A factor in a wild isolated *Neurospora crassa* strain enables a chromosome segment duplication to suppress repeat-induced point mutation

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Repeat-induced point mutation (RIP) is a sexual stage-specific mutational process of *Neurospora crassa* and other fungi that alters duplicated DNA sequences. Previous studies from our laboratory showed that chromosome segment duplications (*Dp*s) longer than ~300 kbp can dominantly suppress RIP, presumably by titration of the RIP machinery, and that although *Dp*s <200 kbp did not individually suppress RIP, they could do so in homozygous and multiply heterozygous crosses, provided the sum of the duplicated DNA exceeds ~300 kbp. Here we demonstrate suppression of RIP in a subset of progeny carrying the normally sub-threshold 154 kbp *Dp(R2394)* from a cross of *T(R2394)* to the wild isolated Carrefour Mme. Gras strain (CMG). Thus, the CMG strain contains a factor that together with *Dp(R2394)* produces a synthetic RIP suppressor phenotype. It is possible that the factor is a cryptic *Dp* that together with *Dp(R2394)* can exceed the size threshold for titration of the RIP machinery and thereby causes RIP suppression.

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1. Introduction

Repeat-induced point mutation (RIP) is a sexual stage-specific mutational process of *Neurospora* and other fungi that induces G:C to A:T mutations and cytosine methylation in duplicated DNA segments longer than 400 bp and sharing >85% sequence identity (Cambareri *et al.* 1989; Selker 1990; Watters *et al.* 1999; Galagan *et al.* 2003; Galagan and Selker 2004; Clutterbuck 2011). We showed previously that the frequency with which a short, gene-sized (i.e. 1–2 kbp) duplication undergoes RIP in *Neurospora crassa* is decreased if the cross is also heterozygous for another longer (i.e. >~300 kbp) chromosome segment duplication (Bhat and Kasbekar 2001; Fehmer *et al.* 2001; Singh and Kasbekar 2008; Singh *et al.* 2009). Adjacent 1 segregation in a cross of a strain bearing an insertional or quasiterminal translocation (*T*) with a normal sequence strain (*N*) can generate progeny that now carry a duplication (*Dp*) of the translocated segment (for a figure, *see* Singh *et al.* 2009 or 2010). Perkins *et al*. (1997) first demonstrated that long *Dp*s also are substrates for RIP. The long duplication apparently outcompetes the short duplication for the RIP machinery when both duplications are present in a cross, and the competition is effective regardless of whether the long and short duplications are in the same nucleus or in separate nuclei of the ascogenous dikaryon. Long duplications thus behave as dominant suppressors of RIP. [The term 'duplication' (*Dp*) is used to designate either a chromosome segment that is present as two non-tandem copies or a strain that contains such a segment.] Three *Dp*s, (*Dp(B362i)*, *Dp(EB4)* and *Dp(R2394)*), that are only 118–154 kbp in size (Singh *et al.* 2009, 2010), were RIP non-suppressors in heterozygous crosses, but could suppress RIP in homozygous and multiply heterozygous crosses, provided the sum of the duplicated DNA was >~300 kbp (Singh and Kasbekar 2008). This suggested that *Dp*s might titrate out the RIP machinery, and the 'equivalence point' of titration is ~300 kbp.

The RIP non-suppressor phenotype of *Dp(B362i)*, *Dp(EB4)* and *Dp(R2394)* was established using the *Dp* segregants from

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crosses of the *Dp*-generating translocations *T(B362i)*, *T(EB4)* and *T(R2394)* with the standard laboratory Oak Ridge (OR) strains of the opposite mating type (Singh *et al.* 2009, 2010). We now report that RIP can be suppressed by a subset of *Dp(R2394)* progeny from a cross of *T(R2394) a* with the wild isolated strain from Haiti, Carrefour Mme. Gras (abbreviated hereafter to CMG).

