Nonmycorrhizal (Myc^-) Mutants of *Hebeloma cylindrosporum* Obtained Through Insertional Mutagenesis

Jean-Philippe Combier, Delphine Melayah, Colette Raffier, Régis Pépin, Roland Marmeisse, and Gilles Gay

Université Lyon 1, UMR CNRS 5557 d'Ecologie Microbienne Bât. A. Lwoff, 43 boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

Submitted 5 January 2004. Accepted 26 April 2004.

Polyethylene glycol-mediated transformation of protoplasts was used as a method for insertional mutagenesis to obtain mutants of the ectomycorrhizal fungus *Hebeloma cylindrosporum* impaired in symbiotic ability. Following restriction enzyme-mediated integration or conventional plasmid insertion, a library of 1,725 hygromycin-resistant monokaryotic transformants was generated and screened for the symbiotic defect, using *Pinus pinaster* seedlings as host plants. A total of 51 transformants displaying a dramatically reduced mycorrhizal ability were identified. Among them, 29 were nonmycorrhizal (myc^-), but only 10 of them had integrated one or several copies of the transforming plasmid in their genome. Light and scanning electron microscopy observations of pine roots inoculated with myc^- mutants suggested that we selected mutants blocked at early stages of interaction between partners or at the stage of Hartig net formation. Myc^- mutants with plasmid insertions were crossed with a compatible wild-type monokaryon and allowed to fruit. Monokaryotic progenies were obtained in three independent crosses and were analyzed for symbiotic activity and plasmid insertion. In all three progenies, a 1:1 myc^- : myc^+ segregation ratio was observed, suggesting that each myc^- phenotype resulted from a single gene mutation. However, for none of the three mutants, the myc^- phenotype segregated with any of the plasmid insertions. Our results support the idea that master genes, the products of which are essential for symbiosis establishment, do exist in ectomycorrhizal fungi.

Fungi have evolved tight links with plants, including mutualistic associations such as the mycorrhizal symbiosis. This symbiosis has appeared independently several times during both plant and fungal evolution (Hibbet et al. 2000; Wilkinson 2001). This symbiotic association essentially leads to nutrient exchanges between the two partners. The fungus improves the mineral nutrition of its host plant and increases the plant ability to tolerate and survive under adverse conditions. In counterpart, the plant provides the fungus with photosynthetates required for vegetative growth and development, including completion of the sexual cycle (Smith and Read 1997).

Ectomycorrhizal fungi are commonly found interacting with roots of trees in temperate forests. Ectomycorrhiza is a specialized mixed structure resulting from a finely tuned interac-

tion between the plant root and a compatible fungus. It includes three structural components, each playing a specific role. Extraradical hyphae actively explore the soil, thus allowing the efficient transfer of water and nutrients to the plant root. The mantle is a parenchyma-like fungal tissue made of packed hyphae surrounding the root. The Hartig net, made of highly branched fungal hyphae proliferating between root cortical cells, constitutes a large interface of nutrient exchange between the host plant and its fungal partner.

Ectomycorrhiza formation has been extensively studied at both cytological and biochemical levels, but the genetic and molecular mechanisms that control this interaction remain largely unknown. Recent data suggest that the ability of fungal hyphae and root tissues to switch to symbiotic organ formation could be basically controlled by master genes (Martin 2001; Martin and Tagu 1999). The activity of these genes during ectomycorrhiza differentiation is associated with extensive changes in gene transcription (Voiblet et al. 2001) and protein biosynthesis (Hilbert et al. 1991). Differential screening of cDNA libraries obtained from mycorrhizas using mRNA from plant and fungus grown separately (Kim et al. 1999; Sundaram et al. 2001; Tagu et al. 1993) as well as the use of microarrays (Johansson et al. 2004; Voiblet et al. 2001) allowed the identification of numerous genes being transcriptionally regulated during the symbiotic process. However, molecular demonstration that any of these symbiosis-regulated genes is essential for mycorrhiza differentiation is still needed.

A method of choice to identify genes necessary for symbiosis establishment would be to obtain nonmycorrhizal (myc^-) mutants and to subsequently identify the corresponding mutated genes. Insertional mutagenesis is a powerful tool for this purpose because it usually results in loss-of-function mutations giving unambiguous phenotype and the gene of interest is physically tagged, thus making easy the characterization of the genomic regions flanking the site of insertion by, for example, plasmid rescue or inverse polymerase chain reaction. In filamentous fungi, plasmid DNA-mediated transformation has been the preferred method for insertional mutagenesis. It has been used to identify genes involved in pathogenicity in several species, including *Magnaporthe grisea* (Clergeot et al. 2001; Shi and Leung 1995; Sweigard et al. 1998; Urban et al. 1999), *Cochliobolus heterostrophus* (Lu et al. 1994, 2003; Yang et al. 1996), *Colletotrichum* species (Dufresne et al. 1998; Thon et al. 2000; Yakobi et al. 2001), and *Ustilago maydis* (Basse et al. 2000; Bölker et al. 1995). The addition of restriction enzymes has been shown to trigger the integration of the transforming DNA molecule to corresponding restriction

Corresponding author: G. Gay; E-mail: gay@univ-lyon1.fr; Telephone: +33 (0)4 72 44 80 47; Fax: +33 (0)4 72 43 16 43.

sites in several filamentous fungi. This process, originally described in *Saccharomyces cerevisiae* (Schiestl and Petes 1991) and referred to as restriction enzyme mediated integration (REMI), is of interest when generating large collections of insertional mutants, since it generally results in higher transformation efficiency and mostly in single-site integration of the transforming DNA molecule (Kahmann and Bass 1999; Kuspa and Loomis 1992; Schiestl and Petes 1991). Furthermore, REMI facilitates the recovery of flanking sequences.

To date, the basidiomycetous fungus *Hebeloma cylindrosporum* Romagnesi is one of the few ectomycorrhizal species suitable for insertional mutagenesis. Uninucleate protoplasts can be obtained from monokaryotic mycelia and viable mycelium can be regenerated from protoplasts (Durand et al. 1992; Hébraud and Fèvre 1987). A genetic transformation system has been developed (Marmeisse et al. 1992), thus allowing the selection of mutants. As haploid monokaryotic strains are able to form mycorrhizas (Debaud et al. 1988), both recessive and dominant mutations can be directly selected. Finally, the complete life cycle of this fungal species, including fruitbody and meiotic spore (basidiospore) production, can be obtained under controlled axenic conditions (Debaud and Gay 1987), thus allowing segregation analysis of mutations among haploid progenies.

