Ascochyta blights of grain legumes

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Reprinted from *European Journal of Plant Pathology*, Volume 119 Issue 1, 2007

Springer

A C.I.P catalogue record for this book is available from the library of Congress

ISBN 978-1-4020-6064-9 (HB)
ISBN 978-1-4020-6065-6 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA, Dordrecht, The Netherlands

Printed on acid-free paper

Cover photos:
From top to bottom: Ascochyta blight (*Didymella rabiei*) on chickpea leaflets; Faba bean seeds infected by *Ascochyta fabae*; *Mycosphaerella pinodes* colony on Petri dish; Ascochyta blight (*Mycosphaerella pinodes*) on pea; Pycnidia of *Ascochyta fabae* in leaf tissue

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Printed in the Netherlands

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Eur J Plant Pathol (2007) 119:1–2
DOI 10.1007/s10658-006-9086-4

Foreword

Robert (Bob) A. Henson

Received: 9 November 2006 / Accepted: 20 November 2006 / Published online: 2 February 2007
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We dedicate this special issue to our friend and colleague Dr. Bob Henson who unexpectedly passed away during the first international workshop on Ascochyta blight entitled 'Ascochyta 2006.' Dr. Henson of Carrington, North Dakota, USA was noted for his research on management for Ascochyta blight and was also instrumental in establishment of mist nurseries for evaluation of several crops for resistance to Sclerotinia white mold in collaboration with plant breeders. Dr. Henson's degrees include a B.A. in Chemistry from Macalester College in St. Paul, Minnesota, and a Master of Agriculture in Plant and Soil Technology and Ph.D. in Agronomy from the University of Minnesota, St. Paul. He was an active member of the American Society of Agronomy, the Crop Science Society of America and the Soil Science Society of America as well as numerous industry organizations. He was a member of the North American Pulse Improvement Association and was currently serving on the Board of Directors. Prior to joining the Carrington Research and Extension Center in 1998, he worked as a bean Agronomist and Physiologist in Brazil and Ecuador and as a consultant to the World Bank in Mexico and Bolivia. Bob was well recognized as a hard worker and productive researcher with a friendly outgoing manner and smile for everyone. He is survived by his wife Soraia, two sons, Robert and Peter, and a daughter, Gabriella. Bob was a good friend of the Ascochyta and Sclerotinia communities and will be sorely missed.

Robert (Bob) A. Henson

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Ascochyta blights of grain legumes

Ascochyta blights of the cool season food legumes (peas, lentils, chickpeas and faba beans) are important production constraints in all regions where these crops are grown and in severe cases result in complete crop loss. The global importance of Ascochyta as a production constraint to these legumes is well documented. To review past research and to develop plans for overcoming this production constraint, the first international workshop on Ascochyta blight entitled 'Ascochyta 2006' was organized and conducted at Le Tronchet, France during the week of 2–6 July 2006. The workshop brought together experts on all aspects of the problem to review current knowledge and to formulate plans for future research and collaboration. Plenary sessions were devoted to pathogen biology, plant resistance, epidemiology and integrated disease management. Presentations of posters of current research complimented these sessions and provided additional insights into the disease. Four additional talks followed by informal round tables were given in order to enlarge the thematic sessions: interest in *Medicago truncatula* for disease resistance in grain legumes(Alain Baranger, INRA, France); the *Ascochyta* genus (Ivan Sache, INRA, France); grain legume research and extension (Robert Morrall, University of Saskatchewan, Canada); the place of legumes in crop rotations (Raphaël Charles, University of Changins, Switzerland). The goal of the workshop was to identify gaps in knowledge, identify new research approaches and to establish collaborative relationships among Ascochyta blight researchers. Sixty-five participants from 13 countries were in attendance. Most of the participants reported on their current research on Ascochyta on one or more of the cool season food legumes.

The workshop was very successful in achieving its goals set out by its organizers, and the participants enjoyed the venue and hospitality provided by the local organizing committee. This special issue will serve as a useful reference for years to come. Recommendations of the group were formulated to stimulate future collaborative research on the Ascochyta blight problem as it affects the cool season food legumes. A committee was established for developing a follow-up workshop to be held at Washington State University in Pullman, Washington, USA in June of 2009 entitled 'Ascochyta 2009.'