Crosses of strains bearing a duplication of the *erg-3* gene, *Dp(erg-3)*, with seven wild isolated strains (of ~460 tested), namely, Adiopodoumé (FGSC 430), Adiopodoumé-7 (P4305), Bayan Lepas (P2663), Coon (P0881), Fred (P0833), Golur-1 (P0334) and Sugartown (P0854), also were suppressed in RIP (Noubissi *et al.* 2000; 2001; Bhat *et al.* 2003). RIP suppression by the Adiopodoumé-7, Bayan Lepas, Coon and Fred strains showed complex inheritance (Vyas *et al.* 2006). That is, less than 1 in 6 of the f_1 progeny from the crosses of the OR strains with the Adiopodoumé-7, Bayan Lepas, Coon and Fred strains showed the RIP suppressor phenotype, suggesting that suppression required the inheritance of more than one locus from the wild parent. We now report that a larger fraction of the f_1 progeny from crosses of CMG with RIP suppressor derivatives of Coon and Fred can suppress RIP. Presumably, the CMG strain contains a factor that can produce a RIP suppressor phenotype along with factors from Coon and Fred.

2. Materials and methods

2.1 *Neurospora crassa strains, their general genetic manipulation*

Neurospora genetic analysis was done essentially as described by Davis and De Serres (1970). The *N. crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO 64110, USA. They included the standard laboratory OR strains 74-OR23–1 *A* (FGSC 987) and OR8–1 *a* (FGSC 988); and the translocation strain *T(IIL>IVR)R2394 A* (FGSC 2757, henceforth referred to as *T(R2394) A*). The *T(R2394) A* strain was crossed with OR8-1 *a* and a *T(R2394) a* segregant was obtained from this cross. The *Sad-1 A* (FGSC 8740) and *Sad-1 a* (FGSC 8741) strains were kindly provided by the late Robert L Metzenberg.

Dp(R2394) strains were obtained as segregants from crosses of the *Dp*-generating translocation *T(R2394) a* with OR A and CMG A, and were recognized by the barren phenotype of the *Dp*-heterozygous crosses (i.e. *Dp*×OR) (Perkins 1997). Barren crosses make normal-looking perithecia but produce exceptionally few ascospores. The barrenness of *Dp*–heterozygous crosses is caused, at least in part, by a gene-silencing process called meiotic silencing by unpaired DNA, a presumed RNAi-mediated elimination of the transcripts of any gene not properly paired with a homolog in meiosis (Aramayo and Metzenberg 1996; Shiu *et al.* 2001, 2006). The semi-dominant *Sad-1* suppressor of meiotic silencing was used to increase the productivity of *Dp*–heterozygous crosses.

2.2 *The Dp(erg-3)-based RIP assay*

Strains *Dp(erg-3) A* and *Dp(erg-3) a* carrying a duplicated *erg-3* gene were used to assay for RIP as previously described (Bhat *et al.* 2003; Prakash *et al.* 1999). The strains contain the transgene *Dp(erg-3)* tagged with the bacterial *hph* gene for resistance to hygromycin, and that duplicates a 1.2 kbp segment of the LG IIIR gene *ergosterol-3* (*erg-3*) coding for the ergosterol biosynthetic enzyme sterol C-14 reductase. The ectopically duplicated fragment serves to target RIP to *erg-3*. RIP-induced *erg-3* mutant progeny ascospores from crosses involving *Dp(erg-3)* strains produce colonies with a distinct morphology on Vogel's sorbose agar medium, which allows them to be easily distinguished from their erg^+ siblings under a dissection microscope (Noubissi *et al.* 2000). Typically, more than 200 colonies were scored when determining the frequency of RIP-induced *erg-3* mutations. Crosses of *Dp(erg-3)* with the wild-type and the non-suppressor *Dp*s yield RIP-induced *erg-3* mutants at frequencies of 2–25%, but in crosses with the suppressor *Dp*s, this frequency was <0.5 %. Conclusions made using the *Dp(erg-3)*-based RIP assay have previously been validated using another test gene, *dow*, and supposedly apply generally (Vyas *et al.* 2006). Gene symbols are italicized, while phenotype symbols are not. The non-italicized symbol Srp^+ signifies non-suppressor phenotype (frequency of RIP-induced *erg-3* mutants >1 %), and non-italicized Srp^- signifies suppressor phenotype (frequency of RIP-induced *erg-3* mutants <0.5 %). In some crosses we used the *Sad-1; Dp(erg-3) a* strains described by Vyas *et al.* (2006).

