(A)$^-$ RNA and poly(A)$^+$ RNA isolated from RI-64 (AG4) using the cloned fragment of pRS64–2. Each RNA sample was denatured, separated by agarose gel electrophoresis and hybridized with probe H-12 (**A** lanes 1–4), probe BE-3 (**B** lanes 1–4) or probe BE-9 (**C** lanes 1–4) from pRS64-2, as shown in Fig. 1. Lanes 1, total RNA; lanes 2, poly(A)$^-$ RNA; lanes 3, poly(A)$^+$ RNA; lanes 4, cloned fragments, H-12, BE-3 and BE-9, respectively

Results

Detection of poly(A)$^-$ RNA hybridizing to DNA plasmid pRS64

The transcription of pRS64 plasmids was examined by Northern blot analysis of RNA extracted from *R. solani* using hybridization probes derived from isolated regions of pRS64-1. In an initial experiment, one of the cloned fragment, BE-3, was used as a hybridization probe (see Materials and methods). One transcript was detected as a readily visible band of about 0.5 kb in total RNAs of all the plasmid-containing isolates, RI-64, R101, and GM-11 of *R. solani* AG4 (Fig. 2). On the other hand, the transcript was not found in the total RNAs of isolates C-527 (AG1), H-16 and C-148 (AG2), ST-9 (AG3), ST-8 (AG5) and KNB2-2 (AG6), which contain different plasmids (Fig. 2).

Three cloned fragments of pRS64-2 were next used as probes (Fig. 3). Probe BE-3 hybridized to total RNA and poly(A)$^-$ RNA, but not to poly(A)$^+$ RNA (Fig. 3B, lane 3). Probes H-12 and BE-9 did not hybridize to total RNA, poly(A)$^-$ or poly(A)$^+$ RNA (Fig. 3A, C).

Probe BE-3 hybridized strongly and probe XB-1 hybridized weakly to the 0.5 kb transcript in the poly(A)$^-$ RNA fraction (Fig. 4B, C, lanes 1), whereas probe HE-1 did not hybridize to the poly(A)$^-$ RNA fraction (Fig. 3A, lane 1). This result suggests that the region of pRS64-2 DNA hybridizing with the 0.5 kb transcript is

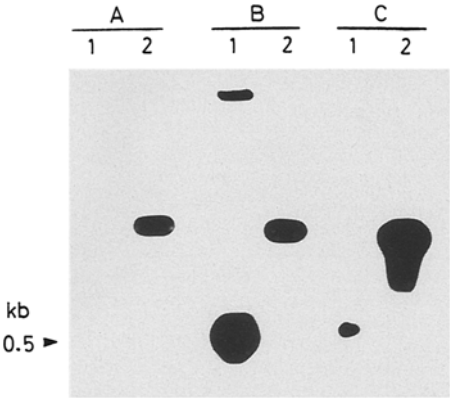

Fig. 4A–C Northern hybridization analysis of poly(A)$^-$ RNA isolated from RI-64 to the cloned fragments of pRS64. The RNA was hybridized with probe HE-1 (**A** lanes 1 and 2), probe BE-3 (**B** lanes 1 and 2) and probe XB-1 (**C** lanes 1 and 2). These probes are derived from pRS64-2 shown in Fig. 1. Lanes 1, poly(A)$^-$ RNA; lanes 2, cloned fragment

located near the *Eco*RI site and to the left of the *Bam*HI site on the restriction map of pRS64-2.

Cloning and nucleotide sequencing of the cDNA of a unique poly(A)$^-$ RNA hybridizing to pRS64 plasmids

To investigate the origin and function of the poly(A)$^-$ RNA hybridizing to a cloned fragment of pRS64, cDNA to the poly(A)$^-$ RNA was synthesized. From the cDNA library prepared, ca. 5000 colonies were screened by hybridization with the labeled cloned fragment of pRS64-2. Fourteen cDNA clones showed strong hybridization. The nucleotide sequence of the insert in clone pMHO4, derived from plasmid pRS64-1, was 439 nucleotides long (Fig. 5). Those of all other clones were virtually identical.

