JOURNAL OF PROTEINS AND PROTEOMICS
Vol. 1, No. 1, January-June 2010, pp. 15-23

Genome Defense Mechanisms in *Neurospora* and Associated Specialized Proteins

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Abstract: *Neurospora crassa, the filamentous fungus possesses widest array of genome defense mechanisms known to any eukaryotic organism, including a process called repeat-induced point mutation (RIP). RIP is a genome defense mechanism that hypermutates repetitive DNA sequences; analogous to genomic imprinting in mammals. As an impact of RIP, Neurospora possesses many fewer genes in multigene families than expected. A DNA methyltransferase homologue, RID was shown to be essential for RIP. Recently, a variant catalytic subunit of translesion DNA polymerase zeta (Pol ζ) has been found to be essential for dominant RIP suppressor phenotype. Meiotic silencing and quelling are two other genome defense mechanisms in Neurospora, and proteins required for these two processes have been identified through genetic screens.*

Keywords: *Argonaute-like Protein, DNA Methyltransferanse, Meiotic Silencing, Quelling, Repeat-induced Point Mutation, RNA-dependent RNA Polymerase, Translesion DNA Polymerase.*

I INTRODUCTION

Neurospora crassa has become a model for the assemblage of over 250,000 species of non-yeast fungi, was first documented in 1843 as a contaminant of bakeries in Paris and developed as an experimental organism in the 1920s (Payen, 1843; Shear and Doge, 1927; Lindegren, 1936). *N. crassa* provided the first example of the genome defense systems, called repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD), and provided an early example of RNAi-based gene silencing in the process called quelling.

Neurospora possesses active processes that shape its own genome, unlike the cases where genome shapes an organism. In both the sexual phase and in the vegetative phase of *Neurospora*, defense mechanisms scan the genome to counteract the invasion of viruses, retrotransposons, and insertion sequences, which, if unchecked, can have deadly consequences to the organism (Shiu and Metzenberg, 2002). Any sequence of ~1 kb or larger that is present in more than a single copy is likely to be a retrotransposon capable of damaging the integrity of the genome (Pratt et al., 2004). Filamentous fungal genomes are at a greater risk than those of plants and animals because the same cytoplasm is shared by many nuclei in these organisms. Possibly due to this reason, *Neurospora* genome encodes specialized proteins that on one hand protect it against invading parasitic elements and on the other hand, prevent evolution of new protein through gene duplication. Therefore, *Neurospora* is also the first example where proteins trim genome (Borkovich et al., 2004; Galagan et al., 2003). An up-to-date understandings of repeat-induced point mutation, meiotic silencing, quelling, and the protein products predicted to be required for these processes have been discussed in this review.

II REPEAT-INDUCED POINT MUTATION (RIP)

This process was first described as “rearrangement induced premeiotically” (Selker et al., 1987a). In strains transformed with single copies of a plasmid that included host sequences, both elements of the duplication, that from the plasmid and that from the host, became altered in the sexual phase of the life cycle by multiple C:G to T:A mutations and the remaining cytosine residues typically became methylated *de novo*. Subsequent to this finding the process was renamed “repeat-induced point mutation” (Cambareri et al., 1989; Selker, 1990). Results from tetrad analyses indicated that RIP occurs before meiosis, during a stage between fertilization and karyogamy.

III ACTION OF RIP

RIP operates on duplicated sequences that are > 400 base pairs in length and have > 80% sequence identity (Cambareri et al., 1991; Watters et al., 1999). The C:G to T:A mutations within the duplicated sequence preferentially occurs at cytosines immediately 5’ of adenines (CpA). In a segment of RIP altered linked