This special issue of EJPP contains invited presentations and contributed papers by workshop participants. The workshop was organized by INRA (French National Institute of Agronomical Research), Agrocampus Rennes (College of Agronomy), AEP (European Association of Grain Legumes), USDA (United States Department of Agriculture) and SFP (French Plant Pathology Society).

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Eur J Plant Pathol (2007) 119:3–12
DOI 10.1007/s10658-007-9122-z

FULL RESEARCH PAPER

Towards identifying pathogenic determinants of the chickpea pathogen *Ascochyta rabiei*

David White · Weidong Chen

Received: 30 October 2006 / Accepted: 8 March 2007 / Published online: 3 April 2007
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Abstract Ascochyta blight is a serious disease of cool-season grain legumes (chickpea, faba bean, lentil and pea) caused by fungal species of the anamorphic genus Ascochyta and related genera. Despite extensive studies on the biology, ecology, epidemiology and management of the disease, little is known about the pathogenic determinants of these pathogens. This research aims at using *Ascochyta rabiei* as a model for the genus in investigating genetic factors of pathogenicity, with the ultimate goal of elucidating pathogenic mechanisms. Three advances were made: (1) insertional mutants with altered pathogenicity were identified through in vivo screening, and genomic regions adjacent to the insertion sites in selected mutants were determined; (2) a phage library of *A. rabiei* genomic DNA was constructed, and the library was estimated to provide complete coverage of the *A. rabiei* genome. This library was used successfully to recover clones with DNA adjacent to insertional mutation sites and to isolate specific genes; (3) DNA probes specific for an acyl-CoA ligase (*cps*1) and a polyketide synthase gene (*pks*1) were developed and library clones containing the corresponding genomic regions were identified from the phage library. These advances provide the foundation and necessary tools for experimentation of ectopic complementation assays and targeted mutagenesis to elucidate the genetic mechanisms of pathogenicity of *A. rabiei*.

Keywords *Agrobacterium*-mediated transformation · Gene disruption · Phage library

Introduction

Ascochyta blight is an important disease of cool-season grain legume crops including chickpea, faba bean, lentil, and pea. The pathogens are often host-specific, each species causing the disease with economical significance only on specific crops, e.g. *Ascochyta rabiei* on chickpea, *A. fabae* on faba bean, *A. lentis* on lentil, and *A. pisi Mycosphaerella pinodes*, and *Phoma medicaginis* var. *pinodella* on pea (Peever 2007). Extensive studies have been conducted on a number of the species on pathogen ecology (Taylor and Ford 2007), epidemiology and management (Tivoli and

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Banniza 2007; Davidson and Kimber 2007). However, research on pathogenic determinants of *Ascochyta* spp. in general has received little attention.

Ascochyta blight pathogens are all necrotrophic, killing plant cells in advance of mycelial development. Therefore, toxins and cell-wall degrading enzymes are often presumed to be important biochemical determinants of pathogenesis. Among the ascochyta pathogens, *A. rabiei* on chickpea is probably the most intensively studied pathosystem in terms of biochemical interactions between the host and the pathogen. *Ascochyta rabiei*, causal agent of chickpea ascochyta blight, produces toxin solanopyrones through the polyketide synthesis pathway (Alam et al. 1989; Hohl et al. 1991), and hydrolytic or cell-wall degrading enzymes (Tenhaken and Barz 1991; Tenhaken et al. 1997). Several lines of evidence show the roles of the phytotoxins in causing blight (Chen and Strange 1991; Kaur 1995). The hydrolytic enzymes are considered necessary for fungal nutrition and to facilitate spatial spread of fungi (Walton 1994). *Ascochyta rabiei* was first transformed with the protoplast/PEG protocol with a GUS reporter gene for observing the infection process (Kohler et al. 1995), and later transformed with *Agrobacterium*-mediated transformation (AMT) for studying pathogenicity factors (White and Chen 2006; Morgensen et al. 2006). However, little information is currently available about the genetic determinants of pathogenicity of the ascochyta pathogens. Knowledge of pathogenic determinants will allow us to develop a better understanding of host-pathogen interactions to devise novel or more effective measures in managing the disease.