In this study, we obtained, for the first time, nonmycorrhizal fungal mutants, using plasmid-mediated transformation of *H. cylindrosporum* as an insertional mutagenesis procedure. A detailed phenotypic characterization of some *myc*⁻ mutants suggested that we selected mutants impaired at different stages of the symbiotic process.

RESULTS

Insertional mutagenesis in *H. cylindrosporum*.

Protoplasts from the monokaryotic strain h7 of *H. cylindrosporum* were transformed with the integrative plasmid pGHGHT, using either conventional plasmid integration or REMI procedure. In several independent conventional transformations using either circular or prelinearized plasmid, hygromycin-resistant transformants were obtained at frequencies of 14.0 ± 7.0 and 10.5 ± 6.1 transformants per microgram of transforming DNA, respectively (Table 1). Southern blot analysis of 51 randomly selected hygromycin-resistant mycelia recovered following conventional transformation using circular plasmid indicated that 13 (25.5%) of them did not integrate transforming DNA in their genomes (data not shown). The frequency decreased to 0% (0/33) when the plasmid was

prelinearized by *Hind*III prior to transformation. Each transformant showing plasmid integration had a unique restriction pattern, indicating that the transforming molecule had no preferential insertion site in the fungal genome. Irrespective of the plasmid conformation, the average copy number integrated in the genome was approximately 2.5 (Table 1). The use of restriction enzyme *Hind*III or *Sal*I in variable amounts during REMI transformation significantly decreased the transformation efficiency, which ranged from 0.4 ± 0.3 to 3.8 ± 2.7 transformants per microgram of DNA, depending on the nature and concentration of the restriction enzyme used (Table 1). REMI transformation using *Hind*III and prelinearized plasmid yielded 12.8% (5/39) of untaged hygromycin-resistant mycelia or 7.4% (2/27) when transformation was performed in the presence of *Sal*I. REMI did not significantly reduce the number of plasmid copy per genome, which ranged from 1.3 ± 0.7 to 3.0 ± 0.5 , depending on the REMI protocol used. No true REMI integrations (i.e., single plasmid insertion with restored restriction sites at both plasmid and genomic DNA junctions) were observed, but tandem integrations of plasmid were evidenced (data not shown). Finally, 11 (8.5%) of the 130 plasmid-tagged transformants we characterized at the molecular level, obtained either by conventional or REMI transformation procedure, had integrated a single copy of the plasmid in their genome.

Identification of mycorrhiza-defective mutants.

Following 59 independent transformation experiments, a library of 1,725 putative insertional mutants was generated. Mycorrhizal activity of the transformants was assessed by performing in vitro mycorrhizal syntheses with *Pinus pinaster*, one of the common host plants of *H. cylindrosporum*. Cocultures were performed on three different media derived from Melin-Norkrans medium (MNM). For this, MNM was supplemented with either 2.7 mM fructose, 2.7 mM glucose, or 13.8 mM glucose as carbohydrate source. We identified 51 (approximately 3%) transformants dramatically impaired in their ability to form mycorrhizas. In contrast to the wild-type strain, these mutants were unable to form ectomycorrhizas when cocultures were performed on the MNM supplemented with 2.7 mM fructose or glucose. However, mycorrhizal ability could be partially restored for 22 of the 51 transformants by increasing the glucose concentration to 13.8 mM. Nevertheless, on this high glucose concentration, these transformants formed 3 to 30 times fewer mycorrhizas as compared with the wild-type strain (Fig. 1). For this reason, these latter mutants were qualified as poorly mycorrhizal in the sense

Table 1. Transformation efficiency (TE) and average copy number (ACN) of integrated plasmid in the genome of the haploid strain h7 of *Hebeloma cylindrosporum* following either conventional or REMI transformation^a

Nature	Enzyme	Transforming plasmid			
		Circular		Linear	
	Amount	TE	ACN	TE	ACN
None		14.0 ± 7.0 (15/969) ^b	2.8 ± 0.4 ($n = 34$) ^c	10.5 ± 6.1 (8/321) ^b	2.3 ± 0.3 ($n = 21$) ^c
<i>Hind</i> III	5 U	—	—	3.8 ± 2.7 (2/54)	3.0 ± 0.5 ($n = 10$)
	10 U	—	—	2.5 ± 1.8 (2/36)	1.8 ± 0.6 ($n = 8$)
<i>Sal</i> I	20 U	1.1 ± 0.7 (10/123)	1.3 ± 0.7 ($n = 4$)	1.5 ± 0.4 (5/93)	1.9 ± 0.3 ($n = 27$)
	40 U	0.6 ± 0.4 (3/19)	2.0 ± 1.0 ($n = 5$)	0.8 ± 0.5 (1/8)	1.7 ± 1.4 ($n = 3$)
	60 U	0.4 ± 0.3 (1/5)	1.7 ± 1.2 ($n = 3$)	0.7 ± 0.3 (4/28)	1.6 ± 0.8 ($n = 5$)
	80 U	—	—	1.0 ± 0.4 (7/70)	1.6 ± 0.8 ($n = 5$)
	100 U	—	—	0.9 ± 0.3 (1/9)	2.0 ± 0.5 ($n = 5$)

^a TE is expressed as the number of transformants obtained per μ g of transforming DNA. The number of plasmid copies integrated per genome was estimated by digesting genomic DNAs with a restriction enzyme that cuts once into the plasmid. *Hind*III or *Sal*I was used depending on the transformation procedure used. Data are given $\pm$ standard deviation of the mean ($P \leq 0.05$).

^b The first number indicates the number of independent transformation experiments, and the second number indicates the total number of hygromycin-resistant colonies used to calculate TE.

^c The number of randomly selected transformants used to calculate ACN.

they do not form any mycorrhiza on the MNM supplemented with 2.7 mM fructose or glucose, whereas they occasionally form mycorrhizas on the MNM supplemented with 13.8 mM glucose. In contrast, no detectable ectomycorrhiza was observed when pine roots were cocultured on this glucose-rich medium with the remaining 29 transformants. Since these transformants were found unable to differentiate any ectomycorrhiza on the three media tested, they were considered *myc*⁻. We focused on this class of mutants for further phenotypic characterization.

As mentioned above, mutants classified as *myc*⁻ were unable to form mycorrhizas when cocultures were performed on MNM supplemented with either 2.7 mM fructose or glucose at 2.7 or 13.8 mM. As illustrated in Figure 2, fungal survival and growth in the vicinity of the root seemed to differ between these *myc*⁻ mutants. Mycelia of nine mutants, exemplified by 1F25, 2E6, and 14D22, moderately proliferated in the rhizosphere when cocultures were performed in the presence of 2.7 or 13.8 mM glucose, whereas 14 mutants (illustrated for mutant 40B1) seemed to be unable to colonize the root surface when observed under a compound microscope (at 50× magnification).

