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Titration of repeat-induced point mutation (RIP) by chromosome segment duplications in *Neurospora crassa*

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Abstract Repeat-induced point mutation (RIP) is a hypermutational process that alters duplicated DNA sequences in *Neurospora crassa*. In previous studies, five of six large (>100 kb) chromosome segment duplications (*Dp*'s) examined were shown to dominantly suppress RIP in smaller (<5 kb) duplications. The suppressor duplications were >270 kb, whereas the lone non-suppressor duplication was ~117 kb. We have now screened another 33 duplications and found 29 more suppressors and four more non-suppressors. All 22 suppressor duplications whose size could be estimated were >270 kb, whereas two newly identified non-suppressor duplications examined were 140–154 kb. RIP was suppressed in a subset of crosses heterozygous for more than one ordinarily non-suppressor duplication. These results strengthen the hypothesis that large duplications titrate out the RIP machinery and suggest the "equivalence point" for the titration is close to 300 kb.

Keywords Chromosome segment duplications · Dominant suppression of RIP · Neurospora genome

Introduction

Repeat-induced point mutation (RIP) is a genome defense process of Neurospora and other fungi that alters DNA sequences that are duplicated in an otherwise haploid genome, via hypermutation of G:C basepairs into A:T, and methylates many of the remaining C residues. It is presumed that RIP protects the genome against the proliferation of transposable elements and other repeated DNA sequences (Galagan and Selker 2004). RIP occurs during the sexual stage of the life cycle. For an introduction to Neurospora and a description of its life cycle see the Neurospora Home Page (http://www.fgsc.net/Neurospora/neurospora.html). Briefly, during the sexual stage colonies of the two mating type (*mat A* and *mat a*) come into contact and form fruiting bodies called perithecia. Within the perithecia, fusion of *mat A* and *mat a* nuclei results in the formation of transient diploid nuclei that immediately undergo meiosis. The four haploid products of one meiosis stay together in a sac called an ascus. In *Neurospora crassa* each of the four products of meiosis undergoes a further mitotic division, resulting in an octad of eight ascospores within each ascus. The ascospores are forcibly ejected from the perithecia and upon germination they produce hyphae of the progeny colonies. RIP occurs in the haploid nuclei of the premeiotic dikaryon that forms before the fusion of the *mat A* and *mat a* nuclei. Duplications >400 bp and sharing >85% sequence identity are substrates for the RIP machinery (Watters et al. 1999).

Previous studies from our laboratory showed that in *Neurospora crassa* a small gene-sized duplication (typically <5 kb) can escape RIP if another larger ($\gtrsim$270 kb) chromosome segment duplication (*Dp*) is present in the cross (Bhat and Kasbekar 2001; Fehmer et al. 2001; Bhat et al. 2003; Vyas et al. 2006). Duplication strains can be obtained in the laboratory as segregants from crosses between some translocation and normal sequence strains (see Perkins 1997, for a review). Depending on the translocation, the duplicated segment can be hundreds of

This article is dedicated to the memory of Robert L. Metzenberg.

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kilobases (kb) in size and it can include many genes. The duplication segregants are identifiable based on the barren phenotype of *Dp* × wild type (WT) crosses. Barren crosses make normal looking perithecia but produce exceptionally few progeny ascospores. Barrenness is due to a presumably RNAi-based process called meiotic silencing by unpaired DNA, that silences duplicated genes, including those required for the completion of meiosis and ascus development (Shiu et al. 2001; Shiu and Metzenberg 2002). Semi-dominant mutations of the *Suppressor of ascus dominance-1* (*Sad-1*), *Sad-2* and *Suppressor of meiotic silencing-2* (*Sms-2*) genes can suppress meiotic silencing and thus can enhance the productivity of *Dp* × normal (i.e., duplication-heterozygous) crosses (Shiu et al. 2001, 2006; Lee et al. 2004; Srividhya Iyer and D. P. Kasbekar, unpublished results). The semi-dominant suppressors of meiotic silencing do not interfere with the RIP suppressive effect of large duplications.

Our RIP assay used a gene-sized (∼1.2 kb) probe duplication called *Dp*(*erg-3*) to target RIP to the *ergosterol-3* (*erg-3*) gene (Noubissi et al. 2000). Crosses of *Dp*(*erg-3*) strains with non-duplication strains yielded RIP-induced *erg-3* mutant progeny at frequencies typically in the 2–25% range, but in crosses with the duplication strains this frequency was <0.5%. Dominant suppression of RIP by duplications was also demonstrated using *Dp*(*dow*), a small duplication of the *downy* (*dow*) gene (Vyas et al. 2006). More recently, Perkins et al. (2007) attributed the suppression by *Dp*(*OY329*) and *Dp*(*S1229*) of $eas^{UCLA191}$—induced recurrent *cya-8* mutation to a dominant suppression of RIP targeted by a presumptive insertion of *cya-8* sequence into the $eas^{UCLA191}$ allele.