There are two approaches to investigate pathogenic determinants of fungal pathogens. One is targeted gene disruption to construct mutants defective of a defined gene of interest. In this instance, target genes could be previously-reported pathogenicity genes in other pathosystems. The role of the gene in infection can be assessed by comparing the pathogenicity of the mutant with that of the wild-type. The other approach is to generate random and tagged mutations within the pathogen genome. The modern technique in this approach is through insertional mutagenesis of either restriction-enzyme-mediated integration (Oliver and Osbourn 1995; Kahmann and Basse 1999) or AMT (Michielse et al. 2005). This approach does not require a priori knowledge of gene function, and it involves generation of a library of random mutations, screening the library for altered phenotypes or pathogenicity, and characterization of disrupted genomic regions. This strategy is powerful in the identification of previously unknown pathogenicity factors.

There are a number of previously reported conserved fungal virulence factors that could be explored in *A. rabiei*. Lu et al. (2003) described a general fungal virulence factor (an acyl-CoA ligase *cps*1) in several plant-pathogenic ascomycetes. Disruption of the *cps*1 homolog in several plant pathogens produced no observable growth phenotype, but showed reduced virulence. Production of melanin has also been shown to be a virulence factor in some pathogenic fungi (Henson et al. 1999; Kawamura et al. 1999). *Ascochyta rabiei* produces melanin through the 1,8-dihydroxynaphthalene pathway via polyketide synthesis (Chen et al. 2004b). Thus, polyketide synthases could potentially be pathogenicity factors in *A. rabiei* through their involvement in melanin biosynthesis or in the synthesis of phytotoxin solanapyrones (Hohl et al. 1991).

The goal of our research is to use *A. rabiei* as a model for the other ascochyta pathogens to open the door for investigating pathogenic determinants. Our research hypotheses are (1) insertional mutagenesis can be applied to *A. rabiei* to elucidate pathogenic determinants, and (2) some of the previously-reported pathogenicity factors from other plant pathogens could be identified and isolated from *A. rabiei*. Here we report successful identification and characterization of tagged mutants with reduced or lost pathogenicity, development of gene-specific probes, construction of a phage library of the *A. rabiei* genome, and the isolation of clones containing potential pathogenicity factors through screening the library. The research provides the foundation and necessary tools for further assessing the roles of the respective genes in causing ascochyta blight.

Materials and methods

Fungal strains, transformation, and pathogenicity screening of transformants

The pathotype II strain AR628 (Chen et al. 2004a) of *A. rabiei* was used in transformation experiments. The transformation was carried out as previously described (White and Chen 2006). Briefly, conidia of strain AR628 were co-cultured with cells of *Agrobacterium tumefaciens* carrying T-DNA. The co-cultivation was spread on a membrane and incubated on medium containing timentin and hygromycin to select against bacteria and select for hygromycin-resistant transformants. Transformed conidia that grew on the selective medium were further purified by single-conidium isolation. After confirming resistance to hygromycin, the transformants were screened for altered pathogenicity before further characterization.

The mini-dome bioassay (Chen et al. 2005) was used to screen transformants for reduced pathogenicity. The transformants were always compared with wild-type strains AR19 (pathotype I) and AR628 in the pathogenicity assays on chickpea cvs Dwelley and Spanish White (six plants in three replicates of each cultivar per strain). Disease severity was assessed according to the 1–9 rating scale (Chen et al. 2004a). The transformants that showed reduced pathogenicity in the first assay were tested again in a second assay. Nine transformants that showed significantly reduced pathogenicity in both assays were selected for further study. In addition, two transformants that lost ability to produce conidia were also selected for further characterization.

Southern hybridization, inverse-PCR and sequence analysis of transformants

Southern hybridization was used to determine the number of insertions in transformants. Genomic DNA from transformants and wild-type AR628 were digested with XhoI (New England Biolabs, Ipswich, MA, USA), separated on an agarose gel, and transferred to a nylon membrane. A DIG-labelled DNA probe was synthesized from an internal region of the hygromycin B gene using PCR (White and Chen 2006). Probed membranes were processed according to the manufacturer's instructions, and detected using the anti-DIG-alkaline phosphatase conjugate antibody and the chemiluminescent substrate CSPD (Roche) by exposure to autoradiograph film to visualize hybridized fragments.

