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EXPERIMENTAL ARTICLES

Agrobacterium tumefaciens-mediated Transformation of the Plant Pathogenic Fungus *Rosellinia necatrix*[1]

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Abstract—*Rosellinia necatrix* is a soil-borne root pathogen affecting a wide range of commercially important plant species. The mycelium of *R. necatrix* was transformed to hygromycin B resistance by an *Agrobacterium tumefaciens*-mediated transformation system using a binary plasmid vector containing the hygromycin B phosphotransferase (*hph*) gene controlled by the heterologous fungal *Aspergillus nidulans* P-*gpd* (glyceraldehyde 3-phosphate dehydrogenase) promoter and the *trpC* terminator. Co-cultivation of *R. necatrix* strain W1015 and *A. tumefaciens* strain AGL-1 at 25°C using the binary vector pAN26-CB1300, which contained the hygromycin B resistance cassette based on pAN26 and pCAMBIA1300, resulted in high frequencies of transformation. The presence of the *hph* gene in the transformants was detected by PCR, and single-copy integration of the marker gene was demonstrated by Southern b lot analy s is. This report of an *Agrobacterium*-mediated transformation method should allow the development of T-DNA tagging as a system for insertional mutagenesis in *R. necatrix* and provide a simple and reliable method for genetic manipulation.

Keywords: *Agrobacterium tumefaciens*, hygromycin B, *Rosellinia necatrix*, T-DNA, transformation.

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The filamentous ascomycete *Rosellinia necatrix* (Hartig) Berlese is a commercially important, soil-borne, root pathogen affecting a wide range of plant species. It is the causal agent of white root rot disease, and host plants infected by the fungus quickly wither and die. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grapevines, apple and pear trees, and other crops. Among the few effective control methods for white root rot are fungicides and biological control. However, long-term prevention of this root disease is difficult.

In filamentous fungi, the presence of double-stranded RNA (dsRNA) elements [1, 2] has been reported. The elements are known to reduce the virulence of phytogenic fungi. Such dsRNA elements in *R. necatrix* have also been reported [3]. Moreover, a novel bipartite dsRNA has been isolated and characterized from *R. necatrix* [4]. Valuable knowledge about the molecular basis of the pathogenicity of *R. necatrix* could be gained from genetic studies such as gene insertional mutagenesis. However, understanding the genetic basis of its pathogenicity has been limited by the lack of a suitable transformation system.

Agrobacterium tumefaciens-mediated transformation (ATMT), which has long been a workhorse in plant science, has been exploited for fungal transformation. *A. tumefaciens* has the ability to deliver its T-DNA into chromosomes of the budding yeast, *Saccharomyces cerevisiae* [5], and diverse filamentous fungi [6–9]. Besides ascomycetes and basidiomycetes, this technique has been successfully applied to transform zygomycetes also [10]. In comparison with Restriction Enzyme Mediated Integration (REMI), ATMT does not require protoplasts and allows a broad spectrum of starting material to be transformed. Protoplasts, hyphae, spores and even blocks of mushroom mycelial tissues [6] were transformed through ATMT with a higher efficiency than through REMI. DNA transfer from *A. tumefaciens* has been used for both gene knock-out and gene transformation studies in filamentous fungi [5, 7, 9] and is being developed as a system for insertional mutagenesis in filamentous fungi [8, 9].

In this study, we described a successful procedure for the genetic transformation of mycelium from *R. necatrix*. Transformation efficiency was optimized based on *Agrobacterium* strain and co-cultivation temperature. Single-copy T-DNA insertions into fungal genomes suggest that ATMT is a useful insertional mutagenesis and gene disruption tool in *R. necatrix*.

MATERIALS AND METHODS

Strains and culture conditions. The *R. necatrix* strains (W97, W1015, W370T1 and W779) used in this study

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Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics (locality)	Origin or provider
Rosellinia necatrix		
W97	Field-isolated original strain (Japan)	Dr. H. Nakamura
W1015	W779 protoplast-regenerated strain	Dr. H. Nakamura
W370T1	W370 single hypha-isolated strain	Dr. H. Nakamura
W779	Field-isolated original strain (Japan)	Dr. H. Nakamura
Agrobacterium tumefaciens (*Rhizobium radiobacter*)		
AGL-1	AGL0 *recA::bla* pTiBo542DT-region Mop^+ Cb^R	ATCC[11]
EHA105	L,L-succinamopine type, derivative of pTiBo542	Dr. Y. Niimi [12]
LBA4404	Ti plasmid pAL4404, derivative of pTiAch5	Dr. Y. Niimi [12]
Plasmid		
pAN26	*hph* gene	FGSC [13]
pBI121	Binary plasmid	Dr. Y. Niimi [14]
pCAMBIA1300	Binary plasmid	CAMBIA [15]
pAN26-BI121	Binary plasmid	This study
pAN26-CB1300	Binary plasmid	This study

