Conversion of the Pathogenic Fungus *Colletotrichum magna* to a Nonpathogenic, Endophytic Mutualist by Gene Disruption

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Hygromycin-resistant transformants of the cucurbit pathogen *Colletotrichum magna* (teleomorph: *Glomerella magna*) were generated by restriction enzyme-mediated integration (REMI) transformation. A rapid pathogenicity assay involving watermelon (*Citrullus lanatus*) seedlings was developed and 14,400 REMI transformants were screened and assessed for their ability to cause disease, colonize plant tissues, and confer disease resistance against wild-type *C. magna*. A total of 176 nonpathogenic REMI mutants capable of colonizing cucurbit plants were isolated and assigned to three groups based on their ability to confer disease resistance: phenotype A, 80 to 100% disease protection; phenotype B, 10 to 65% disease protection; and phenotype C, 0 to 4% disease protection. Molecular and genetic analyses of one REMI mutant (R1) indicated that the nonpathogenic phenotype A resulted from a single-site integration. R1 showed a 1:1 segregation of hygromycin resistance and nonpathogenicity and all hygromycin-resistant progeny were nonpathogenic. The integrated vector and 5.5 kb of flanking fungal genomic DNA were isolated from R1 and designated pGMR1. To verify that pGMR1 contained pathogenicity gene sequences, a wild-type isolate of *C. magna* was transformed with pGMR1 to induce gene disruptions by homologous integration. Approximately 47% of the pGMR1 transformants expressed phenotype A, indicating homologous integration and gene disruption.

Additional keywords: plant pathogen, plasmid rescue.

Colletotrichum magna is one of the causal agents of anthracnose disease on cucurbit plants (Jenkins and Winstead 1964). The disease process of fungi belonging to this genus begins with the adhesion of spores to the plant cell surface, spore germination, appressorium formation, and penetration into the first subcuticular cell, followed by production of secondary hyphae that quickly disseminate throughout the plant,

causing plant cell death and hence disease symptoms (Bailey et al. 1992). Although many studies have focused on the developmental, biochemical, and molecular analyses of plant-pathogen interactions, the genetic basis for the disease process is still largely undefined (Sweigard et al. 1998; Jackson and Taylor 1996; Hammond-Kosack and Jones 1996; Kuc 1990; Keen 1986; Dickman and Patil 1986; Hammerschmidt et al. 1982, 1984).

To understand the mechanisms and processes involved in pathogenicity, a nonpathogenic mutant of *C. magna* was generated through UV mutagenesis and denoted path-1 (Freeman and Rodriguez 1993). The mutant path-1 was capable of colonizing cucurbit plants without causing disease. Once plants were colonized, they were protected from disease when exposed to *C. magna*, *Fusarium oxysporum* (Freeman and Rodriguez 1993, 1992), and *C. orbiculare* (Redman et al. 1999). Genetic analysis of path-1 indicated that the mutation of a single locus allowed conversion of a plant pathogen to a nonpathogenic, endophytic mutualist (Freeman and Rodriguez 1993). Analysis of plant biochemical indicators of localized (peroxidase, phenylalanine ammonia-lyase, and lignin) and systemic (salicylic acid) "plant defense responses" collectively indicated that disease protection in path-1 colonized plants was nonsystemic and correlated with the ability of these plants to mount a defense response more rapidly and to greater levels than plants exposed to *C. magna* alone (Redman et al. 1999). We propose that the protection from disease afforded to plants colonized by the path-1 mutant is an endophyte-associated response (EAR) and involves an interaction between the mutant and plant resulting in the priming of the host defense systems. Once primed, the host defenses are activated rapidly and to high levels when the plants are challenged with virulent fungi (Redman et al. 1999).