To isolate DNA regions flanking the insertion sites in the transformants, an inverse-PCR technique was used. Genomic DNA of selected transformants was digested with XhoI to isolate DNA flanking the right border, and digested with either SacI, SalI or KpnI to isolate DNA flanking the left border. Digested DNA was ligated to itself and used as template for the inverse PCR using primers LB5IP and RB5IP (White and Chen 2006). Products were isolated from agarose gels and ligated to the pGEM-T Easy vector (Promega, Madison, WI, USA) for further analysis.

To determine the gene disrupted by the T-DNA in each transformant, the ends of each cloned inverse-PCR product were sequenced using the M13F (-20) and M13R (-21) primers (New England Biolabs). Forward and reverse sequences were joined after removing all vector and T-DNA border sequences. Assembled sequences were compared to each other to verify that each contained a unique region of the *A. rabiei* genome and translated in all six reading frames for comparison to the GenBank database as well as the *Stagonospora nodorum* genome (http://www.broad.mit.edu). The *S. nodorum* genome was selected because, for the fungi with genomes available, *S. nodorum* is the closest phylogenetically related to *A. rabiei* (Peever et al. 2007).

Development of gene-specific probes for *A. rabiei*

Specific probes were developed for genes that could be potential virulence factors in *A. rabiei*. The genes encoding the polyketide synthase (*pks*1) from *Glarea lozoyensis* (Zhang et al. 2003) and an acyl-CoA ligase (*cps*1) from *Cochliobolus heterostrophus* (Lu et al. 2003) were selected as candidate virulence factors. These gene products were compared to and aligned with

the translated genome of *S. nodorum*, and conserved locations were selected to design PCR primers. PCR primers pksF2 (5′-CACTACCACTGCCGTCGCAT) and pksR2 (5′-TAGACTTGACCATGCCACTGCA) were designed to amplify a 562-bp region of the *pks*1 gene, and primers cpsF (5′-GGGACAAGAGCAACCTCTA) and cpsR (5′-TGGTAGTTGTATGCAGC) to amplify a 683-bp region of the *cps*1 gene. PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced using the M13F and M13R primers as described above.

Construction and screening of a genomic library of *A. rabiei*

To construct a genomic library of *A. rabiei*, genomic DNA of strain AR628 was digested with ApoI and fragments between 7,000 and 10,000 bp were eluted from agarose gels, desalted, and ligated to pre-digested and phosphatased (*Eco*RI) Lambda ZAPII vector arms (Stratagene, La Jolla, CA, USA), packaged using Gigapack III extracts, and amplified in *E. coli* strain XL1-Blue. The efficiency of the ligation and packaging reactions were determined using X-GAL and IPTG. To determine the average insert size of the recombinant phage library, plasmid rescue (in vivo excision) was performed on phage collected from ten random plaques using the *ExAssist*® helper phage and *E. coli* strain SOL-R. Recovered plasmids were digested with ApoI and separated on an agarose gel. A single round of library amplification was performed and the phage suspension stored in 7% DMSO at –80°C until use.

To isolate clones from the library that contain either *pks*1 or *cps*1 homologs, probes were constructed using PCR with the *pks* or *cps* primers and labelling procedures described above. Approximately 80,000 plaques were transferred from NZY agar to nylon membranes (Amersham) and probed sequentially, first with the *cps*1 probe, then with the *pks*1 probe. Single plaques that hybridized with each probe were recovered from the corresponding NZY plate and in vivo excision reactions were performed to rescue phagemid DNA. Recovered phagemid DNA was used as template for PCR with the corresponding primer pairs used to generate the probe, and were also digested with ApoI to estimate the insert size by agarose gel electrophoresis.

To isolate clones from the phage library that contain DNA adjacent to the T-DNA insertion sites, approximately 80,000 plaques were screened with probes generated from inverse-PCR products. Probes from the transformants were mixed together for the primary hybridization and detection screen. Phage from positive plaques from the primary screen was harvested and pooled in SM buffer to make an enriched phage stock for infecting *E. coli* XL-1 Blue cells. Plaques generated from the enriched phage stock were transferred to nylon membranes and screened with individual probes. Phagemids were recovered by in vivo excision as described above from phage collected from three plaques identified by each probe and analyzed by restriction digestion and sequencing.

