Development of *Colletotrichum gloeosporioides* Restriction Enzyme-Mediated Integration Mutants as Biocontrol Agents Against Anthracnose Disease in Avocado Fruits

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Accepted for publication 30 October 2000.

ABSTRACT

Yakoby, N., Zhou, R., Kobiler, I., Dinoor, A., and Prusky, D. 2001. Development of *Colletotrichum gloeosporioides* restriction enzyme-mediated integration mutants as biocontrol agents against anthracnose disease in avocado fruits. Phytopathology 91:143-148.

Reduced-pathogenicity mutants of the avocado fruit pathogen *Colletotrichum gloeosporioides* isolate Cg-14 (teleomorph: *Glomerella cingulata*) were generated by insertional mutagenesis by restriction enzyme-mediated integration (REMI) transformation. Following seven transformations, 3,500 hygromycin-resistant isolates were subjected to a virulence assay by inoculation on mesocarp and pericarp of cv. Fuerte avocado fruits. Fourteen isolates showed a reduced degree of virulence relative compared with wild-type Cg-14. Two isolates, Cg-M-142 and Cg-M-1150, were further characterized. Cg-M-142 produced appressoria on avocado pericarp similar to Cg-14, but caused reduced symptom development on the fruit's pericarp and mesocarp. Isolate Cg-M-1150 did not produce appressoria; it caused much reduced maceration on the mesocarp and no symptoms on the pericarp. Southern blot analysis of Cg-M-142 and Cg-M-1150 showed REMI at different *Xba*I sites of the fungal genome. Pre-inoculation of avocado fruit with Cg-M-142 delayed symptom development by the wild-type isolate. Induced resistance was accompanied by an increase in the levels of preformed antifungal diene, from 760 to 1,200 µg/g fresh weight 9 days after inoculation, whereas pre-inoculation with Cg-M-1150 did not affect the level of antifungal diene, nor did it delay the appearance of decay symptoms. The results presented here show that reduced-pathogenicity isolates can be used for the biological control of anthracnose caused by *C. gloeosporioides* attack.

Additional keywords: pathogen–fruit interaction, pathogenicity factors, postharvest decay.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. (teleomorph: *Glomerella cingulata*) is an important postharvest fungal pathogen attacking a wide variety of tropical and subtropical fruits (7,22). *C. gloeosporioides* spores germinate on avocado fruits in the orchard by growing a germ tube that develops an appressorium (3). The appressorium produces infection hyphae that remain quiescent in the cells of the fruit's epidermal layer until the fruit ripens and softens during storage (20). The importance of *Colletotrichum* spp. as a worldwide economic problem and the search for biological means to control quiescent infection have led to the development of tools to control decay.

The search for biocontrol strategies against fruit and vegetable diseases after harvest has intensified in recent years, particularly with the banning of several pesticides (32). Most biocontrol strategies are directed toward wound pathogens and involve the use of antagonist bacterial strains that produce antibiotics, compete successfully for nutrients, or produce lytic enzymes that affect germinating fungal hyphae by possible direct degradation of cell wall-degrading enzymes or direct action against the pathogen's hyphae (6,8,9,32,33). The search for biocontrol agents against pathogens causing quiescent infections has been more difficult because the infecting hyphae are protected from microorganisms once the pathogen has penetrated the plant cuticle. Thus, few reports describe the presence of naturally occurring strains for biological control of quiescent infections in general and in avocado in particular (12,14,16). In the absence of a potentially direct interaction between the antagonist and quiescent-infecting pathogens, and the need to understand the nature of the genetic alteration accrued in the used strain, it was justified to developed reduced-pathogenicity mutants of *C. gloeosporioides*, isolated from avocado fruit, which induce resistance to anthracnose, as an approach to controlling quiescent *Colletotrichum* infections.

In *Colletotrichum* spp., transformation-mediated genetic manipulation was established to enable heterologous gene expression (34), targeted gene disruption (5), and random gene disruption by restriction enzyme-mediated integration (REMI) (25). REMI involves the transformation of an organism with a linear fragment of DNA in the presence of the restriction enzyme used to linearize the fragment (19). Under these conditions, the linear DNA integrates into the chromosome at the restriction sites recognized by that particular enzyme (13,18,28). REMI was first used in yeast (28) to study the mechanism of illegitimate recombination. REMI protocols were adjusted to other systems, facilitating increased transformation-mediated gene disruption in *Dictyostelium discoideum* (18), *Cochliobolus heterostrophus* (35), *Magnaporthe grisea* (29), *Ustilago maydis* (4), *Alternaria alternata* (1), *Colletotrichum* spp. (25), and *Penicillium paxilli* (11).

We describe the development of reduced virulence mutants generated by REMI and their use to elevate the natural resistance mechanism of avocado fruit, as a way of reducing the severity of anthracnose during storage.

