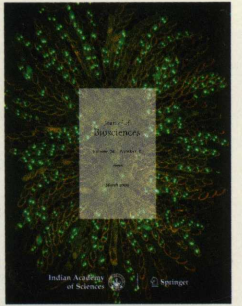

Journal of
Biosciences
Volume 34 Number 1
March 2009
Indian Academy
of Sciences
Springer



Cover: A rosette of maturing asci of *Neurospora crassa* from a wild type x *histone H1-GFP* cross. Histone H1, being a chromosomal protein, allows the GFP-tagged nuclei (two per spore at this stage) to fluoresce in four of the eight ascospores; the remaining four ascospores carry untagged nuclei from the wild type parent. *HH1-GFP* is silenced during meiosis in heterozygous asci and re-expressed in the autonomously developing ascospores. The rosette was dissected out from an eight-day-old perithecium (~0.5 mm diameter). (*See* article by Namboori B Raju, pp 139– 159.)

Edited and Published by N Mukunda for the Indian Academy of Sciences, Bangalore 560 080
Typeset by EMBOSS, Bangalore and printed at Tholasi Prints India, Bangalore

Review

Neurospora as a model fungus for studies in cytogenetics and sexual biology at Stanford[1]

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Dodge's early work (1927–1940) on *Neurospora* genetics and sexual biology inspired Beadle and Tatum at Stanford to use *N. crassa* for their landmark discovery that genes specify enzymes. *Neurospora* has since become a model organism for numerous genetic, cytogenetic, biochemical, molecular and population biology studies. *Neurospora* is haploid in the vegetative phase with a transient diploid sexual phase. Its meiotic cells (asci) are large, allowing easy examination of dividing nuclei and chromosomes under a light microscope. The haploid meiotic products are themselves the sexual progeny that grow into vegetative cultures, thus avoiding the cumbersome testcrosses and complex dominance–recessive relationships, as in diploid organisms. The Perkins' laboratory at Stanford (1949–2007) played a pivotal role in advancing our knowledge of *Neurospora* genetics, sexual biology, cytogenetics and population biology. Since 1974, I have taken advantage of various chromosome-staining methods to examine ascus and ascospore development in wild type and in numerous mutant strains. In addition, I have used GFP-tagged genes to visualize the expression or silencing of unpaired genes in a post-transcriptional gene silencing process (meiotic silencing by unpaired DNA) that operates specifically during meiosis. The genome of *N. crassa* contains over 10 000 protein-coding genes. Gene knockouts or mutations in specific sequences may now be readily correlated with the observed cytological defects in the sexual stage, thus advancing our molecular understanding of complex processes during ascus and ascospore development.

[Raju N B 2009 *Neurospora* as a model fungus for studies in cytogenetics and sexual biology at Stanford; *J. Biosci.* **34** 139–159]

1. Introduction and historical background

Biologists have long adopted a select group of organisms as models for detailed analyses of morphological, developmental, physiological, genetic and evolutionary processes. The filamentous fungus *Neurospora* is one such model. Approximately 10% of all known living organisms are fungi (~250 000 species); they are found in all niches, some in symbiotic association with plants (mycorrhiza) and algae (lichens), many causing serious plant and animal diseases, and most others playing a beneficial role in the decomposition of organic matter and thereby recycling nutrients. Some fungi are used for food (mushrooms, morels), in bread-making and fermentation (*Saccharomyces*), and others in industry for producing antibiotics and secondary metabolites (*Aspergillus* and *Penicillium*). The Perkins

Keywords. Ascus development; ascospore; chromosome rearrangements; *Cochliobolus heterostrophus*; *Coniochaeta tetraspora*; GFP-tagged genes; *histone H1-GFP*; meiotic drive; meiotic silencing; *Neurospora* sexual biology; spore killers

[1]This article is dedicated to the memory of David Perkins (1919–2007) and Robert Metzenberg (1930–2007) for their seminal contributions to *Neurospora* genetics, sexual biology and meiotic silencing.

Abbreviations used: dsRNA, double-stranded RNA; GFP, green fluorescent protein; IT, insertional translocation; MSUD, meiotic silencing by unpaired DNA; NOR, nucleolus organizer region; PTGS, post-transcriptional gene silencing; RdRP, RNA-directed RNA polymerase; RIP, repeat-induced point mutation; RISC, RNA-induced silencing complex; RNAi, RNA interference; RT, reciprocal translocation; siRNA, small interfering RNA; SPB, spindle pole body

laboratory at Stanford (1949–2007) played a pivotal role in the development of *Neurospora* as a model for genetic, cytogenetic, cytological and population biology studies and, more recently, for the molecular analysis of its sexual cycle (*see* Perkins 1992; Alshire and Selker 2007; Raju 2007). The US National Institutes of Health lists *Neurospora* as one of 12 eukaryotic models (organisms having true nuclei) for biomedical research (*http://www.nih.gov/science/models*), and at least one publication touts '*Neurospora*: a model of model microbes' (Davis and Perkins 2002).

Neurospora possesses numerous characteristics that make it a suitable model for genetics and sexual biology: (i) simple nutritional requirements – salts, sugar and biotin; (ii) fast vegetative growth and a 2- to 3-week life cycle; (iii) two distinct mating types for making genetic crosses; (iv) many recognizable stages during sexual development; (v) large asci (meiotic cells) for studying meiotic processes and ascospore (sexual spore) development under a light microscope; (vi) linearly ordered ascospores within the elongated narrow ascus, reflecting the underlying crossover events; (vii) groups of 8 ascospores forcefully ejected from asci allowing easy isolation and tetrad analysis; (viii) differential ascospore pigmentation – dark, viable normal ascospores and unpigmented, inviable deficiency ascospores – allowing easy detection of chromosome rearrangements; and (ix) haploid vegetative phase, and a transient diploid phase limited to the young ascus. The haploid meiotic products (normally thought of as gametes) are themselves the sexual progeny that grow into haploid vegetative cultures. Thus, every meiotic product can be scored directly without cumbersome testcrosses, or complex dominance–recessive relationships, as in diploid organisms.

The first record of *Neurospora* was in 1843, when an orange mould infestation in French bakeries was investigated (*see* Perkins 1992). In the early 1900s, the Dutch plant physiologist FAFC Went experimented with the same fungus in Indonesia and Surinam, where it was commonly used to prepare 'oncham' and an alcoholic beverage from cassava meal. In nature, *Neurospora* is often found on scorched vegetation after wildfires or agricultural burns (figure 1A, B). Profuse orange-coloured masses of *Neurospora* are also found on the sugar-rich filter mud at sugar factories (figure 1C; Rashmi *et al* 2003); honeybees have been observed to visit the orange masses and collect *Neurospora* conidia (asexual spores) in their 'pollen baskets' (Shaw 1998).

Shear and Dodge (1927) discovered the sexual cycle of this orange 'bread mould' and named the fungus *Neurospora* because of its characteristic 'nerve-like' ascospore wall ornamentation (figure 1D). They found two mating types (*mat A* and *mat a*), and described the life histories of two 8-spored outcrossing (heterothallic) species *N. crassa* and *N. sitophila*, and one 4-spored selfing (homothallic, later called pseudohomothallic) species *N. tetrasperma*. Dodge (1927) also pioneered cytological investigations of *Neurospora* and described the nuclear phenomena associated with heterothallism (distinct mating types in different thalli or bodies leading to obligate outcrossing) in the 8-spored species. In addition, he was the first to discover and describe the unique fungal mating system of pseudohomothallism in the 4-spored species, where the mating types are present in separate nuclei as in heterothallics, but single ascospores contain both mating-type nuclei giving rise to self-fertile cultures as in true homothallics (figure 2). Dodge also showed that the linearly ordered ascospore pairs in the elongated asci of the 8-spored species reflect the underlying genetic events during meiosis, thus introducing *Neurospora* to a wider audience. It was Dodge's work on *Neurospora* and its simple nutritional requirements that inspired George Beadle and Edward Tatum of Stanford University to use *N. crassa* for their landmark discovery, that genes specify enzymes (Beadle and Tatum 1941), for which they shared the Nobel Prize in Physiology or Medicine in 1958.

Subsequent cytological studies by McClintock (1945) and Singleton (1953) showed that meiosis and chromosome behaviour in *Neurospora* are very similar to those of higher plants and animals. Perkins' former graduate student Edward Barry was trained in McClintock's aceto-orcein squash method for pachytene chromosome analysis. Barry visited Stanford every summer for collaborative cytogenetic work with Perkins for over 30 years. With these humble beginnings, *Neurospora* has now become a model organism for numerous discoveries on vegetative incompatibility (self/non-self recognition), recombination, gene conversion, intragenic complementation, biological clocks, genome-defence mechanisms, signal transduction, chromosome rearrangements, meiotic drive, post-transcriptional gene silencing and population biology (*see* Perkins 1992; Davis and Perkins 2002). For general information on *Neurospora* genetics and biology, *see* Davis (2000), and Perkins *et al* (2001).

My basic training was in agriculture, and genetics and plant breeding at Banaras Hindu University, Varanasi. However, I became a fungal cytologist by chance in 1968, after entering the University of Guelph, Canada, for graduate studies. My PhD supervisor, Benjamin Lu, had just started working on the mushroom fungus *Coprinus* (= *Coprinopsis*) mainly because of its synchronous meiotic divisions. He also developed an iron-haematoxylin staining procedure, which stains chromosomes, nucleoli, spindles and spindle pole bodies, for observing meiotic divisions in the developing basidia (meiotic cells of Basidiomycota). It became possible to take advantage of both the meiotic synchrony and the iron-haematoxylin staining method to time meiosis in the developing mushrooms of four species of *Coprinus* (Lu and Raju 1970; Raju and Lu 1970).

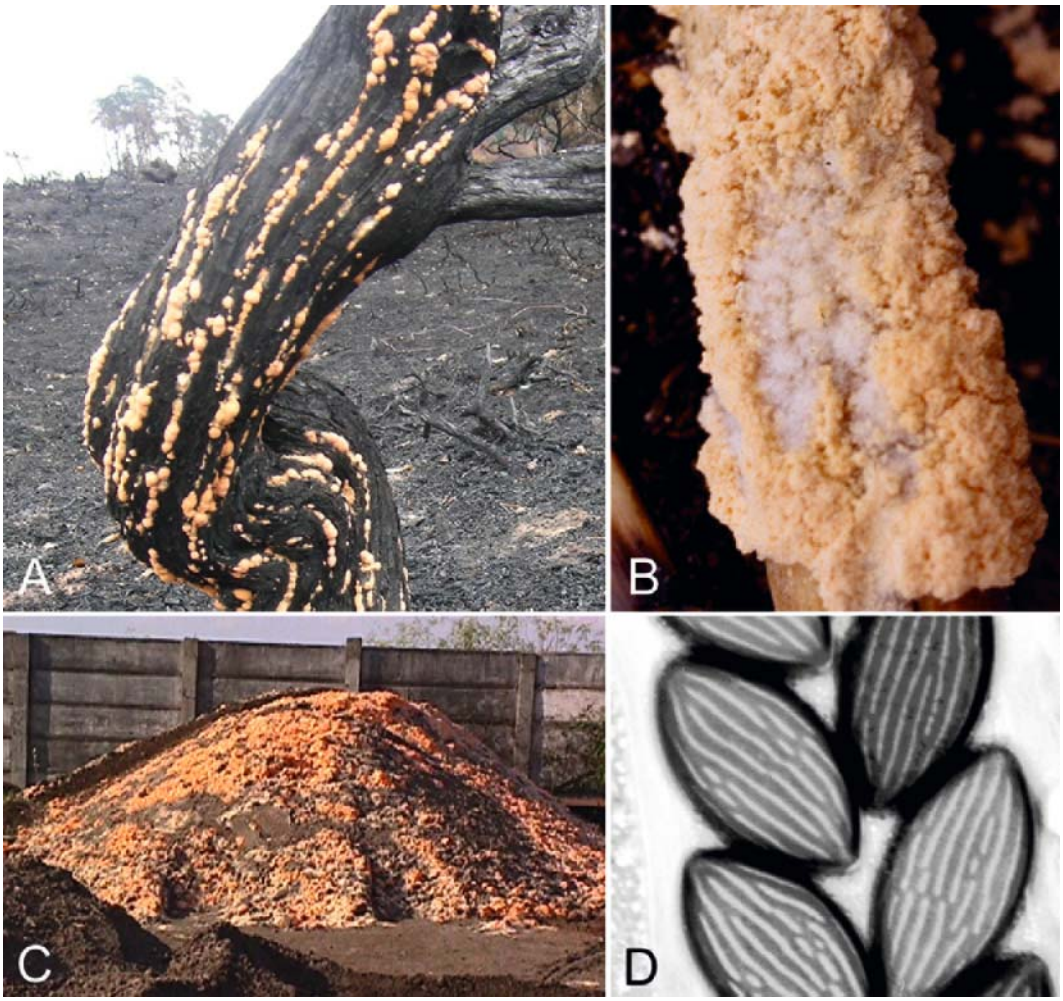

Figure 1. (**A**) *Neurospora* growing on a scorched tree trunk following a forest fire in Surrey, England. (**B**) *Neurospora* growing on a sugarcane stem in Louisiana, USA. (**C**) *Neurospora* blooms on filter mud at a sugar factory in southern India. (**D**) Immature ascospores of *N. crassa* showing nerve-like striations, hence the genus name *Neurospora*. (**A**, courtesy of Martin Bidartondo; **B**, courtesy of David Jacobson; **C**, from Rashmi *et al* 2003, with permission of P Maruthi Mohan)

Beginning in 1974, I adapted the iron-haematoxylin staining method to *Neurospora* and concentrated on studies of its cytogenetics and sexual biology. My more recent work has employed the DNA-specific fluorochrome acriflavin, and green fluorescent protein (GFP)-tagged genes for visualizing meiotic chromosomes, meiotic gene silencing and its suppression. Most of my focus has been on light microscopy studies of ascus and ascospore development relevant to our laboratory's genetic and cytogenetic interests in *Neurospora*. I have previously reviewed the cytogenetic work on *Neurospora* sexual biology using both wild type, and ascus and ascospore mutant strains (Raju 1980, 1992a, 1994). The present paper contains a summary of the first 25 years of my *Neurospora* research and reviews my more recent work on meiotic silencing using GFP-tagged genes.

