Expression and localization of the linear DNA plasmid-encoded protein (RS224) in *Rhizoctonia solani* AG2-2

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Abstract

Expression of the linear DNA plasmid-encoded protein (RS224) from the plant-pathogenic fungus *Rhizoctonia solani* isolate H-16, anastomosis group 2-2, and its localization were studied. Extracts from *Escherichia coli* cells expressing the open reading frame (ORF) of RS224 (RS224ORF in pRS224) contain a 92-kDa T7.Tag-RS224orf fusion protein. Antisera raised against the fusion protein obtained from *E. coli* cells cross-reacted with a 90-kDa protein in the mycelia. To analyze the subcellular localization of the 92-kDa protein, mycelia of *R. solani* were disrupted and fractionated. Antibodies against RS224 proteins specifically reacted to the mitochondrial fraction, suggesting that RS224 is localized in mitochondria.

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1. Introduction

The plant-pathogenic fungus *Rhizoctonia solani* has a wide range of hosts and is one of the most serious fungal pathogens in the world. Isolates of the fungus are assigned to incompatibility groups based on their affinities for hyphal fusion with members of designated anastomosis groups (AGs). Japanese isolates have been divided into 11 AGs [15]. Linear plasmid DNAs have been identified in isolates of these AGs [2,4,7,13] that have hairpin loops at their termini instead of free ends with a 5'-bound terminal protein. Miyasaka et al. [13] used Southern blot analysis to examine the sequence homology among plasmid DNAs found in representative isolates, with nick-translated plasmid DNAs as probes. Plasmids from the same fungal AG often contained homologous regions. In addition, total RNAs obtained from six AGs of *R. solani* were analyzed by Northern blot analysis using the cloned fragment of the plasmid DNAs as probes. There was considerable sequence homology among plasmid-encoded transcripts obtained from isolates of the same AG [12].

By comparison, isolate RI-64 of AG4 of *R. solani* possesses three linear DNA plasmids (pRS64-1, -2, and -3) [14]. These plasmids had more than 69% homology to each other, and sequence homology to pRS64 was also maintained within the chromosomal DNA of isolate 1271 of AG4, which does not possess the plasmid [18]. Hongo et al. [6] found transcripts of the *R. solani* plasmid pRS64 and used immunologic methods to detect the protein product. Extracts from *Escherichia coli* cells expressing ORF1-1 gave a specific 7-kDa protein, and anti-ORF1-1-encoded protein (RS64) antibodies raised against the synthetic fusion peptide cross-reacted with the specific 7-kDa protein from the mycelia. Linear DNA plasmids (pRS224-1, -2, and -3) were also found in mycelial cells of *R. solani* AG2-2 isolates H-16 [9], and Katsura et al. [9] found transcripts of the *R. solani* plasmid pRS224-1. One ORF (RS224ORF) in pRS224-1 encoded a 887-amino acid protein and a potential coding capacity of 102 kDa.

To understand the function of pRS224-1 in *R. solani*, it was essential to analyze the plasmid-encoded protein.
(RS224). In this study, we describe the isolation of RS224 fused with T7.Tag that was expressed in E. coli and its localization in R. solani.

2. Materials and methods

2.1. Fungal isolates

R. solani isolate H-16 (plasmid-containing isolate) belonging to AG2-2 was used. Cultures were grown and maintained on potato/sucrose agar medium (2.4% (w/v) Potato Dextrose Broth, Becton, Dickinson and Co., NY, USA), 2% (w/v) agar).

2.2. Preparation of total protein

Cultures were grown without shaking in PPD liquid medium (2.4% (w/v) Potato Dextrose Broth, 1% (w/v) poly-pyrene) for 2–6 days. Mycelia were harvested on filter paper and stored at −80°C. Frozen mycelia (1 g) were crushed in 6 ml lysis buffer (100 mM Tris–HCl, pH 6.8, 8.3% 2-mercaptoethanol, 10 mM phenyl-methyl sulfonyl fluoride (PMSF)) with Polytron PT10-35 (Kinematica, Lucern, Switzerland) at 12000 rpm for 5 min. Sodium dodecyl sulfate (SDS) and glycerin were added to a final concentration of 2.5% and 10%, respectively. The sample was boiled for 3 min and then centrifuged at 20000 × g for 5 min.

