AAL-Toxin-Deficient Mutants of *Alternaria alternata* Tomato Pathotype by Restriction Enzyme-Mediated Integration

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ABSTRACT

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Host-specific toxins are produced by three pathotypes of *Alternaria alternata*: AM-toxin, which affects apple; AK-toxin, which affects Japanese pear; and AAL-toxin, which affects tomato. Each toxin has a role in pathogenesis. To facilitate molecular genetic analysis of toxin production, isolation of toxin-deficient mutants utilizing ectopic integration of plasmid DNA has been attempted. However, the transformation frequency was low, and integration events in most transformants were complicated. Addition of a restriction enzyme during transformation has been reported to increase transformation frequencies significantly and results in simple plasmid integration events. We have, therefore, optimized this technique, known as restriction enzyme-mediated integration (REMI), for *A. alternata* pathotypes. Plasmid pAN7-1, conferring resistance to hygromycin B, with no detectable homology to the fungal genome was used as the transforming DNA. Among the three restriction enzymes examined, *Hind*III was most effective, as it increased transformation frequency two- to 10-fold depending on the pathotype, facilitating generation of several hundred transformants with a 1-day protocol. *Bam*HI and *Xba*I had no significant effect on transformation frequencies in *A. alternata* pathotypes. Furthermore, the transforming plasmid tended to integrate as a single copy at single sites in the genome, compared with trials without addition of enzyme. Libraries of plasmid-tagged transformants obtained with and without addition of restriction enzyme were constructed for the tomato pathotype of *A. alternata* and were screened for toxin production. Three AAL-toxin-deficient mutants were isolated from a library of transformants obtained with addition of enzyme. These mutants did not cause symptoms on susceptible tomato, indicating that the toxin is required for pathogenicity of the fungus. Characterization of the plasmid integration sites and rescue of flanking sequences are in progress.

Since the first finding of host-specific toxicity in culture filtrates of the *Alternaria alternata* (Fries) Keissler Japanese pear pathotype (formerly *A. kikuchiana*) by Tanaka in 1933 (18), studies on host-specific toxins (HSTs) have contributed to a great advance in research on plant-microbe interactions. The search for HSTs associated with *A. alternata* diseases resulted in the finding of seven toxins, and the chemical structures of five of these have since been determined (4,7,11). Extensive studies on the mode of action of these HSTs have resulted in an understanding of the real importance of these HSTs, not only in inducing necrosis, but in suppressing host defenses, thereby preparing the plant for infection by the fungus (9,15). Subsequently, with the advance of molecular biology, the biggest concern in HST research has been to clone the genes involved in the biosynthesis of toxin.

Genes involved in the biosynthesis of HSTs have been cloned from *Cochliobolus carbonum* and *C. heterostrophus* race T, producers of HC- and T-toxin, respectively. HC-toxin synthetase, *HTS1*, was cloned through a traditional genetic approach utilizing protein purification and library screening (10,22,23). In contrast, several approaches have been taken to mutagenize *Tox1*, a genetic locus involved in the biosynthesis of T-toxin (25,28). Among the approaches taken, restriction enzyme-mediated integration (REMI) insertional mutagenesis gave a high transformation frequency, simple plasmid integration events, and yielded T-toxin-deficient mutants. Flanking genomic DNA was rescued from one mutant; its sequence identified a polyketide synthase required for T-toxin biosynthesis (6).

REMI was first reported in yeast by Schiestl and Petes (16) for studying the mechanism of illegitimate recombination. Later, addition of restriction enzyme to the transformation reaction protocol was reported to increase transformation frequency in *Dictyostelium discoideum* (5) and the filamentous fungi *C. heterostrophus* race T (6), *Magnaporthe grisea* (17), and *Ustilago maydis* (1). Furthermore, the linearized transforming vector was integrated at the restriction sites in the genome via the sticky ends, suggesting a simple protocol for cloning genes by plasmid rescue.

