What Have We Learned by Doing Transformations in *Neurospora tetrasperma*?

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3.1 Introduction to *Neurospora tetrasperma* and Comparison with *N. crassa*

The germination of an ascospore marks the "birth" of a *Neurospora* strain. Ascospores are the end products of a sexual cross between two strains, one of mating type *mat A*, and the other *mat a*. Heat, or chemicals produced by heating the substrate, induces ascospores to germinate and to send out a germ tube that becomes the first hypha. Ascospores also serve as units of dispersal, although dispersal can additionally occur during vegetative growth, via powdery windborne vegetative spores (called conidia), that bud off from the tips of aerial hyphae (Pandit and Maheshwari 1996).

Conidia are spheroid cells containing 2–10 nuclei that can live for up to several weeks, whereas ascospores are more resistant to stress and longer lived (months to years). Under favorable conditions a conidium sends out a germ tube to produce a new hypha. Conidia also function as the paternal fertilizing element during a sexual cross. The maternal element is the protoperithecium, a specialized knot of hyphae that is produced from the vegetative hyphae following nutrient deprivation. Specialized hyphae called trichogynes emanate from the protoperithecia and in response to mating-type-specific sex hormone from conidia of the opposite mating type show chemotropic growth towards the conidia (Bistis 1996). Fertilization of protoperithecia by conidia of the opposite mating type is the prelude to their differentiation into perithecia. Within the perithecia, the dikaryotic ascogenous hyphae undergo several rounds of karyogamy (nuclear fusion) between nuclei of opposite mating types, and the diploid zygote nucleus produced by each nuclear fusion immediately undergoes meiosis in a cell, called the penultimate cell, that then differentiates to become an ascus. The four haploid nuclei from meiosis then undergo a post-meiotic mitosis. In *Neurospora crassa*, the resultant eight nuclei (4 *mat A* + 4 *mat a*) are then partitioned into the eight ascospores that develop within an ascus. Additional mitotic divisions occur within each ascospore and produce more nuclei and the ascospore then matures and becomes dormant. Finally, octets of ascospores produced in each ascus are shot out through the ostiole, an aperture at the top of the perithecium. All the nuclei in a strain (mycelium) produced by germination of an individual ascospore are of the same genotype (i.e., the mycelium is homokaryotic), therefore to complete the sexual cycle mycelium from an ascospore of the opposite mating type is needed.

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Since the products of two ascospores are required for completion of the sexual cycle, *N. crassa* is designated as a heterothallic species.

In contrast, in *N. tetrasperma* the eight haploid nuclei produced by meiosis and post-meiotic mitosis are packaged as four non-sister pairs (1 *mat A* + 1 *mat a*) into each of four ascospores that form per ascus. Each ascospore thus contains nuclei of both mating types, and the resulting mycelium is heterokaryotic (i.e., having nuclei of more than genotype) and it is competent to complete the sexual cycle without the need for a mycelium from another ascospore. Since the sexual cycle can be completed by the mycelium from a single heterokaryotic ascospore, *N. tetrasperma* is a pseudohomothallic species.

A subset of the vegetative conidia produced by a heterokaryotic *N. tetrasperma* mycelium can by chance be homokaryotic, and germination of such conidia can give rise to vegetatively derived single-mating-type strains that are self-sterile. Homokaryotic strains can cross with like strains of opposite mating type. *N. tetrasperma* asci occasionally produce five or more (up to eight) ascospores instead of the normal four by replacement of a dikaryotic ascospore by a pair of homokaryotic ascospores, each homokaryotic ascospore being slightly smaller in size than a dikaryotic ascospore. The mycelium generated from the small ascospores is self-sterile, but it can cross with a homokaryon of the opposite mating type. The dominant *Eight-spore* (*E*) mutation can substantially increase the frequency of replacement of dikaryotic ascospores by pairs of smaller homokaryotic ascospores and a majority of asci from crosses heterozygous for the *E* mutation contain five to eight ascospores. Therefore, *N. tetrasperma* is actually a facultatively heterothallic species.

The webpage http://www.fgsc.net/Neurospora/sectionB2.htm provides excellent figures to explain the differences between the heterothallic, pseudohomothallic, and homothallic lifecycles.

In *N. tetrasperma*, a marker that has undergone second-division segregation can become segregated into both the *mat A* and *mat a* nuclei of a subset of progeny ascospores. A self-cross of the resulting culture would thus be homozygous for the marker. In *N. crassa*, a novel mutation arising in one of the haploid parents of a cross can be made homozygous only in a subsequent cross between f1 segregants of the opposite mating type, whereas in *N. tetrasperma* homozygosity for a newly arisen mutation that recombines with the centromere can be automatically achieved.

