An ATP-binding Cassette Multidrug-Resistance Transporter Is Necessary for Tolerance of *Gibberella pulicaris* to Phytoalexins and Virulence on Potato Tubers

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The necrotrophic pathogen *Gibberella pulicaris* infects potato tubers through wounds that contain fungitoxic secondary metabolites such as the phytoalexins rishitin and lubimin. In order to colonize tuber tissue, the fungus must possess a mechanism to tolerate potato defense compounds. In this paper, we show that a gene, *Gpabc1*, that codes an ATP-binding cassette (ABC) transporter is required for tolerance to these phytoalexins and for virulence on potato. The *Gpabc1* gene, isolated in the course of a differential cDNA screen, shares high sequence homology with the *ABC1* gene of *Magnaporthe grisea*. *G. pulicaris* mutants deficient in *Gpabc1* were still able to metabolize rishitin but lost their tolerance to this phytoalexin as well as their virulence on potato. These results strongly suggest that the *Gpabc1*-encoded ABC transporter is necessary for tolerance of *G. pulicaris* to rishitin and that this tolerance is required for virulence on potato.

Since the term “phytoalexin” was introduced by Müller and Börger about 60 years ago (Müller and Börger 1940), the production of antimicrobial secondary metabolites by plants in response to wounding and microbial infection has been considered a major defense mechanism of plants. Phytoalexins constitute a structurally diverse group of chemicals, including phenylpropanoids, terpenoid, and fatty acid derivatives, and they have been found to accumulate to high levels in damaged tissue of many plants (Ebel 1986). In the 1960s, work by Cruickshank (1962; Cruickshank and Perrin 1965) indicated that tolerance to phytoalexins might be an important requirement for phytopathogenic fungi. Since then, much effort has been invested in research involving the detoxification of phytoalexins by degradation (Morrissey and Osbourn 1999; VanEtten et al. 1995). Genetic studies with *Nectria haematococca* (Miao and VanEtten 1992; VanEtten et al. 1989) and *Gibberella pulicaris* (Desjardins and Gardner 1989, 1991; Desjardins et al. 1992) supported the correlation between the ability of these fungi to metabolize their host phytoalexin and virulence on their host plants.

The application of molecular genetic techniques to phytopathogenic fungi led to the isolation of two genes from *N. haematococca* that code for phytoalexin-detoxifying enzymes. The *PDA1* gene coding for pisatin demethylase and the *MAK1* gene coding for maackiain 1a-hydroxylase are involved in detoxifi-

cation of the phytoalexins pisatin from pea and maackiain from chickpea, respectively (Covert et al. 1996; Weltring et al. 1988). Disruption mutants of both genes showed reduced virulence on pea and chickpea, indicating that metabolism of phytoalexins to less toxic products contributes to, but is not essential for, virulence of *N. haematococca* on these host plants (Enkerli et al. 1998, Wasmann and VanEtten 1996). These data raised the possibility that other mechanisms of phytoalexin tolerance might be important for virulence of phytopathogenic fungi.

The necrotrophic fungus *G. pulicaris* (anamorph *Fusarium sambucinum*) causes potato dry rot disease by infecting tubers through wounds. Since wounding of potato tubers has been shown to induce production of phytoalexins, especially rishitin and lubimin, the fungus is likely to be exposed to high levels of these phytoalexins during infection (Kuc 1982). Genetic studies indicated a correlation between tolerance to rishitin and virulence of the fungus (Desjardins and Gardner 1989, 1991; Desjardins et al. 1992). This correlation made *G. pulicaris* an ideal candidate to identify rishitin-induced genes involved in phytoalexin tolerance via a differential cDNA screen.

