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QUINIC ACID-MEDIATED INDUCTION OF HYPOVIRULENCE AND A HYPOVIRULENCE-ASSOCIATED DOUBLE-STRANDED RNA IN *RHIZOCTONIA SOLANI*

By

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B.S. Wuhan University, 1989

M.S. Wuhan University, 1992

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Biochemistry and Molecular Biology)

The Graduate School

The University of Maine

August, 2001

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QUINIC ACID-MEDIATED INDUCTION OF HYPOVIRULENCE AND A HYPOVIRULENCE-ASSOCIATED DOUBLE-STRANDED RNA (DSRNA) IN *RHIZOCTONIA SOLANI*

By Chunyu Liu

Thesis Advisor: Dr. Stellos Tavantzis

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Biochemistry and Molecular Biology)
August, 2001

This study is a part of a project focused on the relationship between dsRNA and hypovirulence in *R. solani*. Hypovirulence refers to a condition in which a pathogen has a drastically reduced ability to cause disease. *Rhizoctonia solani* is a soil-borne pathogen causing diseases in numerous plants. Phenyl acetic acid (PAA), a metabolite of phenylalanine, causes Rhizoctonia disease symptoms on potato in the absence of the pathogen itself. The amount of PAA produced by a hypovirulent isolate is 10% of that produced by virulent isolates. A 3.6 kb dsRNA (M2) has been shown to be associated with hypovirulence in *R. solani*. Polypeptide A (pA) encoded by the M2 dsRNA is phylogenetically related to the pentafunctional polypeptide AROM of the shikimate pathway and QUTR, repressor of the quinate pathway in fungi. A hypothesis has been proposed to explain the mechanism of the

M2 dsRNA-mediated hypovirulence. Polypeptide A may interfere with both the shikimate and quinate pathways, leading to a decreased production of aromatic amino acids and PAA, thus leading to a decreased virulence.

Results of this study 1) supported this hypothesis, and 2) verified the relationship between the M2 dsRNA and hypovirulence in *R. solani*. A protein band of the expected size (83 kDa) was detected only in M2-containing isolates. The hypovirulent isolate Rhs 1A1 has a constitutive quinate pathway whereas the virulent isolate Rhs 1AP has an inducible quinate pathway. Moreover, Rhs 1AP has a higher level of expression for the shikimate pathway than Rhs 1A1. Data also sowed that phenylalanine levels were positively correlated with virulence in Rhs 1AP. The addition of quinate converted virulent Rhs 1AP to hypovirulent, and induced the synthesis of 1) a polypeptide of the same size as pA and reacting with anti-pA antibodies, and 2) the respective M2-specific transcript.

For the first time, the *arom* gene has been cloned from Basidiomycetes. The *R. solani arom* gene has five introns as compared to one intron found in *arom* genes from other fungi. The deduced *R. solani* AROM polypeptide contains all of the highly conserved motifs and enzyme domains found in AROM polypeptides from other fungal species.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS....................ii

LIST OF TABLES....................vi

LIST OF FIGURES....................vii

Chapter

1. INTRODUCTION....................1

 1.1. Double-stranded RNA (dsRNA) and hypovirulence in plant pathogenic fungi....................1

 1.2. Cytoplasmic hypovirulence in *Rhizoctonia solani*....................3

 1.3. The Rhs 1AP, 1, 2, 3 model....................5

 1.4. The shikimate pathway and quinate pathway....................7

 1.5. The connection between hypovirulence and shikimate-quinate pathways....................14

2. MATERIALS AND METHODS....................17

 2.1. Media....................17

 2.2. Fungal isolates....................18

 2.3. Culture conditions for western blotting....................18

 2.4. Culture conditions for enzyme assays and polysome purification....................19

 2.5. Culture conditions for assessment of virulence....................20

 2.6. Production and partial purification of antibodies against polypeptide A....................21

2.7. Protein extraction for western blotting..23

2.8. SDS-polyacrylamide gel electrophoresis..23

2.9. Western blot analysis..24

2.10. Preparation of cell-free extracts for enzyme assays..25

2.11. Enzyme assays..25

2.12. Assessment of virulence..26

2.13. Amino acid analysis by high performance liquid chromatography (HPLC)..27

2.14. Immunoprecipitation of polysomes..28

2.15. Reverse transcription polymerase chain reaction (RT-PCR)..29

2.16. Total RNA extraction..29

2.17. Complementary DNA (cDNA) cloning of the *arom* mRNA..30

2.18. Genomic DNA cloning of the *arom* sequence..31

2.19. Statistical analysis..32

3. RESULTS..36

3.1. Quinate down-regulates virulence in *R. solani*; shikimate, chorismate and the aromatic amino acids enhance the virulence in Rhs 1AP..36

3.2. Quinic acid overrides the effect of chorismate on the virulence..37

3.3. Relationship between relative concentration of phenylalanine [Phe] and virulence in *R. solani* isolates Rhs 1A1 and Rhs 1AP..40

3.4. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies in M2-containing isolates but not in M2-lacking isolates....43

3.5. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies in quinate-induced Rhs 1AP....43

3.6. Detection of M2 transcripts in quinate-induced Rhs 1AP....46

3.7. Quinate dehydrogenase activity is constitutive in the M2-containing, hypovirulent Rhs 1A1, and inducible in the M2-lacking, virulent Rhs 1AP....50

3.8. Shikimate kinase (SK) activity is higher in the M2-lacking Rhs 1AP than in the M2-containing Rhs 1A1....52

3.9. Sequence analysis of the *arom* gene from *R. solani*....53

4. DISCUSSION....65

BIBLIOGRAPHY....71

BIOGRAPHY OF THE AUTHOR....84

LIST OF TABLES

Table 1-1. DsRNA occurring in the *R. solani* cultures
Rhs 1AP, Rhs 1A1, Rhs 1A2, and Rhs 1A3....................................6

Table 2-1. Media on which *R. solani* isolates Rhs 1A1 and Rhs 1AP
were cultured before their virulence was tested on
potato sprouts..22

Table 2-2. Nucleotide sequences of primers used for PCR-mediated
amplification of the *arom* gene from
genomic DNA of the *R. solani* culture Rhs 1AP 35

Table 3-1. Comparison of the pentafunctional AROM proteins
from *A. nidulans*, *P. carinii*, *S. cerevisiae* and *R. solani*......................60

LIST OF FIGURES

Figure 1-1. The genes, enzymes and metabolites comprising the quinate and shikimate pathways in *A. nidulans*.........................9

Figure 1-2. Modular structure of the enzymes and regulatory proteins comprising the quinate and pre-chorismate shikimate pathways in *A. nidulans*.........................11

Figure 2-1. Schematic of overview of the strategy used in cDNA cloning from *arom* gene of *R. solani*.........................33

Figure 2-2. Schematic of overview of the strategy used to amplify the *arom* gene from genomic DNA of *R. solani*..............34

Figure 3-1. Effect of quinate, shikimate, chorismate, aromatic amino acids on virulence of the *R. solani* isolates Rhs 1AP and Rhs 1A1...........38

Figure 3-2. Quinate overrides chorismate-induced virulence enhancement in *R. solani* isolates.........................39

Figure 3-3. Relationship between relative [Phe] and virulence in Rhs 1AP grown on the media indicated.........................41

Figure 3-4. Relationship between relative [Phe] and virulence in Rhs 1A1 grown on the media indicated.........................42

Figure 3-5. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies in M2 dsRNA-containing isolates by western blot analysis.........................44

Figure 3-6. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies in quinate-induced Rhs 1AP by western blot analysis......................45

Figure 3-7. Detection of an M2-specific transcript by RT-PCR in polysome fractions from quinate-induced Rhs 1AP.......................47

Figure 3-8. Alignment of the sequences of the M2 dsRNA sense strand and RT-PCR product from quinate-induced Rhs 1AP polysome fraction..48

Figure 3-9. Specific activity of quinate dehydrogenase in *R. solani* isolates Rhs 1A1 and Rhs 1AP..51

Figure 3-10. Specific activity of shikimate kinase (SK) in *R. solani* isolates Rhs 1A1 and Rhs 1AP..54

Figure 3-11. DNA sequence and the predicted amino acid sequence of the *R. solani arom* gene including introns and the 5' and 3' untranslated regions.....................................56

Figure 3-12. Comparison of the amino acid sequences of four AROM polypeptides and the corresponding *E.coli* monofunctional enzymes...61

INTRODUCTION

1.1. Double-stranded RNA (dsRNA) and hypovirulence in plant pathogenic fungi

Hypovirulence refers to a condition in which a fungal pathogen has a reduced ability to cause disease. Studies have shown that hypovirulence is caused by nuclear or cytoplasmic genetic factors (Elliston, 1982). Fungal dsRNAs have been reported to be associated with hypovirulence in several fungi. *Ophiostoma (Ceratocystis) ulmi* (Brasier, 1983; Rogers *et al.*, 1987) is the causative agent of Dutch elm disease. Hypovirulent isolates of the pathogen displayed a slow growth rate, reduced viability of conidia, and impairment of sexual reproduction (Brasier, 1983). Transmission of the diseased state coincides with transmission of a specific set of dsRNA elements (Rogers *et al.*, 1987). *Gaeumannomyces graminis* var. *tritici*, the incitant of wheat take-all, is another fungal pathogen in which cytoplasmic elements have been implicated in the pathogenicity variation found in natural populations. It has been shown that hypovirulent isolates contain viral dsRNAs, whereas virulent isolates are free of those dsRNAs (Rawlinson *et al.*, 1973).

dsRNA-associated hypovirulence has been intensively studied in *Cryphonectria parasitica*, the causative agent of chestnut blight. Early in the 20th century, American and European chestnut forests were devastated by *C. parasitica*. Later, the disease was arrested in many European regions by the natural spread of hypovirulent strains of the pathogen (Grente, 1965). Subsequent studies showed that hypovirulence was caused by viral dsRNAs (Day *et al.*, 1977).

In *C. parasitica*, the most widely studied of the viruses responsible for hypovirulence, is the *Cryphonectria hypovirus 1*-713, designated as CHV1-713. It causes a distinct range of symptoms (hypovirulence-associated traits), including reduced virulence (hypovirulence), reduced pigmentation, poor asexual sporulation, and female infertility. In 1992, Choi and Nuss provided direct evidence demonstrating CHV1-713 dsRNA caused hypovirulence in *C. parasitica* (Choi and Nuss, 1992). A full-length complementary DNA (cDNA) copy of this dsRNA was introduced into a virulent, dsRNA-free strain of *C. parasitica*. It was shown that the cDNA was integrated into chromosomal DNA. Transformants contained the respective 12.7-kb dsRNA, were converted to hypovirulence, and exhibited all of the characteristics of hypovirulent strains in culture.

CHV1-713 dsRNA has been reported to suppress transcription of a number of fungal genes in *C. parasitica* (Powell and Van Alfen, 1987a, 1987b). Genes *Mf2/1* and *Mf2/2*, formerly named *Vir1* and *Vir2*, encode the pheromone precursors of the Mat-2 mating type of the fungus. Studies have shown *Mf2/1* and *Mf2/2* produce abundant transcripts in noninfected strains of the fungus, but the transcripts are not detectable in virus-infected strains (Zhang *et al.*, 1998). CHV1-713 infection also represses transcription of the *Mf1/1* gene of a Mat-1 strain of the fungus (Zhang *et al.*, 1998). Deletion of the *Mf2/2* gene in virulent *C. parasitica* results in hypovirulence-associated traits (Zhang *et al.*, 1993). In fungi, the *Lacc1* gene encodes an extracellular laccase (Clutterbuck, 1972), and which appears to be involved in lignin degradation (Ander and Eriksson, 1976), virulence (Geiger *et al.*, 1986), sporulation (Leatham and Stahmann, 1981), and pigmentation (Clutterbuck, 1972). It

has been found that laccase biosynthesis was down-regulated by reducing laccase mRNA accumulation in dsRNA-containing hypovirulent *C. parasitica* (Rigling and Van Alfen, 1991). Gene *Crp* encodes a cell wall hydrophobin, which is specific to aerial hyphae and fruiting bodies of *C. parasitica*. Northern blot analysis showed that the transcripts of *Crp* are down regulated 70% in CHV1 infected *C. parasitica* (Zhang *et al.*, 1994). Heterotrimeric G proteins are known to be involved in signal transduction pathways. The α-subunits of G proteins have been cloned in a number of filamentous fungi and have been shown to be important in sporulation and development (Hicks *et al.*, 1997; Lui *et al.*, 1997; Ivey, *et al.*, 1999). It has been shown that a GTP-binding protein α-subunit is down-regulated by viral dsRNA infection in *C. parasitica* (Choi *et al.*, 1995).

1.2. Cytoplasmic hypovirulence in *Rhizoctonia solani*

Rhizoctonia solani is the anamorph of *Thanatephorus cucumeris*, a basidiomycete. It is a collective species consisting of at least 13 anastomosis groups (AGs) in which hyphal fusion occurs between isolates within the same AGs (Carling, 1996). *R. solani* exists in mycelial form on plants or in culture and does not produce sexual spores. As a fungal pathogen, *R. solani* attacks numerous plants (over 500 hosts in the United States alone), including alfalfa, peanut, soybean, lima bean, cucumber, papaya, eggplant, corn, and potato. The most common symptoms caused by *R. solani* are damping off of seedlings, root rot, stem rot, or stem canker. Extensive rots cause plant decline and reduced yields. It has been found that AG3 of *R. solani* is the major cause of the Rhizoctonia disease in potato crops throughout

North America (Bandy *et al.*, 1988). Rhizoctonia disease occurs wherever potatoes are grown, but is most severe where soils are moist and cool (16-23 ^{o}C). Adding compost and organic fertilizers can decrease disease levels (Hoitink and Boehm, 1999). The fungicide pentachloronitrobenzene (PCNB) is used to treat seed potatoes to control Rhizoctonia disease. PCNB has a wide range of antimicrobial activity; thus it is harmful to beneficial microorganisms living in the rhizosphere of potato.

A cytoplasmically controlled degenerative disease (termed *Rhizoctonia* decline) was reported to affect strain 189a in *R. solani* (Castanho Butler, 1978a, 1978b). The disease was characterized by a loss of mycelial pigmentation, a reduced growth rate and sclerotia production. The above condition was associated with the presence of hypovirulence similar to that of *C. parasitica*. Attempts to isolate a mycovirus or virus-like particles were unsuccessful. However, dsRNA was consistently extracted from strain 189a (Castanho and Butler 1978a). Subsequent studies showed that three out of 13 strains of *R. solani* which had dsRNA were hypovirulent, whereas 10 virulent isolates contained no detectable dsRNA (Castanho *et al.*, 1978). In contrast, Finkler *et al.* (1985) reported that dsRNA was detectable only in virulent strains of *R. solani.* Zanzinger *et al.* (1984) reported that 49 of 50 isolates of *R. solani* examined contained dsRNAs. These isolates ranged from being highly virulent to being essentially non-pathogenic (Zanzinger *et al.*, 1984). Bharathan and Tavantzis (1990, 1991) reported that dsRNAs from *R. solani* isolates obtained from the same field were significantly different genetically. The same was true of dsRNAs from various continents. Several lines of indirect evidence showed that dsRNAs may carry different genetic information and have varying effects on the

respective host systems (Tavantzis, 1989; Bharathan and Tavantzis, 1990, 1991; Tavantzis and Lakshman 1995; Jian *et al.*, 1997; Lakshman *et al.*, 1998).

In order to determine if hypovirulent *Rhizoctonia solani* isolates could reduce the disease symptoms caused by virulent isolates, field experiments have been conducted (Bandy and Tavantzis, 1990). Rhs 1A1 is a hypovirulent isolate of *R. solani* that induces a slight discoloration on potato stems at the point of entry. In contrast, virulent isolates cause dark, sunken necrotic lesions (Lakshman and Tavantzis, 1994). Plants inoculated with Rhs 1A1 alone exhibited a greater growth response expressed as a 4-fold increase in dry weight of stolons (yield predictor), 1.7-fold increase in dry weight of stems (including foliage), full bloom of plants (onset of tuberization) occurred 7-10 days earlier than their respective uninoculated controls (Bandy and Tavantzis, 1990). In plants inoculated with both Rhs 1A1 and the virulent isolate Rhs 27, disease was reduced by 56%, as compared to the plots inoculated only with the virulent Rhs 27 (Bandy and Tavantzis, 1990). Sneh *et al.* (1986) also reported an increased plant growth response induced by nonpathogenic *R. solani*.

1.3. The Rhs 1AP, 1, 2, 3 model

In the last decade, a genetic model of *R. solani* has been used to study the relationship between dsRNA and the host virulence (Lakshman and Tavantzis, 1994). Rhs 1AP (P stands for parental) is a virulent field isolate belonging to AG3. In the course of several years, Rhs 1AP has given rise to three morphologically distinct sectors, designated as Rhs 1A1, Rhs 1A2, Rhs 1A3. The three sectors exhibited slower growth, loss of mycelial pigmentation and sclerotia production, and were

hypovirulent when they were inoculated on potato sprouts (Lakshman and Tavantzis, 1994). Rhs 1AP, Rhs 1A1, Rhs 1A2 and Rhs 1A3 possess different dsRNA elements (Table 1-1) that are visible in ethidium bromide-stained agarose gel electrophoretograms of dsRNA extracted from fungal tissue (Lakshman and Tavantzis, 1994).

Table 1-1. DsRNAs occurring in the *R. solani* cultures Rhs 1AP, Rhs 1A1, Rhs 1A2 and Rhs 1A3.

	dsRNA element (kb)				
Isolates	25 kb (L1)	23 kb (L2)	6.4 kb (M1)	3.6 kb (M2)	1.2 kb (S1)
1AP		+	+		
1A1	+	+	+	+	+
1A2				+	+
1A3	+				+

The phenotype and dsRNA content of the four isolates are stable. All five dsRNA elements have been found in the cytoplasm, but none have been found in the nucleus. All five dsRNA elements are also transmissible through hyphal anastomosis (Jian *et al.*, 1997). Northern blot hybridization analysis has shown that L2 and M1, occurring in Rhs 1AP and Rhs 1A1, are genetically related (probably identical), but that the 5 dsRNAs are not related to one another. In Rhs 1AP, L1, M2, and S1 are not detectable by ethidium bromide staining or northern blot hybridization. However, they are found in a DNA or RNA form and are detectable only by PCR or RT-PCR, respectively (Lakshman and Tavantzis, 1994).

Indirect evidence suggests that the M2 dsRNA is associated with hypovirulence in *R. solani* (Jian *et al.*, 1997). All of the isolates harboring M2 dsRNA (M2-containing or M2$^+$) are hypovirulent. M2 can be transmitted from M2$^+$ to M2$^-$ (M2-lacking) cultures through hyphal anastomosis, and converts M2$^-$ strains from virulent to hypovirulent (Jian *et al.*, 1997). The M2 dsRNA is located mainly in the cytoplasm. The M2 cDNA has been cloned and sequenced (Lakshman *et al.*, 1998). The largest open reading frame (ORF A) of the M2 dsRNA sense strand encodes a putative protein of 754 amino acids, designated as polypeptide A or pA. Polypeptide A possesses all four motifs of a viral double-stranded RNA-dependent RNA polymerase (RDRP) (Koonin *et al.*, 1991; Bruenn, 1993; Lakshman *et al.*, 1998) and is phylogenetically related to the RDRP of a mitochondrial dsRNA associated with hypovirulence in strain NB631 of *C. parasitica* (Polashoch *et al.*, 1994). It has been shown that viral dsRNAs evolve very rapidly. The only gene that is common to all viral dsRNAs is their RDRP, which shows homology with short stretches of conserved amino acids or motifs (Koonin *et al.*, 1991; Bruenn, 1993). A stretch of ORF A (190-517 aa) is also homologous to two C-terminal domains of the pentafunctional polypeptide AROM of the shikimate pathway in fungi (Lakshman *et al.*, 1998).

1.4. The shikimate pathway and quinate pathway

The shikimate pathway has been described as a metabolic tree with many branches (Bentley, 1990). This pathway is found in prokaryotes, microbial eukaryotes and higher plants, but not in animals (including mammals) that have been studied to

date. The shikimate pathway begins with erythrose-4-phosphate and phosphoenolpyruvate and leads to the biosynthesis of an extensive range of primary essential and secondary metabolites. Chorismate, the end product of this pathway, is the common precursor for the aromatic amino acids (Phe, Tyr, Trp), ρ-aminobenzoic acid and a number of other important metabolites (Figure 1-1). The primary metabolites include metal chelators, vitamins E and K, ubiquinone, and plastoquinone. The secondary metabolites include compounds that have antibacterial and antifungal activities and miscellaneous antibiotics (Bentley, 1990).

Two intermediates of the shikimate pathway, 3-dehydroquinate (DHQ) and dehydroshikimate (DHS), are also intermediates in the quinate pathway that is responsible for the catabolism of quinate to protocatechuate (PCA) in many fungi and bacteria. Quinate comprises approximately 10% (w/w) of decaying leaf matter and can be used as an important carbon source by soil-borne microbes. In wild-type strains, utilization of quinate is completely dispensable. The presence of quinate is necessary for the induction of the quinate pathway genes. Other carbon sources (such as glucose) can repress the induction of this pathway by quinate (Chaleff, 1974a).