A method for transforming *A. alternata* was established by Tsuge et al. (19) using the plasmid pDH25, which confers hygromycin B resistance. However, the number of transformants generated by this method was low, and plasmid integration events were complex. Later, an efficient transformation system was developed using a plasmid containing the gene encoding rRNA (19), but transformation mediated by homologous recombination made it inadequate for gene tagging. Thus, efficient transformation in *A. alternata* by ectopic integration has not been possible. This paper assesses the prospect of addition of restriction enzyme to the transformation protocol of *A. alternata* from various standpoints and describes the construction of REMI-generated libraries and subsequent detection of HST-deficient mutants.

MATERIALS AND METHODS

Fungal strains. Three strains of *A. alternata* (apple pathotype, Japanese pear pathotype, and tomato pathotype [*A. alternata* f. sp. *lycopersici*]) were used in this work and designated M-71, O-276, and As-27, respectively. All strains were purified by single spore isolation and were stored in 20% glycerol at -80°C .

Preparation of protoplasts. Fungal protoplasts were prepared through modifications of the methods described by Yelton et al.

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(26) and Tsuge et al. (19). Fungi were grown in 50 ml of potato dextrose (PD) broth, inoculated with hyphal blocks cut from agar-based cultures, and incubated at 26°C with vigorous reciprocal shaking (80 strokes/min) for 4 days. Mycelia were harvested by centrifugation at $3,000 \times g$ for 5 min and washed with sterilized H₂O followed by sterilized OM buffer (1.2 M MgSO₄ and 10 mM Na₂HPO₄, pH 5.8). Washed mycelia were mixed with 5 ml of sterile enzyme solution containing 10 mg of Novozym 234 (Novo Nordisk, Bagsvaerd, Denmark)/ml and 15 mg of Driselase (Kyowa Hakkou, Tokyo)/ml in OM buffer. This mixture was shaken (80 strokes/min) for 4 to 5 h at 30°C. The culture was then transferred to a centrifuge tube and overlaid with 2 ml of ST buffer (0.6 M sorbitol and 100 mM Tris-HCl, pH 8.0). Protoplasts were banded at the interface of OM buffer and ST buffer by centrifuging at $750 \times g$ for 5 min, transferred to a new tube, mixed with 5 ml of STC buffer (1 M sorbitol, 50 mM CaCl₂, and 50 mM Tris-HCl, pH 8.0), and separated from cell debris and hyphae by centrifuging at $80 \times g$ for 5 min. Protoplasts were pelleted by centrifugation at $750 \times g$ for 5 min, and then washed twice with 5 ml of STC buffer and resuspended in STC buffer to a final concentration of 1.25×10^8 per milliliter. About 1.0×10^8 protoplasts were generally obtained from 50 ml of a 4-day-old culture.

Transformation. Transformations were carried out in 1.5-ml Eppendorf tubes according to procedures modified from those of Vollmer and Yanofsky (21). For transformation, purified plasmid pAN7-1 (12) was initially linearized by digestion with *Bam*HI, *Hind*III, or *Xba*I, with each being cut once, extracted with phenol/chloroform, extracted with chloroform, and precipitated with ethanol. Plasmid pAN7-1 contains a single recognition site for *Hind*III and *Xba*I at the end of the terminator region of the *Aspergillus nidulans trpC* gene (*TrpC*) and a single site for *Bam*HI between the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) and *TrpC* (12). Twenty microliters of 40% polyethylene glycol (PEG) 4,000 solution in STC buffer and linearized pAN7-1 (1 or 5 µg) were added to 80 µl of protoplast suspension in STC buffer containing 1.0×10^7 protoplasts. In the case of transformation with addition of restriction enzyme, 0.01 to 100 units of restriction enzymes were simultaneously added with plasmid. The solution was mixed gently and incubated on ice for 30 min. A further 900 µl of 40% PEG 4,000 solution was added, and the solution was mixed and incubated at room temperature for 10 min. Approximately 1 ml of this reaction mixture was mixed with 5 ml of regeneration medium (0.1% yeast extract, 0.1% casein enzymatic hydrolysate, and 1 M sucrose) (19) containing 0.8% agar main-

