Neurospora Heterokaryons with Complementary Duplications and Deficiencies in Their Constituent Nuclei Provide an Approach to Identify Nucleus-Limited Genes

Dev Ashish Giri,*† S. Rekha,* and Durgadas P. Kasbekar*†

*Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500001, India and †Graduate Studies, Manipal University, Manipal 576104, Karnataka, India

ORCID ID: 0000-0002-5269-8020 (D.P.K.)

ABSTRACT

Introgression is the transfer of genes or genomic regions from one species into another via hybridization and back-crosses. We have introgressed four translocations (EB4, IB5, UK14-1, and B362i) from Neurospora crassa into N. tetrasperma. This enabled us to construct two general types of heterokaryons with mat-A and mat-a nuclei of different genotypes: one type is [T + N] (with one translocation nucleus and one normal sequence nucleus), and the other is [Dp + Df] (with one nucleus carrying a duplication of the translocation region and the other being deleted for the translocation region). Self-crossing these heterokaryons again produced [T + N] and [Dp + Df] progeny. From conidia (vegetative spores) produced by the heterokaryotic mycelia, we obtained self-fertile (heterokaryotic) and self-sterile (homokaryotic) derivative strains. [T + N] heterokaryons produced homokaryotic conidial derivatives of both mating types, but [Dp + Df] heterokaryons produced viable conidial homokaryons of only the mating type of the Dp nucleus. All four [T + N] heterokaryons and three [Dp + Df] heterokaryons produced both self-sterile and self-fertile conidial derivatives, but the [Dp(B362i) + Df(B362i)] heterokaryons produced only self-sterile ones. Conceivably, the Df(B362i) nuclei may be deleted for a nucleus-limited gene required for efficient mitosis or nuclear division, and whose deficit is not complemented by the neighboring Dp(B362i) nuclei. A cross involving Dp(EB4) showed repeat-induced point mutation (RIP). Because RIP can occur in self-crosses of [Dp + Df] but not [T + N] heterokaryons, RIP alteration of a translocated segment would depend on the relative numbers of [Dp + Df] vs. [T + N] ancestors.

KEYWORDS

chromosome translocation introgression pseudo-homothallism RIP MSUD

Perkins (1997) described three elementary chromosome translocation (T) types in Neurospora crassa, namely, insertional (IT), quasiterminal (QT), and reciprocal (RT). ITs transfer a segment of a donor chromosome into a recipient chromosome without any reciprocal exchange (Figure 1); QTs transfer a distal segment of a donor chromosome to the tip of a recipient chromosome, distal to any essential gene, and presumably the donor chromosome breakpoint is capped with the tip from the recipient chromosome; and RTs reciprocally interchange the terminal segments of two chromosomes. Other chromosome rearrangements are essentially variants of these, e.g., an intrachromosomal transposition (Tp) is an IT in which the same chromosome is both donor and recipient, an inversion (In) is a Tp in which a chromosome segment is re-inserted in opposite orientation into the site from which it was derived, and there are complex rearrangements such as linked RT and IT. Three breakpoint junctions define an IT; junction A created by the deletion on the donor chromosome, and junctions B and C (proximal and distal), created by the insertion into the recipient chromosome; however, two breakpoint junctions define a QT or RT; junction A, between the breakpoint-proximal segment on the donor...
the two chromosomes that underwent reciprocal interchange of their
obtaining 8B:0W = 0B:8W signals an
one pole and
their normal sequence homologs are designated as N0 and N1. In
alternate segregation (ALT), T0 and T0 segregate to one spindle pole,
and N0 and N0 segregate to the other. Subsequently, meiosis II and
postmeiotic mitosis generate eight parental-type nuclei, namely, four
IT + four N. In adjacent 1 segregation (ADJ), N0 and T0 and
segregate to one pole and T0 and N0 segregate to the other to ultimately produce
eight nonparental nuclei: four Dp + four Df.

Figure 1 Alternate and adjacent 1 segregation in IT × N. The donor
and recipient chromosomes of the IT are designated as T0 and T0
and their normal sequence homologs are designated as N0 and N0. In an
alternate segregation (ALT), T0 and T0 segregate to one spindle pole,
and N0 and N0 segregate to the other. Subsequently, meiosis II and
postmeiotic mitosis generate eight parental-type nuclei, namely, four
IT + four N. In adjacent 1 segregation (ADJ), N0 and T0 and
segregate to one pole and T0 and N0 segregate to the other to ultimately produce
eight nonparental nuclei: four Dp + four Df.

chromosome and the tip from the recipient chromosome, and junction
B, between the breakpoint-proximal sequence on the recipient chromo-
some and the donor segment grafted onto it (Singh et al. 2010). In
the cross of a translocation strain with normal sequence, the chromo-
somes can segregate in one of two ways in meiosis I: alternate or
adjacent 1 (see Figure 1 for IT × N). Alternate segregation produces
eight parental-type ascospores (i.e., 4 IT + 4 N), whereas adjacent 1
segregation produces eight nonparental ascospores, namely, four
viable ascospores containing a duplication (Dp) of the translocation
segment and four inviable ones with the complementary deficiency
(Df) (Figure 1). Viable ascospores blacken (B), whereas inviable ones
remain white (W). Therefore, alternate and adjacent 1 segregation
produce, respectively, 8B0W and 4B4W asci (Perkins 1997). Because
both segregations are equally likely, IT × N and QT × N crosses are
characterized by 8B0W = 4B4W, whereas isosequential crosses (i.e.,
N × N or T × T) produce mostly 8B0W ascis (Perkins 1997). In an RT,
the two chromosomes that underwent reciprocal interchange of their
terminal segments can be designated as T1 and T2 and their normal
sequence homologs can be designated as N1 and N2. In an RT × N
cross, alternate segregation moves T1 and T2 to one spindle pole and
N2 to the other to produce eight parental-type ascospores (i.e.,
4 RT + 4 N) that are viable and black. Adjacent 1 segregation moves
N1 and T2 to one pole and N2 and T1 to the other to generate only inviable
white ascospores bearing complementary duplications and deficiencies
(i.e., Dp2/Df1 and Dp1/Df2), and the ascis are 0B:8W. In other words,
obtaining 8B0W = 0B:8W signals an RT × N.

Dp strains (i.e., the viable segreants from 4B4W ascis) are recognizable
by the characteristic barren phenotype of Dp × N crosses, wherein
normal-looking perithecia are made, but only a few exceptional as-
cospores are produced (Perkins 1997). Barrenness is caused by meiotic
silencing by unpaired DNA (MSUD), an RNAI-mediated process that
eliminates the transcripts of any gene not properly paired with a ho-
monog during meiosis (Shiu et al. 2001). Presumably, Dp-borne genes,
including those underlying ascus and ascospore development, fail to be
properly paired in a Dp × N cross, and their silencing by MSUD renders the cross barren. The breakpoint junctions of several ITs, QTs, and RTs were defined in our laboratory (Singh 2010; Singh et al. 2010). PCR with breakpoint junction-specific primers can now be used to distinguish the Dp progeny from their T and N siblings. IT progeny contain all three breakpoints (A, B, and C), Dp progeny contain B and C, but not A, and N progeny contain none. Although Dps have been extensively studied (Perkins 1997; Kasbekar 2013), the use of Dfs was limited to flagging the Dp-bearing 4B:4W asci. We now report the generation of [Dp + Df] heterokaryons with complementing duplications and deficiencies in their constituent nuclei. They were obtained by introgressing N. crassa ITs (and a QT) into N. tetrasperma. Introggression is the transfer of genes or genomic regions from one species into another (Rieger et al. 1991).