In most bacteria, steps 2 to 6 of the pre-chorismate shikimate pathway are catalyzed by 5 monofunctional enzymes that are encoded by 5 separate genes located throughout the genome (Pittard, 1987). In higher plants, the 3-dehydroquinase and shikimate dehydrogenase enzymatic activities are fused into a single bifunctional protein (Koshiba, 1978; Mousdale *et al.*, 1987). In yeast, filamentous fungi and *Euglena*, steps 2 to 6 of the shikimate pathway are catalyzed

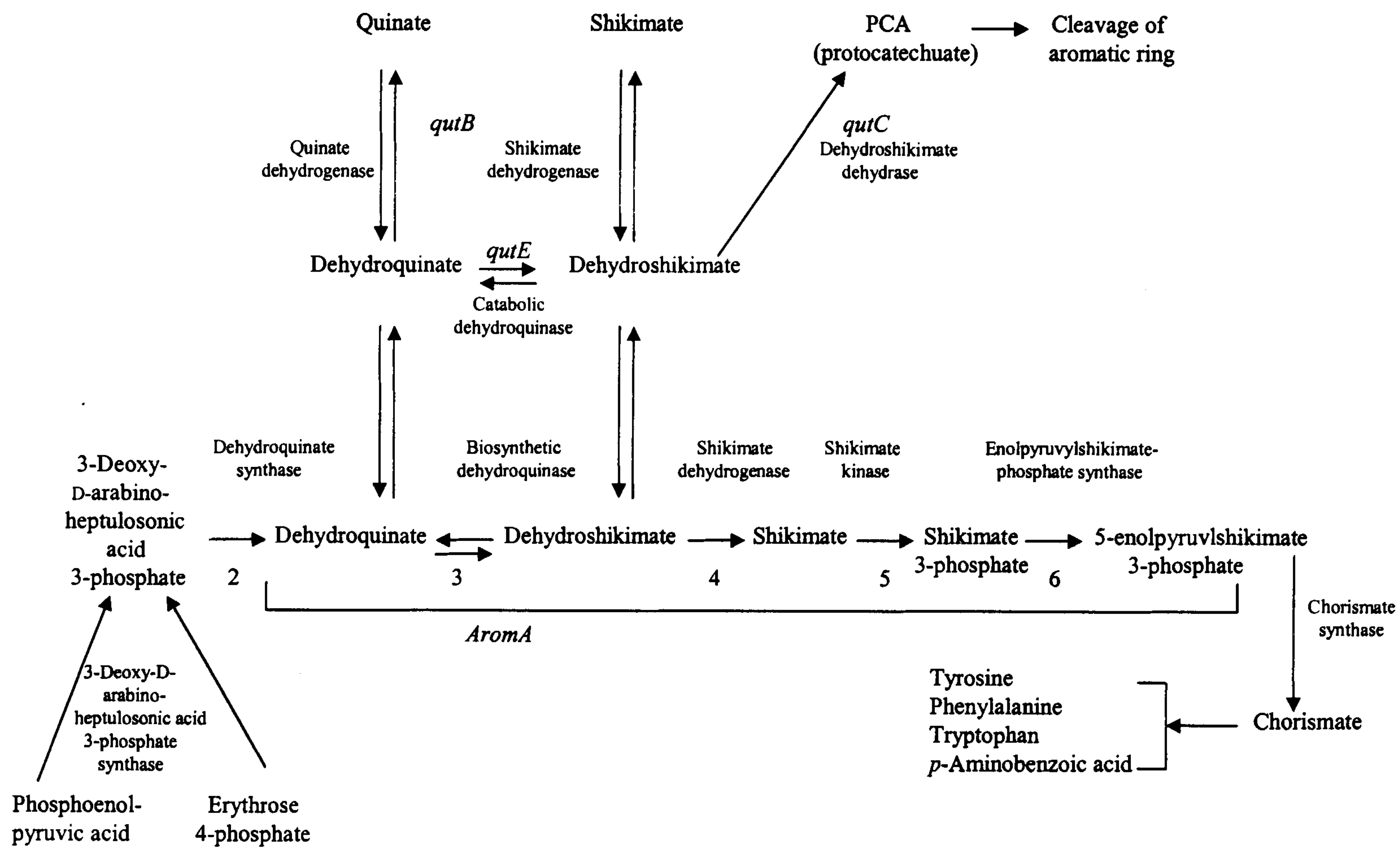


Figure 1-1. The genes, enzymes and metabolites comprising the quinate and shikimate pathways in *A. nidulans* (Lamb *et al.*, 1992)

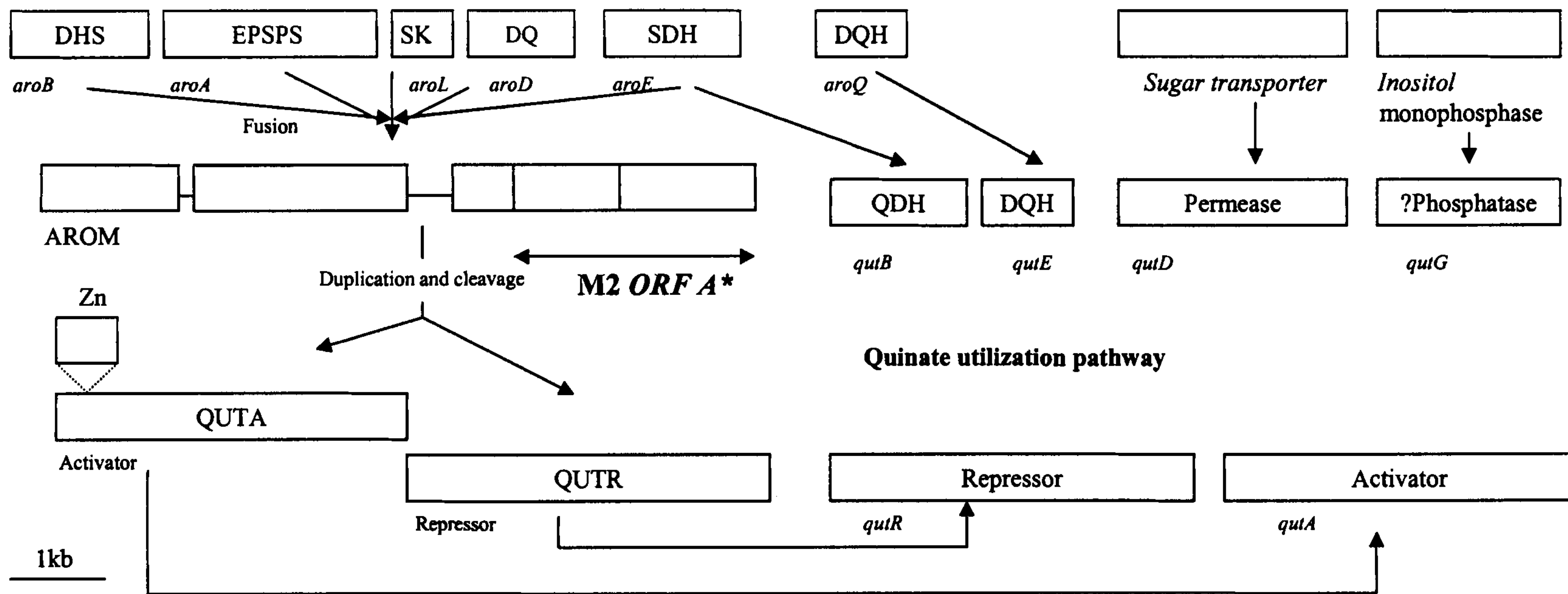
by a pentafunctional enzyme, the AROM protein, that is encoded by a single gene, the *arom* gene (Charles *et al.*, 1986; Hawkins *et al.*, 1993a).

Arom genes have been cloned and sequenced from *Saccharomyces cerevisiae* (Duncan *et al.*, 1987), *Aspergillus nidulans* (Charles *et al.*, 1986) and *Pneumocystis carinii* (Banerji *et al.*, 1993). Comparison of the predicted amino acid sequences encoded by *arom* genes with those of corresponding genes from bacteria strongly suggest that the *arom* gene has evolved from the fusion of the respective corresponding enzymes in bacteria (Hawkins, 1987). The AROM polypeptide possesses five enzymatic activities and contains the five corresponding domains of the respective monofunctional enzymes from bacteria. The five domains (starting from N-terminal) in AROM are dehydroquinate synthase (DHS), enolpyruvylshikimate-phosphate synthase (EPSPS), shikimate kinase (SK), dehydroquinase (DQ) and shikimate dehydrogenase (SDH). The modular structure of AROM polypeptide is shown in Figure 1-2 (Hawkins *et al.*, 1993a).

The quinate pathway has been studied in more detail in *Neurospora crassa* (Grant *et al.*, 1988) and *Aspergillus nidulans* (Hawkings *et al.*, 1988). Eight genes, comprising the quinate pathway (*qut*) gene cluster, are involved in quinate catabolism. Six genes have clear functions (Grant *et al.*, 1988). The six *qut* genes and their products are *qutA*-activator (QUTA), *qutB*-quinate/shikimate dehydrogenase; *qutC*-dehydroshikimate dehydrase, *qutD*-quinate permease, *qutE*-3-dehydroquinase (catabolic), and *qutR*-repressor (QUTR). All quinate pathway genes are regulated at the transcriptional level (Grant *et al.*, 1988). The activator

Figure 1-2. Modular structure of the enzymes and regulatory proteins comprising the quinate and pre-chorismate shikimate pathways in *A. nidulans* (Hawkins *et al.*, 1993b). The boxes denoted *aro* designate genes from bacteria encoding monofunctional shikimate pathway enzymes: *aroA, E. coli* EPSP synthase (EPSPS); *aroB, E. coli* DHQ synthase (DHS); *aro D, E. coli* type I 3-dehydroquinase (DQ); *aroE, E. coli* shikimate dehydrogenase (SDH); *aro L, E. coli* shikimate kinase (SK); and *aro Q, M. tubeculosis* type II 3-dehydroquinase (DQH). AROM designates the modular structure of the AROM protein of *A. nidulans* which is specified by the *arom A* gene that arose by the fusion of the bacterial *aro A , B, D,* E and *L* genes. The boxes denoting the genes of the *qut* pathway are: *qut A*, activator; *qut B*, quinate/shikimate dehydrogenase (QDH); *qut D,* permease; *qut E*, type II 3-dehydroquinase (DQH); *qut G*, possibly a phosphatase; and *qut R*, repressor. The genes encoding the quinate pathway activator and repressor proteins are proposed to have arisen by duplication of the *arom A* gene followed by cleavage in the DNA sequence specifying the C-terminus of the EPSP synthase domain (*aro A* equivalent) (Hawkins *et al.*, 1993b). Zn, a pre-formed zinc binuclear cluster motif.

Double-headed arrow '*' indicates that two domains (DQ-like and SDH-like) included in the *ORF A* of the M2 dsRNA (Lakshman *et al.*, 1998).



DHS
EPSPS
SK
DQ
SDH
DQH
aroB
aroA
aroL
aroD
aroE
aroQ
Sugar transporter
Inositol monophosphase
Fusion
AROM
QDH
DQH
Permease
?Phosphatase
qutB
qutE
qutD
qutG
Duplication and cleavage
M2 ORF A*
Zn
Quinate utilization pathway
QUTA
Activator
QUTR
Repressor
Repressor
Activator
qutR
qutA
1kb

protein (QUTA) is required for its own mRNA synthesis (autoregulation) and for synthesis of other quinate pathway mRNAs (Grant *et al.*, 1988). The QUTR repressor protein mediates its repressing activity through a direct interaction with the QUTA activator protein, thereby indirectly controlling its own expression (Lamb *et al.*, 1996). The repressing effect of the QUTR is likely to be negated by the binding of the inducer quinate or other *qut* pathway metabolites (Lamb *et al.*, 1996).

Comparison of the predicted amino acid sequences of the *arom*, *qutA* and *qutR* genes suggests that *qutA* and *qutR* originated from the splitting of a duplicated copy of the *arom* gene or its precursor (Hawkins *et al.*, 1993b) (Figure 1-2). The deduced amino acid sequences of the QUTA and the QUTR peptides show homology with the two N-terminal domains (dehydroquinate synthase and EPSP synthase) and the three C-terminal domains (shikimate kinase, 3-dehydroquinase, and shikimate dehydrogenase) of the AROM protein, respectively (Hawkins *et al.*, 1993b). Cross-linking studies strongly suggested that the native AROM protein is active as a dimer of identical subunits that fold into a compact globular shape with the N- and C-terminal domains (dehydroquinate synthase and shikimate dehydrogenase, respectively) closely linked (Smith and Coggins, 1983). The above evidence might explain the mechanism for the recognition of the activator QUTA by the repressor QUTR. As in the native AROM protein, QUTA and QUTR juxtapose closely so that the N-terminus of the activator, dehydroquinate synthase-like domain, is in close proximity with the C-terminus, shikimate dehydrogenase-like domain, of the repressor (Smith and Coggins, 1983).

The QUTA protein of the quinate pathway has at least four functional domains: the DNA-binding domain, a dimerization domain, a transcription activation domain, and a domain for interaction with the repressor (Giles *et al.,* 1991). The DNA-binding domain, located at the N-terminal of the activator, is a conserved 28 amino acid sequence containing a six-cystein zinc-binding motif (Baum *et al.*, 1987). The transcription activation domain is located at the C-terminal of the activator (Geever *et al.*, 1989). The domain for interaction with the repressor overlaps with the transcription activation domain at the C-terminal of the activator (Giles *et al.*, 1991). The N-terminal region of the QUTR is thought to occlude the transcription activation domain of QUTA and prevent it from interacting with the promoter regions of quinate pathway genes (Hawkins *et al.*, 1993a)

1.5. The connection between hypovirulence and shikimate-quinate pathways

Phenylacetic acid (PAA) is a metabolite of phenylalanine. Cultures of *R. solani* produce PAA *in vitro* (Frank and Francis, 1976) and PAA acts as a plant growth regulator (auxin) at physiological levels (Chamberlain and Wain, 1971; Milborrow *et al.*, 1975). However, at high concentrations it causes the Rhizoctonia disease symptoms on potato in the absence of the pathogen itself (Frank and Francis, 1976). The increased growth response of potato to the hypovirulent isolate Rhs 1A1 appeared to be phytohormome-induced (Bandy and Tavantzis, 1990). The amount of PAA (μg/g dry weight of mycelium) produced by hypovirulent isolate Rhs 1A1 was only 10% of that produced by virulent AG3 isolates, and hypovirulence is

related to a reduced level of toxin (PAA) production (Tavantzis *et al.*, 1989; Tavantzis and Lakshman, 1995).

As discussed above, the polypeptide A shows homology with the two C-terminal domains of AROM (Figure 1-2). Polypeptide A is also related to QUTR (Figure 1-2). Amino acid comparison between polypeptide A and repressor QUTR of the quinate pathway shows that polypeptide A lacks the portion of the EPSPS-like and the SK-like domains that comprise the N-terminal of QUTR (Lakshman *et al.*, 1998). This important difference between polypeptide A and QUTR is the basis of the hypothesis stated in the following paragraph as to the potential mechanism used by the M2 dsRNA to interfere with the normal functions of the shikimate and quinate pathways. It is predicted that polypeptide A interacts with the activator but still allows transcription of the quinate pathway genes to proceed.

The ultimate goal is to control the Rhizotonia disease biologically by exploiting the relationship between dsRNA and hypovirulence. However, the mechanism that causes hypovirulence in *R. solani* AG3 isolates is unknown. Based on the genetic information carried by the M2 dsRNA (Lakshman *et al.*, 1998), data from PAA analysis (Tavantzis *et al.*, 1989; Tavantzis and Lakshman, 1995), the intensive studies on the relationship of shikimate and quinate pathways in fungi (Geever *et al.*, 1989; Giles *et al.*, 1991; Hawkins *et al.*, 1993a), and the fact that the M2 dsRNA replicates only in hypovirulent isolates (Tavantzis and Lakshman, 1994), a hypothesis has been put forth to explain the mechanism of the M2 dsRNA-mediated hypovirulence in *R. solani.* It has been observed that M2 dsRNA, which is capable of self-replicating, produces a high amount of polypeptide A (Lakshman and Tavantzis,

1994). This polypeptide may act as a truncated, inactive repressor that could out-compete QUTR in binding the transcriptional activator QUTA while still allowing transcription of the quinate pathway gene cluster. This would cause a constitutive expression of the quinate pathway. In tum, the shikimate pathway would be down-regulated and PAA levels reduced, thus resulting in the hypovirulence phenotype of the $M2^+$ isolates (Lakshman *et al.*, 1998).

In order to test this hypothesis, this project addressed the following questions: 1. Is pA detectable in $M2^+$ isolates? 2. What are the expression levels of the shikimate and quinate pathways in $M2^+$ and $M2^-$ isolates? 3. Could induction of the quinate pathway bring about hypovirulence in Rhs 1AP? 4. Do the intermediates or end-product (s) of the shikimate pathway affect the virulence of Rhs 1AP and Rhs 1A1? 5. Is pA or its respective mRNA detectable in quinate-induced Rhs 1AP? 6. Is the *arom* gene pentafunctional in *R. solani* (Basidiomycetes)?

MATERIALS AND METHODS

2.1. Media

Vogel's minimal salts (MS) solution was described By Davis and deSerres (1970). One liter of MS solution contained 150 g of sodium citrate (5 1/2 H_2O), 250 g of KH_2PO_4 (anhydrous), 100 g of NH_4NO_3 (anhydrous), 10 g of $MgSO_4$ (7 H_2O), 5 g of $CaCl_2$ (2 H_2O), and 5 ml of trace element solution (see below). This gives a 50x strength stock solution, pH 5.8. Vogel's minimal salts solution containing 20 mM glucose is referred to as liquid 'glucose minimal media.' Similarly, Vogel's minimal salts solution containing either quinate (26 mM) or glycerol (20 mM) as a sole carbon source is referred to as liquid 'quinate minimal media' or liquid 'glycerol minimal media' (Lamb *et al.*, 1992). Solid media contained 15 g of agar in 1000 ml of the above liquid media.

In 100 ml, the trace element solution contained 5 g of citric acid (1 H_2O), 5 g of $ZnSO_4$ (7 H_2O), 1 g of Fe $(NH_4)_2(SO_4)$ (6 H_2O), 0.25 g of $CuSO_4$ (5 H_2O), 0.05 g of $MnSO_4$ (1 H_2O), 0.05 g of H_3BO_3 (anhydrous), 0.05 g of Na_2MoO_4 (2 H_2O). The reagents contained in MS and trace element solutions were dissolved in the order listed above.

Potato dextrose yeast extract (PDY) (Difco Laboratories, Detroit) liquid media contains 29 g of potato dextrose and 1 g of yeast extract dissolved in 1000 ml of distilled water. Solid media contains 15 g of agar in 1000 ml of the above liquid media.

2.2. Fungal isolates

Isolates Rhs 1AP, Rhs 1A1, Rhs 1A2, Rhs 1A3 were described in the Introduction. Rhs 23-3b was generated by pairing Rhs 1A2 and Rhs 1A3 (Jian *et al.*, 1997). Rhs 1A3 was used as a potential recipient of M2 dsRNA. A mycelial explant from the Rhs 1A3 side at position b was taken 48 hrs after hyphal fusion and gave rise to culture Rhs 23-3b (Jian *et al.*, 1997). Rhs 23-3b contains all three dsRNA elements from Rhs 1A2 and Rhs 1A3 (L1, M2, S1). Thus, Rhs 23-3b is a $M2^+$, hypovirulent culture. All of the fungal cultures were maintained on PDYA at room temperature.

2.3. Culture conditions for western blotting

The hypothesis is based on the presence of polypeptide A in $M2^+$ isolates. To test if $M2^+$ isolates contain polypeptide A, western blot analysis was carried out using total proteins extracted from three $M2^+$ isolates (Rhs 1A1, Rhs 1A2, and Rhs 23-3b) and two $M2^-$ isolates (Rhs 1AP and Rhs 1A3). Plugs of Rhs 1AP, Rhs 1A1, Rhs 1A2, Rhs 1A3, and Rhs 23-3b were transferred from PDYA plates to one-liter flasks containing 150 ml PDY liquid media. Inoculated flasks were incubated at room temperature for 10 days. Mycelia pads were harvested, frozen in liquid nitrogen, and stored at -70 °C until use.

To study the effect of quinate induction on expression of polypeptide A, western blot analysis was carried out using total proteins extracted from untreated and quinate-induced Rhs 1A1 ($M2^+$) and Rhs 1AP ($M2^-$). Plugs of Rhs 1A1 and Rhs 1AP were transferred from PDYA plates to one-liter flasks containing 150 ml liquid

media with glucose or glycerol as a carbon source. Inoculated flasks were incubated at room temperature for 10 days. Mycelial pads grown in glucose minimal media were harvested on a sterilized Buchner funnel and washed twice with 500 ml sterile minimal media (no carbon source). For quinate induction, washed mycelial pads were transferred to 150 ml of fresh glycerol minimal media for 1 hour. Quinic acid (Sigma Chemicals, St. Louis) (pH 6.0, adjusted with sodium hydroxide) was then added to a final concentration of 5.2 mM. For control, the washed mycelial pads were transferred to fresh glycerol minimal media with no quinate. Mycelia grown on glycerol minimal media were directly induced by the addition of quinate to a final concentration of 5.2 mM. Mycelia from the above three treatments were harvested after 5 hours, frozen in liquid nitrogen, and stored at -70°C until use.

2.4. Culture conditions for enzyme assays and polysome purification

Mycelial plugs of Rhs 1A1 and Rhs 1AP cultures were transferred from PDYA plates onto solid glucose minimal media covered with cellophane (Fisher Scientific Co., Dayton, OH). Young mycelia were harvested and homogenized in a blender (Commercial blender, No. 31BL91, Dynamics Co., New Hartford, CO) three times at high speed for 10 seconds each time. The mycelial suspension was used for inoculation of the liquid media.

The induction method described by Lamb *et al.* (1992) was used in this study with some modifications. A 20-µl aliquot of the mycelial suspension was transferred to one-liter flasks containing 150 ml of glucose minimal media. The flasks were incubated at 25°C for 2 days on a gyratory shaker (Model G25, New Brunswick

Scientific Co., Inc., Edison, NJ) at 250 rpm. Mycelia were harvested on a sterilized Buchner funnel and washed twice with 500 ml of sterile minimal media containing no carbon sources. For quinate induction, washed mycelia were resuspended in 150 ml of glycerol minimal media for 1 hour. Quinic acid (pH 6.0, adjusted with sodium hydroxide) was then added to a final concentration of 5.2 mM. In control experiments, washed mycelia were resuspended in glycerol minimal media containing no quinate. Induced and control mycelia were incubated for either 5 hrs or 18 hrs on a gyratory shaker at 250 rpm.

In experiments where quinate was used as a sole carbon source, mycelia were cultured initially in the same manner as described above using glucose as the carbon source to facilitate growth. Harvesting and washing steps were the same as those described above. Washed mycelia were then resuspended in 150 ml of minimal media without a carbon source for 1 hour. Quinic acid (pH 6.0, adjusted with sodium hydroxide) was added to a final concentration of 26 mM. The treated mycelia were incubated for 18 hrs on a gyratory shaker at 250 rpm.

All of the treated mycelia were harvested and frozen in liquid nitrogen and stored at -70°C until use.

2.5. Culture conditions for assessment of virulence

Mycelial plugs of Rhs 1AP and Rhs 1A1 were transferred from PDYA plates onto solid glucose minimal media. Mycelial plugs of both isolates were then taken from glucose minimal media to fresh plates with different media. Mycelial plugs were used to inoculate potato sprouts after three transfers on the designated media.

Cultures were allowed to grow for 5 days and mycelial plugs were taken from the newly growing mycelial tips. The different media are listed in Table 2-1. Quinate, chorismate, shikimate, phenylalanine, tryptophane, and tyrosine were purchased from Sigma Chemicals.

2.6. Production and partial purification of antibodies against polypeptide A

A region of 15 amino acids (DKPQLYPFSKEWNRE, positions 685-699) near the carboxyl terminal end of polypeptide A (pA) was selected for antibody production based on hydropathy index, most favorable protein secondary structure, accessible surface probabilities and regions of flexibility. The selected polypeptide was synthesized, and antibodies to polypeptide A (polypeptide A-specific antibodies or anti-pA antibodies) were raised using a rabbit (Zymed Laboratories Inc., California). Immunoglobulins (Igs) were partially purified by ammonium sulfate precipitation (Hampton *et al.* 1990). The IgG fraction was further purified by Affi-Gel protein A agarose column chromatography (Bio-Rad, Hercules, CA). A partially purified antibody preparation in binding buffer (10 mM sodium phosphate, 150 mM sodium chloride, pH 8.4) was applied to the pretreated column (equilibrated with the binding buffer) according to the manufacturer's directions (Bio-Rad). Columns were thoroughly washed with the binding buffer until the absorbance (280 nm) of the eluant approached zero. Immunoglobulins were eluted with 1 ml of 0.1 M acetic acid, pH 3.0 according to the manufacturer's directions (Bio-Rad). Protein concentration was determined by the Bradford method (Bradford, 1976).

Table 2-1. Media on which *R. solani* isolates Rhs 1A1 and Rhs 1AP were cultured before their virulence was tested on potato sprouts.