3.2 An *ERG-3* Mutant Enables Transformation of *N. tetrasperma*

Transformation experiments in *N. crassa* for the most part use the bacterial hygromycin (*hph*) gene as a selectable marker and select for transformants on medium supplemented with the antibiotic hygromycin. However, this protocol did not work in *N. tetrasperma* because it was found to be naturally resistant to hygromycin. We discovered that *ergosterol-3* (*erg-3*) mutations increased hygromycin-sensitivity in *N. crassa* (Bhat et al. 2004), which led us to surmise that *erg-3* mutations might also increase hygromycin-sensitivity in *N. tetrasperma*. The *erg-3* gene encodes the enzyme sterol C-14 reductase, which is essential for ergosterol biosynthesis. We crossed a strain bearing an *erg-3* mutation in the *N. crassa* gene with the *N. crassa* / *N. tetrasperma* hybrid strain C4, T4 (Metzenberg and Ahlgren 1969; Perkins 1991), and then used an *erg-3* mutant segregant to initiate a series of backcrosses with the *N. tetrasperma* reference strains *85 A* or *a*. We anticipated that an *erg-3* strain of *N. tetrasperma* would possess dual mating specificities; that is, it would be capable of crossing with both *85 A* and *85 a*; however, it would be self-sterile, since *erg-3* strains of *N. crassa* 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 was designated Te-4 and adopted as the reference *N. tetrasperma erg-3* strain. UV spectroscopy confirmed the absence of ergosterol from Te-4, and we confirmed that Te-4 was self-sterile but

that it could cross with both *85 A* and *a*. The wild-type and Te-4 differed strikingly in their sensitivity to hygromycin. Strain *85* conidia could grow in the presence of as much as 220 μg/mL of hygromycin whereas Te-4 conidia were sensitive, and the sensitivity phenotype segregated with the *erg-3* mutation in crosses. The hygromycin-sensitive phenotype made it feasible to use the Te-4 strain to select for transformants on hygromycin medium by complementation with the *hph* gene (Bhat et al. 2004).

3.3 Screening for RIP-Defective Mutants in *N. tetrasperma*

The genome defense process called repeat-induced point mutation (RIP) occurs during a sexual cross, in the haploid nuclei of the premeiotic dikaryon, and subjects duplicated DNA sequences to G:C to A:T mutations and cytosine methylation (Selker 1990). Only a few recessive RIP-defective mutants have been reported and they were identified by a "candidate gene" approach in *N. crassa* (Freitag et al. 2002). Isolation of additional mutants would define additional genes required for RIP. The difficulty of achieving homozygosity for unknown mutations affecting a diplophase-specific process such as RIP makes it impractical to use *N. crassa* to screen for such mutations, but such screens are feasible, at least in principle, in *N. tetrasperma* wherein a novel mutation can automatically become homozygous via second-division segregation. Our approach was to create a tagged duplication of the *erg-3* gene by transformation. In a sexual cross the duplication would target RIP to the endogenous *erg-3* gene. Ascospores bearing *erg-3* mutations produce colonies with a distinct morphology on Vogel's-sorbose agar medium, thereby allowing RIP efficiency to be determined by simply counting the number of wild-type and mutant progeny colonies under a dissection microscope. RIP in self-crosses of a *N. tetrasperma* strain duplicated for *erg-3* sequences would produce mutations in *erg-3*, and following this, crossing over between the centromere and the *erg-3* mutation results in a fraction of the ascospores becoming homoallelic for the mutation. Alternatively, some of the small ascospores might be homokaryotic for the mutation. In either case, if a self-cross failed to produce any *erg-3* mutant progeny, it signaled a potential homoallelism for a novel mutation conferring a RIP defect. We used transformation of Te-4 to construct self-fertile strains that contained a mutant *erg-3* allele at the endogenous locus and were homoallelic for an ectopic *erg-3*$^{+}$ transgene. RIP-induced *erg-3* mutant ascospores were generated in self-crosses of these strains at frequencies in the 2–20 % range, and they produced colonies with the mutant morphology on Vogel's sorbose agar. By screening for self-crosses that failed to produce *erg-3* mutant progeny, presumably due to homozygosity for novel recessive RIP-deficient mutations, we isolated a UV-induced mutant with a putative partial RIP defect (Bhat et al. 2004).

We also performed co-transformations of Te-4 with the *hph* gene together with PCR-amplified DNA of other genes to construct strains duplicated for the amplified DNA. In this way, we isolated RIP-induced mutants in *rid-1* and *sad-1*, which are essential genes, respectively, for RIP and another genome defense mechanism called meiotic silencing by unpaired DNA (Bhat et al. 2004).

