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TECHNICAL NOTE

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Genetic transformation of *Ascochyta rabiei* using *Agrobacterium*-mediated transformation

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Abstract In order to study pathogenic mechanisms of the plant pathogen *Ascochyta rabiei*, conditions for efficient transformation using *Agrobacterium*-mediated transformation were investigated. Hygromycin B resistance (*hph*) was superior to geneticin resistance (*nptII*) for selecting transformants, and the *hph* gene was more efficiently expressed by the *Aspergillus nidulans trp*C promoter than by the Cauliflower mosaic virus 35S promoter *CaMV35S*. Co-cultivation on solid media for 72 h was optimal for generating transformants, but increasing the ratio of bacterial cells to conidia did not affect transformation efficiency. All hygromycin B-resistant transformants carried transfer-DNA (T-DNA) as determined by polymerase chain reaction (PCR) and the T-DNA integrations appeared to be random and in single copy as detected by Southern hybridization. Transformants remained resistant to hygromycin B in the absence of selection. Variations in colony morphology were observed in the presence of hygromycin B under different culture conditions, and a variety of altered phenotypes including reduced virulence were observed among 550 transformants. Inverse PCR was more efficient than TAIL-PCR in identifying flanking genomic sequences from T-DNA borders, and the possible causes are discussed. This transformation technique and recovery of flanking DNA using inverse PCR will provide a useful tool for genetic studies of *A. rabiei*.

Keywords Ascochyta blight · Fungus · Insertional mutagenesis · Plant pathogen

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Introduction

The ascomycete fungus *Ascochyta rabiei* (Pass.) Lab. (teleomorph: *Didymella rabiei*) causes Ascochyta blight, an important disease of chickpea worldwide. *A. rabiei* exhibits considerable variation in pathogenicity and two pathotypes of *A. rabiei* are found in the US (Chen et al. 2004). The mechanisms by which *A. rabiei* infects and colonizes chickpea plants remain incompletely understood. Investigations indicate that *A. rabiei* can enter plants by direct penetration (Höhl et al. 1990). The pathogen also produces solanapyrone toxins, and these toxins can induce symptoms similar to Ascochyta blight (Köhler et al. 1995; Hamid and Strange 2000). In addition, this pathogen produces cutinase (Tenhaken et al. 1997), which has been shown to be a virulence factor in other fungi (Dickman et al 1989). However, very little is known about the genetic control of pathogenicity. Generation and isolation of fungal mutants defective in pathogenicity has been very useful for understanding the genetic and enzymatic processes responsible for infectivity of a number of pathosystems (Indnurm and Howlett 2001).

Although chemical-induced, irradiation-induced, and spontaneous mutations may lead to identifying non-pathogenic phenotypes, isolating the resultant genetic mutation is difficult for two reasons. First, mutations generated by these methods are usually the result of single base mutations are thus untagged and difficult to isolate. Second, these methods favor multiple mutations within a single genome, adding to the difficulty of linking the altered phenotype to a particular genetic locus. Insertional mutagenesis, using known DNA fragments, generates tagged mutations and has become a common method for manipulating many diverse genomes. Restriction enzyme-mediated integration (REMI) has been used to identify and isolate a number of pathogenicity-related genes in plant pathogenic fungi (Maier and Schäfer 1999). More recently, insertional mutagenesis via *Agrobacterium*-mediated transformation (AMT) has

been shown to function outside of plant kingdom (de Groot et al. 1998; Mullins and Kang 2001). Owing to its wide applicability and relative simplicity, AMT has been used to transform a variety of fungal species (Michielse et al. 2005). However, the efficiency of the process is species dependent and must be optimized for each species (Abuodeh et al. 2000; Fang et al. 2004; Gouka et al. 1999; Mullins et al. 2000; Tanguay and Breuil 2003; Sun et al. 2002; Vijn and Govers 2003).

In general, the methodologies of REMI and AMT are similar. Recipient cells, usually spores, receive foreign DNA capable of integrating into the host genome. In the case of REMI, naked DNA is propelled into chemically treated spores in the presence of a restriction enzyme The restriction enzyme will digest both the introduced and the host genomic DNA and during DNA repair the foreign DNA may be integrated into the genome. AMT involves the co-cultivation of *Agrobacterium tumefaciens* bacteria containing transfer-DNA (T-DNA) with untreated fungal cells, usually spores. Bacteria move the T-DNA into the spore, where it is directed to the host nucleus through the combinatorial action of bacterial and host proteins, presumably during early germination. Once in the nucleus, the T-DNA can integrate into the host genome. In many cases, the DNA used in REMI transformations contains a bacterial replication origin allowing the recovery of genomic DNA flanking insertions. With AMT, a more common method for recovery is through inverse PCR or TAIL-PCR methods (Combier et al. 2003; Gardner et al. 2005); however, a limited number of binary vectors have been designed with bacterial replication origins (Mullins et al. 2000).