Media
Glucose minimal medium (GluMM): Vogel's minimal salts + glucose (20 mM)
GluMM + shikimate (2.2 μM)
GluMM + chorismate (1.7 μM)
GluMM + quinate (5.2 mM) (for induction)
GluMM + aromatic amino acids [Trp (25 μg/ml)/ Tyr (125 μg/ml)/ Phe (25 μg/ml)]
GluMM + chorismate (1.7 μM) + quinate (5.2 mM)
Quinate minimal media: Vogel's minimal salts + quinate (26 mM)
Glycerol minimal media (GlyMM): Vogel's minimal salts + glycerol (20 mM)
GlyMM + quinate (5.2 mM) (for induction)
PDYA: 29 g potato dextrose + 1 g yeast extract + 15 g agar

2.7. Protein extraction for western blotting

Total protein samples were prepared from *R. solani* isolates Rhs 1AP, Rhs 1A1, Rhs 1A2, Rhs 1A3, and Rhs 23-3b according to the direction of the 'Xpress Protein Purification System' (Invitrogene Co., Carlsbad, CA) with some modification. Frozen mycelia were pulverized in liquid nitrogen and incubated with 3 volumes of pre-warmed guanidinium lysis buffer (6M guanidine hydrochloride, 20 mM Na_3PO_4, 500 mM NaCl, pH 7.8) at 37°C for one hour. Crude extracts were centrifuged at 4,000 x *g* for 10 minutes. The supernatant was passed through mira-cloth (Calbrichem brand, Behring Diagnostics, La Jolla, CA) and dialyzed against 0.5 x PBS buffer (13.8 mM NaCl, 3 mM KCl, 11.6 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) containing protease inhibitors [leupeptin (1μg/ml), pepstatin A (1μg/ml), EDTA mM] at 4°C overnight. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.8. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels (8%) were prepared according to Sambrook *et al.* (1989). Each lane was loaded with approximately 50 μg of protein extracted from *R. solani* isolates. Before loading onto the gel, protein samples were mixed with the appropriate amount of loading buffer and boiled for 5 min. Proteins of the Benchmark Prestained Protein Ladder (Gibco BRL, Gaithersburg, MD) were used as size standards. Electrophoresis was carried at 20 V/cm overnight in running buffer (192 mM glycine, 25 mM Tris base, and 0.1% SDS, pH 8.3) (Sambrook *et al.* 1989).

2.9. Western blot analysis

After fractionation on SDS-polyacrylamide gels, fractionated proteins were electrotransferred onto Zeta-probe blotting membranes by a Trans-blot cell at 20 V overnight at room temperature (Manufactures' manual, Bio-Rad). The transferring buffer contained 25 mM Tris base, and 192 mM glycine, pH 8.3 (Sambrook *et al.*, 1989). Purified Anti-pA antibodies were used for detecting the M2-encoded polypeptide A. Western blot hybridization was carried out according to Sambrook *et al.* (1989) with minor modifications. Following protein transfer onto Zeta-probe blotting membrane, membranes were incubated in 50 ml of TBS buffer (20 mM Tris-HCl, and 500 mM NaCl, pH 7.5) with 3% (v/w) nonfat dry milk for 1 hr. After washing in 100 ml of TBST buffer (TBS buffer + 0.05% Tween-20, w/v) with 1% nonfat dry milk (w/v) for 5 min, the membranes were incubated with anti-pA antibodies (1: 200 dilution) in fresh TBST buffer for 3 hrs. Membranes were then washed twice with 100 ml of TBST for 30 min and incubated with anti-rabbit alkaline phosphatase labeled secondary antibody (1: 30,000 dilution, Sigma) in fresh TBST buffer containing 1% nonfat dry milk for 30 min. Membranes were then washed twice with 100 ml of TBST buffer for 30 min, rinsed twice in 10 ml of alkaline buffer (100 mM diethanolamine, 100 mM NaCl, and 5 mM $MgCl_2$), and incubated in 10 ml of freshly prepared substrate solution [a nitroblue tetrazolium (NBT)/5'-bromo-4-chloro-3-indoxyl phosphate (BCIP) tablet dissolved in 10 ml of alkaline buffer] (NBT/BCIP tablets were purchased from Sigma Chemicals). One-half milliliter of 0.5 M EDTA (pH 8.0) was added to stop the color reaction. All of the procedures were performed at room temperature with gentle shaking.

2.10. Preparation of cell-free extracts for enzyme assays

Cell-free extracts were prepared using a method described by Chaleff (1974b) with minor modifications. Mycelia from the different treatments were harvested in liquid nitrogen. After lyophilization, mycelia were ground to a fine powder and extracted in 3 volumes of 50 mM Tris-HCl buffer (pH 7.1) containing 1 mM EDTA, 1mM α-thioglycerol, 2 μg of leupeptin/ml and 1 μg of pepstatin A/ml. The mixture was incubated on ice for 30 min with gentle stirring. Cellular debris was removed by centrifugation (J2-21, Beckman Instruments, Inc., Palo Alto, CA) at 40,000 x *g* for 1 hour and the supernatant was transferred to a sterile tube. Protein concentrations were determined by the Bradford method (Bradford, 1976). Glycerol was added to a final concentration of 20% (w/v), and small aliquots were stored at –20°C.

2.11. Enzyme assays

All assays were performed spectrophotometrically in a total volume of 1 ml in disposable cuvettes of 1.0 cm light path (HP 8425A UV-Visible Spectrophotometer, Hewlett Packard Co.). Specific activities were defined as μmol of substrate consumed (or of product formed)/min/mg protein.

Analysis of the shikimate kinase (SK) activity and quinate dehydrogenase (QDHase) activity was carried out to determine the expression levels of the shikimate pathway and the quinate pathway, respectively.

Shikimate kinase activity was measured as described by Coggins *et al.* (1987) at 25°C by coupling two reactions catalyzed by pyruvate kinase (PK) and

lactate dehydrogenase (LDH) reactions. Oxidation of NADH was monitored at 340 nm. SK, PK, and LDH catalyze the three following reactions, respectively (Bergmeyer, 1974):

1) Shikimic acid + ATP $\longrightarrow$ shikimate-3-phosphate + ADP
2) ADP + Phosphoenolpruvate $\longrightarrow$ ATP + Pyruvate
3) Pyruvate + NADH + H^+ $\longrightarrow$ Lactate + NAD^+

The reaction mixture contained 1 mM shikimic acid (Sigma Chemicals, St. Louis), 2.5 mM ATP (Sigma Chemicals), 1mM phosphoenolpyruvate (Sigma Chemicals), 200 μM NADH (Sigma Chemicals), 2.5 mM $MgCl_2$, 50 mM KCl, 50 mM triethanolamine-HCl (pH 7.0), 3 units of PK/ml (Boehringer, Mannheim, Germany), 2.5 units of LDH/ml (Boehringer), 0.3 mM KCN, and 1/10 volume of tissue extract.

Quinic acid dehydrogenase (QDHase) activity was assayed at 37°C by monitoring the reduction of NAD^+ (Chaleff, 1974b). The reaction mixture contained 85 mM Tris-HCl (pH 8.6), 6.7 mM quinate (Sigma Chemicals), 2.5 mM NAD (Sigma Chemicals), 0.3 mM KCN (Sigma Chemicals) and 1/10 volume of tissue extract. Quinate dehydrogenase catalyzes the following reaction:

2.12. Assessment of virulence

After three transfers on the designated media (Table 2-1), cultures of Rhs 1AP or Rhs 1A1 were used to inoculate potato sprouts. Mycelial plugs from uninoculated media were used as controls. Pathogenicity tests were conducted as described by Bandy *et al.* (1988). Inoculated potato sprouts were covered with

sterilized soil at room temprature for 5 days. The lesion area on the sprouts was measured to determine the relative virulence. Two sets of experiments were carried out. The first set was to determine whether virulence of Rhs 1AP and Rhs 1A1 are affected by quinate, shikimate (shikimate pathway intermediates), chorismate (end-product of the shikimate pathway) or the three aromatic amino acids (Phe, Try, Tyr, major metabolites of the shikimate pathway). The second set of experiments was designed to examine if quinate could override the effect of the metabolites used in the first set of experiment.

2.13. Amino acid analysis by high performance liquid chromatography (HPLC)

Mycelia of Rhs 1AP and Rhs 1A1 were grown on the designated media described in Table 2-1. To facilitate harvesting of the mycelia, cellophane was placed on the media prior to the final mycelial transfer. Mycelia were harvested, lyophilized, and ground to a fine powder. Sample hydrolysis and amino acid labeling were as described by Lin *et al.* (1975) with minor modifications. Samples (2.0 ± 0.5 mg) were hydrolyzed in vacuum hydrolysis tubes (No. 29560, Picerce, Rockford, IL) at 115°C for 24 hours in vacuum as described by Simpson *et al.* (1976). The hydrolysate was neutralized partially with 0.5 ml of 3.5 N NaOH. Neutralized solution aliquots (10 μl) were transferred to an Eppendorf tube and freeze-dried. The following reagents were added to the above Eppendorf tube: 130 μl of 0.1 N $NaHCO_3$ and 130 μl of dabsyl chloride (4 nmol/μl acetone) (Sigma Chemicals). This mixture was heated at 70°C for 10 min and filtered using a cellulose nitrate filter (0.45 μm). A 5-μl aliquot of the filtrate was used for HPLC analysis. Standard amino acids (Sigma Chemicals)

were labeled with dabsyl chloride using the same method described above for generating standard curves. Amino acids were fractionated by HPLC using Supelcosil LC-DABS columns (Supelco, Ballefonte, PA) on a Hewlett Packard Series 1050 HPLC machine (Hewlett Packard Co., Burlington, MA) described by Stocchi *et al.* (1989).

2.14. Immunoprecipitation of polysomes

Polysomes were isolated as described by Hampton *et al.* (1990) with minor modifications. Frozen induced and uninduced Rhs 1AP and Rhs 1A1 mycelia were pulverized in the presence of liquid nitrogen using a mortar and pestle. The pulverized mycelium was mixed well with 5 volumes of Buffer A (0.2 M Tris-HCl, pH 8.5, 0.2 M sucrose, 30 mM $MgCl_2$, 60 mM KCl). The mycelial extract was clarified by centrifugation (J2-21, Beckman Instruments, Inc., Palo Alto, CA) at 29,000 x *g* for 20 min. The supernatant was layered on 6 ml cushions of 1.5 M sucrose in Buffer B (20 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 20 mM KCl) and centrifuged at 95,000 x *g* for 90 min (L8-M Ultracentrifuge, Beckman Instruments, Inc.) The pellet of polysomes was rinsed and resuspended in 1 volume of Buffer C (10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 50 mM KCl, 3 mg of heparin/ml). The polysomes were then mixed with purified anti-pA antibodies for 4 hours in Binding buffer. This suspension was loaded onto equilibrated columns containing Affi-Gel protein A agarose (Bio-RAD). Columns were equilibrated with Binding buffer (Buffer C amended with 150 mM sodium chloride). The flow-through and the first 3 ml of rinse were reapplied to the column, which was then rinsed with 20 bed volumes of polysome Binding buffer. Anti-pA-

specific polysomes were eluted from the column after applying 1 ml of dissociation buffer (25 mM Tris, pH 7.5, 20 mM EDTA, 0.2 mg of heparin/ml). RNA from anti-pA-specific polysomes was obtained by phenol/chloroform extraction and ethanol precipitation, and then dialyzed overnight against DEPC-treated water to remove heparin.

2.15. Reverse transcription polymerase chain reaction (RT-PCR)

Anti-pA-specific polysome RNA from induced and uninduced Rhs 1AP and Rhs 1A1 was used as a template in RT-PCR experiments. Primer M2-P33 (5' TTGCTCTCTTGCAGATCGAC 3', positions 1263 to 1242) was used for reverse transcription of the first strand of cDNA. Primer M2-P33 with primer M2-P34 (5' ATCTCCTTCCACCAATGCCT 3' positions 2474 to 2493) were then used for PCR. PCR products were fractionated by agarose gel electrophoresis and confirmed by sequencing (Sambrook *et al.*, 1989).

2.16. Total RNA extraction

Total RNA was extracted from *R. solani* using the method of Logemann *et al.*, (1987). Mycelial tissue was pulverized in liquid nitrogen using a mortar and pestle, mixed with 2 volumes of guanidine buffer (8 M guanidine HCl, 20 mM MES, 20 mM EDTA, 50 mM β-mercaptoethanol, pH 7.0), and incubated on ice for 30 min. Cell debris was removed by centrifugation for 10 min at 10,000 x *g*. To remove proteins, one volume of phenol/chloroform (1:1) was added to the supernatant, and the phases were separated by centrifugation at 10,000 x *g* for 45 min. The RNA was

precipitated from the aqueous phase by the addition of 0.7 volumes of ethanol and 0.2 volumes of 1 M acetic acid and incubated at –20°C overnight. RNA was collected by centrifugation at 10,000 x *g* for 30 min. The pellet was dissolved in DEPC treated water, and the RNA suspension was again phenol/chloroform extracted and then precipitated with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. After storing at -20°C overnight, RNA was recovered by centrifugation at 10,000 *g* for 30 min, washed with 70% ethanol, dried, and resuspended in DEPC treated water and stored at -80°C.

2.17. Complementary DNA (cDNA) cloning of the *arom* mRNA

Degenerate primers (EPSP1 and EPSP2) were derived from consensus sequences of three AROM polypeptides (*Saccharomyces cerecisiae*, *Aspergillus nidulans* and *Pneumocystis carinii*). EPSP1 was derived from the consensus sequence GNAGTA (residues 586-591 from the *A. nidulans* AROM protein), and EPSP2 from the consensus sequence KECNRI (residues 752-758 from the *A. nidulans* AROM protein) of the EPSP domains. The nucleotide sequence of EPSP1 was 5' GGNAAYGCNGGNACNGC 3', and that of EPSP2 5' DATNCKRTTRCAYTCYTT 3'. RT-PCR was carried out using total RNA from *R. solani* and the above degenerate primers. A PCR product of 850 bp was cloned and sequenced. Blast N and Blast X searches in the GENBANK confirmed that this was a partial *arom* clone. Using other upstream and downstream conserved primers as well as end-specific primers from the above clone, the rest of the *arom* mRNA was cloned and sequenced. One pair of primers included a degenerate primer DHQ3 (upstream,

5' ACHGGNGGNGGNGTNATHGG 3') from the consensus sequence GGGVIGD (residues 113-118 from the *A. nidulans* AROM protein) of the DHS domain and an end-specific primer EPSP3 (downstream, 5' AGACAGTGGTGAGGAAGCGA 3'). Another pair included an end-specific primer EPSP4 (upstream, 5' GTATTGCCAACCAACGTGTC 3') and an anchor primer 5' AAGCTTTTTTTTTTTTTTTA 3'. A 5'-RACE (rapid amplication of cDNA ends) reaction was employed with SuperScript^{TM} II RT polymerase for reverse transcription in two-step RT-PCR (Gibco BRL) to clone the 5'-end of *arom* gene. The strategy for cDNA cloning is illustrated in Figure 2-1.

2.18. Genomic DNA cloning of the *arom* sequence

PCR of genomic DNA from *R. solani* was carried out using selected overlapping primers from the *R. solani arom* cDNA sequence. Genomic DNA was partially digested with endonuclease *EcoR*I since no restriction sites were detected in the *R. solani arom* cDNA sequence to improve the effciency of PCR. *Arom*-specific PCR products were separated and eluted from agarose gels using the QIA quick PCR Purification Kit (QIAquick, Quagen Inc., Mississauga, Ontario, Canada) and subjected to sequencing. The strategy for cloning the *arom* genomic DNA is illustrated in Figure 2-2. All of the primers used in genomic DNA cloning are listed in Table 2-2.

The upstream sequence from the transcription initiation site of *arom* gene was cloned by a designed PCR technique (Sambrook *et al.*, 1989). Genomic DNA of *R. solani* was digested with restriction endonucleases *Xho*I and *Sac*I (New England

Biolabs Inc., Beverly, MA). Plasmid pBSK(-) was also digested with the same enzymes. The double-digested plasmid and genomic DNA were ligated at the *SacI* site. PCR reactions were carried out using a primer (PBSK2) from the plasmid and a primer (AROMP1) near the 5' end of the *arom* gene. This procedure allowed determination of an additional sequence of 199 bp located upstream of the *R. solani* *arom* gene. Sequences of PBSK2 and AROMP1 are listed in Table 2-2.

2.19. Statistical analysis

In experiments involving enzyme assays and HPLC analysis, five samples were analyzed for each treatment. Similarly, in virulence determination experiments, five sprouts were infected with the respective inoculum to measure the lesion area for each treatment. SigmaPlot 4.0 (SPSS Inc.) was employed to calculate the means and standard deviations. Paired t-tests (between selected pairs) were performed to determine whether or not significant differences occurred between the treatments. A P-value of <0.05 was considered to be a significant difference between two treatments.

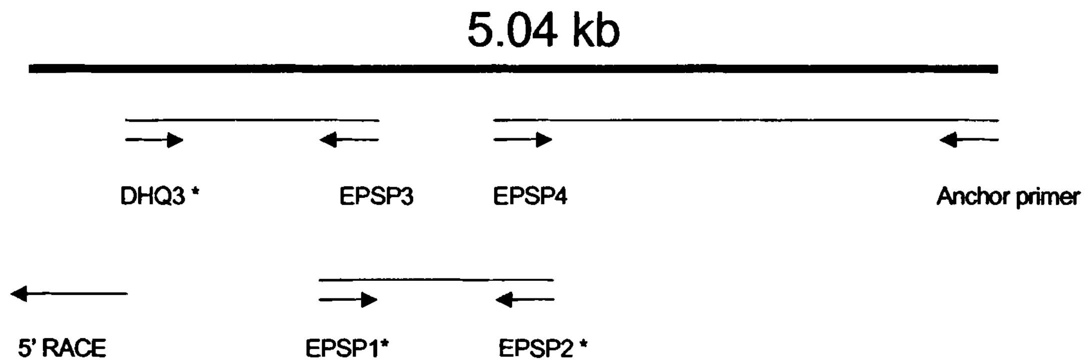


Figure 2-1. Schematic of overview of the strategy used in cDNA cloning from *arom* gene of *R. solani.* Degenerate primers '*' were selected from the consensus sequences of three AROM polypeptides (*Saccharomyces cerecisiae*, *Aspergillus nidulans* and *Pneumocystis carinii).* The 5.04 kb line denotes the *R. solani* cDNA. The initial PCR product was obtained by RT-PCR using two degenerated primers, EPSP1 and EPSP 2. This sequence was extended to the 5' end by RT-PCR using degenerate primer DHQ 3 and the end-specific primer EPSP3 (selected from the initial cDNA clone). 5' RACE reactions were carried out to clone the 5' end of the *arom* mRNA. The initial sequence was extended to the 3' end by RT-PCR using the end-specific primer EPSP4 (selected from the initial cDNA clone) and a anchor primer. Total RNA from the *R. solani.* isolate Rhs IAP was used as a template in the RT-PCR reactions.

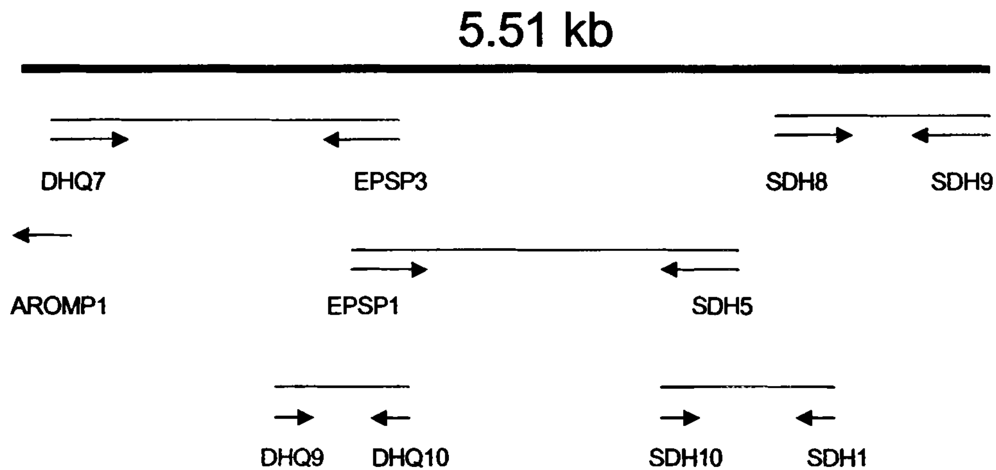


Figure 2-2. Schematic of overview of the strategy used to amplify the *arom* gene from genomic DNA of the *R. solani*. The 5.51 kb line denotes the *R. solani arom* gene. The overlapped lines denote PCR reactions using the end-specific primers selected from the *R. solani* cDNA clone. The upstream promoter region was cloned by a specifically designed PCR reaction (see text for details). Primer sequences used in the above PCR reactions are listed in Table 2-2.

Table 2-2. Nucleotide sequences of primers used for PCR-mediated of the *arom* gene amplification of the *arom* gene from genomic DNA of the *R. solani* culture Rhs 1AP.

Primers	Nucleotide sequence (5' to 3')
DHQ7	CGATTCCAACGACAGCGCAC
DHQ9	CAAGAGCATCTCCAACCGTG
DHQ10	TGAGACCGCCAGCACCAATG
EPSP1	TCGCTTCCTCACCACTGTCT
EPSP3	AGACAGTGGTGAGGAAGCGA
SDH1	GTACCAAACAGACTCGTGGA
SDH5	GCCACGATGCGATGATCTGA
SDH8	CTCAGATCATCGCATCGTGG
SDH9	GCGCCGAAACGCTGATCTTG
SDH10	TTGCGCCACACCTCGTCTCT
AROMP1	CGCGTGTCGGTAATAAGAAC
PBSK2	CTTCCGGCTCGTATGTTGTG

RESULTS

3.1. Quinate down-regulates virulence in *R. solani*; shikimate, chorismate and the aromatic amino acids enhance the virulence in Rhs 1AP

The virulence (indicated as lesion areas on inoculated potato sprouts) of the Rhs 1A1 and Rhs 1AP isolates grown on the media indicated is shown in Figure 3-1. The glucose minimal medium treatment was used as a control in this set of experiments. The PDYA treatment was used to ensure that the virulence of the two isolates remained at the expected levels in the presence of complete media.

In addition to quinate induction, the *qut* pathway enzymes are subject to a second type of regulation (carbon catatolite repression) when glucose or other carbon sources are present in the growth medium (Pall, 1981; Grant *et al.*, 1988). *R. solani* showed a faster and thicker growth when quinate was added to glucose minimal medium (data not shown). When quinate was used as a sole carbon source, *R. solani* showed a faster but more sparse growth pattern than that of glucose minimal media (data not shown). More importantly, quinate, used as a sole carbon source or as an amendment in glucose minimal medium, brought about a dramatic reduction in the virulence of both isolates. The virulence of Rhs 1AP decreased by 90% (66 mm^2 vs. 7 mm^2, $p < 0.05$) and 94% (66 mm^2 vs. 4 mm^2, $p < 0.05$), respectively, and that of Rhs 1A1 decreased by 95% (57 mm^2 vs. 3 mm^2, $p < 0.05$) and 70% (57 mm^2 vs. 17 mm^2, $p < 0.05$), respectively.

The addition of shikimate, chorismate, and aromatic amino acids (AAA) to the glucose minimal medium increased the virulence of Rhs 1AP by 233% (66 mm^2 vs.

221 mm^2, $p < 0.05$), 244% (66 mm^2 vs. 228 mm^2, $p < 0.05$) and 456% (66 mm^2 vs. 369 mm^2, $p < 0.05$), respectively (Figure 3-1). In contrast, the virulence of Rhs 1A1 was not affected dramatically by shikimate, chorismate (Figure 3-1). In fact, the virulence of Rhs 1A1 decreased about 60% ($p < 0.05$) in the AAA treatment (Figure 3-1).