ATP-binding cassette (ABC) multidrug-resistance transporters, which have been found in all prokaryotic and eukaryotic organisms examined so far, are a likely candidate for an alternative tolerance mechanism. Most ABC transporters in higher eukaryotes consist of two transmembrane domains (TMD), each containing six membrane-spanning regions and two nucleotide binding folds (NBF) in a two times two domain configuration. The transporter proteins can have either a [TMD-NBF]₂ or an [NBF-TMD]₂ configuration, depending on the location of the NBF. In addition, ABC transporters are characterized by the presence of a Walker A and Walker B motif separated by 120 to 170 amino acids, including an ABC signature (Walker et al. 1982; Hyde et al. 1990).

By definition, multidrug-resistance ABC transporters have a broad substrate range. The best characterized examples are the yeast Pdr5p and the human MDR1 transporters (Balzi et al. 1994; Endicott and Ling 1989). MDR1 has 93 known substrates of various chemical classes (Gottesman and Pastan 1993). The physiological role of these transporters is the active secretion of toxic substances in order to protect the cell from damage by these compounds. This function may be especially important for plant pathogenic fungi, as proposed by De Waard (1997) and Del Sorbo and associates (2000), because they may provide protection against fungitoxic defense compounds produced by plants in response to infection. This hypothesis is supported by the observation that a wide variety of natural compounds can act as substrates or inducers of ABC transport-

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ers (del Sorbo et al. 1997; Gottesman and Pastan 1993; Seelig 1998) and by the reduced virulence on grapevine and the increase of sensitivity to the grapevine phytoalexin resveratrol of mutants of *Botrytis cinerea* in which the ABC transporter *BcatrB* had been disrupted (Schoonbeek et al. 2001). The latter result in particular suggests that ABC transporters can contribute to virulence of phytopathogenic fungi by reducing the toxic effect of phytoalexins.

Additional indirect evidence for the role of membrane transporters in phytoalexin tolerance was provided by the presence of an energy-dependent, nondegradative tolerance mechanism against the pea phytoalexin pisatin in *N. haematococca* involving reduced retention of pisatin in the mycelium (Denny and VanEtten 1983a, 1983b; Denny et al. 1987).

In this paper, we describe the isolation of a gene encoding a multidrug-resistance ABC transporter by differential cDNA screening. Replacement of the gene in *G. pulicaris* dramatically reduced tolerance to potato phytoalexins as well as virulence on potato tubers. These data demonstrate that multidrug-resistance transporters are necessary for both tolerance to phytoalexins and pathogenicity of *G. pulicaris* on potato tubers.

RESULTS

Isolation and characterization of the *Gpabc1* gene.

A cDNA library in λ -ZAP prepared from RNA isolated from mycelium exposed to rishitin on agar plates for 2 to 4 h was screened with radiolabeled cDNA from rishitin-induced (2 h) and noninduced mycelia. Two partial cDNA clones with homology to multidrug-resistance ABC transporters of other fungi were obtained and used as probes to isolate full-length cDNAs and genomic sequences from a cosmid library. Sequencing of these DNA fragments and comparison of the genomic sequence with that of the cDNAs confirmed that both isolated partial cDNA clones belonged to the same 4,576-bp open reading

frame (ORF) that encoded a predicted protein of 1,491 amino acids. The ORF was interrupted by two introns of 47 and 56 bp, and an additional 50-bp intron was located in the 5'-untranslated portion of the transcript. In a BLAST search, the gene, designated *Gpabc1* (accession number AJ306607), showed high homology to multidrug-resistance transporters of yeast and other fungi, especially to *ABC1* of *Magnaporthe grisea* (Urban et al. 1999). *ABC1* and *Gpabc1* share 65% identity at the amino acid level and possess identical Walker motifs and ABC signature sequences (N-terminal: WalkerA GPPGS-GCST, WalkerB QCWDNSTRGLD, ABC signature VSGG-ERKRVITIA. C-terminal: WalkerA GVSGAGTTL, WalkerB LFFVDEPTSGLD, ABC signature NVEQRKRLTIGV).

