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Genetic Transformation of *Neurospora tetrasperma*, Demonstration of Repeat-Induced Point Mutation (RIP) in Self-Crosses and a Screen for Recessive RIP-Defective Mutants

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ABSTRACT

The pseudohomothallic fungus *Neurospora tetrasperma* is naturally resistant to the antibiotic hygromycin. We discovered that mutation of its *erg-3* (sterol C-14 reductase) gene confers a hygromycin-sensitive phenotype that can be used to select transformants on hygromycin medium by complementation with the *N. crassa erg-3*$^+$ and bacterial *hph* genes. Cotransformation of *hph* with PCR-amplified DNA of other genes enabled us to construct strains duplicated for the amplified DNA. Using transformation we constructed self-fertile strains that were homoallelic for an ectopic *erg-3*$^+$ transgene and a mutant *erg-3* allele at the endogenous locus. Self-crosses of these strains yielded *erg-3* mutant ascospores that produced colonies with the characteristic morphology on Vogel's sorbose agar described previously for *erg-3* mutants of *N. crassa*. The mutants were generated by repeat-induced point mutation (RIP), a genome defense process that causes numerous G:C to A:T mutations in duplicated DNA sequences. Homozygosity for novel recessive RIP-deficient mutations was signaled by self-crosses of *erg-3*-duplication strains that fail to produce *erg-3* mutant progeny. Using this assay we isolated a UV-induced mutant with a putative partial RIP defect. RIP-induced mutants were isolated in *rid-1* and *sad-1*, which are essential genes, respectively, for RIP and another genome defense mechanism called meiotic silencing by unpaired DNA.

NEUROSPORA tetrasperma and *N. crassa* are closely related fungal species that are virtually indistinguishable during their vegetative growth phase but differ during the sexual phase in a way that profoundly affects their genetics. In both species a sexual cross involves the fusion of two nuclei of different mating types (*A* and *a*) followed by a meiosis and a postmeiotic mitosis that give rise to eight haploid nuclei. In *N. crassa* these nuclei get partitioned into the eight initially uninucleate ascospores that are produced per ascus. Each ascospore upon germination generates a homokaryotic mycelium (*i.e.*, containing genetically identical nuclei). A homokaryotic mycelium can mate with a mycelium of the opposite mating type, which in *N. crassa* has to be generated from another ascospore. Since the products of two different ascospores are required to complete the sexual cycle, this life cycle is called "heterothallic." Other heterothallic species of this genus are *N. discreta*, *N. intermedia*, and *N. sitophila*. In contrast, in *N. tetrasperma* the eight nuclei produced by the postmeiotic mitosis are sequestered into the four initially binucleate ascospores that are produced per ascus, each ascospore receiving one nucleus of each mating type (Dodge 1927; Raju and Perkins 1994). In this species the *mat* idiomorphs are tightly linked to the centromere and almost always undergo first-division segregation. Ascus development is programmed so that the pair of nuclei delivered to each ascospore receives both alleles of all markers that have undergone first-division segregation. The mycelium generated from such ascospores is thus heterokaryotic (heteroallelic) for mating type and consequently it can be self-fertile. Since the sexual cycle can be completed by the nonidentical nuclei derived from a single ascospore, this life cycle is called "pseudohomothallic." The true homothallic species (*N. africana, N. dodgei*, *N. galapagosensis*, *N. lineolata*, *N. pannonica*, *N. sublineolata*, and *N. terricola*) produce eight-spored asci and the homokaryotic mycelium issuing from each ascospore is capable of completing the sexual cycle. The homothallic species are incapable of outcrossing; therefore, they are unsuitable for genetic studies. Significantly, in *N. tetrasperma* it is possible for markers that have undergone second-division segregation to become segregated into both nuclei of a subset of progeny ascospores (see Merino *et al.* 1996 for an explanatory figure). Self-crosses of the resulting cultures would thus become homozygous for such markers. It should be noted that a novel mutation arising in one of the haploid parents of a cross with *N. crassa* can be made homozy-

This article is dedicated to the memory of C. Vaishnavi.

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gous only in a subsequent cross between f_1 segregants of opposite mating type whereas homozygosity for a newly arisen mutation that recombines with the centromere can be achieved automatically in *N. tetrasperma*.

Occasionally *N. tetrasperma* asci do produce five or more (up to eight) ascospores per ascus and the proportion of eight-spored asci is dramatically increased by the dominant *Eight-spore* (*E*) mutation. In such asci, one or more large ascospores are replaced by pairs of small, uninucleate ascospores that are homoallelic for mating type. The small, uninucleate ascospores can be easily distinguished from the large, binucleate, heteroallelic ascospores and they produce self-sterile cultures that can mate only with cultures of the opposite mating type. Self-sterile, homoallelic cultures of *N. tetrasperma* can also be obtained from conidia (vegetative spores) of a heterokaryotic culture (Raju 1992).

The genome defense process called repeat-induced point mutation (RIP) was discovered in *N. crassa* (reviewed in Selker 1990). RIP occurs during a sexual cross in the haploid nuclei of the dikaryons, which form between fertilization and karyogamy, and subjects duplicated DNA sequences to multiple G:C to A:T mutations and cytosine methylation. Only one recessive "*RIP-d*efective" mutant, *rid-1*, has been reported thus far, and it was isolated by a "candidate gene" approach (Freitag *et al.* 2002). Isolation of additional mutants would define additional *rid* genes, but the difficulty in achieving homozygosity for a novel mutation affecting a diplophase-specific process makes it impractical to use *N. crassa* (or other heterothallic species) for the mutant screens. However, such screens would be feasible, at least in principle, in *N. tetrasperma* wherein a novel mutation can automatically become homozygous via second-division segregation.