The impact of *myc*⁻ mutations on the saprophytic growth of mutants presented in Figure 2 was investigated by studying their radial growth rate on the media used for mycorrhizal syntheses. In the absence of host plant, mutant strains cultivated in the presence of glucose were hardly discriminated from the wild-type strain (Fig. 3). In contrast, in the presence of 2.7 mM fructose, the transformant 40B1 showed a twofold reduction of its radial growth rate as compared with the wild-type strain, whereas the growth rate of mutant 14D22 was slightly reduced compared with that of the wild type. These results show that the *myc*⁻ mutations we selected can have different impacts on the saprophytic growth of the fungus but the inability of mutants to form mycorrhizas on the three studied media cannot be ascribed to a growth defect.

Detailed light microscope observations (400× magnification) of squashed short roots in contact with hyphae of *myc*⁻

mutants were performed, using roots grown on the three MNM media we used. For 19 of the mutants (such as 1F25, 14D22, 2A9, 10F11, and 40B1), neither fungal sheath nor Hartig net could ever be detected, indicating that these mutants are probably blocked at a very early stage of the symbiotic process. For 10 mutants (such as 2E6), no Hartig net was discernable, but we could occasionally observe, on roots grown in the presence of 13.8 mM glucose, some rare patches of loosely packed labyrinthic hyphae that may indicate the presence of discontinuous fungal sheath on the root surface (data not shown). This suggests these mutants could be blocked at a later stage of symbiosis establishment than the former mutants.

A scanning electron microscopy (SEM) study was performed to clarify at which stage of symbiosis establishment the above-described *myc*⁻ mutants are blocked. When mycorrhizal syntheses were performed in the presence of 2.7 mM fructose, hyphae of the wild-type strain were shown to adhere to *P. pinaster* root within two days after inoculation (Fig. 4A), and a typical mantle was visible three days after inoculation (Fig. 4B). Mycorrhizas showed a typical Hartig net 7 days postinoculation (Fig. 4C). For mutant 2E6, despite the repeated observation of hyphae growing inside the mucilage covering the roots, no Hartig net was ever observed (Fig. 4D). For this mutant, patches of mantle-like hyphae we observed under light microscope on roots grown in the presence of 13.8 mM glucose could not be evidenced on roots grown on the MNM medium supplemented with 2.7 mM fructose. Seven days after inoculation with the *myc*⁻ mutant 14D22, cortical cells of *P. pinaster* taproots became disorganized (compare the colonized left part of the taproot in Figure 4E with the right part, which is not colonized), whereas hyphae did not aggregate to form a mantle (Fig. 4F). Similar observations were made in mutants 1F25 and 40B1 (Fig. 4G and H, respectively). Figure 4G shows that the outer root cortical cells are entirely dissociated from each other and appear as rows of cells that have lost their longitudinal orientation and are curled. This suggests that longitudinal walls of cortical cells have been partly disorganized. Figure 4H shows noticeable disorganization of cortical cells in the

Fig. 1. Mycorrhizal activity of eight randomly selected poorly mycorrhizal mutants. Mycorrhizal syntheses were performed on Melin-Norkrans medium (MNM) supplemented with 13.8 mM glucose, using *Pinus pinaster* as host plant. Mycorrhizas were counted three weeks after inoculation. As opposed to the original wild-type strain h7, these poorly mycorrhizal mutants unequivocally did not form any mycorrhiza on MNM medium containing either 2.7 mM glucose or fructose as carbon source. Data are given $\pm$ standard deviation of the mean ($P \leq 0.05$).

Fig. 2. Phenotypic characterization of four representative nonmycorrhizal (*myc*⁻) mutants. Mycorrhizal syntheses were performed, using *Pinus pinaster* as host plant on Melin-Norkrans medium containing 2.7 mM fructose or 2.7 to 13.8 mM glucose as sole carbon source. Root systems were photographed 3 weeks after inoculation. Control corresponds to uninoculated pine roots. The whitish discs on the agar surface correspond to the fungal inoculum plugs. Root hairs are visible on most short roots of control plants and plants inoculated with *myc*⁻ mutants. They are absent from roots inoculated with the control wild-type strain h7. Bar = 2 mm.

colonized left part of the taproot as compared with the noncolonized right part. With mutant 40B1, hyphae were embedded in a partly disorganized root mucilage (Fig. 4I). The disorganization of the root cortical cells when colonized by these mutants was not detrimental to the plants, since we did not observe any significant reduction in root or shoot biomass when compared with plants inoculated with the wild-type strain (data not shown). In the case of two other *myc*⁻ mutants, namely 2A9 and 10F11, as early as day 3 postinoculation, the hyphal tips seemed to burst in contact with the taproot (Fig. 4J). The hyphae progressively broke open, releasing their cellular content (Fig. 4K), and finally collapsed entirely (Fig. 4L). Consequently, fungal colonization did not proceed further.

Molecular and genetical analyses of the *myc*⁻ mutants.

Southern blot hybridizations showed that only 10 out of the 29 *myc*⁻ mutants had integrated transforming DNA, the plasmid copy number varying from one to three per genome (Fig. 5). All the mutants harboring transforming plasmid in their genome were shown to contain the hygromycin phosphotransferase (*hph*) sequence after more than a 1-year growth on a nonselective medium (data not shown), indicating that mitotically stable plasmid insertions into the genome had occurred.

To determine whether the *myc*⁻ mutations were tagged, each transformant was crossed with a sexually compatible wild-type monokaryon. All reconstituted dikaryons displayed mycorrhizal activity (data not shown), indicating that the *myc*⁻ mutations we selected are recessive. After *in vitro* mycorrhizal syntheses, sporulating fruit bodies were recovered for three different crosses involving the *myc*⁻ mutants 2A9, 14D22, and 40B1. *Myc*⁻ homokaryotic mycelia were recovered in the haploid progenies of all three crosses (Table 2). A chi-square comparison showed that the segregation ratios of the *myc*⁻/*myc*⁺ phenotypes within the three progenies were not significantly different from a 1:1 ratio, indicating that the *myc*⁻ phenotypes that characterize transformants 2A9, 14D22, and 40B1 were probably the result of single gene mutations.

At the molecular level, the two copies of pGHGHT inserted in the genome of 2A9 were shown to segregate independently in the progeny of the cross (data not shown). However, no cosegre-

gation could be evidenced between the *myc*⁻ phenotype and either of the two plasmid insertions. Noticeably, some *myc*⁻ sib-monokaryons did not carry any of the two plasmid insertions in their genomes. This indicates that the *myc*⁻ phenotype did not result from an integrative event. Similar results were obtained with mutant 14D22, which contained two genetically independent insertions of pGHGHT. In case of mutant 40B1, the unique plasmid insertion was recovered in less than 10% of the progeny. This suggests that this plasmid insertion may affect spore germination. As for this mutant, we also identified several *myc*⁻ sib-monokaryons having no plasmid integration and concluded that the mutation at the origin of the *myc*⁻ phenotype of 40B1 was not tagged.