Northern analysis of *Gpabc1* induction by rishitin revealed that the expression of the gene is induced within 5 to 10 min after addition of 150 μ g of rishitin per ml to mycelium in liquid culture (Fig. 1) and the transcript is detectable for at least 10 h. A rishitin content of as little as 25 μ g per ml was still sufficient to turn on the transcription of the gene.

Replacement of *Gpabc1*.

In order to test the importance of the ABC transporter for tolerance to potato phytoalexins, we conducted gene replacement of *Gpabc1*. The replacement vector pABCko was constructed by cloning a 1,039-bp *SacI* fragment of the 5'-region of *Gpabc1* and a 1,035-bp *XbaI* fragment of the 3'-region into the vector pGPC1 (Desjardins et al. 1992). The *G. pulicaris* wild-type isolate R6380 was transformed with a linear *Sall/SpeI* fragment containing the hygromycin cassette flanked by the 5'- and 3'-transporter gene fragments (Fig. 2).

Transformants in which the internal part of the *Gpabc1* gene was replaced with the hygromycin cassette were identified by Southern analysis (Fig. 3). We obtained four different types of transformants. Of 55 transformants resulting from two independent transformation experiments, 24 had one or more ectopic integrations, as indicated by the presence of the 8-kb wild-type fragments and one or more other hybridizing fragments (type 1, T47).

In 31 of the transformants, homologous integrations occurred at the *Gpabc1* locus. This was indicated by the replacement of the 8-kb *HindIII* wild-type fragment by one or more fragments of different sizes. Two of these transformants contained the expected 7.1-kb fragment characteristic for a simple replacement of the internal part of the *Gpabc1* gene by the hygromycin cassette (type 2, T76) and an additional integration

Fig. 1. Time course of the induction of *Gpabc1*. The expression of *Gpabc1* after adding rishitin (150 μ g/ml) to liquid cultures was tested by northern analysis using total RNA. Lane 2, 0 min; lane 3, 5 min; lane 4, 10 min; lane 5, 15 min; lane 6, 20 min; lane 7, 30 min; lane 8, 40 min; lane 9, 50 min; and lane 10, 60 min after adding rishitin. Lane 1, untreated control, 30 min incubation. The equal loading of each lane with 15 μ g of RNA per lane was verified by hybridization with a part of the rDNA cluster of *Gibberella pulicaris*.

Fig. 2. Construction of the transformation vector pABCko. A 1,039-bp *SacI* fragment and a 1,035-bp *XbaI* fragment from the 5' and 3' regions, respectively, of the *Gpabc1* coding region were cloned into plasmid pGPC1 on either side of the hygromycin cassette. The linear *Sall/SpeI* fragment containing the hygromycin cassette flanked by these *Gpabc1* fragments was used for transformation.

Fig. 3. Southern analysis of *Gibberella pulicaris* transformed with the *Gpabc1*-hygromycin cassette transformation fragment. *HindIII*-digested DNA of transformants was probed with the entire linear fragment used for transformation (Fig. 2). Lane 1, R6380 (wild type); lane 2, T30; lane 3, T74; lane 4, T77; lane 5, T76; and lane 6, T47. The wild type DNA contains the expected 8-kb fragment. The hybridization pattern for T30, T74, and T77 indicates homologous integration of the transformation fragment with internal rearrangements. The pattern for T76 indicates both homologous and ectopic integration of the transformation fragment, while the pattern for T47 indicates ectopic integration only.