3.4 Meiotic Silencing by Unpaired DNA in *N. tetrasperma*

Meiotic silencing by unpaired DNA (also MSUD, or simply, meiotic silencing) is a gene silencing mechanism discovered in *N. crassa* that is presumed to employ RNAi to eliminate the transcripts of any gene that does not pair properly in meiosis with a homolog in the same chromosomal position (Aramayo and Metzenberg 1996; Shiu et al. 2001, 2006). Meiotic silencing can be assayed by using tester strains such as ::*Bml*^{r} and ::*mei-3* that contain an extra copy of the β−tubulin, or *mei-3* (RAD51 ortholog) gene inserted ectopically into the *his-3* locus on chromosome 1. In the cross of a tester with a standard laboratory OR strain of opposite mating type, the ectopically

duplicated gene lacks a homolog to pair with, and therefore it silences itself as well as its endogenous copies, regardless of the latter being paired. Since the gene product is essential for ascus development, the silencing results in an ascus-development defect and reduced ascospore production (Raju et al. 2007; Kasbekar et al. 2011). In a *tester A* × *tester a* homozygous cross the ectopic gene is paired, and consequently it is not silenced, and the cross shows normal ascus development and ascus production. Semi-dominant *Sad-1*, *Sad-2*, and *Sms-2*, and other mutations suppress meiotic silencing, presumably by disrupting the normal pairing of their wild-type alleles (i.e., *sad-1*$^+$, *sad-2*$^+$, *sms-2*$^+$, etc), and induce the latter to silence themselves. Crosses of the testers with the *Sad-1* and *Sad-2* suppressors also show normal ascus development (Raju et al. 2007; Kasbekar et al. 2011).

When 80 wild-isolated *N. crassa* strains were examined by crossing them with the::*Bml*^{r} and ::*mei-3* testers, only eight behaved like OR and showed silencing in crosses with both the testers (Ramakrishnan et al. 2011). These eight strains were designated the "OR" type. Four wild strains showed suppression of meiotic silencing of the *bml* and *mei-3* genes, and typified the "Sad type". Crosses with the 68 other wild strains showed suppression of silencing in *mei-3*, but not *bml*, and additional results suggested that in crosses of these strains, the silencing is greater in perithecia produced early in the cross relative to that in the perithecia produced later in the cross. We designated them an intermediate "Esm type" (for early silencing in meiosis). We hypothesized that the Sad or Esm phenotype arises from heterozygosity for sequence polymorphism in the cross with the OR-derived testers. The polymorphisms might reduce pairing and thereby silence meiotic silencing genes. These results prompted us to ask whether *N. tetrasperma* is of Sad or Esm type, since earlier results of Jacobson et al. (2008) had already suggested that meiotic silencing is greatly reduced or absent in *N. tetrasperma*. Jacobson et al. (2008) had hypothesized that structural differences between the mating-type chromosomes [chromosome 1 which bears the *mat* idiomorphs] might result in a substantial region to remain unsynapsed during normal meiosis and thus cause self-silencing of the *sad-1* gene.

We used the *asm-1* (*ascus maturation-1*) gene to study whether self-crosses in strain 85 were *Esm* or *Sad* type. The *asm-1* gene extends from nucleotide 3,977,115 to 3,980,557 of chromosome 5 and encodes a key regulator of sexual development. In *N. crassa*, a cross heterozygous for a deletion mutant (i.e., *asm-1*$^+$ × Δ*Asm-1*) silences the *asm-1* gene and a large majority of the asci contain ascospores that remain white, immature, and inviable (Aramayo and Metzenberg 1996). We amplified a 1,724 bp segment (nucleotides 3,977,199–3,979,922) of *N. crassa asm-1* by PCR, then purified it by gel electrophoresis and gel elution, and cloned it into the vector pCSN43 which carries the bacterial *hph* gene (Staben et al. 1989). The resulting plasmid was transformed into the Te-4 strain and transformants were selected on Vogel's-sorbose agar medium supplemented with hygromycin. One transformant was crossed with the single-mating-type *85 A* strain and self-fertile progeny (i.e., containing both *mat A* and *mat a* nuclei), were identified. Southern analysis identified six strains that also contained the *Dp*(*asm*) transgene. If we disregard second-division segregation, self-crosses of these progeny are expected to be heterozygous for the transgene. A large proportion of asci from self-crosses of these progeny were white-spored, whereas white-spored asci were never seen from self-crosses of the 85 strain. By analogy with *N. crassa*, we presume that during meiosis *Dp*(*asm*) remains unpaired and this triggers *asm-1* silencing causing all the four ascospores of the ascus to remain white, immature, and inviable. The proportion of white-spored asci was greater (range 31–76 %) among the early tetrads and then showed a decline (range 4–33 %) after about a day. These results suggested that self-crosses in the strain 85 background are of the Esm type.

Additionally, we crossed the initial *Dp*(*asm*) transformant with the *E A* strain. As mentioned above, the *E* mutation increases the proportion of small homokaryotic ascospores. We identified two self-sterile progeny that presumably were of single-mating type, and that by Southern analysis were verified to contain the *Dp*(*asm*) transgene.