Our research is directed toward understanding the genetic factors controlling pathogenesis of *A. rabiei*. Currently, studying the genetics of *A. rabiei* virulence is limited by the lack of appropriate tools to investigate pathogenesis at the molecular level. Here, a molecular genetic approach of insertional mutagenesis, via AMT, is applied to generate tagged mutants that can be screened for the loss of pathogenicity. This paper describes the development of suitable plasmid vectors and appropriate conditions for efficient transformation of *A. rabiei*. In addition, we compared the growth phenotype of transformants on different media and under different selection conditions, and we determined that inverse PCR was more effective than TAIL-PCR in recovering flanking sequences from *A. rabiei* transformants.

Materials and methods

Strains, plasmids, and culture conditions

Microbial strains used in this study included *Escherichia coli* strain DH10B (Invitrogen, Carlsbad, CA, USA), *Agrobacterium tumefaciens* strains AGL-1, and *A. rabiei* strain AR628 (Table 1). *E. coli* was cultured in Luria Bertani (LB) or SOB medium (Sambrook et al. 1989) at 37°C. *Agrobacterium tumefaciens* was grown in YEP (Lichtenstein and Draper 1986), minimal medium (MM), induction medium (IM) (Bundock et al. 1995), or LB medium at 25–28°C. *A. rabiei* was cultured on Difco potato dextrose agar (PDA), acidified PDA (APDA) containing 0.5% lactic acid, or V8 agar (Barve et al. 2003) at 20°C. Plasmids pCAMBIA1300 (Cambia, Melbourne, Australia), pGEM-T (Promega, Madison, WI, USA), pII99 (Namiki et al. 2001), and pSH75 (Kimara and Tsuge 1993), kindly provided by Takashi

Table 1 Bacteria strains, fungal isolates, and plasmids used in this study

Strains	Relative genotype	Relative phenotype	Function	Reference
Bacteria				
DH10B	N/A	Nal^R	General cloning	Invitrogen
AGL-1	N/A	Rif^R	T-DNA delivery	Lazo et al. (1991)
AT1	pCAMBIA1300	Kan^R Rif^R Hyg^R	T-DNA delivery	CAMBIA
AT3	AGL-1::pDJW2	Kan^R Rif^R Gen^R	T-DNA delivery	This study
AT5	AGL-1::pDJW5	Kan^R Rif^R Hyg^R	T-DNA delivery	This study
Fungi				
AR628	Wild-type	Pathotype II	Transformation target	Chen et al. (2004)
ArW76	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW80	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW95	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW96	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW97	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW99	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW101	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW102	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW103	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW104	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW105	AR628::*trpC-hph*	Hyg^R	Transformant	This study
ArW106	AR628::*trpC-hph*	Hyg^R	Transformant	This study
Plasmids				
pCAMBIA1300	*CaMV35s-hph, nptII*	N/A	Binary vector	Cambia
pDJW2	*trpC-nptII, nptII*	N/A	Binary vector	This study
pDJW5	*trpC-hph, nptII*	N/A	Binary vector	This study

Tsuge, were maintained in *E. coli*. The 9,646 bp plasmid pDJW2 was created by ligating a 2,812 bp *Xho*I–*Eco*RI fragment containing the *trpC-nptII* region from pII99 to pCAMBIA1300 digested with the same two enzymes. The 9,851 bp plasmid pDJW5 was created by ligating a 3,017 bp *Xho*I–*Eco*RI fragment from pSH75 containing the *trpC-hph* region to pCAMBIA1300 digested with the same enzymes (Table 1). Restriction and modification enzymes were obtained from Invitrogen or New England Biolabs (Beverly, MA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA). For maintenance of plasmids and T-DNA, antibiotics were used at the following concentrations: kanamycin, 50 μg/ml, *E. coli* and *Agrobacterium tumefaciens*; ampicillin, 100 μg/ml, *E. coli*; hygromycin B, 200 μg/ml (V8 agar and PDA), 150 μg/μl (V8 broth) 100 μg/ml (PDB), *A. rabiei*; rifamycin, 20 μg/ml, *Agrobacterium tumefaciens*. To select against all other microorganisms, the following antibiotics were used: choloramphenicol, 100 μg/ml; cefotaxime, 200 μM; or timentin, 150 μg/ml. Plasmids were introduced into *E. coli* and *Agrobacterium tumefaciens* by electroporation as described by Taketo (2004), or using the freeze–thaw method for *Agrobacterium tumefaciens* (Chen et al. 1996). Plasmids were transferred from *E. coli* to *Agrobacterium tumefaciens* via tri-parental mating with a helper strain carrying plasmid pRK2013 (Figurski and Helinski 1979).