We developed an assay for RIP in *N. crassa* that made use of a tagged duplication that targeted RIP to the *ergosterol-3* (*erg-3*) gene. Ascospores mutant in *erg-3* produce morphologically distinct colonies on Vogel's sorbose agar medium (see Noubissi *et al.* 2000 for an image), thereby allowing RIP efficiency to be determined by simply counting the number of mutant and wild-type progeny colonies under a dissection microscope. The availability of a similar assay in *N. tetrasperma* would make it possible to undertake screens for novel *rid* mutants. RIP in self-crosses of a *N. tetrasperma* strain duplicated for *erg-3* sequences would produce mutations in *erg-3*. Following this, if crossing over occurred between the centromere and the mutant locus, the mutation could become homoallelic in a fraction of the progeny ascospores. Alternatively, some of the spontaneously produced "small" ascospores might be homokaryotic for the mutation. In either case, if a self-cross failed to produce any *erg-3* mutant progeny, it would signal a potential homoallelism for a novel mutation conferring a defect in RIP.

This prospect motivated us to try to develop an *erg-3*-based RIP assay in *N. tetrasperma*. However, no RIP studies had been reported to date using this species. This was probably because most studies of RIP have employed DNA sequence duplications that were constructed by transformation and the protocols developed for transformation in *N. crassa* did not appear to work in *N. tetrasperma*. Thus, at the beginning of this study we were not sure whether RIP was efficient enough in *N. tetrasperma* for the feasibility of mutant hunts. In *Podospora anserina*, the only pseudohomothallic fungus in which RIP had been reported, its efficiency was far too low for use in screens for RIP-defective mutants (Hamann *et al.* 2000; Graia *et al.* 2001). In this article we report the construction of an *erg-3* mutant of *N. tetrasperma* and our finding that the mutant ascospores produce morphologically distinct colonies like their *N. crassa* counterparts do. Most significantly, we discovered that it was possible to transform the *N. tetrasperma erg-3* strain by an electroporation protocol developed for *N. crassa*. By transformation with a complementing *erg-3*$^+$ gene we constructed a strain that was duplicated for *erg-3* gene sequences. Self-crosses of this duplication strain yielded RIP-induced *erg-3* mutant progeny at a high-enough frequency (~2%) to enable us to undertake screens for RIP-induced mutants in the *N. tetrasperma rid-1* homolog. We also screened UV-mutagenized populations for mutants in novel *rid* genes.

MATERIALS AND METHODS

Strains: The following strains were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas Medical Center, Kansas City: *N. tetrasperma* single-mating-type strains *85 A* (FGSC 1270) and *85 a* (FGSC 1271), the *E* mutant strains *lwn; al(102); E A* (FGSC 7283) and *lwn; E a* (FGSC 7284), the *N. crassa*/*N. tetrasperma* hybrid strain C4,T4 *a* (FGSC 1778), the *N. crassa* strains of the standard Oak Ridge background 74-OR23-1 *A* (FGSC 987) and OR8-1 *a* (FGSC 988), and the *N. crassa* mutant strain *erg-3 a* (FGSC 2725).

Single-mating-type derivatives of the wild-isolated *N. tetrasperma* strains P 556 (Hanalei, Hawaii), P 2356 (Ahipara, New Zealand), and P 2361 (Ahipara, New Zealand) were kindly provided by David J. Jacobson, Stanford University. Approximately equal numbers of conidia of each single-mating-type derivative were mixed to reconstruct the wild-isolated strains. The *N. crassa* *m*eiotic *s*ilencing by *u*npaired *D*NA (MSUD)-suppressor strain 96-01 (*Sad-1 A*) was kindly provided by Robert L. Metzenberg (University of California, Los Angeles). The *sad-1* locus is in linkage group (LG) I (Shiu and Metzenberg 2002). We obtained a *Sad-1; erg-3 A* segregant from the cross *Sad-1 A* × *erg-3 a*.

The *N. crassa* strains *Dp 1.3*^{ec} *hph A* and *a* were constructed previously in our laboratory and have been described by Prakash *et al.* (1999). They contain the wild-type allele at the *erg-3* locus in LG IIIR and a duplication of a 1.3-kb *Hin*dIII fragment from *erg-3* inserted as a single-copy transgene linked to *al-3* in LG V (Bhat *et al.* 2003). The *erg-3* gene codes for the ergosterol biosynthetic enzyme sterol C-14 reductase. The duplicated segment does not encode a functional enzyme but serves to target RIP to *erg-3*. The transgene is marked with the bacterial *hph* gene, which encodes the enzyme hygromycin

phosphotransferase whose expression confers resistance to the antibiotic hygromycin B.

The *N. tetrasperma erg-3* mutant strain, designated Te-4, and six other strains (121, 122, 123, 131, 132, and 133) were constructed in this study and deposited in the FGSC with strain numbers FGSC 9077–9083.

Growth conditions: Crossing and maintenance of Neurospora strains was essentially as described by Davis and de Serres (1970). Antibiotic resistance was scored by streaking conidia onto 1.5% agar plates containing Vogel's N medium plus "sorbose" (0.05% fructose, 0.05% glucose, and 2% sorbose) and supplemented with the antibiotic. The antibiotics tested were α-tomatine (Sigma, St. Louis) at up to 90 μg/ml, made from a 25 mg/ml stock solution in dimethyl formamide, and hygromycin B (Sigma) at up to 200 μg/ml, made from a 100 mg/ml aqueous stock solution. Among *N. crassa* strains, only the *erg-3* mutant strains are able to grow on tomatine-supplemented medium after an overnight incubation at 30° (Sengupta *et al.* 1995), and only those strains expressing the *hph* gene could grow on hygromycin medium.

Ascospore collection and scoring for *erg-3* mutants: Crosses were performed by confrontation between mycelia inoculated as plugs on synthetic crossing medium in petri dishes. Generally, ascospores began to be shot within 16–18 days in *N. crassa* crosses and within 12–14 days in *N. tetrasperma* crosses. Ascospores were harvested by washing the lids with ~1 ml water. The frequency of *erg-3* mutants was scored by determining the proportion of colonies with the mutant growth morphology under a dissection microscope. Reliability of identifying the *erg-3* mutant phenotype in this way was established by confirming the ability of the conidia to germinate and grow on tomatine medium (for *N. crassa*) or by UV spectroscopic analysis of the sterols (for *N. tetrasperma*).