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duplication that was sequenced, ~64%, ~18%, ~13% and ~5% of the mutations were in, CpA, CpT, CpG and CpC dinucleotides respectively (Cambareri et al., 1989). The preference for mutating CpA to TpA, increases the prevalence of the stop codons TAA and TAG and computer simulations indicate that after a duplication, each copy has an 80% probability of acquiring an in-frame stop codon after only a single round of RIP and a 99.5% probability that RIP would mutate the copies to less than 85% nucleotide similarity (Galagan et al., 2003). In addition to suffering mutations, RIP-mutated sequences are frequent targets for DNA methylation that interferes with gene expression by preventing transcription elongation and therefore, can cause gene silencing as in the mammals (Rountree and Selker, 1997). RIP thus mutates and epigenetically silences repetitive DNA. However, RIP associated cytosine methylation is not restricted to the duplicated segments; it can also extend beyond the duplicated region into flanking single-copy sequences (Irelan and Selker, 1997; Prakash et al., 1999; Selker et al., 1993; Vyas and Kasbekar, 2005). The extent of methylation and severity of RIP are correlated: greater the number of RIP-induced mutations, heavier the methylation (Singer et al., 1995a, b). Sequences once altered by RIP remain susceptible to it in subsequent generations, even after six generations. However, if both copies have diverged by ~30% they become resistant to RIP (Cambareri et al., 1991). RIP-induced mutations are generally restricted to the duplicated region but there is evidence that mutations can infrequently "spill" over into the adjoining single copy DNA (Foss and Selker, 1991; Irelan and Selker, 1997; Irelan et al., 1994; Perkins, 1997; Vyas and Kasbekar, 2005).

IV MECHANISM OF RIP

RIP occurs in a microscopic ascogenous tissue, which limits biochemical approaches to reveal the mechanism of RIP. On the other hand, this tissue contains nuclei from each parent that prevents recognition of recessive defects by classical genetic studies. A screen for RIP efficiency among 446 wild-isolated *Neurospora* strains resulted in the identification of seven strains that could dominantly suppress RIP (Bhat et al., 2003). One of these seven strains is the Adiopodoumé strain and it was isolated from rice and sedge leaves collected near the biological research station at Adiopodoumé, Ivory Coast, in 1955.

The dominant RIP suppressor of the Adiopodoumé strain (hereafter referred to as "*Srp*" for Suppressor of RIP) displays sufficient penetrance and mapping studies have shown that the *Srp* is identical to the variant catalytic subunit of translesion DNA polymerase ζ (Pol ζ). A suppressor Pol ζ catalytic subunit interferes with the assembly or function of a putative RIP polymerase (Tamuli and Kasbekar, 2008). Translesion DNA polymerases Pol ζ, Pol η, Pol ι, Pol κ, and Rev1 are dispensable for RIP in *N. crassa* (Tamuli et al., 2006). The unprecedented polymorphisms of the Pol ζ catalytic subunit among *Neurospora* wild-isolates may indicate yet unknown functions of Pol ζ (Figure 1; Tamuli et al., 2006).

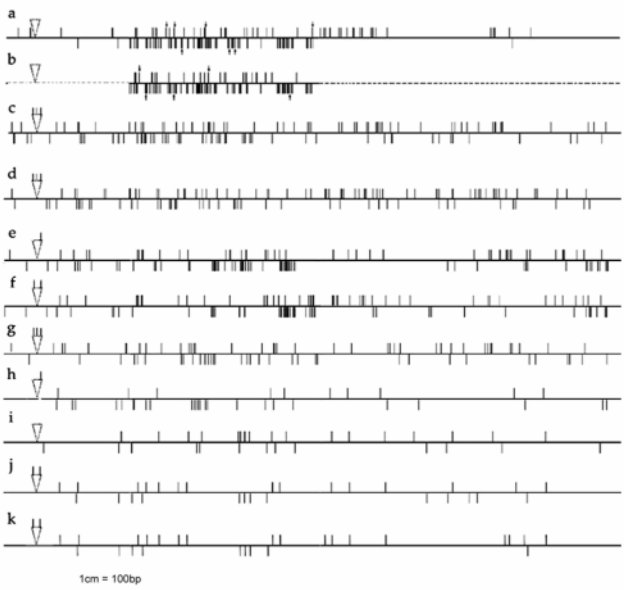

Figure 1: Sequence differences in *upr-1* alleles of different strains relative to the OR sequence. Synonymous and non-synonymous are shown as tick marks, respectively, above and below the corresponding lines. The triangles represent the 101 base intron sequences. (a) Adiopodoumé -1 (FGSC 430), (b) Adiopodoumé –7 (P4305), (c) *N. tetrasperma* 85 *a*, (d) *N. tetrasperma* 85 *A*, (e) Makaba-2 (P3816), (f) Dagguluru (P3360), (g) Golur (P0334), (h) Colonia Paraiso (P4212), (i) Franklin (P4467), (j) Fred (P0833) and (k) Coon (P0881). For the *upr-1* allele of Adiopodoumé –7 only the intron and a 1830 bp segment was sequenced. The unsequenced portion of the ORF is indicated by the dotted line. The arrows in (a) and (b) identify sequence changes that are specific to the Adiopodoumé -1 and Adiopodoumé –7 strains, respectively. The two strains were identical at all the other sites in the 1830 bp segment. (See Tamuli et al., 2006 for details).