Eight ascospores form per ascus in N. crassa, whereas four are
formed in N. tetrasperma. In both species the parental mat A and
mat a nuclei fuse in the ascogenous cell to produce a diploid zygote
nucleus that immediately undergoes meiosis and a postmeiotic mitosis
to generate eight haploid nuclei (four mat A + four mat a). In N. crassa
the nuclei are partitioned into the eight initially uninucleate ascospores
(Raju 1980). In contrast, N. tetrasperma ascospores are initially bina-
culate, receiving a pair of nonsister nuclei (one mat A + one mat a)
(Raju and Perkins 1994). Thus, N. crassa ascospores produce homo-
karyotic mycelia that are either mat A or mat a in mating type, whereas
N. tetrasperma ascospores can produce heterokaryotic mycelia contain-
ning nuclei of both mating types. In N. crassa, a sexual cross perforce
requires mycelia from two different ascospores, one mat A and the
other mat a, thus making the lifecycle “heterothallic”; but a heterokary-
otic N. tetrasperma mycelium from a single ascospore bearing nuclei of
both mating types is competent to undergo a self-cross, making the lifecycle “pseudo-homothallic.” However, a subset of conidia (vegetative
spores) produced by a heterokaryotic N. tetrasperma mycelium can be
homokaryotic by chance, and N. tetrasperma ascogogenesis occasionally
produces five or more (up to eight) ascospores by replacement of one or
more dikaryotic ascospore by a pair of smaller homokaryotic ones
(Raju 1992). The dominant Eight-spore (E) mutant increases the frequency
of such replacement, although E-homoyxogous crosses are infertile
(Calhoun and Howe 1968). Mycelia from homokaryotic conidia or
ascospores can cross with like mycelia of the opposite mating type.
Therefore, N. tetrasperma is actually a facultatively heterothallic species.

Although [mat A + mat a] heterokaryons can form quite easily in
N. tetrasperma, their formation is prevented in N. crassa by mating
type-mediated vegetative incompatibility (Newmeyer 1970). The
N. crassa allele of the tolerant gene (tofC) is a key regulator of this
incompatibility (Shiu and Glass 1999). If tofC is replaced either by a
recessive allele tol or by the N. tetrasperma allele (tolF), then
heterokaryons of genotype [tol mat A + (tol mat a)] can form in
N. crassa and are stable, provided that they are homokaryotic for het
loci that determine other vegetative heterokaryon incompatibilities
(Smith and Lafontaine 2013). N. crassa strains of the same mating type
and sharing the same alleles at the other het loci can fuse to form a
culture that has both nuclear types in a common cytoplasm. Hetero-
karyon formation between two strains with different auxotrophic muta-
tions can be “forced” by plating them together on minimal medium.
The helper-1 strain (genotype amI ad-3B cyh-1) is capable of forming
vigorous heterokaryons with either of the mating type strains of the
standard laboratory Oak Ridge (OR) background, because the mat
a allele amI is inactive and does not elicit mating type-mediated vege-
tative incompatibility. The amI allele also makes the helper-1 compo-
nent of such a heterokaryon a passive partner when the heterokaryon
is used in a cross (Perkins et al. 2001). We have used helper-1 to genetically
map the fmf-1 mutation (Iyer et al. 2009), which has a unique female-sterile and male-sterile phenotype. Heterokaryons have also been used for the analysis of complementation, and to “rescue” recessive lethal mutations. Additionally, heterokaryosis in N. crassa (Davis 1960) and Penicillium cyclopium (Links 1952) has long been known to confer phenotypic plasticity that enables the fungus to respond to changes in environmental conditions by changes in the ratios of the constituent nuclei, but how this happens is still not understood. Recent studies from Dr. Hanna Johannesson’s laboratory have shown that the mat A/mat a nuclear ratio can change during the N. tetrasperma lifecycle (Samils et al. 2014). The ratio is biased for mat A nuclei during mycelial growth and becomes more balanced only during sexual development. There was also a bias in expression for mat A-linked genes during mycelial growth that switched during the sexual stage into a bias for genes of the mat a nuclei. These findings were interpreted to suggest that N. tetrasperma mat A and mat a nuclei have co-evolved to optimize their relative fitness in the heterokaryon by altering their ratios and by regulating gene expression. Previous studies from this group showed that that wild-isolated N. tetrasperma strains from United Kingdom generally produced a greater proportion of homokaryotic conidia than strains isolated from New Zealand, and some even showed a strong bias in the homokaryotic conidia for one of the mating types (e.g., 0 mat A: 17 mat a; 7 mat A: 0 mat a; and 0 mat A: 9 mat a). However, all the strains made self-fertile conidia (Corcoran et al. 2012). The high proportion of single-mating-type conidia attributed to a putative nonrandom distribution of nuclei in the heterokaryotic mycelium and/or stronger ability of certain nuclei to be packaged into conidia.

Here, we have introgressed three N. crassa ITs (EB4, IB5, and B362) and one QT (UK14-1) into N. tetrasperma, and we have shown that $T \times N$ crosses can produce both $[T + N]$ and $[Dp + Df]$ heterokaryotic progeny. The $[Dp + Df]$ progeny allowed us to ask whether the Df deletes any gene with a nucleus-limited function. A gene may be considered to be nucleus-limited if nuclei bearing its null allele ($\Delta$) are not complemented by wild-type nuclei (WT) in a $[\Delta + WT]$ heterokaryon (Kasbekar 2014). If a Df deletes a nucleus-limited gene, then one might expect to see a phenotype difference between $[T + N]$ and $[Dp + Df]$ heterokaryons. Although no nucleus-limited genes have been reported as yet, their existence in fungi is not ruled out, especially given the putative nucleus-limited behavior of the N. crassa scor1 mutant (Burton and Metzenberg 1972), the DNA damage checkpoint signal in Saccharomyces cerevisiae (Demeter et al. 2000), and the MatS gene silencing process in Aspergillus nidulans (Czaja et al. 2013). We found that although $[T(B362i) A + N a]$ heterokaryons could produce both heterokaryotic and homokaryotic conidia, the $[Dp(B362i) A + Df(B362i) A]$ heterokaryons produced only homokaryotic conidia, possibly because a putative “nucleus-limited” gene required for efficient packaging of nuclei into conidia is absent from the Df($B362i$). Second, we found evidence that suggests that the N. tetrasperma E mutant contains a recessive mutation affecting alternate but not adjacent 1 segregation. Third, we show that a Dp-heterozygous cross can exhibit RIP (repeat-induced point mutation), the sexual stage-specific process that induces Gc to A:T mutations in duplicated DNA (Selker 1990).