3.2. Quinic acid overrides the effect of chorismate on the virulence

Figure 3-2 shows the overriding effect of quinic acid over chorismate on virulence of Rhs 1AP and Rhs 1A1. In this experiment, four treatments were used as controls, glucose minimal medium (GluMM), glucose minimal media amended with quinate (GluMM+Qui) or chorismate (GluMM+Cho) and PDYA. The virulence of Rhs 1A1 was decreased by 87% (30 mm^2 vs. 4 mm^2, $p < 0.05$) when it was grown on glucose minimal media amended with both quinate and chorismate as compared to that of glucose minimal media amended only with chorismate. The virulence of Rhs 1AP was decreased by 77% (123 mm^2 vs. 27 mm^2, $p < 0.05$) when it was grown on glucose minimal media amended with both quinate and chorismate as compared to that of glucose minimal media amended only with chorismate.

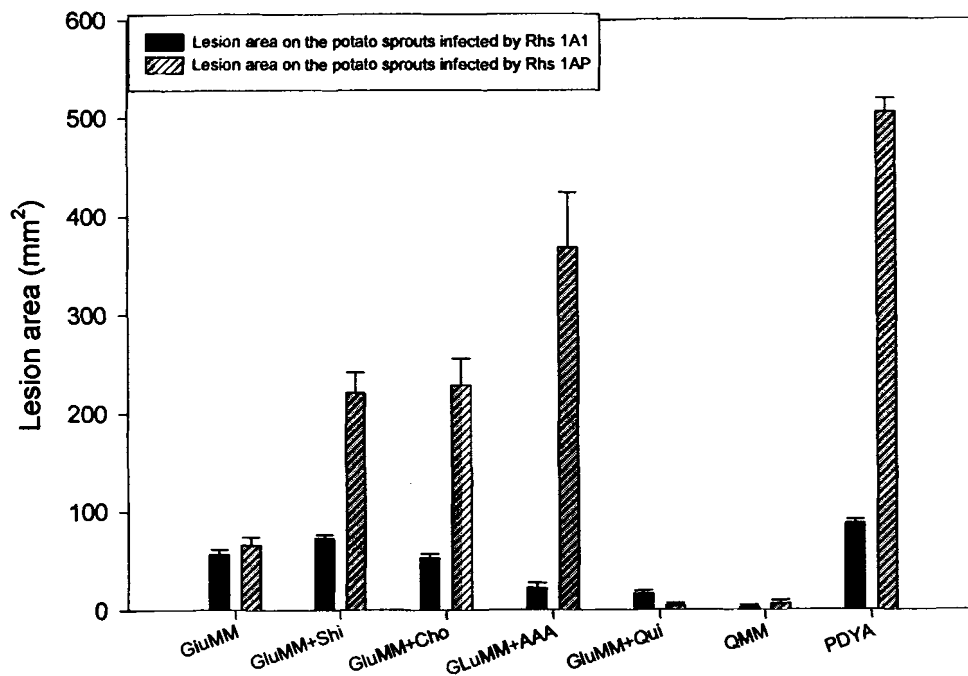


Figure 3-1. Effect of quinate, shikimate, chorismate, aromatic amino acids (AAA) on virulence (expressed as lesion area on potato sprouts) of the *R. solani* isolates Rhs 1AP and Rhs 1A1. Rhs 1AP and Rhs 1A1 were grown on the media indicated. After three transfers on the respective medium, mycelial plugs were taken from the growing area of Rhs 1AP and Rhs 1A1 to inoculate 5 potato sprouts per treatment. The inoculated sprouts were placed in sterilized soil for 5 days. Error bars indicate standard deviations. See Table 2-2 for the composition of the different media. GluMM, glucose minimal medium; QMM, quinate minimal medium; PDYA, potato dextrose yeast extract agar; GluMM+Shi (Cho, or Qui), GluMM amended with shikimate, chorismate, and AAA, respectively.

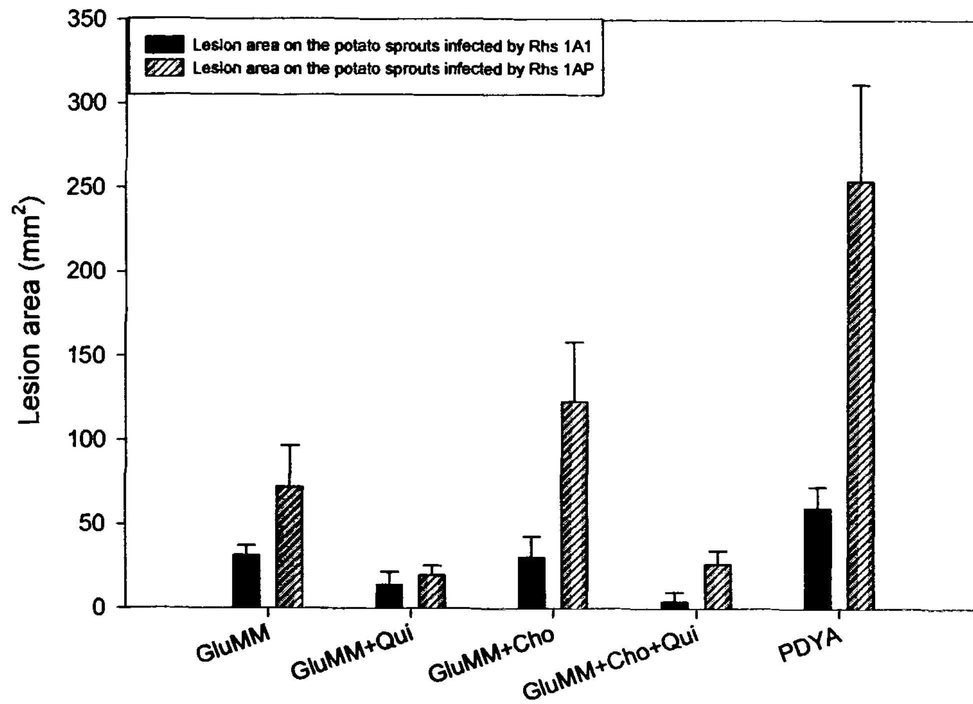


Figure 3-2. Quinate overrides chorismate-induced virulence enhancement in *R. solani* isolates. Rhs 1AP and Rhs 1A1 were grown on the different media indicated. After three transfers on the respective medium, mycelial plugs were taken from the growing area of Rhs 1AP and Rhs 1A1 to inoculate 5 potato sprouts per treatment. The inoculated sprouts were placed in sterilized soil for 5 days. Error bars indicate standard deviations. See Table 2-2 for the composition of the different media. GluMM, glucose minimal medium; PDYA, potato dextrose yeast extract agar; GluMM+Cho (Qui), GluMM amended with chorismate or quinate; GluMM+Cho+Qui, GluMM amended with chorismate and quinate.

3.3. Relationship between relative concentration of phenyalanine [Phe] and virulence in *R. solani* isolates Rhs 1A1 and Rhs 1AP

The concentrations of 17 individual amino acids were determined by HPLC analysis. Although it was reported that tryptophan could be restored by this procedure (Simpson *et al.*, 1976), no tryptophan-specific signal was identified on the HPLC profile. Asparagine and glutamine were converted into aspartate and glutamate by hydrolysis (Simpson *et al.,* 1976). The concentration of total amino acids (designated as [Total]) was calculated by adding the concentrations of individual amino acids. The relative phenylalanine concentration (designated as [Phe]) or tyrosine concentration (designated as [Tyr]) were expressed as ratios of [Phe]/[Total] or [Tyr]/[Total], respectively. The relative [Tyr] was used as a control aromatic amino acid in this study. The relationship between the relative [Phe] and [Tyr] and virulence of Rhs 1AP and Rhs 1A1 is shown in Figures 3-3 and 3-4, respectively. In Rhs 1AP, both [Phe]/[Total] and virulence were increased when GluMM amended with shikimate, chorismate, or AAA (Figure 3-3), but a similar relationship between [Phe]/[Total] and virulence was not observed in Rhs 1A1 (Figure 3-4) or when quinate was present in the media (Figures 3-3, 3-4). In contrast, the relative amount of tyrosine was not related to virulence changes in either isolate.

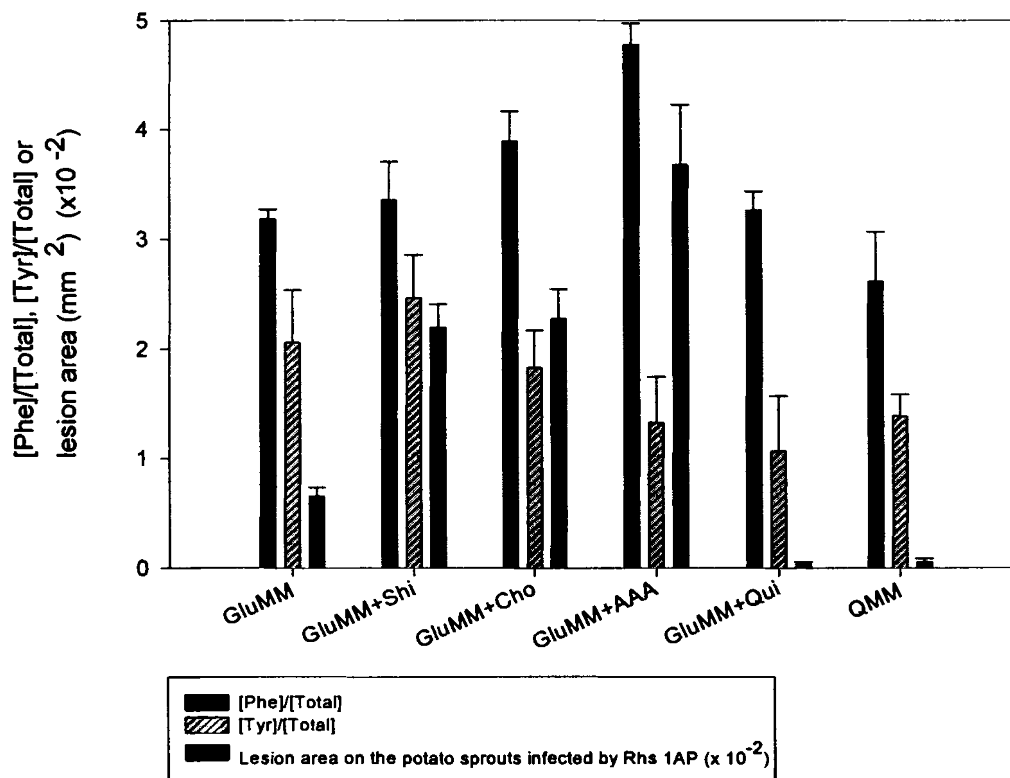


Figure 3-3. Relationship between relative [Phe] and virulence in Rhs 1AP grown on the media indicated. Amino acid concentration was determined by HPLC analysis. Total amino acid concentration (designated as [Total]), was calculated by adding the concentrations of the individual amino acids (see text for details). Relative phenylalanine or tyrosine concentrations (designated as [Phe] or [Tyr]) are expressed by the ratio [Phe]/[Total] or [Tyr]/[Total], respectively. Relative virulence of Rhs 1AP is expressed as lesioned areas ($x10^{-2}$) on the inoculated potato sprouts. Error bars indicate standard deviations. GluMM, glucose minimal medium; GluMM+Shi (Cho, AAA, or Qui), glucose minimal media amended with shikimate, chorismate, aromatic amino acids or quinate; QMM, quinate minimal medium. See Table 2-2 for the composition of the different media.

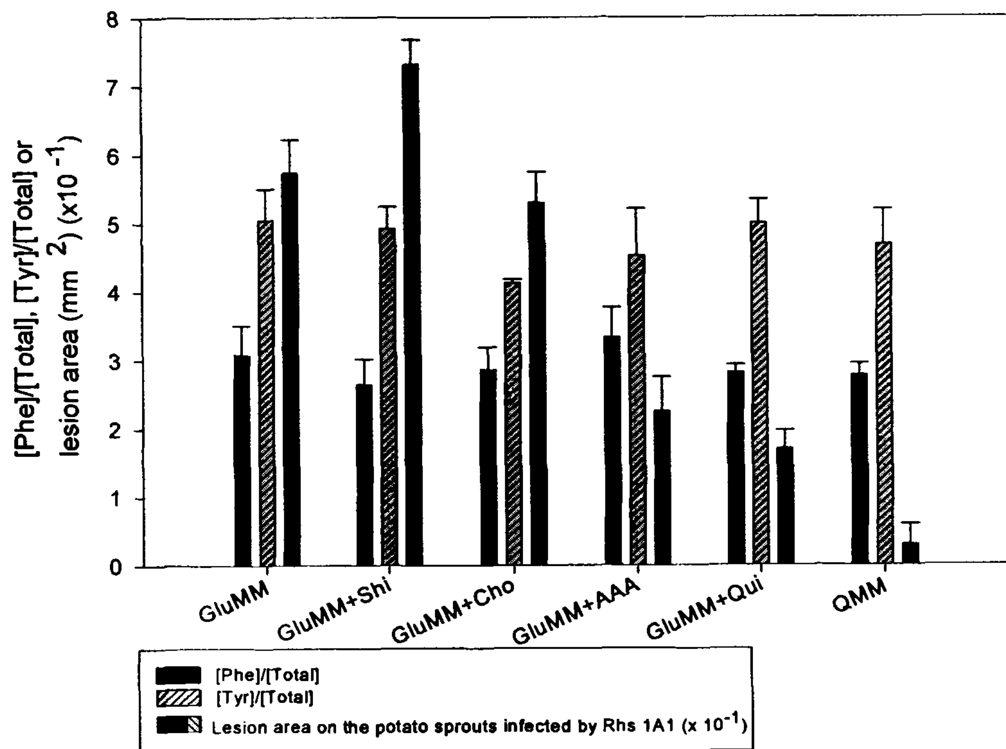


Figure 3-4. Relationship between relative [Phe] and virulence in Rhs 1A1 grown on the media indicated. Amino acid concentration was determined by HPLC analysis. Total amino acid concentration (designated as [Total]) was calculated by adding the concentrations of the individual amino acids (See text for details). Relative phenylalanine or tyrosine concentrations (designated as [Phe] or [Tyr]) are expressed by the ratio [Phe]/[Total] or [Tyr]/[Total], respectively. Relative virulence of Rhs 1A1 is expressed by lesioned areas ($x10^{-1}$) on the inoculated potato sprouts. Error bars indicate standard deviations. GluMM, glucose minimal medium; GluMM+Shi (Cho, AAA, or Qui), glucose minimal media amended with shikimate, chorismate, aromatic amino acids or quinate. QMM, quinate minimal medium. See Table 2-2 for the composition of the different media.

3.4. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies in M2-containing isolates but not in M2-lacking isolates

Western blot analysis (Figure 3-5) showed that a protein band of the size (83 kDa) expected for polypeptide A was detected in three M2 dsRNA containing isolates Rhs 1A1 (lane2), Rhs 1A2 (lane 3) and Rhs 23-3b (lane 4), but not in two M2 dsRNA lacking isolates Rhs 1A3 (lane 5) and Rhs 1AP (lane 6). The purpose of using Rhs 23-3b in this study was to see if there was a positive relationship between detection of polypeptide A and the transmission of the M2 dsRNA (Rhs 1A3 was a potential recipient of M2 dsRNA when Rhs 23-3b was generated by the pairing of Rhs 1A2 and Rhs 1A3). A large band of approximately 180 kDa was also found in Rhs 23-3b. The size of this band is twice that of polypeptide A but the identity of this band is unknown.

In this study, only crude protein extracts (50 μg) were used in SDS-polyacrylamide gel electrophoresis. The signal of the 83 kDa band is strong, indicating that $M2^{+}$ isolates contain relatively high amounts of this polypeptide.

3.5. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies in quinate-induced Rhs 1AP

Figure 3-6 shows that a polypeptide band of the expected size (83 kDa) was detected in quinate-induced Rhs 1AP. This band, however, is not detectable in uninduced Rhs 1AP. In contrast, both induced and uninduced Rhs 1A1 showed the expected polypeptide band.

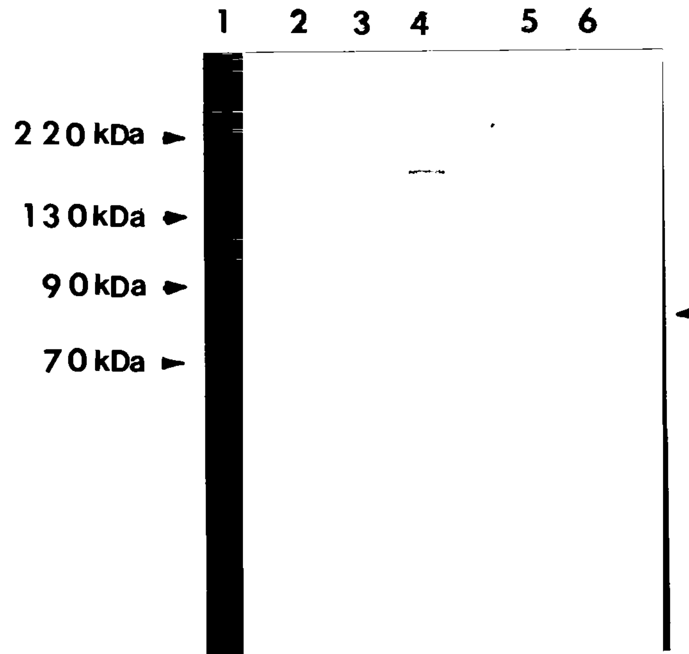


Figure 3-5. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies (anti-pA antibodies) in M2 dsRNA-containing isolates *R. solani* isolates by western blot analysis. Rhs 1A1, Rhs 1A2, Rhs 1A3, and Rhs 23-3b were grown in potato dextrose yeast extract liquid media for 10 days at room temperature. SDS-polyacrylamide gel (8%) electrophoresis was used to fractionate total protein samples. Each lane was loaded with approximate 50 μg of total protein. Antibodies against a fifteen-residue peptide (positions 685-699) selected from polypeptide A reacted with a protein of the expected size (83 kDa) in M2-containing cultures Rhs 1A1 (lane 2), Rhs 1A2 (lane 3), Rhs 23-3b (lane 4), but not in M2-lacking cultures Rhs 1A3 (lane 5) and Rhs 1AP (lane 6). Lane 1 was loaded with 10 μl Benchmark Prestained Protein Ladder (1 μg/band) (Gibco BRL). The protein bands of the prestained protein ladder are 70 kDa (pink), 90 kDa, 130 kDa and 220 kDa, respectively. The arrow on the right shows the position of the 83 kDa protein band reacting to the anti-pA antibodies. A large band of unknown identity was detectable in Rhs 23-3b.

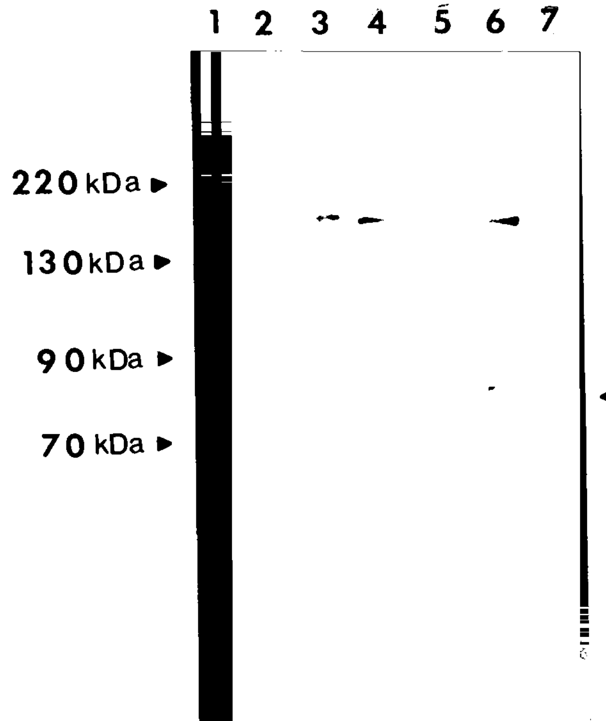


Figure 3-6. Detection of an 83 kDa protein band reacting to polypeptide A-specific antibodies (anti-pA antibodies) in quinate-induced Rhs 1AP by western blot analysis. Growth and induction conditions of the *R. solani* isolates, Rhs 1AP and Rhs 1A1, were described in Materials and Methods. SDS-polyacrylamide gel (8%) electrophoresis was used to fractionate total protein samples. Each lane was loaded with about 40 μg of proteins. Antibodies prepared against a fifteen-residue peptide (positions 685-699) from polypeptide A reacted with a protein of the expected size (83 kDa) in uninduced Rhs 1A1 (lane 2), induced Rhs 1A1 (initially grown in glucose minimal media) (lane 3), induced Rhs 1A1 (initially grown in glycerol minimal media) (lane 4), induced Rhs 1AP (initially grown in glucose minimal media) (lane 6), induced Rhs 1AP (initially grown in glycerol minimal media) (lane 7), but not in uninduced Rhs 1AP (lane 5). Lane 1 was loaded with 10 μl Benchmark Prestained Protein Ladder (1 μg/band) (Gibco BRL). The sizes of the prestained proteins are 70 kDa (pink), 90 kDa, 130 kDa and 220 kDa, respectively. The arrow on the right shows the position of the 83 kDa protein band reacting to the anti-pA antibodies. A large size (180 kDa) protein band reacting to the anti-pA antibodies was detected in quinate-induced Rhs 1A1 and Rhs 1AP.

Glucose is routinely used as a carbon source to initially grow fungus in quinate-induction studies because fungal cultures grow faster and large mycelial yields are obtained. Glycerol is routinely used as an alternative carbon source in quinate-induction media (Chaleff, 1974a, 1974b; Lamb *et al.*, 1992). It has been shown that quinate pathway enzymes exhibited reduced activities when glucose or glycerol was present in the induction media (Chaleff, 1974a, 1974b; Hawkins *et al.*, 1984; Grant *et al.*, 1988). The suppressing effect of glucose was much higher than that of glycerol (Grant *et al.*, 1988). It was interesting to see if the initial carbon sources could make a difference in the expression level of the 83 kDa polypeptide. Figure 3-6 shows that the use of glucose or glycerol as initial carbon sourses does not result in a significant difference in the expression of this polypeptide.

3.6. Detection of M2 transcript in quinate-induced Rhs 1AP

M2-specific polysomes were purified from induced and uninduced Rhs 1A1 and Rhs 1AP by immunoprecipitation using anti-pA antibodies. RT-PCR experiments were carried out using the respective polysome RNA. The RT-PCR products were analyzed on a 1.2% agarose gel (Figure 3-7). An RT-PCR product of the expected size was present in reactions containing polysome RNA from Rhs 1A1 (both induced and uninduced) and induced Rhs 1AP. The PCR product from induced Rhs 1AP was sequenced and found to be nearly identical to that of the sense strand of the M2 dsRNA (Figure 3-8). All of the nucleotide (nt) differences (6 out of 1170 nts) were located on the third position of the respective codon, and the respective amino acid remained the same.