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Publication no. P-2000-1218-01R

MATERIALS AND METHODS

Avocado fruits, fungal isolates, and growth media. Avocado fruits (*Persea americana* Miller var. *drymifolia* (Schidl. and Cham.) S. F. Blake cv. Fuerte) were obtained from K. G. Brenner, Israel. A single-spore isolate of *C. gloeosporioides*, Cg-14, was obtained from a decayed avocado fruit (cv. Fuerte), and spores were maintained in 10-mM sodium-phosphate buffer (pH 7.2), 40% glycerol at –80°C. Three-week-old conidia were harvested from Mathur's medium (M_3S) plates (30) and used for culture and fruit inoculation. Spore production was estimated on 3-week-old colonies on M_3S plates at 20°C by spore-counting with a hemacytometer (Brand, Wertheim, Germany).

Vector construction, fungal transformation, and isolate selection. The *hph-B* cassette (hygromycin phosphotransferase), conferring resistance to hygromycin, was subcloned from pHA-1.3 (25) by restriction with *Hin*dIII and *Xba*I (Promega, Madison, WI), generating a 2.13-kb fragment that was ligated into pGEM-7Z (Promega), restricted with the same enzymes. The product, pGH-1 (5.13 kb), was used as a transformation vector. For transformation, 4-day-old mycelia were harvested and 3 g were digested with Novozyme 234 (Calbiochem-Novabiochem Corp., La Jolla, CA) and β-glucuronidase (Sigma Chemical, St. Louis) to obtain fungal protoplasts that were transformed with pGH-1 (1 µg) in the presence of 24 units of *Xba*I-restriction enzyme, as initially described by Redman and Rodriguez (25). The transformation suspension was incubated for 48 h at 22°C and selected on media containing 100 µg/ml of hygromycin (25). Hygromycin-resistant colonies were collected 4 to 8 days later and transferred to new hygromycin plates. Colonies were transferred to M_3S plates to enhance sporulation, and a single-spore culture was obtained. The mutants were sporulated by transferring the colonies to M_3S media plates, and in order to test transformation stability, spores were transferred back to hygromycin plates.

Hygromycin-resistant colonies were tested for their ability to induce symptoms on the pericarp and maceration of the mesocarp of peeled fruits. A single freshly harvested fruit was spot inoculated on both sides of its longitudinal axis. Each fruit was inoculated at three points with *C. gloeosporioides* isolate Cg-14 and at three points with the transformed isolate. Inoculation was carried out on the pericarp and mesocarp (after peeling a 1- to 2-mm thickness of pericarp) by placing a 3-mm^2 disk of media taken from the edge of a 4-day-old colony on hygromycin plates and from hygromycin without supplemented hygromycin for Cg-14. Mesocarp maceration (2 to 3 mm deep, dark spots) occurred within 48 h, and pericarp symptoms occurred (darkening) within 10 to 12 days at 22°C. The diameters (millimeters) of the decay and maceration caused by the transformed isolates and Cg-14 were compared. Colonies that showed a lack of, or reduced, macerating abilities were re-inoculated on 20 fruits for further analysis (described previously).

TABLE 1. Phenotypic analysis of *Colletotrichum gloeosporioides* restriction enzyme-mediated integration

Isolate	Growth on M_3S[a]	Sporulation in vitro[b]	Pericarp attack[c]	Mesocarp attack[c]	Autotroph growth[d]
Cg-M-6	Well	Normal	4	1	+
Cg-M-25	Well	Normal	4	2	+
Cg-M-104	Well	Normal	3	1	–
Cg-M-114	Well	Normal	3	2	+
Cg-M-142	Well	Enhanced	3	2	+
Cg-M-154	Well	Normal	3	2	+
Cg-M-182	Slow	Very low	4	3	–
Cg-M-392	Well	Normal	3	1	+
Cg-M-627	Slow	Very low	4	3	+
Cg-M-807	Well	Normal	3	1	+
Cg-M-809	Slow	Very low	4	3	–
Cg-M-1150	Well	Low	4	3	+
Cg-M-1210	Well	Normal	3	1	–
Cg-M-1230	Well	Normal	2	1	+

[a] Growth on Mathur's medium (M_3S) was compared with that of Cg-14: well = similar growth; and slow = reduced growth.

[b] Sporulation was estimated by spore counting relative to Cg-14: enhanced = more spores; low = less spores; normal = the same amount; and very low = just a few spores.

[c] Decay development on mesocarp and pericarp was estimated with the following scale: 1 = 0 to 9% reduction; 2 = 10 to 50% reduction; 3 = 51 to 90% reduction; and 4 = >90% reduction.

[d] Colonies were grown on minimal media for auxotrophic (–) or autotrophic (+) growth detection.