2. Normal ascus and ascospore development

The best-known *Neurospora* species, *N. crassa*, is heterothallic with two mating types, *mat A* and *mat a*. Haploid strains are hermaphroditic (having both male and female structures) but self-sterile, and the sexual cycle is initiated only when fruiting body initials (protoperithecia) of one mating type are fertilized by cells of a different mating type (Bistis 1981). Fertilized protoperithecia develop into perithecia (sexual fruiting bodies) within which asci (meiotic cells) are formed from successively developing ascogenous croziers (hook-shaped cells) (figure 3A–C). The croziers are three-celled, and the binucleate (*mat A* + *mat a*) subapical cell develops into an ascus. In the young asci, the two haploid nuclei fuse and the resulting diploid zygote nucleus immediately undergoes meiosis (two divisions)

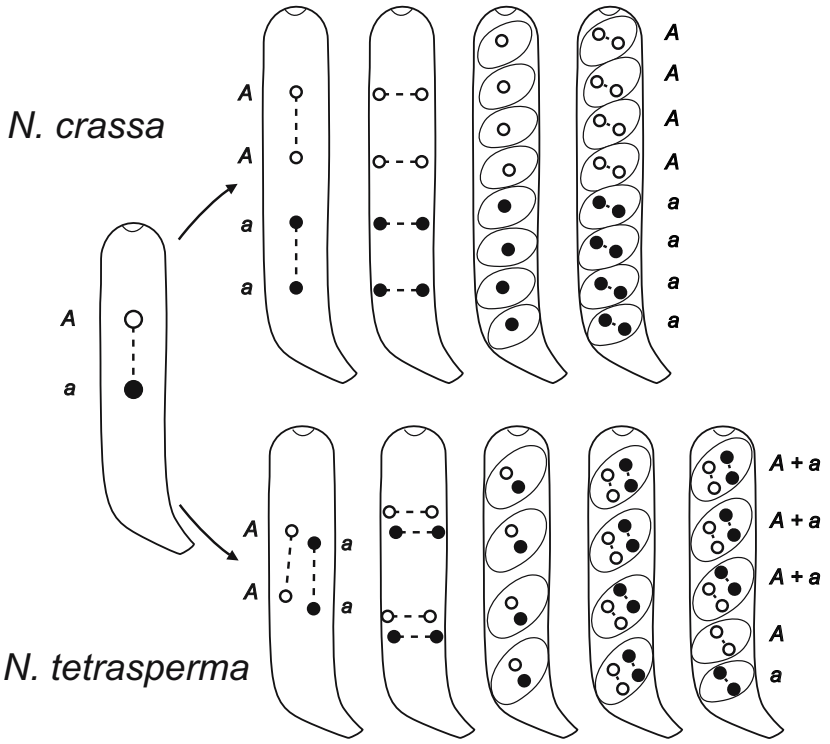

Figure 2. A schematic diagram of ascus development in the heterothallic species *N. crassa*, and in the pseudohomothallic species *N. tetrasperma*. Mating types (*mat A* and *mat a*) segregate at the first division of meiosis in both species. In *N. crassa*, the second-division spindles are aligned in tandem, and the four spindles at the third division (mitosis) are aligned equidistant and across the ascus. Ascospores are uninucleate at inception, 4 *mat A* and 4 *mat a*. In *N. tetrasperma*, the second- and third-division spindles overlap, and each of the 4 ascospores encloses two nuclei of opposite mating type (from Jacobson *et al* 2008). The photographic images in figures 3, 4, 7–9 are mostly from *N. crassa*, except where stated otherwise.

and a postmeiotic mitosis. There is no free-living diploid phase in *Neurospora*. The ascus is the largest cell (20 × 200 μm) in the entire life cycle of *Neurospora*; nuclei, chromosomes, spindle and the associated organelles are also correspondingly large and clearly seen with the light microscope. Meiosis and a postmeiotic mitosis occur in the common cytoplasm of the ascus prior to ascospore delimitation. Each perithecium (~400 x 500 μm) produces 200–400 asci, and each ascus produces 8 linearly ordered haploid ascospores (~15 x 30 μm). The nuclear divisions and other processes in the developing asci and ascospores are summarized below (*see* Raju 1980, 1992a for details and references).

Premeiotic DNA replication is known to occur in the crozier or in the ascus initial prior to karyogamy, fusion of the two haploid nuclei. Chromosomes are short at karyogamy as they soon begin to pair, but they elongate throughout the zygotene and pachytene stages (7–18 μm long; figure 3C–E). This is in contrast to the chromosome behaviour in *Coprinus*, where the chromosomes are fully extended at karyogamy (Lu and Raju 1970; Raju and Lu 1970; Raju 1980). The paired and extended pachytene chromosomes in *Neurospora* appear as railroad tracks (figure 3D). Following a diffuse diplotene stage, chromosomes condense and segregate in a manner similar to higher eukaryotes (figure 3F–H; Raju 1980, 1986a). The spindles at the two meiotic divisions are oriented longitudinally and in tandem at the second division, parallel to the ascus wall (figure 3G, H). A second round of DNA replication occurs during a prolonged interphase II, following meiosis, in preparation for the postmeiotic mitosis. It is during this interphase that the ascospore-delimiting double membranes are formed around the ascus cytoplasm, and spindle pole bodies (SPBs) duplicate and form greatly enlarged outer plaques (figure 3J). The SPB plaques separate and migrate to opposite sides of the ascus to form transverse spindles during the postmeiotic mitosis (figure 3K). The four pairs of sister nuclei, which are initially on opposite sides of the ascus, realign in single file, with the sister nuclei located adjacent to one another, and all 8 SPB plaques facing the same side of the ascus (figure 3L). Preformed ascospore wall membranes invaginate around individual nuclei and cut out 8 uninucleate ascospores (*see* Raju 1980; Read and Beckett 1996). Actin microfilaments and microtubules emanating from the SPB plaques are

shown to play a major role in the realignment of nuclei and ascospore delimitation (Thompson-Coffe and Zickler 1992, 1993, 1994).

A second postmeiotic mitosis occurs in the young ascospores soon after they are delimited, and several additional mitoses occur in the mature, black ascospores (figure 3M, N, P). The multinucleate condition is clearly visible in the unpigmented ascospores of the perithecial colour mutant *per-1,* and with GFP-tagged histone H1 (figures 3P, 8E) (Freitag *et al* 2004; Raju 1980). Nuclear divisions within an ascus and in ascospores of each ascus are generally synchronous (*see* figure 3H, K, N). In contrast, there is no such synchrony of mitoses within the multinucleate vegetative cells. The vegetative nuclei of *Neurospora* are very small (~2 μm in diameter); they divide asynchronously, and the chromosomes during mitosis are too small for detailed observations using the light microscope. I made a serious attempt to study mitosis in *N. crassa* soon after learning that the vegetative spores (macroconidia) and their nuclei can be enlarged several-fold in liquid medium

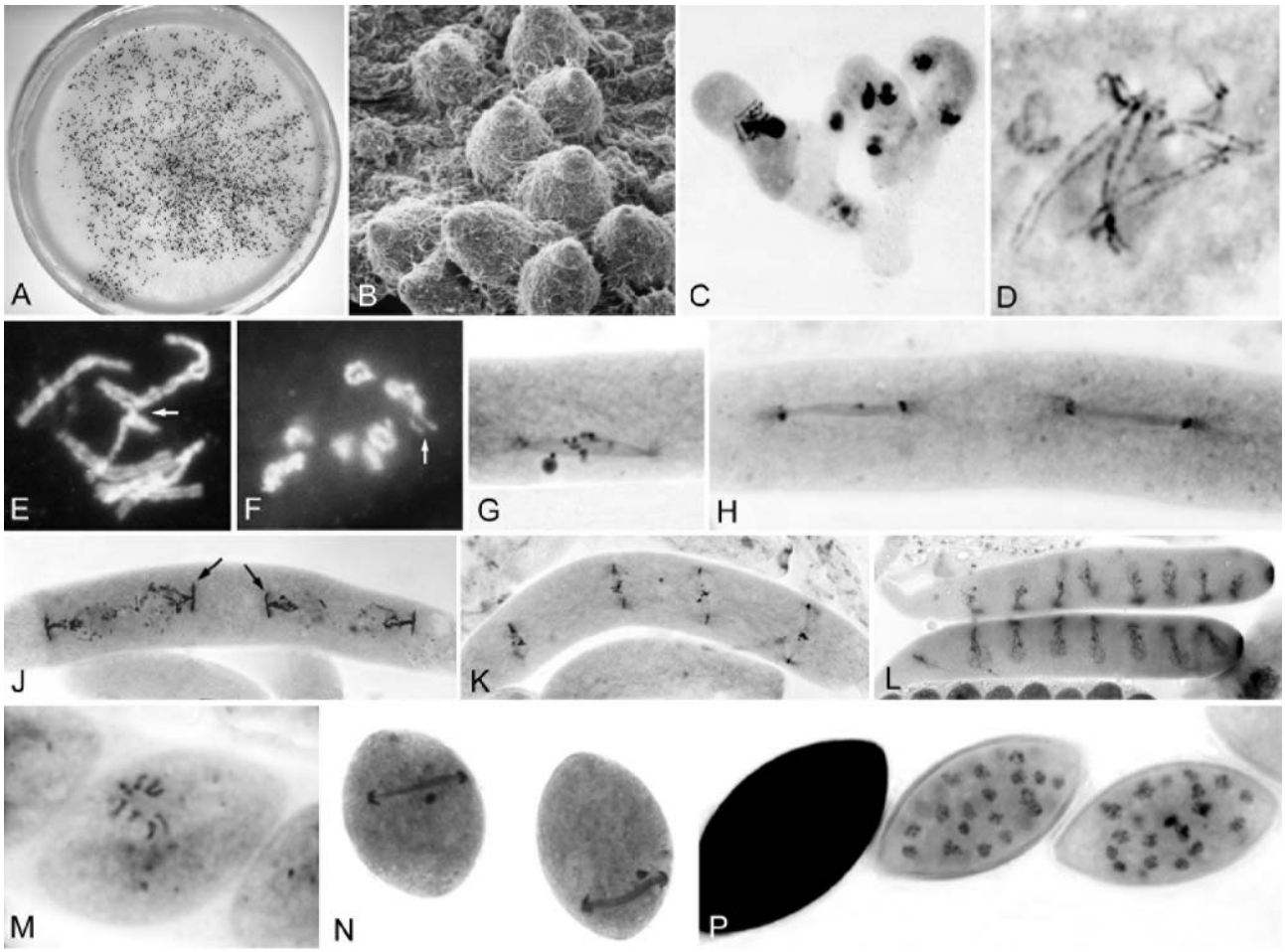

Figure 3. Sexual development and ascospore genesis in *N. crassa*. Haematoxylin staining except where noted otherwise. (**A**) Perithecia in a 9 cm plate. (**B**) Scanning electron micrograph of perithecia at high magnification. (**C**) Croziers and a young ascus. The binucleate cell in the middle crozier develops into an ascus (left). (**D**) Pachytene chromosomes stained with aceto-orcein; the paired chromosomes appear as railroad tracks. (**E**) Pachytene chromosomes from Normal x Reciprocal translocation, showing the chromosome rearrangement (arrow). The attenuated nucleolus organizer strands are discernable under the upper left chromosome (acriflavin staining). (**F**) Seven condensed chromosomes at diakinesis, one chromosome shows attenuated nucleolus organizer strands (arrow; acriflavin staining). (**G**) Metaphase I. (**H**) Telophase II. (**J**) Interphase II following meiosis II. Note the enlarged, duplicated spindle pole body (SPB) plaques (arrows). (**K**) Metaphase III, the four spindles are aligned across the ascus. (**L**) Interphase III asci in the homothallic species *N. terricola*, with all eight nuclei lined up in single file at ascospore delimitation. (**M**) Metaphase IV in a young ascospore, polar view. (**N**) Telophase IV that results in binucleate ascospores; the old nucleolus is in the spindle region (*N. discreta*). (**P**) Wild type x *per-1*. The unpigmented *per-1* ascospores contain ~32 nuclei, following several mitoses. (**B**, courtesy of Matthew Springer; **C**, from Raju and Newmeyer 1977; **D**, from Raju 2008b; **F**, **P**, from Raju 1992a; **H**, **J**, **M**, **N**, from Raju 1980; **L**, from Raju 1978)

containing ethylene glycol (3.22 M; Bates and Wilson 1974). To my delight, I found that the division stages and the mitotic chromosomes in the enlarged nuclei are readily seen with the light microscope. The division stages generally resemble those at the postmeiotic mitoses in the ascus and ascospores, and those in higher eukaryotes (*see* Raju 1984 for photos).