(Table 1) were originally obtained from Dr. Hitoshi Nakamura (National Institute of Agro-Environmental Sciences, Japan) and were routinely grown on potato dextrose agar (PDA; potato extract, 2% glucose, 2% agar) at 25°C. Mycelia for DNA extraction were grown on a cellophane sheet overlaid on PDA. *A. tumefaciens* strain AGL-1 (ATCC BAA-101) was supplied by the American Type Culture Collection [11]. The *A. tumefaciens* strains EHA105 and LBA4404 [12] were kindly provided by Dr. Yoshiyuki Niimi (Prefectural University of Hiroshima, Japan), and are listed in Table 1. These *Agrobacterium* strains were routinely grown on Luria-Bertani (LB) agar (1% sodium chloride, 1% tryptone, and 0.5% yeast extract, pH 7.0) containing 50 μg/ml kanamycin to maintain the plasmid vector for transformation experiments.

Plasmid construction. The plasmid vectors pAN26 [13], pBI121 [14] and pCAMBIA1300 [15] were supplied by the Fungal Genetics Stock Center (Kansas City, KS, USA), Dr. Yoshiyuki Niimi (Japan) and the Center for Application of Molecular Biology to International Agriculture (Canberra, Australia), respectively (Table 1). The plasmids pAN26-BI121 and pAN26-CB1300 contain a hygromycin B resistance cassette, which is based on pAN26 for the selection of resistant fungal clones (Fig. 1), and a kanamycin resistance gene for selection in *Agrobacterium*. The hygromycin B resistance cassette contains the hygromycin phosphotransferase gene (*hph*) under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter (P-*gpd*) and the trpC terminator (T-*trpC*) from *Aspergillus nidulans* (Eidam) Winter. *A. tumefaciens* strains AGL-1, EHA105 and LBA4404 were transformed by electroporation with constructed binary vectors using a Gene Pulser system (Bio-Rad, Tokyo, Japan) according to the supplier's protocol [16].

***A. tumefaciens*-mediated fungal transformation.** The transformation procedure was based on the protocol described by Chen et al. [6], with some modifications. A single colony of *A. tumefaciens* (strains AGL-1, EHA105 and LBA4404) carrying the plasmid pAN26-BI121 or pAN26-CB1300 was grown in 30 ml LB broth containing 50 μg/ml kanamycin overnight at 28°C with shaking at 120 rpm. The cells were collected again and suspended in induction medium [5] to an optical density at 600 nm of 0.5–0.8. The bacterial suspension was incubated for 5–6 h at 28°C under agitation (120 rpm) to preinduce the virulence of *A. tumefaciens*.

The *R. necatrix* strains were grown in PD (potato extract and 2% glucose) ' medium for 1 week or on the surface of a cellophane sheet on PDA for 4 days at 25°C. The culture broth containing the mycelial mat was homogenized with a Nissei Homogenizer (AM-12) at 8,000 rpm for 2 min. The homogenized mycelium and the virulence-preinduced *A. tumefaciens* were mixed and collected by centrifiigation. The resultant pellet containing *R. necatrix* mycelium and *A. tumefaciens* was transferred onto a cellophane sheet overlaid on IM agar medium (IM + 2% agar). After 3 days of co-culture on these IM agar media at each temperature, 17°C, 20°C and 25°C, the cellophane sheets were transferred onto selection medium (SM: PDA containing 40 μg/ml hygromycin B for selection of transformants and 25 μg/ml meropenem for killing *A. tumefaciens* [17]). Hygromycin-resistant colonies appear after approximately 2 weeks. These colonies were transferred to and maintained on PDA containing 50 μg/ml hygromycin B.