In this article the frequency of *erg-3* mutant progeny is used as a measure of RIP efficiency. It is known in *N. crassa* that the frequency of RIP increases with the age of the cross; late harvests show greater RIP frequencies than early harvests (Singer *et al.* 1995). Therefore, in some experiments the *erg-3* mutation frequencies were determined for both an early (18 days) and a late (25 days) harvest.

Construction of the *N. tetrasperma erg-3* mutant strain: A cross was performed between the *N. crassa* strain *Dp1.3^{ec} hph a* and the *N. tetrasperma* strain *85 A*. As expected, this interspecies cross was quite unproductive and after >2 months we were able to obtain ~80 viable progeny. Of these, 15 (18.8%) were RIP-induced mutants in *erg-3* (Bhat and Kasbekar 2001). An *erg-3^{+} a* segregant that also contained the *Dp1.3^{ec} hph* transgene was used to initiate a series of backcrosses with *85 A* as the recurrent parent. RIP-induced *erg-3* mutants could be recovered from each backcross but after even 12 backcrosses we were unable to detect any four-spored asci.

Our inability to introgress the *Dp1.3^{ec} hph* transgene into the four-spored background prompted us to explore whether an *erg-3* mutation from one of the backcrosses can instead be introgressed. An *erg-3* mutant from the sixth backcross was crossed with the hybrid strain C4,T4 (Metzenberg and Ahlgren 1969; Perkins 1991), and a mutant segregant (designated Te4-17) was used to initiate a new series of backcrosses with *85 A* or *a*. We anticipated that a *N. tetrasperma erg-3* strain would possess dual mating specificities; that is, it would be capable of crossing with both *85 A* and *85 a*; however, it would be expected to be self-sterile, since *N. crassa erg-3* strains are female sterile. We began recovering *erg-3* strains with dual mating specificity in the third backcross. A dual mating specificity *erg-3* mutant strain from the fourth backcross, designated provisionally as *Te*4-17-17-8-1-*4* (Te-4) was adopted as the *N. tetrasperma erg-3* strain. Southern analysis of Te-4 confirmed that it did not contain any *Dp1.3^{ec} hph* transgene sequences (data not shown).

Sterol analysis: Mycelia were grown in 30 ml Vogel's glucose medium for 3 days, harvested by filtration, lyophilized, and powdered with glass beads. About 50 mg of the mycelial powder was extracted for 7–8 hr with 500 μl each of methanol and chloroform in a 1.5-ml microfuge tube. The organic supernatant was separated from the mycelial debris by centrifugation and washed once with 0.9% NaCl and twice more with 2 M KCl. The organic phase was dried in a rotary evaporator and redissolved in 10 μl of chloroform, 2 μl of the chloroform solution was diluted in 1 ml of ethanol, and its UV spectrum (200–300 nm) was recorded using a Hitachi spectrophotometer. Ergosterol has UV absorption maxima at 272, 282, and 293 nm whereas the *erg-3* mutant sterols absorb maximally at ~250 nm (Prakash *et al.* 1999).

PCR amplification of *erg-3*, *rid-1*, and *sad-1* sequences: PCRs were performed using oligonucleotide primers synthesized at the Centre for Cellular and Molecular Biology (Hyderabad, India) oligonucleotide synthesis facility. The reaction conditions used were a 4-min denaturation at 94° followed by 30 cycles of 1 min denaturation at 94°, 1 min annealing at 55°, and 1 min elongation at 72°. The *erg-3* gene fragment was amplified using the 21-mer oligonucleotide 5′-GCTCTCCG TGTTGTCGTCTAC-3′ corresponding to nucleotides 347–367 of the *N. crassa erg-3* gene sequence (accession no. X77955) and the 24-mer 5′-GCGAAGAGGATATTGGCGCTGATG-3′ complementary to nucleotides 964–987. The resulting 0.64-kb product was purified by gel electrophoresis and used for automated DNA sequencing with the same primers, the Big Dye Terminator Ready Reaction kit (Perkin-Elmer, Norwalk, CT), and an ABI Prism 3700 DNA analyzer.

The primers 977 and 980 (described by Freitag *et al.* 2002) were used to amplify an ~2.5-kb fragment from the coding region of the *N. tetrasperma rid-1* gene, and the primers SADF1 (5′-ATGTCAGGAATACCCAACTGGG-3′) and SADR1 (5′-GTC AATATATTGGTGGTTCTCGG-3′), made on the basis of the *N. crassa sad-1* gene sequence (accession no. AY029284), were used to amplify an ~1-kb fragment of the *N. tetrasperma sad-1* gene. These fragments were purified by agarose gel electrophoresis and used for cotransformation experiments.

Partial nucleotide sequences (738 bp) corresponding to the nucleotides 577–1314 of the *rid-1* gene sequence (accession no. AF500229) were determined for the *rid-1* mutant alleles constructed in this work. This was done by amplifying a 1.5-kb segment of the mutant allele with the 21-mer primers RIDF1 (5′-TCCGCGTCCTACCATTCCCGC-3′) and RIDR1 (5′-CCATG AGTTGGTGAGGGAAAR-3′), and the RIDR1 primer was used for the sequencing reactions. The RIDF1 primer annealed to sequences that are not included the duplicated segment; this ensured that the sequence obtained was from the endogenous *rid-1* locus and not from the ectopically duplicated *rid-1* fragment used to target RIP to *rid-1*.

Partial nucleotide sequences of the *sad-1* mutant alleles were determined by amplifying a 1.5-kb segment of the mutant allele with primers SADF1 and SADR3 (5′-CGGTGGCTTACC TATTGGATTG-3′) and both primers were used for the sequencing reactions. The SADR3 primer anneals to sequences that are not included in the duplicated segment, which ensured that the sequence obtained was from the endogenous *sad-1* locus and not from the ectopically duplicated *sad-1* fragment used to target RIP to *sad-1*.