In several homothallic species of *Neurospora*, where there are no mating type distinctions and single ascospore cultures are self-fertile, ascus development and nuclear divisions follow exactly the same course as in the heterothallic *N. crassa*, including the fusion of two haploid nuclei and the formation of linearly ordered ascospores (Raju 1978). The exception is *N. pannonica*, where the immature asci are broad and the young ascospores are not linearly ordered, although mature asci show linearly arranged ascospores (Raju 2002a). A similar behaviour is found in several large-spored species of *Gelasinospora* (Glass *et al* 1990). In the naturally outcrossing *N. crassa*, most of the asci mature normally in crosses between unrelated parents. In contrast, crosses between highly inbred laboratory strains result in a high proportion of ascus abortion following ascospore delimitation, somewhat resembling inbreeding depression in maize. Hence, it is imperative that we use unrelated parents for obtaining images of rosettes of asci for display (Raju *et al* 1987).

N. tetrasperma is a 4-spored pseudohomothallic species (also called secondarily homothallic). Ascus development is reprogrammed so that each of the 4 ascospores encloses two nuclei of opposite mating type; single-ascospore cultures are thus self-fertile (figures 2, 10B). This is accomplished by the complete linkage of the mating-type locus to the centromere (no crossing over), overlapping spindles at the second and third divisions, and precise alignment of non-sister pairs of nuclei for sequestration into 4 ascospores (Dodge 1927; Raju 1992b). A large, genetically determined recombination block is positively correlated with a cytologically detectable, long unpaired region in linkage group I (Gallegos *et al* 2000; Jacobson *et al* 2008).

Of six pseudohomothallic species examined across five genera, only *N. tetrasperma* turned out to have evolved a recombination block in the mating-type chromosome and overlapping spindles at the second division of meiosis. Five other species (*Apiosordaria verruculosa, Coniochaetidium savoryi, Gelasinospora tetrasperma, Podospora anserina* and *P. tetraspora*) have apparently evolved an obligate crossing over proximal to mating type, and tandem spindles at the second division to accomplish the same end result -- self-fertile ascospore progeny. Pseudohomothallic ascus programming may have evolved independently because it shows much variation among different species (Raju and Perkins 1994, 2000). In all six pseudohomothallic species, pairs of non-sister nuclei are sequestered into 4 heterokaryotic ascospores. However, in some asci, pairs of small, single-mating-type homokaryotic ascospores replace one or more large, heterokaryotic ascospores. Such 5–8 spored asci are more common (up to 10%) in highly inbred laboratory strains than in wild-collected strains (1–2%). Several mutant strains produce mostly 5–8-spored asci (e.g. *Eight spore*), and certain wild strains produce all 8-spored asci in outcrosses (Jacobson 1995). The self-sterile small-ascospore progeny, as well as the cultures from the homokaryotic conidia, can outcross. Apparently, *N. tetrasperma* and other pseudohomothallic species have the best of both worlds: self-fertile cultures routinely undergo a sexual cycle without needing a compatible sexual partner, and the self-sterile cultures can outbreed, bringing in a new gene pool (Raju and Perkins 1991, 1994; Raju 1992b).

3. Mutants that affect ascus and ascospore development

Numerous *Neurospora* mutants that affect the sexual phase of the life cycle are known; most have been analysed genetically and cytologically but not molecularly (*see* Raju 1992a). Mating-type mutants, as well as female- and male-sterile mutants (e.g. *fmf-1*), are usually defective in haploid sexual functions or in early perithecial development (Griffiths 1982; Johnson 1979). *fmf-1* is a homologue of *Schizosaccharomyces pombe ste11*, which is a master regulator of sexual development (Iyer *et al* 2009). Many other mutants affect post-fertilization events such as ascus differentiation, karyogamy, meiosis, chromosome pairing and segregation, postmeiotic mitosis, ascospore delimitation and development. Some affect ascus or ascospore morphology, the size and number of ascospores, or ascospore pigmentation and viability. Spore killer strains show meiotic drive, causing the death of ascospores not carrying the spore killer haplotype (a set of alleles of linked genes that are transmitted together) (*see* Raju 1992a, 1994).

Dodge (1934, 1939) studied the first ascus mutants in *N. tetrasperma*, and Srb *et al* (1973) at Cornell University studied similar mutants in *N. crassa*. The first *N. crassa* mutant I examined is *Banana* (*Ban*), which produces giant ascospores that enclose all eight nuclei of the ascus, following meiosis and a postmeiotic mitosis (figure 4A; Raju and Newmeyer 1977). We have shown that meiosis and postmeiotic mitosis are apparently normal, and the four pairs of sister nuclei that are initially on both sides of the ascus fail to line up in single file, or cut out 8 uninucleate ascospores. The giant ascospores mature normally, undergo three or four additional mitoses and become highly multinucleate, and are ejected forcefully just as normal ascospores. The ascospores germinate and give rise to mixed cultures that often contain 2–4 different meiotic progeny nuclei (Raju 1979). *Ban* shows abnormal vegetative morphology and other

pleiotropic effects in the sexual phase (Raju and Newmeyer 1977). I have used the heterokaryotic giant ascospores of *Ban* in studies of spore killer analysis and meiotic silencing (*see later*).

Another less extreme giant-ascospore mutant, *Perforated* (*Prf*), shows multiple apical pores at the ascus apex, rather than a single normal pore (figure 4B, C). *Prf* carries a vegetative lethal, and shows abnormal crozier phenotype (Raju 1987). Two conditional *Four-spore* (*Fsp*) mutants of *N. crassa* are temperature sensitive in the sexual phase and delimit ascospores at the 4-nucleate stage, without undergoing a postmeiotic mitosis. In heterozygous crosses, *Fsp-1* produces mostly 2–4 spored asci at high temperature (>25°C) and *Fsp-2* produces all 4-spored asci at low temperature (15–20°C). However, an intercross of *Fsp-1* x *Fsp-2* produces mostly 4-spored asci and shows no temperature effects. Similarly, the *Fsp-1 Fsp-2* double mutant is temperature independent and produces all 4-spored asci both in heterozygous and homozygous crosses (Raju 1986b).

Several other ascus mutants affect ascus shape (*peak*), ascospore shape (*Round spore*), ascospore pigmentation (*per-1, asco, cys-3*), or ascospore viability. *peak* (*pk*) is colonial in the vegetative phase and produces swollen asci in crosses (*pk* x *pk*). In the swollen asci, meiosis and postmeiotic mitosis are normal but the ascospores are not linearly ordered (figure 4D). The asci fail to form an apical pore and consequently the ascospores are not ejected forcefully out of perithecia (Raju 1988). The interaction of the dominant allele of *peak* (*Pk*) and *Banana* (*Pk* × *Ban*) results in swollen asci that produce single, swollen giant ascospores. *Round spore* (*R)* is ascus dominant, meaning that in heterozygous crosses ($R^+ \times R$), all 8 ascospores are phenotypically round (figure 4E, F). Thus, the round ascospore phenotype is determined by the genotype of the zygote (ascus) rather than the genotype of the individual ascospores. *R* shows abnormal vegetative morphology and is female sterile.

Mature ascospores of *Neurospora* are black, and normally only black ascospores are viable. Ascospores of *asco* (*lysine-5*) and *cys-3* fail to pigment or mature, and are not viable. The images of mature asci of these two mutants have been widely used in textbooks to show the segregation of alleles either at the first division (4 black:4 white ascospores) or at the second division of meiosis (2B:2W:2B:2W or 2:4:2) (figure 4G). The perithecial colour mutant *per-1* is an exception, where the ascospores are unpigmented but are

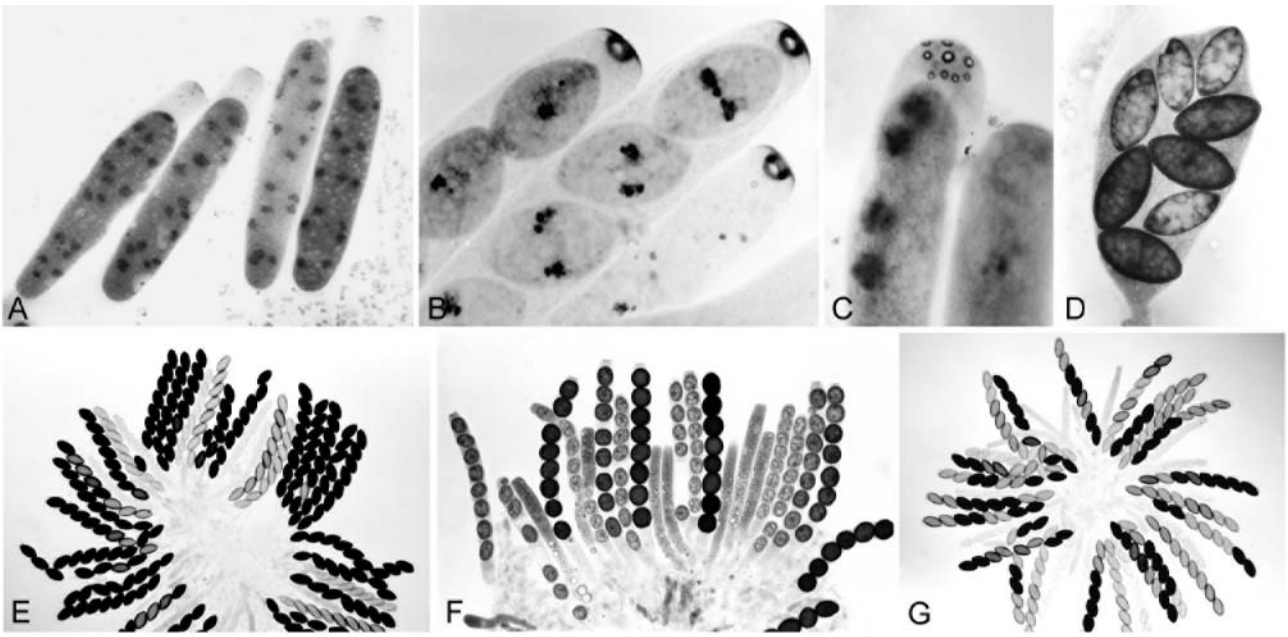

Figure 4. Ascus and ascospore phenotypes in several mutant strains. (**A**) In the giant ascospore mutant *Banana* (wild type x *Ban*), each ascus cuts out a single giant ascospore that encloses all eight nuclei. The ascospores shown here contain 16 nuclei, following a mitosis. (**B**) Normal ascus apical pores in wild-type asci (WT x WT). (**C**) Ascus apex showing multiple pores in the *Perforated* mutant (WT x *Prf*). Ascospores are nevertheless ejected forcefully by rupturing the perforations. (**D**) A swollen ascus showing unordered ascospores of the dominant allele of the mutant *peak* (WT x *Pk*). The swollen asci fail to differentiate apical pores or eject ascospores. (**E**) A rosette of maturing wild-type asci for comparison with mutant asci in **A**, **D**, **F** and **G**. (**F**) Wild type x *Round spore*. All 8 ascospores (*R* as well as R^+) are round, because the *Round spore* mutation is ascus dominant. (**G**) A rosette of maturing asci from wild-type x *cys-3*. Ascospores that received the mutant *cys-3* allele fail to pigment or mature. The linearly ordered ascospores show the segregation of *cys-3* and *cys-3*$^+$ alleles either at the first division (4 black: 4 white) or at the second division (2:2:2:2 or 2:4:2) of meiosis. (**A**, from Raju and Newmeyer 1977; **B**, **C**, from Raju 1987; **D**, **G**, from Raju 2008b; **E**, from Raju 1980; **F**, from Raju 1992a)

fully viable. The unpigmented *per-1* ascospores germinate spontaneously without the need for heat shock; in fact, heat shock kills them. Johnson (1976) used *per-1* to analyse the ontogeny of perithecial wall tissue, and I used it to visualize the multinucleate condition of mature ascospores. When *per-1* is used as the protoperithecial parent, the perithecia are unpigmented, often hyaline or light orange. This behaviour is dramatically shown in Petri plate crosses, where the two parents (e.g. *per-1 mat A* and *per-1*$^{+}$ *mat a*) are inoculated on opposite sides of the plate. Where the two cultures meet at about the midline of the plate, two lines of perithecia are formed with a clear zone devoid of perithecia in the middle; the perithecia on the *per-1* side are colourless and the perithecia on the *per-1*$^{+}$ side are darkly pigmented (*see* Perkins *et al* 2001). The asci in all perithecia on both sides are biparental in origin, however, and contain 4 black and 4 hyaline ascospores. These results clearly show the ontogeny of perithecial wall tissue from the protoperithecial (female) parent. I have used *per-1* in a cross with wild type to observe multiple nuclei (~32) in mature but unpigmented *per-1* ascospores (figure 3P; Raju 1980).