2.3. Fractionation of microorganelles

Fresh mycelia (20 g) were disrupted in an isotonic buffer (0.44 M sucrose, 10 mM Tris–HCl, pH 7.6, 5 mM EDTA) with the Polytron PT10-35 at 14000 rpm for 5 min. Disruptants were filtered with a steel sieve and centrifuged at 1000 × g for 5 min to remove cell debris (crude cell wall fraction). The supernatant was centrifuged at 6000 × g for 5 min, and the pellet was considered as the crude nuclear fraction. The supernatant was then centrifuged at 20000 × g for 20 min, and the pellet was considered as the crude mitochondrial fraction. After mitochondria were sedimented for further purification, the resulting supernatant was centrifuged at 30000 × g for 30 min to obtain the membrane fraction. The supernatant of the latter centrifugation was again centrifuged at 120000 × g for 3 h to obtain the high-speed pellet, and the final resulting supernatant was considered the cytosolic fraction.

2.4. Purification of nuclei

The crude nuclear fraction was purified further according to Blobel and Potter (1966) [1] with modifications. Sucrose buffer (10 mM Tris–HCl pH 7.6, 5 mM EDTA, 2.1 M sucrose) was added to the crude nuclear fraction to a final concentration of 1.6 M of sucrose. The mixture was layered over a 2.1 M sucrose buffer. After centrifugation for 1 h at 26000 × g in a swinging bucket rotor SRP228SA (Hitachi, Tokyo, Japan), at 4°C, the supernatant was poured off. The precipitates were saved as the nuclear fraction.

2.5. Preparation of mitochondria

The crude mitochondrial fraction was resuspended in mitochondrial extraction buffer (0.8 M sucrose, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% bovine serum albumin (BSA)). The diluted crude mitochondria were layered onto 1–2 M linear sucrose density gradients. Loaded gradients were centrifuged at 80000 × g for 90 min at 6°C. The material that moved into the 1.18 g ml−1 density region of the gradient was collected and diluted with extraction buffer. The diluted material was centrifuged at 20000 × g for 15 min. The precipitates contained the mitochondria. The mitochondria were evaluated by fluorescence microscopy after staining with DAPI.

2.6. Solubilization of the crude cell wall fraction

For solubilization, Triton X-114 was added to the crude cell wall fraction to a final concentration of 1%. After shaking on ice for 1 h, the suspensions were centrifuged at 3000 × g for 5 min to sediment the Triton-insoluble material. Pellets were washed five times by suspending in 40 ml of extraction buffer (50 mM Tris–HCl, pH 7.6, 100 µM EDTA, 5 mM 2-mercaptoethanol, 100 µM PMSF), and centrifuged at 15000 × g for 5 min. Pellets were used for protein blot analysis.

2.7. Cloning of the entire ORF of pRS224

To amplify the entire ORF (RS224ORF) of pRS224, we performed polymerase chain reactions (PCR) using the EXPAND® high fidelity PCR system (Boehringer Mannheim, Mannheim, Germany). The first PCR amplification used the primers RS2241stF (5’-CGCTGTATCAACA-CGCTAGA-3’) and RS2241stR (5’-TTGGTCTCTGACTGAGCTCAAGA-3’). Then 1 µl of the 50-µl sample of the first PCR product was amplified by a second PCR with the nested primers BamHI-RS224orfF which contain a recognition site for BamHI followed by nucleotides 1–25 of RS224ORF (including the initiation codon) (5’-CC-GGGATCCATGAAAAATTATTACGACAA-3’) and BamHI-RS224orfR which contains a recognition site for BamHI followed by complement any nucleotides 2640–2664 of RS224ORF (including the stop codon) (5’-CC-GGGATCCATATCTTCTCATCCCTGAA-3’) as described in the manufacturer’s protocol. The reactions were performed for 30 cycles, beginning with an initial 5-min denaturation step at 94°C, followed by 50 s at
94°C, 45 s at 63°C, 1 min at 72°C, and a final extension step at 72°C for 5 min. The amplification product was analyzed by 1.0% agarose gel electrophoresis, purified, inserted into pT7A Blue(R) T-vector (Novagen, Madison, WI, USA), and used for transformation of XL1-Blue host cells. Promising clones were sequenced and analyzed further.

2.8. Construction of pETlla-RS224orf

The RS224ORF was ligated into pET11a (Novagen). The sequences cloned into the pET11a vector were specifically expressed at the T7.Tag-RS224orf fusion proteins in E. coli cells.