Since both large (i.e., >100 kb) and small duplications are substrates for RIP (Perkins et al. 1997), Bhat and Kasbekar (2001) hypothesized that large duplications might titrate out the RIP machinery. An alternative hypothesis was that RIP might contribute to the barren phenotype of the duplication-heterozygous crosses and that the few ascospores generated in the barren crosses may be the ones in which RIP either had not occurred or was very inefficient, thus accounting for the observed low frequency of RIP-induced mutants amongst the survivors. The latter hypothesis was undermined by the demonstration that duplication-heterozygous crosses that were either heterozygous for a dominant mutation conferring a RIP defect or homozygous for the recessive *RIP-defective* (*rid*) mutation were as barren as their RIP-competent controls (Noubissi et al. 2000; Bhat and Kasbekar 2004). On the other hand, the titration hypothesis was based on examination of only six duplications, of which five were suppressors and ≳270 kb in size, whereas the lone non-suppressor duplication was ∼117 kb (Vyas et al. 2006). We have now screened 33 more duplications, of which 29 were suppressors and four non-suppressors. It was meaningful to now ask whether, in general, non-suppressor duplications are smaller than suppressor duplications. Also, we could address whether crosses heterozygous for multiple non-suppressor duplications can suppress RIP if the combined size of the duplications exceeds that of one or more suppressor duplications. Affirmative answers to these questions would significantly strengthen the titration hypothesis.

Materials and methods

Neurospora growth and crosses

Neurospora strains were maintained and crossed essentially as described by Davis and De Serres (1970). Crosses were performed by confrontation between mycelia inoculated as plugs on synthetic crossing medium in petri dishes. Ascospores began to be shot within 16–18 days and were harvested by washing the lids with ∼1 ml water.

Strains from other collections

Unless otherwise indicated, *N. crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC, University of Missouri, Kansas City, MO 64110). They included the standard Oak Ridge (OR) strains 74-OR23–1 A (FGSC 987) and OR8-1 a (FGSC 988); the mutant strain *erg-3 a* (FGSC 2725); the translocation strains *T*(*AR17*) *A* (FGSC 2442), *T*(*AR17*) *a* (FGSC 1463), *T*(*B362i*) *A* (FGSC 2935), *T*(*B362i*) *a* (FGSC 2988), *T*(*IBj5*, *cpc-1*) *A* (FGSC 4433), *T*(*IBj5*, *cpc-1*) *a* (FGSC 4434) and *T*(*UK3-41*) *A* (FGSC 6869).

The FGSC strain numbers for the 34 translocation strains listed in Table 1 are (in the serial order used in the table); 2676, 3826, 6869, 3046, 7037, 6958, 7080, 2021, 2133, 2270, 2466, 2928, 6298, 2637, 1483, 1752, 2264, 2279, 2469, 1610, 2595, 3635, 3666, 3669, 3881, 4641, 2599, 5011, 2757, 1828, 2064, 767, 2104 and 3209. Five translocations (*Tp*(*T54M94*), *T*(*AR173*), *T*(*OY337*), *T*(*4540 nic-2*) and *T*(*7442 mo*)) were obtained in *mat a* strains and the remaining 29 in *mat A* strains. The 34 translocations and the duplications derived from their crosses with the wild type are described in Perkins (1997). Experiments to map the breakpoints of *Dp*(*Y112M4i*) were done using the *T*(*Y112M4i*) *ad-3B a* strain (FGSC 2638).

The wild-isolated strains Aarey-1 (0679), Fred (P1138), Mauriceville-1c (FGSC 2225) and Mugalsarai-2 (P0736) were used in RFLP mapping experiments to determine the extent of duplication coverage.

The semi-dominant suppressor of meiotic silencing by unpaired DNA, *Sad-1*, was used to increase the

Table 1 RIP-induced *erg-3* mutants from crosses heterozygous for various translocation (*T*) and duplication (*Dp*) strains