I have also examined many other mutants of *N. crassa* that affect meiosis (e.g. *mei-1*, *mei-2*, *mei-3*), postmeiotic mitosis (*mus-8*), and other sexual phase processes (*see* Raju 1992a; Perkins *et al* 2001 for references). The ascus mutants described above are all laboratory-induced, and we have subsequently shown that many of the wild-collected isolates carry cryptic recessive sexual phase mutations. The isolation and analysis of such recessive mutations, however, requires recombining any putative recessive mutation into the opposite mating type and backcrossing the progeny with the original wild isolate for making the mutation homozygous (Leslie and Raju 1985; Raju and Leslie 1992). In *N. tetrasperma*, the *bud* mutant was isolated from a 4-component heterokaryon (*bud*$^{+}$ *mat A* + *bud*$^{+}$ *mat a* + *bud mat A* + *bud mat a*) collected in Florida. *bud* is recessive and shows abnormal ascospore buds when homozygous (*bud mat A* x *bud mat a*) (Raju and Burk 2004).

4. Chromosome rearrangements

David Perkins pioneered the detection and analysis of chromosome rearrangements in *Neurospora*. He analysed more than 350 rearrangements over a period of 40 years, and consequently *Neurospora* is now the best studied of any fungus for this enquiry (*see* Perkins 1986, 1997; Perkins and Barry 1977). In a major paper describing 135 chromosome rearrangements, he laid out the theoretical basis for the behaviour of reciprocal translocations (RT), in which chromosome segments are exchanged reciprocally, and insertional translocations (IT), in which a segment from a donor chromosome is translocated to a recipient chromosome, but there is no reciprocal exchange (Perkins 1974). One third of the random viable progeny from IT x Normal sequence are duplicated for the inserted chromosome segment. The duplication progeny (*Dp*) are typically barren both in heterozygous and homozygous crosses (i.e. the crosses produce perithecia but their development is blocked in meiosis or ascospore formation, *see below*). Perkins' inquiry did not involve tedious meiotic chromosome analysis (*see* figure 3D–F). Instead, he simply examined under a dissecting microscope unordered groups of 8 ascospores that were ejected from individual asci. This was made possible by the knowledge that the deficiency ascospores fail to pigment and are inviable. In crosses of RT x Normal sequence, equal numbers of asci produced 8 black: 0 white ascospores and 0 black: 8 white ascospores, and any crossovers between the translocation breakpoint and the centromere produced 4 black: 4 white ascospores. In contrast, IT x Normal produced 8B: 0W and 4B: 4W asci in equal numbers and 6B: 2W asci represented crossovers. The frequencies of various ascus types indicate the type of rearrangement and how far the breakpoint is from the centromere (*see* Perkins 1974, 1986, 1997 for details).

I have participated in at least four chromosome rearrangement studies that required detailed cytological examination of nuclei and chromosomes during meiosis, or the arrangement of black and white ascospores in mature intact asci. In the earliest study, nine duplication (*Dp*) strains (from IT x Normal) were examined cytologically in crosses of *Dp* x wild type or *Dp* x *Dp* for recognizable blocks in meiosis or ascospore development (Raju and Perkins 1978). The duplications differed widely in their size and gene content, but all showed characteristic developmental blocks – the majority of them at the crozier or ascus differentiation stage, and others during meiosis in the developing asci. In the second study, 36 RT strains with breakpoints at varying distances from the centromere were examined in crosses of RT x Normal sequence for the arrangement of black and white ascospores in intact asci containing 4B: 4W ascospores. The expectation, based on chromosome segregation, was that such 4B: 4W asci must all result from crossing over in the interstitial region between the breakpoint and the centromere, and that the crossover asci must show 2B: 2W: 2B: 2W or 2B: 4W: 2B (also 2W: 4B: 2W) for the ascospore arrangements. This was generally the case for the RT where breakpoints are far away from the centromere. However, in at least 20 RT strains, a vast majority (>70%) of 4B: 4W asci showed a BBBBWWWW ascospore arrangement. Such asci could have come only from 3:1 segregation of the translocation quadrivalent rather than from crossing over in the interstitial region (Perkins and Raju 1995).

The last two studies examined the behaviour of the nucleolus organizer region (NOR) in the second-longest chromosome (linkage group VL). In crosses of IT x

Normal sequence, the progeny nuclei in the developing asci and ascospores contained either two NORs or only a partial NOR, or no NORs at all (Perkins *et al* 1980, 1984, 1995). The IT strain AR33 has a segment of linkage group VL containing the NOR inserted at the distal end of IVL. When AR33 is crossed by Normal sequence, one third of the viable progeny contain a non-tandem duplication with two NORs per nucleus, which often showed two nucleoli or a larger fusion nucleolus. The NOR-deficient complementary meiotic products are inviable (Perkins *et al* 1980). In another IT strain, OY321, a distal portion of the NOR is interchanged with a long terminal segment of IL. Thus, each nucleus now has the two NOR segments on different chromosomes. In crosses of OY321 x OY321, each meiotic product is capable of making two nucleoli, and some nuclei do show two nucleoli and other nuclei show a single fusion nucleolus (Perkins *et al* 1984). In the last study, we examined four RT strains, in which a chromosome segment is translocated to the end of NOR on VL. I used the DNA-specific fluorochrome acriflavine, which 'stains' the NOR as attenuated (less brightly staining) strands at one end of the second-longest chromosome (figure 3E, F). When an NOR rearrangement strain was made homozygous (e.g. *UK1-35* x *UK1-35*), a brightly fluorescing translocated segment could be readily seen attached to the less brightly fluorescing NOR strands (Raju 1986a; Perkins *et al* 1995).

5. Natural populations

Most of Perkins' early work (1950–1968) on *Neurospora* genetics utilized a few laboratory wild-type strains or mutations induced in them. He soon realized that natural populations would provide greater diversity, and collected thousands of wild isolates from many parts of the tropics following both wildfires and agricultural burns, mainly in sugarcane plantations. Each of these isolates was crossed with the standard species testers in the laboratory and each was assigned to one of four heterothallic species based on fertility. A vast majority of the isolates belonged to *N. intermedia*, followed by *N. crassa*, *N. sitophila* and *N. discreta* (Perkins *et al* 1976; Perkins and Turner 1988). More recent collections in the temperate regions of North America and Europe following forest fires, however, gave a different population structure: *N. discreta*, *N. crassa* and *N. sitophila* were more common than *N. intermedia*. Subsequent analyses of wild strains provided insights into the diversity and evolution of this model organism (Jacobson *et al* 2004, 2006). To this day, the wild isolates continue to serve as a valuable resource and studies with them have contributed to several major discoveries: vegetative incompatibility genes, transposable elements, senescence-causing mitochondrial plasmids, recessive sexual phase mutants and meiotic drive-inducing spore killers, to name a few. There were several cytological studies involving the wild isolates and their derivatives (Raju 1979; Leslie and Raju 1985; Raju and Leslie 1992; Jacobson 1995; Raju and Burk 2004). Perkins and Raju (1986) described a new 8-spored heterothallic species, *Neurospora discreta*, based on crossing behaviour of certain wild isolates from the USA; this species is most prevalent among isolates from scorched trees, following forest fires in western North America (Jacobson *et al* 2004). *Neurospora* population studies were often limited to observation and sampling of conidial masses on scorched vegetation. A notable exception was the work of Pandit and Maheshwari (1996), who reported finding protoperithecia and perithecia under the epidermis of old, dried sugarcane stems in fields in Karnataka, southern India (*see also* Maheshwari 2007).

6. Spore killers as meiotic drive elements

Neurospora spore killers (*Sk*) are meiotic drive elements that closely resemble the segregation distorters in *Drosophila*, *t* complexes in mouse, pollen killer in wheat, and female gamete eliminator in tomato (Turner and Perkins 1979, 1991). They distort genetic ratios of *Sk*-linked genes in heterozygous crosses. In crosses of *Killer* x *Sensitive* (normal), 4 of the 8 ascospores of each ascus that contains the sensitive allele fail to mature and are inviable (figure 5). The discovery of *Neurospora* spore killers in Perkins' laboratory was by no means accidental. The laboratory was well aware of various causes of ascospore death, such

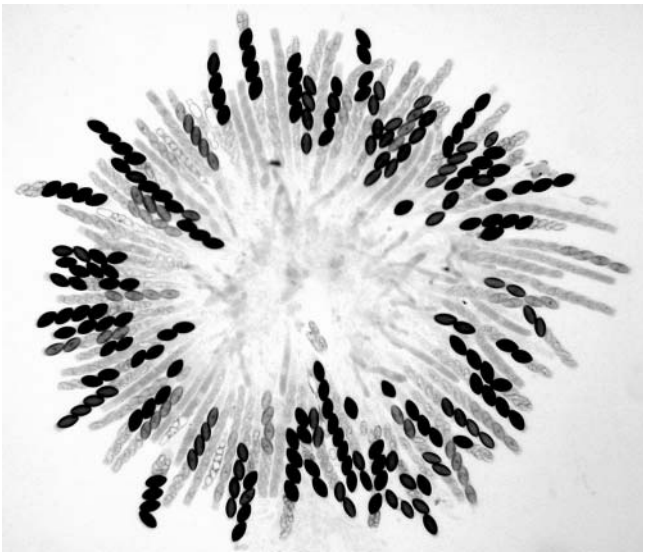

Figure 5. A rosette of maturing asci of *N. sitophila* from *Spore killer-1* x wild type (*Sk*-sensitive) showing the death of 4 ($Sk\text{-}1^{S}$) ascospores in almost all asci. All survivors from 4:4 asci carry $Sk\text{-}1^{K}$. Ascospore development is completely normal in *Killer* x *Killer* or *Sensitive* x *Sensitive* crosses.

as recessive mutations that affect ascospore pigmentation and maturation, and chromosome rearrangements where deficiency ascospores fail to mature and are inviable. For example, in crosses of *cys-3* x wild type, every mature ascus shows 4B: 4W ascospores, and the white ascospores that carry *cys-3* are inviable (figure 4G). As we have seen earlier, chromosome rearrangements produce asci with characteristic patterns of black and white ascospores (e.g. 8B: 0W, 6B: 2W, 4B: 4W, 0B: 8W). The ascospore death in *Killer* x *Sensitive*, and subsequent progeny analysis did not fit into any known segregation patterns resulting from recessive ascospore-maturation mutants or chromosome rearrangements. Turner and Perkins (1979), with their prior knowledge of segregation distorters in *Drosophila* and other causes of meiotic drive in various organisms, immediately recognized that the Sk-induced ascospore death must be due to meiotic drive.

Meiosis, postmeiotic mitosis and ascospore genesis are completely normal in *Killer* x *Sensitive* crosses. A second mitosis occurs in all 8 ascospores before there is any sign of death in 4 of the 8 ascospores. All survivors are killers (Raju 1979, 1994, 2002b). Fungal spore killers were first discovered in *Neurospora* among natural populations of *N. sitophila* (*Sk-1*) and *N. intermedia* (*Sk-2* and *Sk-3*). *Sk-2* and *Sk-3* have been found in only four *N. intermedia* isolates from Borneo (Brunei, Sabah), Java and Papua New Guinea among ~2500 isolates of this species from around the world, whereas *Sk-1* killer is found in up to 30% of *N. sitophila* isolates from many parts of the world (Turner and Perkins 1979; Turner 2001; Jacobson *et al* 2006).

Sk-2 and *Sk-3* have been introgressed into *N. crassa* and *N. tetrasperma* for detailed genetic analysis. When either *Sk* is heterozygous in a cross (*Sk-2* or *Sk-3* x wild type), crossing over is blocked in a 30 map-unit region that includes the centromere of linkage group III (Campbell and Turner 1987). There is neither killing nor recombination block when either killer is homozygous. When *Sk-2* is crossed with *Sk-3*, all 8 ascospores are killed because of mutual killing, i.e. *Sk-2* is sensitive to killing by *Sk-3*, and *Sk-3* is sensitive to killing by *Sk-2*. Sensitive nuclei are sheltered from killing when a killer nucleus is also enclosed in the same ascospore. This was first shown in *N. crassa* by using *Ban*, where all 8 nuclei of each ascus (4K + 4S) are enclosed in a single giant ascospore, and subsequently, in the naturally heterokaryotic 4-spored asci of *N. tetrasperma*. Progeny cultures from the heterokaryotic ascospores contain both killer and sensitive nuclei, which remain unchanged through subsequent generations (Raju 1979, 1994; Raju and Perkins 1991). The sheltering of sensitive nuclei in the heterokaryotic ascospores prompted us to suggest that pseudohomothallism in *N. tetrasperma* may have evolved to circumvent the deleterious effects of spore killers in heterothallic species (Raju and Perkins 1991). The exact chromosomal location of *Neurospora* spore killers is not known because of the recombination block in linkage group III (Campbell and Turner 1987), and none of the spore killers have yet been cloned for molecular analysis (*see* reviews by Raju and Perkins 1991; Turner and Perkins 1991; Raju 1994, 2002b).

Sk-2 and *Sk-3* have since been used for determining centromere distances of marker genes by simple scoring of ejected, unordered, half-tetrads (Perkins *et al* 1986), and in studies of meiotic silencing and its suppression (Raju *et al* 2007, *see below*). Use of a tightly centromere-linked *Sk* reduces the labour, by several-fold, required for obtaining centromere distance of any gene not linked to *Sk*. For example, in a cross of Sk^{K} *cys-3*$^{+}$ x Sk^{S} *cys-3*, 4 Sk^{S} ascospores at one end of each ascus are killed. The remaining 4 viable ascospores at the other end are all Sk^{K}, because Sk^{K} segregates from Sk^{S} at the first division of meiosis. The 4 viable Sk^{K} and 4 aborted Sk^{S} ascospores of each ascus are forcefully ejected from the perithecium as a disordered group. If both *cys-3* and *cys-3*$^{+}$ alleles are represented in the surviving products, they must therefore have segregated from one another at the second division of meiosis, because of a crossover between the gene and the centromere. The use of *Sk* thus effectively converts unordered tetrads into ordered tetrads for determining centromere distances (Perkins *et al* 1986).