The goal of this study was to identify the gene(s) that when mutated resulted in conversion of wild-type *C. magna* to a nonpathogenic endophyte, and to determine the genetic complexity of this phenomenon. Although there are several different approaches for obtaining the gene(s) of interest, earlier studies conducted in our laboratory indicated that REMI (restriction enzyme-mediated integration) transformation was a viable option for the efficient generation of insertional mutants in *Colletotrichum* spp. (Redman and Rodriguez 1994). There are many advantages to REMI transformation. Transformation efficiencies are often elevated by the REMI proto-

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col. Gene(s) of interest are tagged by the transformation vector and may be isolated by "plasmid rescue," and retrieved plasmids with flanking genomic DNA may be used for gene disruption studies. In addition, utilization of REMI for the successful generation of mutants and gene isolation has been shown in many species including *Dictyostelium discoideum* (Kuspa and Loomis 1992, 1994), *Neurospora crassa* (Kang and Metzenberg 1993), *Ustilago maydis* (Bolker et al. 1995), *Alternaria alternata* (Akamatsu et al. 1997), *Candida albicans* (Brown et al. 1995), *Coprinus cinereus* (Granado et al. 1997), and *Magnaporthe grisea* (Sweigard et al. 1998; Shi et al. 1995).

In this study, we present the isolation and characterization of 176 nonpathogenic REMI mutants. One of the genes responsible for the nonpathogenic phenotype was isolated from REMI mutant R1 and its function verified through gene disruption studies in a wild-type isolate of *C. magna*. The significance of the R1 mutant in defining the genetic basis of fungal symbiotic lifestyles is discussed.

RESULTS

Isolation and characterization of nonpathogenic mutants.

Optimization of parameters involved in the transformation protocol, such as concentrations of cell wall-degrading enzymes, regeneration buffer, and length of time of regeneration, contributed to increase the efficiencies from 0–50 (Redman and Rodriguez 1994) to 1,000–2,000 transformants μg^{-1} of plasmid DNA in *C. magna* (data not shown). REMI transformation was optimized by the addition of 0 to 200 U of different restriction enzymes (*Xba*I, *Hind*III, *Sac*I, *Sal*I, *Nde*I, *Apa*I, *Pvu*II) and linearized plasmid (digested with the same restriction enzyme added to the transformation reaction) to the protoplast transformation cocktail (data not shown). The highest transformation efficiencies (2,000 to 3,000 transformants μg^{-1} of plasmid DNA) were obtained with reactions containing 5 μg of *Hind*III-linearized pHA1.3 (Powell and Kistler 1990; Rodriguez and Redman 1992) and 40 to 80 U of *Hind*III restriction enzyme. All subsequent REMI transformations were conducted under these optimized conditions.

Over 20,000 REMI transformants were generated with a vector that conferred hygromycin resistance (pHA1.3) and 14,400 transformants that exhibited stable hygromycin resistance were screened for pathogenicity. Each transformant was individually screened for its ability to cause disease on three anthracnose-susceptible watermelon seedlings. Transformants (288) were identified that did not cause plant disease. These were subjected to a secondary screening that involved assessing their ability to colonize 30 anthracnose-susceptible watermelon seedlings. From the 288 putative nonpathogenic transformants, 176 were identified that did not cause disease in the secondary screening but did colonize cucurbit plants. The remaining 112 nonpathogenic REMI mutants appeared to be defective in the ability to colonize plants.

To determine if any of the nonpathogenic REMI mutants expressed the same phenotype as the original path-1 mutant, the mutants were screened for the ability to confer disease resistance. Anthracnose-susceptible watermelon seedlings were colonized with REMI mutants and then exposed to lethal concentrations of spores of the wild-type *C. magna* isolate L2.5 (Freeman and Rodriguez 1992, 1993). The 176 nonpathogenic

REMI mutants were placed into one of three phenotypic categories based on disease protection: phenotype A, 60 REMI mutants affording 80 to 100% disease protection; phenotype B, 55 REMI mutants affording 10 to 65% disease protection; and phenotype C, 61 REMI mutants affording 0 to 4% disease protection (Fig. 1). It is possible that the mutants placed at the extremes of the phenotypic classes may be improperly designated. However, this would apply to a low percentage of the REMI mutants.

The ability of REMI mutants expressing phenotypes A, B, and C to protect plants against disease is shown in Figure 2. Initial characterization of the REMI mutants was performed to assess the three nonpathogenic phenotypes for differences in growth rates, initial developmental processes (spore adhesion, germination, appressorial formation, and plant colonization), or extracellular enzyme production (protease and cellulase). Extensive characterization of these physiological attributes indicated that the variation between the REMI mutants and the wild-type isolate L2.5 was $\leq 10\%$ (data not shown). In fact, the only notable difference between the nonpathogenic mutants and the wild type was that the mutants generally produced less pigmentation (data not shown).