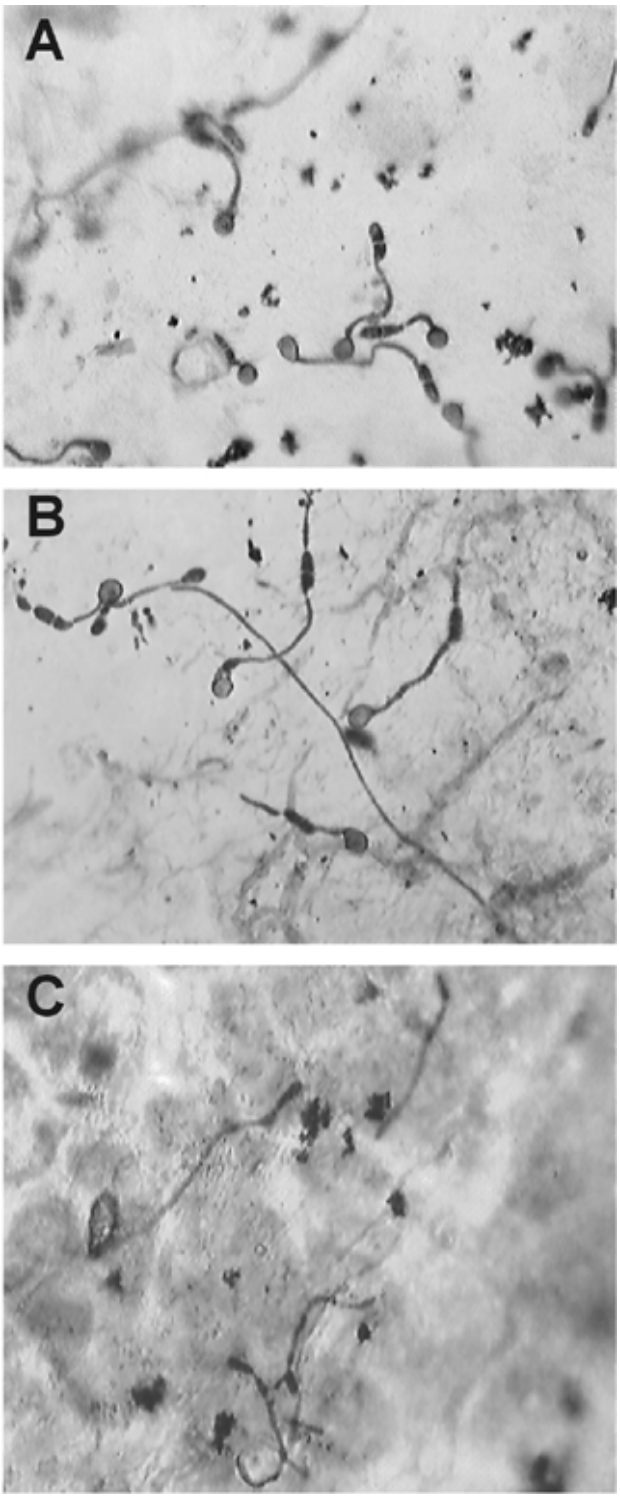

Fig. 1. Spore germination and appressorium formation by *Colletotrichum gloeosporioides* isolates 48 h after cv. Fuerte avocado fruit pericarp inoculation with **A,** Cg-14; **B,** Cg-M-142; and **C,** Cg-M-1150.

Fruit protection assays and phenotypic characterization of transformed isolates. Fruit protection assays were carried out by dipping 20 freshly harvested avocado fruits for 30 s in a spore suspension (5×10^5 spores per ml) of Cg-M-142 or Cg-M-1150 and storing for 24 h at 90% relative humidity (RH), 20°C. Fruits were then inoculated with 10 µl of Cg-14 spore suspension (1×10^6 spores per ml) at six points, three on each side of the longitudinal axis of the fruit, and incubated at 22°C in 90% RH for 15 days. Isolates were characterized for appressorium formation by spot inoculation of the different isolate spores (1×10^6 spores per ml) on the fruit's pericarp and 48-h later were observed under a light microscope (BH-2; Olympus, Tokyo, Japan) after cutting a 0.5-mm-deep slice of the inoculated pericarp with a scalpel. Appressorium formation was photographed by video camera (APPLITEC MSV-800; Azor, Israel). Isolate growth rates were measured on three replicates of M_3S plates or five replicates of the pericarp by inoculation with 10 µl of spore suspension as described previously. Growth rate on the mesocarp was measured by inoculating at six points with a 3-mm^2 square of media with growing isolate (described previously). All experiments were repeated at least three times over two consecutive avocado seasons.