Fungal transformation

Agrobacterium tumefaciens strain AGL-1 containing plasmid pCAMBIA1300, pDJW2, or pDJW5 was grown for approximately 20 h in LB containing kanamycin and rifamycin. Cultures were washed with MM, diluted 1:5 with IM and pre-cultivated for 6–12 h at 25°C. Following pre-cultivation, bacterial cells were centrifuged at 3,000 rpm, washed once with fresh IM, and suspended in 1 volume of fresh IM. Conidia of *A. rabiei* strain AR628 from 14-day V8 agar cultures were washed once with H_2O and suspended in IM to a final concentration of 1×10^6 conidia/ml. Washed bacteria suspensions were mixed with an equal volume of *A. rabiei* conidia suspensions, added to prepared dialysis (MWCO 12,000, Spectrapore, Rancho Dominguez, CA, USA) or nylon (0.22 μm, Amersham) membranes on IM agar (ca. 200 μl/2 cm^2 membrane), and co-cultivated under various conditions. Each culture condition was replicated five times and the experiments were performed three times. Following co-cultivation, the membranes containing the co-cultivate were inverted and transferred to either PDA or V8 agar plates containing 200 μg/ml of hygromycin B with chloramphenicol and cefotaxime. The membranes were removed after an additional 2 days of co-cultivation at 20°C. *A. rabiei* transformants that arose 4–10 days after transfer were immediately moved to fresh selection plates followed by transfer to APDA or V8 plates containing timentin to eliminate bacteria. Transformants were maintained on PDA or V8 agar with or without selection.

Characterization of transformants

DNA extraction and analysis

DNA was isolated from *A. rabiei* transformants and wild-type strain using the method of Lee and Taylor (1990) with modifications. Approximately 2 g of filtered, frozen mycelia from a liquid culture was ground to a powder and lysed using lysis buffer (50 mM Tris–HCl, pH 7.4, 50 mM EDTA, 3% SDS, 10 mM β-mercaptoethanol) at 65°C for 1 h. The lysate was extracted once using 1 volume of Tris-buffered phenol followed by two extractions with 1 volume of chloroform/isoamyl alcohol (24:1). RNA was removed by treating with RNase (10 μg/ml) for 1 h at 37°C. DNA was precipitated and suspended in Tris–HCl (10 mM). Sometimes a modified microwave method was used to isolate DNA for PCR (Goodwin and Lee 1993). Briefly, small amounts (ca. 10–50 mg) of mycelia harvested from PDA or V8 agar plates were heated in a standard microwave oven for 5 min at 700 W. After adding TE buffer, samples were vortexed for 30 s, and centrifuged at 13,000 rpm for 5 min. The supernatant-containing DNA was transferred to a new tube and stored at −20°C until use in PCR.

Molecular confirmation of transformantion events

Two methods were used to confirm transformation events. PCR was used to confirm the presence of T-DNA by amplifying an internal 854 bp region of the *hph* gene of T-DNA using primers hph-F (5′-GAGCCTGACCTATTGCATCTC) and hph-R (5′-CCGTCAACCAAGCTCTGATAG), while Southern hybridizations were performed to determine the frequency and randomness of T-DNA integration. PCR reactions consisted of approximately 10 pg of genomic DNA, 1 U *Taq* DNA polymerase, 1× polymerase buffer, 2 μM $MgCl_2$, and 2 μM primers using an ABI 96-well Thermocycler (Perkin-Elmer, Boston, MA, USA). Conditions for amplification were an initial denaturation of 5 min at 95°C followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Amplified products were compared to a product amplified from plasmid pDJW5 on 0.8% agarose gels. For Southern analysis genomic DNA (ca. 10 μg) was digested with *Xho*I, fragments were separated on a 0.8% agarose gel for 1 h and for 4 h, and transferred to a Nylon membrane (Millipore, Billerica, MA, USA). A probe was generated by PCR using the hph-F and hph-R primers with the PCR DIG Probe Synthesis Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Aqueous hybridizations were performed at 65°C overnight and detected using the DIG Luminescent

Detection Kit (Roche) according to the manufacturer's instructions.