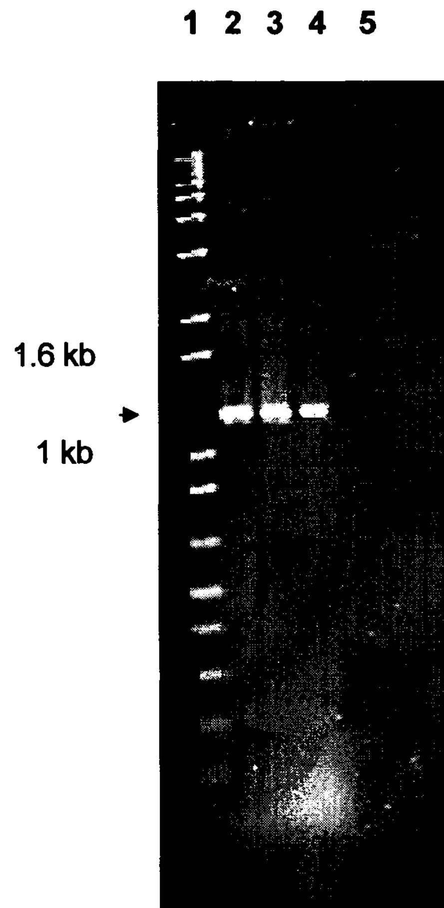


Figure 3-7. Detection of an M2-specific transcript by RT-PCR in polysome fractions from quinate-induced Rhs 1AP. Lane 1, 1 kb plus molecular ladder (Bio-Rad); lane 2, quinate- induced 1A1; lane 3, quinate-uninduced Rhs 1A1; lane 4, quinate-induced Rhs 1AP; and lane 5, quinate-uninduced Rhs 1AP. M2-specific polysomes from induced and uninduced Rhs 1AP and Rhs 1A1 were purified by immunoprecipitation using anti-pA antibodies (see Materials and Methods for details). RT-PCR was carried out using respective polysome RNAs as templates. The numbers on the left indicate the size of selected bands from 1kb plus ladder. The arrow shows the position of the RT-PCR products (1.17 kb).

```
P33-34     1  ------------------------TAAATCATTTGATGATAAAGTTGATTCGCAGGGTCA
m2      1261  TGCTTGCTCTCTTGCAGATCGACTTAAATCATTTGATGATAAAGTTGATTCGCAGGGTCA

P33-34    37  ATGGATAAGACAAGTCTCGAAATCGGGACAAGTTCTTACTCAAGTTGACTCAATGAAATC
m2      1321  ATGGATAAGACAAGTCTCGAAATCGGGACAAGTTCTTACTCAAGTTGACTCAATGAAATC

P33-34    97  GAAAGATTCCATTAGAGGTCATGGTATTGGTCCTGGACTTGGTCTTTCGCAATTTGCGCT
m2      1381  GAAAGATTCCATTAGAGGTCATGGTATTGGTCCTGGACTTGGTCTTTCGCAATTTGCGCT

P33-34   157  CAAAGAAGAAGCTGCTGGAAAAATCCGGCTCTTCGCTTTGATGGATTCAATAACACAAAG
m2      1441  CAAAGAAGAAGCTGCTGGAAAAATCCGGCTCTTCGCTTTGATGGATTCAATAACACAAAG

P33-34   217  TGTTATGAGTCCTCTCCATGATTACATGTTTGCCATCTTAAGGAATATTCCTAACGATGG
m2      1501  TGTTATGAGTCCTCTCCATGATTACATGTTTGCCATCTTAAGGAATATTCCTAACGATGG

P33-34   277  GACATTTGATCAAGAGGCATCAATTGCTAGATCTCAAGAAAAGGCCGTTACCGCGGGCAA
m2      1561  GACATTTGATCAAGAGGCATCAATTGCTAGATCCCAAGAAAAGGCCGTTACCGCGGGCAA

P33-34   337  AGCATTCAGTTATGATCTGACTGCTGCTACGGATCGACTACCTGTAATCCTTACTGCGTT
m2      1621  GGCATTCAGTTATGATCTGACTGCTGCTACGGATCGACTACCTGTAATCCTTACTGCGTT

P33-34   397  CATACTCTCCACAATCGTGGGGATTAGAACGTTTGGGGGTCTYTGGAGGTCAATTTTGGT
m2      1681  CATACTCTCCACAATCGTGGGGATTAGAACGTTTGGGGGTCTTTGGAGGTCAATTTTGGT

P33-34   457  CAAAAGACCTTTTGGTTTCAACAGCAATGTTGCTGAGAAATTGAAAGTCTCTGACGGTCC
m2      1741  CAAAAGACCTTTTGGTTTCAACAGCAATGTTGCTGAGAAATTGAAAGTCTCTGACGGTCC
```

Figure 3-8. Alignment of the sequences of the M2 dsRNA sense strand and RT-PCR product from quinate-induced Rhs 1AP polysome fraction. Y stands for C or T. M2-specific polysomes from induced and uninduced *R. solani* isolates Rhs 1AP and Rhs 1A1 were purified by immunoprecipitation using anti-pA antibodies. RT-PCR was carried out using the respective polysome RNA and primers P33 and P34. The RT-PCR product from induced Rhs 1AP was sequenced (see text for details). All of the nucleotide differences (6 out of 1170 nts) were located on the third position of the respective codon.

```
P33-34   517   GTACTTCTACGAGGTAGGGCAACCTATGGGTGCTCTATCTTCTTGGCCCGGTCTTGCCTT
m2       1801  GTACTTCTACGAGGTAGGGCAACCTATGGGTGCTCTATCTTCTTGGCCCGGTCTTGCCTT

P33-34   577   AACGCATCACTGGATTGTCCAAGTTGCCGCTTTTAGAGTTACAAATTCTAAGAGTTGGAA
m2       1861  AACGCATCACTGGATTGTCCAAGTTGCCGCTTTTAGAGTTACAAATTCTAAGAGTTGGAA

P33-34   637   CACCGAGTATGAAATACTTGGTGATGATATCGTAATATTCAATGAACTTATTGCTCAAGA
m2       1921  CACCGAGTATGAAATACTTGGTGATGATATCGTAATATTCAATGAACTTATCGCTCAAGA

P33-34   697   ATATCTAAATATCATGGCTGTAATCGGGTGTGAGATTAATTTAAATAAATCAATCTCYTC
m2       1981  ATATCTAAATATCATGGCTGTAATCGGGTGTGAGATTAATTTAAATAAATCAATCTCCTC

P33-34   757   CCGATGCCGTCCCGTTTTCGAATTCGCAAAACGAACCTGTTGGGGCTTYGCAATAGTAAG
m2       2041  CCGATGCCGTCCCGTTTTCGAATTCGCAAAACGAACCTGTTGGGGCTTTGCAATAGTAAG

P33-34   817   TGGAATCTCCCTCGCTCAAATAAGAGCGGGTTGGAGAATCGGGGGTCGTGTAGCTAATGC
m2       2101  TGGAATCTCCCTCGCTCAAATAAGAGCGGGTTGGAGAATCGGGGGTCGTGTAGCTAATGC

P33-34   877   TCTGCAATTTGCAAGAGCAGGACTACTAGAACCCGGTGAATCCCTGTTGCAAGCTATCCT
m2       2161  TCTGCAATTTGCAAGAGCAGGACTACTAGAACCCGGTGAATCCCTGTTGCAAGCTATCCT

P33-34   937   TTCTAGAAATACCTTTTCAAAAGGTAGAGTATTGCCAGGTTACAAGACGGAGTCAGTAAC
m2       2221  TTCTAGAAATACCTTTTCAAAAGGTAGAGTATTGCCAGGTTACAAGACGGAGTCAGTAAC

P33-34   997   CTCACAAAAGGCTCTAGCCTTAGGGGTACTGGCGTTGTTAGGGGAGAGATTCCGATCTGG
m2       2281  CTCACAAAAGGCTCTAGCCTTAGGGGTACTGGCGTTGTTAGGGGAGAGATTCCGATCTGG

P33-34   1057  AATCATCCCGCTAAGAACGGTAATGCACGCGATCATAGATCCTATTTCTAAGAATCTAGA
m2       2341  AATCATCCCGCTAAGAACGGTAATGCACGCGATCATAGATCCTATTTCTAAGAATCTAGA

P33-34   1117  TCTGAAAGGGGATGCTATCGCTATCCCTATCAAAGCGTCATTACATGCAGCTTA------
m2       2401  TCTGAAAGGGGATGCTATCGCTATCCCTATCAAAGCGTCATTACATGCAGCTTACCAGGC
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Figure 3-8 (Continued)

3.7. Quinate dehydrogenase activity is constitutive in the M2-containing hypovirulent Rhs 1A1, and inducible in the M2-lacking, virulent Rhs 1AP

Glucose minimal medium was used initially to grow *R. solani* to facilitate mycelial growth. Induction was by addition of quinate after mycelial cultures were washed and transferred to glycerol minimal medium (see Materials and Methods for details). Glucose minimal medium treatment was used as a control in enzyme assay experiments (quinate dehydrogenase assay and shikimate kinase assay).

Quinate dehydrogenase (QDH) assay experiments (Figure 3-9) show that specific QDH activities were 1.61 μmol of NAD^+ consumed/min/mg protein in Rhs 1A1, and 0.25 μmol of NAD^+ consumed/min/mg protein in Rhs 1AP when the isolates were grown in glucose minimal media without quinate induction. This corresponds to a QDH activity 7-fold higher ($p < 0.05$) in Rhs 1A1 than that in Rhs 1AP. Compared to the specific QDH activity in Rhs 1A1, Rhs 1AP possesses only a basal level of QDH activity. Upon quinate induction, the specific QDH activity was increased dramatically in Rhs 1AP as compared to uninduced Rhs 1AP, about 5 times higher (1.26 vs. 0.22 μmol of NAD^+ consumed/min/mg protein, $p < 0.05$) after a 5-hr-induction period and about 6 times higher (1.49 vs. 0.22 μmol of NAD^+ consumed/min/mg protein, $p < 0.05$) after an 18-hr induction period. In Rhs 1A1, upon quinate induction, QDHase activity showed a 70% (1.61 vs. 2.74 μmol of NAD^+ consumed/min/mg protein, $p < 0.05$) and 50% (1.61 vs. 2.40 μmol of NAD^+ consumed/min/mg protein, $p < 0.05$) increase (superinduction) after induction periods of 5 hrs and 18 hrs, respectively. When mycelia were grown in quinate as the sole carbon source, QDHase activity was 95% higher in Rhs 1A1 (3.15 vs. 1.61 μmol of

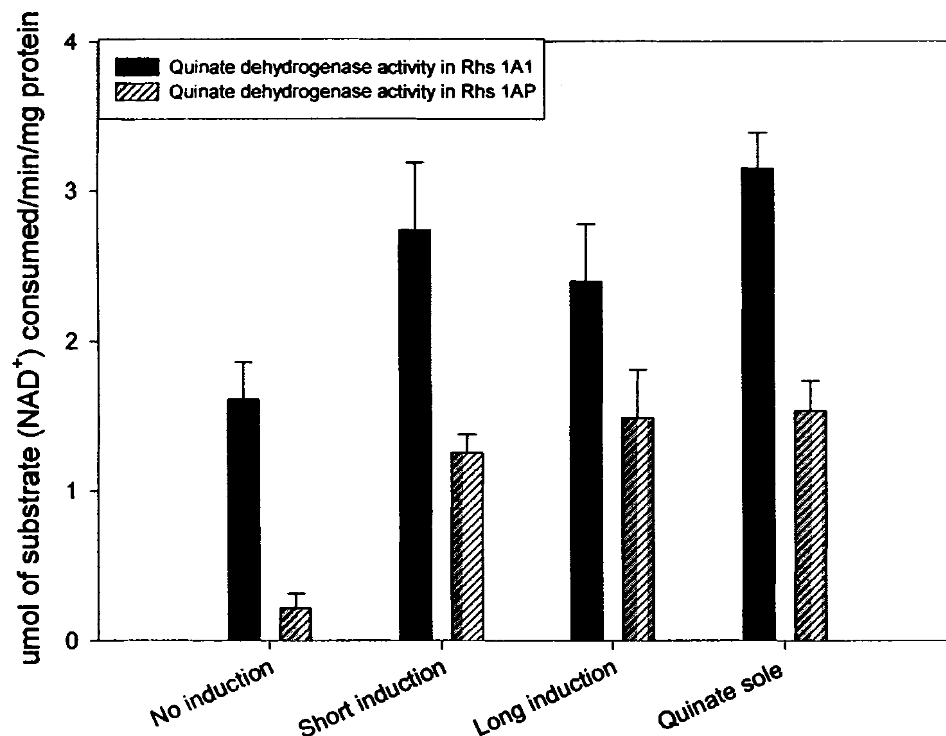

Figure 3-9. Specific activity of quinate dehydrogenase in *R. solani* isolates Rhs 1A1 and Rhs 1AP. Rhs 1AP and Rhs 1A1 were initially grown in glucose minimal media. After 48 hrs, mycelia were harvested, washed with a minimal salts solution and transferred to quinate minimal media or glycerol minimal media. See Materials and Methods for the induction procedure. Enzyme activities were measured spectrophotometrically at 37°C in a total volume of 1 ml in disposable cuvettes of 1.0 cm light path. Specific QDH activity is determined as μmol of NAD^+ consumed/min/mg protein. Five samples per treatment were assayed. Error bars indicate standard deviations. No induction, mycelia were grown in glucose minimal media without quinate; short induction, mycelia were induced with quinate for 5 hrs; long induction, mycelia were induced with quinate for 18 hrs; quinate sole, mycelia were grown on quinate minimal media.

NAD^+ consumed/min/mg protein, $p < 0.05$), and 6-fold higher in Rhs 1AP (1.53 vs. 0.22 μmol of NAD^+ consumed/min/mg protein, $p < 0.05$) as compared to that of uninduced cultures. These results indicate that the quinate utilization pathway is constitutive in hypovirulent isolate Rhs 1A1 and superinducible upon quinate induction, and as expected, inducible in the M2-lacking, virulent isolate Rhs 1AP.

A previous study showed that *qut* pathway enzyme levels reached a maximum value 4-5 hrs after the addition of quinate in *A. nidulans* (Levesley *et al.,* 1996). This was the first study conducted on the kinetics of induction of quinate pathway enzymes in *R. solani.* Induction periods of 5 hrs and 18 hrs were used to measure the DHQ enzymatic activities. The data showed that there was no significant difference in DHQ activities between 5-hr-induction and 18-hr-induction in Rhs 1AP (1.26 vs. 1.49 μmol of NAD^+ consumed/min/mg protein, $p > 0.05$) or Rhs 1A1 (2.74 vs. 2.40 μmol of NAD^+ consumed/min/mg protein, $p > 0.05$).

3.8. Shikimate kinase (SK) activity is higher in the M2-lacking Rhs 1AP than in the M2-containing Rhs 1A1

SK activity is one of the 5 enzymatic activities of the pentafunctional protein AROM of the shikimate pathway. Enzyme analysis (Figure 3-10) showed that the specific SK activity was about 60% higher ($p < 0.05$) in Rhs 1AP than that in Rhs 1A1 without quinate induction (2.13 μmol of NADH consumed/min/mg of protein in Rhs 1AP and 1.32 μmol of NADH consumed/min/mg of protein in Rhs 1A1). Upon quinate induction (5 hr), SK activity was not significantly different from that of glucose minimal media in either Rhs 1AP (2.09 vs. 2.13 μmol of NADH consumed/min/mg

protein, $p > 0.05$) or Rhs 1A1 (1.68 vs. 1.32 μmol of NADH consumed/min/mg protein, $p > 0.05$). Also, there was no significant difference in SK activity between the 5-hr-induction and the18-hr-induction in SK activities in either Rhs 1AP (2.09 vs. 2.16 μmol of NADH consumed/min/mg protein, $p > 0.05$) or Rhs 1A1 (1.68 vs. 1.56 μmol of NADH consumed/min/mg protein, $p > 0.05$).

3.9. Sequence analysis of the *arom* gene from *R. solani*

The nucleotide sequence of the *R. solani arom* gene and its predicted amino acid sequence are shown in Figure 3-11. The cloned *R. solani arom* cDNA consists of 5459 bp including a poly (A) tail. The *R. solani* arom gene consists of 5097 bp. It possesses five introns that are distributed throughout the entire region of the gene (Figure 3-11). The first intron consists of 51 base pairs, the rest are 48 base pairs each. All of the introns start with GT and end with AG. A comparison of length of the genes, length of the polypeptides, intron positions, and molecular weight of the deduced polypeptides of the four *arom* genes is shown in Table 3-1.

Alignment of the deduced AROM polypeptide of *R. solani* with the three known AROM polypeptides and the corresponding *E. coli* monofunctional enzymes is shown in Figure 3-12. The *R. solani* AROM protein shows a significant homology with the other fungal AROM proteins. The *R. solani* protein has 48% identities and 31% similarities with the AROM protein from *A. nidulans*. The *R. solani* AROM protein contains most of the highly conserved motifs and the five domains of the respective enzymes found in AROM polypeptides from other fungi.

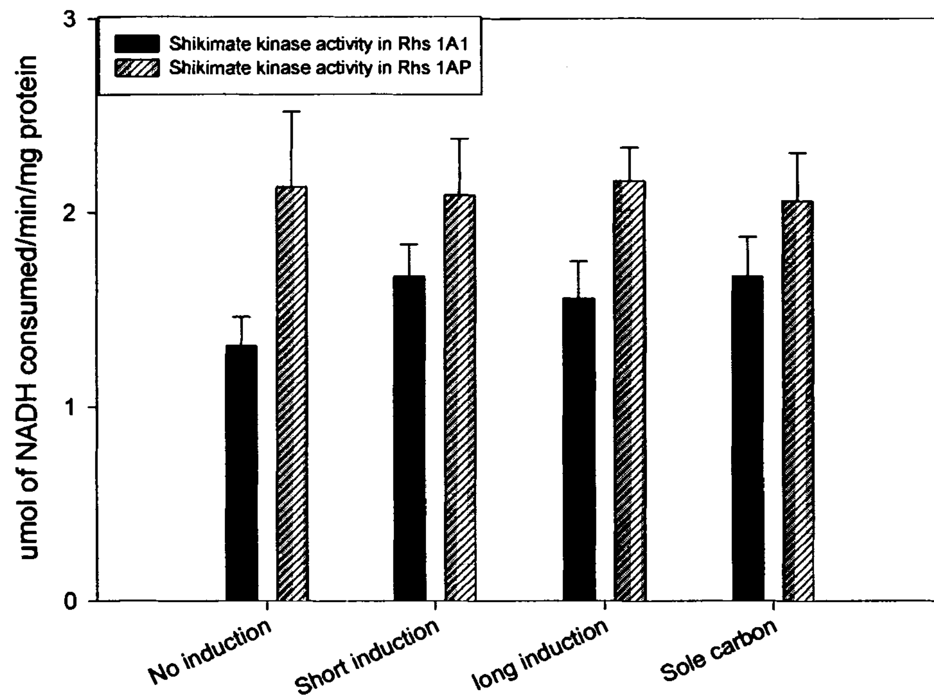

Figure 3-10. Specific activity of shikimate kinase (SK) in *R. solani* isolates Rhs 1A1 and Rhs 1AP. Rhs 1AP and Rhs 1A1 were initially grown in glucose minimal media. After 48 hrs, mycelia were harvested, washed with a minimal salts solution and transferred to quinate minimal media or glycerol minimal media. See Materials and Methods for the induction procedure. Enzyme activities were measured spectrophotometrically at 25°C in a total volume of 1 ml in disposable cuvettes of 1.0 cm light path. Specific SK activity is determined as µmol of NADH consumed/min/mg protein. Five samples per treatment were assayed. Error bars indicate standard deviations. No induction, mycelia were grown in glucose minimal media without quinate; short induction, mycelia were induced with quinate for 5 hrs; long induction, mycelia were induced with quinate for 18 hrs; quinate sole, mycelia were grown on quinate minimal media.

A GC box (GGGCGG) sequence (located at –176 to –171 in reference to the 5'-methionine codon) has been found in the promoter region, but a TATA box sequence has not been found in this region. Interestingly, two repeats (GTATTAGA) were found just before the transcription initiation site. Another interesting phenomenon is a three-base deletion (GAT) at the 5'-end of the genomic DNA sequence (position –131 in reference to the 5'-methionine codon) as compared to that of the cDNA clone. A possible explanation is that the three-base sequence (GAT) was added to the mRNA by a transcriptional or post-transcriptional editing process. A dinucleotide binding motif (GXGXXG) (Rossman *et al.*, 1974) and a purine dinucleotide binding motif (G/AXXXXGKT/S) (Walker *et al.*, 1982) were found in the DHS and SK domains, respectively. A universally conserved glycine in the motif LGNAGTA in the EPSP domain was also found. This glycine is thought to be involved in the interaction with the substrate phosphoenolpyruvate (Padgette *et al.*, 1991).

```
   1  GCTATGTCATTGGGCGTCTGATATGTCAAGTCCGCGGGCGAGCGAGGCATGGTGGTCGAC  60

  61  GTGTCTGGCAGTGTGTCGACAGATACGAGGACGGCCTCGGCATTTCTCTCGTATTTCGCA  120

 121  ACACTGGCGTCTATTGCACCTGGTTCGGAACTCAGGGCGGAAGTATTAGATCCGGTATTG  180
                         *
 181  GACGGGTATTAGACCCTACTTTCCCGCGATTCCAACGACAGCGCACTTGAGGCAGCATTC  240

 241  GGTCGCACGGCCCCTGTAGAACTCCCACAGTACGAAGATATATAGAGGCTATCTGCCATC  300

 301  GAAGTTTCCACACTTTATACGACTTGCGCCATGGCCACTGCCAGCGTCGAACAACCCGAT  360
                                       M  A  T  A  S  V  E  Q  P  D
 361  CTTACCAAGGTTTCTATCCTCGGCAAGGATTCCATTCACTGCGGCTTCCACTTGAGCCCG  420
       L  T  K  V  S  I  L  G  K  D  S  I  H  C  G  F  H  L  S  P
 421  TACATTGCCGATACAGTCCTCACTAATCTCCCTGCATCTACTTATGTTCTTATTACCGAC  480
       Y  I  A  D  T  V  L  T  N  L  P  A  S  T  Y  V  L  I  T  D
 481  ACGCGCGTTGCCAAGTTCCATCTCGAGTCGTTCGAGGCCGCATTTACCGACGCTTTGGCG  540
       T  R  V  A  K  F  H  L  E  S  F  E  A  A  F  T  D  A  L  A
 541  GCTAGGCCCAATACCTCTGCTCGCTTCTTGACCCATGTGATTCCCCCTGGCGAGACCAGC  600
       A  R  P  N  T  S  A  R  F  L  T  H  V  I  P  P  G  E  T  S
 601  AAAAGCCGCGAGGGCAAGGCCGAAATCGAAGACTTTTTGCTCGACAACTCGTGCACGCGT  660
       K  S  R  E  G  K  A  E  I  E  D  F  L  L  D  N  S  C  T  R
 661  GATACGGTCGTTCTTGCCCTGGGTGGTGGAGTTATCGGAGACCTCGTTGGGTTTGTGGCC  720
       D  T  V  V  L  A  L  G  G  G  V  I  G  D  L  V  G  F  V  A
 721  GCGACCTTgtaagtctaattcatgcggggaaatgacgcacttactgactgttctatcagC  780
       A  T  F
 781  ATGCGTGGTGTGCGCTTTGTTCAGATCCCAACGACTCTATTGGCCATGGTCGACTCGTCG  840
       M  R  G  V  R  L  V  Q  I  P  T  T  L  L  A  M  V  D  S  S
 841  GTTGGGGGCAAGACTGCGATTGATACGCCTCATGGCAAGAACCTGATCGGAAGCTTCTGG  900
       V  G  G  K  T  A  I  D  T  P  H  G  K  N  L  I  G  S  F  W
 901  CAGCCATCCTATGTGTTCATCGACGCCGCATTCCTCGAGACACTTCCCCAGCGCGAGTTT  960
       Q  P  S  Y  V  F  I  D  A  A  F  L  E  T  L  P  Q  R  E  F
 961  GTAAACGGTATGGCCGAAGTGATTAAAgtgagtgacatgatggtctcggccgatcagttc  1020
       V  N  G  M  A  E  V  I  K
1021  tcacgtgactgctagACGGCCGCGATCTGGAACGAAGACGAGTTTTCGATGCTCGAGGCT  1080
                      T  A  A  I  W  N  E  D  E  F  S  M  L  E  A
1081  TCGGCGCCTGCGCTATTTGCCGCCATTGGATCCTCCTCGTCCACGACCTCTGCGGGCCGC  1140
       S  A  P  A  L  F  A  A  I  G  S  S  S  S  T  T  S  A  G  R
1141  ACTACCGCCACCCGGTCAGAGGCCCAGTCTCTTCTGTTGCACGTGATTACCGAGAGCATT  1200
       T  T  A  T  R  S  E  A  Q  S  L  L  L  H  V  I  T  E  S  I
1201  GGAGTCAAGGCACACATTGTGACCCTCGACGAACGCGAAACGGGTCTGCGCAATCTGGTC  1260
       G  V  K  A  H  I  V  T  L  D  E  R  E  T  G  L  R  N  L  V
1261  AACTTTGGACACACTATCGGTCATGCGATCGAGGCCGTGCTCACCCCGACTATTCTCCAC  1320
       N  F  G  H  T  I  G  H  A  I  E  A  V  L  T  P  T  I  L  H
1321  GGCGAATGTGTTGCCATTGGCATGGTTCTCGAAGGCGAAGTTGCCCGACAACTCGGCGTA  1380
       G  E  C  V  A  I  G  M  V  L  E  G  E  V  A  R  Q  L  G  V
```

Figure 3-11. DNA sequence and the predicted amino acid sequence of the *R. solani arom* gene including introns and the 5' and 3' untranslated regions. Introns are represented by lower-case characters. The untranslated 5' end (positions 1 to 330) and 3' end (positions 5428 to 5522) are represented by italic capital characters. The coding region is represented by capital characters. Bold characters represent the GC box and two repeat sequences before the transcription initiation site. The asterisk '*' shows the position of the GAT deletion on the chromosome DNA. See text for details.