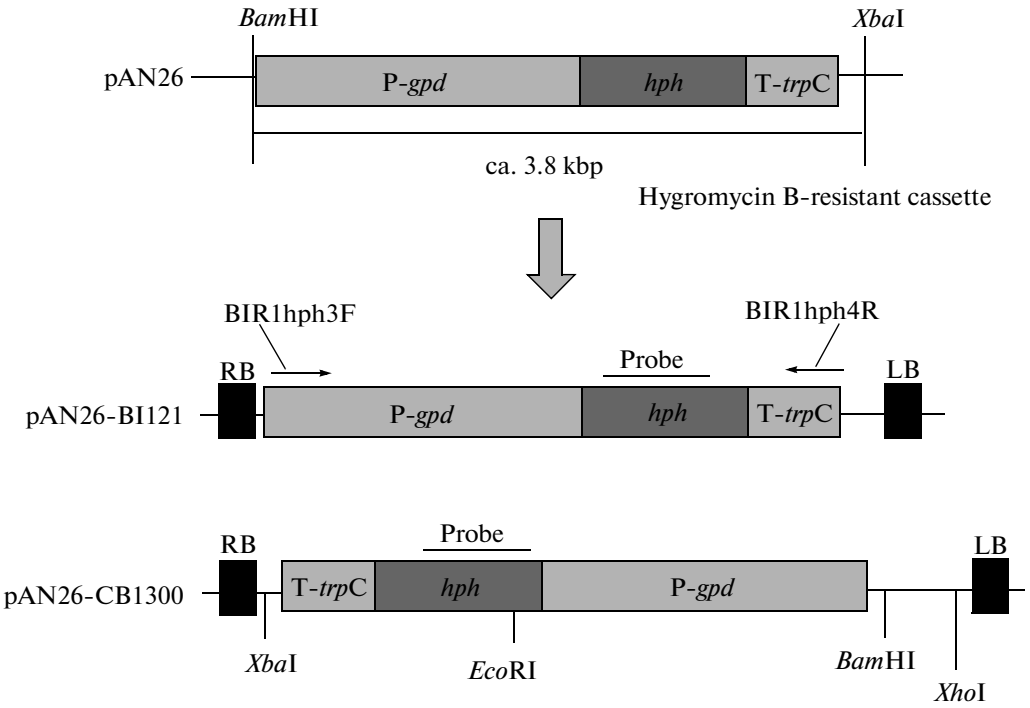

Fig. 1. Schematic representation of pAN26 and construction of pAN26-BI121 and pAN26-CB1300 used in the transformation of *R. necatrix*. pAN26-BI121 vector was constructed with the In-Fusion PCR Cloning System using the primers BIRlhph3F and BIRlhph4R which are indicated by arrows. pAN26-CB1300 was constructed using a restriction enzyme-based method. Bars correspond with hybridization signals, which are shown in Fig. 4. Abbreviations: *P-gpd* and T-*trp*C, promoter and terminator of *A. nidulans*, respectively; *hph*, the hygromycin B resistance gene; *RB* and *LB* represent the right and left borders of T-DNA, respectively.

To calculate the efficiency of the transformation, small mycelial cubes cut with a Pasteur pipette from the margins of young *R. necatrix* mycelium were inoculated onto cellophane sheets on PDA [18]. After overnight incubation at 25°C, the cellophane sheets with the young mycelial fragments were transferred to IM agar medium (Fig. 2B-a, b). Young mycelial fragments were co-cultivated by pouring the virulence-preinduced *A. tumefaciens* onto the IM agar medium containing the young mycelial fragments. The cellophane sheets were transferred onto SM after 3 days at 25°C, as described above. Hygromycin-resistant colonies on SM were counted to calculate the efficiency.

PCR and Southern blot analysis. Genomic DNA from *R. necatrix* strains was isolated using the hexadecyltrimethylammonium bromide (CTAB) procedure as described previously [19] and digested with the appropriate restriction endonucleases, separated by agarose gel electrophoresis, and blotted onto Hybond-N^+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). DNA hybridization probes were labeled and detected using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations.

To amplify the DNA of *hph* which was used to analyze the transformant strains and also used as a probe, the oligonucleotide primers hph1 (5'-TTC GAT GTA GGA GGG CGT GGA-3') and hph2 (5'-CGC GTC TGC TGC TCC ATA CAA G-3') were designed based on the nucleotide sequence of *hph* in pAN26 [20]. PCR was carried out according to the manufacturer's recommendations (Promega, Madison, Wis., USA). The amplified DNA fragments encoding *hph* were purified with a Wizard SV Gel and PCR Clean-Up System (Promega) and used as a DNA template for direct sequencing and as a probe for Southern blot analysis. DNA sequencing was carried out in an ABI 310 Genetic Analyzer (Applied Biosystems) by a chain-termination procedure with a BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) using the hph2 primer.