DISCUSSION

In this work, we showed that nonectomycorrhizal fungal mutants can be obtained through insertional mutagenesis, thus providing the starting point for the identification of essential symbiotic genes. This represents a major advance in our ability to investigate the molecular mechanisms underlying mycorrhizal symbiosis establishment. To our knowledge, this is the first report of nonsymbiotic mutants of a mycorrhizal fungus. To date, only nonarbuscular mycorrhizal plants have been identified (David-Schwartz et al. 2001, 2003; Duc et al. 1989; Marsch and Schultze 2001). Such mutants have proven to be very useful tools to dissect plant functions involved in mycorrhizal symbiosis (Ané et al. 2004; Bonfante et al. 2000; Lévy et al. 2004).

Using the integrative plasmid pGHGHT, the transformation efficiency of *H. cylindrosporum* was low, from one to ten transformants per microgram of plasmid DNA were selected. It was, however, of the same order of magnitude as that reported for conventional transformation in *Aspergillus nidulans* (Sanchez et al. 1998), *Colletotrichum graminicola* (Thon et al. 2000), or *U. maydis* (Bölker et al. 1995). Irrespective of the enzyme used and of its concentration, REMI did not significantly improve *H. cylindrosporum* transformation. In theory, REMI increases both transformation efficiency and single copy plasmid integration, as exemplified in *Aspergillus oryzae* (Yaver et al. 2000), *Coprinus cinereus* (Cummings et al. 1999;

Fig. 3. Growth rate of nonmycorrhizal mutants. Mycelia were cultivated on the different Melin-Norkrans media (MNM) used for mycorrhizal syntheses. Growth rate was calculated on the basis of growth between days 7 and 14. Data are given $\pm$ standard deviation of the mean ($P \leq 0.05$). Means with the same letters were not significantly different ($P \leq 0.05$) on the basis of the Fischer's protected least significant difference test, performed using Statview software (Abacus Concept Inc., Berkeley, CA, U.S.A.).

Fig. 4. Scanning electron microscopy study of the early interactions between some nonmycorrhizal (myc^-) mutants and *Pinus pinaster* roots. Roots were observed after 3 to 7 days of cocultivation on Melin-Norkrans medium containing 2.7 mM fructose as sole carbon source. **A**, One day after inoculation, hyphae of the wild-type monokaryon h7 adhere (arrows) to the epidermal cells of taproots. **B**, Hyphae of the wild-type strain rapidly proliferate at the root surface and form a mantle (star). **C**, Cross section in a mature mycorrhiza formed by the wild-type strain h7 sampled 7 days after inoculation, showing a mantle (M) and a typical Hartig net (arrows) between plant cortical cells (CC). **D**, Fracture in a short root 7 days after inoculation with the myc^- mutant 2E6. Fungal hyphae (arrows) can be seen growing in the mucilage covering the root. Hyphae do not proliferate between plant cortical cells (CC). **E**, *P. pinaster* taproot inoculated with the myc^- mutant 14D22. The left half of the root is colonized by the fungus, the right half is not. The plant cortical cells are noticeably disorganized (arrows) in the root parts colonized by the fungus, whereas they are not disorganized in the parts of the root free from fungal hyphae. **F**, Higher magnification of the taproot shown in E, revealing that fungal hyphae (arrows) do not branch and do not form any mantle in contact with the root. Stars indicate remains of mucilage covering the taproot. **G**, Taproot 7 days after inoculation with the myc^- mutant 1F25. The outer cortical cells are completely disorganized (as compared with the noncolonized right part of the root shown in H), appearing as rows of cells that get curled (arrows), suggesting that their longitudinal walls have been disorganized. **H**, Taproot 7 days after inoculation with the myc^- mutant 40B1. The right part of the root, which is not colonized by the fungus, shows the typical aspect of a normal *P. pinaster* taproot. The left side of the root is colonized by the fungus and shows disorganized cortical cells (arrows). **I**, Higher magnification of the colonized part of the root shown in H. Hyphae are mainly nonbranching (arrows) and do not aggregate to form a mantle. They are embedded in remains of mucilage covering the root (stars). **J**, Hypha of mutant 2A9 growing on the root surface 3 days after inoculation (arrow). Its apex seems to burst (double arrowhead) in contact with a cortical cell. **K**, Hyphae of mutant 10F11 growing in contact with a host root break open 7 days after inoculation and release their cellular content (arrows). **L**, Hyphae of mutant 10F11 finally collapse entirely (arrows) 7 days after inoculation, and colonization does not proceed further. Bars = 2 μm (D, J, K, L), 5 μm (A, B), 10 μm (C, F, I), 50 μm (H), 100 μm (G), and 200 μm (E).

Granado et al. 1997), *M. grisea* (Shi et al. 1995), *Cochliobolus heterostrophus* (Lu et al. 1994), and *Colletotrichum graminicola* (Thon et al. 2000). However, REMI transformation efficiency is known to depend on fungal species, vector conformation, restriction enzyme, and transformation procedure (Maier and Schäfer 1999; Mullins and Kang 2001). Complex plasmid integration patterns were usually observed in *H. cylindrosporum* genome. They included plasmid rearrangements, tandem repeat insertions, and systematic loss of one or both flanking restriction sites, as previously reported in other species (Chung et al. 2003; Sanchez et al. 1998). This situation suggests that nonhomologous end-joining mechanisms (Riggle and Kumamoto 1998) are likely to operate in *H. cylindrosporum*. From these results, we concluded that REMI does not help in generating *H. cylindrosporum* insertional mutants suitable for the straightforward recovery of inactivated genes by plasmid rescue. However, the fact that all the transformants clearly harbored distinct hybridization patterns was consistent with random ectopic integrations in the *H. cylindrosporum* genome, which is a prerequisite for efficient insertional mutagenesis.