event at an ectopic site. In all other transformants the single hybridizing *Hind*III fragment varied in size but was always larger than 7.1 kb. The size of these *Hind*III fragments indicated either integration of several fragments at the *Gpabc1* locus, as in T30 (type 3), or rearrangement of the integrated DNA leading to a partial duplication within the integrated fragment (type 4, T74 and T77). Hybridizations of *Pst*I-digested DNA of eight of these latter transformants with the complete transformation fragment detected three hybridizing bands in each lane (Fig. 4). Rehybridization of the Southern blot both with border fragments alone and with the hygromycin cassette revealed that the 6.6-kb fragment represents the 5' border and that the 3.2-kb fragment represents the 3' border (data not shown). These two fragments are present in the DNA of six transformants, whereas two transformants showed an increase in size of the 5' *Pst*I fragment. The DNA of all eight transformants contained an additional fragment that varied in size and specifically hybridized to the hygromycin probe. This variation in size indicated that the rearrangement occurred within the internal part of the transformation fragment involving the hygromycin cassette, possibly leading to a partial duplication of this gene. This result and the presence of the two *Pst*I fragments of the correct size in six of the transformants prove that the sequences around the integration site are obviously unaffected, at least in these six transformants. Therefore, we chose two of these transformants (T74 and T77, type 4), one with several integrations at the homologous site (T30), and T76 with a perfect replacement and one additional ectopic integration for further experiments.

To determine whether gene replacement successfully inactivated *Gpabc1*, northern analyses of the four representative replacement transformants (T76, type 2; T30, type 3; and T74 and T77, type 4) and one with an ectopic integration (T47, type

Fig. 4. Southern analysis of transformants in which the transformation fragment integrated only via homologous recombination at the *Gpabc1* locus (Fig. 3). To analyze the type of rearrangement within the integrated DNA, *Pst*I-digested DNA of eight transformants was probed with the whole linear fragment used for transformation (Fig. 2). Lane 1, R6380 (wild type); lane 2, T74; lane 3, T75; lane 4, T77; lane 5, T91; lane 6, T101; lane 7, T108; lane 8, T115; and lane 9, T121.

1) were performed. After exposure to rishitin for 30 min, the *Gpabc1* gene transcript was not detectable in the replacement mutants. The transformant with the ectopic integration showed gene expression identical to that of the wild type (Fig. 5). This analysis confirmed that the gene is not expressed at detectable levels in the transformants with a disrupted *Gpabc1* gene.

Phenotypic characterization of *Gpabc1* replacement mutants.

The four replacement mutants were analyzed for tolerance to rishitin by placing a mycelial plug on agar medium supplemented with 200 μ g of the phytoalexin per ml. As shown in Figure 6, growth of the mutants was dramatically reduced by 90% on rishitin-amended medium compared to the wild type after 3 days of incubation. On medium without rishitin, mutants and wild type grew equally well, covering the whole plate within the incubation time. Growth of the ectopic mutant T47 was the same as the wild type on both the amended and non-amended medium.

In two separate experiments, all transformants were tested for their ability to metabolize rishitin by inoculating mycelial mats of the transformants and R6380 on rishitin-amended Czapek-Dox agar medium for 24 h. All transformants and the wild-type strain completely degraded the substrate within this time period, as indicated by the lack of rishitin in all agar extracts except for that of the noninoculated control plates (data not shown). This strongly suggests that the multidrug-resistance transporter encoded by *Gpabc1* is necessary for the tolerance of *G. pulicaris* to rishitin.

In order to test the impact of the inactivated ABC transporter gene on virulence of *G. pulicaris* on potato, all four replacement mutants, the ectopic transformant, and the wild-type progenitor strain R6380 were tested for their ability to colonize potato tuber tissue by placing a mycelial plug at the edge of a potato disc and incubating it for 5 days at 20°C. As shown in Figure 7, the untransformed strain R6380 and the ectopic transformant colonized more than half of the potato slice. In contrast, growth of the *Gpabc1* replacement mutants was restricted to the inoculation site. Extended incubation did not result in further colonization of the potato slice by the mutants in contrast to strain R6380, which eventually macerated the entire tuber disc. Obviously, growth of the replacement mutants is completely stopped, which strongly suggests that the reduction in virulence is caused by the inability of the mutants to escape the toxic effect of rishitin by excreting the phytoalexin.

Complementation of a *Gpabc1* replacement mutant with the wild type gene.