It was of interest to determine if the variation in disease protection conferred by the nonpathogenic phenotypes was due to differences in colonization ability. The colonization of plants by REMI mutants representing each phenotype was assessed 7 days after inoculation (Table 1). Although the plant colonization studies are a qualitative measure of colonization, these studies revealed that all of the 176 nonpathogenic REMI mutants were able to colonize the roots and majority of plant

Fig. 1. Distribution of nonpathogenic restriction enzyme-mediated integration (REMI) mutant phenotypes in relation to disease protection level conferred to susceptible Crimson Sweet watermelon plants. REMI mutants expressing phenotypes A, B, and C conferred 80–100, 10–65, and 0–4% protection against *Colletotrichum magna* wild-type isolate L2.5, respectively. Data indicate disease protection levels (as percentage of 30 mutant colonized seedlings that survived exposure to lethal concentrations of wild-type L2.5 spores) conferred by each of the individual 176 REMI mutants.

stem sections. There was little variation in the extent and pattern of colonization in the middle (M) and upper (U) stem sections by REMI phenotypes A (M, 92% and U, 22%) B (M, 93% and U, 22%), and C (M, 98% and U, 5%).

Molecular analysis of nonpathogenic REMI mutants.

Genomic DNA from 20 REMI mutants including representatives of groups A, B, and C was digested with *Xho*I, which does not digest pHA1.3, and with *Hind*III, which digests once within pHA1.3, and subjected to Southern blot analysis (data not shown). Figure 3 shows three representative REMI mutants of phenotype A (Tx01, Tx02, and Tx03) exhibiting varying integration patterns. There appeared to be three types of integration events involving either single-site integration into *Hind*III sites (Tx01); multiple integrations into multiple *Hind*III sites (Tx02); and single and/or tandem integration(s) into *Hind*III, non-*Hind*III, or modified *Hind*III sites made non-

Fig. 2. Seven-day-old, anthracnose-susceptible watermelon plants exposed to the following five treatments (left to right): treatment 1, colonization with restriction enzyme-mediated integration (REMI) mutant phenotype A (R1) for 48 h followed by challenge with the *Colletotrichum magna* wild-type isolate L2.5; treatment 2, colonization with REMI phenotype B (R4) for 48 h followed by challenge with L2.5; treatment 3, colonization with REMI phenotype C (R5) for 48 h followed by challenge with L2.5; treatment 4, water control; and treatment 5, water control plants challenged with L2.5. Challenge conditions involved exposure to lethal concentrations (3.0×10^6 conidia ml^{-1}) of isolate L2.5. Plant mortality was assessed after 5 to 7 days and was 0, 33, 100, 0, and 100% for treatments 1, 2, 3, 4, and 5, respectively.

Table 1. Pathogenicity and colonization of *Colletotrichum magna* restriction enzyme-mediated integration (REMI) mutants

REMI mutants (no.) ^b	Phenotype ^c	Plant colonization ^a			
		Root (%) ^d	Lower stem (%) ^d	Middle stem (%) ^d	Upper stem (%) ^d
60	A	100	100	92	22
55	B	100	100	93	22
61	C	100	100	98	5

^a Three Crimson Sweet seedlings colonized by exposing lower stems and roots to a spore suspension of each of 176 REMI mutants.

^b Number of nonpathogenic REMI mutants screened in each phenotypic group.

^c Phenotypes based on percent disease protection (number of mutant-colonized plants surviving pathogen challenge) afforded to 30 anthracnose-susceptible watermelon seedlings by the mutants: (A) 80 to 100%; (B) 10 to 65%; and (C) 0 to 4%.