Results

Identification and characterization of transformants with altered pathogenicity

Approximately 800 transformants were generated and screened for pathogenicity in this study. The transformants exhibited a wide range of variation in colony morphology, growth rate, and conidial production. For example, six transformants had lost ability to produce conidia. Some transformants produced constitutively black mycelium. In general, under selection conditions (V8 agar with 200 μg ml^{-1} hygromycin) many transformants produced less conidia than when grown in the absence of selection (V8 agar without hygromycin). The wild-type strain AR628 consistently produced about 3.7×10^7 conidia per plate, 63 transformants produced about 10% of conidia of WT, seven transformants produced about 1.5% of conidia of the wild type. Two transformants produced 5× more conidia than the wild type.

In pathogenicity screening, the transformants that lost ability to produce conidia were not screened because the screening procedure uses conidia as inoculum (Chen et al. 2004a). Most of the transformants screened were about equally

virulent as the parental wild-type strain, producing disease scores above 6 (Fig. 1). Some transformants showed reduced pathogenicity in the first screening, but produced higher levels of disease severity in a second pathogenicity assay possibly due to heterogeneity of nuclei (co-existence of transformed and non-transformed nuclei). To date, 21 of the transformants produced significantly lower disease severity (score <4 on a 1–9 rating scale) than that of the wild-type in at least two independent pathogenicity assays (Fig. 1). Nine of the 21 transformants plus two transformants that lost ability to produce conidia were selected for further characterization (Table 1).

Southern hybridization, inverse PCR and sequence analysis of selected transformants

Southern hybridization of digested transformant DNA probed with the hygromycin-resistance gene (*hph*) showed single hybridization bands of various sizes (data not shown), confirming that the T-DNA was integrated into the genome of *A. rabiei* and that each transformant contained a single insertion. Inverse PCR amplified single products from transformants, ranging in size from 850–2500 bp (Table 1). Sequences adjacent to the insertion sites from the transformants were first compared among themselves, and comparison showed that two pairs of the 11 transformants, ArW520 vs ArW525, and ArW247 vs ArW251, were identical in insertion locations. This reduced the number of characterized transformants from 11 to 9.

The sequences flanking the T-DNA from each transformant were used as queries in tBLASTx searches of the GenBank database as well as the genome database of *S. nodorum*. DNA recovered from two (ArW8 and ArW540) of the transformants shared a high degree of similarity with known proteins while the sequence from another transformant (ArW247/ArW251) shared significant similarity with a hypothetical protein of *A. nidulans* (Table 1). The translated DNA (576 bp) from transformant ArW8 shared 71% identity (91/128 aa) with the kinesin of *C. heterostrophus* (accession AY230433). Translated DNA from transformant ArW540 (440 bp) shared 66% identity (86/130 aa) with the transposase protein of the *S. nodorum* transposon *molly*. Three additional sequences shared minimal sequence similarity with proteins in the database as indicated by the low E values (Table 1). The remaining three sequences (from transformants ArW522, ArW524 and ArW529) did not have any similarity to known proteins (Table 1). In searching the *S. nodorum* genome, sequences of three transformants (ArW8, ArW247 and ArW540) shared significant similarity to translated regions (hypothetical proteins) of the genome, while sequences of the remaining six transformants did not have any similarity with any translated region of the genome (Table 1).

Construction of genomic library and screening with gene-specific and transformant-generated probes

A phage library consisting of 1.7×10^6 recombinants containing *A. rabiei* DNA was constructed with a background (phage without insertion) of less than 2%. The average insert size of the recombinants was about 6,300 bp (data not shown). Thus, this DNA library contains more

Fig. 1 Screening transformants for altered pathogenicity using a mini-dome bioassay. 1 = non-inoculated control; 2 = inoculated with parental wild-type strain AR628; 3, 4 and 5 = transformants ArW1, ArW8 and ArW16, respectively

Table 1 Characterization of insertion sites in selected transformants of *A. rabiei*