### MATERIALS AND METHODS

**Neurospora strains and general genetic manipulations**

All Neurospora strains were obtained from the Fungal Genetics Stock Center, Kansas State University, Manhattan, Kansas USA, unless otherwise indicated. N. crassa OR A (FGSC 987) and OR a (FGSC 988) are the standard laboratory Oak Ridge strains; the translocation strains are $T(VR \rightarrow VI)EB4 A$ (FGSC 3046), $T(VIL \rightarrow IR) IB5 cpc-1 A$ (FGSC 4433), $T(VIR > VL) UK14-I A$ (FGSC 6958), and $T(VI \rightarrow I) B362i A$ (FGSC 2935) [abbreviated T(EB4), T(IB5), T(UK14-I), and T(B362i)], T(EB4), T(IB5), and T(B362i) are ITs, whereas T(UK14-I) is a QT. These translocations have been described by Perkins (1997) and Singh (2010). Perkins (1997) reported that T(IB5) translocates a chromosome VII segment to IR, linked to al-2 and un-18. However, Singh et al. (2010) showed that the VII segment is, in fact, inserted into a chromosome IVR sequence. Therefore, T(IB5) might include an additional rearrangement (possibly an RT) involving IVR and IR, whose breakpoints are as yet undetermined. Because T(B362i) also involves chromosomes I and IV, the cross T(IB5) $\times$ T(B362i) would resemble an RT-heterozygous cross.

The semi-dominant MSUD suppressor strains Sad-1 A (FGSC 8740) and Sad-1 a (FGSC 8741) were gifted by the late Robert L. Metzenberg and are described by Shiu et al. (2001). The Sad-1 locus encodes an RNA-dependent RNA polymerase essential for MSUD, and the Sad-1 suppressor allele is presumed to prevent proper pairing of its wild-type homolog, thus inducing it to autogonously silence itself (Shiu et al. 2001). The MSUD testers $pan-2$; his-3;his-3* $Bml^a$ A (FGSC 8755); $pan-2$; his-3;his-3* $Bml^a$ a (FGSC 8756); $pan-2$; his-3;his-3* mei-3* A (FGSC 8759); and $pan-2$; his-3;his-3* mei-3* a (FGSC 8760) (hereafter designated as :: $Bml^a$ A; :: $Bml^a$ a; :: $mei-3$ A; and :: $mei-3$ a) are described by Raju et al. (2007). Another tester, rid his-3; VII::$\rho'^{-}$-lhp $A$ (ISU3117) (hereafter ::$\rho'^{-}$), was a gift from Dr. Tom Hammond (Illinois State University). The ::$Bml^a$ and ::$mei-3$ testers have an extra copy of the $bml$ (β-tubulin) or mei-3 gene inserted ectopically in the his-3 locus in chromosome 1, whereas the tester strain ::$\rho'^{-}$ has a copy of the $r$ (round spores) gene inserted ectopically into chromosome 7. In a cross of the tester with wild-type, the ectopic copy remains unpaired in meiosis and results in elimination of all its homologous transscripts, including from the paired endogenous copies. In crosses of ::$Bml^a$, ::$mei-3$, and ::$\rho'^{-}$ with the wild-type, the bml, mei-3, and $r$ genes, respectively, are silenced. Silencing of the bml or mei-3 gene arrests normal ascus development (Raju et al. 2007; Kasbekar et al. 2011), and silencing of $r$ causes all eight ascospores to be round instead of having the normal spindle shape. Homozygous tester $A \times$ tester $a$ crosses do not show MSUD, nor do crosses of the testers with the Sad-1 suppressor of MSUD, and the ascii developed normally (Raju et al. 2007; Kasbekar et al. 2011).

N. tetrasperma has standard strains 85 A (FGSC 1270) and 85 a (FGSC 1271); the E mutants are hwn; al(102); E A (FGSC 2783) and hwn; al(102); E a (FGSC 2784) (hereafter E A and E a). N. crassa / N. tetrasperma hybrid strain is C4,T4 a (FGSC 1778). The C4,T4 a strain has four N. crassa great-grandparents and four N. tetrasperma great-grandparents (Metzenberg and Ahlgren 1969). The N. crassa great-grandparents were of the OR background, whereas the N. tetrasperma great-grandparents were of the 343.6 A E background (Metzenberg and Ahlgren 1969).

Neurospora genetic analysis was performed essentially as described by Davis and De Serres (1970). The alternative recipe of Metzenberg and Ahlgren (1969).

### Outline of the introgression crosses and characterization of the resultant strains

Crosses between N. crassa and N. tetrasperma strains are almost completely sterile. However, both N. crassa strain OR A and N. tetrasperma...
strain 85 A can cross with the N. crassa/N. tetrasperma hybrid strain C4T4 a and produce viable progeny (Perkins 1991; also see Table 3 of this article); therefore, we used the C4T4 a strain as a bridging strain for the initial introgression crosses. The N. crassa T strains were crossed with C4T4 a and T progeny from these crosses (designated T\(C4T4\)) were distinguished from their \(Dp\) and \(N\) siblings by PCR with breakpoint junction-specific primers. Nominally, 50% of the genome of T\(C4T4\) progeny is derived from the C4T4 a parent. The T\(C4T4\A strains were crossed with C4T4 a to obtain the T\(C4T4\) progeny in a similar manner. Crosses of T\(C4T4\) with the opposite mating-type derivative of strain 85 were productive, and their T progeny were designated as T\(185\). Likewise, T\(185\times 85\) yielded T\(285\), etc. After two to three iterations of the crosses with 85, we recovered progeny ascospores that produced mycelium of dual mating specificity characteristic of N. tetrasperma. That is, the resulting mycelium could cross with both 85A and a, and it could also undergo a self-cross. A heterokaryotic strain containing all three breakpoints (A, B, and C) is potentially of genotype \([T + N]\) or \([Dp + Df]\). The salient features of the genotype via self-crosses I1-I5 were self-fertile heterokaryons obtained from the cross of homo-

RESULTS

The different self-fertile strains listed in Table 2 were named using the following rules. The letters E, I, U, or B in the name identify strains derived from introgressions of, respectively, the N. crassa translocations T\(EB4\), T\(IBj5\), T\(UK14-1\), and T\(B362i\). The strains E1, U9, B7, and I1-I5 were self-fertile heterokaryons obtained from the cross of homokaryotic T\(EB4\), T\(UK14-1\), T\(B362i\), and T\(IBj5\) strains with either 85 a or 85 A (see Figure 2). Strains E1, E1, E1, E1, etc., were derived from the self-cross of strain E1; likewise, strains I1, I1, I1, I1, etc., were from the self-cross of strain I1; U9, U9, U9, etc., were from the self-cross of strain U9; and 11B7, 18B7, and 19B7 were from self-crosses of strain B7. Further, strains 1(1U9), 2(1U9), 3(1U9), etc., were from self-cross of strain 1U9; 6(19B7) was from self-cross of strain 19B7; and 1[6(19B7)], 2[6(19B7)], etc., were from the self-cross of strain 6(19B7).

A note on strain nomenclature

The different self-fertile strains listed in Table 2 were named using the following rules. The letters E, I, U, or B in the name identify strains derived from introgressions of, respectively, the N. crassa translocations T\(EB4\), T\(IBj5\), T\(UK14-1\), and T\(B362i\). The strains E1, U9, B7, and I1-I5 were self-fertile heterokaryons obtained from the cross of homokaryotic T\(EB4\), T\(UK14-1\), T\(B362i\), and T\(IBj5\) strains with either 85 a or 85 A (see Figure 2). Strains E1, E1, E1, E1, etc., were derived from the self-cross of strain E1; likewise, strains I1, I1, I1, I1, etc., were from the self-cross of strain I1; U9, U9, U9, etc., were from the self-cross of strain U9; and 11B7, 18B7, and 19B7 were from self-crosses of strain B7. Further, strains 1(1U9), 2(1U9), 3(1U9), etc., were from self-cross of strain 1U9; 6(19B7) was from self-cross of strain 19B7; and 1[6(19B7)], 2[6(19B7)], etc., were from the self-cross of strain 6(19B7).