RESULTS AND DISCUSSION

***Agrobacterium*-mediated transformation of *R. necatrix*.** To establish whether *A. tumefaciens* transfers T-DNA to *R. necatrix*, we constructed the binary vectors pAN26-BI121 and pAN26-CB1300 (Fig. 1). pAN26-BI121 and pAN26-CB1300 were constructed by inserting into pBI121 and pCAMBIA1300, respec-

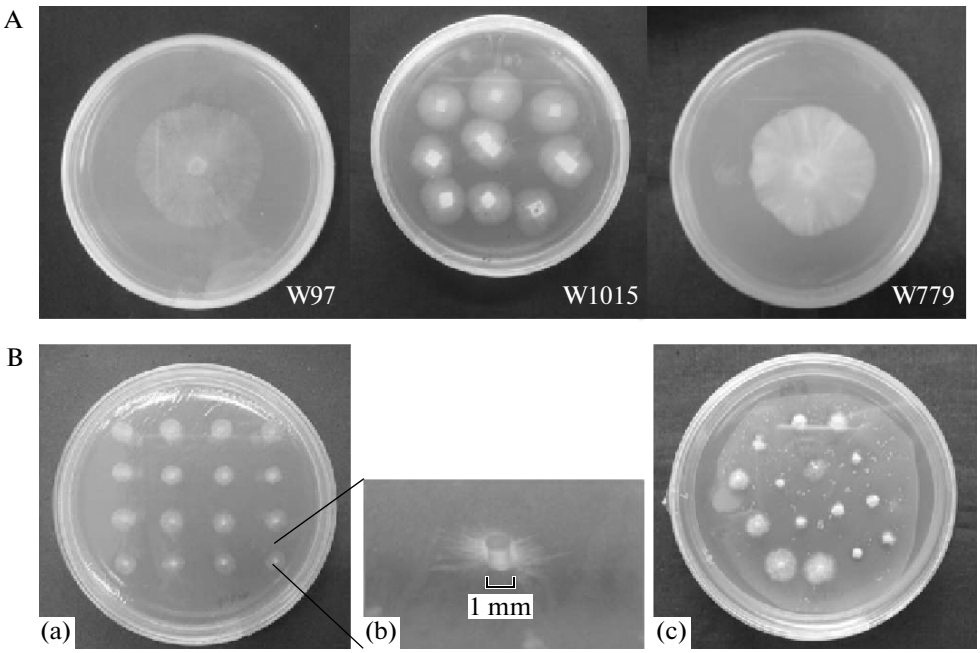

Fig. 2. Example of *A. tumefaciens*-mediated transformation of *R. necatrix*. (A) *A. tumefaciens* strain AGL-1 containing pAN26-CB1300 was co-cultivated with mycelium of *R. necatrix* strains W97, W1015 and W779. Cultures were grown for 4–7 days at 25°C on selection medium (SM) containing 50 μg/ml hygromycin B. (B) Young mycelia fragments of *R. necatrix* obtained by use of a Pasteur pipette were grown on PDA overnight at 25°C (a, b). Growth of putative transformants of *R. necatrix* on SM containing 50 μg/ml hygromycin B (c).

tively. Both constructs were introduced to three *A. tumefaciens* strains (Table 1).

The cocultivation temperature has also been shown [21, 22] to have an influence on transformation efficiency. The temperatures tested were 17°C, 20°C and 25°C; in the *Agrobacterium* strain AGL-1, the most reproducible frequencies of transformation were obtained when co-cultivation was at 25°C (Table 2). This finding is consistent with observations made in ATMT of *A. awamori* and *Botrytis cinerea* [21, 22]. These results indicate that the optimal temperature for transformation of *R. necatrix* is around 25°C, a similar temperature found to be optimal for T-DNA transfer machinery by *A. tumefaciens* [23].

We observed resistant fungal clones of the W97, W1015 and W779 strains on SM; however, we did not obtain resistant colonies from W370T1 (Table 2). Subsequently, we tried to inoculate the mycelium using a Pasteur pipette containing young mycelial fragments for transformation (Fig. 2B-a, b). The efficiency of the transformation, based on the number of hygromycin resistant colonies per 10^2 mycelial fragments, was easily determined for each co-cultivation condition, which used pAN26-CB1300 (Fig. 2 B-c).