Fig. 1. Effect of addition of restriction enzymes on the transformation frequencies in the *Alternaria alternata* tomato pathotype (As-27). Transformation with addition of restriction enzymes was carried out with protoplasts prepared from As-27 and A, 5 µg and B, 1 µg of pAN7-1. Enzymes used in this experiment were *Bam*HI, *Hind*III, and *Xba*I. Each value indicates the average and standard deviation of three duplicate experiments. Transformation frequencies with addition of *Hind*III are significantly different at $P = 0.05$ according to Fisher's least significant differences (LSD) test. LSD value is indicated by LSD bar.

Fig. 2. Effect of addition of *Hind*III on the transformation frequencies in *Alternaria alternata*. Transformation with addition of *Hind*III was carried out by using protoplasts prepared from wild type of *A. alternata* and A, 5 µg and B, 1 µg of pAN7-1. The *A. alternata* used in this experiment were the apple pathotype, the Japanese pear pathotype, and the tomato pathotype. Each value indicates the average and standard deviation of three duplicate experiments. Transformation frequencies with addition of *Hind*III on the apple pathotype are significantly different at $P = 0.05$ according to Fisher's least significant difference (LSD) test. LSD value is indicated by LSD bar.

tained at 47°C and overlaid onto a plate containing regeneration medium (1.5% agar). After incubation at 26°C overnight, 5 ml of 1.5% agar containing 400 µg of hygromycin B (Wako Pure Chemical Industries, Osaka, Japan)/ml was poured onto the plate.

DNA manipulations. Plasmid pAN7-1 was maintained in *E. coli* HB101, isolated by the alkaline lysis method, and purified by polyethylene glycol precipitation as described by Sambrook et al. (14). Plasmid DNA was linearized with a restriction enzyme (*Bam*HI, *Hind*III, or *Xba*I) and used after phenol/chloroform extraction, chloroform extraction, and ethanol precipitation. Linearized pAN7-1 was finally resuspended to a final concentration of 1 mg/ml in sterile TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Fungal genomic DNA was isolated by the miniprep method described by Yoder (27) from freeze-dried mycelium from cultures grown in 50 ml of PD broth at 26°C with shaking (80 strokes/min) for 4 days. Genomic DNA was digested with restriction enzymes and separated by agarose gel electrophoresis using standard techniques (14). Fractionated DNA was transferred to Hybond N⁺ nylon membranes (Amersham International plc., Buckinghamshire, United Kingdom) by capillary transfer under alkaline conditions (13).

Leaf necrosis assay for AAL-toxin production. All transformants were transferred to fresh selective medium (PD agar containing hygromycin B at 100 µg/ml.). Three hundred of 800 transformants were inoculated individually into 10 ml of Richard's liquid medium, incubated for 2 weeks, and then tested for AAL-toxin production. Host-specificity in these culture filtrates was determined by a necrosis assay on tomato leaves of a susceptible (Earlpack 7) and a resistant (Ace) cultivar. The surfaces of the leaves were scratched near the center with a needle, and culture filtrate samples (200 µl) were placed on each wounded site. Treated leaves were incubated in a moist chamber under irradiation of light (100 µE m⁻² s⁻¹) at 26°C for 4 days, and toxin activity was determined by induction of veinal necrosis on the susceptible cultivar.

Pathogenicity test. Pathogenicity of the three AAL-toxin-deficient mutants was tested on the susceptible (Earlpack 7) and resistant (Ace) tomato cultivars. Young leaves of both cultivars were inoculated by spraying spore suspension (1.0 × 10⁶ spores/ml) with a glass atomizer. After the leaves were incubated in a moist chamber at 26°C for 5 days, lesion development on the leaves was evaluated.