RESULTS

N. tetrasperma \([T + N]\) and \([Dp + Df]\) strains can switch genotype via self-crosses

The salient features of the N. crassa translocations T\(EB4\), T\(IBj5\), T\(UK14-1\), and T\(B362i\) are summarized in Table 1, along with the accession numbers of their breakpoint junction sequences. The introgression of these translocations into N. tetrasperma is outlined in the Materials and Methods section, and Figure 2 schematically presents the actual crosses performed.

Introggression of T\(EB4\) yielded the self-fertile heterokaryotic strain designated E1 (Figure 2). Using E1 genomic DNA as template and T\(EB4\) breakpoint junction-specific primers, all three breakpoint junctions (A, B, and C) of T\(EB4\) could be amplified by PCR (data not shown). A heterokaryon possessing all the three breakpoints is potentially of genotype \([T(EB4) + N]\) or \([Dp(EB4) + Df(EB4)]\). Heterokaryons of genotype \([T(EB4) + +\]\), \([T(EB4) + +\]\), or \([T(EB4) + +\]\) also fulfill this criterion, but, they were deemed to be less likely because one or more crossover is required to generate them. Eight self-fertile progeny from the self-cross of E1 were analyzed and the results, which are summarized in Table 2 (serial numbers 1–8), established that progeny 2E1, 4E1, and 6E1 were of the \([T(EB4) + +\]\) genotype, whereas 1E1, 9E1, and 12E1 were of \([Dp(EB4) + +\]\). Only six self-sterile conidial derivatives were obtained for 3E1, and PCR revealed all to be type \(T(EB4)\ a\), suggesting that 3E1 has the genotype \([T(EB4) + +\]\ A). However, given the small numbers tested, and given the possibility of

![Figure 2](image-url)
skewed segregation, our results do not exclude the \([T(EB4)\ a + N\ A]\) genotype. No self-sterile conidial derivatives were obtained from 13E1; therefore, its genotype was not determined.

From self-crosses of strains 6E1 and 9E1 (see above), we examined 39 and 24 progeny and found that 12 and 9, respectively, were self-sterile. From a subset of self-sterile progeny, we obtained self-sterile (i.e., homokaryotic) conidial derivatives and determined their mating type by crossing with 85 a and 85 A. Of three self-sterile progeny tested from strain 6E1, two appeared to be \([T(EB4)\ a + N\ A]\) and one was \([Dp(EB4)\ a + Df(EB4)\ A]\); of three self-sterile progeny tested from strain 9E1, one appeared to be \([Dp(EB4)\ a + Df(EB4)\ A]\) and the other two appeared to be \([T(EB4)\ a + N\ A]\). We used PCR to confirm the genotype of the \([Dp(EB4)\ a + Df(EB4)\ A]\) progeny of 6E1, and the genotype of one \([T(EB4)\ a + N\ A]\) progeny of 9E1 (Table 2, serial numbers 10 and 12). These results showed that self-crosses of both \([T(EB4)\ a + N]\) and \([Dp(EB4)\ a + Df(EB4)\ A]\) heterokaryons can again generate \([T(EB4)\ a + N]\) and \([Dp(EB4)\ a + Df(EB4)\ A]\) progeny.

The translocations \(T(IBj5), T(UK14-1),\) and \(T(B362i)\) were introgressed in a similar manner (Figure 1), and we recovered heterokaryons of genotypes \([T(IBj5)\ a + N\ A]\) and \([Dp(IBj5)\ a + Df(IBj5)\ A]\) (Table 2, serial numbers 15, 18-21, and 25), \([T(UK14-1)\ a + N\ A]\) and \([Dp(UK14-1)\ a + Df(UK14-1)\ A]\) (Table 2, serial number 26, 27, 31, 33, and 34), and \([T(B362i)\ a + N\ A]\) and \([Dp(B362i)\ a + Df(B362i)\ A]\) (Table 2, serial numbers 35-41, 43, 45, and 47). Again, self-crosses of the \([T + N]\) and \([Dp + Df]\) heterokaryons produced progeny of the alternative genotype. Specifically, self-cross of the \([Dp(IBj5)\ a + Df(IBj5)\ A]\) type strain I1 produced the \([T(IBj5)\ a + N\ A]\) type progeny strain III (Table 2, serial numbers 15 and 20); of the \([T(UK14-1)\ a + N\ A]\) type strain, U9 produced the \([Dp(UK14-1)\ a + Df(UK14-1)\ A]\) type progeny strain 1U9, whose self-cross, in turn, produced the \([T(UK14-1)\ a + N\ A]\) type strain 2U9 (Table 2, serial numbers 26, 27, and 33); and of the \([Dp(B362i)\ a + Df(B362i)\ A]\) type strain, B7 produced the \([T(B362i)\ a + Df(B362i)\ A]\) type progeny strain 1B9, whose self-cross, in turn, produced the \([T(B362i)\ a + Df(B362i)\ A]\) type progeny strain 6(1B9), whose self-cross produced the \([T(B362i)\ a + N\ A]\) type progeny 2(6(1B9)) and 5(6(1B9)) (Table 2, serial numbers 35, 38, 43, 45 and 47). In sum, our results show that \([T + N]\) and \([Dp + Df]\) genotypes can be interchanged through self-crosses.

The genotype of two heterokaryons was found to be putatively \([Df(IBj5)\ a + N\ A]\) and \([Df(IBj5)\ a + Df(IBj5)\ A]\) (Table 2, serial numbers 16 and 17), but our results do not rule out the possibility that skewed segregation in small numbers might account for the absence of self-sterile \(T(IBj5)\) or \(N\) conidial types, respectively, from what in fact might be \([T(IBj5)\ a + N\ A]\) heterokaryons. One heterokaryon was found to contain three nuclear types, and its genotype was \([T(IBj5)\ a + N\ A + Df(IBj5)\ A]\) (Table 2, serial number 22). It got flagged because two of the seven \(mat\ A\) self-sterile conidial derivatives examined possessed only the \(A\), but not \(B\), junction of \(T(IBj5)\), whereas the other five did not possess either junction. A homokaryon bearing only the \(A\) junction is not expected to be viable because it contains an uncomplemented \(DF\) chromosome; therefore, we presume the two self-sterile \(mat\ A\) derivatives were \([NA + Df(IBj5)\ A]\) heterokaryons and we infer that the genotype of the self-fertile strain was \([T(IBj5)\ a + NA + Df(IBj5)\ A]\). We defer to the Discussion section a consideration of how such a strain might have arisen.

**Table 1 Translocations used in this study**

| Translocation | Size (bp) | Genes (N) | Breakpoint Junction Sequence (Accession Number) |
|---------------|----------|----------|-----------------------------------------------|
| T(VIR > VII) EB4 | 145,282 | 39 | GQ504681 GQ504682 GQ504683 |
| T(VIL > IRI) IB5 | 405,319 | 120 | GQ504684 GQ504685 NA |
| T(VIR > V) UK14-1 | 490,958 | 126 | GQ504703 |
| T(VIR > II) B362i | 118,782 | 36 | GQ504697 GQ504698 GQ504699 |

NA, not applicable.

* \(T(IBj5)\) may be a complex rearrangement (see text).