Spore killers have also been found in *P. anserina*, *Gibberella fujikuroi* and *Cochliobolus heterostrophus* (Raju 1994). In *G. fujikuroi* and *C. heterostrophus*, crosses between killer and sensitive strains result in the death of 4 of the 8 ascospores that do not contain the killer allele, just as in *N. crassa*. In *P. anserina*, killing of 2 of the 4 ascospores occurs when the killer and sensitive alleles segregate at the first division of meiosis. Sensitive nuclei are sheltered in the heterokaryotic ascospores, following second-division segregation resulting from crossing over in the centromere proximal region (Raju 2002b).

7. Sexual biology of other Ascomycota

The work discussed thus far is mainly about *Neurospora* because of our laboratory's focus on the genetics and cytogenetics of this model fungus. Nevertheless, I have examined ascus biology of several other members of Ascomycota, either for comparative purposes or for better understanding of a specific problem that we were already interested in in *Neurospora*. Here I summarize my work on the ascus biology of *Coniochaeta tetraspora* and *Cochliobolus heterostrophu*s. Mature asci of *C. tetraspora* always contained 4 black ascospores; hence the species name "*tetraspora*" (figure 6A; Cain 1961). Cytological studies with *C. tetraspora* were initiated with the assumption that it is pseudohomothallic, as are *N. tetrasperma* and *P. anserina*. However, the asci of *C. tetraspora* initially

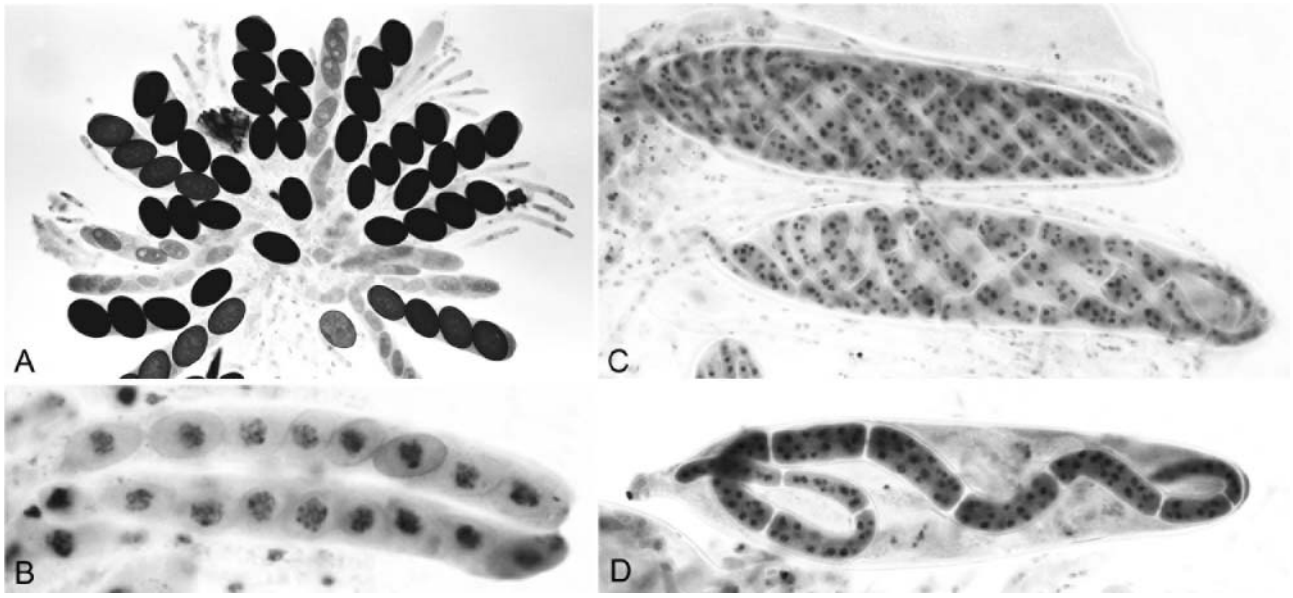

Figure 6. Ascospore development in *Coniochaeta tetraspora* (**A, B**), and *Cochliobolus heterostrophus* (**C, D**). (**A**) A rosette of maturing asci in *C. tetraspora*. Immature asci are 8-spored but the mature asci are 4-spored, because of death of 4 ascospores in each ascus. (**B**) Two immature 8-spored asci; all ascospores are uninucleate. The top ascus is beginning to show second-division segregation (2:2:2:2) for ascospore death, and the lower ascus is showing the early signs of first-division segregation (4:4). (**C**) Ascospores of *C. heterostrophus* are multinucleate, multisegmented and helically coiled. All 8 ascospores are mature in the top ascus, but only 4 are mature in the bottom ascus (from Raju 2008b). (**D**) A mature ascus showing a single multinucleate, multisegmented ascospore; the remaining 7 ascospores have aborted early in development.

contained 8 linearly ordered uninucleate ascospores, similar to those of *N. crassa* (Raju and Perkins 2000). The 4-spored condition resulted only secondarily by disintegration of two pairs of sister ascospores, leaving only two pairs of mature ascospores, which showed either the first (4 viable: 4 inviable) or the second-division-segregation patterns (2:2:2: 2 or 2:4:2) for ascospore death (figure 6A, B). Arrangement of viable and dead ascospore pairs in intact linear asci reflects segregation patterns of a chromosomal gene located at some distance from the centromere – thus producing non-crossover asci (4 viable: 4 inviable ascospores) as well as crossover asci (2:2:2:2 or 2:4:2). Progeny analysis showed that single-ascospore cultures are self-fertile, and again produce 4 viable and 4 inviable ascospores generation after generation.

These observations indicate that differentiation of the two nuclear types occurs *de novo* in each generation, that it involves alteration of a specific chromosome locus, and that the specific change occurs early in the sexual phase. One of the two haploid nuclei entering each functional zygote must carry the altered element, which is segregated into two of the four meiotic products and is eliminated when ascospores that contain it disintegrate. Fusion of haploid nuclei cannot be random – a recognition mechanism must exist. The ascospore death in *C. tetraspora* superficially resembles that of *Neurospora* spore killers, but the death cannot be due to interaction of *Sensitive* and *Killer* haplotypes as in *Neurospora*, because *C. tetraspora* is homothallic and there are no such genotypic differences. *C. tetraspora* is primarily an 8-spored homothallic species, and not a pseudohomothallic species like *N. tetrasperma*. Raju and Perkins (2000) discussed similar but less drastic phenomena in several other fungi (e.g. *Sclerotinia trifoliorum* and *Chromocrea spinulosa*), and attributed them to epigenetic mutational changes in one of the two nuclei that go into meiosis.

Olen Yoder and Charlotte Bronson spent their sabbaticals in the Perkins laboratory in the 1970s and 1980s, respectively, and they are responsible for my introduction to *Cochliobolus heterostrophus*, which causes southern corn leaf blight. In the sexual phase, it produces 8 filiform ascospores per ascus, following meiosis and a postmeiotic mitosis (figure 6C, D). The species has two distinct mating types, *mat 1-1* and *mat 1-2*. Single-mating-type strains are self-sterile, but cross-fertile. Early ascus development and nuclear divisions in *C. heterostrophus* resemble those of *N. crassa*. However, the two fungi differ in several important details owing to differences in ascus and ascospore shape, spindle pole body behaviour during spore delimitation and ascospore development (Raju 2008a). The two spindles at meiosis II, and the four spindles at the postmeiotic mitosis are aligned irregularly, unlike the tandem or ladder rung-like orientation

of spindles in *N. crassa*. Prior to ascospore delimitation, all 8 nuclei reorient themselves but remain in the middle of the ascus while their SPB plaques migrate toward the base of the ascus. The SPB plaques facilitate demarcation of the lower end of each incipient ascospore. Ascospores are uninucleate and unsegmented at inception but they become highly multinucleate (>100), multisegmented (~10), and helically coiled when mature (figure 6C, D). A mature ascospore is normally longer than the ascus itself and usually folds on itself at both ends. Ascospore morphology is readily observed in asci that contain one or a few mature ascospores rather than in 8-spored asci, where the ascospores are tightly coiled (figure 6D). A *Neurospora Sk*-like spore killer is found in certain race-O field isolates. In crosses of Sk^K x Sk^S, a vast majority of asci contain 4 or fewer mature ascospores (Sk^K); the 4 ascospores that carry Sk^S nuclei abort shortly after spore delimitation (Raju 1994). An illustrated account of ascus and ascospore development in *C. heterostrophus* is now available (Raju 2008a).

8. Meiotic silencing in *N. crassa*

Twenty years ago, experiments on transgenic plants for antibiotic resistance or for flower colour genes had shown that ectopically inserted transgenes suppress their own expression as well as that of the native homologous gene (Mazke *et al* 1989; Napoli *et al* 1990; van der Krol *et al* 1990). This phenomenon was called 'co-suppression'. A similar phenomenon was discovered in *N. crassa* at about the same time. Conidia of wild type *N. crassa* contain bright orange carotenoid pigments that are synthesized by different enzymes encoded by three structural genes (*albino-1* [*al-1*], *al-2*, *al-3*). Ectopic insertions of any of the three genes into wild-type (orange) strains often resulted in severe impairment of expression of the inserted al^+ gene(s) as well as its native homologue (Nelson *et al* 1989; Schmidhauser *et al* 1990; Romano and Macino 1992). This phenomenon in *Neurospora* was called 'quelling', but is very similar to co-suppression in plants. Both quelling and co-suppression occur in vegetative tissue and are reversible. The mechanism underlying quelling and co-suppression was later shown to be a post-transcriptional gene silencing (PTGS) process involving RNA interference (RNAi). A similar silencing process in *N. crassa* operates in the sexual phase. This recently discovered phenomenon will be covered in somewhat greater detail, not only because of its novelty and significance but also because the subject has not been adequately reviewed and illustrated for the benefit of general biologists.

Meiotic silencing, first discovered in *N. crassa*, is related to quelling and co-suppression, but its effects are expressed only during meiosis and postmeiotic mitosis. Any gene whose expression is required for meiosis and that is without a homologue in the same chromosomal position during meiotic prophase generates a sequence-specific signal that prevents expression of all copies of that gene (Aramayo and Metzenberg 1996; Shiu *et al* 2001). For example, when a normal strain containing a meiosis-specific gene (e.g. $asm\text{-}1^+$, for ascospore maturation) is crossed with a strain in which $asm\text{-}1^+$ is deleted ($asm\text{-}1^+$ x $asm\text{-}1^\Delta$), the normal $asm\text{-}1^+$ fails to function during meiosis. Consequently, all 8 ascospores that contain either $asm\text{-}1^+$ or $asm\text{-}1^\Delta$ fail to mature. This result shows that $asm\text{-}1^\Delta$ is ascus dominant in its effect. Furthermore, when a second copy of the normal gene is inserted into one of the parents but not the second parent, the newly inserted gene at the ectopic location as well as the native copies of the gene fail to function in a heterozygous cross (e.g. $asm\text{-}1^+$ x $asm\text{-}1^+$ $asm\text{-}1^{+ect}$). There is no silencing when the ectopic gene inserts are paired in homozygous crosses ($asm\text{-}1^+$ $asm\text{-}1^{+ect}$ x $asm\text{-}1^+$ $asm\text{-}1^{+ect}$). Aramayo and Metzenberg (1996) first used the term 'meiotic transvection' to describe this trans-sensing meiotic phenomenon. Shiu *et al* (2001) have further extended these studies with four additional genes whose functions are required for normal meiosis and found the same result. Apparently, homologous gene pairing is essential for its normal expression during meiosis and ascus development, and the unpaired sequences at the homologous site trigger meiotic silencing. They named the phenomenon meiotic silencing by unpaired DNA (MSUD). Early observations on MSUD utilized ectopic inserts of $asm\text{-}1^+$, $actin^+$, $\beta\text{-}tubulin^+$, $mei\text{-}3^+$, and $pma\text{-}1^+$, and their effects on ascus development, when silenced, could be readily monitored cytologically. For example, in crosses of wild type x $actin^{+ect}$ (i.e. $actin^+$ x $actin^+$ $actin^{+ect}$) the unpaired $actin^{+ect}$ insert as well as the paired native copies of the $actin^+$ gene are silenced during meiosis, and in the absence of actin filaments the asci are swollen like balloons (figure 7A). There is much less silencing when the ectopic inserts are paired in homozygous crosses ($actin^+$ $actin^{+ect}$ x $actin^+$ $actin^{+ect}$), where most of the asci elongate and develop normally (figure 7B; Shiu *et al* 2001). Similar results were obtained with ectopic inserts of $\beta\text{-}tubulin^+$, $mei\text{-}3^+$, $pma\text{-}1^+$ and two GFP-tagged genes (figure 7C, D). The GFP gene was cloned from the jellyfish *Aequorea victoria*. The use of *histone H1-GFP* and *β-tubulin-GFP* ectopic inserts to visualize their expression or silencing during meiosis, and the suppression of meiotic silencing using mutants of certain genes that are required for silencing, are described next.