Previously, only one component of the molecular machinery for RIP, called *rid* (RIP-defective), had been reported and the question whether RID has DMT and/or deaminase activity remains unanswered (Freitag et al., 2002). This gene is predicted to encode a DNA methyltransferase (DMT), RID, that contains all six highly conserved eukaryotic DMTs motifs and crosses homozygous for the *rid* mutant display no RIP. RID predicted to be an 845-aa tripartite protein and the center domain has all motifs found in known eukaryotic DMTs in the conventional order, including the AdoMet-binding and catalytic Pro-Cys sites; motifs I and IV, respectively (Figure 2). The *rid* gene was identified based on the homology with a putative DNA methyltransferase in *Ascobolus immersus*, *Masc1*, which is required for a

related gene-silencing process, methylation-induced premeiotically (MIP) triggered by repeat sequences during the sexual cycle in *Ascobolus* (Malagnac et al., 1997). Unlike RIP, MIP causes only methylation of the duplicated sequences without any associated mutations (Rhounim et al., 1992; Rossignol and Faugeron, 1995; 1994).

Figure 2: Alignment of the catalytic DMT domain of RID homologues from *N. crassa* (RID), *A. fumigatus* (DmtA), and *A. immersus* (Masc 1), DMT motifs are indicated by Roman numerals. Residues identical in all eukaryotic DMTs are marked by asterisks; positions that have conservative substitutions are indicated by dots. Reproduced with permission from Freitag et al., 2002.

Analyses of RIP in nuclei harboring more than two copies of a gene showed that RIP operates in a pair-wise manner (Selker and Garrett, 1988; Fincham et al., 1989). This suggested that RIP involves direct DNA-DNA pairing akin to homologous recombination. However, RIP was not impaired neither by a mutation in *mei-2* that abolishes chromosome pairing and meiotic recombination nor by a mutation in *rec-2* that reduces excision of tandem duplications (Bowring and Catcheside, 1993; Foss and Selker, 1991). Since in the G2 phase of cell cycle all sequences are present in duplicate, recognition of duplications probably occurs in G1 and stable pairs of homologous sequences sharing a minimum length would accumulate. The replication machinery, with its accessory RIP-apparatus, would then modify and dissolve the paired sequences (Selker, 1990).

Three different possible mechanisms of RIP were proposed and kinetic behaviour of the enzymatic models were theoretically analyzed (Selker, 1990; Mautino and Rosa, 1998). The C:G to T:A mutation could occur by (a) enzymatic deamination of cytosine to form uracil followed by DNA replication and if C→U transitions escape the DNA repair system such as uracil DNA glycosylase, they would be fixed as C→T transitions in successive DNA replication cycles. The altered sequences could later direct *de novo* DNA methylation. (b) Alternatively, cytosine methylation followed by deamination by a hypothetical DNA- (5-methylcytosine) deaminase (5m-cytosine deaminase) that would catalyse the conversion of 5m-cytosine to T (Rossignol and Faugeron, 1994). (c) The third model was proposed based on the proposed mechanism of cytosine methylation (Santi et al., 1983; Wu and Santi, 1987). Enzymatic methylation of cytosine that involves a nucleophilic attack at the 6 position of cytosine to form a reactive 5, 6-dihydrocytosine intermediate which results in a negative charge at the 5 position that activates this previously inert carbon to accept a methyl group from S-adenosylmethionine (SAM) and convert into 5-methylcytosine. Alternatively, it can tautomerize to an enamine capable of suffering a subsequent spontaneous hydrolysis, giving rise to uracil. As the tautomerization of the intermediate is a unimolecular reaction, the probability of deamination would depend on the half-life of the complex, which in turn depends on the cellular concentration of SAM. Under limiting concentrations of SAM, cytosine deamination would be favored than methylation. The G:U mismatch generated during this process, if not repaired before replication, would be fixed as a C→T transition mutation (Yang et al., 1996). However, a DNA-cytosine deaminase remains to be shown in *Neurospora*. That RID encodes a putative DMT is consistent with the hypothesis that RIP can involve methylation of cytosines and subsequent enzymatic deamination to yield thymines (Hagemann and Selker, 1996; Mautino and Rosa, 1998; Selker, 1990; Watters et al., 1999).