Analysis of regions flanking T-DNA insertions in A. rabiei

Two methods were attempted to identify genomic DNA sequences flanking T-DNA insertions: inverse PCR (Gardner et al. 2005) and TAIL-PCR (Combier et al. 2003). To sequence regions downstream from the right border by inverse PCR, DNA was digested with *Xho*I, whereas to sequence upstream from the left border DNA was digested with *Sac*I. Aliquots of each digest were used in ligation reactions overnight at 10°C. PCR was performed using the ligation reactions as DNA templates using Elongase or *Taq* DNA polymerase, primers LB5IP (5′-AGTCGTTTACCCA GAATGCACAGGTACACT) and RB5IP (5′-CTTGC ACAAATTGGATGTCCATCTTCGAAAC) for 35 cycles of the conditions 94°C, 30 s; 55°C, 30 s; 68°C, 3 min. Products were separated on 1% agarose gels (Nuseive, IBC BioExpress). For TAIL-PCR, genomic DNA was used as template in successive reactions using nested left border-specific primers LB1, LB2, LB3 or nested right border-specific primers RB1, RB2, RB3, and degenerate primer AD1 as described by Mullins et al. (2000) or degenerate primers AD2 and AD3 as described by Combier et al. (2003). Primary reactions consisted of transformant genomic DNA as template and either LB1 or RB1 and a degenerate primer. Secondary reactions used product from the first reaction, the appropriate nested primer (LB2 or RB2), and the same degenerate primer used in the primary reaction. Tertiary reactions used product from the second reaction, nested primers LB3 or RB3, and the same degenerate primer used in the first two rounds of amplification. Secondary and tertiary products were compared for predicted size shifts on 1.2% agarose gels corresponding to amplification of border regions. Reactions yielding single products with the expected size shift were either sequenced directly using primer LB5IP or RB5IP (for inverse PCR-derived products) or LB3 or RB3 (for TAIL-PCR-derived products) after clean-up using the Exo-Sap-It Kit (USB, Cleveland, OH, USA) or cloned into plasmid pGEM-T Easy and then sequenced using primer T7 or SP6 at the Washington State University Bioinformatics Core, Pullman, WA, USA.

Pathogenicity assay

Transformants were maintained on APDA and initially screened for the ability to produce conidia on V8 agar containing hygromycin B. Conidia suspensions (2×10^5 spores/ml) in sterile water were used to inoculate 2-week-old chickpea plants using a mini-dome assay (Chen et al. 2005). Disease severity was scored using a 1–9 rating scale and compared to the pathogenicity of the parent strain AR628 (Chen et al. 2004). Transformants that showed reduced virulence were tested again for a second time.

Results

Sensitivity of *A. rabiei* to the antibiotics hygromycin B and geneticin

The sensitivity of wild strains of *A. rabiei* to hygromycin B and geneticin was determined by transferring fungal cultures from V8 agar onto PDA, APDA, V8 agar, and acidified V8 agar amended with various concentrations of hygromycin B (up to 200 μg/ml) and geneticin (up to 100 μg/ml). Growth of *A. rabiei* was completely inhibited for 2 weeks at 200 μg/ml hygromycin B in all media tested, although some growth occurred after 18 days of incubation. Growth of *A. rabiei* was only inhibited for 4–8 days by geneticin at 100 μg/ml. The growth response of *A. rabiei* in the presence of hygromycin B is media dependent. In general *A. rabiei* was more sensitive to hygromycin B in PDA than in V8 agar, and in PDB than in V8 broth. Near-complete inhibition of growth was observed in PDB with 100 μg/ml hygromycin B, while near-normal growth still occurred in V8 broth with 150 μg/ml hygromycin B. However, the response of *A. rabiei* to geneticin was the same on both media. To ensure that recovered transformants were not false positive for hygromycin B resistance, *A. rabiei* growth was selected during the first 10 days post co-cultivation on V8 agar containing 200 μg/ml hygromycin B and broth cultures of transformants were maintained with sufficient concentrations of hygromycin B, whereas transformants arising in the presence of 100 μg/ml geneticin were only selected during the first 4 days of exposure.

AMT of *A. rabiei*

Agrobaterium tumefaciens strains AT1 (AGL-1::pCAMBIA1300), AT3 (AGL-1::pDJW2), and AT5 (AGL-1::pDJW5) were induced under various cultivation conditions including prolonged pre-cultivation, liquid media co-cultivation, and solid media co-cultivation on membranes (a minimum of five membranes per condition) to determine the transformation efficiency of each construct. In all the conditions tested, stable transformants could only be recovered from co-cultivations of AT5 and AR628, whereas stable transformants were never recovered from co-cultivation containing AT1 and AR628. In the case of co-cultivations containing AT3 and AR628, fungal growth did appear within 3–4 days after placement on selective medium; however, this outgrowth was unable to grow when transferred to fresh plates containing geneticin. In addition, co-cultivation on a solid IM support medium was more efficient in transferring T-DNA to *A. rabiei* than co-cultivation in liquid IM as stable transformants were never recovered from any liquid co-cultivation.

Aliquots of liquid co-cultivate could be re-cultivated on a solid support (i.e., dialysis membrane on IM agar) to generate transformants. Transformants were only recovered from co-cultivations on dialysis membranes and never from co-cultivations on nylon membranes. As a result, all experiments to determine the optimal co-cultivation conditions were performed using mixtures of AT5 and AR628 using five membranes per cultivation condition.