2.3 *RIP-suppressor strains derived from the wild isolates Coon and Fred*

The strains Coon (P0881) and Fred (P0833) were two wild isolates previously identified to have the dominant suppressor of RIP phenotype (Bhat *et al.* 2003). The original strains were lost, therefore we used their RIP suppressor progeny #54 and 22 obtained from crosses of the Coon and Fred strains with *mat A* strains of the OR background. Segregant 54 is a dominant RIP suppressor segregant from the cross Coon a×OR A. No RIP-induced *erg-3* mutants were observed in 427 progeny from #54 *a*×*Dp(erg-3) A*. Segregant 54 is therefore indistinguishable in its RIP suppressor phenotype from its wild isolated Coon parent. Segregant 22 is a dominant RIP suppressor segregant from the cross Fred

a×*Dp(erg-3) A*. No RIP-induced *erg-3* mutants were observed in 271 progeny examined from 22 *a*×*Dp(erg-3) A*. Therefore, segregant 22 is indistinguishable in its RIP suppressor phenotype from its RIP suppressor Fred parent.

2.4 *Molecular markers*

Singh and Kasbekar (2008) reported the molecular marker to distinguish *Dp(R2394)* segregants from their non-*Dp* siblings. Briefly, genomic DNA was prepared from the f_1 progeny from the crosses of *T(R2394) a* with OR *A* and CMG *A* (especially those that gave a barren phenotype in crosses with *Dp(erg-3)* strains of opposite mating type). The DNA was used as template in PCR using the oligonucleotide primers 5′ CGAGACGGAGAATGGAGAAC and 5′ ACCTATGGACTGGACGAGGA, and the PCR amplified DNA was digested with *Hae*III. The restriction pattern for the amplicon from the *T(R2394)* strain differs from that of the amplicons from OR *A* and CMG *A*. DNA amplified using *Dp(R2394)* genomic DNA as template gives patterns of both parental alleles, namely, from the translocation (*T*) and the normal sequence (*N*) parent. The marker was also used to confirm that the non-barren segregants from the *T(R2394) a*×CMG *A* cross were either *T* or *N* strains.

3. Results

Dp progeny from a *T*×*N* cross can be distinguished by their barren phenotype in heterozygous crosses (i.e. *Dp*×*N*). Barren crosses make normal-looking perithecia but produce exceptionally few ascospores. The barren phenotype of *Dp*–heterozygous crosses is due to meiotic silencing by unpaired DNA (Shiu *et al.* 2001). Presumably, *Dp*s include one or more genes essential for meiosis and ascus formation and their presence in three copies in a *Dp*–heterozygous cross might cause one (or more) copy to not pair properly in meiosis, thus triggering silencing and rendering the cross barren. Semi-dominant suppressors of meiotic silencing (e.g. *Sad-1*, *Sad-2* and *Sms-2*) can significantly (> 100–1000 times) increase the productivity of *Dp*–heterozygous crosses (Shiu *et al.* 2001, 2006; Lee *et al.* 2003; Singh *et al.* 2009). The *Sad-1*, *Sad-2* and *Sms-2* suppressor alleles are presumed to disrupt the normal pairing of their wild-type homologs (i.e. *sad-1*$^+$, *sad-2*$^+$ and *sms-2*$^+$), and thereby induce them to silence themselves. The *sad-1*, *sad-2* and *sms-2* genes encode, respectively, a putative RNA-dependent RNA polymerase (RdRP) (Shiu *et al.* 2001), a protein required for the proper perinuclear localization of the SAD-1 RdRP (Shiu *et al.* 2006) and an argonaute-like protein used in meiotic silencing (Lee *et al.* 2003). A decrease in the level of any of these proteins might cause an overall lowering of meiotic silencing efficiency, thereby alleviating the silencing of *Dp*-borne genes, and thus increase the productivity of the duplication-heterozygous cross.