**[Dp(B362i) + Df(B362i)]** heterokaryons yield only self-sterile conidial derivatives

The search for self-sterile homokaryotic conidial derivatives (above) was expected to inevitably also identify self-fertile ones. A majority of conidial derivatives from both \([T + N]\) and \([Dp + Df]\) strains of \(EB4\), \(IB5\), and \(UK14-1\) were self-fertile (Table 2, serial numbers 1-33), and self-fertile conidial derivatives (or derivatives heterokaryotic for mating type) were also obtained from \([T(B362i) + N]\) (Table 2, serial numbers 38, 45, and 47). Therefore, we were very surprised to find that all the 117 conidial derivatives examined from seven different \([Dp(B362i) + Df(B362i)]\) heterokaryons were self-sterile homokaryons (Table 2, serial numbers 35-37, 39-41, and 43).

As an additional control we performed the cross \(Dp(B362i)\ a \times 85\ A\) and tested 61 progeny, of which 39 proved to be self-fertile (i.e., heterokaryotic). We examined 10 to 25 conidial derivatives from each of 16 self-sterile progeny, and in every case we found some self-fertile conidial derivatives. Additionally, for three progeny, we also identified self-sterile conidial derivatives of both mating types, and by PCR we could establish that the genotype of one progeny was \([Dp(B362i)\ a + Dp(B362i)\ A + N\ A]\), and the other two were \([Dp(B362i)\ a + Dp(B362i)\ A]\). Thus, unlike their \([Dp(B362i)\ a + Df(B362i)\ A]\) counterparts, the \([Dp(B362i)\ a + N\ A]\) heterokaryons were able to make self-fertile heterokaryotypic conidia. Additionally, among the progeny from the self-cross of a \([Dp(B362i)\ a + Df(B362i)\ A]\) self-fertile heterokaryon, we recovered one that was \([N\ a + Dp(B362i)\ A]\) type (Table 2, serial number 44), and of four conidial derivatives examined, one was self-fertile. Therefore, the absence of self-sterile conidia from \([Dp(B362i)\ a + Df(B362i)\ A]\) heterokaryons appears to be exceptional. The implication of this phenotype is considered in the Discussion section.

**Characterizing the T-type homokaryons**

The homokaryotic \(T\)-type conidial derivatives designated as \(T(EB4)^{N1}\), \(T(IBj5)^{N1}\), \(T(UK14-1)^{N1}\), and \(T(B362i)^{N1}\) were obtained from the self-fertile \([T + N]\) heterokaryons (see Materials and Methods) and were found to be like bona fide \(N.\ tetrasperma\) strains. That is, their crosses with opposite mating-type derivatives of \(N.\ tetrasperma\) strain 85 were fertile, whereas their crosses with \(N.\ crassa\) OR strains of opposite mating type were as infertile as an OR \(\times 85\) interspecies cross (Table 3). Control crosses of the \(N.\ crassa\ T\) strains (\(T^{N}\)) with the OR strains of the opposite mating type were productive, but the crosses of the \(T^{N}\) with the opposite mating type derivatives of strain 85 were sterile (Table 3). The \(C47A\) hybrid strain produced viable ascospores in crosses with both OR A and 85 A (Table 3).
To obtain larger numbers of eight-spored asci, we crossed the T(E41)Nt A and T(EB4)Nt A strains with E strains of the opposite mating type. The T(E41)Nt A × E A and T(EB4)Nt A × E A crosses were productive and produced 8:0 and 4:4 ascus types at comparable frequencies (Table 4), which is a characteristic of IT × N and QT × N crosses in N. crassa. Surprisingly, the crosses T(IB5)Nt A × E A and T(B362)Nt A × E A did not

Table 2 Genotype of self-fertile strains

| Serial No. | Self-Fertile Strain | Conidial Derivatives (N) | Self-Sterile Derivatives (N) | mat a (N) | mat A (N) | Strain Genotype Indicated by PCR |
|------------|---------------------|--------------------------|-------------------------------|-----------|-----------|--------------------------------|
| 1          | 1E1                 | 67                       | 25                            | 0         | 25        | [Df a + Dp A]                  |
| 2          | 2E1                 | 38                       | 10                            | 5         | 5         | [N a + T A]                    |
| 3          | 3E1                 | 28                       | 6                             | 6         | 0         | [T a + Df A]^a                 |
| 4          | 4E1                 | 85                       | 12                            | 1         | 11        | [T a + N A]                    |
| 5          | 6E1                 | 129                      | 30                            | 10        | 20        | [T a + N A]                    |
| 6          | 9E1                 | 62                       | 14                            | 0         | 14        | [Df a + Dp A]                  |
| 7          | 12E1                | 49                       | 16                            | 16        | 0         | [Dp a + Df A]                  |
| 8          | 13E1                | 16                       | 0                             | 0         | 0         | ND                             |
| 9          | 1(6E1)              | 59                       | 9                             | 4         | 5         | ND                             |
| 10         | 3(6E1)              | 63                       | 7                             | 0         | 7         | [Df a + Dp A]                  |
| 11         | 4(6E1)              | 54                       | 10                            | 4         | 6         | ND                             |
| 12         | 1(9E1)              | 40                       | 8                             | 6         | 2         | [T a + N A]                    |
| 13         | 2(9E1)              | 56                       | 10                            | 0         | 10        | ND                             |
| 14         | 3(9E1)              | 65                       | 11                            | 8         | 3         | ND                             |
| 15         | 1                   | 87                       | 17                            | 8         | 0         | [Dp a + Df A]                  |
| 16         | 2                   | 75                       | 6                             | 0         | 6         | [Df a + N A]^b                 |
| 17         | 3                   | 27                       | 5                             | 0         | 5         | [Df a + T A]^f                 |
| 18         | 4                   | 121                      | 34                            | 2         | 8         | [T a + N A]                    |
| 19         | 5                   | 62                       | 23                            | 4         | 0         | [Dp a + Df A]                  |
| 20         | 1(1I1)              | 50                       | 4                             | 0         | 4         | [T a + N A]                    |
| 21         | 2(1I1)              | 24                       | 16                            | 16        | 0         | [Dp a + Df A]                  |
| 22         | 3(1I1)              | 60                       | 9                             | 2         | 7         | [T a + N A + Df A]^e           |
| 23         | 4(1I1)              | 26                       | 0                             | 0         | 0         | ND                             |
| 24         | 1(I1)               | 30                       | 1                             | 0         | 1         | [? a + T A]^c                  |
| 25         | 2(I1)               | 30                       | 18                            | 9         | 9         | [T a + N A]                    |
| 26         | 1(1U9)              | 5                       | 1                             | 4         | 1         | [T a + N A]                    |
| 27         | 1                   | 19                       | 8                             | 8         | 0         | [Dp a + Df A]                  |
| 28         | 2                   | 49                       | 9                             | 1         | 8         | ND                             |
| 29         | 3                   | 56                       | 7                             | 5         | 2         | ND                             |
| 30         | 4                   | 80                       | 7                             | 0         | 0         | ND                             |
| 31         | 5                   | 44                       | 8                             | 3         | 5         | [N a + TA]                     |
| 32         | 1(1U9)              | 0                        | 0                             | 0         | 0         | ND                             |
| 33         | 2(1U9)              | 45                       | 12                            | 3         | 9         | [N a + T A]                    |
| 34         | 3(1U9)              | 10                       | 10                            | 0         | 10        | [Df a + Dp A]                  |
| 35         | 5                   | 13                       | 3                             | 3         | 3         | [Df a + Df A]                  |
| 36         | 11B7                | 30                       | 30                            | 30        | 0         | [Dp a + Df A]                  |
| 37         | 18B7                | 20                       | 20                            | 20        | 0         | [Dp a + Df A]                  |
| 38         | 19B7                | 11                       | 8                             | 3         | 5         | [N a + T A]                    |
| 39         | 24B7                | 7                        | 7                             | 0         | 0         | [Dp a + Df A]                  |
| 40         | 28B7                | 33                       | 33                            | 0         | 0         | [Dp a + Df A]                  |
| 41         | 30B7                | 6                        | 6                             | 0         | 0         | [Dp a + Df A]                  |
| 42         | 31B7                | 0                        | 0                             | ND        | 0         | [Df a + T A]                   |
| 43         | 6(19B7)             | 11                       | 11                            | 11        | 0         | [Dp a + Df A]                  |
| 44         | 1[6(19B7)]          | 4                        | 3                             | 2         | 1         | [N a + Dp A]                   |
| 45         | 2[6(19B7)]          | 9                        | 7                             | 2         | 5         | [N a + T A]                    |
| 46         | 4[6(19B7)]          | 24                       | 1                             | 0         | 1         | [? a + T A]^h                  |
| 47         | 5[6(19B7)]          | 15                       | 14^g                         | 12        | 2         | [N a + T A]                    |

