Chapter 3.

Large duplications as dominant suppressors of RIP
Chapter 3

3.1 Introduction

Duplication strains of *N. crassa* can be generated in the laboratory from crosses between some translocation and normal sequence strains (Perkins, 1997). Duplication progeny are generally one-third of the viable progeny produced from the cross (Fig. 3.1). The corresponding deficiency progeny are not viable. Duplication strains can be identified by the characteristic barren phenotype of crosses involving them. A genome defense process called meiotic silencing by unpaired DNA (MSUD, or meiotic silencing) is responsible for the barren phenotype (Shiu *et al.*, 2001). Meiotic silencing is a presumed RNAi-dependent process that silences genes that remain unpaired during meiosis. Large chromosome segment duplications can include many genes including some that are required for ascus development or ascospore maturation. During meiosis in a duplication x euploid cross, one copy of each duplication-borne gene remains unpaired and it triggers the silencing of all three copies, this renders the cross barren (Shiu *et al.*, 2001).

Earlier studies from our lab on the chromosome segment duplications *Dp*(AR17), *Dp*(IBj5), *Dp*(OY329) and *Dp*(S1229) established that large chromosomal duplications can dominantly suppress RIP in smaller gene-sized duplications, possibly by titrating out the RIP machinery (Watters *et al.*, 1999; Bhat and Kasbekar, 2001; Bhat *et al.*, 2003; Fehmer *et al.*, 2001; Vyas *et al.*, 2006). Dominant suppressors of RIP have also been identified among wild-isolated strains of *N. crassa* (Noubissi *et al.*, 2000; Noubissi *et al.*, 2001). One of the suppressor wild-isolates, Sugartown, showed linkage of the suppressor to a barren phenotype. In this strain the barrenness and dominant RIP-suppression ability might both be due to the presence of a chromosome segment duplication (Noubissi *et al.*, 2001; Vyas *et al.*, 2006).

In the present study, I have tested the dominant RIP suppressor phenotype in two more duplications; *Dp*(AR18) and *Dp*(B362i). *Dp*(AR18) behaved as a dominant suppressor of RIP in the probe duplication *Dp*(erg-3/1) but *Dp*(B362i) failed to do so. It was conceivable that the size of the chromosome segment duplication determines whether or not it suppresses RIP in a gene-sized duplication. To investigate this, I determined the size of the chromosome segment duplications. For this I made use of two resources. One was the *N. crassa* genome sequence accessible at
Figure 3.1: Generation of chromosome segment duplication from a translocation by normal sequence cross. Four kinds of progeny are produced. 1. Translocation, 2. Normal sequence, 3. Duplication and 4. Deficiency. Deficiencies are not viable. \( R^T \) and \( D^T \) are respectively the recipient and donor chromosomes of the translocation parent and \( R^N \) and \( D^N \) are the corresponding chromosomes of the normal sequence parent. Segregation of \( R^T \) with \( D^N \) produces duplication progeny. The duplication progeny are generally one third of the viable progeny.
http://www.broad.mit.edu/annotation/genome/neurospora/Home.html and the other was the collection of wild-isolated strains available from the Fungal Genetics Stock Centre (www.fgsc.net). The present assembly of the genome sequence contains ~39 Mb. Using the sequence, practically any genomic fragment can be amplified by PCR and used as a probe for RFLP analysis. I used the genome sequence to design oligonucleotide primers that amplified genomic fragments of approximately 1.5-1.8 kb. The wild-isolated strains were used as a source of RFLP markers for mapping. Information from the literature on the translocation strains (Perkins, 1997), was used to identify genomic regions in which RFLPs were sought. Based on the coverage of several RFLP markers, I estimated the sizes of $Dp(AR17)$, $Dp(B362i)$, $Dp(IBj5)$ and $Dp(0Y329)$ and also localized their breakpoints to within ~5 kb segments. My results showed that $Dp(B362i)$, the only tested chromosome segment duplication that did not suppress RIP in $Dp(erg-3/1)$, was ~117 kb in size. The other three duplications, were all > ~ 350 kb. $Dp(AR18)$ has been shown by others (Smith and Glass, 1996) to be ~ 270 kb and based on the coverage of conventional markers I have estimated $Dp(S1229)$ to be > 270 kb.