Fig. 3. Effect of addition of *Hind*III on the transformation frequencies in *Alternaria alternata*. Transformation with a low amount of *Hind*III was carried out with protoplasts prepared from wild type of *A. alternata* and 5 µg of pAN7-1. The numbers of protoplasts used in this experiment were 10⁷ and 10⁶. Each value indicates the average and standard deviation of three duplicate experiments. Transformation frequencies with addition of *Hind*III on 10⁷ protoplasts are significantly different at *P* = 0.05 according to Fisher's least significant difference (LSD) test. LSD value is indicated by LSD bar.

RESULTS

Transformation of *A. alternata* with circular or linearized non-homologous plasmid DNA yields about five transformants per microgram of DNA per 10⁷ protoplasts. The effects of three restriction enzymes on the transformation frequencies in the *A. alternata* tomato pathotype are shown in Figure 1. When 20 units of *Bam*HI, *Hind*III, or *Xba*I was added separately to the transformation mixture containing 5 µg of pAN7-1 linearized with the same enzyme, *Hind*III significantly increased in the transformation frequency by sevenfold compared with control (Fig. 1A). When 20 units of each enzyme was also added separately to a mixture containing 1 µg of pAN7-1, *Hind*III increased the transformation frequency by sixfold compared with control (Fig. 1B). On the other hand, *Xba*I had no statistically significant effect on transformation efficiencies. In the case of *Bam*HI, only a few transformants were generated. Addition of a higher amount of restriction enzymes (50 and 100 units) in the transformation mixture caused a smaller effect than addition of 20 units. No changes in the viability of protoplasts by addition of *Bam*HI and higher amounts of enzymes were observed in these experiments (data not shown).

Trials to investigate the effects of *Hind*III on the transformation frequencies of the *A. alternata* apple and Japanese pear pathotypes were also carried out (Fig. 2). When 20 units of *Hind*III was added to the transformation mixture containing 5 µg of pAN7-1, the transformation frequency was significantly increased by 10-fold in the apple pathotype (Fig. 2A). In the case of transformation with 1 µg of pAN7-1, addition of restriction enzymes had no significant effect on the transformation efficiencies in apple and Japanese pear pathotypes (Fig. 2B). A higher amount of *Hind*III (50 and 100 units) in the transformation of these two pathotypes resulted in a smaller effect compared with 20 units. The results resemble those obtained with the tomato pathotype (Figs. 1 and 2).

The effect of adding less than 20 units of *Hind*III on the transformation frequency with 5 µg of pAN7-1 with the tomato pathotype was also investigated (Fig. 3). When 10⁷ protoplasts was used, the transformation frequency increased by eightfold with addition of 1 unit of *Hind*III compared with a transformation without en-

Fig. 4. Southern blot showing hybridization of pAN7-1 to genomic DNA digests of *Alternaria alternata* tomato pathotype transformants obtained by addition of restriction enzymes. *Eco*RI, *Hind*III, and *Bam*HI digests of genomic DNA were hybridized with [DIG-11-dUTP]-labeled pAN7-1. Lane 1, 27HR-45; lane 2, 27HR-73; lane 3, 27HR-234; lane 4, 27HR-250; and lane 5, 27HR-265. The numbers on the left of the figure indicate the position and size in kilobase pairs (kb) of *Hind*III-digested λ size marker.

zyme. However, in experiments with 10⁶ protoplasts, addition of 1 unit of *Hind*III resulted in only a twofold increase in the transformation frequency. When 10⁶ protoplasts and less than 20 units of *Hind*III were used in the apple and Japanese pear pathotypes, this increase was not observed (data not shown). Thus, transformations with low amounts of *Hind*III in the three pathotypes were highly efficient, with 200 to 1,000 transformants being generated by a 1-day protocol.