^d Data indicate percent nonpathogenic REMI mutants from each phenotype recovered from various plant tissue sections 7 days after inoculation.

functional by the integration process (Tx03). Single-site integration was represented by REMI mutant Tx01, in which one vector-sized and one larger than vector-sized band (representing pHA1.3 and surrounding flanking DNA) were observed in *Hind*III and *Xho*I digestions, respectively (Fig. 3, lanes 1 and 2). Multiple integration events into genomic *Hind*III sites were observed with TX02 as a single vector-sized band in *Hind*III-digested genomic DNA and as multiple bands larger than vector size in *Xho*I digestions (Fig. 3, lanes 3 and 4). Integration of the plasmid through single and/or multiple integration event(s) into *Hind*III and either modified *Hind*III or non-*Hind*III sites was observed with Tx03 as multiple bands that were vector-sized and/or larger than vector-sized in *Hind*III- and *Xho*I-digested genomic DNA (Fig. 3, lanes 5 and 6). Our molecular studies indicated that 70% of the initial 20 REMI mutants chosen for analysis arose through single-site integration events and approximately half of these mutants arose through integration at *Hind*III sites.

Selection of an REMI mutant for genetic and molecular studies.

To optimize our chances of isolating the disrupted sequences responsible for generating nonpathogenic mutants, REMI mutant R1 expressing phenotype A was chosen for the remainder of these studies. Mutant R1 exhibited colonization of the lower, middle, and upper stem and root tissues comparable to all other phenotype A mutants, and conferred 100% disease protection to plants. In addition, REMI mutant R1 had the same Southern blot hybridization profile as TX01 (Fig. 3, lanes 1 and 2), indicating that R1 resulted from a single integration event into the same *Hind*III site as TX01.

Segregation analysis.

Genetic segregation analyses were performed to test whether the integration of pHA1.3 was likely to be responsible for the nonpathogenic phenotype for REMI mutant R1. Analysis of 86 progeny representing four perithecia derived from crosses between the wild-type *C. magna* isolate (Gm16) and REMI mutant R1 indicated approximately a 1:1 segregation ratio for hygromycin resistance:sensitivity, and patho-

Fig. 3. Southern blot analysis of genomic DNA from three representative restriction enzyme-mediated integration (REMI) mutants of phenotype A possessing single (Tx01 [lanes 1 and 2]) and multiple (Tx02 [lanes 3 and 4]) integrations into *Hind*III restriction enzyme sites, and single and/or tandem integration(s) into *Hind*III, and either non-*Hind*III or modified *Hind*III sites (Tx03 [lanes 5 and 6]). Samples were digested with *Hind*III (lanes 1, 3, and 5) or *Xho*I (lanes 2, 4, and 6) restriction enzymes and probed with ^{32}P -labeled, *Hind*III-linearized pHA1.3 vector. Arrow denotes linear vector-sized DNA (6.4 kb).

genicity:nonpathogenicity (Table 2). In addition, all of the hygromycin-resistant progeny were nonpathogenic and all of the hygromycin-sensitive progeny were pathogenic (data not shown). Exact chi-square tests performed with StatXact 3.1 for Windows (Cytel Software, Cambridge, MA) showed no significant deviation from a 1:1 ratio in the Gm16 × R1 crosses (Table 2). Therefore, REMI mutant R1 was an appropriate candidate for the reisolation of the integrated vector and flanking genomic DNA by “plasmid rescue” (below).

Plasmid rescue, transformation, and gene disruption of *C. magna*.

To recover pHA1.3 and flanking fungal genomic sequences from the integration site, genomic DNA of REMI mutant R1 was digested with *Xho*I (an enzyme that does not digest pHA1.3), ligated, and transformed into *Escherichia coli*. The recovered plasmid, designated pGMR1, was approximately 12.0 kb in length and contained approximately 5.5 kb of flanking genomic DNA. To confirm that the retrieved genomic DNA contained sequences related to pathogenicity, pGMR1 was transformed into the wild-type *C. magna* isolate L2.5 in an attempt to disrupt the wild-type copy of the sequences responsible for the R1 mutation. If pGMR1 integrated by homology then the disrupted gene sequences in pGMR1 would replace the wild-type copy and generate nonpathogenic mutants. Following transformation with pGMR1, 107 hygromycin-resistant transformants were screened for pathogenicity and colonization of anthracnose-susceptible watermelon plants. Approximately 47% (50 mutants designated GkMa1–50) of the 107 transformants were nonpathogenic, colonized plant tissues, and expressed disease protection phenotype A (data not shown). The remaining 57 hygromycin-resistant transformants were pathogenic and were designated EMa1–57. When wild-type *C. magna* was transformed with the pHA1.3 vector alone, 100% of the hygromycin-resistant transformants (100 screened) were pathogenic (data not shown). These results indicated that the pGMR1 vector contained fungal genomic sequences that were involved in pathogenicity.