The most reproducible transformation frequencies were obtained when W1015 was used for transformation (Table 3). W97 and W779 had significantly better reproducible transformation frequencies than W370T1. There appears to be variation in the transformation efficiency between *R. necatrix* strains. In some other fungi, the transformation frequency obtained varied between different isolates of the same fungal species [20, 24]. The cause for this variation was not determined, but it could result from differences in cell wall components or in growth conditions.

The transformation efficiency was compared using three different strains of *A. tumefaciens*. There were differences in transformation efficiencies among the strains. For the W1015 strain, which had the highest transformation efficiency, AGL-1 was found to be the most effective than another strain (Table 3). For the W97 strain, transformation efficiencies of AGL-1 and LBA4404 were higher than EHA105. In contrast, the transformation efficiency of LBA4404 was lower than AGL-1 and EHA105 for W779 transformation. For W370T1, which had the lowest efficiency, we were unable to obtain resistant colonies in co-cultivation with EHA105. The transformation efficiency of AGL-1 and LBA4404 was almost the same. Our results might be caused by host specificity of *A. tumefaciens* and variation in sensitivity of *R. necatrix* to *A. tumefaciens*. In particular, AGL-1 showed high efficiency with all *R. necatrix* strains. Various *Agrobacterium* strains have been used for the transformation of *Cryphonectria parasitica*. In *C. parasitica*, *Agrobacterium* AGL-1 was more efficient for transformation than LBA4404 [25]. These results suggest that the difference in the transformation efficiency was due to the genetic background of the *Agrobacterium* strains [12].

Table 2. Growth inhibition tests on PDA medium and *A. tumefaciens*-mediated transformation of *R. necatrix*

R. necatrix	Hygromycin B (μg/ml)							*A. tumefaciens*	Plasmid	Co-cultivation temp. (°C)
	0	10	20	30	40	50	100			
Wild-type										
W97	+	+	+	−	−	−	−			
W1015	+	+	+	−	−	−	−			
W370T1	+	+	+	−	−	−	−			
W779	+	+	+	−	−	−	−			
Transformant										
W97MT-1	+	+	+	+	+	+	+	AGL-1	pAN26-CB1300	25
W1015MT-1	+	+	+	+	+	+	+	AGL-1	pAN26-CB1300	25
W1015MT-2	+	+	+	+	+	+	+	AGL-1	pAN26-BI121	25
W1015MT-3	+	+	+	+	+	+	+	AGL-1	pAN26-BI121	25
W779MT-1	+	+	+	+	+	+	+	AGL-1	pAN26-CB1300	20

Note: +, could grow; −, could not grow.

In conclusion, by defining the co-cultivation temperature, *R. necatrix* and *Agrobacterium* strain, we determined the optimal T-DNA transfer conditions (W1015 were co-cultivated with AGL-1 containing pAN26-CB 1300 at 25°C).

Test of growth inhibition by hygromycin B on the five transformants are shown in Table 2 and Fig. 2A. All transformants could grow on PDA supplemented with 100 μg/ml hygromycin B. On the other hand, the wild-type strains W97, W1015 and W779 could not grow on the same medium. These results suggest that the introduced *hph* under the control of the *P-gpd* promoter from *A. nidulans* is functional in the *R. necatrix* cells.

Table 3. *A. tumefaciens*-mediated transformation of *R. necatrix* using a Pasteur pipette

A. tumefaciens	Plasmid	Hygromycin^R colonies per 10^2 fragments			
		W97	W1015	W370T1	W779
AGL-1	pAN26-CB1300	1.78	3.57	0.89	1.76
EHA105	pAN26-CB1300	0.89	2.65	0.00	1.78
LBA4404	pAN26-CB1300	1.78	2.65	0.89	0.89

Integration of *hph* in to the genome of *R. necatrix*. The putative hygromycin-resistant transformants of *R. necatrix* were analyzed by PCR and Southern blot analysis to determine the fate of the resistance gene after transformation. In the PCR analysis (using hph1 and hph2), all transformants showed amplification of the expected 626 bp DNA fragment (Fig. 3, lanes *1*, *3–5*, and *7*), whereas no amplified fragments appeared using a template derived from wild-type strains (Fig. 3, lanes *2*, *6*, and *8*). When sequenced, the amplified DNA bands corresponded to the expected 626 bp internal fragment of the *hph* gene (data not shown).