I also developed another probe duplication, $Dp(dow/1)$, to target RIP to the downy (dow) locus, and established that dominant suppression of RIP by large duplications was not peculiar to $Dp(erg-3/1)$. These studies demonstrate that large chromosome segment duplications can dominantly suppress RIP in gene-sized probe duplications and suggest this characteristic depends upon the duplication’s size.

3.2 Results

3.2.1 RIP suppression ability of $Dp(AR18)$ and $Dp(B362i)$.

**Suppression of RIP by $Dp(AR18)$.

$T(II\text{l}\rightarrow II\text{r})\ AR18$, referred to henceforth as $T(AR18)$, is a translocation that includes the het-6 locus responsible for vegetative heterokaryon compatibility. Slow growing duplication progeny, duplicated for het-6 can be obtained from the cross, $T(AR18)\ A \times OR\ a$. The slow growth is an inherent property of the duplication and is not attributable to the het-6 duplication (Perkins, 1997). Twenty-five slow and five fast
growing progeny from the cross $T(AR18) A \times ORa$ were crossed with the standard lab Oak Ridge strains, ORA or ORa (Fig. 3.2). Crosses with all the slow growing progeny were barren, whereas those with the fast growers were fertile. The barren segregants, presumed to be genotypically $Dp(AR18)$, were crossed with $Sad-1; Dp(erg-3/1)$ and the progeny were analyzed for frequency of erg-3 mutants. The $Sad-1; Dp(erg-3/1)$ strain is duplicated for a fragment of the erg-3 gene (See chapter 2 for further details on $Dp(erg-3/1)$). The $Sad-1$ mutation suppresses the barren phenotype of crosses heterozygous for large chromosome segment duplications. The frequency of RIP-induced erg-3 mutants in the segregants was < 0.5% while that in the control cross with the non-duplication sibling it was 5.1% (Table 3.1). These results show that $Dp(AR18)$ is a dominant suppressor of RIP.

$Dp(B362i)$ is a non suppressor of RIP in $Dp(erg-3/1)$

$T(IV \rightarrow I) B362i$ (referred to henceforth as $T(B362i)$) is a translocation of a IV R segment near the methionine tetrahydrofolate reductase ($met-1$) gene to linkage group I (Perkins, 1997) and it can be used to generate $Dp(B362i)$ duplication progeny in crosses with a normal sequence strain. $Dp(B362i)$ strains were generated from the cross of a wild-isolated strain, Lankala Koderu (FGSC#1110) with $T(B362i) A$ (Fig. 3.3A). Of the twenty-two progeny examined from this cross, thirteen segregants were $mat A$ and nine were $mat a$. All the twenty two segregants were analyzed by Southern hybridization for the presence of $Dp(B362i)$ (the translocation is linked to the mating type) using a 1.4 kb fragment of the $met-1$ gene as the probe. Three $mat A$ progeny #3, #8 and #10 (Fig. 3.3B) were identified as $Dp(B362i) A$ strains and used in crosses with $Sad-1; Dp(erg-3/1) a$. $Dp(B362i)$ duplication progeny were also generated from the crosses $T(B362i) A \times ORa$ and $T(B362i) a \times ORA$. The duplications were confirmed by Southern hybridization using $TB6iK$, a junction fragment at the distal breakpoint of $Dp(B362i)$. When used as a probe in Southern hybridization, it reveals an RFLP between the OR and $T(B362i)$ strains thus making it possible to identify duplication progeny (Fig. 3.4 A and B).

In crosses with $Sad-1; Dp(erg-3/1)$, $Dp(B362i)$ strains failed to suppress RIP in $Dp(erg-3/1)$ (Table 3.2). I also examined whether $Dp(B362i)$ suppressed RIP in $Dp(erg-3/1)$ when the two duplications were present in the same nucleus i.e. in cis. Of twenty
Fig. 3.2: Construction of \( Dp(AR18) \) strains. A cross between \( T(AR18) \) and OR produces four different kinds of progeny. 1. Normal sequence, 2. Translocation, 3. Duplication and 4. Deletion. The deletion progeny are inviable. Duplication progeny are barren in crosses with OR whereas normal sequence and translocation are fertile.
Table 3.1: Frequency of RIP-induced erg-3 mutants from crosses between the duplication segregants derived from T(AR18)a x ORA and Sad-1; Dp(erg-3/1) A/a.