RIP-mediated transcriptional gene silencing is independent of RNA interference (RNAi) mechanism (called quelling in *Neurospora*), although, RNAi machinery may contribute to control transposon relics and repeated sequences by targeting degradation of transcripts derived from these regions (Chicas et al., 2004;

Hannon, 2002; Tamuli and Kasbekar, unpublished). Additional factors that determine how RIP detects small duplications efficiently in the context of the entire genome remain to be discovered.

V IMPACT OF RIP

The analysis of the ~42.9 Mb *N. crassa* genome predicted a total of 10,082 protein-coding genes (Galagan et al., 2003; Borkovich et al., 2004). Their examination revealed that *Neurospora* possesses many fewer genes in multigene families than is expected for a genome of its size and this is in marked contrast to other sequenced genomes. *Neurospora* contains only eight genes with top matches of greater than 80% amino acid or coding sequence identity. The small proportion of genes in multigene families and the near absence of highly similar genes are consistent with the actions of RIP. Secondly, although *Neurospora* possesses nearly twice as many genes as *Schizosaccharomyces pombe* (~ 4,800) and *S. cerevisiae* (~ 6,300) and as many as genes as *Drosophila melanogaster* (~14,300) there is a paucity of highly similar gene pairs. Third and most importantly, consistent with the hypothesis that RIP acts as a defense mechanism against selfish DNA (Selker, 1990), no intact mobile elements were identified in the sequenced *Neurospora* genome.

Additionally, the German *Neurospora* Genome Project report for linkage groups II and V of *N. crassa* confirms absence of any chromosome or genome duplication and identified only small DNA sequence stretches in the range 4-10 kb with 57-74% sequence identities (Mannhaupt et al., 2003). The duplicated sequence stretches are all found in non-coding DNA and are very AT rich, none of the repeat regions encode transposon-like elements. For example, a putative conserved eukaryotic class II transposon Fot1, originally detected in *Fusarium oxysporum*, is not only much shorter in *Neurospora* (LGV) but, more importantly, only fragments of the *Fusarium* homolog can be identified and the terminal inverted repeats are missing (Daboussi *et al.*, 1992; Daboussi and Langin, 1994). Therefore, it appears to be an inactive element that has likely fallen victim to RIP (Mannhaupt et al., 2003).

In *Neurospora*, about 1.5% of the cytosines (C's) are methylated (Foss et al., 1993; Russell et al., 1987; Selker et al., 1987b), with an exception of no methylated protein coding genes identified. Three naturally methylated regions are identified, tandemly arranged rDNA (Perkins et al., 1986), the 1.6 kb zeta-eta (ζ-η) region (Miao et al., 2000; Selker and Stevens, 1985; 1987; Selker et al., 1993), and the psi-63 (ψ63) region (Foss et al., 1993; Margolin et al., 1998; Metzenberg et al., 1985; Miao et al., 1994). The last two regions are relics of RIP. It was proposed that almost all DNA methylation in the *Neurospora* genome, including the limited methylation of the rDNA, is a result of RIP (Borkovich et al., 2004). Consistent with this, a survey of methylated *Neurospora* sequences isolated by affinity chromatography, using the methyl binding domain of mammalian MeCP2, revealed clear evidence of RIP in nearly all sequenced fragments (Selker et al., 2003).

VI HOW DO GENES OR PROTEINS EVOLVE IN *NEUROSPORA* AGAINST THE BACKGROUND OF RIP?

The characteristics of RIP immediately suggest that it might have had a significant impact on evolution of *Neurospora*. RIP has had a powerful impact in suppressing the creation of new genes or partial genes through genomic duplication; consistent with the large number of mutations induced in duplicated sequences by RIP. RIP thus appears tailor-made to control "selfish" DNA such as transposons but RIP would also seem to hamper genome evolution considering that duplication of genes is thought to provide raw material for evolution (Ohno, 1970). Together with the scarcity of highly similar gene pairs, Galagan and Selker (2004) argued that most, if not all, paralogous genes in *Neurospora* duplicated and diverged before the emergence of RIP, and since that point the evolution of new genes through gene duplication has been virtually arrested. This conclusion raises the serious question of whether and how *Neurospora* might evolve new genes.