The number of sites and number of copies of integrated *Hind*III-linearized pAN7-1 in *A. alternata* tomato pathotype transformants obtained by transformation with or without restriction enzyme were analyzed as follows. Genomic DNA of 34 and 23 transformants were digested with *Eco*RI, an enzyme that cuts twice in pAN7-1, and *Hind*III and *Bam*HI, which both cut once in pAN7-1, respectively, and Southern blots of these digests were probed with [DIG-11-dUTP]-labeled pAN7-1 (Figs. 4 and 5). All transformants used for Southern hybridization were generated with *Hind*III-linearized pAN7-1. In all transformants except 27HR-250 and 27H-27 (Fig. 4, lane 4, and Fig. 5, lane 4), hybridization of *Eco*RI digests with pAN7-1 showed the 2.5-kb internal sequence of the plasmid containing *Pgpd* and part of *hph*. Transformants 27HR-45 and 234 (Fig. 4, lanes 1 and 3) showed, in addition, two other bands interpreted to represent the internal sequence of pAN7-1 plus flanking genomic sequence. These results indicate that one copy of pAN7-1 integrated into the genome of these two transformants. A Southern blot of *Hind*III and *Bam*HI digests in these transformants showed one band of more than 6.6-kb, one intense band corresponding to the 5.9-kb *Hind*III-*Bam*HI portion of pAN7-1 plus flanking genomic DNA, and one faint band corresponding to the 0.7-kb *Bam*HI-*Hind*III portion of pAN7-1 plus flanking genomic DNA. These signals are consistent with the results of *Eco*RI digests.

A Southern blot of transformants generated without *Hind*III addition indicated that plasmid integration was complex (Fig. 5). In transformants generated with *Hind*III, the percentage of a single copy integration of pAN7-1 into a single site was 41.2%, but, in those without *Hind*III, it was 13.0% (Table 1).

Hybridization signals in *Eco*RI and *Hind*III digests were analyzed to determine whether the plasmid integration site was ran-

dom, and whether transformation with addition of restriction enzyme causes a REMI event. The REMI event was defined as an enzymatically linearized plasmid integrated into an identical restriction site in a genome (5,16). A Southern blot of *Eco*RI digests (Figs. 4 and 5) was different in each transformant, suggesting that integration sites were random. If integration of pAN7-1 occurred at target restriction sites in the genome, then a 6.6-kb band (the size of pAN7-1) should be detected. However, Southern blots of *Hind*III digests of transformants 27HR-45 and 234 (Fig. 4, lanes 1 and 3) showed one band larger than 6.6 kb, indicating that at least one *Hind*III restriction site on either the plasmid, flanking genomic DNA, or both were destroyed or deleted. In almost all transformants analyzed, the single 6.6-kb band in *Hind*III digests was not found, indicating that the frequency of REMI events was only 5.88% (Table 1).

To detect and isolate AAL-toxin-deficient mutants, 300 transformants in a library of 800 generated via pAN7-1 (with or without *Hind*III addition) were tested for AAL-toxin production. Three transformants, 27HR-100, 108, and 215, obtained in the presence of *Hind*III, failed to produce detectable AAL-toxin (Fig. 6). These mutants were purified by single spore isolation. The AAL-toxin-deficient phenotype was stable in all mutants after at least five subcultures on nonselective medium. When these toxin-deficient mutants were grown on PD agar medium, growth rates and colony morphologies were similar to those of wild-type strain As-27. Moreover, it was confirmed by random amplified polymorphic DNA (RAPD) analysis using five random primers that these three mutants were derived from As-27 (data not shown).

Pathogenicity tests by inoculation of spores obtained from these toxin-deficient mutants on tomato leaves resulted in no symptoms, which were distinguishable from those caused by inoculating spores of As-27 on tomato leaves (Fig. 7).

DISCUSSION

The feasibility of gene isolation from *A. alternata* using gene tagging by transformation with addition of restriction enzymes was investigated. The first requirement for such a strategy is to establish a high frequency transformation system. However, the frequency of transformation in some filamentous fungi with non-homologous plasmids is often low (2,19,20) and, despite the fact that pAN7-1 gives good transformation frequencies with many filamentous fungi (3,8,24), this has not been the case with *A. alternata*. By utilizing a transformation protocol with addition of restriction enzyme as first described in yeast (16), *D. discoideum* (5), and a few filamentous fungi (1,6,17) to the transformation in *A. alternata*, an improvement in the transformation frequency has been achieved. This phenomenon is consistent with reports in yeast (16), *D. discoideum* (5), *C. heterostrophus* (6), and *M. grisea* (17), but inconsistent with findings with *U. maydis* (1). The increase in transformation frequency of the *A. alternata* tomato pathotype