Molecular analysis of targeted gene disruption and ectopic integration transformants.

To characterize the molecular basis of REMI phenotype A, genomic DNA from REMI mutant R1, wild-type isolate L2.5, gene disruption transformants GkMa-1 and GkMa-2, and pathogenic transformants EMa-1 and EMa-2 was digested with *Xho*I and analyzed by hybridization analysis with pGMR1 as the probe (Fig. 4, lanes 1–6, respectively). Single hybridization bands of approximately 12.0 kb for REMI mutant R1 (lane 1) and 5.5 kb for wild-type L2.5 (lane 2) indicated that pHA1.3 (6.4 kb in size) had integrated into a single *Hind*III site, resulting in the disruption of a pathogenicity-

related DNA sequence. The nonpathogenic pGMR1 transformants GkMa-1 and GkMa-2 had the same hybridization pattern as REMI R1, indicating that pGMR1 had integrated by homology into the same site as pHA1.3 had in REMI R1. In contrast, both pathogenic pGMR1 transformants EMa-1 and EMa-2 had two hybridizing bands, one at 5.5 kb as observed with wild-type L2.5, and additional bands at >8 kb. These results indicated that pGMR1 had integrated ectopically in EMa-1 and EMa-2.

DISCUSSION

A number of factors produced by plant-pathogenic fungi, such as extracellular enzymes and toxins, have been implicated as key components involved in plant disease. However, the exact mechanisms and/or the genetic and biochemical factors are undefined (Jackson and Taylor 1996; Madamanchi and Kuc 1991; Keen 1986; Kolattukudy 1985; Ciuffetti et al. 1983). Production of gene disruption mutants through REMI mutagenesis provides a powerful tool to investigate genes encoding factors involved in the disease process. In order to understand the basis of pathogenicity in *Colletotrichum* spp., 14,400 REMI mutants were screened and 176 nonpathogenic endophytic mutants were obtained. Although all 176 REMI mutants were nonpathogenic endophytes, approximately 34% could be defined as mutualists, 35% as commensals, and 31% as having an intermediate phenotype based on disease protection.

Molecular studies of 20 nonpathogenic REMI transformants by Southern blot analysis indicated that approximately 70% of the REMI mutants (including REMI R1) resulted from a single integration event (data not shown). Genetic segregation studies showed a 1:1 segregation ratio of the nonpathogenic and hygromycin resistance phenotypes, which confirmed that the disruption of a single gene required for pathogenesis occurred in REMI R1. More importantly, the integrated trans-

Fig. 4. Hybridization analysis of genomic DNA from nonpathogenic restriction enzyme-mediated integration (REMI) R1 (lane 1), wild-type isolate L2.5 (lane 2), nonpathogenic pGMR1 gene disruption transformants GkMa-1 (lane 3) and GkMa-2 (lane 4), and pathogenic ectopic pGMR1 integration transformants EMa-1 (lane 5) and EMa-2 (lane 6). DNA was digested with *Xho*I (does not digest pHA1.3) and probed with ³²P-labeled, linearized pGMR1 as the probe. DNA size in kilobases (kb) indicated on left. + and – denote pathogenic and nonpathogenic phenotypes, respectively, of fungal isolates on watermelon plants.

Table 2. Segregation analysis of nonpathogenic phenotype A

Phenotype	Parental isolates ^a		Progeny	Segregation analysis		
	Gm16	R1	Gm16 × R1	Ratio	Chi-square	P value
Hygromycin	S	R	40R:46S	0.87	0.419	0.590
Pathogenicity ^b	+	–	40–:46+	0.87	0.419	0.590

^a S = hygromycin sensitive; R = hygromycin resistant.

^b Assessed 7 days after inoculation of plants with conidia.

formation vector and flanking genomic DNA were reisolated from REMI mutant R1, designated pGMR1, and tested to verify the presence of a disrupted pathogenicity gene through function. When pGMR1 was transformed into the wild-type isolate L2.5, the plasmid integrated by homology in approximately 47% of the transformants, disrupting sequences responsible for pathogenicity, and converting the transformants into nonpathogenic, endophytic mutualists. These results in conjunction with hybridization analysis indicated that homologous integration and gene disruption can occur at high frequencies in this species and confirmed that the flanking genomic sequences in pGMR1 contained pathogenicity gene sequences. Future studies will involve "plasmid rescue" of integrated vector and flanking DNA from representative REMI phenotypes A, B, and C.