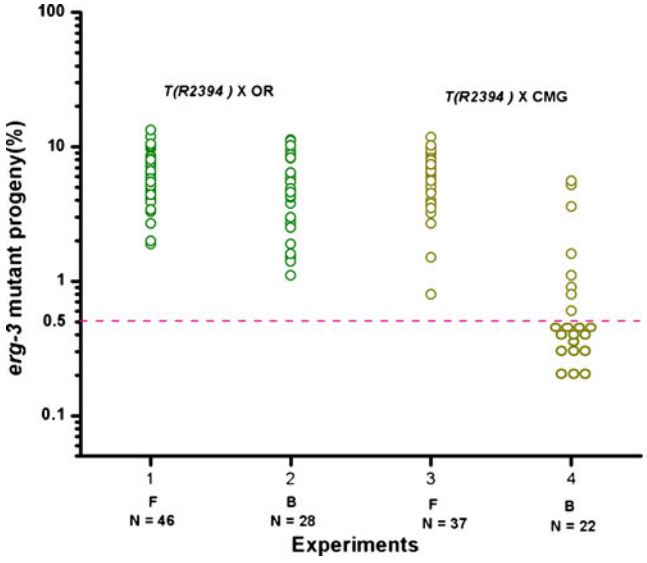

Figure 1. Suppression of RIP by *Dp(R2394)* segregants from *T(R2394)*×CMG. Frequency of RIP-induced *erg-3* mutant progeny obtained in crosses of *Dp(erg-3)* strains with f_1 segregants from *T(R2394) a*×OR *A* (experiments 1 and 2) or *T(R2394) a*×CMG *A* (experiments 3 and 4) expressed as percentages and plotted on a log scale. Frequency <0.5% (dotted line) defines the suppressor of RIP phenotype. Fertile segregants (F) were crossed with *Dp(erg-3)* strains and barren segregants (B) with *Sad-1; Dp(erg-3).* All fertile segregants from both the crosses are RIP non-suppressors (experiments 1 and 3), as are the barren segregants from *T(R2394) a*×OR *A* (experiment 2). However, the barren segregants from *T(R2394) a*×CMG *A* include several that are RIP suppressors (experiment 4). N is the number of segregants crossed with *Dp(erg-3)* strains to determine the frequency of RIP-induced *erg-3* mutants in the progeny.

The translocation strain *T(R2394) a* was crossed with OR *A* and CMG *A*. The f_1 segregants from these crosses were

Table 1. Srp$^-$ progeny from crosses between CMG *A* or OR *A* and strains representing the Coon and Fred RIP suppressor wild isolates

	×CMG *A*	×OR *A*
mat a parent	% Srp$^-$ Progeny (N)	
54 (Coon)	49.0 (92)	27.0 (96)
22 (Fred-2)	29.3 (99)	14.4 (90)

Strains 54 and 22 are derived from the RIP suppressor wild isolates Coon and Fred. The f_1 progeny from 54×CMG *A*, 54×OR *A*, 22×CMG *A*, and 22×OR *A* were crossed with *Dp(erg-3)* strains of opposite mating type and the frequency of RIP-induced *erg-3* progeny was determined. If this frequency was <0.5%, the f_1 progeny was determined to be of Srp$^-$ phenotype.