To determine the T-DNA copy number, we performed restriction analysis of genomic DNA isolated from the five transformants followed by Southern blot analysis with an amplified DNA fragment of *hph* labeled with fluorescein as the probe. Genomic DNAs from wild-type strains W1015, W97 and W779 did not produce a hybridization signal (Fig. 4, lanes *4*, *6*, and *8*). All of the transformants contained a single copy of T-DNA in their genomes (Fig. 4, lanes *1–3*, *5*, and *7*). Southern blot analysis of strain W1015 showed hybridizing bands of different sizes, indicating that the T-DNA was integrated at different chromosomal locations (Fig. 4, lanes *1–3*). Some studies have reported that multiple integrations predominate in T-DNA transfer to filamentous fungi [26]. However, similar results have been reported in *Venturia inaequalis* [24] and *Beauveria bassiana* [8], where a majority of transformants contained a single copy of T-DNA in the genome. Therefore, the genetic manipulation of

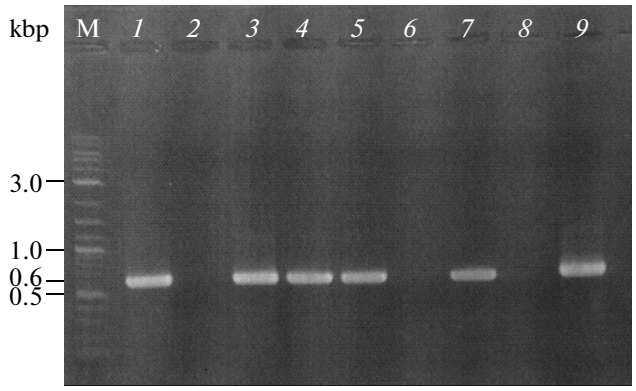

Fig. 3. PCR analysis of the *hph* gene from putative hygromycin-resistant transformants of *R. necatrix*. PCR amplification was carried out using genomic DNA and primers hphl and hph2, amplifying an approx. 0.6 kbp fragment. M, DNA molecular size markers (in kilobases) are indicated on the left; lanes 1, 3-5 and 7, transformant W97MT-1, W1015MT-1, W1015MT-2, W1015MT-3 and W779MT-1, respectively; lanes 2, 6 and 8, wild-type strain W97, W1015, W779, respectively; lane 9, positive control with pAN26-CB1300.

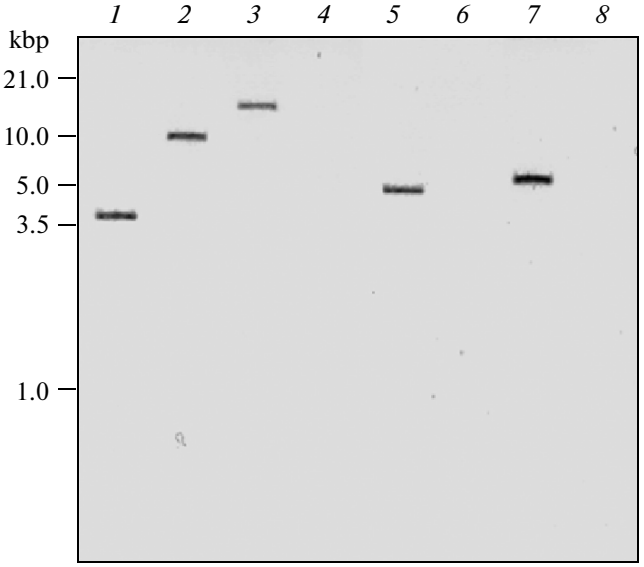

Fig. 4. Southern blot analysis of *R. necatrix* transformants. Genomic DNA (1 μg) was digested with *Xba*I and separated on 0.8% agarose gels. Hybridization was performed at 62°C using a digoxigenin (DIG)-labeled *hph* gene probe. Lanes *1–3*, transformant W1015MT-1, W1015MT-2, and W1015MT-3; lane *4*, wild-type strain W1015; lane *5*, transformant W97 MT-1; lane *6*, wild-type strain W97; lane *7*, transformant W779 MT-1; lane *8*, wild-type strain W779. The positions of molecular DNA size markers (in kilobases) are shown on the left.

R. necatrix may be possible because *A. tumefaciens* transformation resulted in single-copy integration.

Agrobacterium-mediated transformation is simpler and less labor intensive than protoplast-based transformation systems. It should be possible to adapt this approach for gene transfer, gene knock-out and insertional mutagenesis through T-DNA tagging. The development of T-DNA transfer as a deficient and repeatable transformation system for *R. necatrix* will greatly facilitate the molecular analysis of this important fungus.

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