Southern blot analysis and plasmid rescue. Fungal DNA was obtained from 0.2 g of lyophilized hyphae as described by Rodriguez (26). Fungal DNA (5 µg) was restricted for 15 h at 37°C by 20 units of *Hin*dIII or *Xba*I. Restricted DNA was run on a 0.8% agarose gel for 15 h and blotted onto HyBond$^+$ nylon membrane (Amersham, Buckinghamshire, U.K.) according to the capillary method (27).

All hybridizations were carried out at 65°C, and washes were with 0.1 × sodium chloride/sodium citrate. The membrane was probed with either a 4.1-kb *pel* gene (GenBank Accession No. AF052632) or pGH-1. The blotted membrane was exposed to both X-ray film and Fuji BAS sample screen, and the image was captured with the Fuji BAS reader (Fujifilm, Tokyo, Japan). Bands were quantified by MacBAS software (version 2.3, Fujifilm).

For plasmid rescue, 1 µg of fungal DNA was used. DNA was restricted with 10 units of *Xba*I or *Hin*dIII for 15 h. Restriction enzymes were separated from the DNA with phenol/chloroform (50:50, vol/vol) followed by ethanol precipitation. The DNA was eluted and subjected to ligation using ligase (Promega) for 12 h at 4°C. Ligation mixture from each isolate (1 µl) was electroporated, using Gene Pulser II (Bio-Rad Laboratories, Hercules, CA), into delayed-hypersensitivity 5α-competent cells (prepared according to Bio-Rad Laboratories recommendations). Bacteria were grown on Luria-Bertani media (27) supplemented with 100 µg of ampicillin per milliliter at 37°C for 15 h. Randomly selected colonies were grown for plasmid minipreparation with Wizard Plus SV Minipreps (Promega). Plasmids were restricted with the same restriction enzyme used for their rescue and run on a 1% agar gel followed by ethidium bromide staining.

Antifungal diene extraction. A 10-g sample of avocado pericarp (1 to 2 mm thick) was homogenized in 95% ethanol in an Omni-Mixer (Sorvall, DuPont Company, Newtown, CT) at full speed for 3 min. The ethanol extract was dried in a rotary evaporator at 40°C, redissolved in 10 ml of distilled water, and the organic phase was extracted by fractionation with dichloromethane. Following two extractions, the organic phases were pooled, dried with anhydrous $MgSO_4$ (Riedel-deHaen, Seelze, Germany), and evaporated to dryness. Samples were redissolved in 1 ml of ethanol AR (Bio Lab, Jerusalem, Israel) and analyzed by high performance liquid chromatography (23). The average values of three separate extractions are presented. The experiment was repeated twice during each year of two consecutive avocado seasons.

RESULTS

***C. gloeosporioides* reduced-pathogenicity mutants.** A transformation vector was constructed by subcloning the *hph-B* gene into pGEM-7Z, generating pGH-1 with two unique restriction sites, *Xba*I and *Hin*dIII. Transformation of pGH-1 into the fungal protoplasts as REMI (in the presence of 24 units of *Xba*I) elevated the recovered colonies from approximately 50 obtained using native supercoiled DNA to 500 per microgram of plasmid DNA. Following seven transformations, 3,500 recovered colonies were selected on hygromycin and used to inoculate avocado mesocarp and pericarp. Fourteen isolates showed varying levels of symptom development on avocado fruit, and four isolates exhibited auxotrophic growth on minimal media (Table 1).

Two isolates, Cg-M-142 and Cg-M-1150, showed no spore morphological difference to Cg-14, but demonstrated reduced