A genetic screen has identified seven wild-isolated *Neurospora* strains that dominantly suppress RIP (Noubissi et al., 2000; Bhat et al., 2003). It was also discovered that a small gene size duplication can escape RIP if the RIP machinery is titrated out by the presence of a large chromosome segment duplication in the same cross (Bhat and Kasbekar, 2001). Taken together, Kasbekar (1999; 2003) suggested that novel genes can still evolve in contexts where the efficiency of RIP has been tempered, as for instance in the Adiopodoumé strain. Consistent with this idea, the Adiopodoumé strain was the only *Neurospora* strain found possessing active copies of the transposable element *Tad* (Kinsey and Helber, 1989; Kinsey, 1989).

Additionally, if a RIP event does not introduce stop codon in duplicated sequences, a new gene or protein may evolve. Notably, the *Neurospora* genes possess greater structural complexity than *S. cerevisiae* and *S. pombe*, a predicted 17,118 introns (1.7 introns per gene),

compared to roughly 286 (0.04 intron per gene) and 4435 (0.95 intron per gene) in *S. cerevisiae* and *S. pombe*, respectively (Galagan et al., 2003; Borkovich et al., 2004). In contrast to the general case in eukaryotes, in euascomycetes histone genes are few in number and they contain introns that break up the contiguous stretches of homology. This might be a mechanism to escape from RIP and MIP that would otherwise detect sizable duplications and silence the associated genes. One of the best example that confirms this notion is that *hH4-1* and *hH4-2* in *Neurospora* that encode identical proteins and are 96% identical (300/312) at the nucleotide level in their coding regions, contain two introns, at precisely conserved locations, but show no similarity in the introns or in the 5' or 3' untranslated regions (UTRs). The intron lengths are also different. introns 1 and 2 of *hH4-1* are 69 and 68 bp, whereas the introns of *hH4-2* are 316 and 65 bp, respectively (Hays et al., 2002).

The only sizeable repetitive sequences known to persist in spite of RIP in *N. crassa* are 175-200 copies of the tandemly arranged, 9 kb rDNA repeats that give rise to the 17S, 5.8S and 25S rRNAs (Free et al., 1979; Galagan et al., 2003; Selker, 1990). This rDNA, which is located close to one end of Linkage Group V (Davis, 2000), forms a nucleolus organizer region (NOR), and probably this location, and not the rDNA sequence itself, protects them from RIP.

VII AN ASSAY FOR RIP

A convenient assay for RIP was developed that makes use of a duplicated 1.3 kb *Hind III* fragment of the *erg-3* gene (which codes for the sterol biosynthetic enzyme, sterol C-14 reductase). The duplicated fragment, designated *Dp(erg-3)*, is tagged with *hph*, the bacterial hygromycin resistance gene and inserted ectopically into LG VR linked to *al-3*. This transgene was previously described as *Dp1.3^{ec}hph* (Prakash et al., 1999; Bhat et al., 2003). The ectopic copy of *erg-3* does not make a functional enzyme but serves to direct RIP to the endogenous *erg-3* locus on LG IIIR. The RIP-induced *erg-3* mutants are viable, female sterile, and resistant to the steroidal glycoside α-tomatine and sensitive to isoflavonoids (Sengupta et al., 1995). Most importantly, the colonies from *erg-3* mutant ascospores have a characteristic morphology on Vogel's-sorbose medium, which makes them very easy to distinguish from wild-type colonies under a dissection microscope (Figure 3; Noubissi et al., 2000). Thus, even rare RIP events can be readily scored.

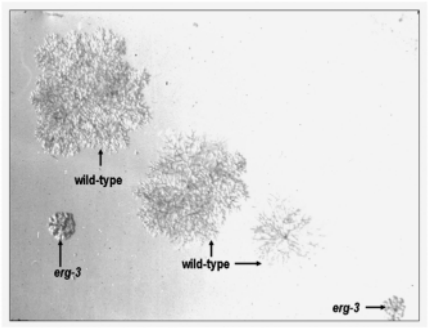

Figure 3: Colonies Derived from Wild-type and erg-3 Mutant Ascospores. The erg-3 and the Wild-type Colonies on Vogel's-sorbose Medium are Indicated.