ND: not determined.

^a,b(T a + N A) and c(N a + T A) genotypes are not excluded, see text.

DNA from two of the seven mat-a conidial derivatives amplified the A junction, but DNA from the other five did not. See text for details.

On only one self-sterile conidial derivative was obtained. It was mat A and its DNA amplified the A and B junction fragments of T(IB5), thus establishing it as type T. The mat a nucleus could be type N, T, Dp, or Df. Compare with footnote d.

All 15 conidial derivatives were self-sterile, but PCR showed that 14 were homokaryotic for mat A or mat a, and one was a [mat a + mat A] heterokaryon. Because the 5(619B7] strain was self-fertile, the self-sterility of the heterokaryotic conidial derivative can be attributed to homozygosity for a secondary mutation.

Only one self-sterile conidial derivative was obtained. It was mat A and its DNA amplified the A, B, and C junction fragments of T(B362), thus establishing it as type T. The mat a nucleus could be type N, T, Dp, or Df. Compare with footnotes d and f.
produce any 8:0 or 6:2 asci, and most ascospores from these crosses (~90 and 70%, respectively) were white and inviable (Table 4). However, the crosses T(IBj5)Nc A × 85 A and T(B362i)Nc A × 85 A produced a few eight-spored asci, and in them the 8:0 and 4:4 frequencies appeared to be comparable (Table 4).

Results summarized in Table 5 show that the crosses of T(EB4)Nc and T(IBj5)Nc with T(UK14-1)Nc and T(B362i)Nc produced many four-spored asci, consistent with the conclusion that the TN strains behave like bona fide N. tetrasperma strains. However, the crosses also produced many non-four spored asci, probably because the TN strains still retain a significant fraction of the ancestral non-85 genetic background (N. crassa T or C4T4 a). All ascospores produced in the cross T (IBj5)Nc a × T (B362i)Nc were white and inviable. A model to explain this result is considered in the Discussion section.

**RIP in a Dp-heterozygous N. tetrasperma cross**

In *N. crassa*, crosses involving Dp strains can generate RIP-induced mutant progeny (Perkins et al. 1997). Therefore, we expected crosses of Dp strains in *N. tetrasperma* also to yield RIP-induced mutants. Dp(EB4) duplicates the ad-7 (adenine requiring-7) gene (Perkins 1997). Ascospores from Dp(EB4) a × E A were germinated on adenine-supplemented Vogel’s FGS medium, 130 germings were picked to adenine-supplemented Vogel’s glucose medium, and then their growth was tested on unsupplemented Vogel’s glucose medium. Three adenine-requiring auxotrophic strains were identified among 125 progeny examined. Two (RIP1 and RIP2) were N-type homokaryons; their ad-7 locus, derived from strain 85 A, was found to be altered by several RIP mutations (G:C to A:T transitions) (Table 6).

The third auxotrophic strain (RIP3) was a heterokaryon for mating type, showing that E has incomplete penetrance. Both the mat A and mat a nuclei of this heterokaryon must contain RIP-induced mutant ad-7 alleles, and its genotype potentially can be [Dp + N], [Dp + Dp], or [N + N], with the latter two resulting from second-division segregation. Both copies of the 145-kb duplicated segment that participated in RIP are “captured” in the [Dp + N] and [Dp + Dp] types. The T recipient chromosome has *N. crassa*-derived sequences, whereas the *N. tetrasperma* homolog of the T donor chromosome has 85 A-derived sequences; therefore, their ad-7 mutant alleles, RIP3C and RIP3T, are distinguishable. Both RIP3C and RIP3T showed evidence of RIP (Table 6). Interestingly, all the RIP3T mutations were G to A changes, whereas those in RIP3C were C to T changes.

**Table 3** Ascospore productivity in crosses of *T* strains with OR and 85

| Strain | Productivity in Cross | OR | 85 |
|--------|-----------------------|----|----|
| T(EB4)Nc | ++++ | — |
| T(IBj5)Nc | ++++ | — |
| T(UK14-1)Nc | ++++ | — |
| T(B362i)Nc | ++++ | — |
| T(EB4)Nt | — | ++++ |
| T(IBj5)Nt | — | ++++ |
| T(UK14-1)Nt | — | ++++ |
| T(B362i)Nt | — | ++++ |
| OR A | ++++ | — |
| 85 A | ++++ | — |
| C4T4 a | ++++ | ++ |

Ascospore yield: – <100; 100 < ++ < 500; 500 < +++ < 5000; ++++ > 5000.

a: T<sup>N</sup> are *N. crassa* T strains and T<sup>Nt</sup> are the translocation breakpoint-bearing homokaryons derived from self-fertile heterokaryons.

**Crosses involving C4T4 a and 85 A/a show relatively weak MSUD**

Ramakrishnan et al. (2011) classified wild-isolated *N. crassa* strains into three types based on the strength of MSUD in their crosses with tester strains, namely, “OR” (which includes the standard laboratory strains OR A and OR a), “Sad” (which includes the semi-dominant Sad suppressors of MSUD obtained in the OR background), and “Esm.” MSUD was strongest in crosses with the OR type, of intermediate strength in crosses with the Esm type, and weakest in crosses with the Sad type. We performed crosses of C4T4 a with the MSUD tester strains. The results summarized in Figure S1 show that, like the Esm-type strains, C4T4 a also partially suppresses MSUD. The Esm phenotype of C4T4 a must have come from its 343.6 A E ancestor.

In *N. crassa*, crosses of Dp(EB4) and Dp(IBj5) strains with the OR-type strains were barren, whereas their crosses with the Sad-type strains were fertile (Ramakrishnan et al. 2011). Most Esm-type strains gave a fertile cross with Dp(EB4) and gave a barren cross with Dp(IBj5). Some Esm-type strains gave barren crosses with both the Dps, and fewer still gave fertile crosses with both the Dps. Other results of Ramakrishnan et al. (2011) suggested that *N. tetrasperma* strain 85 is the Esm type. We crossed the *N. tetrasperma* Dp(EB4) and Dp(IBj5) strains with opposite mating-type derivatives of strain 85 and found the crosses were as productive as crosses of T(EB4) and T(IBj5) with strain 85 (data not shown). This is consistent with the classification of strain 85 as the Esm type or the Sad type.