<table>
<thead>
<tr>
<th>S no.</th>
<th>Cross with Sad-1; Dp(erg3/1)</th>
<th>N</th>
<th>erg-3 mutants</th>
<th>RIP frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dp (AR18)/4</td>
<td>398</td>
<td>0</td>
<td>&lt;0.3%</td>
</tr>
<tr>
<td>2.</td>
<td>Dp (AR18)/11</td>
<td>123</td>
<td>0</td>
<td>&lt;0.8%</td>
</tr>
<tr>
<td>3.</td>
<td>Dp (AR18)/19</td>
<td>292</td>
<td>0</td>
<td>&lt;0.3%</td>
</tr>
<tr>
<td>4.</td>
<td>Dp (AR18)/23</td>
<td>236</td>
<td>1</td>
<td>&lt;0.4%</td>
</tr>
<tr>
<td>5.</td>
<td>Dp (AR18)/5</td>
<td>276</td>
<td>0</td>
<td>&lt;0.3%</td>
</tr>
<tr>
<td>6.</td>
<td>Dp (AR18)/20</td>
<td>239</td>
<td>0</td>
<td>&lt;0.4%</td>
</tr>
<tr>
<td>7.</td>
<td>Dp (AR18)/9</td>
<td>294</td>
<td>0</td>
<td>&lt;0.4%</td>
</tr>
<tr>
<td>8.</td>
<td>Dp (AR18)/1</td>
<td>318</td>
<td>0</td>
<td>&lt;0.3%</td>
</tr>
<tr>
<td>9.</td>
<td>Fertile segregant/1</td>
<td>232</td>
<td>12</td>
<td>5.1%</td>
</tr>
<tr>
<td>10.</td>
<td>T (AR18) a</td>
<td>528</td>
<td>13</td>
<td>3.0%</td>
</tr>
<tr>
<td>11.</td>
<td>C-2 (3)- 50 a*</td>
<td>234</td>
<td>7</td>
<td>3.0%</td>
</tr>
</tbody>
</table>

* Strain C-2 (3)- 50 a* has the het-6 allele of the Panama type (see section 3.3).
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype in crosses with OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal sequence</td>
<td>Fertile</td>
</tr>
<tr>
<td>Translocation</td>
<td>Fertile</td>
</tr>
<tr>
<td>Duplication</td>
<td>Barren</td>
</tr>
<tr>
<td>Deficiency</td>
<td>NA</td>
</tr>
</tbody>
</table>