Culture conditions affect transformation efficiency

A number of co-cultivation conditions were tested in three independent experiments to optimize the transformation efficiency of strain AT5 on AR628. The addition of the synthetic plant hormone acetosyringone (AS), ratio of bacterial cells to recipient fungal conidia, temperature and duration of co-cultivation were tested. Acetosyringone was necessary to induce transformation since no transformants were obtained at 0 and 100 μM, whereas 15 and 20 transformants were generated at 200 and 400 μm, respectively. During temperature experiments, most transformants (15) were obtained at 20°C and the number of transformants decreased as the temperature increased. The ratio of bacterial cells to conidia had little effect; the numbers of transformants generated were 15, 20, and 20 when the bacteria to conidia ratios were 1:10, 1:1 and 10:1. However, the duration of co-cultivation had the most dramatic effect on transformation efficiency. The transformation efficiencies were significantly higher at incubation times between 72 to 96 h (50 and 45 transformants, respectively). Co-cultivation times of less than 72 h produced fewer transformants (25 and 30 transformants at 24 and 48 h respectively) while cultivation times longer than 96 h did not produce more transformants. The optimal combination of conditions for transformation of *A. rabiei* was co-cultivation of bacteria and conidia at 1:1 ratio in the presence of 200 μm of acetosyringone for 72 h at 20 °C.

Characterization of transformants

Putative transformants were purified by single conidium isolation on APDA containing hygromycin B and timentin to eliminate contaminating *Agrobacterium tumefaciens* cells, and maintained on APDA-hygromycin or V8-hygromycin. The *hph* region of T-DNA insertions was detected by PCR in all transformants tested after single conidium isolation, regardless of growth media, and was not detected in the parent strain AR628. Overall, colony morphology differed depending on the medium used in combination with hygromycin B. Most of the transformants exhibited colony morphology similar to the wild-type strain AR628 when cultured on V8-hygromycin (200 μg/ml) agar (Fig. 1b, c), although slight differences in growth rate were observed. When incubated on PDA-hygromycin (200 μg/ml) all transformants produced uniformly dark, restricted growth, punctuated by a delay in the production of conidia (Fig. 1a), whereas the colony morphology and conidia production of transformants on PDA without hygromycin B was identical to the parent strain AR628 (Fig. 1d, f). This growth phenotype was consistent over multiple passages between PDA supplemented with and without hygromycin B, was not affected by the acidification of the media using lactic acid, and consistently observed at hygromycin B concentrations as low as 50 μg/ml.

Similar to wild-type strains, the transformants were more sensitive to hygromycin B in liquid medium than in solid medium. For instance, all transformants were routinely grown on PDA containing 200 μg/ml hygromycin B; however, no transformants were able to grow in PDB containing 200 μg/ml hygromycin B. Transformants produced robust growth in PDB containing

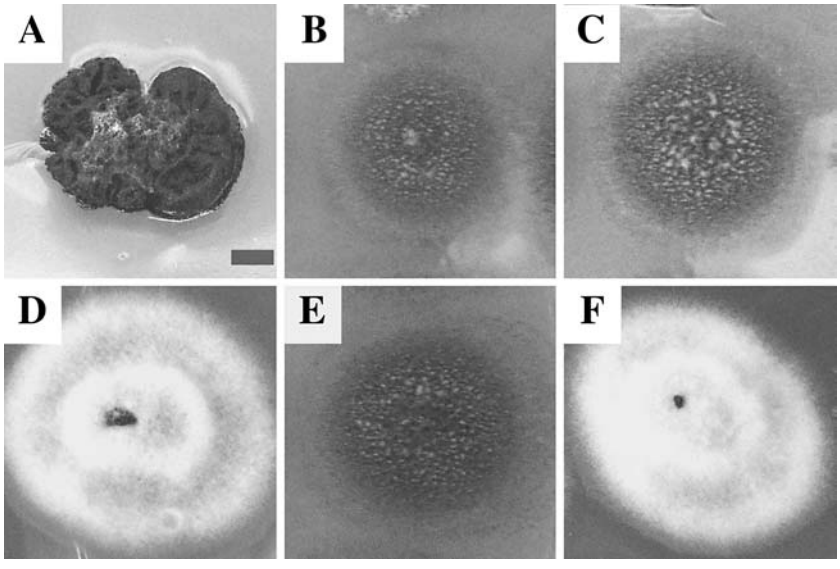

Fig. 1 Colony morphology of *A. rabiei* transformants on solid media. **a** ArW101 PDA/200 μg/ml hygromycin; **b** ArW101 V8/200 μg/ml hygromycin; **c** wild-type strain AR628 V8; **d** ArW101 PDA; **e** ArW101 V8; **f** wild-type strain AR628 PDA. *Bar* = 10 mm