8.1 *Histone H1-GFP*

Histone H1 is a chromosomal protein in eukaryotic nuclei. When the *hH1* gene is tagged with the *GFP* gene (*hH1-GFP*) and introduced into *N. crassa*, by transformation targeted to the *his-3* locus in linkage group I, the nuclei fluoresce because of the expression of hH1-GFP. The fluorescent

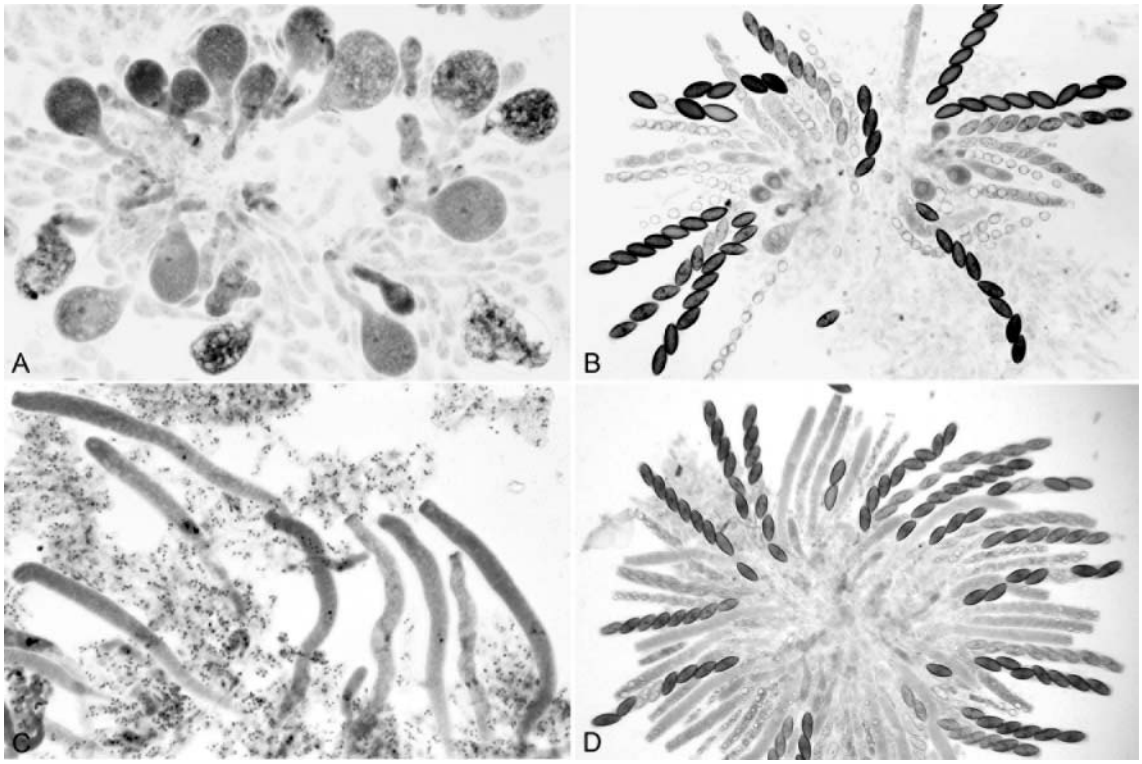

Figure 7. Early observations on meiotic silencing, using *actin*$^{+ect}$ and *β-tubulin*$^{+ect}$ ectopic inserts. Both actin filaments and *β*-tubulin are required for normal cytoskeleton and ascus development. (**A**) Wild type x *actin*$^{+ect}$. A rosette of swollen asci at 6 days after fertilization, arrested in meiotic prophase, resulting from silencing of actin expression in the heterozygous cross. (**B**) *actin*$^{+ect}$ x *actin*$^{+ect}$. Most of the asci show normal development when the *actin*$^{+ect}$ inserts are paired in a homozygous cross. (**C**) Wild type x *β-tubulin*$^{+ect}$. The expression of *β*-tubulin is silenced and the asci are arrested prior to forming metaphase I spindles. (**D**) *β-tubulin*$^{+ect}$ x *β-tubulin*$^{+ect}$. A rosette of maturing asci showing normal ascus and ascospore development when the *β-tubulin*$^{+ect}$ inserts are paired in a homozygous cross. (**A**, from Raju *et al* 2007; **C**, from Shiu *et al* 2001)

nuclei can be readily seen in the vegetative hyphae and conidia under a fluorescence microscope (Freitag *et al* 2004). In heterokaryotic mycelia that contain both *GFP*-tagged and -untagged nuclei, all nuclei fluoresce because hH1-GFP is made in the cytoplasm and incorporated into *all* nuclei in the same cytoplasm. In crosses of ectopic *hH1-GFP*^{ect} x ectopic *hH1-GFP*^{ect}, the nuclei fluoresce throughout meiosis and ascospore development, i.e. *hH1-GFP*^{ect} is expressed in homozygous asci, because the *hH1-GFP* constructs were inserted at the same chromosomal location (*his-3*) and are paired (figure 8A, B). However, in crosses of *hH1-GFP*^{ect} x wild type, the developing asci showed no fluorescing nuclei throughout meiosis until after ascospore delimitation. Thus, the expression of *hH1-GFP*^{ect} is silenced in heterozygous asci, where the ectopically inserted *hH1-GFP*^{ect} does not have a pairing partner (figure 8C). Meiotic silencing is restricted to meiosis and postmeiotic mitosis in the developing asci, and it does not extend into later stages of ascospore development and maturation, however. The silenced *hH1-GFP*^{ect} is reactivated in maturing ascospores, and the nuclei in 4 of the 8 ascospores begin to fluoresce ~1 day after the ascospores are delimited. The nuclei fluoresce brighter as more hH1-GFP is made and incorporated into the chromosomes (figure 8D). The ascospores remain binucleate for about 2–3 days after they are delimited, but they later become highly multinucleate following three or four additional mitoses in the mature, darkly pigmented ascospores (figure 8E).

8.2 *hH1-GFP* x *Banana*

The giant ascospore mutant *Banana* was used to examine the expression of *hH1-GFP*^{ect} in the heterokaryotic giant ascospores of *hH1-GFP*^{ect} x *Ban*, where one-half of the nuclei are genotypically *hH1-GFP*^{ect} and the other one-half are wild type. *Histone H1-GFP*^{ect} is silenced during meiosis in the heterozygous asci but it is re-expressed in the developing giant ascospores. Initially, only 8 of the 16 nuclei at one end of the young ascospore show fluorescence, and later all 16 nuclei show hH1-GFP (figure 8F, G). Apparently, the mRNA from *hH1-GFP*^{ect} is translated in the cytoplasm surrounding the *hH1-GFP*^{ect} nuclei, and the protein is first

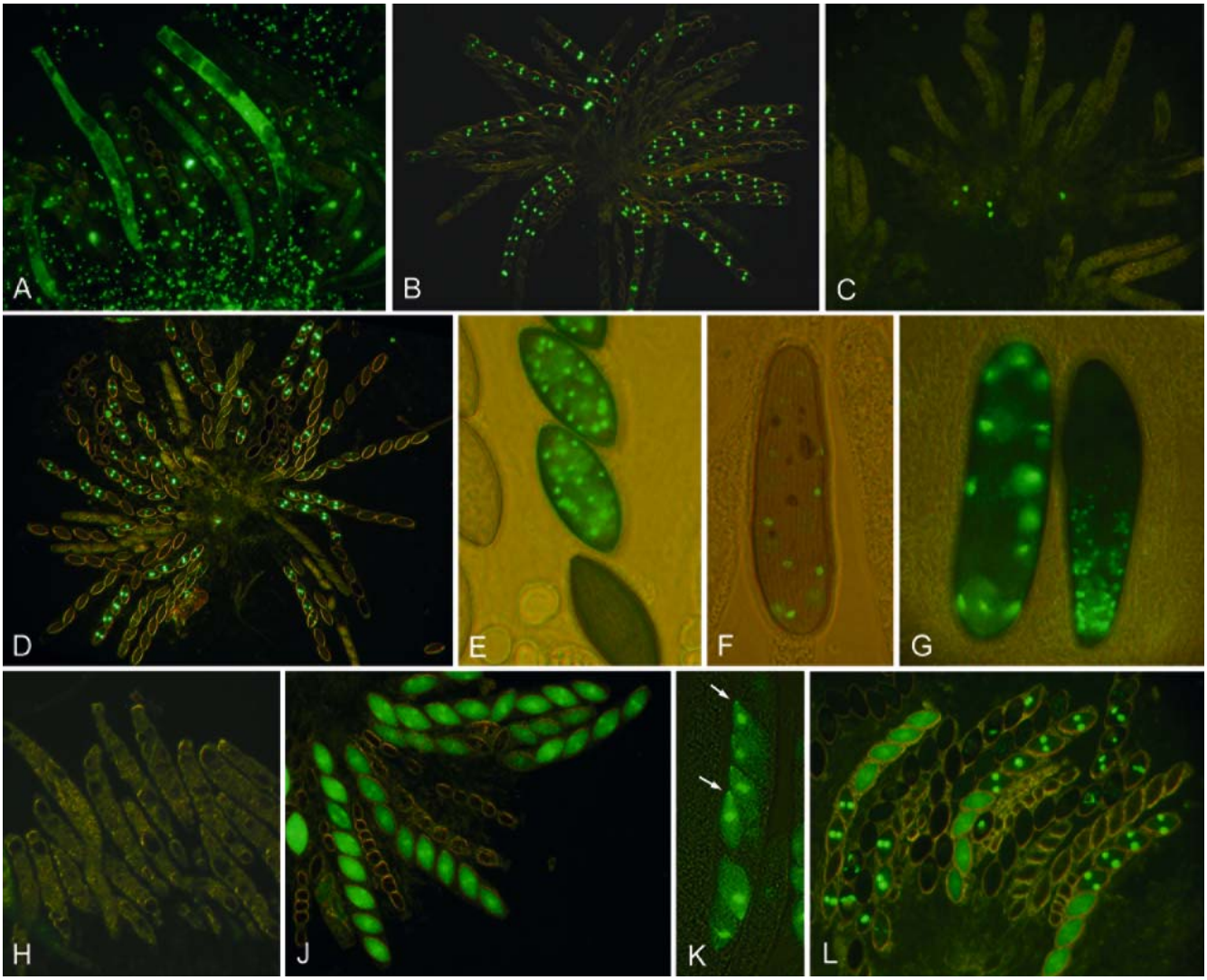

Figure 8. Visualization of meiotic silencing using GFP-tagged *histone H1* and *β-tubulin* ectopic inserts in homozygous and heterozygous crosses. (**A, B**) *hH1-GFP*^{ect} x *hH1-GFP*^{ect} 4–5 days after fertilization. Young asci show normal expression of hH1-GFP throughout meiosis and ascospore development; there is no meiotic silencing when the *hH1-GFP*^{ect} inserts are paired in meiosis. (**B**) A rosette of maturing asci at 7 days after fertilization showing fluorescing binucleate ascospores. (**C–E**) Wild type x *hH1-GFP*^{ect}. (**C**) The expression of *hH1-GFP*^{ect} is completely silenced in the young heterozygous asci in meiosis (5 days after fertilization, compare with A). (**D**) A rosette of maturing asci at 7–8 days after fertilization. The silenced *hH1-GFP*^{ect} is re-expressed in 4 of the 8 ascospores in each ascus (compare with B). (**E**) Mature black ascospores, at 9–10 days after fertilization, showing multiple fluorescing nuclei following 3 or 4 mitoses. (**F, G**) Giant ascospores from *hH1-GFP*^{ect} x *Ban* that enclose all four products of meiosis and their mitotic derivatives (4 *hH1-GFP*^{ect} + 4 non-*hH1-GFP*^{ect}). *hH1-GFP*^{ect} is silenced in the young asci prior to spore delimitation, but it is re-expressed in the developing giant ascospores. (**F**) hH1-GFP is first expressed in the genotypically *hH1-GFP*^{ect} nuclei at one end of the giant ascospore, and the non-*hH1-GFP*^{ect} nuclei at the opposite end show fluorescence later, because of protein migration through the cytoplasm. (**G**) Two maturing giant ascospores. The ascospore at left shows GFP in all 16 nuclei (8 *hH1-GFP*^{ect} + 8 non-*hH1-GFP*^{ect}). The more mature ascospore at the right is highly multinucleate following several mitoses, and the hH1-GFP gradient is clearly visible from one end of the ascospore to the other. (**H**) Wild type x *β-tubulin-GFP*^{ect}. 7 days after fertilization. The expression of *β*-tubulin is silenced in the heterozygous asci, which abort in meiotic prophase. (**J**) *β-tubulin-GFP*^{ect} x *β-tubulin-GFP*^{ect}. *β*-tubulin is expressed in homozygous crosses, where the asci and ascospores develop normally. (**K, L**) *hH1-GFP*^{ect} x *β-tubulin-GFP*^{ect}. Ascus development is nearly normal in the intercross between two strains carrying different ectopic gene inserts at the same chromosomal location. (**K**) A young ascus at spore delimitation showing the expression of *β-tubulin-GFP*^{ect} in the SPB plaques (arrows) and in the nuclear region. (**L**) Maturing asci in which 4 ascospores express hH1-GFP in the nuclei, and 4 ascospores express *β*-tubulin-GFP in the cytoplasm. The asci show first-division segregation (4:4) for the two GFP constructs that are inserted at the same location, close to the centromere of linkage group I.

incorporated into the nearby *hH1-GFP*^{ect} nuclei. The GFP-tagged protein also diffuses throughout the cytoplasm of the giant ascospores and is incorporated into all 16 nuclei causing them to show green fluorescence (figure 8G, left). A gradient of hH1-GFP expression is visibly more pronounced in mature giant ascospores as they undergo three or four additional mitoses in quick succession. In such highly multinucleate ascospores, only the 50% of nuclei containing *hH1-GFP*^{ect} at one end of the ascospore fluoresce initially. Over time, the green fluorescence spreads toward the opposite end until all nuclei of the giant ascospore fluoresce (figure 8G, right).