Fig. 3.3 A: Construction of *Dp(B362i)* strains. A cross between *T(B362i)* and the wild isolated strain Lankala Koderu (FGSC 1110) produces four different kinds of progeny. 1. Normal sequence, 2. Translocation, 3. Duplication and 4. Deletion. The deletion progeny are inviable. Duplication progeny are barren in crosses with OR whereas normal sequence and translocation are fertile.
Figure 3.3 B: Identification of duplications from the cross T(B362i) A x 1110 a. Genomic DNA of progeny from the cross T(B362i) A x Lankala Koderu a (FGSC 1110), digested with BamH1 was probed with the met-1 gene fragment on a Southern blot. T is the T(B362i) parent. Progeny #3, 8 and 10 are duplicated for the met-1 fragment because they represent RFLP patterns of both the parents and these then were identified as the Dp(B362i) segregants. The hazy and diffused pattern of progeny #10 might possibly be due to high salt concentration in the genomic DNA preparation. This strain was later also confirmed by independent means to be a duplication strain.
Figure 3.4 A: Junction fragment of a translocation breakpoint. A, B, C and D are fragments of the donor chromosome that can be amplified by PCR using primers a1, a2; b1, b2; c1, c2 and d1, d2 respectively. These segments flank the breakpoint on either side but do not overlap the breakpoint. JuncL and JuncR are fragments that can be amplified using the primers pairs a1, b2 and c1, d2. These are the junction fragments which overlap the breakpoint and hence can give RFLP with the translocation and the normal sequence.
Figure 3.4 A: Junction fragment of a translocation breakpoint. A, B, C and D are fragments of the donor chromosome that can be amplified by PCR using primers a1, a2; b1, b2; c1, c2 and d1, d2 respectively. These segments flank the breakpoint on either side but do not overlap the breakpoint. JuncL and JuncR are fragments that can be amplified using the primers pairs a1, b2 and c1, d2. These are the junction fragments which overlap the breakpoint and hence can give RFLP with the translocation and the normal sequence.
Figure 3.4 B: Barren progeny from the cross ORa x T(B362i) A and ORA x T(B362i) a as analyzed by Southern hybridization to identify duplications. The enzyme used was Xhol and a junction fragment of the translocation breakpoint was used as the probe. There is an RFLP between the OR and T(B362i) (T) strains. The duplications represent a pattern which has combination of both the OR and T patterns. All the strains are duplication strains. DNA of progeny #1 DNA is degraded and so it was not considered. DNA concentration in progeny #17, 21, 23 and 29 is low so they also have not been used for any further analysis. Segregants #2, 4, 9, 11, 12, 19, 24, 27 and 30 were used for further analysis.
Table 3.2: Frequency of RIP-induced erg-3 mutants from crosses that were trans for the duplication $Dp(B362i)$ and the transgene $Dp(erg-3/1)$. Progeny from $T(B362i) A \times Lankala Koderu$ cross were crossed to $Sad-1; Dp(erg-3/1) a$.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Barren/Fertile with OR</th>
<th>N</th>
<th>RIP frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Dp(B362i) #3$</td>
<td>Barren</td>
<td>284</td>
<td>7.3</td>
</tr>
<tr>
<td>$Dp(B362i) #8$</td>
<td>Barren</td>
<td>240</td>
<td>7.0</td>
</tr>
<tr>
<td>$Dp(B362i) #10$</td>
<td>Barren</td>
<td>208</td>
<td>6.2</td>
</tr>
<tr>
<td>$T(B362i) #5$</td>
<td>Fertile</td>
<td>290</td>
<td>6.5</td>
</tr>
<tr>
<td>$T(B362i) #6$</td>
<td>Fertile</td>
<td>217</td>
<td>6.3</td>
</tr>
<tr>
<td>$T(B362i) #14$</td>
<td>Fertile</td>
<td>165</td>
<td>7.0</td>
</tr>
<tr>
<td>$N(1110) #17$</td>
<td>Fertile</td>
<td>231</td>
<td>8.0</td>
</tr>
<tr>
<td>$T(B362i) #21$</td>
<td>Fertile</td>
<td>187</td>
<td>7.8</td>
</tr>
</tbody>
</table>
progeny examined from each of the crosses of Sad-1; Dp(erg-3/1) a with the Dp(B362i)
A strains #3, #8 and #10, the mat A segregants were screened for hygromycin-
resistance. Hygromycin-resistance could be either due to inheritance of the Dp(erg-3/1)
transgene or that of the Sad-1 mutation (for details of the Sad-1 strain, see chapter 2)
(Fig. 3.5 A). The hygromycin-resistant progeny were analyzed by Southern hybridization
using fragments of erg-3 and met-1 as probes, respectively, for the presence of Dp(erg-
3/1) and Dp(B362i) (Fig. 3.5 B and C). Five Dp(B362i); Dp(erg-3/1) A double
duplication strains 3/4, 3/11, 8/8, 8/26 and 10/8 were identified from this analysis and
used in crosses with Sad-1 a. The >1.0 % frequency of erg-3 mutant progeny from these
crosses showed that Dp(B362i) fails to suppress RIP in Dp(erg-3/1) even in cis (Table
3.3).

3.2.2 Does size of the segmental duplication determine its ability to dominantly
suppress RIP?

The above results suggested that while most large duplications dominantly
suppress RIP in Dp(erg-3/1), there are some like Dp(B362i) that do not. From the
available information, T(B362i) is known to include only one marker, met-1. It was
therefore presumed to be a small duplication and its inability to suppress RIP in Dp(erg-
3/1) might be due to its small size. To test this, I determined the size of the chromosome
segment duplications.