T used to generate *Dp* segregants	Frequency (% (N)) in *T*-heterozygous crosses[a]	Frequency (% (N)) in *Dp*-heterozygous crosses	*Dp* tested in *cis* or *trans* (C or T)[b]	*Dp* phenotype[c]
1. T (VI > IV) CJS1	3.3 (214)	1.6 (60)	C	ND
2. T (I > II) MD2	3.3 (120)	0.2 (409)	C	Srp
3. T (VR > VIL) UK3-41	12.2 (41)	0.3 (707); 0.4 (815)	T	Srp
4. T (VR > VII) EB4	13.3 (15)	2.4 (900); 1.3 (455); 1.1 (890)	C + T	+
5. T (IIIR > IL) UK8-18	23.5 (68)	1.3 (304); <0.5 (210)	C	Srp
6. T (VIR > VL) UK14-1	6.0 (232)	1.9 (377); 2.8 (574); 5.1 (692)	C	+
7. T (III; IV; VII) UK14-5	3.6 (137)	0.2 (379); 0.3 (312)	C	Srp
8. T (VL > IVL) AR33	22.6 (133)	0.3 (310); 0.3 (340); 0.4 (518)	C	Srp
9. T (VIL > IR) T39M777	7.3 (96)	<0.6 (196); 1.2 (467)	T	Srp
10. T (IL > VIL) T51M156un	2.0 (98)	0.2 (551); 0.2 (464)	C	Srp
11. T (VIIL > IVR) T54M50	24.6 (69)	0.6 (1454)	T	Srp
12. T_P (IR > IL) T54M94	5.1 (196)	0.6 (492); 0.7 (134)	C	Srp
13. T (VIIR > IR) Z88	14.3 (140)	0.7 (833); 0.2 (801)	C	Srp
14. T (IR > IIIR) Y112M4i	ND	0.8 (510); 0.7 (757); 0.3 (725)	C	Srp
15. T (IIL > VR) NM149	11.3 (106)	0.8 (259)	C	Srp
16. T (IVR > I) NM152	13.7 (95)	0.2 (555); 0.6 (171); 0. 5 (206)	T	Srp
17. T (IVR > VIR) ALS159	2.5 (80)	<0.5 (203); 0.8 (628)	T	Srp
18. T (IR > VL) NM169d	2.7 (219)	1.9 (1452); 3.0 (1095); 2.0 (461)	C	+
19. T (IR; VR; IR > VII) AR173	ND	<0.5 (200)	C	Srp
20. T (IIR > IL) NM177 mo	13.0 (69)	0.3 (297); 0.1 (767)	T	Srp
21. T (IIL > X; IV; V) AR179	ND	2.1 (531); 0.9 (894)	C	Srp
22. T (VIR > IIIR) OY320	19.2 (99)	0.2 (531); 0.9 (428); 0.9 (232)	C	Srp
23. T (IVR > IL) OY333 met	6.0 (50)	0.3 (347); 1.6 (378)	T	Srp
24. T (IIR > IVR) OY337	10.3 (146)	0.2 (411); 0.3 (312)	T	Srp
25. T (IR > VIR) OY343	1.9 (156)	0.8 (245)	C	Srp
26. T (VIL > IR) OY350	24.7 (81)	0.4 (253)	C	Srp
27. T (IIIR; VR; VII) P1156	4.1 (122)	0.4 (1129)	C	Srp
28. T (I > VIL) S1425	2.9 (140)	0.3 (324); 0.5 (441); 0.4 (262)	C	Srp
29. T (IIL > IV) R2394	20.2 (84)	4.2 (593); 2.7 (288); 12.8 (343)	C + T	+
30. T (IIL > VI) P2869	24.3 (74)	0.5 (191)	C	Srp
31. T (IVR > IIIR) S4342	7.3 (123)	0.9 (758); 0.6 (179); 0.6 (355)	T	Srp
32. T (IR > IIIR) 4540 *nic-2*	ND	0.4 (269)	C	Srp
33. T (VIIR > IL) 5936	18.0 (389)	0.3 (861); 0.4 (496); 0.2 (452)	C	Srp
34. T (IR > VII) P7442 mo	ND	0.3 (313); 2.4 (502)	T	Srp

ND, not determined

[a] The translocation strains were crossed with *Dp*(*erg-3*) strains of the opposite mating type and the frequency of RIP-induced *erg-3* mutants was determined in the progeny

[b] See the text for details

[c] Srp, suppressor of RIP. That is, at least one cross shows <1% frequency of RIP-induced *erg-3* mutant progeny. N = total number of progeny screened

productivity of the duplication-heterozygous crosses (Shiu et al. 2001; Shiu and Metzenberg 2002). The strains *Sad-1 A* (FGSC 8740), *Sad-1 a* (FGSC 8741), *Sad-2* (RIP32) *A* (RLM 30–12) and *Sad-2* (RIP32) *a* (RLM33-12) were kindly provided by R. L. Metzenberg (California State University, Northridge).

Strains made previously in our laboratory

Strains *Dp*(*erg-3*) *A* or *a* were earlier called *Dp1.3*^{ec} *hph A* and *a* (Prakash et al. 1999; Bhat et al. 2003). The transgene *Dp*(*erg-3*) contains a 1.2 kb fragment of the LG IIIR gene *ergosterol-3* (*erg-3*) tagged with the bacterial *hph* gene for

resistance to hygromycin. The *erg-3* fragment serves to target RIP during a cross and the resulting RIP-induced *erg-3* mutant progeny can be easily scored under a dissection microscope by their distinct colony morphology on Vogel's—sorbose agar (Noubissi et al. 2000), thus providing a convenient measure of RIP efficiency. Frequencies of *erg-3* mutants were determined in ascospores harvested at 31 days. Construction of strains *Sad-1*; *Dp*(*erg-3*); *Dp*(*AR17*), *Sad-1*; *Dp*(*AR17*), *Dp*(*B362i*) *a*, *Sad-1*; *Dp*(*B362i*); *Dp*(*erg-3*) *a*, and *Dp*(*IBj5*) *A* was described by Vyas et al. (2006).