Strain	Disease score[a]	Inverse PCR[b]	Sequence length[c]	GenBank tBLAST results (Accession #)	E value	*S. nodorum* genome	E value
ArW8	3.5 ± 0.5	1900	576	*C. heterostrophus* kinesin (AY230433)	$1e^{-46}$	SNOG_04288: hyp. protein	$2e^{-44}$
ArW247/ArW251	NT[d]	500	175	*A. nidulans* hypothetical protein (XM_659106)	$3e^{-10}$	SNOG_01574 hyp. protein	$5e^{-16}$
ArW519	2.0 ± 0.5	1600	433	*M. musculus* p21 activated kinase (AK08851)	0.096	No significant similarity	–
ArW520/ ArW525	1.3 ± 0.3	850	786	*A. erytherum* put. transcript. rep. (AY62365)	0.051	No significant similarity	–
ArW522	1.2 ± 0.3	2200	742	No significant similarity	–	No significant similarity	–
ArW524	2.5 ± 0.9	1500	755	No significant similarity	–	No significant similarity	–
ArW529	1.0 ± 0	2100	818	No significant similarity	–	No significant similarity	–
ArW540	1.8 ± 0.6	2500	440	*S. nodorum* transposon *molly* (AJ488502)	$3e^{-34}$	SNOG_08250 hyp. protein	$8e^{-10}$
ArW541	1.2 ± 0.3	2300	619	*A. oryzae* cDNA, contig sequence: (AoEST1849)	6.6	No significant similarity	–
Ar628 (WT)	8.0 ± 0.8	–[e]		–	–	–	–

[a] Pathogenicity score (± standard deviation, n=3) on chickpea cv. Dwelley using a 1–9 rating scale (1 = healthy, no disease and 9 = dead plant)

[b] Size in base pairs of total product of inverse PCR

[c] Sequence length used in tBlast searches

[d] Not tested due to lack of conidia

[e] Not Applicable

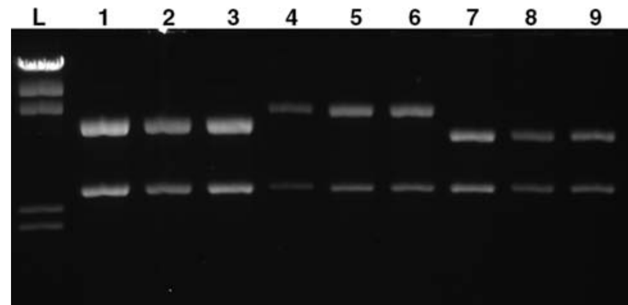

Fig. 2 DNA inserts rescued from positive plaques (three random selected plaques per probe), Lane L, λDNA digested with HinDIII; Lanes 1–3, plaques positive with the ArW8 probe; Lanes 4–6, plagues positive with the ArW519 probe; Lanes 7–9, plaques positive with the ArW541 probe

than 10,000 MB of *A. rabiei* DNA. Assuming a genome size of 40 MB for *A. rabiei* (Akamatsu and Peever 2005), this library would provide more than 250× coverage. After a single round of amplification the final titer of the library was 1×10^9 pfu ml^{-1}.

Screening with gene-specific probes

The amplified *pks*1 DNA fragment had 81% (455/562) identity to the *Bipolaris oryzae* polyketide synthase gene (accession AB176546). The amplified *cps*1 fragment was 82% (560/683) identical to the *cps*1 gene (accession AF332878) of *C. heterostrophus*. The two sequences were deposited into GenBank and assigned the accession numbers EF092313 (*ARcps*1) and EF092314 (*ARpks*1). Two positive clones were identified with the *pks* probe, and the two clones contained 5,800- and 7,000-bp inserts, respectively. The 7,000-bp clone contained an intact DNA region defined by the two PCR primers pksF2 and pksR2. A single positive clone with 5,500-bp insert was identified with the *cps* probe and it contained the entire DNA region defined by the *cps* primers cpsF and cpsR (data not shown).

Screening with transformant-generated probes

After a primary screening of approximately 80,000 plaques with a mixture of the nine probes of the transformants, phage from 55 positive plaques was harvested and pooled to form an enriched phage stock for a secondary screen using individual probes. Each probe in the secondary screening was exposed to approximately 30,000 plaques generated from the enriched phage stock. Phagemid DNAs were rescued from three random plaques identified by each single probe and in each case the three recovered phagemids contained the same sized-DNA inserts (Fig. 2). It was assumed that the three clones represented the same region of *A. rabiei* genomic DNA and only one clone was selected for further analysis.