Transformation: Transformation was done essentially as described by Margolin *et al.* (1997). The Te-4 strain was grown in Vogel's medium N supplemented with 1.5% glucose for 10 days and the conidia were harvested in sterile water and filtered through a cheese cloth. The conidia were pelleted in a Sorvall SS34 rotor at 4000 rpm for 10 min, washed twice in 30 ml of 1 M sorbitol, and resuspended in 1 M sorbitol at a concentration of 3×10^9/ml. Approximately 500 ng of DNA

of the plasmid pEC86-Hph, which contains the *N. crassa erg-3* gene and the *hph* marker (Prakash and Kasbekar 2002b), was added to 40 μl of the conidial suspension and electroporated in a 0.2-cm cuvette (BTX, San Diego) in a Bio-Rad (Richmond, CA) Genepulser. The electroporation conditions used were 1.5 kV, 25 μF, and 600 Ω. After the pulse, 960 μl of 1 m sorbitol was added and 500 μl of the transformation mix was plated on a Vogel's sorbose plate containing 250 μg/ml hygromycin. Transformants could be picked after incubation at 30° for 3–4 days. One of the transformants was used for the construction of the strains 121, 122, 123, and 131 (see results). These strains are phenotypically wild type but contain a duplication of the *erg-3* gene sequence and therefore can generate RIP-induced *erg-3* mutant progeny when self-crossed.

The plasmid pMP6 (M. Plamann, University of Missouri, Kansas City), which also contains the *hph* gene, was used in the cotransformation experiments with the PCR-amplified fragments of the *N. tetrasperma sad-1* or *rid-1* genes. The DNA mixtures were electroporated into Te-4 conidia and hygromycin-resistant transformants were selected as described above.

Mutagenesis and screens for RIP-defective mutants: The *N. tetrasperma* strains 121, 122, 123, and 131 are phenotypically wild type but contain a duplication of the *erg-3* gene sequence and therefore can generate RIP-induced *erg-3* mutant progeny when self-crossed (see results). These strains were grown in flasks for ~10 days, and the conidia were harvested in water and separated from the mycelia by filtering through cheese cloth. They were washed twice by centrifugation for 5 min at 4° in a Sorvall SS-34 rotor at 4000 rpm. A suspension of 10^9 conidia in 10 ml water was poured into a 85-mm petri dish and exposed to UV radiation for 10–20 min in an UV crosslinker (Amersham Life Science). This treatment generally resulted in ~60% killing. The mutagenized conidia were then plated onto crossing medium, allowed to cross *en masse*, and f_1 ascospores were harvested from this mass mating. The ascospores were germinated on Vogel's sorbose medium and *erg-3*$^+$ colonies were individually picked to crossing medium and allowed to grow and self-cross. From the lid of each self-cross plate a reasonably large number (>200) of f_2 ascospores were harvested with an inoculating loop and streaked onto Vogel's sorbose agar medium. The ascospores were allowed to rehydrate on the plate for a few hours, then heat-shocked to induce germination, and incubated overnight at 30°. Each f_1 culture is potentially homoallelic for newly induced mutations that have undergone second-division segregation. Therefore any streak that failed to show at least a few colonies with the *erg-3* mutant morphology signaled a culture that was potentially homoallelic for a novel RIP-defective mutation. Such cultures were retested using additional f_2 ascospores.

RESULTS

The *erg-3* genes of *N. tetrasperma* and a null mutant: The *N. crassa erg-3* gene codes for a 490-residue protein belonging to a family of sterol reductases that is evolutionarily conserved across Arabidopsis, Dictyostelium, and humans (Prakash and Kasbekar 2002a); consequently its *N. tetrasperma* counterpart was expected to have a similar nucleotide sequence. Consistent with this expectation, a PCR performed with oligonucleotide primers made to the *N. crassa erg-3* gene sequence and genomic DNA of the *N. tetrasperma 85 A/a* strain as the template resulted in the amplification of a DNA fragment of the expected size (0.64 kb). A 544-bp sequence was determined from the amplified DNA. This sequence (accession no. AY256902) corresponded to the 548 nucleotides (residues 427–974) of the *N. crassa erg-3* gene (Papavinasasundaram and Kasbekar 1994) and included both intron and exon sequences. The introns of the *N. tetrasperma* and *N. crassa* genes are, respectively, 83 and 87 residues long and are 88.5% identical in nucleotide sequence. A *Dpn*II site is present in the *N. crassa* intron but is absent in the *N. tetrasperma* intron and provided a convenient polymorphism to distinguish the two genes. The remaining 461-nucleotide exonic sequences were 98% identical in nucleotide sequence and coded for 153 amino acid residues, of which only one residue differed between the *N. crassa* and *N. tetrasperma erg-3* proteins (G94A).

The *N. tetrasperma erg-3* strain Te-4 was constructed by doing a RIP mutagenesis of the *erg-3* locus in a *N. crassa/N. tetrasperma* hybrid strain followed by introgression of the mutation into the four-spored *N. tetrasperma* background (see materials and methods for details). The 548-bp partial sequence (accession no. AY256903) of the mutant *erg-3* allele corresponded to residues 427–974 of the *N. crassa* gene and revealed that the mutant allele was in fact derived from *N. crassa*. As expected for RIP, there were 13 C:G to T:A transition mutations in this sequence of which 6 caused nonsynonymous codon changes (Q102*, H110Y, T114I, M133I, C134Y, and Q146*). These results allowed us to conclude that Te-4 is indeed an *erg-3* null mutant strain.

Phenotype of the Te-4 strain: The absence of ergosterol in the Te-4 strain was confirmed by UV spectrophotometry. The UV absorption spectrum of the sterol preparation from the wild-type strain *85 A/a* gave peaks at 271, 281, and 293 nm that are typical of ergosterol. These peaks were absent from the spectrum of the sterol preparation from the Te-4 strain (data not shown). Instead, the spectrum of the mutant sterol preparation showed a broad peak at 250 nm, which is typical of Δ8,14 sterols (data not shown).