Construction of duplication strains bearing *Sad-1* or *Sad-2*

Translocation strains *T*(*EB4*) *A* and *T*(*R2394*) *A* were crossed with *Sad-1*; *Dp*(*erg-3*) *a* strains and the hygromycin-resistant progeny were crossed with Oak Ridge and *Sad-1* strains of the opposite mating type to determine whether or not they had inherited the *Sad-1* allele. The presence of the duplication was verified by examining molecular markers using the RFLPs described in Supplementary Table 2. In this way, we obtained the strains *Dp*(*R2394*) *A* (13, 15, 19 and 37), *Dp*(*R2394*); *Dp*(*erg-3*) *A* (30), *Sad-1*; *Dp*(*R2394*); *Dp*(*erg-3*) *A* (3 and 35), *Sad-1*; *Dp*(*R2394*); *Dp*(*erg-3*) *a* (5 and 22), *Dp*(*EB4*) *a* (50), *Dp*(*EB4*); *Dp*(*erg-3*) *A* (60) and *Sad-1*; *Dp*(*EB4*) *a* (61). These strains were used in the crosses described in Table 4. In a like manner, we constructed the strains *Dp*(*B352i*); *Sad-2 A* (12) and *Dp*(*B362i*); *Dp*(*EB4*) *A* (3, 8 and 11).

PCR, other molecular methods and transformations

PCRs were performed using custom oligonucleotide primers purchased from Bioserve, India. Reaction conditions, other molecular methods and transformation protocols were essentially the same as previously described (Bhat et al. 2004).

Estimating size and extent of duplications's

The size and extent of duplications was determined by testing coverage of linked RFLP markers as described by Vyas et al. (2006). In these tests, an initial molecular marker (an RFLP or PCR-based RFLP) was verified to be covered by the duplication and then additional markers, at various distances from the initial duplicated marker, were tested in a similar manner. The sequence separating the farthest covered markers (designated FCR and FCL, respectively, for the farthest covered markers on the right and left) provides an estimate of the duplication's minimum size and that between the closest flanking uncovered markers (CUR and CUL, respectively, for the closest uncovered markers on the right and left), of its maximum size (see Fig. 1 of Vyas et al. 2006).

Oligonucleotide primers were constructed to PCR amplify ~2–3 kb genome fragments from relevant translocation (T) and wild-isolated (W) strains. RFLPs within the amplified fragment allowed us to distinguish between the T and W fragments (alleles). Alternatively, we identified RFLPs outside the amplified segment by using the amplified fragment to probe Southern blots of restriction-digested genomic DNA from the T and W strains. Duplication (*Dp*) progeny from $T \times W$ were identified by the barrenness of their crosses with Oak Ridge strains and we confirmed the presence in them of the T and W alleles of the initial molecular marker. Usually this marker was chosen from a genome segment close to a genetic marker known to be covered by the duplication and it was given a designation such as *nr*(*un-18*) for "*near un-18*". Additional linked markers at various distances from the initial duplicated marker were tested in a similar way. The presence in the duplication progeny of both *T* and *W* alleles of the new RFLP indicated that the duplication extended to the genomic segment marked by the new RFLP. But if the duplication contained only the *W* allele, then it followed that the marker was not covered by the duplication. These steps were iterated to progressively narrow down the genomic interval bracketed by the covered and uncovered markers to localize the duplication breakpoints to within 5 kb intervals. The primers, enzymes and wild-isolated strains used for the RFLP analysis are given in Supplementary Table 1.

The data presented in Table 2 follows the convention used by Vyas et al. (2006). "D" is the size of the initial duplicated marker; A^L and A^R are the distances between the initial duplicated marker and the farthest nucleotide of the farthest covered RFLPs and B^L and B^R are the distances between the initial duplicated marker to the closest nucleotide of the closest uncovered RFLPs. Thus ($A^L + D + A^R$) defines the duplication's minimum size and ($B^L + D + B^R$) the maximum size. Southern analysis was done to verify the presence of an RFLP between the translocation and Oak Ridge strains, caused presumably by the presence of the breakpoint in the identified interval. Supplementary Table 2 lists the primers and enzymes used to define the RFLPs between Oak Ridge (OR) and the translocation strains *T*(*EB4*), *T*(*Y112M4i*) and *T*(*R2394*). These RFLPs were used to identify *Dp*(*EB4*) and *Dp*(*R2394*) segregants from crosses of the type $T \times$ OR.