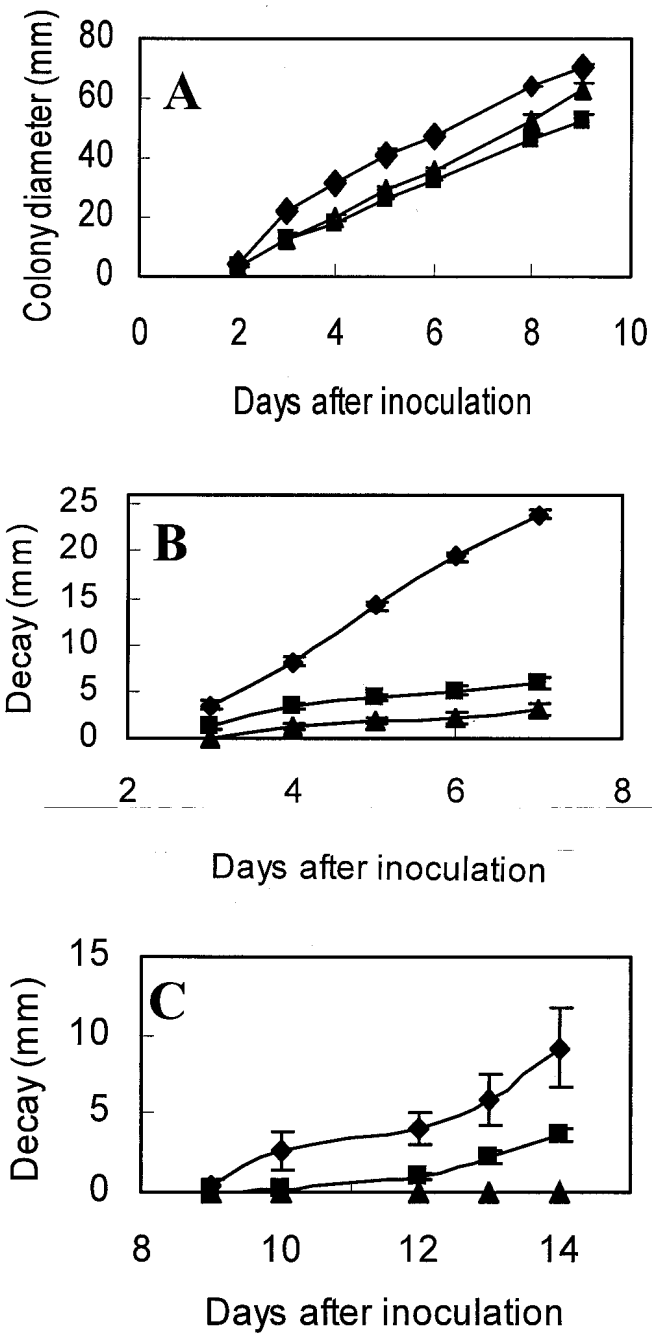

Fig. 2. A, Growth rate in vitro on Mathur's medium plates and **B,** in vivo decay development on pericarp or **C,** mesocarp of avocado fruits (cv. Fuerte) by *Colletotrichum gloeosporioides* isolates Cg-14, Cg-M142, and Cg-M-1150. Experiments were repeated three times over two consecutive harvesting seasons. Standard errors of the means are shown as bars on data points; differences between means were analyzed by analysis of variance and are significantly different ($P < 0.01$).

virulence on avocado fruits. Three-week-old colonies of isolate Cg-M-142 sporulated (approximately 1×10^7 spores per cm^2) approximately 10-fold more than Cg-14, whereas isolate Cg-M-1150 showed (approximately 1×10^5 spores per cm^2) an approximate 10-fold reduction in spore production. Isolates Cg-14, Cg-M-142, and Cg-M-1150 generated similar germination tubes on the pericarp (Fig. 1A, B, and C, respectively) after 48 h; however, only Cg-14 and Cg-M-142 formed 100% appressoria (Fig. 1A and B, respectively). Isolate Cg-14 formed 60% of its appressoria within 24 h, whereas isolate Cg-M-142 showed delayed appressorium formation during that timeframe. No significant differences on the size, pigmentation, and wall thickness of appressoria between Cg-14 and Cg-M-142 were observed. Isolate Cg-M-1150 generated no appressoria at all.

Growth on M_3S plates was similar for all three isolates (Fig. 2A). However, isolate Cg-14 was the fastest growing on the fruit mesocarp and pericarp (Fig. 2B and C). Isolate Cg-M-1150 was growth-retarded on fruit, especially on the pericarp where no decay symptoms developed (Fig. 2C). Isolate Cg-M-142 showed statistically significant reduced virulence relative to Cg-14 but enhanced virulence relative to Cg-M-1150 (Fig. 2B and C).

Molecular characterization of the two REMI mutants. Southern blot analysis using either pGH-1 (Fig. 3A) or the 4.1-kb *pel* gene (Fig. 3B) showed Cg-M-142 and Cg-M-1150 REMI as a band at 5.13 kb, the size of pGH-1, when restricted with *Xba*I; isolate Cg-M-1150 showed additional ectopic integration as a band at 5.5 kb (Fig. 3A). This was confirmed by running *Hin*dIII-restricted DNA of Cg-M-1150, which revealed the same 5.13-kb band and an additional three bands representing the ectopic integration and the two end plasmids with the flanking fungal DNA (Fig. 3A). Isolate Cg-M-142 restricted with *Hin*dIII produced three bands: two bands at 4.5 and 6.0 kb, consisting of the integrated plasmids with a genomic flanking region, and a stronger band at 4.3 kb, representing the two *hph-B* cassettes (Fig. 3A). Isolate Cg-14 did not hybridize at all with the pGH-1. The membrane was probed with the single-copy 4.1-kb *pel* to show that the strong bands appearing at 5.13 kb were a result of the integration of 10 plasmids in tandem sequence for Cg-M-1150, two in tandem sequence for Cg-M-142 (Fig. 3B), and not due to different amounts of loaded DNA that was quantified with MacBAS software.