To obtain final proof that the observed phenotype was the result of the functional inactivation of the ABC transporter, we

Fig. 5. Northern analysis of the *Gpabc1* replacement mutants. To test the expression of *Gpabc1* in mutant strains, total RNA was isolated from mycelia incubated for 30 min with rishitin (150 μ g/ml) and probed with the 1,053-bp *Xba*I fragment of *Gpabc1*. Lane 1, R6380 (wild type); lane 2, T30; lane 3, T74; lane 4, T77; lane 5, T76; and lane 6, T47. The equal loading of each lane with 15 μ g of RNA per lane was verified by hybridization with a part of the rDNA cluster of *Gibberella pulicaris*.

complemented one of the replacement transformants with a functional copy of *Gpabc1*. The complementation vector was constructed by cloning a genomic 8-kb *HindIII* fragment carrying the *Gpabc1* gene into pAN8-1 (Mattern et al. 1988), which contains a phleomycin resistance cassette. The mutant T74 was used as the recipient strain for the entire circular plasmid. A total of 43 transformants were tested for reconstitution of the wild-type phenotype by inoculation on potato tuber slices. After 5 days of incubation, several transformants had a virulence phenotype comparable to wild-type strain R6380 (Fig. 7). Three of these pathogenic transformants were tested for tolerance to rishitin. All three of them regained tolerance to the phytoalexin (Fig. 6). Northern analysis confirmed that the wild-type phenotypes were due to the expression of a functional *Gpabc1* gene. As shown in Figure 8, all three of the transformants expressed the gene in response to incubation with rishitin. These experiments clearly demonstrate that expression of a functional *Gpabc1* gene is necessary for both tolerance to rishitin and virulence on potato tubers.

DISCUSSION

Over the past few decades, much circumstantial evidence concerning the importance of phytoalexins for plant resistance against fungi has been produced. Genetic studies on the correlation between metabolism of phytoalexins and virulence have indicated that the tolerance of these compounds is essential for virulence of phytopathogenic fungi (Morrissey and Osbourn 1999; VanEtten et al. 1995). The best examples of this hypothesis involve the metabolism of pisatin and maackiain by *N. haematococca* and rishitin degradation by *G. pulicaris* (Desjardins et al. 1992; Miao and VanEtten 1992; VanEtten et al. 1989). In these fungi, high virulence is always correlated with the ability to metabolize phytoalexins. However, the isolation of *G. pulicaris* strains that were avirulent but able to metabolize rishitin indicated that there must be other genes, apart from metabolism genes, with a major impact on virulence (Desjardins and Gardner 1991). De Waard (1997) and Del Sorbo and associates (2000) proposed that such virulence

Fig. 6. Comparison of the tolerance to rishitin of wild type strain R6380 with *Gpabc1* replacement and complemented mutants. Mycelial plugs were placed at the center of Czapek-Dox agar plates without (control) and with rishitin (200 µg/ml) and incubated at 28°C in the dark. After 3 days, the increase in size of the colony was measured from the edge of the inoculating plug to the edge of the colony. Each value represents the mean $\pm$ standard error of at least three replicates. Column 1, R6380 (wild type); column 2, T30; column 3, T76; column 4, T77; column 5, T74 (replacement mutants); column 6, T47 (ectopic); column 7, K4; column 8, K32; and column 9, K38 (complementation mutants). White bars = control. Black bars = on rishitin.