Table 2 Estimates of duplication size based on genome sequence separating known covered genes

Dp		Covered markers	NCU numbers	Minimum size (Mb)
1.	*Dp(UK3-41)*	*pab-1-pyr-6*	ncu06714.3–ncu04323.3	1.2
2.	*Dp(EB4)*	*cot-2-ad-7*	ncu04189.3–ncu04216.3	0.1
3.	*Dp(UK8-18)*	*trp-1-nit-7*	ncu00200.3–ncu00498.3	1.1
4.	*Dp(AR33)*	*NOR*		1.4[a]
5.	*Dp(T39M777)*	*chol-2-lys-5*	ncu04699.3–ncu05526.3	1.2
6.	*Dp(T54M50)*	7.32–7.84 contig		0.7[b]
7.	*Dp(T54M94)*	*nit-1-al-2*	ncu00736.3–ncu00585.3	0.5
8.	*Dp(Z88)*	*arg-10-nt*	ncu08162.3–ncu05752.3	0.1
9.	*Dp(NM149)*	*ro-3-cys-3*	ncu03483.3–ncu03536.3	0.2
10.	*Dp(NM152)*	*rib-2-pyr-2*	*ncu08313.3*–ncu05290.3	2.7
11.	*Dp(ALS159)*	*pyr-1-uvs-2*	ncu06532.3–ncu05210.3	3.8
12.	*Dp(NM177 mo)*	*arg-12*-contig end	ncu01667.3	0.6[c]
13.	*Dp(AR179)*	*cys-3-thr-2*	ncu03536.3–ncu03425.3	0.4
14.	*Dp(OY337)*	*arg-12-fl*	ncu01667.3–ncu08726.3	0.9
15.	*Dp(OY343)*	*wc-2-un-18*	ncu00902.3–ncu08616.3	2.7
16.	*Dp(P2869)*	*pi-cys-3*	–ncu03536.3	0.6[d]
17.	*Dp(S4342)*	*arg-14-uvs-2*	ncu07682.3–ncu05210.3	3.1
18.	*Dp(4540 nic-2)*	*ace-7-un-1*	ncu09111.3–ncu08346.3	0.3
19.	*Dp(5936)*	*arg-11-cpc-2*	ncu02227.3–ncu05810.3	0.6
20.	*Dp(P7442 mo)*	*nic-2-un-1*	ncu03282.3–ncu08346.3	0.5

[a] Butler and Metzenberg (1990)

[b] Covered gene *het-e* is on contig 7.66 whose orientation is not known. The *Dp* is known to cover additional genes in flanking contigs. Therefore one end of contig 7.66 also is covered. This distance is to the closer end and therefore a more conservative estimate of the *Dp's* minimum size

[c] The IIR marker *arg-12* is on contig 7.5. The uncovered gene *aro-1* is distal to *arg-12* and is also on contig 7.5. The *nuc-2* gene is covered and proximal to *arg-12* and located on the unplaced contig 7.25. Therefore the proximal end of contig 7.5 must be covered

[d] Smith and Glass (1996)

Results

Screening of duplications for the dominant RIP suppressor phenotype

Thirty-four insertional or quasiterminal translocation strains (*T*) were crossed with *Dp*(*erg-3*) strains of the opposite mating type and the frequency of RIP-induced *erg-3* mutant progeny was determined for each cross. The results, summarized in Table 1, showed no evidence of a RIP defect in any of the *T* × *Dp*(*erg-3*) crosses. Their RIP occurrences are within the normal 2–25% range, therefore none of the translocation strains tested possessed a dominant RIP suppressor phenotype.

Next, 10–15 hygromycin-resistant f1 progeny from the *T* × *Dp*(*erg-3*) crosses were crossed with OR (wild-type Oak Ridge) or *Sad-1* strains of the opposite mating type. Hygromycin-resistance signals f1 progeny that have inherited the *Dp*(*erg-3*) probe duplication and a subset of these progeny is expected to also be duplicated for the translocated segment (*Dp*). Since the crosses made with the f1 progeny were potentially of the type *Dp*; *Dp*(*erg-3*) × *OR* and *Dp*; *Dp*(*erg-3*) × *Sad-1*, that is, both *Dp* and *Dp*(*erg-3*) were in the same parental nucleus, they were designated as the *cis* crosses. In some cases, we used 10–15 f1 progeny from *T* × *OR* to make crosses with *Dp*(*erg-3*) or *Sad-1*; *Dp*(*erg-3*) strains of the opposite mating type and these crosses were designated as the *trans* crosses because *Dp* and *Dp*(*erg-3*) were in different parental nuclei (i.e., *Dp* × *Dp*(*erg-3*) and *Dp* × *Sad-1*; *Dp*(*erg-3*)). The f1 progeny that displayed a barren phenotype in crosses with OR or *Dp*(*erg-3*) were presumed to represent the duplication segregants, and the frequency of RIP-induced *erg-3* mutants was determined among the f2 progeny from the corresponding, more productive *Sad-1*-heterozygous crosses. The results, summarized in Table 1, show that for 29 duplications, at least one putative duplication-heterozygous cross produced *erg-3* mutants at frequencies <1% (in fact, in the majority of such crosses it was <0.5%). That is, the duplication showed evidence for dominant suppression of RIP. In contrast, RIP was not suppressed in crosses parented by the f1 progeny that gave non-barren crosses with OR or *Dp*(*erg-3*) strains (data not shown). A duplication was typed as a suppressor of RIP