Plasmid rescue from genomic DNA of isolate Cg-M-142 restricted with *Xba*I (p142X4) and of isolate Cg-M-1150 restricted with *Xba*I (p1150X1) or *Hin*dIII (p1150H1) showed the 5.13-kb pGH-1-band size for both isolates (Fig. 4), further supporting *Xba*I REMI in both isolates. The *Hin*dIII-rescued plasmid from Cg-M-142 (p142H1 and p142H3), generating the two upper bands appearing on the Southern blot at 6.0 and 4.5 kb, consisted of a 5.13-kb transformation vector and 0.87 kb of fungal genome flanking area for the former, and the 3.0-kb pGEM-7Z and 1.5 kb of fungal genome flanking area for the latter (Fig. 3A). The 4.3-kb band was not rescued because it consisted of two head-to-head *hph-B* cassettes with no pGEM-7Z. The 5.5-kb band, detected when Cg-M-1150 DNA was restricted with *Xba*I (Fig. 3A), did not appear when the membrane was probed with pGEM-7Z (data not shown), supporting the band's consisting of *hph-B* with no

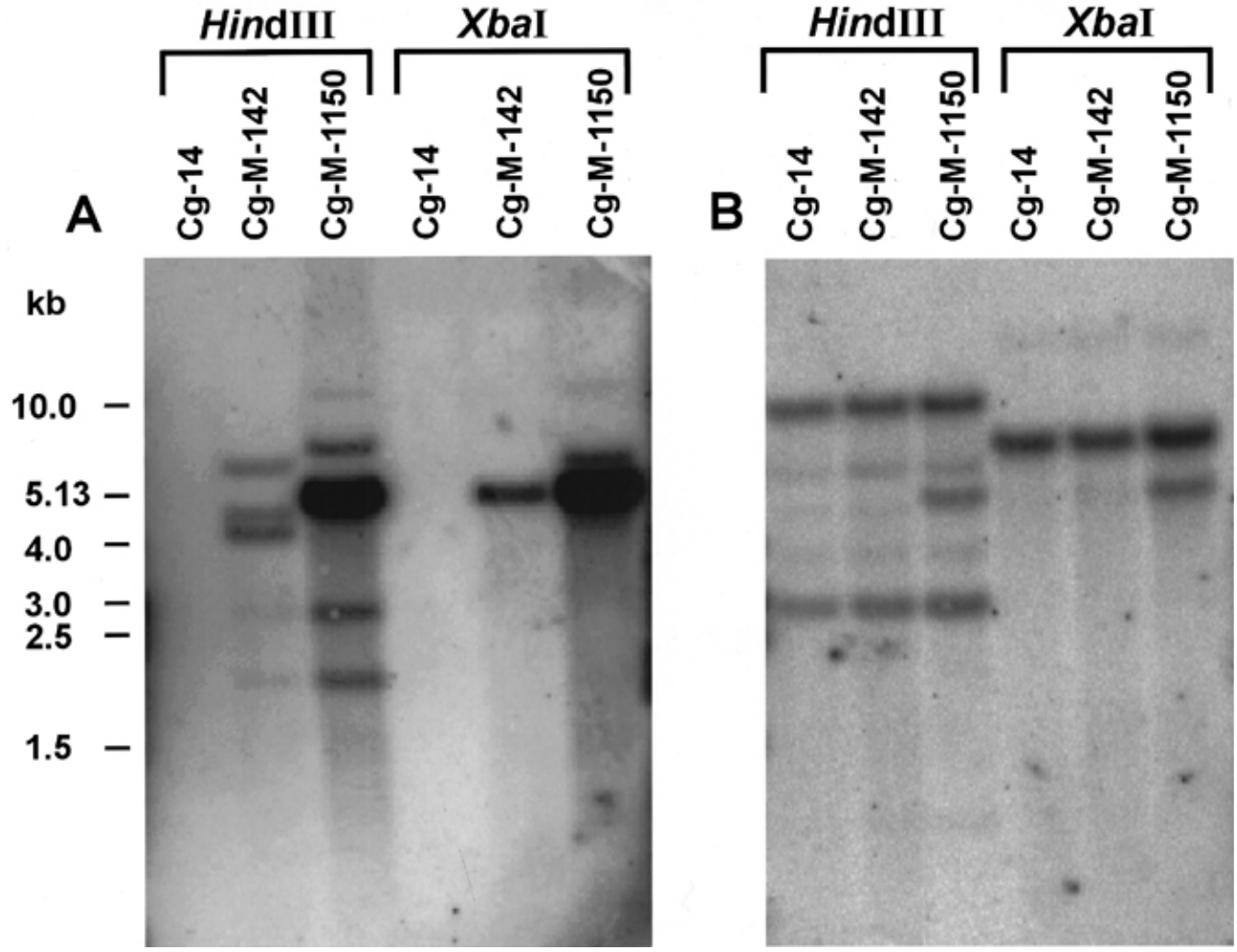

Fig. 3. Pattern of plasmid integration analyzed by Southern blot of *Colletotrichum gloeosporioides* isolates Cg-14, Cg-M-142, and Cg-M-1150. Fungal DNA was restricted with *Xba*I or *Hin*dIII, run on a 0.8% agarose gel and blotted onto a nylon membrane. **A,** The membrane was probed with 5.13-kb pGH-1 or **B,** 4.1-kb *pel*, and exposed to Fuji BAS sample screen.

vector. Sequence analysis of the genomic DNA from the flanking area of Cg-M-142 showed no homology to any known sequences in GenBank.

Fruit protection by pre-inoculation with REMI mutants. Avocado fruits pre-inoculated with a spore suspension of Cg-M-142 before spot inoculation with the wild-type Cg-14 exhibited significantly reduced decay development (Fig. 5). However, pre-inoculation with Cg-M-1150 followed by spot inoculation with the wild-type Cg-14 did not affect decay development (data not shown). Freshly harvested avocado fruits inoculated with Cg-M-142 exhibited an increased level of antifungal diene 9 days later, from 767 ± 97 µg/g fresh weight in noninoculated fruits to 1,189 ± 139 µg/g fresh weight. However, fruit inoculated with Cg-M-1150 showed no increase in diene level or elevation in fruit resistance to Cg-14. Results are the average values of three independent extractions with their standard error.

DISCUSSION

As a mutation tool to generate biocontrol agents, REMI generated 0.4% *C. gloeosporioides* mutants, compared with 2.0% for *C. magna* (24), 1.4% for *U. maydis* (4), and 0.27% in *D. discoideum* (18). Following REMI transformation, the integration of linear DNA molecules into the genome can be either ectopic or restricted-enzyme mediated (into the restriction site generated by the specific restriction enzyme), as one copy or in tandem copies. We found tandem integration of the plasmid in 2 and 10 copies, as reported for *U. maydis* (10,17,31). Unlike other methods of generating random mutations in fungi, where the mutated gene remains an enigma, REMI enables molecular characterization of the isolate in order to find the genotypic cause for the phenotype. Isolate Cg-M-142 yielded three bands when probed with pGH-1 (Fig. 3A); two of these bands were