VIII RIP IN OTHER FUNGI

RIP-like process has been also reported in *Podospera anserina* (Hamman et al., 2000; Graia et al., 2001), *Fusarium oxysporum* (Hua-Van et al., 2001; Daboussi et al., 2002), *Magnaporthe grisea* (Nakayashiki et al., 1999; Ikeda et al., 2002), *Microbotryum violaceum* (Hood et al., 2005), *Leptosphaeria maculans* (Attard et al., 2005), *Aspergillus nidulans* (Clutterbuck, 2004), *A. fumigatus* (Neuveglise et al., 1996), *A. oryzae* (Montiel et al., 2006). *F. oxysporum* is a pathogenic fungus and *M. grisea* is that most destructive rice pathogen. However in other fungi, RIP is very inefficient and has allowed the survival of transposons. In contrast, in *N. crassa* RIP efficiency can be as high as >90% for linked duplications. RIP was also demonstrated in *N. tetrasperma*, a pseudohomothallic species (in which a sexual cross results in the formation of four haploid, binucleate ascospores each of which contains one *mat A* and one *mat a* nucleus and is therefore self fertile), and using *N. tetrasperma* it is possible to identify recessive RIP-defective mutants (Bhat et al., 2004).

IX MEIOTIC SILENCING

Initially termed as meiotic transvection (Aramayo and Metzenberg, 1996), meiotic silencing by unpaired DNA (MSUD) is an RNAi based gene silencing process that causes silencing of genes that are unpaired in meiosis and their homologues (Shiu et al., 2001; Shiu and Metzenberg, 2002). "MSUD" is also the acronym for ''maple syrup urine disease,'' therefore, the term ''meiotic silencing'' is now preferred (Vyas et al., 2006). In duplication-heterozygous crosses the silencing of the duplication-borne genes results in the barren phenotype of such crosses (Shiu et al., 2001). Genes known to be involved in meiotic silencing are *Sad-1* that encodes an RNA-dependent RNA polymerase (Shiu et al., 2001), *Sad-*

2 that recruits SAD-1 to the perinuclear region (Shiu et al., 2006), *Sms-2* that encodes an argonaute-like protein (Lee et al., 2003), *Sms-3* that encodes a Dicer-like protein (McLaughlin and Aramayo, unpublished). Crosses homozygous for *Sad-1*, *Sad-2* and *Sms-2* are completely barren, arrested at meiotic prophase. The meiotic silencing mutants can enhance the productivity of duplication heterozygous cross (Shiu et al., 2001; 2006).

A possible function of meiotic silencing could be defense against spread of transposons at the time of meiosis and reproductive isolation of *Neurospora* species (Shiu et al., 2001). Meiotic silencing also has been discovered in mammals where chromatin regions unpaired in meiotic prophase cells are silenced (Baarends et al., 2005). In *C. elegans*, unpaired chromosomes and chromosomal regions accumulate high levels of histone H3 lysine 9 dimethylation (H3K9me2) that facilitates facultative heterochromatin assembly and results in transcriptional silencing during meiosis (Maine et al., 2005).

X QUELLING

Repeated sequences are detected and inactivated during the haploid, vegetative growth phase by an RNAi-based silencing mechanism called quelling (Cogoni and Macino, 2000; Cogoni, 2001; 2002; Romano, and Macino, 1992). Quelling produces diffusible signals, small interfering RNAs, which interfere with the propagation of the repeated element within nuclei in the same cytoplasm (Catalanotto et al., 2002). In *Neurospora* several genes, *qde-1*, *qde-2*, and *qde-3*, coding for an RNA-dependent RNA polymerase (RdRP), an argonaute-like protein, and a RecQ-like helicase, respectively, underlie quelling (Catalanotto et al., 2000; 2002; Cogoni et al., 1996; Cogoni and Macino, 1997; 1999a; 1999b). Crosses homozygous for either *qde-1* or *qde-3* are sterile, although, *qde-2* homozygous crosses are fertile.

XI CONCLUSIONS

Neurospora is an exceptional organism possessing stringent genome defense mechanisms to maintain genome integrity. The most significant defense mechanism that has an extraordinary impact on genome is repeat-induced point mutation. The protein coding genes required for these mechanisms have been identified despite the difficulties in performing classical biochemical experiment. Thus, *Neurospora* is a classic example where proteins play a vital role in dictating the shape and size of the genome. Further studies will identify additional protein components and uncover their mechanism of action.

XII ACKNOWLEDGEMENTS:

I thank Dr. D. P. Kasbekar, CCMB, for critical readings; Dr.V. K. Dubey and Dr. U. Bora, IITG for suggestions. The Fungal Genetics Stock Centre (FGSC) generously waived charges for strains.

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