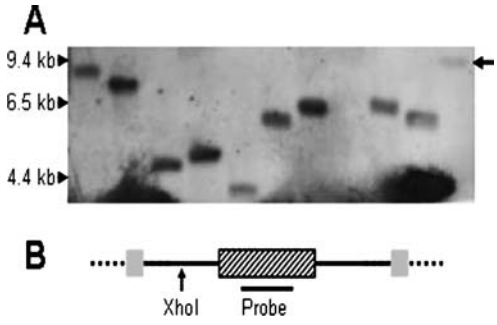

Fig. 2 Random integration of T-DNA into the *A. rabiei* genome. Southern analysis of *A. rabiei* transformant genomic DNA digested with *Xho*I. **a** *Lane 1* ArW95, *lane 2* ArW106, *lane 3* ArW104, *lane 4* ArW103, *lane 5* ArW102, *lane 6* ArW101, *lane 7* ArW99, *lane 8* untransformed wild-type strain AR628, *lane 9* ArW97, *lane 10* ArW96, *lane 11* plasmid pDJW5 control. The plasmid DNA size is indicated by *arrow* on the right. **b** Illustration of T-DNA integration event. *Dark lines* indicate 3,654 bp T-DNA region, *shaded boxes* are left and right border regions, *hatched box* is the *hph* gene. The *line* underneath the *hph* gene represents the 890-bp region used as probe. The *dashed line* represents flanking *A. rabiei* genomic DNA. The *arrow* indicates the single *Xho*I site within the T-DNA

100 μg/ml hygromycin B, a concentration sufficient to inhibit growth of the wild-type AR628. The dark colony morphology of transformants cultured on solid PDA with hygromycin was not consistently observed in liquid PDB containing hygromycin (data not shown), where mycelia morphology ranged from white, fluffy, and identical to the wild-type strain AR628, to small dark mycelia clusters, similar to the phenotype of transformants on solid PDA. The dark colony morphology reappeared in all transformants when the mycelium from PDB cultures was inoculated onto PDA containing 200 μg/ml hygromycin. To determine the stability of transformants, ten transformants were cultured continuously for 8 weeks on V8 agar without selection. The *hph* gene could be detected in all transformants after 8 weeks of culture in the absence of hygromycin B.

The copy number and randomness of T-DNA integration in *A. rabiei* were determined by Southern hybridization. Using part of the *hph* region as a probe and *Xho*I-digested genomic DNA, all of the analyzed transformants produced single hybridizing fragments (Fig. 2) suggesting a single T-DNA insertion. On blots from gels of short run (1 h), no hybridizing bands were detected below 4 kb (gels not shown). The hybridizing bands could be separated only on gels with long run times (4 h). Out of the nine transformants, eight fragments of distinct sizes were detected, ranging from approximately 4.5 to 8 kb, of which 3,200 bp is T-DNA (Fig. 2), suggesting unique insertion positions. Two of the transformants, ArW99 and ArW97 (lanes 7 and 9, respectively), produced hybridizing fragments of approximately the same size, suggestive of insertion into the same genomic position.

The *A. rabiei* sequence flanking each right and left border region was determined using two methods: inverse PCR and TAIL-PCR. Inverse PCR with genomic DNAs from five transformants amplified products ranged in size from approximately 900 to 3,400 bp, of which approximately 700 bp is T-DNA sequence. The predicted post-integration imperfect repeat (Bundock and Hooykaas 1996) at the right border is retained in all five transformants. However, deletions of the T-DNA left border region occurred and ranged from 7 to 9 bp (Fig. 3.). Each T-DNA represents a random insertion as genomic DNA flanking each T-DNA between the five transformants sequenced is dissimilar. Left and right *A. rabiei* sequence flanking each border region were joined, translated in all six reading frames, and used as query in BLAST (tblastx) searches of both the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) as well as the *Stagonospora nodorum* database (blastn and tblastx) (http://www.broad.mit.edu/annotation/fungi/stagonospora_nodorum/background.html); however, no significant similarities were detected. Joined sequences were deposited in the GSS section of the GenBank database