(Srp) even if RIP suppression was seen in only one putative duplication-heterozygous cross, because any non-suppressive cross could have represented a non-duplication segregants mis-scored as barren in the cross with Oak Ridge or *Dp*(*erg-3*). However, for four duplications, namely, *Dp*(*EB4*), *Dp*(*UK14–1*), *Dp*(*NM169d*) and *Dp*(*R2394*) all of the several putative duplication-heterozygous crosses tested showed only the non-suppressor phenotype (Table 1 and additional data not shown). Therefore these four duplications were typed as non-suppressors. We were unable to ascertain the suppressor/non-suppressor status of *Dp*(*CJS1*) because only one putative duplication segregant was obtained and it was non-suppressive in a *trans* cross.

Suppressor duplications are generally larger then ~270 kb

For 20 duplications a minimum size could be estimated based on the genome sequence known to separate pairs of covered genes (Table 2). These included 19 duplications typed as suppressors in Table 1; 16 of them were >400 kb, one (*Dp*(*4540*)) was >300 kb, one (*Dp*(*NM149*)) was >200 kb and one (*Dp*(*Z88*)) was >100 kb. That 200 kb significantly underestimated the minimum size of *Dp*(*NM149*) was inferred from the fact that whereas the *het-6* marker is covered by both *Dp*(*NM149*) and *Dp*(*AR18*), the markers *cys-3* and *het-c* which are, respectively, proximal and distal to *het-6* on LG IIL, are covered by *Dp*(*NM149*) but not by *Dp*(*AR18*) (Perkins 1997); therefore *Dp*(*NM149*) must be larger than *Dp*(*AR18*). *Dp*(*AR18*) is ~270 kb (Smith and Glass 1996), therefore *Dp*(*NM149*) must be $\gtrsim$270 kb.

Tests for coverage of linked RFLP markers were done as described by Vyas et al. (2006) and revealed that *Dp*(*Z88*) was, in fact, >322 kb. In these tests, an initial molecular marker (an RFLP or PCR-based RFLP) was shown covered by the duplication. Then the duplication was tested for coverage of additional markers at various distances from the initial duplicated marker. The sequence separating the farthest covered markers provided an estimate of the duplication's minimum size and that, between the closest flanking uncovered markers, of its maximum size (see Fig. 1 of Vyas et al. 2006). The results of these tests are summarized in Table 3. Table 3 also presents the minimum sizes estimated for three other suppressor duplications for which a covered and sequenced marker was known; *Dp*(*MD2*) (>300 kb), *Dp*(*Y112M4i*) (>372 kb) and *Dp*(*OY350*) (>269 kb). *Dp*(*AR173*), another suppressor duplication was found to be >226 kb, but this is almost certainly an underestimate because the sequence is interrupted by two gaps (Srividhya Iyer and D. P. Kasbekar, unpublished results). For the remaining six suppressor duplications, we did not undertake size determinations because for five duplications (*UK14-5*, *T51M56*, *OY333*, *P1156* and *S1425*), no covered markers were known and the only covered marker reported for *Dp*(*OY320*) was *ws-1* (Perkins 1997), whose sequence is unknown. In summary, our results showed that all 22 suppressor duplications for which a reliable minimum size could be estimated were $\gtrsim$270 kb.

Non-suppressor duplications are smaller then ~200 kb

The non-suppressor duplication *Dp*(*EB4*) was initially estimated to be >100 kb (Table 2); RFLP coverage tests done to localize the proximal and distal breakpoints of *Dp*(*EB4*) revealed that it is, in fact, 140–147 kb (Table 3).

Although no covered marker was known for *Dp*(*R2394*), the "donor" segment of *T*(*IIL > IV*) *R2394* was reported to be tightly linked (0/57) to the IIL marker *pyr-4* (Perkins 1997). The *pyr-4* marker is on contig 7.8 of the sequenced genome. This contig is about 0.98 Mb in size, so we tested four evenly spaced PCR-based RFLPs on this contig and found that the marker designated R3 was in fact covered by *Dp*(*R2394*). Using R3 as the initial duplicated marker, the minimum/maximum sizes for *Dp*(*R2394*) were determined to be 151/154 kb (Table 3). This work placed the distal end of the translocated segment in *T*(*IIL > IV*) *R2394* at 25.8 kb proximal to *pyr-4*. For the non-suppressor duplication *Dp*(*NM169d*), the closest uncovered flanking markers were found to be separated by 196 kb (Table 3), however, as there are two gaps in this sequence, this is possibly an underestimate of *Dp*(*NM169d*)'s maximum size. Only the *ws-1* marker was reported covered by the non-suppressor *Dp*(*UK14-1*) (Perkins 1997), but as noted above, its sequence is unknown, therefore we did not determine minimum and maximum sizes for this duplication. Thus, the two non-suppressor duplications, *Dp*(*EB4*) and *Dp*(*R2394*), for which accurate maximum size estimates were made, were both <200 kb, and our results did not exclude this possibility for *Dp*(*NM169d*).