8.3 β-*tubulin-GFP*

β-tubulin is an essential component of the cytoskeleton, and is also required for spindle formation during nuclear divisions both in mitosis and meiosis. Strains carrying an ectopically inserted *β-tubulin*$^{+ect}$ gene (in addition to its native copy) grow normally. However, in crosses of wild type x *β-tubulin*$^{+ect}$ or wild type x *β-tubulin-GFP*^{ect}, the expression of *β*-tubulin is silenced and the asci are arrested prior to metaphase I. Most of the asci are abnormally shaped with characteristic bends at the distal end of the elongated asci (figures 7C, 8H). In contrast, homozygous crosses of *β-tubulin*$^{+ect}$ inserts (*β-tubulin*$^{+ect}$ x *β-tubulin*$^{+ect}$ and *β-tubulin-GFP*^{ect} x *β-tubulin-GFP*^{ect}) show completely normal ascus and ascospore development, i.e. there is no silencing of *β*-tubulin when the *β-tubulin*$^{+ect}$ inserts are paired in meiosis (figure 8J).

9. Interaction of *hH1-GFP*^{ect} and *β-tubulin-GFP*^{ect} inserts in intercrosses

We have seen that in crosses of wild type x *hH1-GFP*^{ect}, the expression of *hH1-GFP*^{ect} is silenced during meiosis and is re-expressed in the developing ascospores (figure 8C, D). Similar silencing of *β*-tubulin in wild type x *β-tubulin-GFP*^{ect} drastically arrests ascus development prior to metaphase I (figures 7C, 8H). We have also shown that the meiotic silencing of *hH1-GFP*^{ect} and *β-tubulin-GFP*^{ect} is clearly due to the unpaired DNA sequences in heterozygous asci. Both *hH1-GFP* and *β-tubulin-GFP* constructs are ectopically inserted at the *his-3* locus in linkage group I, and both genes are under the control of the same *ccg-1* promoter. An intercross of *hH1-GFP*^{ect} x *β-tubulin-GFP*^{ect} was examined with the assumption that both genes will be silenced because such a cross will be heterozygous for both *hH1-GFP*^{ect} and *β-tubulin-GFP*^{ect} inserts. To my surprise, the asci of the intercross developed normally through meiosis, postmeiotic mitosis and ascospore development, indicating that *β*-tubulin was not silenced completely in the developing asci. Figure 8K shows the expression of *β-tubulin-GFP*^{ect} and its localization in the SPB plaques during spore delimitation. Histone H1-GFP is also expressed, albeit marginally, and the fluorescent nuclei can sometimes be seen during ascus development prior to ascospore delimitation. As the asci mature, 4 ascospores show hH1-GFP in their nuclei and 4 show *β*-tubulin-GFP in their cytoplasm (figure 8L).

These results show that meiotic silencing of *hH1-GFP*^{ect} and *β-tubulin-GFP*^{ect} in the intercrosses is at best partial. A possible explanation for the absence or reduced meiotic silencing in the intercrosses is that the two constructs share homology in the *ccg-1* promoter region and in the *GFP* gene sequences, differing only at the *hH1* and *β-tubulin* portions of the fusion genes. If this difference is not enough to trigger the heterology-based silencing mechanism; it suggests that there is probably a threshold of heterology required for triggering meiotic silencing via RNAi. Similarly, crosses of *β-tubulin*$^{+ect}$ x *β-tubulin-GFP*^{ect} show normal ascus development and *β*-tubulin-GFP expression even though the *GFP* sequence is unpaired, perhaps because the unpaired *GFP* sequence is too small to trigger MSUD (*see* Lee *et al* 2004; Pratt *et al* 2004).

10. Suppression of meiotic silencing by *Sad* mutants

Meiotic silencing is epigenetic, and deletions or mutations in several genes of RNA silencing machinery (*Sad-1*$^{\Delta}$, *Sad-2*^{RIP}, *Sms-2*$^{\Delta}$) suppress MSUD. The wild type *sad-1* gene, which codes for RNA-directed RNA polymerase (RdRP), is an essential component of meiotic silencing. We have recently shown that *sad-2*$^{+}$ is required for the perinuclear co-localization of SAD-1 and SAD-2 proteins and this is necessary for meiotic silencing to occur (Shiu *et al* 2006). In addition, a dicer-like ribonuclease, DCL-1, also co-localizes with SAD-1 and SAD-2 proteins in the perinuclear region and is involved in meiotic silencing. Thus, deletions or extensive mutations in any of these genes (except *dcl-1*) lead to failure of their pairing in meiosis resulting in silencing of the silencer itself (figure 9A–C) (Alexander *et al* 2008). In the early studies on *Sad-1*$^{\Delta}$, we noted that *actin*$^{+ect}$, *β-tubulin*$^{+ect}$ and *mei-3*$^{+ect}$ are not silenced in crosses with *Sad-1*$^{\Delta}$ (or *Sad-2*^{RIP}), because *sad-1* itself is unpaired and self-silenced. GFP-tagged *histone H1* and *β-tubulin* ectopic inserts have since been used for visualizing the suppression of meiotic silencing by *Sad-1*$^{\Delta}$ and *Sad-2*^{RIP}, using fluorescence microscopy (Freitag *et al* 2004; Shiu *et al* 2001, 2006). For example, when MSUD of *hH1-GFP*^{ect} is suppressed by *Sad-1*$^{\Delta}$ or *Sad-2*^{RIP}, hH1-GFP is expressed, and the nuclei fluoresce throughout meiosis and ascus development (figure 9B). Similarly, when *Sad* mutants suppress silencing of *β*-tubulin (e. g. *β-tubulin*$^{+ect}$ x *Sad-1*$^{\Delta}$), *β*-tubulin is expressed and the asci develop normally, with all 8 ascospores initially showing *β*-tubulin-GFP.

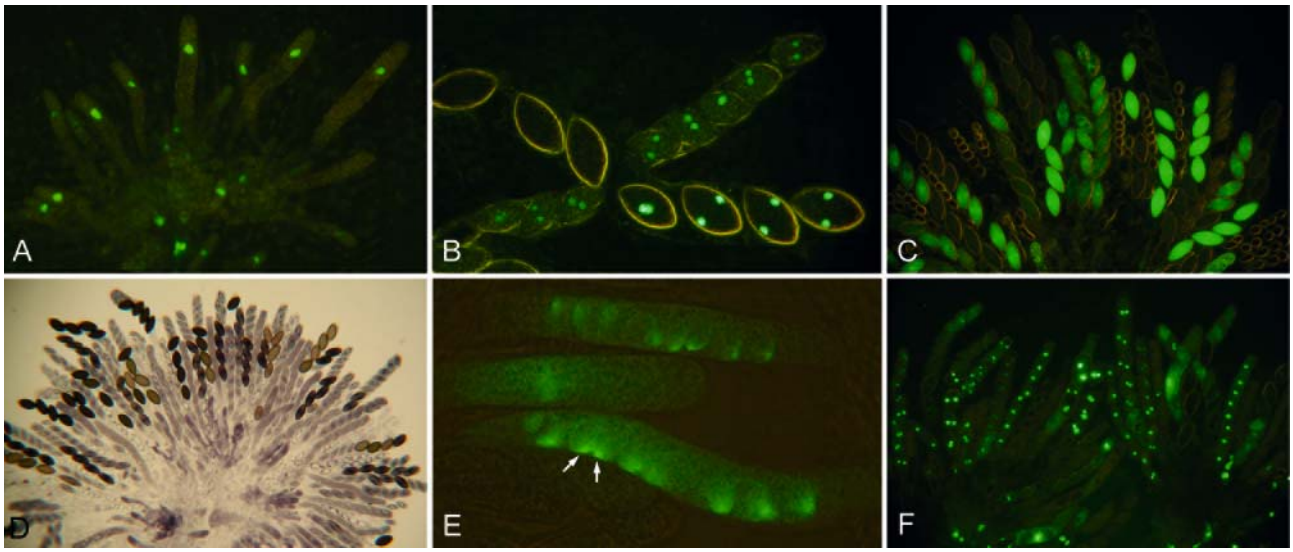

Figure 9. The suppression of meiotic silencing by spore killers and mutations in *sad* genes. (**A, B**) *Sad-1*$^{\Delta}$ x *hH1-GFP*^{ect}. (**A**) *Sad-1* suppresses meiotic silencing, thus young asci show brightly fluorescing nuclei in meiotic prophase. (**B**) An immature ascus shows fluorescing nuclei in all 8 ascospores, but a more mature ascus shows brightly fluorescing nuclei in only 4 (*hH1-GFP*^{ect}) of the 8 ascospores. The remaining 4 ascospores contain non-*hH1-GFP*^{ect} nuclei, and the residual hH1-GFP gradually faded away because of protein turnover. (**C**) A rosette of maturing asci from *Sad-1*$^{\Delta}$ x *β-tubulin-GFP*^{ect} at 8 days after fertilization. The silencing of *β*-tubulin is suppressed by *Sad-1*, and the asci and ascospores develop normally. All 8 ascospores initially show *β*-tubulin-GFP, but the fluorescence later fades away in 4 of the non-*β-tubulin-GFP*^{ect} ascospores. (**D–F**) Meiotic silencing of *actin*$^{+ect}$, *β-tubulin-GFP*^{ect} and *hH1-GFP*^{ect} is suppressed by *Sk-2* and *Sk-3*. (**D**) A rosette of asci from *actin*$^{+ect}$ x *Sk-2*^{K} shows normal ascus development. (**E**) *Sk-3*^{K} x *β-tubulin-GFP*^{ect}. Two of the three immature asci are at the spore delimitation stage, and show *β*-tubulin-GFP in the SPB plaques at one end of the incipient ascospores (arrows). (**F**) A rosette of immature asci from *Sk-3*^{K} x *hH1-GFP*^{ect} at 6 days after fertilization, showing normal hH1-GFP expression throughout meiosis and ascospore formation. (**C**, **E**, from Raju *et al* 2007)

However, as the ascospores mature, only the 4 ascospores that inherited the *β-tubulin-GFP*^{ect} construct continue to produce *β*-tubulin-GFP, and the residual fluorescence in the remaining 4 non-*β-tubulin-GFP*^{ect} ascospores gradually fades away as the ascospores mature because of protein turnover (figure 9C).

11. Suppression of meiotic silencing by spore killers

In an earlier section, we saw spore killer-induced ascospore death in crosses of *Sk* x wild type. In addition to this meiotic drive function, *Sk-2* and *Sk-3* show a seemingly unrelated property of suppressing meiotic silencing of several ectopic gene inserts (figure 9D–F). This behaviour was shown in crosses of *Sk-2* or *Sk-3* with *β-tubulin-GFP*^{ect}, and with *hH1-GFP*^{ect}. In such crosses, where the ectopic gene inserts are heterozygous, the silencing of gene inserts is suppressed, and they are expressed throughout meiosis and ascospore development. Four of the *Sk*^{S} ascospores are killed as expected for an *Sk*^{K} x *Sk*^{S} cross, but the killing is unrelated to the suppression of meiotic silencing (figure 9D). We have shown this in crosses that are homozygous for either of the killer haplotypes, but heterozygous for *hH1-GFP*^{ect} (e.g. *Sk-2*^{K} x *Sk-2*^{K} *hH1-GFP*^{ect}). As expected, there is no ascospore death because of *Sk* homozygosity; however, meiotic silencing is suppressed just as in heterozygous *Sk* crosses, where there is ascospore death. Whereas crosses homozygous for *Sad-1*$^{\Delta}$ or *Sad-2*^{RIP} are completely barren and the asci are arrested in meiotic prophase, all crosses homozygous for *Sk* are fully fertile. Similar suppression of meiotic silencing by *Sk*, as effectively as *Sad-1*$^{\Delta}$ and *Sad-2*^{RIP}, was also observed in crosses of *Sk-2* or *Sk-3* with *asm-1*$^{+ect}$, *actin*$^{+ect}$, *mei-3*$^{+ect}$, *β-tubulin*$^{+ect}$, *β-tubulin-GFP*^{ect} inserts (Raju *et al* 2007). However, unlike the *sad-1* and *sad-2* genes, whose functions and the mechanism of suppression are known, the function(s) of *Sk* haplotypes in either spore killing or in the suppression of meiotic silencing is still a mystery. One possibility is that the *Sk* haplotypes contain *Sad*-like gene(s) that suppress meiotic silencing both in heterozygous and homozygous *Sk* crosses.