Surprisingly, following conventional transformation using circular plasmid, approximately 25% of the hygromycin-resistant colonies we recovered did not contain any transforming DNA. The most trivial explanation for this could be that hygromycin concentration used to select transformants was too low, allowing spontaneous resistant variants to arise. However, no untagged mutant was detected when transformation was performed using prelinearized plasmids, and REMI transformation also decreased the frequency of untagged colonies. This suggests that the occurrence of untagged hygromycin-resistant mycelia depends upon the transformation procedure used and is not simply a consequence of a too low selection pressure. For the three *myc*⁻ mutants we studied in detail, we could show that the *myc*⁻ mutations were not tagged by plasmid insertions, thus indicating that mutational events independent of plasmid integration arose upon transformation. It cannot be ruled out that some of these events also confer hygromycin

resistance. Untagged mutations have already been reported in filamentous fungi transformed by conventional or REMI insertional mutagenesis (Balhadère et al. 1999; Cummings et al. 1999; Idburn and Howlett 2002; Lu et al. 1994; Sanchez et al. 1998; Sweigard et al. 1998; Thon et al. 2000). Kahmann and Basse (1999) estimated that, in most fungal systems, about half of the mutations selected following plasmid-mediated transformation were not the result of the integration of the transforming DNA in the genome. Consequently, it is necessary to experimentally demonstrate that mutant phenotypes indeed result from transforming DNA integrations.

Despite these limitations, insertional mutagenesis in *H. cylindrosporum* allowed us to successfully isolate mutants impaired in symbiotic process. We identified 29 nonmycorrhizal mutants among 1,725 *H. cylindrosporum* transformants, which corresponds to a frequency of 1.7%. Similar frequencies have been reported for nonpathogenic mutants in *Colletotrichum graminicola*, *M. grisea*, and *U. maydis*, in which it was 0.3% (Thon et al. 2000), 0.5% (Sweigard et al. 1998), and 1.4% to 2.8% (Bölker et al. 1995; Kahmann and Basse 1999), respectively. This suggests that insertional mutagenesis is as efficient with symbiotic fungi as it has proven to be with pathogenic fungi.

The screening procedure we used to select *myc*⁻ mutants was designed to specifically identify mutants impaired in symbiosis establishment. The detailed phenotypic characterization of some of them led us to the conclusion that they can fall into three classes.

Some of the mutants (such as 2A9 and 10F11) have a very poor ability to colonize the surface of pine roots. SEM revealed that fungal hyphae in contact with roots seemed to burst, so that the colonization was arrested immediately after the first contact between the two symbionts. For this reason, we called these mutants BAC (blocked after contact). Since hyphal integrity was preserved when the mycelium was not in contact with host root, we hypothesize that the one or more corresponding mutations could affect a transduction pathway required for the fungus to perceive one or more plant signals as soon as it reaches a host root.

Fig. 5. Southern blot analysis of genomic DNAs isolated from the nonmycorrhizal mutants harboring plasmid integrations. Genomic DNAs were digested with *Sa*II, which cuts the transforming plasmid pGHGHT once and were probed with a α^{32} P-labeled pGHGHT. No hybridization signal was observed when probing genomic DNA from the wild-type strain h7 (data not shown). Arrow indicates the size of the linearized pGHGHT plasmid.

Table 2. Segregation ratio of the nonmycorrhizal (*myc*⁻) phenotype within the monokaryotic progenies of dikaryons synthesized from the *myc*⁻ mutants 2A9, 14D22, and 40B1 of *Hebeloma cylindrosporum*^a

Cross	Number of sib-monokaryons	Observed <i>myc</i> ⁻ / <i>myc</i> ⁺ ratio	Expected <i>myc</i> ⁻ / <i>myc</i> ⁺ ratio ^b	Chi square ^c
2A9 × GU 95 I h1	106	47:59	53.0:53.0	1.38*
14D22 × GU 95 I h1	155	66:89	77.5:77.5	3.49*
40B1 × GU 95 I h1	89	36:53	44.5:44.5	3.37*

^a GU 95 I h1 is a wild-type haploid strain sexually compatible with the h7 strain and its derivatives.

^b Expected *myc*⁻/*myc*⁺ segregation ratio indicates theoretical ratio in accordance with a single gene segregation.

^c The chi square values shown are consistent with a 1:1 segregation ratio ($P \leq 0.05$).

Other mutants (e.g., 1F25, 40B1, or 14D22) proliferated at the surface of host roots, but hyphae did not show any modification in their branching pattern. We speculate that these mutants did not perceive one or more plant signals that normally induce hyphal branching and subsequent mantle differentiation. We called this class of mutants MaD (mantle defective). Hyphae of MaD mutants seemed to induce root cortical cell disorganization. Since such a situation has never been observed with control roots or with roots inoculated with the wild-type strain, this could result from the absence of early cross talk between the two partners that prevents the fungus from switching to a symbiotic program. In the absence of symbiotic interaction, the *myc*⁻ hyphae that proliferated on the root surface could induce the disorganization of root cortical cells.

Mutant 2E6 colonized the host root surface but did not induce any disorganization of the root cortical cells. In the presence of 13.8 mM glucose, some hyphae were shown to initiate mantle differentiation, but hyphal aggregation never reached completion, leading only to discontinuous patches of fungal sheath, and no Hartig net was ever observed. From these observations, we conclude that mutants such as 2E6 are blocked at a late stage of symbiosis establishment prior to Hartig net formation; we called this class of mutants HaND (Hartig net defective).

The *myc*⁻ phenotype segregated 1:1 within the progeny of the three mutants we studied in detail, indicating that these phenotypes resulted from single gene mutations. However, the fact that these mutations are untagged will delay the identification of the corresponding altered gene. As an alternative, successful gene tagging following *Agrobacterium tumefaciens*-mediated transformation is a promising method to facilitate molecular cloning (de Groot et al. 1998). As T-DNA tagging operates in *H. cylindrosporum* (Combier et al. 2003), we are currently developing T-DNA insertion lines to expand our *myc*⁻ mutant library. Genetic and molecular characterization of such mutants will allow dissection of the molecular mechanisms underlying symbiosis establishment in fungi.

MATERIALS AND METHODS

Fungal strains and culture conditions.

H. cylindrosporum homokaryotic strain h7 (Debaud and Gay 1987) was used as the recipient host for all genetic transformations. The monokaryotic wild-type strain GU95 I h1 (Guidot et al. 1999) was used as the sexually compatible strain in genetic crosses. All the fungal strains were maintained on YMG agar medium (Rao and Niederpruem 1969) or YMG plus 50 µg per milliliter of hygromycin B at 22 ± 1°C in the dark.

Transformation procedures.