Is RIP suppressed in crosses heterozygous for multiple non-suppressor duplications?

Crosses of the type *Dp*(*B362i*) × *Dp*(*EB4*), *Dp*(*B362i*) × *Dp*(*R2394*) and *Dp*(*EB4*) × *Dp*(*R2394*) are duplication-heterozygous for, respectively, 256–266, 268–274, 291–301 kb. Crosses triply-heterozygous for *Dp*(*B362i*), *Dp*(*EB4*) and *Dp*(*R2394*) are duplication-heterozygous for 407–421 kb. We asked whether crosses double- and triple-heterozygous for the non-suppressor duplications show evidence for dominant suppression of RIP. The strains used

Table 3 Determination of duplication size

Duplication	Initial marker	D	A^L	B^L	A^R	B^R	C^L	C^R	$A^L + D + A^R$ (minimum)	$B^L + D + B^R$ (maximum)
Suppressor *Dp's*										
Dp(MD2)	*un-18*	3918	208404	263513	87734	89879	55109	2145	300056[a]	ND
Dp(Z88)	*arg-10*	1506	45791	ND	274697	350529	ND	75832	321994[a]	ND
Dp(Y112M4i)	*nic-2*	778	269244	ND	102103	103385	ND	1282	372125	ND
Dp(OY350)	*chol-2*	844	225089	ND	43098	ND	ND	ND	269031[a]	ND
Non-suppressor *Dp's*										
Dp(EB4)	*ad-7*	1981	113930	117984	23738	26980	4054	3242	139649	146945
Dp(NM169d)	*un-18*	3918	98205	102481	87734	89879	4276[a]	2173[a]	189829	196278[a]
Dp(R2394)	*R3*	1531	68513	71335	80937	81511	2822	574	150981	154377

Please see Vyas et al. (2006) for explanation of the symbols D, A^L, etc., ND, not determined

[a] Presence of a gap of unknown size in the sequence. The marker *R3* was serendipitously found covered by *Dp(R2394)*. It was among four molecular markers from near the *pyr-4* locus tested for coverage by the *Dp*

to perform these crosses are described in Section "Materials and methods". The results summarized in Table 4 show that RIP was suppressed in 0/3 crosses doubly heterozygous for *Dp(B362i)* and *Dp(EB4)*, 0/2 crosses doubly heterozygous for *Dp(B362i)* and *Dp(R2394)*, 1/2 crosses doubly heterozygous for *Dp(EB4)* and *Dp(R2394)* and 4/6 crosses triply heterozygous for all three duplications.

Duplication-homozygous crosses are barren and, in general, their productivity is not significantly enhanced by the *Sad-1* mutation (Vyas et al. 2006). Nevertheless, sufficient numbers of progeny were obtained from crosses of the type *Sad-1*; *Dp(R2394)*; *Dp(erg-3)* × *Dp(R2394)*, *Sad-1*; *Dp(R2394)*; *Dp(erg-3)* × *Dp(R2394)*; *Dp(erg-3)* and *Sad-1*; *Dp(EB4)*; *Dp(erg-3)* × *Dp(EB4)* possibly because *Dp(EB4)* and *Dp(R2394)* are both relatively small and therefore do not include many genes essential for ascus development. The *Dp(EB4)*-homozygous and *Dp(R2394)*-homozygous crosses are duplicated for, respectively, 279–294 and 302–309 kb and we examined them for suppression of RIP. The results presented in Table 4 show that RIP

Table 4 RIP-induced *erg-3* mutant progeny from crosses multiply heterozygous or homozygous for *Dp(B362i)*, *Dp(EB4)* or *Dp(R2394)*