**DISCUSSION**

We have made self-fertile heterokaryotic [T + N] and [Dp + Df] Neurospora strains by introgressing translocations from *N. crassa* into *N. tetrasperma*. Both types of heterokaryons when self-crossed again produced [T + N] and [Dp + Df] progeny. [T + N] and [Dp + Df] mycelia were distinguishable because the former yielded self-sterile heterokaryotic conidal derivatives of both mating types, whereas the latter produced homokaryons of only the mating type of the Dp component. The introgressions depended on the unambiguous identification, by PCR with breakpoint junction-specific primers, of the translocation (T) progeny from each round of crosses, as represented by the bent arrows in Figure 2. In 1984, N. B. Raju crossed a *N. crassa* rearrangement strain with the giant-spore *Banana* (Bani) mutant with the hope that he could rescue the [Dp + Df] heterokaryon. He had previously used this approach to rescue Sk<sup>b</sup> nuclei from a Sk<sup>b</sup> × Sk<sup>b</sup> cross in [Sk<sup>b</sup> × Sk<sup>b</sup>] heterokaryotic ascospores (Raju 1979); however, analysis of progeny nuclei in the mixed cultures from the later experiment was not easy, and his efforts were inconclusive (N. B. Raju, personal communication). D. D. Perkins also alluded to his obtaining [Dp + Df] heterokaryons (Perkins 1997), but his results remained unpublished. Therefore, to the best of our knowledge, this is the first report of heterokaryons with complementary duplications and deficiencies in their constituent nuclei.

The introgressions were facilitated by use of the C4T4 a bridging strain (Perkins 1991). We found that C4T4 a is a moderate suppressor of MSUD (Supporting Information, Figure S1). Shiu et al. (2001) had shown that the *N. crassa* MSUD suppressor Sad-1 can partially breach the *N. crassa/N. tetrasperma* interspecies barrier. Although C4T4 a does not suppress MSUD as strongly as Sad-1 a, its moderate suppressor phenotype might contribute to increase ascospore production in crosses with both *N. crassa* and *N. tetrasperma* strains. The *N. crassa* T strains were first crossed with C4T4 a, and T A progeny from this cross were crossed with C4T4 a. T progeny from the latter crosses were used to initiate two or three additional rounds of crosses with opposite mating-type derivatives of *N. tetrasperma* strain 85. Although this
sequence of introgression crosses can be indefinitely continued, we stopped once they began yielding self-fertile progeny. Namely, one nucleus in the B7, E1, I1, I2, I3, I4, and U9 self-fertile progeny of Figure 1 derives one-eighth or one-fourth of its genome from the non-
85
nucleus of the B7, E1, I1, I2, I3, I4, and U9 self-fertile progeny of Figure 1. The eight-spored ascus types in IT × N crosses in N. crassa, and now among the eight-spored ascus from IT × N crosses in N. tetrasperma, arise, respectively, from alternate and adjacent 1 segregation. The 6:2 ascus arise from crossing-over in either of the interstitial regions between centromeres and breakpoints, followed by alternate or adjacent 1 segregation (Perkins 1997). The eight-spored ascus from T(IB5)Nt A × 85 a and T(B362)Nt A × 85 a included 8:0, 6:2, and 4:4 types (Table 4), and showed that both alternate and adjacent 1 segregation occur at comparable frequencies. Therefore, the absence of 8:0 and 6:2 ascus from T(IB5)Nt A × E A and T(B362)Nt A × E A was quite surprising (Table 4). In these crosses, the viable ascospores came only from the 4:4 and 2:6 ascus. Because the genome of both the C474a and E strains is partly derived from the N. tetrasperma 343.6 A strain (Myllyharju and Ahlgren 1969), we suggest that the T(IB5)Nt A × E A and T(B362)Nt A × E A ascus types may have become homozygous for a 343.6 A-derived recessive mutation that specifically affects alternate segregation, whereas the mutation may be heterozygous in the crosses T(IB5)Nt A × E A and T(B362)Nt A × E A. To our best knowledge, such a mutant phenotype has not previously been reported. It is not detectable in an N × N cross, and an RT × N cross in the mutant background would produce only inviable ascospores. Given the possibility that T(IB5) might be a complex rearrangement that includes a (I ; IV) RT (see Materials and Methods), homozygosity for the mutation can explain why T(IB5)Nt A × T(B362)Nt A yielded inviable ascospores (Table 5). In principle, it should be possible by genome sequencing to identify a 343.6 A-derived mutation present in T(IB5)Nt A, T(B362)Nt A, and E, but absent in T(E4)Nt A or T(UK14-1)Nt A.

The Dp(EB4)-borne ad-7 gene was alterable by RIP in a Dp(EB4) × E cross. Our results suggest that when a duplicated segment undergoes a single round of RIP, one copy of it suffers G to A mutations, whereas

### Table 4 Ascus (octets) types from T × N crosses in N. crassa (Nc) and N. tetrasperma (Nt)

| Cross          | Octets (N) | 8:0 | 6:2 | 4:4 | 2:6 | 0:8 |
|----------------|------------|-----|-----|-----|-----|-----|
| T Nc × OR a   | T (EB4)Nc A | 56  | 56  | 5   | 36  | 3   | 0   |
|                | T (IB5)Nc A | 72  | 31  | 43  | 19  | 6   | 1   |
|                | T (UK14-1)Nc A | 86  | 21  | 46  | 24  | 7   | 4   |
|                | T (B362)Nc A | 83  | 55  | 13  | 32  | 0   | 0   |

| T Nt × E       | T (EB4)Nt a | 96  | 32  | 29  | 40  | 2   | 0   |
|                | T (IB5)Nt a | 159 | 0   | 0   | 6   | 32  | 67  |
|                | T (IB5)Nt A × 85 a | 275 | 3   | 2   | 1   | 0   | 0   |
|                | T (UK14-1)Nt A | 101 | 47  | 15  | 37  | 1   | 1   |
|                | T (B362)Nt A | 145 | 0   | 0   | 0   | 49  | 19  | 32  |
|                | T (B362)Nt A × 85 a | 124 | 1   | 0   | 0   | 2   | 0   |

Asci (n = 275) were collected on water agar and the fraction of four-, five-, six-, and seven-spored asci, respectively, 58%, 19%, 12%, and 4%. Shown here is the distribution of ascus type among the 6% that were eight-spored.

Asci (n = 124) were collected on water agar and the fraction of four-, five-, six-, and seven-spored asci, respectively, 56%, 25%, 15%, and 2%. Shown here is the distribution of ascus type among the 3% that were eight-spored.