Protoplasts were prepared from actively growing mycelium and were transformed as previously described (Marmeisse et al. 1992), using the plasmid pGHGHT (Schuren and Wessels 1998), which contains the *Escherichia coli* *hph*-encoding gene flanked by the *gpd* promoter and terminator regions from *Schizophyllum commune*. Conventional polyethylene glycol (PEG)-CaCl₂-mediated transformation was performed by incubating 100 µl of protoplast suspension (containing 2 × 10⁷ to 7 × 10⁷ protoplasts) in the presence of 5 µg of plasmid DNA in less than 25 µl of TE buffer (1 mM EDTA, 10 mM Tris HCl, pH 8.0) and of 25 µl of PEG solution (25% PEG 4000 supplemented, 25 mM CaCl₂, 10 mM Tris HCl, pH 7.5). Incubation was first performed on ice for 20 min. PEG solution (1 ml) was subsequently added to the mixture, which was further incubated at room temperature for 5 min. The reaction was stopped by adding 2 ml of STC (1 mM sorbitol, 10 mM Tris

HCl, pH 7.5, 25 mM CaCl₂). Aliquots (500 µl) were then plated onto a selective regeneration medium (YMG medium supplemented with 0.6 M sucrose and 50 µg per milliliter of hygromycin B). This antibiotic concentration is inhibitory for hyphal growth of the wild-type strain. Immediately after protoplasts were seen to regenerate, the surface of the medium was overlaid with the same medium. Mycelia growing through this second layer were isolated and grown successively on two plates containing YMG plus 50 µg per milliliter of hygromycin B and then were propagated onto a nonselective medium. REMI transformation was performed under the same conditions, except *Hind*III or *Sal*I restriction endonucleases were added in varying amounts to the protoplast-plasmid mixture (discussed above).

DNA manipulation and Southern blot hybridization analysis.

Genomic DNA was extracted from freeze-dried mycelia, as previously described (Van Kan et al. 1991), and was digested with either *Bgl*II, which does not cut pGHGHT, or *Hind*III or *Sal*I, which cut once in the plasmid. DNA fragments were separated by electrophoresis in a 0.7% agarose gel and were blotted onto positively charged nylon membranes (Qbiogen, Illkirch, France) using standard techniques (Sambrook et al. 1989). A 5,500-bp fragment corresponding to the entire linearized pGHGHT plasmid was used as a probe to determine the plasmid copy number and the occurrence of tandem repeat integrations. The probe was radiolabeled with α³²P-dCTP, using a random priming kit (Boehringer, Mannheim, Germany). High-stringency hybridizations were performed using standard protocols (Sambrook et al. 1989).

Screening of *myc*⁻ mutants.

In vitro ectomycorrhizal syntheses were performed by inoculating the fungal strain to be tested along the taproot of sterile two-week-old *P. pinaster* seedlings placed on the surface of a solid modified MNM medium (Norkrans 1949) containing 0.45 mM CaCl₂, 0.43 mM NaCl, 3.67 mM KH₂PO₄, 0.22 mM (NH₄)₂HPO₄, 0.61 mM MgSO₄ 7H₂O, Heller's (1953) micronutrients (Gay 1990), 10 g per liter of agar, and 2.7 mM fructose or glucose or 13.8 mM glucose as carbon source and poured into 140-mm petri dishes (Gea et al. 1994). We used fructose instead of glucose as carbon source because the extramatrical mycelium is much less abundant on this former sugar, making the observation of mycorrhizas easier. After a three-week coculture on the MNM medium containing 2.7 mM fructose, root systems were observed under compound microscope to check for the presence or absence of mycorrhizas. At that stage, we defined a *H. cylindrosporum*/*P. pinaster* mycorrhiza as a short root, usually dichotomously branched, devoid of root hair, and surrounded by fungal hyphae. For each transformant, two inoculated plants were initially used. For each transformant being obviously affected in its ability to form mycorrhizas, the initial screening procedure was repeated five times. Transformants identified as putative *myc*⁻ mutants following this screening were again tested in two subsequent runs of mycorrhizal syntheses, each of them involving 40 plants per transformant. All short roots of plants inoculated with putative *myc*⁻ transformants were carefully squashed between two glass slides and were examined under light microscope after staining fungal cells with trypan blue. Presence of fungal hyphae describing typical mantle or Hartig net structures (Smith and Read 1997) was scored. The influence of the carbon source on the expression of the *myc*⁻ phenotypes was evaluated by replacing the 2.7 mM fructose in the culture medium by either 2.7 or 13.8 mM glucose as carbon source. Some of the puta-

tive myc⁻ mutants formed some mycorrhizas in the presence of 13.8 mM glucose. At the end of this screening procedure, we defined a myc⁻ mutant as a mutant that always failed to form any short root having a typical ectomycorrhizal morphology and a Hartig net, whatever the carbon source added to the MNM medium.

SEM observation of myc⁻ mutants.

Root fragments approximately 8 to 10 mm long were excised 3 to 7 days after inoculation and were fixed for 2 days at room temperature in 3% glutaraldehyde dissolved in 0.1 M citrate phosphate buffer. Samples were then dehydrated in a graded series of acetone solutions (5% increments from pure water to pure acetone) and, finally, were processed through CO₂ critical point drying. They were subsequently mounted on SEM stubs with double-faced adhesive tape and were coated with gold-palladium alloy. Observation was performed at the Centre Technologique des Microstructures of the Lyon 1 University (Lyon, France) on a Hitachi S800 scanning electron microscope (Hitachi Scientific Instruments, Sophia-Antipolis, France) operated at 15KV. Micrographs were taken on Kodak PXP films and processed in a Kodak D76 developer (Kodak, Rochester, NY, U.S.A.).

Genetic analysis of myc⁻ mutations.

Each myc⁻ mutant was individually crossed with the sexually compatible wild-type haploid strain GU 95 I h1 to obtain a heterozygous dikaryon. In vitro fruiting of the dikaryons was induced as described by Debaud and Gay (1987). After a 6-month to 1-year coculture period, fruit bodies were obtained, and sib-monokaryotic strains were isolated from single spore germinations.

ACKNOWLEDGMENTS

J. P. Combiér was supported by a grant from the French Ministère de l'Éducation Nationale, de la Recherche et de la Technologie. We thank F. Schuren and J. Wessels for providing pGHGT plasmid clone. We wish to thank S. Cabral and A. Vouilloux for their technical assistance in molecular analysis of the transformants.