The Te-4 strain was self-sterile although it was known to be heteroallelic for mating type because it could be crossed with both *85 A* and *85 a*. The self-sterile phenotype was consistent with the female-sterile phenotype of *N. crassa erg-3* mutants. Of the progeny ascospores from the cross Te-4 × *85 A*, 7.4% (n = 525) produced colonies on Vogel's sorbose agar with the characteristic morphology described previously for *erg-3* mutants of *N. crassa*. These ascospores are presumed to have become homoallelic for *erg-3* following second-division segregation of the mutant allele. However, a minority might represent small homokaryotic ascospores that had inherited the *erg-3* mutant allele. The frequency of *erg-3* progeny from the cross Te-4 × *85 a* was 13.3% (n = 238). The frequency of *erg-3* mutant progeny was increased to 39% (n = 110) and 46% (n = 37), respectively, in crosses of Te-4 with the strains *lwn; al(102); E A* and *lwn; E a*. These increases were not

unexpected because the *E* mutation increases the proportion of small homokaryotic ascospores. We recovered the strains *al(102); erg-3; E A* and *erg-3; E a* from these crosses.

In *N. crassa*, *erg-3* mutants display an increased sensitivity to isoflavonoids such as pisatin, maackiain, and biochanin A and increased resistance to the steroidal glycoside α-tomatine (Sengupta *et al.* 1995). We examined the strains *85* and Te-4 for corresponding differences in drug sensitivity. *N. crassa erg-3* mutants are capable of growth on as much as 90 μg/ml tomatine but this concentration was inhibitory to Te-4. Te-4 conidia grew well on medium supplemented with up to 65 μg/ml α-tomatine but at this concentration even the wild-type *85* conidia showed some residual growth. Thus the difference in tomatine sensitivity between the *erg-3* and wild-type conidia was not as robust in *N. tetrasperma* as it is in *N. crassa*. However, the wild-type but not the *erg-3* conidia could grow on Vogel's sorbose agar medium supplemented with 8 μg/ml biochanin A.

The wild type and Te-4 differed strikingly in their sensitivity to hygromycin. Conidia of strain *85* could grow in the presence of as much as 220 μg/ml hygromycin but those of Te-4 were sensitive. We tested for linkage of the sensitivity phenotype with the *erg-3* mutation. Of 19 wild-type progeny examined from the cross Te-4 × *85 A*, 17 could grow in the presence of 220 μg/ml hygromycin whereas all the 24 *erg-3* mutant progeny tested were hygromycin sensitive. These results indicated that the hygromycin sensitivity phenotype was indeed linked to the *erg-3* mutation.

***N. tetrasperma* bimating-type strains do not participate in outcrosses:** Bistis (1996) reported that bimating-type colonies of *N. tetrasperma* do not put out functional trichogynes and that their perithecia inhibit the formation of other perithecia elsewhere on the same plate. This suggested that bimating-type *N. tetrasperma* strains might not participate in outcrosses even when provided an opportunity to do so. The availability of *E; erg-3 A* and *a* strains made it possible to quantify the effects of such endogamy. Since the *erg-3* mutation confers a female-sterile phenotype, the *E; erg-3 A* and *a* strains can cross only as male parents and, as we noted above, ~50% of the progeny from crosses involving these strains display the *erg-3* colony morphology. Each of the four bimating wild-type strains *85*, P0556, P2361, and P2356 were co-inoculated onto crossing medium together with either *E; erg-3 A* or *E; erg-3 a*, and the frequency of *erg-3* mutants was determined in the progeny. These frequencies were, respectively, <0.3% (386), <0.2% (447), <0.4% (277), and <0.2% (395) in the co-inoculations with *E; erg-3 A* and <0.3% (359), <0.2% (436), <0.3% (375), and <0.2% (451) in the co-inoculations with *E; erg-3 a* (the numbers in parentheses indicate the number of progeny examined). That is, no *erg-3* segregants were seen in a total of 3126 progeny examined, indicating that the frequency of outcross progeny was <0.06%. This result supports the hypothesis that bimating-type strains do not participate in outcrosses.

Transformation of the Te-4 strain: Since the Te-4 mutant was hygromycin sensitive, we explored the feasibility of using it as the recipient strain in transformation experiments. The plasmid pEC86-Hph, which contains both the *N. crassa erg-3*$^+$ allele and the bacterial *hph* gene (Prakash and Kasbekar 2002b), was electroporated into Te-4 conidia and they were then plated for selection of hygromycin-resistant transformants. A total of 11 putative transformants were picked of which 5 retested for the hygromycin-resistant phenotype and 4 also showed resistance to 8 μg/ml biochanin A. The four biochanin-A-resistant transformants were confirmed by Southern analysis to contain the transforming pEC86-Hph DNA (data not shown). Two transformants had also become self-fertile. Among the self-cross progeny of one we identified small ascospores that produced homokaryotic, self-sterile, and phenotypically wild-type cultures. One of these cultures (designated 1) was of the mating type *A* and the other two (2 and 3) were *a*. By Southern analysis of *Eco*RV-, *Xho*I-, and *Dpn*II-digested genomic DNA we confirmed that identical transgenes were present in all three cultures (data not shown).

RIP in self-crosses: Strain 1 was crossed with strains 2 and 3 (see above) and the progeny ascospores harvested after 22 days were found to include *erg-3* mutants at frequencies of 1.9% (n = 212) and 1.7% (n = 179), respectively. Since both the parental strains were duplicated for *erg-3* sequences, these mutants must have arisen because of RIP-induced inactivation of the *erg-3*$^+$ transgene followed by second-division segregation of the mutation. From these crosses we picked up large, self-fertile, phenotypically wild-type progeny designated 121, 122, and 123 (for progeny from 1 × 2) and 131, 132, and 133 (for progeny from 1 × 3). For four strains (121, 122, 123, and 131) we could demonstrate by sterol analysis that both single-mating-type derivatives were phenotypically erg-3$^+$ (data not shown). Thus any *erg-3* progeny produced in self-crosses of these strains would have to result from RIP-induced inactivation of one of the transgenic *erg-3*$^+$ alleles followed by its second-division segregation or by segregation of the inactivated transgene into a homokaryotic small ascospore or possibly even by the simultaneous inactivation by RIP of the transgenic *erg-3*$^+$ alleles in both parental nuclei. Table 1 presents the *erg-3* mutant frequency among self-cross progeny ascospores harvested after 15, 18, and 21 days. As can be seen in Table 1, a substantial number of *erg-3* mutant progeny were obtained, which suggested that RIP inactivation of the *erg-3*$^+$ allele can occur quite efficiently in the self-crosses of these four strains.