Fig. 5. Southern blot showing hybridization of pAN7-1 to genomic DNA digests of *Alternaria alternata* tomato pathotype transformants obtained without addition of restriction enzymes. *Eco*RI and *Hind*III digests of genomic DNA were hybridized with [DIG-11-dUTP]-labeled pAN7-1. Lane 1, 27H-23; lane 2, 27H-24; lane 3, 27H-26; lane 4, 27H-27; lane 5, 27H-28; lane 6, 27H-30; lane 7, 27H-33; and lane 8, 27H-34. The numbers on the left of the figure indicate the position and size in kilobase pairs (kb) of *Hind*III-digested λ size marker.

TABLE 1. Comparison of number of sites and copies of pAN7-1 integrated in transformants of the *Alternaria alternata* tomato pathotype (As-27) obtained from transformation with and without addition of enzyme

Treatment in transformation ^a	No. of transformants analyzed	No. of sites and copies of integration ^b		No. of REMI event ^c
		1 site and 1 copy	Others	
With enzyme	34	14 (41.2%)	20 (58.8%)	2 (5.88%)
Without enzyme	23	3 (13.0%)	20 (87.0%)	0 (0%)

^a One to 5 μ g of pAN7-1 and 0.01 to 100 units of *Hind*III were used.
^b (Number of transformants having a single copy integration into a single site or other integration profile)/(Number of transformants analyzed) are given with percentage in parentheses.
^c (Number of transformants having an integration profile by a restriction enzyme-mediated integration (REMI) event)/(Number of transformants analyzed) are given with percentage in parentheses.

exhibited by addition of *Hind*III was also shown in the *A. alternata* apple pathotype, but not the Japanese pear pathotype. Addition of restriction enzyme presumably increases the transformation frequency by allowing integration of the plasmid where the genome has been nicked by digestion with enzyme. With the *A. alternata* tomato pathotype, the amount of transforming DNA (1 to 5 µg) appeared to have no effect on the frequency of transformation. This was also true for the *A. alternata* Japanese pear pathotype, but not for the *A. alternata* apple pathotype (Fig. 2). In previous studies on *C. heterostrophus* (6) and *M. grisea* (17), large amounts of *Hind*III were used in the transformation. This contrasts with our results, in which low amounts of *Hind*III gave higher transformation frequencies (Fig. 2). The optimal amounts of *Hind*III for *M. grisea* and the *A. alternata* tomato pathotype were 200 and 1 units, respectively. Transformation in the *A. alternata* tomato pathotype with addition of *Bam*HI, which was the most effective in yeast (16), *D. discoideum* (5), and *M. grisea* (17), gave very poor results, with the transformation frequency being similar to the protocol of transformation without addition of restriction enzyme.

Variation in transformation frequency was detected with each enzyme used with the *A. alternata* tomato pathotype, and two interesting points were found: first, a decrease in transformation frequency with high amounts of *Hind*III and *Xba*I; and, second, no significant increase in transformation frequencies by using *Bam*HI for plasmid linearization and transformation. Also, in *C. heterostrophus* (6) and *M. grisea* (17), the first effect was observed. Higher

amounts of enzyme may decrease protoplast viability, and the poor results with *Bam*HI may be a consequence of this enzyme cutting off the pAN7-1 terminator, which may not be reconstructed after integration. This contrasts with the case of *Penicillium paxilli* (3) in which *Bam*HI-linearized pAN7-1-mediated transformants were readily obtained even when only one copy of the plasmid integrated. An investigation into protoplast viability showed that the amount of enzyme had no effect. This result corresponds to those of yeast (16) and *M. grisea* (17). Interestingly, transformation in *M. grisea* with a linearized plasmid treated by phenol/chloroform extraction or heat at 70°C to inactivate the enzyme increased the transformation frequency by seven- or 11-fold, respectively. This was not the case in *C. heterostrophus* (6), the *A. alternata* tomato pathotype (data not shown), and *P. paxilli* (Y. Itoh, *personal communication*), in which a larger effect with nontreated plasmid occurred. Since we found that plasmid before phenol/chloroform extraction was more effective than after extraction, it is possible that, after extraction, the residual enzyme activity is removed.