	Cross	Frequency of *erg-3* progeny [% (N)]	Phenotype
1.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) A* (35) × *Dp(EB4) a* (50)	0.7 (906)	Srp
2.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) A* (3) × *Dp(EB4) a* (50)	2.6 (624)	+
3.	*Sad-2*; *Dp(B362i) A* (12) × *Sad-1*; *Dp(EB4)*; *Dp(erg-3) a* (4)	1.6 (423)	+
4.	*Sad-2*; *Dp(B362i) A* (12) × *Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22)	3.8 (611)	+
5.	*Sad-2*; *Dp(B362i) A* (12) × *Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (5)	3.0 (561)	+
6.	*Sad-1*; *Dp(erg-3) a* × *Dp(EB4)*; *Dp(B362i) A* (3)	2.4 (615)	+
7.	*Sad-1*; *Dp(EB4)*; *Dp(B362i) A* (12) × *Dp(erg-3) a*	2.9 (591)	+
8.	*Sad-2*; *Dp(B362i) A* (12) × *Dp(EB4)*; *Dp(R2394)*; *Dp(erg-3) a* (12)	0.8 (800)	Srp
9.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (21) × *Dp(EB4)*; *Dp(B362i) A* (11)	1.2 (1715)	+
10.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (21) × *Dp(EB4)*; *Dp(B362i) A* (3)	0.3 (609)	Srp
11.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (21) × *Dp(EB4) Dp(B362i) A* (8)	0.3 (654)	Srp
12.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22) × *Dp(EB4)*; *Dp(B362i)*; A (8)	1.4 (1019)	+
13.	*Sad-1*; *Dp(EB4)*; *Dp(B362i)*; *A* (6) × *Dp(R2394)*; *Dp(erg-3)* a (10)	0.4 (770)	Srp
14.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22) × *Dp(R2394) A* (15)	1.7 (232)	+
15.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22) × *Dp(R2394) A* (13)	1.1 (372)	+
16.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22) × *Dp(R2394) A* (37)	1.3 (980)	+
17.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22) × *Dp(R2394) A* (19)	0.8 (463)	Srp
18.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (5) × *Dp(R2394)*; *Dp(erg-3) A* (30)	0.8 (618)	Srp
19.	*Sad-1*; *Dp(R2394)*; *Dp(erg-3) a* (22) × *Dp(R2394)*; *Dp(erg-3) A* (30)	0.6 (351)	Srp
20.	*Sad-1*; *Dp(EB4) a* (61) × *Dp(EB4)*; *Dp(erg-3) A* (60)	0.5 (636)	Srp

was suppressed in 4/7 of the duplication-homozygous crosses.

Discussion

We have identified 29 duplications that could behave as dominant suppressors of RIP in the small probe duplication, *Dp*(*erg-3*), and four duplications that were non-suppressors. Of the 29 suppressor duplications, a minimum size was estimated for 22 and they were all >269 kb. In contrast, two non-suppressor duplications, for which a maximum size was estimated, were <200 kb. These findings, together with those from earlier studies showing five suppressor duplications (*Dp*(*AR17*), *Dp*(*AR18*), *Dp*(*IBj5*), *Dp*(*OY329*) and *Dp*(*S1229*)) to be $\gtrsim$270 kb, and the non-suppressor *Dp*(*B362i*) to be only $\sim$117 kb (Vyas et al. 2006), now constitute a formidable data set of 27 suppressor + 3 non-suppressor duplications which supports the generalization that suppressor duplications are larger than non-suppressor duplications.

Our study has added *Dp*(*EB4*) and *Dp*(*R2394*) to the still modest list of duplications whose breakpoints are now mapped to an imprecision of <5 kb. Other duplications whose breakpoints were mapped as precisely are *Dp*(*AR17*), *Dp*(*B362i*), *Dp*(*IBj5*) and *Dp*(*OY329*) (Vyas et al. 2006). The distal breakpoints of *Dp*(*MD2*) and *Dp*(*Y112M4i*) also were localized to <5 kb. Although we have not yet localized the proximal breakpoint of *Dp*(*Y112M4i*) our results lead us to question the identification of the gene sequence ncu 03235.3 as *cys-13*, because our results show that *Dp*(*Y112M4i*) covers ncu 03235.3, whereas *cys-13* is reportedly not covered by *Dp*(*Y112M4i*) (Perkins 1997).

A major advance of this study was to show that combining multiple non-suppressive duplications in one cross could add up to sufficient duplication to achieve titration of RIP. The duplications *Dp*(*B362i*), *Dp*(*EB4*) and *Dp*(*R2394*) were individually non-suppressing but they could suppress RIP in a subset of double and triple heterozygous crosses. This result cannot be explained easily by the hypothesis that the barren phenotype of the duplication-heterozygous crosses is due to RIP and that the observed low frequency of RIP-induced mutants amongst the surviving progeny is because RIP either had not occurred or was very inefficient in them. RIP was also suppressed in a subset of *Dp*(*R2394*)- and *Dp*(*EB4*)-homozygous crosses. The results from all these different crosses are consistent with the idea that duplication of $\lesssim$270 kb is inadequate for RIP suppression and that RIP suppression begins to be encountered in crosses duplicated for 291–421 kb (median 356 kb). It is noteworthy that *Dp*(*AR17*), the smallest accurately measured suppressor duplication, is 351–357 kb in size (Vyas et al. 2006). In sum, our results strengthen the hypothesis that dominant RIP suppression by duplications occurs via titration of the RIP machinery and suggest that the "equivalence point" is in the relatively narrow range of 270–350 kb.

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