The nonpathogenic mutations described in this study provide insight into the genetic basis of fungal symbiotic lifestyles. The fact that single gene disruptions can alter fungal lifestyles between mutualism, commensalism, and pathogenesis suggests that these genes may represent highly conserved, fundamental aspects of plant-fungal symbioses. More importantly, this phenomenon may reflect the genetic simplicity and flexibility that allow coevolving organisms to achieve an equilibrium of intimate coexistence. It may also reflect a fine balance associated with maintaining nondestructive plant-fungal associations, a balance that may be altered by environmental conditions, habitat alterations, or transportation of plants to new environments. It is well documented that environmental stress may increase the occurrence and severity of plant diseases. It is possible that the potential genetic simplicity of fungal symbiotic lifestyles may allow fungi to optimize their survival under varying environmental conditions even if that means converting from a mutualist to a pathogen. Regardless of this speculation, this system provides a unique opportunity to better understand the ecology, evolution, genetics, and biochemistry of plant-fungal symbioses.

MATERIALS AND METHODS

Fungal strains, media, and plant cultivar.

The pathogenic wild-type isolates L2.5 and Gm16 of *Colletotrichum magna* were obtained from S. Brown and O. C. Yoder (Cornell University, New York). Fungi were cultured on either liquid or solid modified Mathur's (MS) (Tu 1985) and/or hygromycin (HM) media containing 100 µg ml⁻¹ of ampicillin and hygromycin, respectively, as previously described (Redman and Rodriguez 1994). Seeds of Crimson Sweet, an anthracnose-susceptible watermelon cultivar, were purchased from Petoseed (Woodland, CA).

REMI transformation.

Transformations were carried out as previously described (Redman and Rodriguez 1994) with the following exceptions: production of protoplasts was carried out in OM buffer (1.2 M MgSO₄, 10 mM NaPO₄, pH 5.8) containing 3.5 mg ml⁻¹ of Novozyme 234 (Novonordisk, Bagsvaerd, Denmark), 3.5 mg ml⁻¹ of Lysing enzyme (Sigma, St. Louis, MO), and 100 U ml⁻¹ of β-glucuronidase; protoplast regeneration media contained 0.6 M Sucrose, 0.1% casamino acids, and 0.1% yeast extract, (pH 5.3); regeneration time was extended to 24 to 72 h; plasmid pHA1.3 (Powell and Kistler 1990; Rodriguez and

Redman 1992) was linearized with *Hind*III, and 40 to 80 U of restriction enzyme was added to carry out REMI protoplast transformation reactions.

To single-spore purify the transformants, conidia from each REMI transformant were diluted in 1 ml of sterile, distilled water, spread plated on MS medium, and allowed to germinate overnight. A single spore from each transformant was picked and plated onto HM selection medium. All REMI transformants were selected for and maintained on HM selection medium.

Stability of REMI transformants.

The stability of transformants was assessed by serial transfer of mycelial plugs of representative REMI transformants onto HM media containing or devoid of 100 µg ml⁻¹ of hygromycin every 2 weeks. Stability was assessed by the expression of hygromycin resistance after transformants had been serially transferred in the absence of selection for 6 weeks. REMI transformants exhibiting stable hygromycin resistance phenotypes were chosen and screened on plants for pathogenicity.

Characterization of REMI mutants.

Initial characterization of the 176 REMI mutants was performed by comparing growth rates, developmental processes, and extracellular enzyme production of the mutants to the wild-type isolate L2.5.

Growth rates were determined by inoculation of a 3-mm plug of mycelia onto the center of an MS medium plate and measurement of colony diameters 72 and 120 h after inoculation.