Cotransformation of DNA fragments amplified by PCR: In *N. crassa*, cotransformation of an unselected DNA fragment together with a selectable marker can

TABLE 1

RIP-induced *erg-3* mutant progeny from self-crosses

Strain	*erg-3* mutant progeny (%)		
	Harvest 1	Harvest 2	Harvest 3
121	6.2 (160)	9.3 (148)	19.6 (51)
122	8.4 (142)	21.0 (100)	14.7 (156)
123	13.6 (533)	17.3 (138)	15.2 (105)
131	2.1 (142)	7.0 (199)	11.1 (45)

Numbers in parentheses indicate the number of segregants examined. The harvests were made 15, 18, and 21 days after setting up the crosses.

be quite efficient (up to ~80%) and is a very useful procedure. To explore the prospects of cotransformations in Te-4 conidia, we did electroporations using PCR-amplified fragments of the *rid-1* and *sad-1* genes along with the helper plasmid pMP6, which carries a selectable gene for hygromycin resistance (see MATERIALS AND METHODS). The *sad-1* gene encodes a putative RNA-directed RNA polymerase that is required for the post-transcriptional gene-silencing process MSUD (SHIU *et al.* 2001; SHIU and METZENBERG 2002). MSUD is responsible, at least in part, for the poor productivity of interspecies crosses. Crosses between a *N. crassa* semidominant *Sad-1* mutant and a wild-type *N. tetrasperma* show dramatic increases in productivity.

In the cotransformation experiment that used the ~2.5-kb fragment of *rid-1*, we selected 15 hygromycin-resistant primary transformants and crossed each of them with *E* strains. Three transformants were of single mating type and 12 were of dual mating type. Hygromycin-resistant *erg-3* segregants were detected among the progeny of 7 primary transformants, thus confirming that they contained the selected pMP6 DNA. Southern analysis revealed that two hygromycin-resistant *erg-3* progeny (nos. 10-6 and 10-18), derived from 1 primary transformant (no. 10), also contained an ectopically integrated copy of the cotransformed *rid-1* fragment (data not shown).

In the experiment using the 1-kb *sad-1* fragment we selected 10 hygromycin-resistant primary transformants and crossed them with *E* strains of the *A* or *a* mating types. Four transformants were of single mating type and 6 were of dual mating type. The *erg-3* segregants from each cross were examined for inheritance of the hygromycin-resistance marker. The progeny from 6 transformants that showed hygromycin resistance were then examined by Southern analysis for presence of the unselected *sad-1* fragment. The fragment was detected in one hygromycin-resistant *erg-3* segregant that was derived from one primary transformant designated 173 (data not shown). Clearly cotransformation occurs in *N. tetrasperma*, but the frequency is probably lower than that in *N. crassa*.

Isolation of RIP-induced *rid-1* mutants: The two *erg-3* segregants that contained the cotransformed *rid-1* gene fragment (10-6 *A* and 10-18 *a*; see above) were each crossed with single-mating-type derivatives of strain 121. From these crosses eight homokaryotic *erg-3*$^+$*; A* segregants and five *erg-3*$^+$*; a* segregants were obtained. These segregants were mutant at the endogenous *erg-3* locus but carried the ectopic *erg-3*$^+$ allele and by Southern analysis we confirmed that they had also inherited the cotransformed *rid-1* fragment. The segregants were crossed among themselves (40 crosses) and the progeny ascospores from these crosses were germinated on Vogel's sorbose medium. Individual germlings were picked to crossing medium, allowed to undergo self-crosses, and their f_2 ascospores were examined on Vogel's sorbose medium to identify self-crosses that failed to produce any *erg-3* mutant colonies. It was anticipated that a minority of the self-crosses would represent f_1 cultures in which both the *A* and the *a* nuclei carry RIP-induced mutations in the *rid-1* genes. Such self-crosses would be defective for RIP and therefore not yield any *erg-3* mutant progeny. Of 756 self-crosses screened, 5 (0.7%) did not yield any *erg-3* mutant progeny and therefore signaled a potential "homozygosity" for RIP-induced *rid-1* mutants. One culture (no. 6) was derived from the cross 10-18-16 × 10-6-3 and four (nos. 53, 66, 67, and 79) were from 10-18-5 × 10-6-11. We obtained single-mating-type conidial derivatives from each of these cultures and used them to perform complementation tests. Both *A* and *a* derivatives were obtained for cultures 6 or 66, while only *A* derivatives were obtained for 53 and 67 and only *a* derivatives for 79. The representative single-mating-type derivatives used for the complementation tests were designated 6-7 *A*, 6-1 *a*, 66-8 *A*, 66-4 *a*, 53-5 *A*, 67-10 *A*, and 79-9 *a*. Control crosses were done with *A* and *a* derivatives of strain 121 (designated 121-22 *A* and 121-1 *a*). The results, summarized in Table 2, indicate that each culture contains recessive mutations that belong to a single complementation group.

We did partial sequencing of the *rid-1* locus in each of these seven single-mating-type strains to confirm that they were indeed subjected to RIP-induced transition mutations and that many of these mutations introduced in-frame nonsense codons (accession nos. AY490236–AY490239 and AY526722–AY526724). The following nonsynonymous codon changes were found: D418N, W425*, and H432Q in 6-7 *A*; S353L, T364I, L374F, and H432Q in 6-1 *a*; H432Q and Y433D in 66-8 *A*; T364I, Q396*, Q410*, H419Y, H432Y, and Q437* in 66-4 *a*; H432Y in 53-5; Q396*, H419Y, H432Y, and Q437* in 67-10 *A*; and T364I, Q396*, Q410*, H419Y, H432*, Y433N, and Q437* in 79-9 *a*. These results allow us to conclude that the RIP defect was indeed because of homozygosity for mutant *rid-1* alleles. The results also provide proof that RIP is responsible for generating the *erg-3* mutants in the self-crosses.