2.9. Purification of the fusion protein

E. coli BL21 (DE3)-Codon Plus RIL containing pET11a-RS224orf was grown to the late exponential phase at 37°C and then isopropyl-3-thiogalactopyranoside (IPTG) was added to the culture medium to a final concentration of 1 mM. After induction for 3 h, the cells were collected by centrifugation, suspended in 1/10 culture volume of TED buffer (10 mM Tris–HCl, pH 7.0, 0.1 mM EDTA, 1 mM DTT) containing 1% Triton X-100, and sonicated by ultrasonicators. The lysate was centrifuged at 12,000 × g for 10 min, providing a soluble and an insoluble fraction. The insoluble fraction was solubilized by boiling in 1 ml of the sample buffer (65 mM Tris–HCl, pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 10% glycerol). After SDS–polyacrylamide gel electrophoresis (SDS–PAGE), the fusion protein in the gels was stained with 0.2% Coomassie brilliant blue R, and then isopropyl-3-D-thiogalactopyranoside (IPTG) was added to the culture medium to a final concentration of 1 M Tris–HCl, pH 8.8.

2.10. Generation of anti-RS224 antibodies

White rabbits were primed by subcutaneous injection with 200 μg purified fusion protein in a 1:1 mixture of phosphate-buffered saline (pH 7.2) and Freund’s complete adjuvant. This was followed at 2-week intervals by three subcutaneous and intramuscular booster injections with 200 μg of purified protein in a 1:1 mixture of phosphate-buffered saline and incomplete Freund’s adjuvant. Seven days after the last injection, the rabbits were bled, and the sera were collected and stored at −20°C.

The anti-RS224 antibodies were purified according to Pringle et al. (1991) [16] with modifications. Affinity-purified RS224 fusion protein was separated by SDS–PAGE and blotted onto polyvinylidene difluoride (PVDF) membrane. After staining with Ponceau S, the RS224-containing band was cut out, and the membrane strip was incubated with phosphate-buffered saline (pH 7.2) containing 5% anti-RS224 serum, 3% BSA, and 0.05% NaN₃. After the membrane was shaken in 500 μl low-pH buffer (0.2 M glycine–HCl, pH 2.5) for 2 min, the anti-RS224 antibody was recovered in the solution, and the solution was neutralized using 85 μl neutralization buffer (6.9%(w/v) BSA, 1 M Tris–HCl, pH 8.8).

2.11. N-Terminal sequence determination

The fusion protein was separated by SDS–PAGE and blotted electrophoretically onto PVDF membrane. After being stained with 0.2% Coomassie brilliant blue R, protein bands of interest were cut out, and the N-terminal amino acid sequence was determined with an automated 490 precise protein sequence system (Perkin-Elmer, Weiterstadt, Germany).

2.12. Gel electrophoresis and protein gel blot analysis

Crude cell wall fractions, fractionation of microorganelles, and membrane fractions were resuspended in equal volumes of sample buffer. The samples were boiled for 3 min and centrifuged at 20,000 × g for 3 min. All samples were loaded on 10% SDS–polyacrylamide gel and separated by electrophoresis. Proteins were transferred to a PVDF membrane based on the method of Hirano et al. [5]. After incubation with the anti-RS224 antibody and then a second antibody conjugated to peroxidase, the reaction was developed by luminescence using the ECL Western blotting detection reagents (Amersham, Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer’s instructions.

3. Results

3.1. Identification of a 92-kDa polypeptide as the gene product of the ORF in pRS224

To determine if the ORF of pRS224-1 was translated, antibodies were raised against the putative polypeptide coded by RS224orf. We constructed pET11a-RS224orf carrying both the T7.Tag gene and RS224orf cloned in the pET11a vector, which would provide T7.Tag-RS224 fusion protein with a predicted molecular mass of 105 kDa. Expression of the fusion protein was induced with IPTG, and a total protein extract was fractionated by 10% SDS–PAGE. A 92-kDa fusion protein was observed in cells bearing the pET11a-RS224orf (Fig. 1, lane 2). The resultant fusion protein (Fig. 1, lane 3) was purified from cell lysates of E. coli by preparative gel electrophoresis, and used to immunize rabbits. Samples puri-
fied by gel electrophoresis were subjected to N-terminal sequence analysis. The obtained N-terminal sequence of the fusion protein was ASMTG GQQMG RGSMK KKLLR. The N-terminal sequence was coincident with T7.Tag-RS224 fusion protein. Affinity-purified anti-RS224 antibodies from the complete immune serum were used in a Western blot.