Despite the varied transformation frequencies with different enzymes and fungi, this transformation technique appears useful for construction of libraries of plasmid-tagged transformants.

For optimal gene isolation, transformants should have simple plasmid integration events at random sites in the genome. Addition of *Hind*III to the transformation caused pAN7-1 to integrate at a single site more commonly than when enzyme was excluded. In addition, the integration site appeared to be random based on

Fig. 6. Bioassay of culture filtrate of AAL-toxin-deficient mutants of the *Alternaria alternata* tomato pathotype on tomato leaves. As-27 (wild-type strain), 27HR-100, -108, and -215 (toxin-deficient mutants obtained by transformation with addition of restriction enzyme).

Fig. 7. Pathogenicity of wild type and AAL-toxin-deficient mutants of the *Alternaria alternata* tomato pathotype on tomato leaves. Spores of wild type and mutants were inoculated on susceptible ('Earlypak 7') and resistant ('Ace') tomato leaves. Lesions were observed after 5 days of inoculation. As-27 (wild-type strain), 27HR-100, -108, and -215 (toxin-deficient mutants obtained by transformation with addition of a restriction enzyme).

hybridization signals of pAN7-1 to *EcoRI* genomic digests. In the *A. alternata* tomato pathotype, 41.2% of *HindIII* addition transformants had a single copy of pAN7-1 integrated into a single site, indicating that the integration profile is simplified by addition of this enzyme compared with no addition (Figs. 4 and 5 and Table 1). This percentage is not as high as that shown for yeast (16), *D. discoideum* (5), *M. grisea* (17), and *U. maydis* (1). Thus, transformation of the *A. alternata* tomato pathotype with addition of a restriction enzyme simplifies the plasmid integration profile, making gene tagging an attainable goal.

In yeast, Schiestl and Petes (16) reported REMI as an event that recognized both restriction sites generated by cutting the plasmid and genomic DNA. Thus, DNA from transformants digested with the same enzyme as that used in the transformation and for plasmid linearization should yield a band equal to the size of the inserted DNA. In *D. discoideum* (5), *M. grisea* (17), and *U. maydis* (1), this event occurred with a high frequency, indicating that REMI events are common. However, in the *A. alternata* tomato pathotype, REMI events were detected in only two transformants based on this criterion (Table 1). To truly determine whether REMI events are occurring, sequence analysis at the integration site will be required, as was carried out in yeast (16).

The end goal of this research is to isolate a gene involved in HST synthesis by gene tagging using REMI. So far, this method has been used successfully with a number of systems. In *D. discoideum* (5), various developmental mutants have been acquired and, in *M. grisea* (17), 0.8% of transformants examined were mutants that exhibited defects in sporulation, auxotrophy, and reduced pathogenicity. Moreover, in *C. heterostrophus* (6), a producer of T-toxin, two toxin-deficient mutants were isolated by tagging the *Tox1* locus using REMI mutagenesis. In our current study, we succeeded in isolating AAL-toxin-deficient mutants in the *A. alternata* tomato pathotype. All toxin-deficient mutants did not cause symptoms in susceptible tomato. This result supports the hypothesis that AAL-toxin is required for pathogenicity of the tomato pathotype on susceptible tomato. Therefore, the REMI procedure is an effective approach to create useful and interesting mutants in *Alternaria* pathogens, but the mutants may be due to events unrelated to plasmid integration, i.e., the mutated gene may not be tagged with the plasmid. In the future, we hope that these mutants play a major role in isolating the genes concerned.

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