Fig. 7. Pathogenicity assay. Potato slices were inoculated with the following strains and incubated for 5 days at 20°C. Left panel: A, R6380 (wild type); B, T47 (ectopic); C, T30 (replacement). Right panel: A, R6380 (wild type); B, T74 (replacement); C, K38 (complemented).

genes may encode ABC multidrug-resistance transporters. They were able to support this theory to some extent by disrupting the ABC transporter gene *BcatrB* in *B. cinerea*. This gene is induced by the grapevine phytoalexin resveratrol, and its inactivation increased sensitivity to the phytoalexin and slightly reduced virulence of the fungus on grapevine leaves (Schoonbeek et al. 2001). The data on *Gpabc1* presented in this paper further verify this hypothesis and prove that multidrug-resistance transporters not only contribute to, but can be essential for, the virulence of phytopathogenic fungi among the chemical defenses of their host plant. Therefore, these data are a very strong argument in favor of the phytoalexin theory, which proposes that phytoalexins are an important plant defense mechanism that has to be overcome by tolerance mechanisms of phytopathogenic fungi.

The *Gpabc1* gene is induced within minutes at the low threshold of 25 µg of rishitin per ml. This rapid and sensitive reaction of the fungus suggests that the function of the *Gpabc1* encoded transporter is the immediate export of phytoalexins from the infection hyphae. This enables the fungus to outgrow the area of wounded tissue in which these chemicals accumulate. In the replacement mutants, the lack of the transporter and the consequent toxic action of the phytoalexins causes arrest of mutant growth at the initial infection site. This reduced growth, in turn, may enable the host tissue to further restrict the growth of the fungus by activating additional defense reactions, such as lignification or accumulation of polyphenols.

The broad substrate range of ABC multidrug-resistance transporters renders it likely that the GPABC1 transporter also exports other potato phytoalexins, such as lubimin, solavetivone, or phytuberin. Preliminary experiments indicated that lubimin induces *Gpabc1* in the same manner as rishitin and that the *Gpabc1* replacement mutants became sensitive to this compound (A. Fleißner, unpublished data). Isolation of sufficient amounts of lubimin is under way to verify these results.

An ABC transporter is a perfect explanation for the nondegradative pisatin tolerance of *N. haematococca* described by Denny and VanEtten (1983a and 1983b; Denny et al. 1987), who found that tolerance of germinated spores of the fungus to the phytoalexin was mediated by an energy-dependent export mechanism and that the tolerance mechanism was inducible by other structurally related and unrelated substances. Both features are characteristic for ABC multidrug-resistance transporters, which need ATP as an energy source and usually have a very broad substrate range. In these studies, Denny and VanEtten already suggested that the nondegradative tolerance of phytoalexins may be more important than metabolism. This

early hypothesis is confirmed by our results, because the replacement transformants are inhibited in growth, although they are able to metabolize rishitin. This proves that export of the phytoalexin is a prerequisite for tolerance that cannot be substituted by metabolism. This conclusion is indirectly supported by experiments conducted by Enkerli and associates (1998) and Wasmann and VanEtten (1996) in which *N. haematococca* mutants deficient in *PDA1* and *MAK1* genes showed only a 30% reduction in growth compared with the wild type, indicating that, while the detoxification of phytoalexins contributes to tolerance, it is not the major factor.

Loss of virulence of a phytopathogenic fungus as a result of inactivation of an ABC transporter gene has already been described in *M. grisea* (Urban et al. 1999). In this fungus, replacement of the *ABC1* gene, which has the highest sequence homology to *Gpabc1* of *G. pulicaris*, caused a complete loss of virulence on rice plants. However, the *M. grisea ABC1* mutants did not show increased sensitivity to rice phytoalexins or several other chemicals that induced *ABC1* expression. The fact that inactivation of these two closely related ABC transporter genes leads to a marked loss of virulence indicates that ABC transporters have potential as targets for specific fungicides. Analysis of the role of ABC transporters in the virulence of other fungi should shed light on how useful ABC transporters are as fungicide targets.

MATERIALS AND METHODS

Strains, media, and growth conditions.

G. pulicaris strain R6380 was obtained from A. Desjardins (Desjardins and Gardner 1989). For long-term storage and to produce spores, the fungus was grown on V8 juice agar (Stevens 1974). Liquid cultures were grown by inoculating spores from a 7- to 10-day-old V8 juice agar plate culture for 3 days at 28°C on a rotary shaker in 100 ml of Mantle medium (Mantle and Nisbet 1976).