Duplication segregants generated using wild-isolated strains.

Duplication progeny derived from a translocation x normal cross have two
copies of the chromosomal segment that represents the translocated sequence, one from
the normal sequence parent and the other from the translocation parent. If the
translocation and the normal sequence strains are both of OR background, a difference in
the RFLP pattern for the two strains can be expected only at the translocation
breakpoints. Thus duplications generated using strains of the OR background will show
RFLPs only close to the breakpoints. The paucity of RFLPs is overcome by using wild-
isolated strains that have several polymorphisms in the genome sequence. I used several
Figure 3.5 A: Construction of Dp(B362i); Dp(erg-3/1) strains for cis crosses with Sad-1. A cross between Dp(B362i) and Sad-1; Dp(erg-3/1) gives Dp(erg-3/1) transgene inheriting progeny. The normal sequence progeny are generally Sad-1 because of the linkage to LG I. The duplications are obtained mostly in the sad-1+ background with the LGIV varying among these progeny. Type 1 and 2 are normal sequence progeny and type 3 and 4 are duplication progeny. LG IV varies between 1, 2 and 3, 4 respectively.
Figure 3.5 B: Hygromycin-resistant progeny from the crosses of Dp(B362i) A #3, #8 and #10 each with Sad-1; Dp(erg-3/1) screened for the presence of ectopic copy of the erg-3 gene fragment. Dp1.3 is the original (Dp(erg-3/1)) transgenes bearing strain which is used as a positive control. This strain shows an extra copy of the erg-3 fragment. The DNA was digested with Pst1 and the Southern blot was probed with the erg-3 fragment. All progeny except 8/5, 3/7, 10/7 and 10/12 had the transgene. #10/7 and 10/12 may not be considered because of the very low DNA concentration and also weak signal.
Figure 3.5 C: Progeny from the crosses of Dp(B362i) A # 3, # 8 and # 10 each with Sad-1; Dp (erg-3/l) a analyzed on a Southern blot for identifying duplications. A marker already known to be covered by the duplication was used as the probe. BamH1 was used for restriction digestion. There is an RFLP between 1110 and T (T(B362i)). The duplications are expected to show a combination of these two patterns. The segregants, 3/4, 3/11, 3/16, 8/5, 8/8, 10/8 and 8/26 were identified as duplications and used for the cis crosses. 8/5 was a Sad-1 mutant and did not have the Dp(erg-3/l) transgene.
Table 3.3: Frequency of RIP-induced erg-3 mutants from crosses that were cis for the duplication *Dp(B362i)* and the transgene *Dp(erg-3/1)*. Progeny from *Dp(B362i) A x Sad; Dp(erg-3/1)* cross were crossed to *Sad-1; Dp(erg-3/1) a*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Barren/Fertile with OR</th>
<th>N</th>
<th>RIP frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dp(B362i) # 3/4</em></td>
<td>Barren</td>
<td>204</td>
<td>5.4</td>
</tr>
<tr>
<td><em>Dp(B362i) # 3/11</em></td>
<td>Barren</td>
<td>189</td>
<td>5.8</td>
</tr>
<tr>
<td><em>Dp(B362i) # 8/8</em></td>
<td>Barren</td>
<td>259</td>
<td>7.3</td>
</tr>
<tr>
<td><em>Dp(B362i) # 8/26</em></td>
<td>Barren</td>
<td>98</td>
<td>5.1</td>
</tr>
<tr>
<td><em>Dp(B362i) # 10/8</em></td>
<td>Barren</td>
<td>207</td>
<td>5.7</td>
</tr>
</tbody>
</table>
wild-isolated strains as the normal-sequence parent and determined the size of large duplications by RFLP coverage.

To construct duplications in the wild-isolate background, the translocation strains \( T(AR17), T(B362i), T(IBj5) \) and \( T(OY329) \) were each crossed with ten different wild-isolated strains. Twenty \( f_1 \) progeny from each cross were tested for the barren phenotype in crosses with OR. These crosses were monitored for about 25 days for ascospore production. As expected, about one third of the segregants were barren in crosses with the OR strains. Duplication progeny from the cross \( T(AR17) \times OR \) also segregated \( dow \) mutants that result from the action of RIP on \( Dp(AR17) \) which is known to cover the \( dow \) locus. At least two duplication progeny from each of the translocation-heterozygous crosses were crossed with \( Sad-1; Dp(erg-3/1) a \) or \( A \) to test for the RIP suppression ability. The barren segregants chosen indeed suppressed RIP in \( Dp(erg-3/1) \).