LITERATURE CITED

- Ané, J. M., Kiss, G. B., Riely, B. K., Penmetsa, R. V., Oldroyd, G. E. D., Ayax, C., Lévy, J., Debellé, F., Baek, J. M., Kalo, P., Rosenberg, C., Roe, B. A., Long, S. R., Dénarié, J., and Cook, D. R. 2004. *Medicago truncatula DM11* required for bacterial and fungal symbioses in legumes. *Science* 303:1364-1367.
- Balhadère, P. V., Foster, A. J., and Talbot, N. 1999. Identification of pathogenicity mutants of the rice blast fungus *Magnaporthe grisea* by insertional mutagenesis. *Mol. Plant-Microbe Interact.* 12:129-142.
- Basse, C. W., Stumpferl, S., and Kahmann, R. 2000. Characterization of a *Ustilago maydis* gene specifically induced during the biotrophic phase: Evidence for negative as well as positive regulation. *Mol. Cell. Biol.* 20:329-339.
- Bölker, M., Böhnert, H. U., Braun, K. H., Gröl, J., and Kahmann, R. 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme mediated integration (REMI). *Mol. Gen. Genet.* 248:547-552.
- Bonfante, P., Genre, A., Faccio, A., Martini, I., Schausser, L., Stougaard, J., Webb, J., and Parniske, M. 2000. The *Lotus japonicus* *LjSym4* gene is required for the successful symbiotic infection of root epidermal cells. *Mol. Plant-Microbe Interact.* 13:1109-1120.
- Chung, K. R., Ehrenshaft, M., Wetzell, D. K., and Daub, M. E. 2003. Cercosporin-deficient mutants by plasmid tagging in the asexual fungus *Cercospora nicotianae*. *Mol. Genet. Genomics* 270:103-113.
- Clergeot, P. H., Gourgues, M., Cots, J., Laurans, F., Latorse, M. P., Pépin, R., Tharreau, D., Notteghem, J. L., and Lebrun, M. H. 2001. *PLSI*, a gene encoding a tetraspanin-like protein, is required for penetration of rice leaf by the fungal pathogen *Magnaporthe grisea*. *Proc. Natl. Acad. Sci. U.S.A.* 98:6963-6968.
- Combiér, J.-P., Melayah, D., Raffier, C., Gay, G., and Marmeisse, R. 2003. *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the symbiotic ectomycorrhizal fungus *Hebeloma cylindrosporum*. *FEMS (Fed. Eur. Microbiol. Soc.) Lett.* 220:141-148.
- Cummings, W. J., Celerin, M., Crodian, J. K., Brunick, L. K., and Zolan, M. E. 1999. Insertional mutagenesis in *Coprinus cinereus*: Use of a dominant selectable marker to generate tagged, sporulation defective mutants. *Curr. Genet.* 36:371-382.
- David-Schwartz, R., Badani, H., Smadar, W., Levy, A. A., Galili, G., and Kapulnik, Y. 2001. Identification of a novel genetically controlled step in mycorrhiza colonization: Plant resistance to infection by fungal spores but not extra-radical hyphae. *Plant J.* 27:561-569.
- David-Schwartz, R., Gadkar, V., Wininger, S., Bendov, R., Galili, G., Levy, A. A., and Kapulnik, Y. 2003. Isolation of a premycorrhizal infection (*pmi2*) mutant of tomato resistant to arbuscular mycorrhizal fungal colonization. *Mol. Plant-Microbe Interact.* 16:382-388.
- Debaud, J. C., and Gay, G. 1987. In vitro fruiting under controlled conditions of the ectomycorrhizal fungus *Hebeloma cylindrosporum* associated with *Pinus pinaster*. *New Phytol.* 105:429-435.
- Debaud, J. C., Gay, G., Prevost, A., Lei, J., and Dexheimer, J. 1988. Ectomycorrhizal ability of genetically different homokaryotic and dikaryotic strains of *Hebeloma cylindrosporum*. *New Phytol.* 108:323-328.
- de Groot, M. J. A., Bundock, P., Hooykaas, P. J. J., and Beijersbergen, A. G. M. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnol.* 16:839-842.
- Duc, G., Trouvelot, A., Gianinazzi-Pearson, V., and Gianinazzi, S. 1989. First report of non-mycorrhizal plant mutants (Myc⁻) obtained in pea (*Pisum sativum* L.) and fava bean (*Vicia faba* L.). *Plant Sci.* 60:215-222.
- Dufresne, M., Bailey, J. A., Dron, M., and Langin, T. 1998. *Clk1*, a serine/threonine kinase-encoding gene, is involved in pathogenicity of *Colletotrichum lindemuthianum* on common bean. *Mol. Plant-Microbe Interact.* 11:99-108.
- Durand, N., Debaud, J. C., Casselton, L. A., and Gay, G. 1992. Isolation and preliminary characterization of 5-fluorindole-resistant and IAA-overproducer mutants of the ectomycorrhizal fungus *Hebeloma cylindrosporum* Romagnesi. *New Phytol.* 121:545-553.
- Gay, G. 1990. Effect of the ectomycorrhizal fungus *Hebeloma hiemale* on adventitious root formation in deoeroted *Pinus halepensis* shoot hypocotyls. *Can. J. Bot.* 68:1265-1270.
- Gea, L., Normand, L., Vian, B., and Gay, G. 1994. Structural aspects of ectomycorrhiza of *Pinus pinaster* (Ait.) Sol. formed by an IAA-overproducer mutant of *Hebeloma cylindrosporum* Romagnesi. *New Phytol.* 128:659-670.
- Granado, J. D., Kertesz-Chaloupková, K., Aebi, M., and Kües, U. 1997. Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. *Mol. Gen. Genet.* 256:28-36.
- Guidot, A., Lumini, E., Debaud, J. C., and Marmeisse R. 1999. The nuclear ribosomal DNA intergenic spacer as a target sequence to study intraspecific diversity of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* directly on *Pinus* root systems. *Appl. Environ. Microbiol.* 65:903-909.
- Hébraud, M., and Fèvre, M. 1987. Protoplast production and regeneration from mycorrhizal fungi and their use for isolation of mutants. *Can. J. Microbiol.* 34:157-161.
- Heller, R. 1953. Recherches sur la nutrition des tissus végétaux cultivés in vitro. *Ann. Sci. Nat. Bot. Biol. Vég.* 14:1-21.
- Hibbet, D. S., Gilbert, L. B., and Donoghue, M. J. 2000. Evolutionary instability of ectomycorrhizal symbioses in Basidiomycetes. *Nature* 407:506-508.
- Hilbert, J. L., Costa, G., and Martin, F. 1991. Ectomycorrhizal synthesis and polypeptide changes during the early stage of eucalyptus mycorrhiza development. *Plant Physiol.* 97:977-984.
- Idnurm, A., and Howlett, B. J. 2003. Analysis of loss of pathogenicity mutants reveals that repeat-induced point mutations can occur in the Dothideomycete *Leptosphaeria maculans*. *Fungal Genet. Biol.* 39:31-37.
- Johansson, T., Le Quéré, A., Ahren, D., Söderström, B., Erlandsson, R., Lundberg, J., Uhlén, M., and Tunlid, A. 2004. Transcriptional responses of *Paxillus involutus* and *Butleria pendula* during formation of ectomycorrhizal root tissues. *Mol. Plant-Microbe Interact.* 17:202-215.
- Kahmann, R., and Basse, C. 1999. REMI (restriction enzyme mediated integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. *Eur. J. Plant Pathol.* 105:221-229.
- Kim, S. J., Bernreuther, D., Thumm, M., and Podila, G. K. 1999. *LB-AUT7*, a novel symbiosis-regulated gene from an ectomycorrhizal fungus, *Laccaria bicolor* is functionally related to vesicular transport and autophagocytosis. *J. Bacteriol.* 181:1963-1967.
- Kuspa, A., and Loomis, W. F. 1992. Tagging developmental genes in *Dicystostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. U.S.A.* 89:8803-8807.
- Lévy, J., Bres, C., Geurts, R., Chalhou, B., Kulikova, O., Duc, G., Journet, E. P., Ané, J. M., Lauber, E., Bisseling, T., Dénarié, J., Rosenberg, C and Debellé, F. 2004. A putative Ca²⁺ and calmodulin-dependent protein