The nucleotide sequence of the cDNA clone pMHO4 showed 100%, 73%, and 84% homology with sequences of pRS64-1, −2, and −3, respectively. This suggests that, in addition to pRS64-1, pRS64-2, and −3 are also transcribed.

To determine potential coding regions, the nucleotide sequence of clone pMHO4 was analyzed in all possible reading frames. In this analysis, the genetic code for mitochondrial systems of filamentous fungi was used, e.g. TGA codons encode tryptophan (Fox 1987). When ATG was used as the initiation codon, an open reading frame of 117 bp was found, encoding a polypeptide of 39 amino acid residues. When plasmid DNA sequences were analyzed with GTG as the initiation codon, three open reading frames of 204 bp, one in each plasmid (ORF1-1, ORF2-1, and ORF3-1) were detected (Figs. 1, 6, 7), which could encode polypeptides of 68 amino acid residues.

We then compared the nucleotide sequences of ORF1-1, ORF2-1, and ORF3-1 within plasmid se-

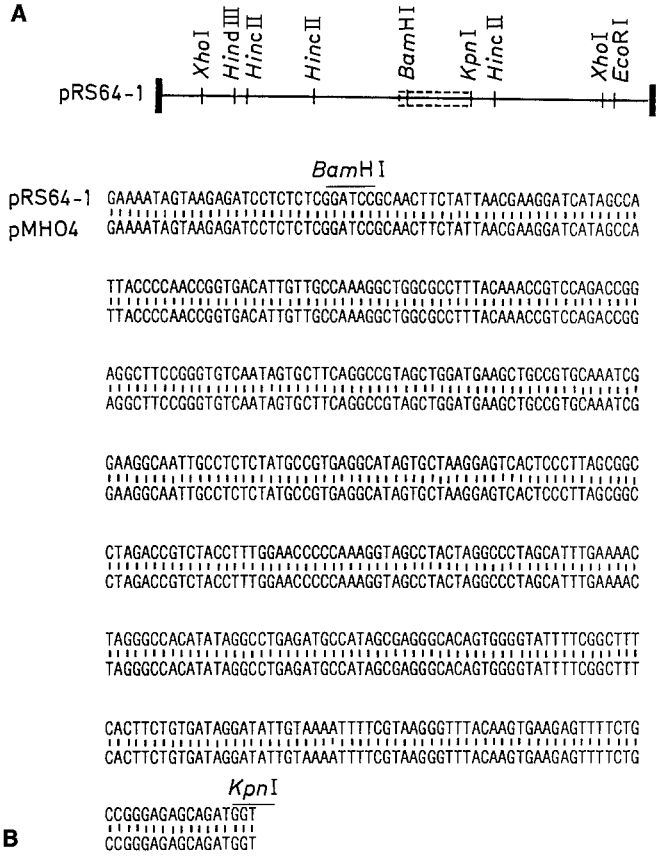

Fig. 5 **A** The region of homology between pRS64-1 and cDNA clone pMHO4 is shown as a *box* with *dotted line*. **B** Sequence similarities between pRS64-1 and cDNA (pMHO4) of poly(A)$^-$ RNA hybridizing to pRS64. Matching nucleotides are shown by a *vertical line*