The two homokaryotic *Dp*(*asm*) progeny were of opposite mating type therefore we could perform a cross that was homozygous for the *Dp*(*asm*) transgene. Only three of the first 15 asci examined from this cross were white-spored, but all the subsequent asci (of >100 examined) were black-spored. These results were consistent with our expectation that the endogenous *asm-1* locus is not subject to meiotic silencing in crosses homozygous for the *Dp*(*asm*) transgene.

3.5 Conclusions and Future Prospects

The foregoing account documents our ability to successfully transform *N. tetrasperma* and use RIP to induce mutations in any gene. This ability opens up the prospect for quickly bringing the insights developed in *N. crassa* into *N. tetrasperma* to take advantage of the latter's pseudohomothallic life cycle. For example, it is now possible to generate RIP-induced mutation in the *mus-51*, *mus-52*, and *mus-53* genes and thereby disable integration of transforming DNA via nonhomologous end joining (NHEJ). In such mutants integration of transforming DNA would occur only by homologous recombination (Ninomiya et al. 2004). NHEJ-defective strains have been used to systematically knockout individual *N. crassa* genes (Dunlap et al. 2007). While the Fungal Genetics Stock Center (FGSC, USA) collection includes strains carrying knock-out mutations in genes involved in NHEJ for diverse species including *N. crassa*, *Magnaporthe grisea*, *Aspergillus nidulans*, *A. niger*, *A. flavus*, and *A. fumigatus*, it does not include a *N. tetrasperma* strain for targeted transformation. NHEJ-defective *N. tetrasperma* strains could be used to make knock-out mutants in *N. tetrasperma*. Indeed, given the high sequence homology between most genes in the two species (>94 %), it might even be possible to knock-out *N. tetrasperma* genes using the same DNA constructs used to make the *N. crassa* knock-out mutants.

NHEJ-defective *N. tetrasperma* strains can also potentially be employed to replace *un-16*$^{+}$ with *un-16*^{ts} to obtain *un-16*^{ts}; *mus* double mutant strains for selection of targeted transformants using the *Magnaporthe* orthologue of *N. crassa un-16*$^{+}$ (ncu01949) as a selective marker. The *un-16*$^{+}$ orthologue complements the "no growth at 37 °C" phenotype of *N. crassa un-16*^{ts} mutants (McCluskey et al. 2007). Insertion of the transforming DNA by homologous recombination will knock-out the target locus, confer temperature-independence to the transformants, and allow their selection at 37 °C. This approach will allow us to use temperature selection in an erg^{+} background instead of hph selection. Development of a selectable marker based on complementation of a temperature sensitive (ts) lethal mutation in *Neurospora* means that transformation can be accomplished while leaving dominant markers such as hygromycin or phosphinothricin resistance for subsequent manipulations. It also avoids the mutagenicity associated with histidine supplementation required for use of the his-targeting system in *N. crassa*. Replacing *un-16*$^{+}$ with *un-16*^{ts} is non-trivial. One approach is to replace the *un-16*$^{+}$ allele in a *mus*; *erg-3* double mutant strain with *erg-3*$^{+}$ and select for *erg-3*$^{+}$ transformants on pisatin-medium. The transformants will include unwanted integrants into *erg-3*. Since *un-16*$^{+}$ is essential for viability, we expect the transformants to be [(*un-16*::*erg-3*$^{+}$) + (*un-16*$^{+}$)] heterokaryons. Next, one could use homologous recombination to replace *un-16*::*erg-3*$^{+}$ with *un-16*^{ts} and select for homokaryotic transformants on nystatin-medium. This strategy makes use of the pisatin-sensitive and nystatin-resistant phenotype of *erg-3* (Grindle 1973, 1974; Papavinasasundaram and Kasbekar 1993).

Another promising area, though not genomic transformation in the strict sense, is the introgression of *N. crassa* translocations into *N. tetrasperma*. The idea is to produce self-fertile [(*T*) + (*N*)] strains whose self-crosses can generate both [(*T*) + (*N*)] and [(*Dp*) + (*Df*)] progeny (Kasbekar 2014; Dev Ashish Giri and Durgadas P. Kasbekar, unpublished results). If the [(*Dp*) + (*Df*)] progeny turn out to be self-sterile, then it might provide the first evidence for the existence of "nucleus-limited" genes required for fertility. A nucleus-limited gene is one in which a wild-type allele

(*WT*) fails to complement a null allele (Δ) in a [(*WT*) + (Δ)] heterokaryon. Such genes have not yet been found, but their existence is predicted based on the putative nucleus-limited phenotype of the *scon*^{c} mutant (Burton and Metzenberg 1972), and the more recently discovered MatIS gene silencing in *A. nidulans* (Czaja et al. 2013).

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