- kinase required for bacterial and fungal symbioses. *Science* 303:1361-1363.
- Lu, S. W., Lyngholm, L., Yang, G., Bronson, C., Yoder, O. C., and Turgeon, B. G. 1994. Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. U.S.A.* 91:12649-12653.
- Lu, S. W., Kroken, S., Lee, B. N., Robbertse, B., Churchill, A. C. L., Yoder, O. C., and Turgeon, B. G. 2003. A novel class of gene controlling virulence in plant pathogenic ascomycete fungi. *Proc. Natl. Acad. Sci. U.S.A.* 100:5980-5985.
- Maier, F. J., and Schäfer, W. 1999. Mutagenesis via insertional—or restriction enzyme-mediated—integration (REMI) as a tool to tag pathogenicity related genes in plant pathogenic fungi. *Biol. Chem.* 380:885-864.
- Marsch, J. F., and Schultze, M. 2001. Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants. *New Phytol.* 150:525-532.
- Marmeisse, R., Gay, G., Debaud, J. C., and Casselton, L. A. 1992. Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. *Curr. Genet.* 22:41-45.
- Martin, F. 2001. Frontiers in molecular mycorrhizal research—genes, loci, dots and spins. *New Phytol.* 150:499-505.
- Martin, F., and Tagu, D. 1999. Developmental biology of plant-fungus symbiosis: The ectomycorrhiza. Pages 51-73 in: *Mycorrhiza: Structure, Molecular Biology and Function*. A. Varma and B. Hock, eds. Springer Verlag, Heidelberg, Germany.
- Mullins, E. D., and Kang, S. 2001. Transformation: A tool for studying fungal pathogens of plants. *Cell. Mol. Life Sci.* 58:2043-2052.
- Norkrans, B. 1949. Some mycorrhiza-forming *Tricholoma* species. *Svensk. Bot. Tidskr.* 43:485-490.
- Rao, P. S., and Niederpruem, D. J. 1969. Carbohydrate metabolism during morphogenesis of *Coprinus lagopus* (*sensu* Buller) *J. Bacteriol.* 100:1222-1228.
- Riggle, P. J., and Kumamoto, C. A. 1998. Genetic analysis in fungi using restriction-enzyme-mediated integration. *Curr. Opin. Microbiol.* 1:395-399.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Sanchez, O., Navarro, R. E., and Aguirre, J. 1998. Increased transformation frequency and tagging of developmental genes in *Aspergillus nidulans* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* 258:89-94.
- Schiestl, R. H., and Petes, T. D. 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* 88:7585-7589.
- Schuren, F. H. J., and Wessels, J. G. H. 1998. Expression of heterologous genes in *Schizophyllum commune* is often hampered by the formation of truncated transcripts. *Curr. Genet.* 33:151-156.
- Shi, Z., and Leung, H. 1995. Genetic analysis of sporulation in *Magnaporthe grisea* by chemical and insertional mutagenesis. *Mol. Plant-Microbe Interact.* 8:949-959.
- Shi, Z., Christian, D., and Leung, H. 1995. Enhanced transformation in *Magnaporthe grisea* by restriction enzyme mediated integration of plasmid DNA. *Phytopathol.* 85:329-333.
- Smith, S. E., and Read, D. J. 1997. *Mycorrhizal symbiosis*. 2nd ed. Academic Press, San Diego, CA, U.S.A.
- Sundaram, S., Kim, S. J., Suzuki, H., Mcquattie, C. J., Hiremath, S. T., and Podila, G. K. 2001. Isolation and characterization of a symbiosis-regulated *ras* from the ectomycorrhizal fungus *Laccaria bicolor*. *Mol. Plant-Microbe Interact.* 14:618-628.
- Swiegar, J. A., Carroll, A. M., Farrall, L., Chumley, F. G., and Valent, B. 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant-Microbe Interact.* 11:404-412.
- Tagu, D., Phytou, M., Crétin, C., and Martin, F. 1993. Cloning symbiosis-related cDNAs from eucalypt ectomycorrhiza by PCR-assisted differential screening. *New Phytol.* 152:339-343.
- Thon, M. R., Nuckles, E. M., and Vaillancourt, L. J. 2000. Restriction enzyme-mediated integration used to produce pathogenicity mutants of *Colletotrichum graminicola*. *Mol. Plant-Microbe Interact.* 13:1356-1365.
- Urban, M., Bhargava, T., and Hamer, J. E. 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:512-521.
- Van Kan, J. A. L., Van Den Ackerveken, F. J. M., and De Wit, P. J. G. M. 1991. Cloning and characterization of the cDNA of avirulence *avr9* of the fungal pathogen *Cladosporium fulvum*, the causal agent of tomato leaf mold. *Mol. Plant-Microbe Interact.* 4:52-59.
- Voiblet, C., Duplessis, S., Encelot, N., and Martin, F. 2001. Identification of symbiosis-regulated genes in *Eucalyptus globulus*-*Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. *Plant J.* 25:181-191.
- Wilkinson, D. M. 2001. Mycorrhizal evolution. *Trends Ecol. Evol.* 16:64-65.
- Yakoby, N., Zhou, R., Kobiler, A., Dinoor, A., and Prusky, D. 2001. Development of *Colletotrichum gloeosporoides* restriction enzyme-mediated integration mutants as biocontrol agents against anthracnose disease in avocado fruits. *Phytopathol.* 91:143-148.
- Yang, G., Rose, M. S., Turgeon, B. G., and Yoder, O. C. 1996. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. *Plant Physiol.* 8:2139-2150.
- Yaver, D. S., Lamsa, M., Munds, R., Brown, S. H., Otani, S., Franssen, L., Johnstone, J. A., and Brody, H. 2000. Using DNA-tagged mutagenesis to improve heterologous protein production in *Aspergillus oryzae*. *Fungal Genet. Biol.* 29:28-37.