Isolation of RIP-induced *sad-1* mutants: The Te-4 cotransformant that contained the PCR-amplified frag-

TABLE 2

Complementation tests of single-mating-type derivatives of RIP-defective cultures

	121-22 *A*	6-7 *A*	53-5 *A*	66-8 *A*	67-10 *A*
121-1 *a*	+ (1.5, 16.2)	+ (0.6, 9.7)	+ (2.1, 10.8)	+ (2.0, 17.0)	+ (2.6, 5.5)
6-1 *a*	+ (1.0, 9.2)	− (<0.1, <0.1)	− (<0.1, <0.1)	− (<0.1, <0.2)	− (<0.1, <0.1)
79-9 *a*	+ (0.7, 1.3)	− (<0.1, <0.1)	− (<0.1, <0.2)	− (<0.1, <0.2)	− (<0.1, <0.2)
66-4 *a*	+ (0.6, 9.5)	− (<0.1, <0.1)	− (<0.1, <0.2)	− (<0.1, <0.1)	− (<0.1, <0.3)

+, *erg-3* progeny detected; −, no *erg-3* progeny detected. Numbers in parentheses indicate frequency (%) of *erg-3* mutants among ascospores harvested at 18 and 25 days, respectively.

ment of *sad-1* was designated 173 (see above). This strain was of dual mating type and yielded hygromycin-resistant *erg-3* segregants in the cross with the *E A* strain. One hygromycin-resistant *erg-3* segregant (designated 173-16), which contained the cotransformed *sad-1* fragment, was crossed with *E A*. Twenty erg-3$^+$ segregants from this cross were examined by Southern analysis, and the *sad-1* duplication, found in the segregant designated 173-16-18, was crossed with *E A*. Two segregants (173-16-18-7, *A* and 173-16-18-9, *E a*) with the *sad-1* duplication were obtained from this cross and crossed with each other. The putatively homokaryotic progeny from this cross were then screened for RIP-induced *sad-1* mutations by crossing them with *N. crassa* semidominant *Sad-1* strains of the opposite mating type: nonmutants show the increased productivity characteristic of *N. tetrasperma* × *N. crassa Sad-1* crosses, whereas a mutant would cause sterility, a characteristic of *sad-1* homozygous crosses (Shiu *et al.* 2001). Two segregants (145 *a* and 163 *a*) of 350 examined did not produce any ascospores in the interspecies crosses after even 50 days. DNA sequencing of the *sad-1* locus in these two strains (accession nos. AY536860 and AY536861) revealed several RIP-induced transition mutations relative to the corresponding sequence of the parental wild-type allele (accession no. AY536859). Several of the mutations introduced nonsynonymous codon changes, including in-frame nonsense codons. They were G125S, S128N, D133N, V139I, D141N, C161Y, C165Y, and W175* in 145 *a* and E123K, G125S, S128N, E131K, D133N, E134K, V139I, D141N, G143S, C161Y, V172I, W175*, E186K, and C201Y in 163 *a* (the codon numbering system follows that of the *N. crassa sad-1* protein). These results confirmed that we had indeed isolated RIP-induced mutations in *sad-1*.

The number of C-to-T changes scored in the partial sequences of the *rid-1* and *sad-1* alleles was 72. Of these, ~78% affected the dinucleotide CpA; ~17%, CpT; ~4%, CpC; and ~1%, CpG. The striking preference of RIP for the CpA dinucleotide is similar to that seen in *N. crassa* (Cambareri *et al.* 1989) and *P. anserina* (Graia *et al.* 2001).

A screen for UV-induced RIP-deficient mutants: Conidia of the strains 121, 122, 123, and 131 were exposed to UV mutagenesis and then plated *en masse* onto crossing medium (see materials and methods for details). The f_1 ascospores from these crosses are potentially homoallelic for newly induced mutations. They were germinated on Vogel's sorbose medium and individual erg-3$^+$ colonies were picked to crossing medium and allowed to grow and self-cross. The f_2 ascospores from these self-crosses were examined for absence of any that produce *erg-3* mutant colonies on Vogel's sorbose plates.

Of 2460 erg-3$^+$ f_1 colonies screened in this way, 204 (~8%) were self-sterile and displayed a single-mating specificity (90 *A*, 114 *a*) and most likely represented the products of small homokaryotic ascospores. However, one self-sterile f_1 colony displayed dual-mating specificities in crosses with *85 A* and *a*. Sterol analysis confirmed that it contained ergosterol (data not shown), which ruled out the possibility that it represented an *erg-3* mutant that was mistakenly scored as erg-3$^+$. Therefore this colony must represent a bimating-type ascospore that had become homoallelic for a novel UV-induced mutation conferring male or/and female sterility. Another f_1 colony was temperature sensitive for growth and although by PCR it was determined to be *mat a*, it failed to cross with *85 A*. Only one f_1 culture (designated m121) was recovered, which yielded very few *erg-3* mutant f_2 progeny. To confirm that this phenotype was reproducible, we performed 20 replicate self-crosses of strain m121 and compared the distribution of *erg-3* progeny frequencies with that of 20 replicate self-crosses of a randomly picked sibling control strain. The results, summarized in Figure 1, showed that although there was some overlap in the distribution of *erg-3* mutant frequencies, the modal frequency for the self-crosses of m121 was in the range 0.5–1.0, whereas for the control self-crosses it was >3.0.

DISCUSSION

In this study we have for the first time achieved the genetic transformation of *N. tetrasperma*, shown that RIP

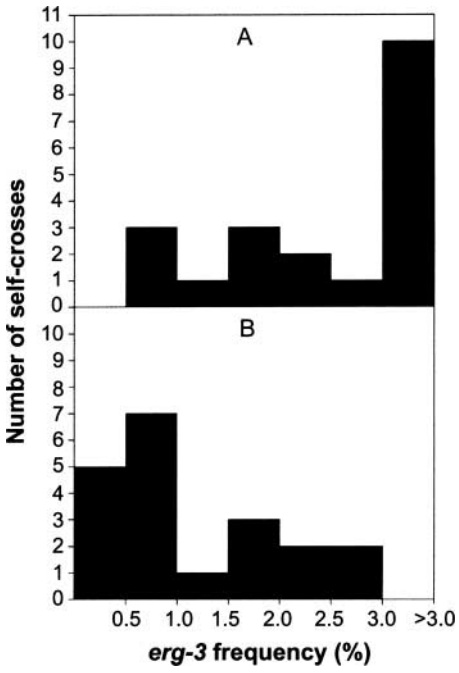

Figure 1.—Distribution of the frequency of *erg-3* mutants in the progeny of 20 replicate self-crosses of a control strain (A) and of the partially RIP-defective mutant strain m121 (B).