Discussion

Three important advances were made towards identifying pathogenicity determinants of *A. rabiei*. First, insertional mutants with altered pathogenicity were identified through in vivo pathogenicity assays, and the DNA sequences adjacent to insertion sites were determined. Second, a phage DNA library of *A. rabiei* was constructed with about 250× coverage of *A. rabiei* DNA. Hybridization with either gene-specific probes or probes generated from random insertion sites of transformants always identified positive clones in the library, proving its utility in isolating other genes. Third, probes for specific genes (*cps*1 and *pks*1) with the potential of being general pathogenic determinants in *A. rabiei* were developed, and positive library clones were identified through Southern hybridization. The positive clones containing the specific genes or the insertion sites will be useful for either ectopic complementation tests or targeted mutagenesis.

Insertion in one of the transformants appears to be within a known fungal gene. Transformant ArW8 is less pathogenic (Table 1), and the T-DNA has disrupted a kinesin-like gene. Kinesins play important roles in the transport of cell organelles, polarized growth, and secretion (Schoch et al. 2003), and the kinesins of the yeast *Schizosaccharomyces pombi* as well as the corn smut fungus *Ustilago maydis* have been studied extensively (Steinberg and Fuchs 2004; Straube et al. 2006). However, this is the first report of a kinesin-like gene potentially being involved in plant pathogenesis. Its role remains to be confirmed and its mechanisms in pathogenesis are not clear.

Diverse DNA sequences are found in the insertion sites, showing the randomness of the insertion mutagenesis. Pathogenesis is a complex biological process involving diverse factors. Pathogenesis of the necrotrophic pathogen *A. rabiei* is predicted to involve a number of processes including attachment and penetration of host plant tissue, as well as production and secretion of extracellular enzymes and phytotoxins, and each process is likely to be controlled by several genes. A mutation in any gene involved in these processes could result in altered pathogenicity.

Many of the sequences recovered from the nine transformants had no significant matches either in the GenBank database or within the *S. nodorum* genome, and the significant *in silico* similarity identified in three of the transformants is primarily with hypothetical proteins. This inability to detect any known sequences with significant similarity could be due to the limited length of query sequences available, to the fact that they are unique pathogenicity factors in *A. rabiei*, or to the fact that small deletions of genomic DNA occurred during T-DNA integration events (Bundock and Hooykaas 1996), resulting in the loss of a coding region flanking the insertion.

Gene-specific probes were developed to isolate a polyketide synthase gene and an acyl-CoA ligase gene from the *A. rabiei* library. These genes were selected because they were shown to be conserved pathogenicity factors in other pathosystems (Kawamura et al. 1999; Lu et al. 2003). Screening using these gene-specific probes also served the purpose to test the completeness and usefulness of the DNA library. Both probes identified positive plaques in the library despite the fact only a portion (80,000 plaques) of the library was exposed to the probes. Thus, this library should be useful for isolating other genes of interest and it will be a valuable resource available to the scientific community for studying *A. rabiei* or other related plant pathogens.

Two pairs of transformants were shown to be identical, likely to have resulted from conidia of the same transformation events, since they were isolated from the same transformation membrane. Although the unintentional inclusion of these transformants resulted in redundancy of work, in retrospect, it provided an important internal control. This result showed that the characterization procedures from pathogenicity screening to inverse PCR are reliable and reproducible, giving us confidence in the techniques developed in this study.

Two approaches need to be taken to unequivocally demonstrate the roles of the identified potential pathogenicity determinants in *A. rabiei*. One approach is to use ectopic complementation tests to prove the role of the genes disrupted in the random insertional mutants. A second shuttle vector carrying the *nptII* gene for geneticin resistance expressed by the *A. nidulans trpC* promoter has been created for delivering library DNA via AMT to hygromycin-resistant transformants, and selection on hygromycin and geneticin has been shown to be stable (unpublished). Additionally, *A. rabiei* is heterothallic (Trapero-Casas and Kaiser 1992). Thus segregation analysis could also be employed.

Another approach is to carry out targeted mutagenesis specifically on the *cps* and *pks* genes to create knockout mutants. To create deletions in the *pks* and *cps* library fragments, a short region of each clone has been removed by restriction digest and replaced with the *trpC-hph* antibiotic resistance cassette. Disruption cassettes containing library clones in a markerless T-DNA shuttle vector are being constructed for delivery into *A. rabiei* wild-type strains via AMT. Integration can occur at the genomic site of interest (homologous recombination) or at other sites (illegitimate recombination), which would be