To measure spore adhesion, germination, and appressorial formation, conidia from each mutant were resuspended in sterile water at a concentration of 1 × 10⁵ spores ml⁻¹ and placed into sterile 60 × 15 mm polystyrene petri plates for 1 h at 22°C. Supernatants were decanted and the petri plates washed five times with 10 ml of sterile water. Spore adhesion was measured by counting the number of spores remaining attached (within four fields of vision at ×400 magnification) to the petri plates. To assess spore germination and appressorial formation, 10 ml of dilute (×0.1) MS medium was added to the petri plates at 22°C. Spore germination was monitored after 4 to 6 h and appressoria formation after 8 to 12 h, microscopically.

Extracellular protease and cellulase enzyme activities were measured by placing a 3-mm mycelial plug at the center of a plate containing either 0.5% gelatin and 1.2% agar (for the detection of protease activity) or 0.5% carboxymethylcellulose (CMC) and 1.2% agar media (for the detection of cellulase activity). Plates were incubated at 22°C for 5 days and measured for extracellular enzyme activities. Gelatin plates were covered with 10 ml of 15% mercuric chloride (wt/vol) and 20% HCL (vol/vol) for 15 min. Extracellular cellulase activity was visualized by staining CMC plates with a 1% Congo Red dye solution for 10 min, and destaining in 1 M NaCl. Protease and cellulase activities were visualized as a halo of clearing surrounding the peripheral edge of the fungal colony and activities were quantitated by measuring the ratio of the clearing diameter to the fungal colony diameter.

Plant pathogenicity assays and inoculation procedures.

Primary pathogenicity screens used three anthracnose-susceptible Crimson Sweet watermelon seedlings per REMI

transformant. Plants were grown in vermiculite for 5 to 7 days in growth chambers (95% humidity, 12-h light regimes, 22°C). Individual REMI transformants were streaked onto 5-ml MS medium slant tubes and grown for 5 to 7 days in a 12-h light regime at 22°C. The spores were resuspended in 5 ml of sterile 0.07% agar solution to a final concentration of 0.5 to 1.5×10^6 conidia ml⁻¹ and three watermelon seedlings were placed into each tube. Plant mortality was assessed after 7 to 10 days. Fluids were maintained at the same levels over the course of the experiments for all assays. Transformants that did not cause disease on any of the three plants were subjected to the secondary pathogenicity screening.

The secondary screening used 30 Crimson Sweet watermelon seedlings. Individual REMI mutants were streaked onto MS media plates and, after 5 days of growth, the conidia were harvested from the plates by gently scraping off the spores with a sterile glass slide. The spores were resuspended in 10 ml of sterile water, filtered through four layers of sterile cotton cheesecloth gauze (Fischer, Pittsburgh, PA) and centrifuged at $3,000 \times g$ for 5 min. The supernatant was decanted and the spores resuspended in sterile water, centrifuged at $3,000 \times g$ for 5 min, and the pellet resuspended in a final volume of 0.07% agar solution such that the conidial concentration was 1.0 to 3.0×10^6 conidia ml⁻¹. Thirty watermelon seedlings were placed into 50-ml glass beakers such that the roots and lower third of the stems were immersed into spore suspensions (1.0 to 3.0×10^6 conidia ml⁻¹), and plant mortality was assessed after 7 to 10 days. Three plants exposed to nonpathogenic REMI transformants were assayed for colonization (see below) and REMI transformants capable of colonizing plants in the absence of disease were chosen for the tertiary screening.

The tertiary screen was conducted in the same manner as the secondary screening with the exception that, after inoculation into REMI mutant spore solutions for 48 h, the plants were challenged with lethal concentrations (1.0 to 3.0×10^6 conidia ml⁻¹) of the wild-type *C. magna* isolate (L2.5) and plant mortality was assessed after 7 to 10 days. All experiments contained a negative control that consisted of 30 plants exposed to 0.07% agar solution devoid of fungal spores for 48 h followed by exposure to lethal concentrations of the wild-type isolate L2.5.

Recovering fungi from plant tissue.

Plants were submerged into 2.0% (vol/vol) sodium hypochlorite for 20 to 30 min with moderate agitation and rinsed with 10 to 20 volumes of sterile, distilled water. With the use of an aseptic technique, plants were cut into sections representing the lower, middle, and upper stem sections, the roots, and the cotyledons. The sections were plated onto HM selection media (containing 100 µg ml⁻¹ of hygromycin and ampicillin) and incubated at room temperature under cool fluorescent lights for 5 to 7 days to allow for the emergence of the fungi. Identification of fungi was verified after conidiation by microscopic analysis.