```
1381  CTCAGCCAAGTCGGTGTTGGCCGTATTACCCGTGCCCTCAAGGCATACGGGCTTCCTACA  1440
       L  S  Q  V  G  V  G  R  I  T  R  A  L  K  A  Y  G  L  P  T
1441  TCGACCAAGGACCCGCGGATCGCCGCTGTGCCAGCATCGCGTCTCTTGACCATCGATAGG  1500
       S  T  K  D  P  R  I  A  A  V  P  A  S  R  L  L  T  I  D  R
1501  TTGCTTGACATTATGAAGATCGACAAGAAGAACTCTGGCCCCGAAAAGAAGATTGTGCTC  1560
       L  L  D  I  M  K  I  D  K  K  N  S  G  P  E  K  K  I  V  L
1561  CTTTCGCGAATTGGCAAGACCTATGAGGAGCGTGCTACGGGCGTCAAGGATGAAGTCATT  1620
       L  S  R  I  G  K  T  Y  E  E  R  A  T  G  V  K  D  E  V  I
1621  CGCCGTGTGTTGGCCGAAGCTTGCCGTGTAATCCCAGGAATCCCTCGGGGCAACCCTGTT  1680
       R  R  V  L  A  E  A  C  R  V  I  P  G  I  P  R  G  N  P  V
1681  CGCATGAGCACCCCCGGCAGCAAGAGCATCTCCAACCGTGCGCTCGTACTAGCCGCTCTC  1740
       R  M  S  T  P  G  S  K  S  I  S  N  R  A  L  V  L  A  A  L
1741  GGCAATGGCACCTGTCGTCTTCGAAATCTTCTCCACAGTGACGACACTCAAGTGATGATG  1800
       G  N  G  T  C  R  L  R  N  L  L  H  S  D  D  T  Q  V  M  M
1801  AGTGCCCTGATTGAACTCAAGgtgcgcgaatgaatcccgtgcattgcatcagaactaact  1860
       S  A  L  I  E  L  K
1861  atgtcacagGGTGCCAAATTTGCTTGGGAAGATGGTGGTGAAACACTCGTGGTTAGTGGT  1920
                G  A  K  F  A  W  E  D  G  G  E  T  L  V  V  S  G
1921  GGCGGAGGTGCCTTTACAATCCCTCCCGCCGGAAAGGAACTCTATCTTGGGAACGCTGGG  1980
       G  G  G  A  F  T  I  P  P  A  G  K  E  L  Y  L  G  N  A  G
1981  ACGGCTGCTCGCTTCCTCACCACTGTCTGCACGCTCGTCGGTCCCGACTCGTCATCGGCA  2040
       T  A  A  R  F  L  T  T  V  C  T  L  V  G  P  D  S  S  S  A
2041  ACTGCTTCTTCCGAGTTCCCCGAAGGTTACACGTTCATCACCGGCAATGCTCGCATGAAG  2100
       T  A  S  S  E  F  P  E  G  Y  T  F  I  T  G  N  A  R  M  K
2101  CAGCGCCCGTGCGGTCCCCTTGTCGATGCTCTCCGCGCCAACGGAAGCAAGGTCAAGTAT  2160
       Q  R  P  C  G  P  L  V  D  A  L  R  A  N  G  S  K  V  K  Y
2161  ATCGAGAGCGAGGGCTGCTTGCCGCTGCACATTGGTGCTGGCGGTCTCAAGGGCGGCACA  2220
       I  E  S  E  G  C  L  P  L  H  I  G  A  G  G  L  K  G  G  T
2221  ATTCAGCTCGCGGCCAGTGTCTCCAGCCAATACGTATCGAGTATTCTTCTATGCGCTCCG  2280
       I  Q  L  A  A  S  V  S  S  Q  Y  V  S  S  I  L  L  C  A  P
2281  TATGCCCGGGATGAAGACGTCGTACTCGAGCTCGTGGGCGGCCAAGTCATCAGCCAGCCG  2340
       Y  A  R  D  E  D  V  V  L  E  L  V  G  G  Q  V  I  S  Q  P
2341  TACATTGACATGACCCTCGCGATGATGAAGACCTTTGGTGTTGAAGTTACCCGCCGCAAG  2400
       Y  I  D  M  T  L  A  M  M  K  T  F  G  V  E  V  T  R  R  K
2401  GCCGAGGACGGTACCCTTCTTGATATCTACGACATTCCACGCGCCCAATATACCAACCCC  2460
       A  E  D  G  T  L  L  D  I  Y  D  I  P  R  A  Q  Y  T  N  P
2461  GAAAAGTATGCCATCGAGAGTGACGCGAGCAGCGCCACCTACCCTCTTGCCGTCGCTGCT  2520
       E  K  Y  A  I  E  S  D  A  S  S  A  T  Y  P  L  A  V  A  A
2521  ATCACCGGAACTACTTGCACTATTGAAAACATCGGTACCTCTTCGCTGCAAGGTGATGCC  2580
       I  T  G  T  T  C  T  I  E  N  I  G  T  S  S  L  Q  G  D  A
2581  GGGTTCGCGGTCAACGTCCTCAAGCGCATGGGCTGCAAGGTCGAGCAAAGCGAGAACGAG  2640
       G  F  A  V  N  V  L  K  R  M  G  C  K  V  E  Q  S  E  N  E
2641  ACGACTGTGACCGGCCCTCCCATCGGCCAGCTCAAGGCGATCGGATTGGTCGATATGGAA  2700
       T  T  V  T  G  P  P  I  G  Q  L  K  A  I  G  L  V  D  M  E
2701  ACCATGACCGACGCATTCTTGACTGCAGTCGTTGTAGCCGCAGTTTGCGGAGAAGGAAGT  2760
       T  M  T  D  A  F  L  T  A  V  V  V  A  A  V  C  G  E  G  S
2761  GCAGAAGGTCTCGAATCCGACGTGCCCAAGAATACCACTCGCATTTTGGGTATTGCCAAC  2820
       A  E  G  L  E  S  D  V  P  K  N  T  T  R  I  L  G  I  A  N
2821  CAACGTGTCAAGGAATGCAACCGTATTCGTGCCATGATCGACGAGCTCGgtaagtgcatt  2880
       Q  R  V  K  E  C  N  R  I  R  A  M  I  D  E  L
2881  cgtatctctcgcgtgcgtCtacttagtagtttgtagCCAAGTTTGGTGTCAAGACCAAG  2940
                                             A  K  F  G  V  K  T  K
2941  GAATTGGACGACGGTCTGGAAGTCTACGGTCAGCCGATCGAGACCTTGAACAAGAACGTG  3000
       E  L  V  D  G  L  E  V  Y  G  Q  P  I  E  T  L  N  K  N  V
3001  TCGGTGCACTGCTACGACGATCACCGCGTGGCGATGGCGTTCTCCGTTCTGGCCACTGTC  3060
       S  V  H  C  Y  D  D  H  R  V  A  M  A  F  S  V  L  A  T  V
3061  GTTCCCGGTACTATTCTGGAAGAGAAGCGTTGCGTCGAAAAGACCTGGCCAAACTGGTGG  3120
       V  P  G  T  I  L  E  E  K  R  C  V  E  K  T  W  P  N  W  W
3121  GACGACCTGACCAACAAGgtacgtcgagtcgattattaatagcataatcactaaccccgt  3180
       D  D  L  T  N  K
```

Figure 3-11. (Continued)

```
3181  ttatagATTGGTATTGATGTCGAAGGTATCGAGCTCGCTCACCAAAGCCCAGCGGTCGCC  3240
             I  G  I  D  V  E  G  I  E  L  A  H  Q  S  P  A  V  A
3241  AAGTACAACCCAGAGGCCACGATCATTATCGTTGGTATGCGCGGAGCTGGCAAGACTCAC  3300
       K  Y  N  P  E  A  T  I  I  I  V  G  M  R  G  A  G  K  T  H
3301  ATTGGAGGCATTGCCGCCGCGTCTCTTGGATGGAAGTTTATCGATGCAGATCATGTGTTT  3360
       I  G  G  I  A  A  A  S  L  G  W  K  F  I  D  A  D  H  V  F
3361  GAAGGCCGCACGGGCGAGCTCGTCTCGCAGTACGTACGCAGCAAGGGTTGGACTGCGTTC  3420
       E  G  R  T  G  E  L  V  S  Q  Y  V  R  S  K  G  W  T  A  F
3421  CGCCAAGCCGAATCTCAAATCATCCAAGACCTTTTGGCTGAAAACCCCACACGCACGATC  3480
       R  Q  A  E  S  Q  I  I  Q  D  L  L  A  E  N  P  T  R  T  I
3481  ATTTCAACTGGAGGCGGGATCGTCGAGTCGCCACTCAACCGCGCGCTCTTGTCCGGTTAC  3540
       I  S  T  G  G  G  I  V  E  S  P  L  N  R  A  L  L  S  G  Y
3541  GGTCGTGCCACAGGCCCTGTCGTGTACGTGATGCGAGACGTGAACGAAATTCTAAGCTAC  3600
       G  R  A  T  G  P  V  V  Y  V  M  R  D  V  N  E  I  L  S  Y
3601  CTCGGCTCGGAGACGGCGCGTCCTGCTTATGGCGAAGAGATCGCAGATGTCTATTGGCGT  3660
       L  G  S  E  T  A  R  P  A  Y  G  E  E  I  A  D  V  Y  W  R
3661  CGCGAACCCTGGTTCCGAGAAGTCTCCACCCACGAGTATATATCTTATACCGGAGGATTC  3720
       R  E  P  W  F  R  E  V  S  T  H  E  Y  I  S  Y  T  G  G  F
3721  CATGCCGCGAATGGAGCGACTCCGGGCGAAATCGCTTCGGCATGCCGCGACGAGACTGCA  3780
       H  A  A  N  G  A  T  P  G  E  I  A  S  A  C  R  D  E  T  A
3781  CGATTCTTCCGCCAGATTACCGGTATCAAGCCTAACCTCTCCGAGGCACTCGCGCAAGGC  3840
       R  F  F  R  Q  I  T  G  I  K  P  N  L  S  E  A  L  A  Q  G
3841  GAACGATCCTTTTTCCTGTCACTCACCTATCCCGACCTTCTCCCTGCCGTCCCTTCGATC  3900
       E  R  S  F  F  L  S  L  T  Y  P  D  L  L  P  A  V  P  S  I
3901  CCGCTCCTCACTGCCGGCGCCGATGCCATCGAACTTCGCGTTGATTTGCTCAACCCTAGC  3960
       P  L  L  T  A  G  A  D  A  I  E  L  R  V  D  L  L  N  P  S
3961  GGTGATCCGGTTACCGGTCCCCCCAACATTCCCTCTTTGGACTTTGTTGCCACCCAGTTG  4020
       G  D  P  V  T  G  P  P  N  I  P  S  L  D  F  V  A  T  Q  L
4021  TCGGCTTTGCGCCACACCTCGTCTCTCCCCGTCGTCTTTACCGTCCGCACTGCGTCCCAG  4080
       S  A  L  R  H  T  S  S  L  P  V  V  F  T  V  R  T  A  S  Q
4081  GGCGGCGCCTTTCCTGACACAGCTGAAAAGGAAGCATTCGCGTTGTACAACCTTGCGCTC  4140
       G  G  A  F  P  D  T  A  E  K  E  A  F  A  L  Y  N  L  A  L
4141  AGGCACGGTGTCGAGTACATTGATGTCGAAATCTCATGGAGCGACAAGAAGATTCAGGAC  4200
       R  H  G  V  E  Y  I  D  V  E  I  S  W  S  D  K  K  I  Q  D
4201  CTCGCTGCGCGCAAGGGCGCTTCTCAGATCATCGCATCGTGGCACGACTGGAGCGGAAAC  4260
       L  A  A  R  K  G  A  S  Q  I  I  A  S  W  H  D  W  S  G  N
4261  ATGAAGTGGAACGGAGCCGTCGTCAAGGAAAAGTATGCTTTGGCCGAGCGAGTAGGAGAC  4320
       M  K  W  N  G  A  V  V  K  E  K  Y  A  L  A  E  R  V  G  D
4321  ATTGTCAAGATCGTCGGCAAAGCGCTCTCGATCGAGGACAACTTTGCGCTCCGGGCATTT  4380
       I  V  K  I  V  G  K  A  L  S  I  E  D  N  F  A  L  R  A  F
4381  GCGGCCGCTCACACCTCCAAGCCGTTTATTGGTATCAACATGGGCGCCGAAGGCCAGCTC  4440
       A  A  A  H  T  S  K  P  F  I  G  I  N  M  G  A  E  G  Q  L
4441  TCGCGTGTGCTTAATACTGCGTTTACTCCCGTTTCGCACCCGCTTTTGCCGACTCGCGCT  4500
       S  R  V  L  N  T  A  F  T  P  V  S  H  P  L  L  P  T  R  A
4501  GCCCCGGGCCAGATGTCTGTGCAAGATATTCACACGGCATTGCACCTCAATGGCCAACTT  4560
       A  P  G  Q  M  S  V  Q  D  I  H  T  A  L  H  L  N  G  Q  L
4561  GCGCCTCAAAAGTTCTACCTGTTCGGCTCACCGATCGCTCATTCGATGTCGCCCCATCTC  4620
       A  P  Q  K  F  Y  L  F  G  S  P  I  A  H  S  M  S  P  H  L
4621  CACAACACAGGGTTCGAGAAGCTCGGCTTGCCCCACAAGTATCATATCTTCGAGACGGCG  4680
       H  N  T  G  F  E  K  L  G  L  P  H  K  Y  H  I  F  E  T  A
4681  ACGATCACCGACGAGGTCAAGGCCGTGATCCGGGCTCCTGAATTTGGCGGCGCGAGCGTG  4740
       T  I  T  D  E  V  K  A  V  I  R  A  P  E  F  G  G  A  S  V
4741  ACGATTCCCCTCAAGCTTGATATTATCCCGCTCCTCGACGAAGTTTCGCCCGAAGCCAAG  4800
       T  I  P  L  K  L  D  I  I  P  L  L  D  E  V  S  P  E  A  K
4801  GCCATTGGCGCGGTCAACACCATCATCCCACGCAAGCGCGCCGACGGTCCACGAGTCTG  4860
       A  I  G  A  V  N  T  I  I  P  R  K  R  A  D  G  S  T  S  L
4861  TTTGGTACCAACACCGATTGGCGGGCGATCCACGACTTGGCGCGCAACAACCTGGTGGTC  4920
       F  G  T  N  T  D  W  R  A  I  H  D  L  A  R  N  N  L  V  V
```

Figure 3-11. (Continued)

4921 GGCATTACCAACGAGACGACCGCGCTTGTTCTCGGTGCCGGCGGAACGGCGCGTGCGGCT 4980
G I T N E T T A L V L G A G G T A R A A

4981 CTGTATGCGATCCATGCGCTCGGCATCAAGACGATCTACTTGTTCAACCGTACACGGGCA 5040
L Y A I H A L G I K T I Y L F N R T R A

5041 GCGGCGCAAGCGCTTGCCGATACGTTCCCCACCTTTGGTATTATTCCTCTCGACTCGCTC 5100
A A Q A L A D T F P T F G I I P L D S L

5101 AACTCGTTCCCCAAGGCTGCTCCCACCGTTGTCGTCTCTGCTATTCCCGCAACAGGTACC 5160
N S F P K A A P T V V V S A I P A T G T

5161 ACGACCGAAAAAGCGAGCGACAGTGCCGGAGTATACCTCCCCCCAAGCCTGTTTGAAGCT 5220
T T E K A S D S A G V Y L P P S L F E A

5221 CCATCCGGTGTAGTGGTCGAGATGGCCTACAAGCCCGCGGTGACGCCTGTGCTCGGACTC 5280
P S G V V V E M A Y K P A V T P V L G L

5281 GCGGCCAAGTCTTCTGGCTGGGTGGGCGTACGTGGGGTGGATATTTTGTGCGAACAAGGG 5340
A A K S S G W V G V R G V D I L C E Q G

5341 TTCTACCAGTACGAGGCTTGGACTGGGCGCAAGGCTCCTCGGGCGGCGATGAAAGCCAAG 5400
F Y Q Y E A W T G R K A P R A A M K A K

5401 GTGATTTCGTTGTATGATAGTCAGCAG*TGATCTTTTTTAGACTGATATTTGTATATGCTT* 5460
V I S L Y D S Q Q

5461 *TTGTATGCGATATTGAATACGATTTAATTCTATGATTTACCTTTTTGCCAATGAAATCAC* 5520

5521 *AA*

Figure 3-11. (Continued)

Table 3-1. Comparison of the pentafunctional AROM proteins from *A. nidulans*, *P. carinii*, *S. cerevisiae* and *R. solani*.

Organism	Length of gene	Length of polypeptide	Intron*	Mr
A. nidulans	4749 bp	1582 aa	53 bp (4685-4737)	174
P. carinii	4788 bp	1596 aa	45 bp (583-628)	176
S. cerevisiae	4767 bp	1588 aa	—	175
R. solani	5097 bp	1618 aa	50 bp (399-449) 48 bp (657-705) 48 bp (1492-1540) 48 bp (2540-2588) 48 bp (2709-2757)	178

The asterisk '*' shows intron positions in reference to the 5'-methionine codon.

```
AnAROM     1   -------MSNPTKISILGRESIIADFGLWRNYVAKDLISDCSSTTYVLVTDTNIGSIYTP
PcAROM     1   -------MKEIIKLSILGKDSIHIGLHLWP-HITNEIFLCIFSPTYVIITDSNIETLYIP
ScAROM     1   -------MVQLAKVPILGNDIIHVGYNIHD-HLVETLIKHCPSSTYVICNDTNLSKVPYY
RsAROM     1   MATASVEQPDLTKVSILGKDSIHCGFHLSP-YLADTVLVNLPASTYVLITDTRVAKFHLE
EcAROM     1   ---------MERIVVTLGERSYPITIASGLFNEPASFLPLKSGEQVMLVTNETLAPLYLD
                        →aroB(1)

AnAROM    54   SFEEAFRKRAAEIT-PSPRLLIYNRPPGEVSKSRQTKADIEDWMLSQNPPCGRDTVVIAL
PcAROM    53   SEKTYEISMAKQRS-INSRLLFFTIPPGEKSKSRKTKALIEDWLLS--EKCTRDTVIIAI
ScAROM    53   QQLVLEFKASLPEG---SRLLTYVVKPGETSKSRETKAQLEDYLLV--EGCTRDTVMVAI
RsAROM    60   SFEAAFTDALAARPNTSARFLTHVIPPGETSKSREGKAEIEDFLLD--NSCTRDTVVLAL
EcAROM    52   KVRGVLEQAGVNVD-----SVILPD--GEQYKSLAVLDTVFTALLQ--KPHGRDTTLVAL

               GGGVIG(DHQ3) (GxGxxG)
AnAROM   113   GGGVIGDLTGFVASTYMRGVRYVQVPTTLLAMVDSSIGGKTAIDTPLGKNLIGAIWQPTK
PcAROM   110   GGGVIGDLVGYVSATFMRGVRFIQIPTTLLAMVDSSIGGKNSINTSYGKNAIGTIWQPER
ScAROM   108   GGGVIGDMIGFVASTFMRGVRVVQVPTSLLAMVDSSIGGKTAIDTPLGKNFIGAFWQPKF
RsAROM   118   GGGVIGDLVGFVAATFMRGVRLVQIPTTLLAMVDSSVGGKTAIDTPHGKNLIGSFWQPSY
EcAROM   103   GGGVVGDLTGFAAASYQRGVRFIQVPTTLLSQVDSSVGGKTAVNHPLGKNMIGAFYQPAS

AnAROM   173   IYIDLEFLETLPVREFINGMAEVIKTAAISSEEEFTALEENAETILKAVRREVTPG--EH
PcAROM   170   IFIDFTFLETLTEKEFINGLAEIIKTIIIWDESEFASLENISEKIAKTVRSMSLTSNKHS
ScAROM   168   VLVDIKWLETLAKREFINGMAEVIKTACIWNADEFTRLESNASLFLNVVNGAKNVKVTNQ
RsAROM   178   VFIDAAFLETLPQREFVNGMAEVIKTAAIWNEDEFSMLEASAPALFAAIGSSSSTTSAGR
EcAROM   163   VVVDLDCLKTLPPRELASGLAEVIKYGIILDGAFFNWLEENLDALLRLDGPAMAYC----

AnAROM   231   RFEGTEEILK-----------ARIIFASARHKAYVVSADEREGGLRNLLNYGHSIGHAIEA
PcAROM   230   KFNEIIKDLK-----------RYIISSIKILKAHIVSIDEKEKDLRTLLNFGHSIGHAIET
ScAROM   228   LTNEIDEISNTDIEAMLDHTYKLVLESIKVKAEVVSSDEREISLRNLLNFGHSIGHAYEA
RsAROM   238   TTATRSEAQS--------LLLHVITESIGVKAHIVTLDERETGLRNLVNFGHTIGHAIEA
EcAROM   219   ----------------------IRRCCELKAEVVAADERETGLRALLNLGHTFGHAIEA

AnAROM   280   ILTP-QILHGECVAIGMVKEAELARHLGILKGVAVSRIVKCLAAYGLPTSLKDARIRKLT
PcAROM   279   VLAP-YILHGESISIGMVKEAELSRHLGILNPNVVSRLIKCLNTWGLPTSFKDRRFKEVI
ScAROM   288   ILTP-QALHGECVSIGMVKEAELSRYFGILSPTQVARLSKILVAYGLPVSPDEKWFKELT
RsAROM   290   VLTP-TILHGECVAIGMVLEGEVARQLGVLSQVGVGRITRALKAYGLPTSTKDPRIAAVP
EcAROM   256   EMGYGNWLHGEAVAAGMVMAARTSERLGQFSSAETQRIITLLKRAGLPVN--GP------
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3-12. Comparison of the amino acid sequences of four AROM polypeptides and the corresponding *E.coli* monofunctional enzymes. AnAROM, *A. nidulans* AROM (Charles *et al.*, 1986); PcAROM, *P. carinii* AROM (Banerji *et al.*, 1993); ScAROM, *S. cerevisiae* AROM (Duncan *et al.,* 1987); RsAROM, *R. solani* AROM; EcAROM, the corresponding *E.coli* monofunctional enzymes which have been arranged in the following order: *aroB*, DHQ synthase residues (1-363) (Millar & Coggins. 1986); *aroA*, EPSP synthase (363-789) (Duncan *et al.*, 1984); *aroL*, shikimate kinase (790-963) (Millar *et al.*, 1986); *aroD*, DHQ dehydratase (964-1215) and *aroE*, shikimate dehydrogenase (1216-1487) (Anton & Coggins,1988). Black boxes indicate identical residues, and gray boxes indicate similar residues. The conserved motifs used in selecting the degenerate primers are also shown.