Metabolism assays.

For an assay of phytoalexin metabolism, a mycelial mat was transferred to Czapek-Dox agar supplemented with 150 µg of rishitin per ml and incubated at 28°C for several hours as previously described (Weltring and Altenburger 1998). The mycelium was removed after 24 h, and the agar medium was extracted with ethyl acetate. The organic phase was analyzed for residual substrate by gas-liquid chromatography as previously described (Gardner et al. 1988).

Tolerance and virulence assays.

Mycelial plugs from the growing margin of a radially grown culture were used as inoculum for both assays. Tolerance of rishitin was analyzed by placing mycelial plugs on Czapek-Dox agar supplemented with 200 µg of rishitin per ml and determining the degree of inhibition of radial growth according to Desjardins and Gardner (1989). To measure virulence, the mycelial plug was placed on a potato tuber slice of the cv. Hansa and the assay was incubated at 20°C for 5 days.

Fungal transformation.

Transformation of *G. pulicaris* was carried out as previously described (Weltring 1995) using a linear *SalI/SpeI* fragment from the replacement vector pABCKo. The fragment was isolated from preparative agarose gels by electroelution, using the Biotrap BT 1000 system (Schleicher und Schuell, Dassel, Germany) according to the supplier's instructions. In the complementation experiment, the entire circular complementation vector carrying the *Gpabc1* gene was used for transformation.

Fig. 8. Northern analysis of the complemented mutants. To test the expression of *Gpabc1* in mutant strains, total RNA was isolated from mycelium incubated for 30 min with rishitin (150 µg/ml) and probed with the replaced internal fragment of the *Gpabc1* gene. Lane 1, R6380 (wild type); lane 2, T74; lane 3, K4; lane 4, K32; and lane 5, K38. The amount of RNA per lane was tested by hybridization with a part of the rDNA cluster of *Gibberella pulicaris*.

Gene libraries.

The cosmid library was constructed by ligating 40- to 45-kb genomic *Sau*3A fragments size-selected through a sucrose gradient into cosmid pGPC1 (Desjardins et al. 1992); standard procedures were applied (Sambrook et al. 1989). The library consisted of approximately 3.5×10^4 CFU. The cDNA library was prepared as described in Loser and Weltring (1998).

DNA isolation and manipulation.

Plasmid DNA was prepared using the JETstar Plasmid Midi Kit (Genomed, Bad Oeynhausen, Germany) according to the supplier's instructions. Sequences were determined using the Thermo Sequenase Cycle Sequencing Kit (Amersham Buchler, Braunschweig, Germany). Fungal DNA was isolated from mycelium harvested from liquid culture using the method described by Doyle and Doyle (1990). For Southern analysis, the DNA was digested with restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany), blotted on nylon membrane Hybond N⁺ (Amersham) in 0.4 M NaOH, and hybridized with ³²P-labeled DNA probes under stringent conditions according to standard protocols (Sambrook et al. 1989).

RNA isolation and manipulation.

For northern analysis, RNA was prepared using the RNeasy Total RNA Isolation System (Promega, Mannheim, Germany). After photometric quantification, 15 µg of the isolated RNA per lane were separated on a denaturing gel, transferred to Hybond N⁺ nylon membrane by downward blotting in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 20 mM NaOH for 1.5 h and hybridized at 65°C as described by Church and Gilbert (1984).

To prepare radiolabeled induced (+) and noninduced (−) cDNA, mRNA was isolated directly from lyophilized mycelium that had been incubated on Czapek-Dox agar with (+) or without (−) rishitin with Dyna Beads (Dyna, Oslo, Norway) according to the supplier's instructions. Reverse transcription into cDNA was accomplished by standard protocols (Sambrook et al. 1989) in the presence of radiolabeled dCTP (Amersham).

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