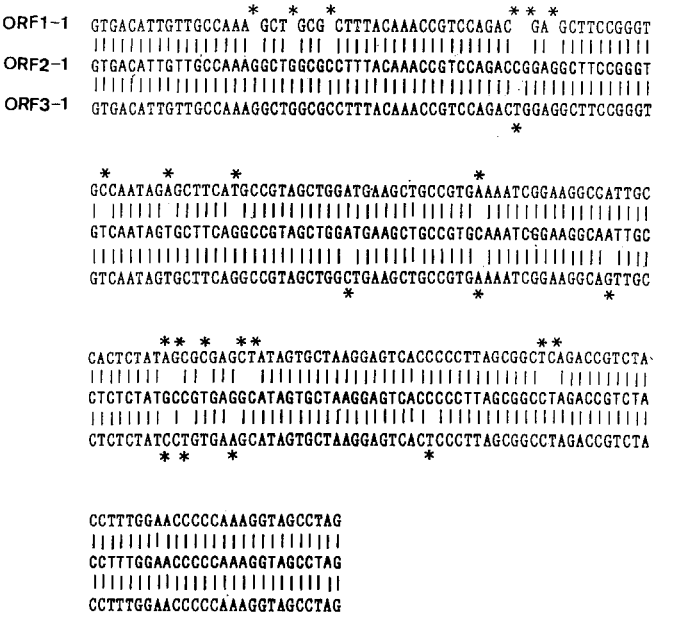

Fig. 6 Alignment of the nucleotide sequences of ORF1-1, ORF2-1 and ORF3-1. Matching nucleotides are shown by a *vertical line*. Mismatching nucleotides are shown by an *asterisk*

```
961  ATTCAGCTTTGCTGAGTGTGCGGTGGCTCCTTGAGGACACCCCAATAGTGGCAGTCCCTG  1003
1104 AGACTTTATCTCAGGGGCCCCTATTGTGGGTGCTATTGACACCATTCAAGGGGGTAATGG  1063
1064 AAAGATCCGAGATAAAAGTGACTGCTTTATTCATCTGAAAATAGTAAGAGATCCTCTCTC  1123
1124 GGATCCGCAACTTCTATTAACGAAGGATCATAGCCATTACCCCAACCGGTGACATTGTTG  1183
                                                  M  T  L  L
1184 CCAAAGGCTGGCGCCTTTACAAACCGTCCAGACCGGAGGCTTCCGGGTGTCAATAGTGCT  1243
     P  K  A  G  A  F  T  N  R  P  D  R  R  L  P  G  V  N  S  A
1244 TCAGGCCGTAGCTGCATGAAGCTGCCGTGCAAATCGGAAGGCAATTGCCTCTCTATGCCG  1303
     S  G  R  S  W  M  K  I  P  C  K  S  E  G  N  C  L  S  M  P
1304 TGAGGCATAGTGCTAAGGAGTCACTCCCTTAGCGGCCTAGACCGTCTACCTTTGGAACCC  1363
     W  G  I  V  L  R  S  H  S  L  S  G  L  D  R  L  P  L  E  P
1364 CCAAAGGTAGCCTAGTAGGCCCTAGCATTTGAAAACTAGGGCCACATATAGGCCTGAGAT  1423
     P  K  V  A  *
```

Fig. 7 Nucleotide sequence of the functional region of pMHO4 containing an open reading frame (ORF1-1). The presumed amino acid sequence encoded by ORF1-1 is shown from left to right below the nucleotide sequence. The consensus 'core enhancer' sequence, TGGAAAG, identified from higher eukaryotes is located 33 bp upstream of the start (▲). The consensus promoter sequence of *Escherichia coli*, TTGACA, is shown by a *bold line*. Numbers at the beginning indicate the portion of the sequence from the terminal *Xho*I site of the left side

quences pRS64-1, −2, and −3. The overall similarities at the nucleotide level were 78% between ORF1-1 and ORF2-1 and 90% between ORF1-1 and ORF3-1 (Fig. 6). When compared at the protein level, the similarities were 89% between the putative protein products of ORF1-1 and ORF2-1, and 90% between the putative products of ORF1-1 and ORF3-1 (data not shown).

Based on the sequence analysis, the theoretical molecular weight of the putative protein is 7.2 kDa. The promoter consensus sequence of *E. coli*, TTGACA, is located 55 nucleotides upstream of the transcription initiation site (Fig. 7). The consensus 'core enhancer' sequence, TGGAAAG, identified from higher eukaryotes, is located 33 nucleotides upstream of the transcription initiation site (Fig. 7). Using the DNASIS software, the aminoacid sequence of the putative polypeptide was compared with those of known proteins. The translation product of ORF1-1 did not show significant homology with known proteins in the data banks.

Identification of a 7-kDa polypeptide as the gene product of ORF1-1 in pRS64

To determine if the ORF1-1 of pRS64-1 is translated, antibodies were raised against the putative polypeptide coded by ORF1-1. We first constructed pGEM-ORF1-1 carrying both the T7 gene 10 and ORF1-1 cloned in the pGEM vector. Transformed *E. coli* cells were induced with IPTG, and a total protein extract was fractionated by SDS-PAGE. The T7 gene 10 protein, of approximately 32 kDa, was present (Fig. 8, lane 3) in extracts from cells containing only the vector while an additional, hybrid protein of 39 kDa was observed in cells bearing pGEM-ORF1-1.