crossed with *Dp(erg-3)* and *Sad-1; Dp(erg-3)* strains of the opposite mating type. The frequency of RIP-induced *erg-3* mutant progeny in the latter crosses was determined, and the results are summarized in figure 1. Progeny from the crosses with OR *A* and CMG *A* that gave fertile crosses with *Dp(erg-3)* were non-*Dp(R2394)* in genotype (i.e. *T* or *N*; data not shown), and as expected, they were RIP non-suppressor in phenotype. The f_1 progeny that gave barren crosses with *Dp(erg-3)* were *Dp(R2394)* in genotype (data not shown), and to establish their RIP suppressor/non-suppressor phenotype, we scored the ascospores from the corresponding more productive crosses with *Sad-1; Dp(erg-3)*. All the *Dp(R2394)* progeny from *T(R2394) a*×OR *A* also were RIP non-suppressor in phenotype. This was consistent with previous results (Singh and Kasbekar, 2008). In contrast, 14 of the 22 *Dp(R2394)* progeny examined from *T(R2394) a*×CMG *A*, had the RIP suppressor phenotype, which suggested that the CMG strain contains a genetic factor that enables the 154 kbp *Dp(R2394)* to suppress RIP.

Strains #54 and #22 are Srp^- *mat a* segregants from, respectively, the crosses Coon *a*×OR *A* and Fred a×*Dp(erg-3) A* (*see* Materials and methods). We crossed these strains with OR *A* and CMG *A* and the f_1 progeny from these crosses were scored for their RIP suppressor/non-suppressor phenotype based on the frequency of RIP-induced *erg-3* progeny produced in their crosses with *Dp(erg-3)* strains of opposite mating type. The results, summarized in table 1, revealed that almost twice as many progeny from the crosses of #54 and #22 with CMG *A* than with OR *A* showed the RIP suppressor phenotype.

4. Discussion

We have found that although the wild isolated CMG strain is a RIP non-suppressor, it contains a genetic factor(s) that can enable the 154 kbp chromosome segment duplication *Dp(R2394)* to suppress RIP, possibly by reducing the titre of the RIP machinery to below the threshold required to support RIP. Intriguingly, the CMG strain also contains factors that, with other factors from RIP suppressor wild strains isolated from other geographical locations (Coon and Fred), can generate a synthetic RIP suppressor phenotype. We have also recently reported that crosses of CMG with testers derived from the OR background are moderately suppressed in meiotic silencing and consequently crosses of CMG with some *Dp*s (e.g. *Dp(R2394)* and *Dp(EB4)*) showed increased productivity, whereas the crosses of these *Dp*s in the OR background were barren (Kasbekar *et al.* 2011; and unpublished results of BKR, MR and DPK). Therefore, it is conceivable that *Dp*s comparable in size with *Dp(R2394)* can potentially lurk cryptically in the CMG strain (i.e. their crosses with OR-derived strains would remain non-barren). One attractive hypothesis is that the CMG strain contains *Dp*s that are individually below the size threshold required to titrate out the RIP machinery but that, together with *Dp(R2394)*, the amount of duplicated DNA exceeds this threshold to produce a synthetic RIP suppressor phenotype. Of course, just because meiotic silencing is compromised in crosses of CMG with OR-derived strains, the factors need not be *Dp*s. An alternative possibility is that the factors are alleles that encode elements of the RIP machinery that misrecognize DNA segments with less than ~85 % homology, and thereby increase the effective RIP substrate. *Dp* and non-*Dp* factors that instigate an ~300 kbp (i.e. <0.75 % of the genome) increase in RIP substrate would be sufficient to titrate out the RIP machinery and thus bring about a dominant RIP suppressor phenotype.

Crosses of the RIP suppressor strains Coon and Fred with OR-derived strains were non-barren, but because the original wild isolates are lost, we do not know whether their crosses with OR-derived strains were suppressed in meiotic silencing. Recent studies in our laboratory have revealed that crosses of OR-derived tester strains with a surprising majority of wild isolated *N. crassa* strains are at least as suppressed in meiotic silencing as are their crosses with CMG (MR, TNS, BKR and DPK, manuscript in preparation). Thus, the factors from Coon and Fred inherited by the strains #54 and #22 also might be *Dp*s, but given that these strains are themselves RIP suppressors, it is not as easy to test whether their factors can impart a synthetic RIP suppressor phenotype to *Dp(R2394)*.

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