Molecular analysis of REMI transformants.

Fungal genomic DNA was isolated from lyophilized mycelia grown in 100 ml of MS liquid media as previously described (Rodriguez 1993; Freeman et al. 1993). Standard procedures were used for restriction enzyme digestion of fungal DNA (Sambrook et al. 1989). Digested and undigested DNA

samples were separated on 0.5 to 0.7% agarose gels and transferred onto nitrocellulose membranes (Micron Separations, Westboro, MA) by capillary migration (Sambrook et al. 1989). Membranes were vacuum dried for 30 min at 80°C and placed into hybridization solution—5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (Sambrook et al. 1989), 0.1% Sarkosyl, 0.02% SDS (sodium dodecyl sulfate), 0.5% blocking reagent (Boehringer-Mannheim, Indianapolis, IN)—for 2 to 12 h at 65°C. Plasmid pHA1.3 and pGMR1 were linearized with *Hind*III and/or *Xho*I restriction enzymes and were radioactively labeled with the T7 QuickPrime Kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Unincorporated nucleotides were removed with Sephadex G-100 columns (Sambrook et al. 1989) and membranes were hybridized with the probe in hybridization buffer for 24 to 48 h at 65°C. Membranes were then subjected to washes with decreasing salt concentrations (2×, 1×, 0.5× SSC) in the presence of 0.2% SDS. Membranes were wrapped in plastic and exposed to X-OMAT AR X-ray film (Kodak, Rochester, NY) at -70°C with intensifying screens until desired exposures were achieved.

Segregation analysis.

Genetic crosses were conducted between the nonpathogenic REMI mutant R1 (mating type B) and *C. magna* isolate Gm16 (mating type A). The mating type designations are arbitrary based on the initial description of this species (Jenkins and Winstead 1964). Crosses were established by inoculating a small mycelial plug of each mating type on opposite sides of a sterile corn leaf section placed on 1/10 potato dextrose agar. Inoculated plates were incubated at 22°C for 2 weeks to allow for the development of perithecia. The corn leaves were obtained from young plants (3 weeks old), cut into sections approximately 5 cm long, and autoclaved. Segregation analysis was conducted by removing perithecia from the corn leaves, washing them in sterile water to eliminate contaminating conidia, and breaking them open in 100 µl of sterile water on MS agar medium. After the ascospores were released, the perithecium was removed from the plate, the ascospores were spread over the surface of the medium, and the plates were incubated at 22°C for 24 h. Pure cultures of the germinated ascospores were obtained by transferring them to individual plates with a scalpel.

Plasmid rescue.

The pHA1.3 transformation vector and flanking genomic DNA surrounding the insertion site were retrieved by "plasmid rescue" as follows: The genomic DNA (3 to 5 µg) of fungal transformant R1 was digested with a restriction enzyme (*Xho*I) that does not digest the transformation vector but does digest in the flanking genomic DNA. The digested DNA was heat treated at 80°C for 30 min to inactivate the restriction enzyme, ethanol precipitated (0.1 M NaCl, 2.5× (vol/vol) 95% ethanol), and ligated (T4 DNA Ligase, GIBCO BRL, Life Technologies, Gaithersburg, MD) in a 100-µl reaction volume. Five microliters of the ligation reaction was used for transformation into the *E. coli* strain DH10B (ElectroMAX cells, GIBCO BRL, Life Technologies) via electroporation with a Cell Porator (GIBCO BRL, Life Technologies) according to the manufacturer's instructions with the exception of the following modifications: transformed bacterial cells

were grown in media devoid of ampicillin with moderate agitation at room temperature for 6 to 8 h; bacterial cells were plated onto medium supplemented with 100 µg ml⁻¹ of ampicillin and incubated at room temperature for 24 to 48 h to help facilitate "plasmid rescue."

Transformation of pGMR1 into *C. magna* L2.5.

C. magna L2.5 protoplasts were transformed with pGMR1 in the same manner as REMI transformation (described above) with the following modifications: pGMR1 was used as the transformation vector; pGMR1 was transformed as circular and *Xho*I-digested plasmid DNA in the absence of restriction enzymes.

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