12. A model for meiotic silencing based on RNA interference

In *N. crassa*, meiotic silencing is based on unpaired regions of heterology during meiosis, and it shares many of the

components of the post-transcriptional gene silencing process in various plants and animals. The unpaired DNA sequences during meiotic prophase likely trigger the production of aberrant RNA, which is converted into double-stranded RNA (dsRNA) by the RdRP from *sad-1*. Dicers (SMS-3/DCL-1) apparently cut dsRNA into small interfering RNA (siRNA), which subsequently interfere with the functioning of mRNA from all sequences that are homologous to the unpaired sequences. At least two other essential RNAi components of meiotic silencing have been identified in *Neurospora*. Lee *et al* (2003) have shown that SMS-2 is an Argonaute-like protein, which is an essential component of the RNA-induced silencing complex (RISC). SAD-2 protein is also required for meiotic silencing, and it recruits SAD-1 RdRP and localizes it to the perinuclear region, where the aberrant RNA is converted to dsRNA by the action of RdRP (Shiu *et al* 2006). We have shown that when an essential component of the silencing machinery is rendered non-functional (e.g. *Sad-1*$^{\Delta}$) and made heterozygous in a cross (*Sad-1*$^{\Delta}$ x *hH1-GFP*^{ect}), the *sad-1*$^{+}$ gene itself is unpaired and self-silenced, resulting in the normal expression of *hH1-GFP*^{ect}. Thus, deletions or extensive mutations in *sad-1*, *sad-2*, *sms-2* behave as dominant suppressors of meiotic silencing by the various unpaired ectopic gene inserts (Shiu *et al* 2001, 2006; Kelly and Aramayo 2007). In addition, we have recently shown that *Neurospora* spore killers *Sk-2* and *Sk-3* function as suppressors of meiotic silencing although nothing is known about their mode of action in the silencing process (Raju *et al* 2007).

13. Meiotic silencing in other organisms

Since the discovery of post-transcriptional gene silencing in plants and *Neurospora* in the early 1990s, similar examples of silencing or inactivation phenomena in nematodes and mammals have been re-examined in terms of RNAi silencing (*see* Kelly and Aramayo 2007 for references). These include X chromosome inactivation or imprinting during spermiogenesis in *Caenorhabditis elegans*, mouse and other mammals. Meiotic silencing and X chromosome inactivation do occur in mammals even though there is no obvious RdRP orthologue in mammalian genomes. *C. elegans* contains RNAi in germ cells and shows meiotic silencing; a putative RdRP (EGO-1 protein) is required for normal meiosis and fertility. In contrast, *Drosophila* neither contains the required RdRP, nor shows meiotic silencing. Yet, *Drosophila* X chromosome is transcriptionally inactivated in male meiosis in a process named meiotic sex chromosome inactivation (*see* Kelly and Aramayo 2007). Although meiotic silencing is evident in diverse organisms, it is apparently absent in *N. tetrasperma* and several other fungi (Jacobson *et al* 2008, *see below*).

14. The absence of meiotic silencing in several fungi

We have recently used *N. tetrasperma* to evaluate both the generality of meiotic silencing within the *Neurospora* genus and its possible evolutionary significance. Several *hH1-GFP* constructs were introgressed from *N. crassa* into various chromosome locations in *N. tetrasperma*. *N. tetrasperma* differs from *N. crassa* in showing a long unsynapsed (unpaired) region in linkage group I (longest chromosome), and the consequent recombination block in the same linkage group (Gallegos *et al* 2000). The *sad-1*$^{+}$ gene is located in the long unpaired middle region of this chromosome, which only shows synapsis at both ends. Even when both parents carry *sad-1*$^{+}$, the sequences are physically and, perhaps, functionally unpaired. Our results indicate that there is no meiotic silencing of *hH1-GFP*^{ect} in this pseudohomothallic species (figure 10A, B), presumably because *sad-1*$^{+}$ is naturally unpaired and self-silenced during meiosis by structural differences between *N. tetrasperma* mating-type chromosomes (Gallegos *et al* 2000; Jacobson *et al* 2008). Thus, when RdRP is absent or present in limited amounts, the silencing mechanism is not operational, resulting in the expression of hH1-GFP throughout meiosis and ascospore development. Similar absence of meiotic silencing has been observed in an *Eight-spore* mutant of *N. tetrasperma*. For example, in a cross of *E* x *hH1-GFP*^{ect}, where most of the asci produce 8 ascospores, the developing asci show normal expression of hH1-GFP (Raju 1992b; Jacobson *et al* 2008). We do not yet know the universality of the meiotic silencing phenomenon in the 8-spored species of *Neurospora*, or the universality of its absence in other pseudohomothallic species. Here I briefly summarize the work on several other fungi that do not show meiotic silencing.

Another 4-spored pseudohomothallic species, *P. anserina*, has been extensively studied and its ascus development programme closely resembles that of *N. tetrasperma*. Bonnet *et al* (2006) used GFP-tagged gene inserts in heterozygous crosses and found no evidence of meiotic silencing during ascus development. However, it is not known whether there is a suppressor mechanism of meiotic silencing in *P. anserina*, and there is also no evidence for unsynapsed regions in *P. anserina,* as is the case with *N. tetrasperma* (*see above*). There is indirect evidence that two other 8-spored Ascomycota (*Aspergillus nidulans* and *Sordaria macrospora*) also do not show meiotic silencing (van Heemst *et al* 1999). Although *A. nidulans* has lost an orthologue of RdRP (QDE-1), it does show inverted repeat transgene-induced RNA silencing in the vegetative cells (Hammond and Keller 2006). Two other fungi, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have been extensively characterized genetically and molecularly. The budding yeast *S. cerevisiae* neither contains RNAi machinery nor shows meiotic silencing or

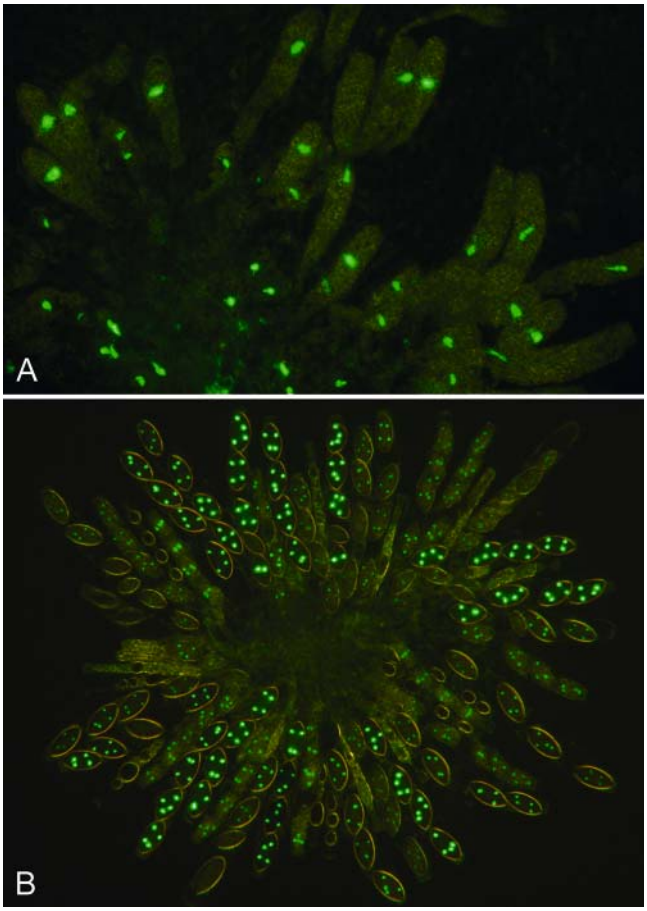

Figure 10. Rosettes of asci of *Neurospora tetrasperma* from wild type x *hH1-GFP*^{ect}, showing the absence of meiotic silencing. (**A**) Young asci at 3–4 days after fertilization, showing brightly fluorescing nuclei during meiosis I. (**B**) A rosette of maturing asci, at 6 days after fertilization, shows mostly 4-spored asci. The ascospores are heterokaryotic for *hH1-GFP*^{ect} but all 4 nuclei (2 *hH1-GFP*^{ect} + 2 non-*hH1-GFP*^{ect}) show hH1-GFP, because they share the same cytoplasm.

quelling. The fission yeast *S. pombe* contains all of the RNAi components required for silencing and shows other forms of chromatin silencing, yet it shows no meiotic silencing or quelling in the vegetative phase (*see* Allshire and Selker 2007; Kelly and Aramayo 2007, for references).

15. Role of silencing in genome defence

To date, three silencing mechanisms have been found in *N. crassa*. (i) Repeat-induced point (RIP) mutation recognizes duplicated sequences in the genome and causes multiple C to T transitions in the premeiotic ascogenous cells. The resulting mutations in the genome are permanent (Selker 2002; Allshire and Selker 2007). (ii) Quelling detects multiple sequences in the haploid genome following transformation, and silences the expression of these sequences in the vegetative phase. Quelling is often reversible resulting in wild-type or intermediate phenotypes (Romano and Macino 1992). (iii) Meiotic silencing is similar to quelling mechanistically but it is based on heterology of DNA sequences that fail to pair during meiosis; it operates only in the developing asci prior to ascospore delimitation. All three silencing phenomena have been implicated to play significant roles in genome defence against invading transposable elements and viruses during specific stages of the life cycle of *N. crassa*. Quelling and meiotic silencing in *Neurospora* share RNAi-mediated genome defence mechanisms, which have also been identified in other organisms (*see* Kelly and Aramayo 2007). Meiotic silencing prevents the expression of novel insertions and their native homologues in the genome during the sexual cycle. This heterology-based meiotic silencing is especially effective in restricting fertility in naturally outbreeding heterothallic species such as *N. crassa*, because such outcrosses are likely to be heterozygous for the inserted sequences, silenced and unproductive. When a major component of meiotic silencing is deleted or mutated (e.g. *Sad-1*$^{\Delta}$, *Sad-2*^{RIP}), heterology-based meiotic silencing is suppressed, because *sad-1* itself is self-silenced. In addition, *Sad-1*$^{\Delta}$ partially removes fertility barriers in certain interspecific crosses or in crosses that are otherwise barren (e.g. *Dp* x *Sad-1*$^{\Delta}$; Shiu *et al* 2001).

16. Conclusion

Dodge's discovery of *Neurospora* mating types and its sexual biology, Beadle and Tatum's demonstration that genes specify enzymes, and David Perkins' nurturing of *Neurospora* since 1949 have made this filamentous fungus a pre-eminent model for genetic, developmental, cytogenetic and cytological studies and, more recently, for the molecular analysis of its sexual cycle (*see* Perkins 1992, 1997; Perkins *et al* 2001; Jacobson *et al* 2008; Raju *et al* 2007; Shiu *et al* 2001). The genome of *N. crassa* has been sequenced (43 Mb), and found to contain over 10 000 protein-coding genes (Galagan *et al* 2003; Colot *et al* 2006). Gene knockouts or mutations in specific sequences could now be readily correlated with observed cytological defects in the sexual stage. Most of the *Neurospora* mutants, deletion strains and gene knockout strains are available from the Fungal Genetics Stock Center (*http://www.fgsc.net*). I am happy to have contributed to the elucidation of the normal processes underlying ascus and ascospore development, abnormal processes in numerous mutant strains, chromosome rearrangements, spore killers and meiotic silencing. It is hoped that the recent discovery of meiotic silencing in *Neurospora*, the use of immunofluorescence and GFP-tagged genes for studying gene expression (or silencing), and the availability of gene sequences for knockout studies,

will pave the way for the molecular analysis of complex processes during ascus and ascospore development.

I would like to end this review with a tribute to David Perkins (1919–2007) and Robert Metzenberg (1930–2007), who contributed much to the fungal genetics community during the many decades of their illustrious careers at Stanford University and at the University of Wisconsin, respectively. In addition to their numerous scientific contributions, they both helped many *Neurospora* researchers, near and far, by sharing knowledge, new methods, strains, advice, enthusiasm and encouragement until a couple of weeks before their deaths (*see* Raju 2007; Selker 2008). In early 1974, an unexpected letter arrived from David Perkins, whom I had never met or communicated with before, asking me to arrange our meeting in Hyderabad. He wrote – "*I have learned from Dr Benjamin Lu that you are now working in the area of Hyderabad. Since I have admired the cytological work you and he have done on meiotic chromosomes in fungi, it would be a pleasure to meet you if this can be arranged during my visit.*" The ensuing meeting on 18 February at the Osmania University (Hyderabad) guesthouse resulted in my pilgrimage to the legendary Perkins' *Neurospora* laboratory a few months later. His guiding light inspired me throughout my 33-year tenure at Stanford. Benjamin Lu trained me in fungal cytology in 1968, and to this day he is my unsung hero.

Acknowledgements

I thank David Jacobson (University of California, Berkeley) for helpful suggestions during the preparation of this manuscript, and Martin Bidartondo (Imperial College, London), David Jacobson, P Maruthi Mohan (Osmania University, Hyderabad), and Matthew Springer (University of California, San Francisco), for the use of their *Neurospora* images reproduced here as figures 1A–C and 3B. I am indebted to Michael Freitag (Oregon State University, Corvallis) for making available several of his *hH1-GFP* and *β-tubulin-GFP* constructs for my meiotic silencing studies.

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MS received 17 October 2008; accepted 6 November 2008

*e*Publication: 19 January 2009

Corresponding editor: DURGADAS P KASBEKAR