### Table 5 Ascus types from N. tetrasperma T × T crosses

| Cross                      | Asci (N) | 4   | 5   | 6   | 7   | 8:0 | 6:2 | 4:4 | 2:6 | 0:8 |
|----------------------------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| T (EB4)Nt a × T (UK14-1)Nt A | 101      | 47  | 28  | 14  | 9   | 1   | 0   | 2   | 0   | 0   |
| T (EB4)Nt a × T (B362)Nt A   | 118      | 24  | 29  | 20  | 13  | 0   | 1   | 8   | 1   | 4   |
| T (IB5)Nt a × T (UK14-1)Nt A | 144      | 72  | 22  | 4   | 2   | 0   | 0   | 0   | 0   | 0   |
| T (IB5)Nt a × T (B362)Nt A   | 149      | 28  | 33  | 26  | 10  | 0   | 0   | 0   | 3   | 0   |
Table 6 RIP-induced ad-7 mutants from Dp(Eb84) × E

| Nt | Nc | RIPI | RIPE | RIPTd |
|----|----|------|------|-------|
| 85 A | 6 (2) | 69 (42) | 45 (6) | 40 (6) | 11 |
| RIPEC | — | 4 | — | — | — |

Comparison of ad-7 gene sequences from 85 A (N. tetrospora FGSC 1270 K0006652), Nt (N. tetrospora FGSC 2508 sequence ID GL919302, nucleotides 1022253 to 1024228), Nc (N. crassa OR74A FungDB database (fungidb.org) NCU04216, nucleotides S230097 to S240080), and the RIP-induced mutant alleles RIP1 (K0006653), RIP2 (K0006654), RIP3C (KR349720), and RIPE5 (KR349721). The RIP3C sequence corresponds to nucleotides S230097 to S240184 of the Nc sequence and RIPE5 to nucleotides 1022550 to 1024228 of the Nt sequence. Numbers in parenthesis indicate nucleotide differences in the introns, and altered amino acid residues are listed below in bold. Super-scripts indicate accession numbers of sequences determined in this work. 85 A /N4 (4); T40T, S216S, I306I, F475F, G506G, T522T, A858A, G181S, G192S.

self-crosses are not possible once all the Dp(B362) nuclei are lost. One model to account for the putative nuclear division defect of Dp(B362) A nucleus in the [Dp(B362) a + Df(B362) A] hyphae is that introgression of T(B362)-linked chromosome 1 sequences from N. crassa disrupts the intracellular interactions of the N. tetrospora mat A and mut a nuclei that were presumed to have co-evolved to optimize their fitness in [mat A + mat a] heterokaryons (Samils et al. 2014). The mating-type chromosome of N. tetrospora has a recombination (crossing over) block over most of its length that ensures that ascospores get one nucleus of each mating type (Gallegos et al. 2000). The block has been correlated with large inversions on the mat A chromosome relative to the mat a chromosome, which has a gene arrangement like that of N. crassa (Ellison et al. 2011). The introgression of T(B362) A chromosome 1 sequences might produce a N. tetrospora mat A chromosome with more synteny with the mat a chromosome over most or all of its length, thereby disrupting the interactions between the mat A and mat a nuclei. Another model is that the Df(B362) nuclei might simply lack a gene required for efficient nuclear division and whose null phenotype is not complemented by the wild-type allele in the neighboring Dp(B362) nuclei, that is, a nucleus-limited gene. Studies showing the nucleus-limited nature of DNA damage checkpoint signal in S. cerevisiae (Demeter et al. 2000) are germane to the second model because they demonstrated that two of these nuclei sharing the same cytoplasm, the one with damaged DNA arrests in mitosis without impedting progression through mitosis of the other with undamaged DNA. If the “disruption of co-evolved interactions” model is correct, then we might expect to see the same or similar phenotype following the introgression of another N. crassa IT whose recipient chromosome is chromosome 1, but if the “deletion of nucleus-limited gene” model is correct, then this phenotype might be T(B362) specific.

Introggression of additional N. crassa translocations into N. tetrospora holds out the prospect of uncovering more genotypes with nucleus-limited effects. Alternatively, N. tetrospora can now be transformed (Kasbekar 2015); therefore, a quicker way to screen for nucleus-limited genes might be to engineer targeted integration of yeast Prf sites into the different chromosome arms, and then use FLP recombinase to induce crossover and produce defined RTs. The [Dp1/D2 + Dp2/D1] and [RT + N] heterokaryons emerging from RT × N crosses would enable us to screen two (or more) DfN in a single experiment.

ACKNOWLEDGMENTS

The authors thank K. Sriethi Reddy for preparing Figure 1, and B. Navitha for technical assistance. D.A.G. was supported by a CSIR-UGC Junior Research Fellowship. D.P.K. holds the Haldane Chair of the Centre for DNA Fingerprinting and Diagnostics (CDFD). This work was supported by a grant from the Department of Science and Technology, Government of India, and by CDFD Core Funds (to D.P.K.).

LITERATURE CITED

Burton, E. G., and R. L. Metzenberg, 1972 Novel mutation causing derepression of several enzymes of sulfur metabolism in Neurospora crassa. J. Bacteriol. 109: 140–151.

Calhoun, F. and H. B. Howe, Jr., 1968 Genetic analysis of eight-spored ascus produced by gene E in Neurospora tetrasperma. Genetics 60: 449–459.

Corcoran, P. D. Jacobson, M. I. Bidartondo, P. C. Hickey, J. F. Kerekes et al., 2012 Quantifying functional heterothallism in the pseudohomothallic ascomycete Neurospora tetrasperma. Fungal Biol. 116: 962–975.

Czaja, W., K. Y. Miller, and B. L. Miller, 2013 Novel sexual-cycle specific gene silencing in Aspergillus nidulans. Genetics 193: 1149–1162.

Davis, R. H., 1960 Adaptation in pantothenate-requiring Neurospora. II. Nuclear competition during adaptation. Am. J. Bot. 47: 648–654.

The other suffers C to T mutations. Because self-crosses of [Dp + Df], but not [T + N], heterokaryons can undergo RIP, the extent to which a translocated segment is altered would depend on the number of adjacent 1 vs. alternate segregations in "ancestral self-crosses." All four [T + N] heterokaryons (i.e., Eb4, Ib5, Uk14-1, and B362) and three of the [Dp + Df] heterokaryons (i.e., Eb4, Ib5, and Uk14-1) produced both self-fertile and self-sterile conidial derivatives; in most cases, the proportion of self-fertile conidial derivatives was >50% (Table 2). The [N a + T(B362) A] and [N a + Dp(B362) A] heterokaryons also produced self-fertile and self-sterile conidial derivatives, although in the [N a + T(B362) A] strains the proportion of self-fertile conidial derivatives was <50%. Therefore, the inability of all seven [Dp(B362) a + Df(B362) A] strains examined to produce any self-fertile conidial derivatives was exceptional. It may be that the Df(B362) A nuclei divide much less efficiently than the Dp(B362) a nuclei; therefore, their fraction rapidly dwindles in the [Dp(B362) a + Df(B362) A] hyphae, making them increasingly less likely to be packaged along with the Dp(B362) a nuclei during conidiation. Even if a few heterokaryotic [Dp(B362) a + Df(B362) A] conidia did form, the Df(B362) A nucleus would be less likely to divide on conidial germination, thus biasing even the heterokaryotic conidia to produce homokaryotic Dp(B362) a germlings. This hypothesis provides an explanation for an otherwise perturbing result that we had obtained in the early stages of this study. A strain was initially scored as [Dp(B362) + Df(B362)], because of its self-fertility and the ability of its DNA to support PCR amplification of all three B362-specific junction fragments. However, later it behaved like a Dp(B362) homokaryon, because it had become self-sterile and its newly isolated DNA failed to amplify the A fragment (D. A. Giri, unpublished results). A self-cross selectively restores the Df(B362) nuclear fraction back to 50%, but