The 39-kDa hybrid protein (Fig. 8, lane 2) was purified from cell lysates of *E. coli* JM109/pGEM by preparative gel electrophoresis, and used to immunize a rabbit.

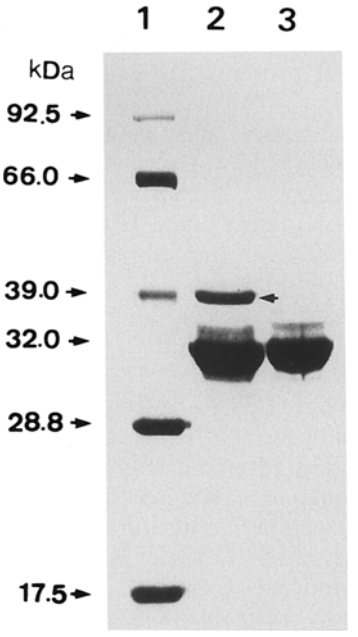

Fig. 8 Detection of the ORF-1 gene product. Protein extracts were electrophoresed in a 10% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 1, size marker; lane 2, products expressed from pGEM-ORF1-1 (containing T7 gene 10 fused to ORF1-1); lane 3, product of T7 gene 10 expressed from pGEMEX1. The fusion protein of approximately 39 kDa in lane 2 is indicated with an *arrow*

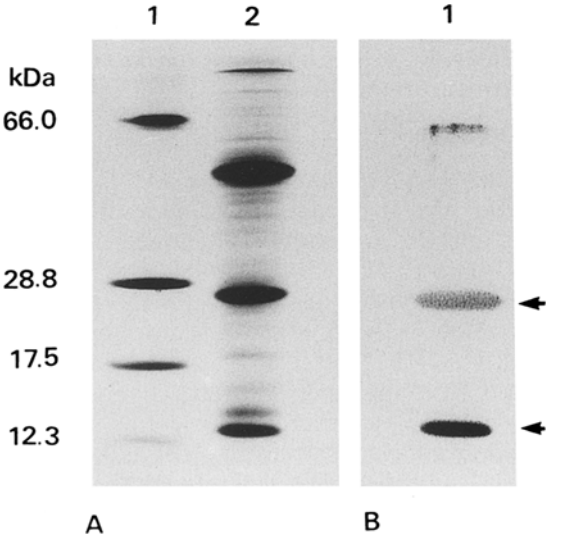

Fig. 9A, B Immunodetection of the ORF1-1 gene product. **A** Coomassie blue-stained 10% SDS-polyacrylamide gel containing total fungal proteins of RI-64 (AG4) **B** Western blot of a gel identical to that in **A** treated with immune serum raised against the gene 10/ORF1-1 fusion protein. Molecular weight standards are indicated on the left. Positions of the 14- and 28-kDa polypeptides are indicated with *arrows*

An aliquot (40 µl) of an IgG fraction purified from the complete immune serum was used in a Western blot containing total proteins from mycelia of the isolate, RI-64 (AG4). By this Western blotting assay we detected two proteins with molecular weights of approximately 14 and 28 kDa (Fig. 9). The two proteins may represent dimers and tetramers, respectively, or the expected 7-kDa fungal protein may occur in complexes with other cellular components of the fungus.

Discussion

To our knowledge, this is the first report on the nucleotide sequence of the RNA transcribed by a fungal linear DNA plasmid. Most of the linear plasmid DNAs of filamentous fungi are thought to reside within mitochondria (reviewed in Samac and Leong 1989). We have shown that the pRS64 DNA of *R. solani* is also located in the mitochondria (Wako et al. 1991).