3.2. Detection and time course of the RS224 protein from isolate of R. solani AG2-2

The RS224 protein in the H-16 isolate of R. solani was estimated by protein gel blot analysis, using a polyclonal antibody raised against RS224. A single band of about 90 kDa was detected (Fig. 2B). To determine the expression kinetics of the 90-kDa molecule, aliquots from different time points of growth were taken from a culture medium, and adjusted for the wet weight of the pellet. The 90-kDa protein was detected in 2-day-old cultures, and increased in concentration thereafter as detected on Western blots (Fig. 2B).

3.3. Detection of RS224 protein from microorganelles

Five-day-old mycelia were crushed in isotonic buffer with a Polytron and centrifuged (see Section 2). The distribution of the RS224 protein was determined by protein gel blotting. The mitochondrial fraction (Fig. 3B, lane 3) contained relatively high concentrations of the RS224 protein, while the protein was not detected in the nuclear fraction (Fig. 3B, lane 2). Suspensions of the crude cell wall fraction were treated with Triton X-114 (see Section 2). RS224 protein was detected in a Triton-non-treated fraction, but not in the Triton-treated fraction (Fig. 3B, lane 1). The membrane, high-speed pellet and cytosolic fractions contained no detectable protein (Fig. 3B, lanes 4–6). We concluded that the RS224 protein was localized in the mitochondria.

4. Discussion

The complete nucleotide sequence of the hairpin-loop linear DNA plasmid (pRS224-1) from the plant-pathogen-
ic fungus *R. solani* AG2-2 isolate H-16 was determined, and its unique RNA transcripts were characterized. One ORF (RS224ORF) found in pRS224-1 encoded a protein with an approximate molecular mass of 102 kDa [9]. To determine whether the ORF of pRS224-1 is translated, we constructed pET11a-RS224orf carrying both the T7.Tag gene and the RS224ORF. Transformed *E. coli* cells were induced with IPTG, and the total extract was fractionated using SDS–PAGE. The fusion protein (T7.Tag-RS224orf, 92 kDa) was obtained in cells bearing pET11a-RS224orf. We detected a single protein with a molecular mass of approximately 92 kDa, a size which is slightly smaller than the 102 kDa calculated from the amino acid sequence coded by the ORF.

Results from the immunoblotting experiment demonstrated that the antiserum raised against the RS224ORF product recognized a specific polypeptide in the total protein extracts of mycelial cells. The putative polypeptide was associated with the mitochondrial fraction, and the pRS224-1 DNA of *R. solani* is also located in the mitochondria [2].

The *R. solani* AG4 contains linear DNA plasmids pRS64-1, -2, -3 [14]. These plasmids were analyzed in detail and possess the following properties [6,8,13]: (1) they have the same size (2.7 kb), (2) the ORFs found in pRS64-1, -2, and -3 all encode proteins that are 68 amino acids long, (3) extracts from *E. coli* cells expressing the ORF contained a specific 7-kDa protein (RS64), (4) antiserum raised against the ORF product obtained from *E. coli* cells cross-reacted with the specific proteins in the mycelia. These plasmid DNAs from *R. solani* are also associated with mitochondria [2], but the 7-kDa protein was detected in the cell wall fraction (unpublished data). A computer search using the DNA/SIS program revealed no significant homology between RS64 and RS224. In addition, RS64 and RS224 were found in the cell wall and mitochondrial fractions, respectively. Thus RS64 and RS224 clearly are different proteins with different functions.

The pRS224-1 ORF contains the seven conserved domains characteristic of a reverse transcriptase [9]. Reverse transcriptase activity was assayed by incorporation of \([\alpha-32P]dTTP\) into trichloroacetic acid-precipitable material, as described previously [3]; however, putative reverse transcriptase activity is not present in mitochondrial lysates from *R. solani* isolate H-16 (unpublished data). In contrast, two linear mitochondrial plasmids (pFOXC2 and pFOXC3) of the plant-pathogenic fungus *Fusarium oxysporum* contain a single, long ORF that encodes a protein of 527 amino acids, and that is highly conserved between the two plasmids [10,17]. This ORF contains conserved amino acid sequence blocks (I–VII) characteristic of retroviral and other reverse transcriptases [11]; and pFOXC2 and pFOXC3 encode an 81-kDa protein that has reverse transcriptase activity [17].

We conclude that pRS224-1 encodes an as yet unidentified protein that is located in the mitochondria of *R. solani* AG2-2. Further studies will be necessary to determine the functions of RS64 and RS224 and their sequences.

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**References**