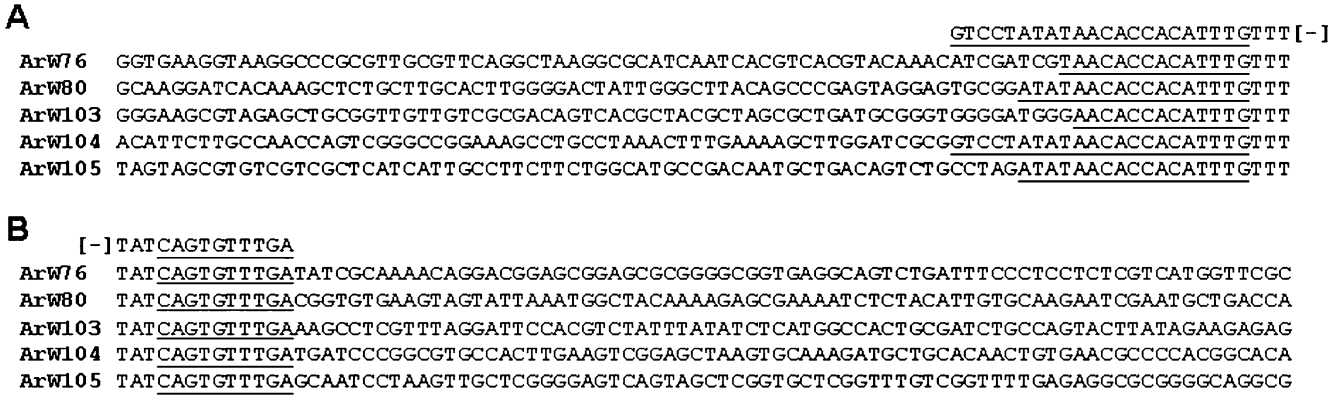

Fig. 3 Alignment of sequences at junction of T-DNA integration recovered using inverse PCR from five *A. rabiei* transformants. Topmost are the left and right T-DNA border sequences. The remaining T-DNA sequence between the borders is represented by the minus symbol. The imperfect repeat regions are *underlined*; *A. rabiei* DNA flanking sequences in *normal font*; all sequences are in the 5′–3′ orientation

(DQ296124, ArW76; DQ296125, ArW80; DQ296126, ArW103; DQ296127, ArW104; DQ296128, ArW105).

For TAIL-PCR, genomic DNA of ten transformants was used as template with a left or right border-specific primer in combination with a degenerate primer. Three rounds of PCR were performed and border region amplification was enriched using three different and increasingly internal left and right border primers (LB1, LB2, LB3 or RB1, RB2, RB3) in combination with a degenerate primer (AD1, AD2, or AD3). Products from the third round of TAIL-PCR were sequenced if the predicted size shift between the second and third rounds of amplification had occurred. The recovered sequence of these products, however, did not correspond to the left or right border regions where the border primers anneal (data not shown), suggesting that these products represented random amplification from the *A. rabiei* genome and not specific amplification of border regions.

In addition to growth phenotypes of transformants observed in different media and under different selection conditions, other altered phenotypes were also observed. Some transformants lost the capacity to produce conidia. Because the pathogenicity assay uses conidia as inoculum, transformants that do not produce conidia could not be screened. Among 550 transformants screened for pathogenicity on chickpea, eight showed reduced virulence. The parent strain always caused disease ratings of 7 or 8 on the 1–9 rating scale, whereas some transformants caused disease ratings of 4 or 5 (data not shown). Transformants with reduced virulence will undergo additional screening such as toxin production and will be genetically characterized.

Discussion

A number of cellular components of *A. rabiei* including solanapyrone toxins (Bahti and Strange 2004) and cutinase (Tenhaken et al. 1997) are shown to be related to virulence. However, these factors have not been conclusively demonstrated to be virulence or pathogenicity factors. One way to demonstrate the specific roles of these factors in causing Ascochyta blight is to generate mutants deficient in each factor followed by assaying for their pathogenicity. An efficient transformation technique would allow this approach to be applied to *A. rabiei*. Previously, *A. rabiei* was transformed with the β-glucuronidase gene using a protoplast-based transformation method (Köhler et al. 1995), albeit at very low efficiency. The protoplasting step has been a limiting factor in applying both REMI and direct gene replacement techniques because efficient protoplasting enzymes are not readily available. DNA transfer mediated by *Agrobacterium tumefaciens* has been shown to be effective in a variety of hosts including plants, yeast, filamentous fungi, and mammalian cells (Sullivan et al. 2002; Mullins et al. 2000; Kunik et al. 2001). An optimal condition for transforming *A. rabiei* with *Agrobacterium tumefaciens* was developed in this study. This protocol bypasses the protoplasting step of REMI and provides a moderately efficient method to introduce foreign DNA into *A. rabiei*. This technique would allow the generation of random tagged mutations to study potential virulence factors and may allow for targeted disruption of specific genes using homologous recombination (Bundock et al. 1995; Gouka et al. 1999).