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AnAROM   339   AGKHCSVDQLVFNMALDKKNDGPKKKIVLLSAIGTPYETRASVVANEDIRVVLAPSIEVH
PcAROM   338   LGKKHLIEDILEIMSIDKKNDSNNKKIVLLSAIGKPYEKKASSVSDDDIRTILSQNILLY
ScAROM   347   LHKKTPLDILLKKMSIDKKNDGSKKKVVILESIGKCYGDSAQFVSDEDLRFILTDETLVY
RsAROM   349   ASRLLTIDRLLDIMKIDKKNSGPEKKIVLLSRIGKPYEERATGVKDEVIRRVLAEACRVI
EcAROM   308   --REMSAQAYLPHMLRDKKVLAGEMRLVLPLAIGKSEVRSCVSHELVLNAIADCQSAVES
                                                            aroB(362)┘
                                                                   →
                                                                aroA(1)

AnAROM   399   --PGVAHSSNVICAPPGSKSISNRALVLAALGSGTCRIKNLLHSDDTEVMLNALERLGAA
PcAROM   398   GIPLNAFQKHTTITLPGSKSISNRALVLASLSNGICYLKNLLHSDDTYYMLSALEKLNAA
ScAROM   407   PFKDIIPADQQKVVIPPGSKSISNRALVLAALGEGQCKIKNLLHSDDTKHMLTAVHELKGA
RsAROM   409   PG--IIPRGNPVRVSTPGSKSISNRALVLAALGNGTCRLRNLLHSDDTQVMMSALIELKGA
EcAROM   366   LTLQPIARVDGTINLPGSKSVSNRALLLAALAHGKTVLTNLLDSDDVRHMLNALTALGVS

                                            *******
                                         GNAGTA(EPSP1)
AnAROM   457   TFSWEDEGEVLVVNGKG--GNLQASSSPLYLGNAGTASRFLTTVATLAN-SST-------
PcAROM   458   EFKWEQDGDVLVVKGKS--GYLENPQMELYLGNSGTTARFLTSICTLVQPNSR-------
ScAROM   467   TISWEDNGETVVVEGHG-GSTLSACADPLYLGNAGTASRFLTSLAALVNSTSS-------
RsAROM   467   KFAWEDGGETLVVSGCGGAFTIPPACKELYLGNAGTAARFLTTVCTLVGPDSSSATASSE
EcAROM   426   YTLSADRTRCEIIGNGG--PLHAEGALELFLGNAGTAMRPLAAALCLGS-----------

AnAROM   507   --VDSSVLTGNNRMKQRPIGDLVDALTANVLPLNTSKGRASLPLKIAAS--GGFAGGNIN
PcAROM   509   --ENHLIILTGSNRMKQRPIGPLVDALKNNGCCIEYLELENCLPLLIKPK-EIGLYGGNIN
ScAROM   519   --QKYIVLTGNARMQQRPIAPLVDSLRANGTKIEYLNNEGSLPIKVYTDS--VFKGGRIE
RsAROM   527   FPEGYTFITGNARMKQRPCGPLVDALRANGSKVKYIESEGCLPLIIGAG---GLKGGTIQ
EcAROM   473   ---NDIVLTGEPRMKERPIGHLVDALRLGGAKITYLEQENYPPLRLQGG----FTGGNVD

AnAROM   563   LAAKVSSQYVSSILMCAPYAKEP-VTLRLVGGKPISQPYIDMTTAMMRSFGIDVQKSTTE
PcAROM   566   LSATVSSQYVSSILMCSPYAKTQ-VTLSLIGGKPISQPYIDMTISMMSSFGIKVTRSHSK
ScAROM   575   LAATVSSQYVSSILMCAPYAEEP-VTLALVGGKPISKLYVDMTIKMMEKFGINVETSTTE
RsAROM   584   LAASVSSQYVSSILLCAPYAKEDVVLELVGGQVISQPYIDMTIAMMKTFGVEVTRRKAE
EcAROM   526   VDGSVSSQFLTALLMTAPLAPED--TVIRIKGDLVSKPYIDITLNLMKTFGVEIENQHYQ

AnAROM   622   E----HTYHIPQGRYVNPAEYVIESDASCATYPLAVAAVTGTTCTVPNIGSASLQGDARF
PcAROM   625   E----NTYYIPKGQYTCPSEYIIEGDATSATYPLAIAAITGGSCTISNVGSASLQGDSKF
ScAROM   634   P----YTYYIPKGHYINPSEYVIESDASSATYPLAFAAMTGTTVTVPNIGFESLQGDARF
RsAROM   644   DGTLLDIYDIPRAQYTNPEKYAIESDASSATYPLAVAAITGTTCTIENIGTSSLQGDAGF
EcAROM   584   Q-----FVVKGGQSYQSPGTYLVEGDASSASYFLAAAAIKGGTVKVTGIGRNSMQGDIRF

AnAROM   678   AVEVLRPMGCTVEQTETSTTVTGPSDGILRATSKRGYGTNDRCVPRCFRTGSHRPMEKSQ
PcAROM   681   SEYILKPMGCEVVQSPTTTYIKGPPKGKLKSIGSINMESMTDTFLTAAVLASVAYEESKP
ScAROM   690   ARDVLKPMGCKITQTATSTTVSGPPVGTLKPLKHVDMEPMTDAFLTACVVAAISHDSDPN
RsAROM   704   AVNVLKRMGCKVEQSENETTVTGPPIGQLKAIGLVDMEPMTDAFLTAVVVAAVCGEGSAE
EcAROM   639   ADVLEKMGATICWGDDYISCTRGELNAIDMDVNHIPDAAMTIATAALFAKGTTRLRNIYN

                                 KECNRI(EPSP2)
AnAROM   738   --------TTPPVSSGIANQRVKECNRIKAMKDELAKFGVICREHDDGLEIDGIDRSNLR
PcAROM   741   --------YVTKITG-ISNQRIKECNRINAMVCELKKFGIEAGEIPDGIYVKALNTSNLP
ScAROM   750   ------SANTTTIEG-IANQRVKECNRILAMATELAKFGVKTTELPDGIQVHGLNSIKDL
RsAROM   764   GLESDVPKNTTRILG-IANQRVKECNRIRAMIDELAKFGVKTKELVDGLEVYGQPIETLN
EcAROM   699   ---------WRVKET--------DR--LFAMATELRKVGAEVEEGHDYIRITPPEKLNFA
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Figure 3-12. (Continued)

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AnAROM   790   QP------VGGVFCYDDHRVAFSFSVLSLVTPQ---------PTTLILEKECVGKTWPGWW
PcAROM   792   YS------VEGINCYNDHRIAMSFSVLACISSK---------PTTILDKACVNKTWPYWW
ScAROM   803   KVPSDSSGPVGVCTYDDHRVAMSFSLLAGMVNSQNERDEVANPVRILERHCTGKTWPGWW
RsAROM   823   KN-------VSVHCYDDHRVAMAFSVLATVVPG----------TIDEEKRCVEKTWPNWW
EcAROM   740   EI----------ATYNDHRMAMCFSLVALSDTP----------VTILDPKCTAKTFPDYF

                                                  (G/AxxxxGKT/S)
AnAROM   835   DTLRQLFKVKLEGKELEEEPVAASGPDRG--NASIYIIGMRGAGKSTAGNWVSKALNRPF
PcAROM   837   DILNSTFKVQMKGIEFDLNPTINSSILHHPSECTIFVIGMRGAGKTTLGQLAANFLGREF
ScAROM   863   DVLHSELGAKLDGAEPLECT------SKKNSKKSVVIIGMRAAGKTTISKWCASALGYKL
RsAROM   866   DDFTNKIGIDVEGIELAHQSP---AVAKYNPEATIIIVGMRGAGKTHIGGIAAASLGWKF
EcAROM   780   EQLARISQAAVT--------------------QPLFVICPRGCGKTTVGMALADSLNRRF
               aroA(427)┘
                     →aroL(1)

AnAROM   893   VDLDTELETVEG-MTIPDIIKTRGWQGFRNAELEILKRTLKER-SRGYVFACGGGVVEMP
PcAROM   897   IDLDSHIEEDIK-TTILEFIQKYSWDVFRRKELHFLKTTLTEK-KENYIISCGGGIIETE
ScAROM   917   VDLDELFEQQHNNQSVKQFVVENGWEKFREEETRIFKEVIQNYGDDGYVFSTGGGIVESA
RsAROM   923   IDADHVFEGRTG-ELVSQYVRSKGWTAFRQAESQILQDLLAEN-PTRTIISTGGGIVESP
EcAROM   820   VDTDQWLQSQLN-MTVAEIVEREEWAGFRARETAALEAVTAPS----TVIATGGGIILTE
AnAROM   951   EARKLLTD----YHKTKGNVLLLMRDIKKIMDFLSIDKSRPAYVEDMMGVWLRRKPWFQE
PcAROM   955   EARDLLIS----YIEANGIVIHIHRNIQDIIKYLNTDKTRAPYQDNIMHVWERRKRWYNL
ScAROM   977   ESRKALKD----FASSGGYVLHLHRDIEETIVFLQSDPSRPAYVEEIREVWNRREGWYKE
RsAROM   981   LNRALLSG----YGRATGPVVYVMRDVNEILSYLGSETARPAYGEEIADVYWRREPWFRE
EcAROM   875   FNRHFMQNNGIVVYLCAPVSVLVNRLQAAPEEDLRPTLTGKPLSEEVQEVLEERDALYRE

AnAROM  1007   CSNIQYYSRDASPSGLAR----------ASEDFNRFLQVATGQIDSLSIIKEKEHSFFAS
PcAROM  1011   CSSHQFHMTSTDPTEFKKNIPLN-----LKSSFNNFLRTITGKNNFFSLILKKKRSYFFS
ScAROM  1033   CSNFSFFAPHCSAEAEFQALR-------------RSFSKYIATITGVREIEIPSGRSAFVC
RsAROM  1037   VSTHEYISYTGGFHAANGATPGEIASACRDETARFFRQFTGIKPNLSEATAQGERSFFFS
EcAROM   935   VAHIIIDATNEPSQVISEIR---------SALAQTINC*KTVTVKDLVIGTGAPKIIVSL
                                                 aroL(174)┘
                                                        →aroD(1)

AnAROM  1057   LTLPDLREAGDILEEVCVGSDAVELRVDLLKDPASN----NDIPSVDYVVEQLSFLR-SR
PcAROM  1066   LAFSDLENIFSLLDIITAGCDAIELRIDLFQKPEEI----DKYPSLEYIAEKIFLLR-QK
ScAROM  1081   LTFDDLTEQTENLTPICYGCEAVEVRVDHLAN------------YSADFVSKQLSILRKAT
RsAROM  1097   LTYPDLLPAVPSLPLITAGADAIELRVDLLNPSGDPVTGPPNIPSLDYVATQLSALR-HT
EcAROM   986   MAKDIASVKSEALAYREADFDILEWRVDHYAD------------ISNVESVMAAAKILRETM

AnAROM  1112   VTLPLIFTIRTQSQGGRFPDNAHDAALELYRLAFRSGCEFVDLDIAFPEDMLRAVTEMKG
PcAROM  1121   TSLPIIYTLRTTNHGGSFLSSEKKLAKEYILHCAKWGHEFLDIELDIASELFKTINNSWP
ScAROM  1130   DSIPIIFTVRTMKQGGNFPDEEFKTLRELYDIALKNGVEFLDLELTLPTDIQYEVINKRG
RsAROM  1156   SSLPVVFTVRTASQGGAFPDLAEKEAFALYNLALRHGVEYIDVEISWSDKKIQDLAARKG
EcAROM  1036   PEKPLLFTFRSAKEGGEQAISTEAYIALNRAAIDSGLVDMIDLELFTGDDQVKETVAYAH