retrieved by plasmid rescue but the low molecular weight band was not. Furthermore, when probed with *hph-B* the two upper bands did not appear (data not shown). These data suggest that Cg-M-142 has head-to-head, two-plasmid REMI (one band when restricted with *Xba*I), where the two top bands are pGEM with flanking regions and the lower band represent the two *hph-B* cassettes. Isolate Cg-M-1150 yielded two bands when probed with pGH-1. The upper band did not appear when the membrane was probed with pGEM (data not shown), suggesting it to be the *hph-B* cassette in ectopic integration. The main band appearing in both *Xba*I- and *Hin*dIII-restricted Cg-M-1150 DNA at 5.13 kb suggests head-to-tail tandem integration of pGH-1. This was further confirmed by plasmid rescue, which retrieved pGH-1 when the plasmid was *Xba*I- and *Hin*dIII-rescued. The ability to rescue plasmids can supply information as to what genes are important for direct (pathogenicity factors) or indirect (regulatory genes) fungal attack, as has been demonstrated with other fungi (19). We are currently working on a genotypic characterization of the putative-disrupted genes in order to link them to the phenotypic appearance of the different isolates.

The development of biocontrol agents against quiescent pathogens has been unsuccessful because of the lack of a direct interaction between the biocontrol agent and the pathogen. Thus, there are few reports describing the biological control of quiescent infections (12,14,16). Korsten et al. (15) used protective spray treatments with *Bacillus subtilis* against avocado anthracnose disease caused by *C. gloeosporioides.* For effective control, continuous protective sprays are necessary because fruits are continually spore-infested in the orchard (15).

Postharvest treatment of avocado fruits with the reduced-pathogenicity strain Cg-M-142 increased their antifungal diene level and enhanced their resistance to Cg-14 attack. Prusky et al. (21) reported that pre-inoculation of avocado fruits with Cg-14 stimulates the host for a short time only, and thus, does not increase diene level or fruit resistance, whereas the higher level of diene induced by Cg-M-142 is proposed to occur because of a vigorous stimulation of the host-antifungal compound, as seen 9 days after inoculation. This hypothesis is based on postharvest inoculation treatment of avocado fruits with a nonpathogenic mutant (path-1) of *C. magna* that shows minor symptoms on mature avocado fruit, enabling an increase in the level of antifungal diene, concomitant-enhanced resistance to *C. gloeosporioides* attack (21). The inability of Cg-M-1150 to increase fruit resistance with no symptom development on the pericarp cannot be explained by the 15% reduction in growth on M_3S media. The lack of appressorium formation with no symptom development on the fruit by Cg-M-1150 and no increase in the antifungal diene level may further support the importance of appressorium formation as the initial stage to breaching the wax and the establishment of early interaction between the fungus and the fruit, as suggested by Beno-Moualem and Prusky (2).