A combination of factors was identified as critical in transforming *A. rabiei*: hygromycin B resistance as a selection marker expressed by the *trp*C promotor; co-cultivation duration of 72–96 h; and co-cultivation on a solid medium. Although both hygromycin B and geneticin inhibited growth of wild-type *A. rabiei* on various media, only hygromycin B was deemed suitable as a reliable selection marker of transformants. The differences between hygromycin B and geneticin in selection efficiency were likely due to different stabilities of the compounds within the culture conditions assayed. Although both the *CaMV35s* promoter and the *trpC* promoter were used successfully in transforming other fungi (Takahara et al. 2004; Kimura and Tsuge 1993), stable transformants of *A. rabiei* were recovered only when the *trpC* promoter was used. The inability to generate transformants using the *CaMV35s* promoter is likely due to insufficient expression of *hph* in *A. rabiei*. It has been shown previously that the fungal promoter GPD-1 from the fungus *Cochliobolus heterostrophus* is sufficient to express both *gusA* and *hph* simultaneously in *A. rabiei* (Köhler et al. 1995).

An important consideration for efficient AMT of *A. rabiei* is the co-cultivation time. The highest efficiency of transformation was found to be between 72 and 96 h of co-cultivation, generating about 10 transformants/10^5 conidia and is consistent with other published summaries of fungal transformation using AMT (Michielse et al. 2005). It appeared that 72 h are necessary for optimal transformation to occur. However, the reason for the decrease in efficiency after 96 h of co-cultivation is unknown, but may be explained by changes in the germination state of the conidia or a decrease in bacterial fitness.

Unlike previous studies (Mullins et al. 2000), variation in the bacteria/conidia ratio did not affect the efficiency of *A. rabiei* transformation. The increase in the number of *Agrobacterium tumefaciens* in co-cultivation did not result in increasing transformation efficiency while increasing concentrations of *A. rabiei* resulted in increased number of false positive transformants (data not shown).

Another important observation is the effect of culture media on morphology of transformants of *A. rabiei*. PDA (nutrient rich) and V8 agar (nutrient limiting) gave similar transformation efficiency. However, dramatic differences were observed in colony morphology between the two media. On PDA containing 50–200 μg/ml hygromycin B, all transformants exhibited the dark and condensed colony morphology, suggesting an increase in the production of a pigment (hyperpigmentation) such as melanin. The same transformants on either PDA

without hygromycin B or on V8 agar with or without hygromycin B exhibited colony morphology indistinguishable from that of the parent strain. The hyperpigmentation, however, was not conserved among all transformants when grown in PDB containing hygromycin B. Although the significance of the enhanced melanin production is unknown, the persistent production of the black pigments of *A. rabiei* transformants on PDA with hygromycin may complicate the selection of melanin-deficient mutants.

An ideal transformation technique is to generate single, random integrations into the target genome. Southern analysis showed that most transformants contained a single copy of the T-DNA molecule integrated in random positions. The binary vector pDJW5 contains a single *Xho*I site, located within the T-DNA region itself, such that digestion of binary vector (in the event of contaminating *Agrobacterium tumefaciens* cells within transformant cultures) produces a 9,851 bp fragment, while the smallest hybridizing fragment of digested transformant DNA would contain approximately 3,200 bp of T-DNA (Fig. 2b). It is important to point out that some of the transformants chosen for Southern analysis were recovered from extended co-cultivation times, where the frequencies of multiple insertions within a single genome can be significantly higher (Mullins et al. 2000).

Inverse PCR was more efficient than TAIL-PCR in identifying flanking sequences in *A. rabiei*. Sequence analysis of five recovered border regions from inverse PCR revealed minimal degradation of left border regions while the right borders remained intact. The small 7–9 bp deletions of the left border region agree with previous reports that the molecular mechanisms of T-DNA illegitimate recombination in plants are most likely conserved in fungi (Bundock and Hooykaas 1996; de Groot et al. 1998). Consequently, any deletions of *A. rabiei* DNA during T-DNA integration should be short. Such deletions can be resolved by the recovery of the intact region from the wild-type strains. Products recovered using TAIL-PCR were not border regions and instead were most likely random regions of the *A. rabiei* genome. Degradations of the borders during T-DNA integration can result in failure of TAIL-PCR as the tertiary primers (LB3 and RB3) anneal within approximately 55 bp of the border cleavage site. Additionally, the reliance on degenerate primers to consistently anneal to target in successive reactions limits the reliability of the TAIL-PCR method. These limitations are bypassed using inverse PCR, where two border-specific primers are used to amplify target in a single reaction. This finding may have wide applications in other transformation systems where TAIL-PCR is problematic.

Altered phenotypes such as decreased conidia production and reduced virulence in repeated mini-dome bioassays were observed among the transformants. These transformants provide materials for studying genetic factors related to virulence. The isolation of transformants unable to produce conidia may be significant because production of solanapyrone toxins is linked to conidiation. Further screening the transformants for phytotoxin production may identify additional altered phenotypes related to virulence.

This study identified optimal conditions for efficiently transforming *A. rabiei* using AMT. The method is reproducible and allows for other mutagenesis techniques including the targeted disruption of specific genes as well as ectopic complementation of loss-of-function strains. This technique will facilitate investigations into the genetics of pathogenicity factors of *A. rabiei*.

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