AnAROM  1172   FSKIIASHHDPKGELSWANMSWIKFYNKALEYG-DIIKLVGVARNIDDNTALRKFKNWAA
PcAROM  1181   YTKIIASYHNIEKPISCDDFEWIQKYKEAQHYG-DIIKLVGTSSSIEDNFFLEEFKSKFI
ScAROM  1190   NTKIIGSHHDFQGLYSWDDAEWENRFNQALTLDVDVVKFVGTAVNFEDNLRLEHFRDTHK
RsAROM  1216   ASQIIASWHDWSGNMKWNGAVVKEKYAIAERVG-DIVKLVGKALSIEDNFALRAFAAAHT
EcAROM  1096   AHDVKVVMSNHDFHKTPEAEEIIARLRKMQSFDADIPKIALMPQSTSDVLTLLAATLEMQ
```

Figure 3-12. (Continued)

AnAROM 1231 EA-HDVPLIAINMGDQGQLSRILNGFMTPVSHP-SLPFKAAPGQLSATEIRKGLSLMGEI
PcAROM 1240 N--KKVPSIIINTCIKGQLSRIMNTFMTPVTHP-SLPSKIAPGQLSIKEINTALHIMGLL
ScAROM 1250 N----KPFIIAVNMTSKGSISRVLNNVLTPVTSD-LLPNSAAPGQLTVAQINKMYTSMGGI
RsAROM 1275 S----KPFIIGINMGAEGQLSRVLNTAFTPVTHP-LLPTRAAPGQMSVQDIHTALHINGQL
EcAROM 1156 EQYADRPIITTVSMAKTGVISRLAGEVFGSAATFGAVKKASAPGQISVNDLRTVLTIHQA
aroD(240)┘

AnAROM 1289 KPKKFAIFGSPISQSAPQLSTTPYLPRSASPITTPAWRLRTPKMCRSSSAL--LTSAAPS
PcAROM 1297 PEKKYFIFGKPIKHSQSPNIHNLGFEIILGLPYKYQLFETDSISELKEIIHL--EEFGGAS
ScAROM 1305 EPKELFVVGKPIGHSRSPILHNTGYEILGLPHKFDKFETESAQLVKEKLLDGNKNFGGAA
RsAROM 1330 APQKFYIFGSPIAHSMSPHLHNTGFEKLGLPHKYHIFETATITDEVKAVIR-APEFGGAS
EcAROM 1216 MET-YAVFGNPIAHSKSPFIHQQFAQQLNIEHPYGRVLAPINDFINTLNAFFSAGGKGAN
→aroE(1)

AnAROM 1347 VTIRSSSTSCPFSTKIPRKPRSSELLTQSFPCRLAR-TLHHAYVGRNTDWQGMILSLRKA
PcAROM 1355 VTIPLKTNISILLDEIS--DHAALIGSVNTITRTYN-NGQYILKGENTDWQGIIKAIKNF
ScAROM 1365 VTIPLKLDIMQYMDELT--DAAKVIGAVNTVIP----LGNKKFKGDNTDWLGIRNALINN
RsAROM 1389 VTIPLKLDIIPLLDEVS--PEAKAIGAVNTIIPRKRADGSTSLFGTNTDWRAIHDLARNN
EcAROM 1275 VTVPFKEEAFARADELT--ERAALAGAVNTLMR----LEDGRLLGDNTDGVGLLSDLERL

AnAROM 1406 GVYGPKRKDQEQSALVVGCGGTARAAIYALHNMGYSPIYIVGRTPSKIENMVSTFPSSYN
PcAROM 1412 NKFEKSFEN--FSGFIIGAGGASRAAIYALLSLGISPIYIINRSKDKINKLYHFFNNNHI
ScAROM 1419 GVPEYVGHT---AGLVIGAGGTSRAALYALHSLGCKKIFIINRTTSKLKPLIESLPSEFN
RsAROM 1447 LVVGITNET---TALVIGAGGTARAALYAIHALGIKTIYIFNRTRAAAQALADTFPIFG-
EcAROM 1329 SFIRPGLRI-----LLIGAGGASRGVLLPLLSLDCA-VTITNRTVSRAEELAKLFAHTGS

AnAROM 1466 IR-IVESPSS--FESVPHVAIGTIPADQPIDPTMRETLCHMFERAQEADAEAVKAIEHAP
PcAROM 1470 IP-ITEYHELNNINFDIRIGISTIPTDNPIDPSVLEIAKIEFN----------LKRKSSE
ScAROM 1476 IIGIESTKSIEEIKEHVGVAVSCVPADKPLDDELLSKLERFLVKG---------AHAAFV
RsAROM 1503 -I-IPLDSLNSFPKAAPTVVVSAIPATGTTTEKASDSAGVYIPPS---------LFEAPS
EcAROM 1383 IQALSMDELEG-----HEFDLIINATSSGISGDIPAIPSSLIHPG--------------

AnAROM 1523 RILLEMAYKPQVTALMRLASDS-GWKTIPGLEVLVGQGWYQFKYWTGISPLYESARACSS
PcAROM 1519 GIFLDMAYGSNTTDLTIIAKAC-NWKIIHGLEIIIEQGSEQFLLWTETYIPYNQVKYAIL
ScAROM 1527 PTLLEAAYKPSVTPVMTISQDKYQWHVVPGSQMLVHQGVAQFEKWTGFKGPFKAIFDAVT
RsAROM 1552 GVVVEMAYKPAVTPVIGLAAKSSGWVGVRGVDIICEQGFYQYEAWTGRKAPRAAMKAKVI
EcAROM 1423 IYCYDMFYQKGKTPFLAWCEQRGSKRNADGLGMLVAQAAHAFLLWHGVLPDVEPVIKQLQ

AnAROM 1582 PLI----
PcAROM 1578 GPNK---
ScAROM 1587 KE-----
RsAROM 1612 SLYDSQQ
EcAROM 1483 EELSA--

AroE (272)┘

Figure 3-12. (Continued)

DISCUSSION

This study showed that an 83-kDa protein reacting to polypeptide A-specific antibodies (anti-pA antibodies) is present only in M2-containing cultures but not in M2-lacking ones (Figure 3-5). This study also showed that the cultures containing the 83 kDa protein also contain M2-specific polysome-associated mRNAs (Figures 3-5, 3-6, and 3-7). These results, in conjunction with the size and the antibody specificity data, strongly suggest that the 83-kDa polypeptide (p83) found in M2-containing cultures is polypeptide A. Another important finding in this study is that the quinate-induced, virulent strain Rhs 1AP contained relatively high amounts of the 83 kDa protein. A polypeptide A-specific transcript was also detected in quinate-induced Rhs 1AP using RT-PCR (Figure 3-7). The results indicate that quinate, directly or indirectly, tums on transcription, and translation of the M2 dsRNA.

The presence of a larger anti-pA specific protein band in 23-3b in westem blot analysis is interesting. This band is about double the size of polypeptide A. It is possible that this band could be a cross-linked dimer of polypeptide A. The sample loading buffer contained SDS and 2-mercaptoethanol, and protein samples were boiled for 5 min before they were loaded on the SDS-polyacrylamide gel. Thus, dimers, formed from covalently bound monomers, are not expected to remain intact.

One of the most important findings in this study is that a virulent *R. solani* isolate became hypovirulent when grown on quinate-containing media. It is possible that quinate acts as a regulation molecule that signals up- or down regulation of a pathway (s), thus leading to a drastic decrease in virulence. As expected, the

quinate utilization pathway is one of the processes that were turned on. When the quinate pathway is persistently on (this would happen when quinate is consistently available to the fungus in the media at induction levels), dehydroquinate (DHQ) and dehydroshikimate (DHS), two intermediates shared with the shikimate pathway, would be drawn from the shikimate pathway to the quinate pathway. Thus, the shikimate pathway is down regulated, leading to a lower level of production of the three aromatic amino acids (AAAs). The above process was observed in *Aspergillus nidulans* by Lamb *et al.* (1992).

The virulence of Rhs 1AP was drastically increased upon the addition of shikimate, chorismate or AAAs to the glucose minimal medium but that of Rhs 1A1 was not. In fact, the virulence of Rhs 1AP was almost fully restored when the AAAs were added into glucose minimal medium (as compared with the virulence data from PDYA treatment) (Figure 3-1). However, the AAA amendment caused a virulence reduction in Rhs 1A1, and a possible physiological change in potato sprouts expressed as increased vigor and branching of the sprouts inoculated with Rhs 1A1 (data not shown). This is a phenomenon similar to that observed in field studies, in which inoculation of potato plants with Rhs 1A1 brought about significantly increased plant vigor and induction of blooming 7-10 days earlier than in control potato plants (Bandy and Tavantzis, 1990). However, the cause of the phenomenon is unknown.

To determine which method should be used in the pathogenisity tests, parallel virulence assessment experiments were carried out using either mycelium with an agar plug (referred to as 'mycelial plug' method) or only mycelium cultured on the cellophane (referred to as 'cellophane' method) and grown on the same media as in

Table 3-1. Although the trend was the same, virulence data for both Rhs 1AP and Rhs 1A1 showed that the 'cellophane' method gave inconsistent results. For example, only two thirds of the quinate treated Rhs 1AP cultures showed reduced virulence, whereas one third of the cultures had the same virulence as those grown on the glucose minimal media. In contrast, the 'mycelial plug' method gave more consistent results than the 'cellophane' method. A possible reason for the above phenomenon is that agar plugs containing the different amendments are needed to maintain the physiological impact of the respective media on the fungus.

HPLC data showed a positive correlation between virulence and relative concentration of phenylalanine [Phe] in Rhs 1AP but not in Rhs 1A1 when the isolates were grown on GluMM amended with shikimate, chorismate or AAAs (Figures 3-3, 3-4). In contrast, such a trend was not observed between virulence and relative concentration of tyrosine, another aromatic amino acid, in either Rhs 1AP or Rhs 1A1. This is consistent with previous results suggesting that PAA was associated with virulence in *R. solani* (Frank and Francis, 1976; Tavantzis *et al.*, 1989). However, when quinate was present in the media, virulence was diminished even when [Phe] was relatively unchanged (Figures 3-3, 3-4). These results are in agreement with data showing chorismate-induced virulence overturned by quinate (Figures 3-2).

Enzyme assay experiments showed that shikimate kinase (SK) activity is higher in Rhs 1AP than in Rhs 1A1 when they were grown on glucose as the carbon source. SK activity is one of the five AROM activities. This result might explain why the ratio [Phe]/[Total] is higher in Rhs 1AP than in Rhs 1A1 when the isolates were

grown in the media amended with shikimate, chorismate, or AAA (Figures 3-3, 3-4). This result might also explain why hypovirulent isolates produce less PAA, which is a metabolite of phenyalanine (Tavantzis *et al.*, 1989; Tavantzis and Lakshman, 1995). Down-regulation of the shikimate pathway gives rise to less chorismic acid, less phenyalanine, and therefore, a lower amount of PAA.

Enzyme assay experiments also showed that quinate dehydrogenase (QDHase) is constitutively expressed in Rhs 1A1 in the absence of environmental quinate (Figure 3-9). QDHse catalyzes the conversion of quinate to 3-dehydroquinate, the first step in the quinate utilization pathway which includes the three enzymes (quinate/shikimate dehydrogenase, dehydroshikimate dehydrase, and catabolic 3-dehydroquinase) involved in the degradation of quinate to protocatechuit acid (PCA). It is reasonable to assume that the other two quinate pathway enzymes are constitutively expressed in Rhs 1A1 as well, because the three enzymes are encoded by three tightly linked structural genes subjected to transcriptional regulation by the same regulatory proteins (Hawkins *et al.*, 1984; Giles *et al.*, 1985). In wild-type fungi, when quinate is not available, repressor QUTR suppresses the expression of the quinate pathway genes by binding to the transcription activator QUTA (Grant *et al.*, 1988). External quinate is needed for the induction of the quinate pathway (Giles *et al.*, 1967; Hawkins *et al.*, 1982). When quinate binds to QUTR, QUTR changes its conformation and dissociates from QUTA, allowing the induction of quinate pathway (Lamb *et al.*, 1996). What causes constitutive expression of the quinate pathway in Rhs 1A1 in the absence of quinate? Indirect evidence from previous reports (Jian *et al.*, 1997; Lakshman *et al.*, 1998) and

evidence from this study show that M2 dsRNA is a good candidate. A possible mechanism of M2-dsRNA associated hypovirulence in *R. solani* has been described in the hypothesis that was stated in the Introduction. Polypeptide A contains four conserved motifs of an RNA-dependent RNA polymerase that is common to all of the RNA viruses (Lakshman *et al.*, 1998). Thus, the polypeptide A could reach high concentrations and out-compete the native *Rhizoctonia* QUTR (r-QUTR) in binding QUTA, but still allow QUTA to interact with the transcription apparatus for expression of quinate pathway genes. Polypeptide A lacks a portion of the EPSP-like domain and the SK-like domain which comprise the N-terminal of QUTR (Lakshman *et al.*, 1998). It is this N-terminal of QUTR that occludes the negatively-charged region of QUTA and prevents it from interacting with the transcription apparatus (Hawkins *et al.*, 1993a). Thus, binding of polypeptide A to QUTA would still allow QUTA to interact with the transcription apparatus and result in constitutive expression of quinate pathway genes in the absence of external quinate. This hypothesis is also supported by experimental data from *N. crassa* and *A. nidulans* studies showing that constitutive QUTR mutants possess all above three enzymatic activities in the absence of quinate (Huiet, 1984; Grant *et al.*, 1988). Complementation analysis has shown that the constitutive phenotype results from a mutation in the QUTR gene which is part of the *qut* gene cluster (Grant *et al.*, 1988). As expected, the quinate pathway is inducible in the M2-lacking, virulent Rhs 1AP. A basal level QDHase activity was detected when Rhs 1AP was grown on glucose media. Upon quinate induction, QDHase activity was increased approximately 7 times in Rhs 1AP.

This is the first time that an *arom* gene from Basidiomycetes has been sequenced. The unique characteristic of the *R. solani arom* gene is that it contains five introns throughtout the entire region. No introns have been reported in the *S. cerevisiae arom* gene (Duncan *et al.*, 1987). The *A. nidulans arom* gene had previously been reported to be a single 4812 bp open reading frame (Charles *et al.*, 1986). More recent work showed that a 53bp intron is present in the extreme C-terminus of the shikimate dehydrogenase domain (Lamb *et al.*, 1996). In the *P. carinii arom* gene, only one intron was identified in the middle of the dehydroquinate synthase domain (Banerji e*t al.*, 1993)

The most important finding of this study is that, in the virulent Rhs 1AP, quinate induces replication of the M2 dsRNA and a simultaneous dramatic reduction in virulence. Quinate comprises 10% of decaying leaf matter and might be used as a less expensive source of carbon utilization than overcoming the defense mechanisms of a host plant. Although this is another piece of strong indirect evidence suggesting an association between hypovirulence and the M2 dsRNA, a direct proof that M2-dsRNA causes hypovirulence in *R. solani* will be provided only when a virulent M2-free isolate is converted to hypovirulent after cDNA or RNA copies of the M2-dsRNA are introduced into it.

BIBLIOGRAPHY

1. Ander, P., and K. E. Eriksson. 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentrum*. Archives in Microbiology 109: 1-8.
2. Anton, I. A., and J. R. Coggins. 1988. Sequencing and overexpression of the *Escherichia coli aroE* gene encoding shikimate dehydrogenase. Biochemical Joumal 249: 319-326.
3. Bandy, B. P., S. S. Leach, and S. M. Tavantzis. 1988. Anastomosis group 3 of *Rhizoctonia solani* is the major cause of Rhizoctonia disease in Maine. Plant Disease 72: 596-598.
4. Bandy, B. P., and S. M. Tavantzis. 1990. Effect of hypovirulent *Rhizoctonia solani* on Rhizoctonia disease, growth, and development of potato. American Potato Joumal 67: 189-199.
5. Banerji, S., A. E. Wakefield, A. G. Allen, D.J. Maskell, S. E. Peters, and J. M. Hopkin. 1993. The cloning and characterization of the *arom* gene of *Pneumocystis carini*. Joumal of General Microbiology 139: 2901-2914.
6. Baum, J. A., R. Geever, and N. H. Giles. 1987. Expression of *qa-1 F* activator protein: identification of upstream binding sites in the *qut* gene cluster and localization of the DNA-binding domain. Molecular and Cell Biology 7: 1256-1266.
7. Bentley, R. 1990. The shikimate pathway- a metabolic tree with many branches. Critical Reviews in Biochemistry and Molecular Biology 25: 307-385.

8. Bergmeyer, H. U. 1974. Methods of enzymatic analysis, pp. 784-788. Edited by Forster, G., D. Bemt and H. U. Bergmeyer. Academic Press, New York.

9. Bharathan, N., and S. M. Tavantzis. 1990. Genetic diversity of double-stranded RNAs in *Rhizoctonia solani*. Phytopathology 80: 631-635.

10. Bharathan, N., and S. M. Tavantzis. 1991. Assessment of genetic relatedness among double-stranded RNA components from *Rhizoctonia solani* isolates of diverse geographic origin. Phytopathology 81: 411-415.

11. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

12. Brasier, C. M. 1983. A cytoplasmically transmitted disease of *Ceratocystis ulmi*. Nature 305: 220-222.

13. Bruenn, J. A. 1993. A closely related group of RNA-dependent RNA polymerase from double-stranded RNA viruses. Nucleic Acids Research 21: 5667-5669.

14. Carling, D. E. 1996. Grouping in *Rhizoctonia solani* by hyphal anastomosis interactions, pp. 37-47. In "*Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control". Edited by Sneh, B., S. Jabaji-Hare, S. Neate, and D. G. Kluwer. Academic Publishers, Dordrecht.

15. Carpenter, C. E., R. J. Muller, P. Kazmierczak, L. Zhang, D. K. Villalon, and N. K. van Alfen. 1992. Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. Molecular Plant Microbe Interaction 4: 55-61.

16. Castanho, B., and E. E. Butler. 1978a. Rhizoctonia decline: A degenerative disease of *Rhizoctonia solani*. Phytopathology 68: 1505-1510.

17. Castanho, B., and E. E. Butler. 1978b. Rhizoctonia decline: Studies on hypovirulence and potential use in biological control. Phytopathology 68: 1511-1514.

18. Castanho, B., E. E. Butler, and R. J. Shepherd. 1978. The association of double-stranded RNA with Rhizoctonia decline. Phytopathology 68: 1515-1518.

19. Chaleff, R. S. 1974a. The inducible quinate-shikimate catabolic pathway in *Neurospora crassa*: Genetic organization. Journal of General Microbiology 81: 337-355.

20. Chaleff, R. S. 1974b. The inducible quinate-shikimate catabolic pathway in *Neurospora crassa*: induction and regulation of enzyme synthesis. Journal of General Microbiology 81: 357-372.

21. Chamberlain, V. K., and R. L. Wain. 1971. Studies on plant growth-regulating substances-the influence of ring substituents on the plant growth regulating activity of phenylacetic acid. Annals of Applied Biology 69: 65-72.

22. Charles, I. G., J. W. Keyte, W. J. Brammar, M. Smith, and A. R. Hawkins. 1986. The isolation and nucleotide sequence of the complex AROM locus of *Aspergillus nidulans*. Nucleic Acids Research 14: 2201-2213.

23. Choi, G. H., and D. L. Nuss. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257: 800-803.

24. Choi, G. H., B. Chen, and D. L. Nuss. 1995. Virus-mediated or transgenic suppression of a G-protein alpha subunit and attenuation of fungal virulence. Proceedings of the National Academy of Sciences (USA) 92: 305-309.

25. Clutterbuck, A. J. 1972. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. Journal of General Microbiology 70: 423-435.

26. Coggins, J. R., M. R. Boocock, S. Chaudhuri, J. M. Lambert, J. Lumsden, G. A. Nimmo, and D. D. S. Smith. 1987. The *arom* multifunctional enzyme from *Neurospora crassa*. Methods in Enzymology 142: 325-341.

27. Davis R. H., and F. J. deSerres. 1970. Genetic and Microbiological Research Techniques for *Neurospora crassa*. Methods in Enzymology 17a: 79-143.

28. Day, P. R., J. A. Dodds, J. E. Elliston, R. A. Jaynes, and S. L. Anagnostakis. 1977. Double-stranded RNA in *Endothia parasitica*. Phytopathology 67: 1392-1396.

29. Duncan, K., A. Lewendon, and J. R. Coggins. 1984. The complete amino acid sequence of *Escherichia coli* 5-enolpyruvylshikimate-3-phosphate synthase. FEBS Letters 170: 59-63.

30. Duncan, K., R. M. Edwards, and J. R. Coggins. 1987. The pentafunctional *arom* enzyme of *Saccharomyces cerevisiae* is a mosaic of monofunctional domains. Biochemical Journal 246: 375-386.

31. Elliston, J. E. 1982. Hypovirulence. Advances in Plant Pathology 1: 1-33.

32. Finkler, A., Y. Holtin, L. Barash, B. Sneh, and B. Pozinak. 1985. Isolation of a virus from virulent strains of *Rhizoctonia solani*. Journal of General Virology 66: 1221-1232.

33. Frank, J. A., and S. K. Francis. 1976. The effect of a *Rhizoctonia solani* phytotoxin on potatoes. Canadian Journal of Botany 54: 2536-2540.

34. Geever, R. F., L. Huiet, J. A. Baum, B. M. Tyler, V. B. Patel, B. J. Rutledge, M. E. Case, and N. H. Giles. 1989. DNA sequence, organization and regulation of the *qa* gene cluster of *Neurospora crassa*. Journal of Molecular Biology 207: 15-34.

35. Geiger, J. P., M. Nicole, and D. Nandris. 1986. Root rot disease of *Hevea brasiliensis*. I. Physiological and biochemical aspects of aggression. European Journal of Pathology 16: 22-37.

36. Giles, N. H., C. W. H. Partridge, S. E. Ahmed, and M. E. Case. 1967. The occurrence of the dehydroquinases in *Neurospora crassa*, one constitutive and one inducible. Proceedings of the National Academy of Sciences (USA) 58: 1930-1937.

37. Giles, N. H., M. E. Case, J. Baum, R. Geever, L. Huiet, V. B. Patel, and B. Tyler. 1985. Gene organization and regulation, in the *qa* (quinic acid) gene cluster of *Neurospora crassa*. Microbiological Reviews 49: 338-358.

38. Giles, N. H., R. F. Geever, D. K. Asch, J. Avalos, and M. E Case. 1991. Organizaton and regulation of the *qa* (quinic acid) genes in *Neurospora crassa* and other fungi. Journal of Heredity 82: 1-7.

39. Grant, S., C. F. Roberts, H. K. Lamb, M. Stout, and A. R. Hawkins. 1988. Genetic regulation of the quinic acid utilization (*qut*) gene cluster in *Aspergillus nidulans*. Journal of General Microbiology 134: 347-358.

40. Grente, J. 1965. Les formes hypovirulentes d' Endothia parasitica et les espoirs ds lutte contre le chancre du chataignier. Comptes Rendus Hebdomadaires des Seances. Academic d'Agriculture de France 51:1033-1037.

41. Hammar, S., D. W. Fulbright, and G. C. Adams. 1989. Association of double-stranded RNA with low virulence in an isolate of *Leucostoma persoonii*. Phytopathology 79: 568-572.

42. Hampton, R., E. Ball, and S. de Boer. 1990. Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens: A Laboratory Manual. APS Press, St. Paul, MN.

43. Hawkins, A. R., N. H. Giles, and J. R. Kinghom. 1982. Genetical and biochemical aspects of quinate breakdown in the filamentaous fungus *Aspergillus nidulans*. Biochemical Genetics 20: 271-286.

44. Hawkins, A. R., S. J. Da Silva Francisco, and C. F. Robert. 1984. Evidence for two control genes regulating expression of the quinic acid utilization (*QUT*) gene cluster in *Aspergillus nidulans*. Joumal of General Microbiology 130: 567-574.

45. Hawkins, A. R. 1987. The complex *arom* locus of *Aspergillus nidulans*: evidence for multiple gene fusions and convergent evolution. Current Genetics 11: 491-498.

46. Hawkins, A. R., H. K. Lamb, M. Smith, J. W. Keyte, and C. F. Roberts. 1988. Molecular orgnization of the quinic acid utilization (*qut*) gene cluster in *Aspergillus nidulans*. Molecular and General Genetics 214: 224-231.

47. Hawkins, A. R., H. K. Lamb, J. D. Moore, I. G. Charles, and C. F. Roberts. 1993a. The prechorismate (shikimate) and quinate pathways in filamentous fungi: theoretical and practical aspects. Journal of General Microbiology 139: 2891-2899.

48. Hawkins, A. R., H. K. Lamb, J. D. Moore, and C. F. Roberts. 1993b. Genesis of eukaryotic transcriptional activitor and repressor proteins by splitting a multidomain anabolic enzyme Gene 136: 49-54.

49. Hicks, J. K., J. H. Yu, N. P. Keller, and T. H. Adams. 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the *Fad A* G alpha protein-dependent signaling pathway. EMBO Journal 16: 4916-4925.

50. Hoitink, H. A. J., and M. J. Boehm. 1999. Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. Annual Review of Phytopathology 37: 427-446.

51. Huiet, L. 1984. Molecular analysis of the *Neurospora* qa-1 regulatory region indicates that two interacting genes control *qa* gene expression. Proceedings of the National Academy of Sciences (USA) 81: 1174-1178.

52. Ivey, F. D., A. Yang, and K. A. Borkovich. 1999. Positive regulation of adenyl cyclase activity by a G alpha (I) homolog in *Neurospora crassa*. Fungal Genetics and Biology 26: 48-55.

53. Jian, J., D. K. Lakshman, and S. M. Tavantzis. 1997. Association of distinct double-stranded RNAs with enhanced or diminished virulence in *Rhizoctonia solani* infecting potato. Molecular Plant-Microbe Interactions 10: 1002-1009.

54. Koonin, E. V., G. H. Choi, D. L. Nuss, R. Shapira, and J. C. Carrington. 1991. Evidence for common ancestry of a chestnut blight hypovirulence-associated double-stranded RNA and a group of positive-strand RNA plant viruses. Proceedings of the National Academy of Sciences (USA) 88: 10647-10651.

55. Koshiba., T. 1978. Purification of two forms of the associated 3-dehydroquinate hydro-lyase and shikimate: NADP+ oxidoreductase in *Phaseolus mungo* seedlings. Biochemica et Biophysica Acta 522: 10-18.

56. Lakshman, D. K., and S. M. Tavantzis. 1994. Spontaneous appearance of genetically distinct double-stranded RNA elements in *Rhizoctonia solani.* Phytopathology 84: 633-639.

57. Lakshman, D. K., J. Jian, and S. M. Tavantzis. 1998. A novel mitochondrial double-stranded RNA found in a hypovirulent strain of *Rhizoctonia solani* occurs in DNA form, and is phylogenetically related to the pentafunctional AROM protein of the shikimate pathway. Proceedings of the National Academy of Sciences (USA) 95: 6425-6429.

58. Lamb, H. K., J. P. T. W. van der Hombergh, G. H. Newton, J. D. Moore, C. F. Roberts, and A. R. Hawkins. 1992. Differential flux through the quinate and shikimate pathways: implications for the channeling hypothesis. Biochemical Journal 284: 181-187.

59. Lamb, H. K., G. H. Newton, L. J. Levett, E. Caims, C. F. Roberts, and A. R. Hawkins. 1996. The QUTA activator and QUTR repressor proteins of *Aspergillus nidulans* interact to regulate transcription of the quinate utilization pathway genes. Microbiology 142: 1477-1490.

60. Lemtham, G. F., and M. A. Stahmann. 1981. Studies on laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. Journal of General Microbiology 125: 147-157.

61. Levesley, L., G. H. Newton, H. K. Lamb, E. van Schothorst, R. W. M. Dalgleish, A. C. R. Samson, C. F. Roberts and A. R. Hawkins. 1996. Domain structure and function within the QUTA protein of *Aspergillus nidulans*: implications for the control of transcription. Microbiology 142: 87-98.

62. Lin, J. K., and J. Y. Chang. 1975. Chromophoric labeling of amino acids with 4-dimethylaminoazobenzene-4'-sulfonyl chloride. Analysis in Chemistry 47: 1634-1637.

63. Logemann, J., J. Schell, and L. Willmitzer. 1987. Improved method for the isolation of RNA from plant tissues. Analytical Biochemistry 163: 16-20.

64. Lui, S., and R. A. Dean. 1997. G protein alpha subunits control growth, development and pathogenicity of *Magnaporthe grisea*, Molecular Plant-Microbe Interactions 9: 1075-1081.

65. Milborrow, B. V., J. G. Purse, and F. Wightman. 1975. On the auxin activity of phenylacetic acid. Annals of Botany 39: 1143-1146.

66. Millar. G., and J. R. Coggins. 1986. The complete amino acid sequence of 3-dehydroquinate synthase of *Escherichia coli* K12. FEBS Letters 200: 11-17.

67. Mousdale, D. M., M. S. Campbell, and J. R. Coggins. 1987. Purification and characterization of bifunctional dehydroquinase-shikimate: NADP oxidoreductase from pea seedlings. Phytochemistry 26: 2665-2670.

68. Padgette, S. R., R. E. D. Biest, C. S. Gasser, D. A. Eichholtz, R. B. Frazier, C. M. Hironaka, E. B. Levine, D. M. Shah, R. T. Fraley, and G. M. Kishore. 1991. Site-directed mutagenesis of a conserved region of the 5-enolpruvylshikimate-3-phosphate synthase active site. Journal of Biological Chemistry 266: 22364-22369.

69. Pall, M. L. 1981. Adenosine 3', 5'-phosphatase in fungi. Microbiological Reviews 45: 462-480.

70. Pittard, A. J. 1987. Biosynthesis of the aromatic amino acids, pp. 368-394. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, Vol. 1. Edited by Niedhardt, C. F., J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. B. Umbarger. American Society for Microbiology, Washington, DC.

71. Polashock J. J., and B. I. Hillman. 1994. A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. Proceedings of the National Academy of Sciences (USA) 91: 8680-8684.

72. Powell, W. A., and N. K. Van Alfen. 1987a. Two non-homologus viruses of *Cryphonectria (Endothia) parasitica* reduce accumulation of specific virulence-associated polypeptides. Journal of Bacteriology 169: 5324-5326.

73. Powell, W. A., and N. K. Van Alfen. 1987b. Differential accumulation of poly $(A)^+$ RNA between virulent and double-stranded RNA-induced hypovirulent strains of *Cryphonectria (Endothia) parasitica*. Molecular and Cellular Biology 7: 3688-3693.

74. Rawlinson, C. J., D. Hornby, V. Pearson, and J. M. Carpenter. 1973. Virus-like particles in the Take-all fungus *Gaeumannomyces graminis*. Annual Application of Biology 74: 209-243.

75. Rigling, D., and N. K. Van Alfen. 1991. Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. Journal of Bacteriology 173: 8000-8003.

76. Rogers, H. J., K. W. Buck, and C. M. Brasier. 1987. A mitochondrial target for double-stranded RNA in diseased isolates of the fungus that causes Dutch elm disease. Nature 329:558-560.

77. Rossman, M. G., D. Moras, and K. W. Olsen. 1974. Chemical and biological evolution of a nucleotide binding protein. Nature 250: 194-199.

78. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual, Second Edition. Cold Spring Harbor Laboratory Press, New York.

79. Simpson, R. J., M. R. Neuberger, and T. Y. Liu. 1976. Complete amino acid analysis of proteins from a single hydrolysate. The Journal of Biological Chemistry 251: 1936-1940.

80. Smith, D. D. S., and J. R. Coggins. 1983. Isolation of a biofuntional domain from the pentafunctional AROM enzyme complex of *Neurospora crassa*. Biochemical Journal 213: 405-415.

81. Sneh, B., M. Zeidan, M. Ichielevich-Auster, I. Barash, and Y. Koltin. 1986. Increased growth responses induced by a nonpathogenic *Rhizoctonia solani*. Canadian Journal of Botany 64: 2372-2378.

82. Stocchi, V., G. Piccoli, M. Magnani, F. Palma, B. Biagiarelli, and L. Cucchiarini. 1989. Reverse-phase high-performance liquid chromatography separation of dimethylaminoazobene sulfonyl- and dimethylaminoazobenzene thiohydantoin-amino acid derivatives for amino acid analysis and microsequencing studies at the picomole level. Analytical Biochemistry 178: 107-117.
83. Tavantzis, S. M., B. L. Perkins, R. J. Bushwa, and B. P. Bandy. 1989. Correlation between in vitro synthesis of phenylacetic acid and virulence in *Rhizoctonia solani*. Phytopathology 79: 1199.
84. Tavantzis, S. M., and D. K. Lakshman. 1995. Virus-like double-stranded RNA elements and hypovirulence in phytopathogenic fungi, vol. III, Chapter 17, pp. 249-267. In Pathogenesis and Host-Parasite Specificity in Plant Disease: Histopathological, Biochemical, Genetic and Molecular Basis. Edited by Kohmoto, K., R. P. Singh, and U. S. Singh. Elsevier (Pergamon) Press, Oxford, UK.
85. Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay. 1982. Distantly related sequence in the α and β subunits of ATP synthase, myosin. Kinases, and other ATP- requiring enzymes and a common nucleotide binding fold. EMBO Journal 1: 945-951.
86. Zanzinger, D. H., B. P. Bandy, and S. M. Tavantzis. 1984. High frequency of finding double-stranded RNA in naturally occurring isolates of *Rhizoctonia solani*. Journal of General Virology 65: 1601-1605.

87. Zhang, L., A. C. L. Churchill, P. Kazmierczak, D. Kim, and N. K. Van Alfen. 1993. Hypovirulence-associated traits induced by a mycovirus of *Cryphonectria parasitica* are mimicked by targeted inactivation of a host gene. Molecular and Cellular Biology 13: 7782-7792.

88. Zhang, L., D. Villalon, Y. Sun, P. Kazmiercazak, and N. K. Van Alfen. 1994. Virus-associated down-regulation of the gene encoding cryparin, an abundant cell-surface protein from the chestnut blight fungus, *Crphonectria parasitica*. Gene 139: 59-64.

89. Zhang, L., Baasiri, R. A., and N. K. Van Alfen. 1998. Virul repression of fungal pheromone precursor gene expression. Molecular and Cellular Biology 18: 953-959.

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