Northern hybridization analysis showed that poly(A)$^-$ RNA hybridizing to pRS64 plasmids is present in the isolates RI-64 (containing all three plasmids), R101 (containing plasmid pRS64-1), and GM-11 (containing pRS64-2) (Chen et al. 1992). Comparison of the nucleotide sequence of the cDNA derived from poly(A)$^-$ RNA and those of pRS64-1, −2, and −3 showed 100%, 73%, and 84% homology, respectively. This suggests that the poly(A)$^-$ RNA hybridizing to pRS64 represents the transcription products of pRS64-1, −2, and −3, and that the genetic information in pRS64 DNAs is expressed. However, as chromosomal DNA sequences that hybridize to pRS64 DNA are found in the isolate RI-64 (Wako et al. 1991), there remains the possibility that the hybridizing poly(A)$^-$ RNA is derived from the genomic DNA. Oeser et al. (1993) have provided evidence for integration of linear plasmids into the mitochondrial genome.

The coding capacity of plasmid DNA in fungi has been inferred from sequence data. In the plant pathogenic fungus *Claviceps purpurea*, it was suggested that the putative ORF1 and 2 of plasmid pCIK1 encoded DNA and RNA polymerases, respectively (Oeser and Tudzynski 1989). However, no nucleotide sequences of plasmid-derived *transcripts* from plant pathogenic fungi have been published. We determined the cDNA sequence of the putative transcript derived from pRS64 in *R. solani*, and identified the potential coding region in the cDNA using the following codons as the initiation codon: ATG (Fox 1987; Waring et al. 1984), and GTG or TTG, which are used in bacteria (Clark and Marcker 1966; Kozak 1983). The longest possible ORF (68 amino acid residues) begins with the GTG codon.

Genetic and molecular approaches have recently elucidated two major classes of pathogenicity determinants in bacteria. The first class contains the *hrp* genes, which have been found among such genetically diverse species as *Pseudomonas syringae*, *P. solanacearum*, *Xanthomonas campestris*, and *Erwinia amylovora* (Willis et al. 1991). The second class contains the avirulence (*avr*) genes, which determine the host range (Keen et al. 1990). The biochemical mechanisms by which the *hrp* and *avr* genes determine the reaction of the plant are presently unknown. For fungi the pathogenicity determinants are also poorly understood.

The immunoblotting results demonstrated that the antiserum raised against the ORF1-1 product recognized a specific polypeptide in the total protein extracts of mycelial cells. A computer search using the DNASIS program failed to reveal any significant sequence homology between the ORF1-1 product and known proteins. However, there is a striking correlation between the production of the ORF1-1 protein and the host specificity of infection in *R. solani* isolates. The isolates of AG4, carrying the DNA plasmid pRS64, produce the 7-kDa polypeptide and infect radish plants.

This correlation indicates that the plasmid may encode a gene that specifies the host range. This is indirectly supported by our finding that the distribution of plasmid DNAs correlates with the distinct host range of AGs and ISGs of *R. solani* (Miyasaka et al. 1990). Linear plasmids have also been found in the Lolium pathogen, *Epichloë typhina* (Mogen et al. 1991). Further studies are necessary to determine the function of the ORF1-1, ORF2-1, and ORF3-1 products in the host specificity of plant infection.

Acknowledgements The authors wish sincerely to thank Dr. T. Ishikawa, Teikyo University, Japan for helpful suggestions and critical reading of the manuscript, Dr. Y. Ehara for kind help, and Dr. I. Havukkala, National Institute of Agrobiological Resources, Japan for critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Science and Culture and the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Chen CL, Miyasaka A, Miyashita S, Ehara Y, Hashiba T (1992) Genetic relatedness of plasmid-like DNA in isolates from anastomosis group 4 of *Rhizoctonia solani*. Ann Phytopathol Soc Jpn 58:286–291

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Clark BFC, Marcker KA (1966) The role of N-formyl-methionyl-sRNA in protein biosynthesis. J Mol Biol 17:394–406

Düvell A, Hessberg-Stutzke H, Oeser B, Rogmann-Backwinkel P, Tudzynski P (1988) Structural and functional analysis of mitochondrial plasmids in *Claviceps purpurea*. Mol Gen Genet 214:128–134