can occur in self-crosses, and demonstrated the use of this system to screen for recessive RIP-defective mutants. *N. tetrasperma* had remained "undomesticated" with respect to genetic transformation techniques until now, possibly because the wild type was already quite hygromycin resistant, thus precluding the application of *N. crassa* transformation protocols involving a selection for hygromycin-resistant transformants (Staben *et al.* 1989). Against this background, the discovery of a hygromycin-sensitive phenotype for the *N. tetrasperma erg-3* mutant represents a very significant result. A difference in hygromycin sensitivity between the wild-type and *erg-3* strains was not previously reported in *N. crassa* (although such a difference has since been observed in unpublished work in our laboratory) and was reported only recently for sterol mutants of yeast (Page *et al.* 2003). This phenotype provided us the means to select for hygromycin-resistant transformants and opened up the possibility of doing cotransformations. It is now quite straightforward to use cotransformation to construct strains that are duplicated for any genomic segment that is amplifiable by PCR and to use these strains in crosses to target RIP mutations to the duplicated segment. Using this approach, which can obviously be extended to other markers, we obtained mutants in the *rid-1* and *sad-1* genes and a start has been made with a few other loci [*e.g.*, *ro-10* (IL tip), *acr-2* (IIIC), *pyr-1* (IVC), *qa-4* (VIIC), and *met-7* (VIIC); R. Tamuli and D. P. Kasbekar, unpublished results]. Many additional candidate loci, including visibles, drug resistants, and auxotrophs, will become available as the annotation and assignment of genetically mapped *N. crassa* genes to the physical map progresses to the next release. Since crossing over is suppressed on much of LG I (Gallegos *et al.* 2000), the RIP-based approach might provide the only convenient way of constructing strains with multiple markers on this linkage group.

We confirmed that the *N. tetrasperma erg-3* mutant lacks ergosterol and that mutant ascospores produce colonies with the characteristic morphology on Vogel's sorbose agar described previously for *erg-3* mutants of *N. crassa*. The colony phenotype allowed us to verify the proposal of Bistis (1996) that bimating-type strains tend not to outcross. By transformation of the *erg-3* mutant strain with an *erg-3*$^+$ allele we could construct strains that were phenotypically wild type yet duplicated for *erg-3* sequences. These strains were homoallelic for the mutant allele at the endogenous locus and homoallelic for an ectopically located *erg-3*$^+$ allele. When these strains were allowed to self-cross, they yielded *erg-3* mutant progeny due to RIP inactivation of the *erg-3*$^+$ allele followed by second-division segregation of the RIPed allele, and the mutant progeny could be easily scored by their colony phenotype. When, in addition, the genotype was also made homoallelic for a *rid-1* duplication, then a small fraction (5/756) of the progeny lost the ability to generate *erg-3* mutants in self-crosses because they had now become homozygous for RIP-induced *rid-1* mutations. In screening for these mutants we did not rely on second-division segregation to automatically make a RIP-induced *rid-1* mutation homozygous because *rid-1* is located on LG I (Freitag *et al.* 2002), and as noted earlier, LG I markers tend not to undergo second-division segregation in this species.

Our success in identifying *rid-1* homozygous crosses emboldened us to extend this approach to isolate UV-induced mutants with a RIP-defective phenotype. We recovered one strain that appeared to produce fewer *erg-3* progeny in self-crosses. Admittedly its phenotype was not as robust as that of the canonical *rid-1* mutants. Thus it might have only a partial defect in RIP or in second-division segregation. We also identified one self-sterile bimating-type culture in these screens. Many female- or male-sterile loci are known in *N. crassa* (Perkins *et al.* 2001), and their number very likely exceeds that of recessive RIP-defective loci. Given that only one self-sterile mutant was found, our failure to recover additional RIP defectives does not appear that surprising; it only underscores the necessity of doing an enrichment for RIP-deficient mutants before embarking on future screens.

To enrich for RIP-deficient mutants, we used the *sad-1* mutant and the transgenic *sad-1* duplication to construct [*sad-1*$^+$ + *sad-1*] heterokaryotic strains that are homoallelic for the ectopically duplicated *sad-1* fragment. These strains are self-fertile and in a self-cross they are liable to RIP inactivate the sole *sad-1*$^+$ allele, in which

case the resulting ascus would be rendered infertile and not yield any ascospores (Shiu *et al.* 2001). This, together with the fact that *sad-1* is on LG I and hence immune to second-division segregation, ensures that the self-crosses will not produce *sad-1* "homoallelic" progeny and that all the progeny will carry the un-RIPed *sad-1*$^+$ allele. If progeny from each self-cross generation are germinated and grown *en masse* on the plate and allowed to mate at random, we would expect the successively later generations to become progressively enriched for RIP-defective mutants. Mutations from this "enriched" culture will be recovered by crossing single-mating-type derivatives with single-mating-type derivatives of an *erg-3*-duplication strain that is homoallelic for *erg-3*$^+$ at the endogenous locus and by obtaining large (*i.e.*, self-fertile) progeny ascospores. These ascospores are now potentially homoallelic for a *rid* mutation from the enriched culture and heteroallelic for the *erg-3* duplication, in which case their self-cross progeny will not include any *erg-3* mutants.

As a bonus, the "enrichment" crosses will also result in the induction of additional RIP mutation of the already mutated *sad-1* allele, thus increasing the severity of its sequence alteration by RIP. Severely altered *sad-1* alleles are expected to display a dominant phenotype like that of their *N. crassa* counterparts (Shiu and Metzenberg 2002), and they would be detectable by their ability to increase the productivity of *N. tetrasperma* × *N. crassa* crosses. By helping to overcome MSUD-dependent barriers to interspecies gene flow, the dominant *sad-1* alleles obtained in this way will facilitate the introgression of mutations and rearrangements from *N. crassa* into *N. tetrasperma*.

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