Sufficient duration and efficiency of the defense induction of *Colletotrichum* strains is mandatory to prevent termination of the quiescent period in wild-type *C. gloeosporioides*. The use of a

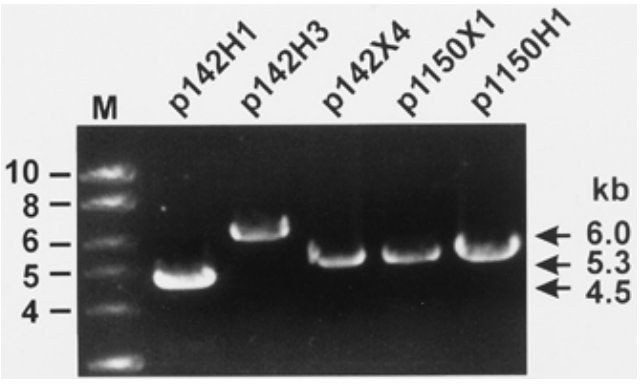

Fig. 4. Plasmid rescue analysis of *Colletotrichum gloeosporioides* isolates Cg-M-142 and Cg-M-1150 restricted with *Hin*dIII (H) and *Xba*I (X). Fungal DNA was restricted with *Xba*I or *Hin*dIII and subjected to ligation. One microliter of the ligation mixture of each isolate was electroporated into delayed-hypersensitivity 5α-competent cells. Colonies were selected (last number on plasmid name) on Luria-Bertani media supplemented with 100 µg/ml of ampicillin. Randomly selected colonies were grown for plasmid minipreparation. Plasmids (p) were restricted with the same restriction enzyme used for their rescue and run on a 1% agarose gel.

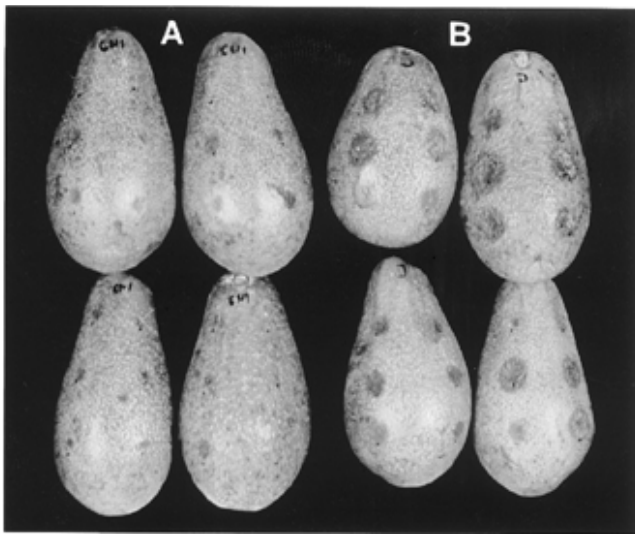

Fig. 5. Decay development of *Colletotrichum gloeosporioides* isolate Cg-14 following preinoculation of cv. Fuerte avocado fruit with **A,** reduced virulence mutant Cg-M-142, or **B,** dipped in water as a control. Twenty freshly harvested fruit were dipped for 30 s in a spore suspension of Cg-M-142 or water as a control. Fruits were inoculated 24-h later at six points and incubated for 15 days, as described in text. Experiments were repeated three times during two consecutive harvesting seasons.

REMI mutant to increase the natural resistance mechanism of avocado fruit demonstrates that this approach may be adopted to control decay in other postharvest pathosystems. Data presented here demonstrates that genetic manipulation can be used for the construction of a local biocontrol agent. Furthermore, the possibility of easily exploring the mutation by plasmid rescue could simplify the isolate's registration as a biocontrol agent.

ACKNOWLEDGMENTS

This research was supported by Research Grants IS-2686-96 to D. Prusky from The U.S.-Binational Agricultural Research and Development Fund, the German-Israel Agricultural Research Agreement for the Benefit of the Third World, and the U.S.-Israel Cooperative Development Research Program. N. Yakoby was granted personal scholarships from the Israel Fruit Marketing Board/Ministry of Agriculture and Rural Development and from The Association of Trustees and Friends of the Agricultural Research Organization in the name of "Avigad and his daughter Dovrat".

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