Esser K, Kuck U, Lang-Hinrichs C, Lemke P, Osiewacz HD, Stahl U, Tudzynski P (1986) Plasmids of eukaryotes. Fundamentals and applications. Springer, Berlin Heidelberg New York

Fox TD (1987) Natural variation in the genetic code. Annu Rev Genet 21:67–91

Gessner-Ulrich K, Tudzynski P (1992) Transcripts and translation products of a mitochondrial plasmid of *Claviceps purpurea*. Curr Genet 21:249–254

Gubler U, Hoffman BJ (1985) A simple and very efficient method for generating cDNA libraries. Gene 25:263–276

Hashiba T (1988) Role of linear DNA plasmids in plant pathogenic fungi. 5th International Congress of Plant Pathology (abstract), p 12

Hermanns J, Osiewacz HD (1992) The linear mitochondrial plasmid pAL2-1 of a long-lived *Podospora anserina* mutant is an invertron encoding a DNA and RNA polymerase. Curr Genet 22:491–500

Keen NT, Tamaki S, Kobayashi D, Gerhold D, Thordal-Christensen H, Dahlbeck D, Staskawicz B (1990) Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. Mol Plant-Microbe Interact 3:112–121

Kistler HC, Leong SA (1986) Linear plasmid-like DNA in the plant pathogenic fungus *Fusarium oxysporum* f. sp. *conglutinans*. J Bacteriol 167:587–593

Kozak M (1983) Comparison of initiation of protein synthesis in prokaryotes, eukaryotes, and organelles. Microbiol Rev 47:1–45

Meinhardt F, Kempken F, Kamper J, Esser K (1990) Linear plasmids among eukaryotes: fundamentals and application. Curr Genet 17:89–95

Miyasaka A, Chen CL, Hashiba T (1990) Detection and properties of plasmidlike DNA in isolates from nine anastomosis and intraspecific groups of *Rhizoctonia solani*. J Gen Microbiol 136:1791–1798

Miyashita S-I, Hirochika H, Ikeda J-E, Hashiba T (1990) Linear plasmid DNAs of the plant pathogenic fungus *Rhizoctonia solani* with unique terminal structures. Mol Gen Genet 220:165–171

Mogen KL, Siegel MR, Schardl CL (1991) Linear DNA plasmids of the perennial ryegrass choke pathogen, *Epichloë typhina* (Clavicitaceae). Curr Genet 23:315–322

Oeser B, Tudzynski P (1989) The linear mitochondrial plasmid pClK1 of the phytopathogenic fungus *Claviceps purpurea* may code for a DNA polymerase and an RNA polymerase. Mol Gen Genet 217:132–140

Oeser B, Rogmann-Backwinkel P, Tudzynski P (1993) Interaction between mitochondrial DNA and mitochondrial plasmids in *Claviceps purpurea*: analysis of plasmid-homologous sequences upstream of the lrRNA gene. Curr Genet 23:315–322

Ogoshi A (1976) Studies on the grouping of *Rhizoctonia solani* Kühn with hyphal anastomosis and on the perfect stages of groups. Bull Natl Inst Agric Sci [Series C] 30:1–63

Sakaguchi K (1990) Invertrons, a class of structurally and functionally related genetic elements that includes linear DNA plasmids, transposable elements and genomes of adeno-type viruses. Microbiol Rev 54:66–74

Samac DA, Leong SA (1989) Characterization of the termini of linear plasmids from *Nectria haematococca* and their use in construction of an autonomously replicating transformation vector. Curr Genet 16:187–194

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. Proc Natl Acad Sci USA 76:4350–4354

Wako T, Ishikawa T, Hashiba T (1991) Unique DNA plasmid pRS64 associated with chromosomal DNAs of the plant pathogenic fungus *Rhizoctonia solani*. J Gen Microbiol 137:2817–2821

Waring RB, Brown TA, Ray JA, Scazyochio C, Davies RW (1984) Three variants of the same general class in the mitochondrial gene for cytochrome oxidase subunit 1 in *Aspergillus nidulans*. EMBO J 3:2121–2128

Willis DK, Rich JJ, Hbrabak EM (1991) *hrp* genes of phytopathogenic bacteria. Mol Plant-Microbe Interact 4:132–138