*Estimating the extent of the duplications \( Dp(AR17), Dp(B362i), Dp(IBj5) \) and \( Dp(OY329) \).*

Duplication progeny are expected to segregate the translocated segment as depicted in Fig.3.6A. Therefore RFLP markers that are included or not included in the translocated segment are expected to segregate as shown in Figures 3.6B and C. The genome sequence was used to first identify an RFLP at a locus known to be included in the translocated segment. We confirmed that the duplications showed evidence for coverage of the RFLP. Further localization of the breakpoints was done by analyzing additional RFLP markers on either side of the initial RFLP (Fig.3.7) until markers were found on either side that showed evidence of not being covered by the duplication. Bracketing between the covered and uncovered RFLPs was done as represented in Figure 3.7. The breakpoints of the duplications were localized with respect to the initial RFLP, \( D \) until the distance between the farthest covered marker FC and the closest uncovered marker CU (CUL and FCL for markers on the left breakpoint; CUR and FCR for markers on the right breakpoint) was bracketed to less than 3 kb on either side of the duplication (Fig. 3.7). The primers used have been listed in Table 3.4. The minimum and maximum size of the duplication was determined by summation of \( AL+D+AR \) and \( BL+D+BR \) respectively (Table 3.5)
Fig. 3.6A: Segregation of markers associated with the translocation chromosome among duplication progeny. a^T and a^N are the translocation and the normal sequence alleles of a marker included in the translocated segment. b^T and b^N are alleles of a marker outside the translocated segment. Duplication progeny generated from this cross would show both alleles of marker ‘a’.

Fig. 3.6B: Detection of coverage of markers by RFLP analysis. Southern analysis done using marker ‘a’ as the probe would show the duplication (Dp) contains a combination of the RFLP patterns of translocation (T) and the normal sequence (N).

Fig. 3.6 C: The marker ‘b’ would show only the N pattern in the duplication (Dp).
Fig. 3.7: Estimating the size and extent of duplications. Genome segments used to determine the size and extent of the duplications in Table 3.4. The initial duplicated marker ORF is represented by D as the blue box in the center. The left and right breakpoints are bracketed by boxes with pink and purple colors which represent, respectively, genome segments used to define the farthest RFLP markers covered by the duplication and the closest uncovered markers. Thus the minimum and maximum sizes of the duplication are, respectively, $A^L + D + A^R$ and $B^L + D + B^R$. 
Table 3.4: Primers, wild-isolates and enzymes used for RFLP analysis of duplications.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Primers</th>
<th>RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild isolate</td>
</tr>
<tr>
<td>dow</td>
<td>5’CATTCAAGCTTCGACAGGACA 5’CTGGCGGGTATCTTCTTCAGC</td>
<td>Mughalsarai-2</td>
</tr>
<tr>
<td>FCL(A)</td>
<td>5’GTTGATGATTTTGAGACTTCGCTAC 5’TCCCATCAGAGGAAAATTCTGTA</td>
<td>Mughalsarai-2</td>
</tr>
<tr>
<td>CUL(A)</td>
<td>5’GGAGGGTAAGGTAACCTTGTTG</td>
<td>Franklin (4467)</td>
</tr>
<tr>
<td>FCR(A)</td>
<td>5’ACCCTTTGGACTAGGGAATTACA 5’TACCGGTTCAGTCGGGAGCATTA</td>
<td>Bichpuri-1</td>
</tr>
<tr>
<td>CUR(A)</td>
<td>5’TCAAGGATAGGGTGAAGAAAT 5’GGGACAAAGATCAAAGGAGCTT</td>
<td>Fred</td>
</tr>
<tr>
<td>met-l</td>
<td>5’CAAGGCTAGCTTCTTTGTCGTACAC 5’GGGACAGAGACAGTACAC</td>
<td>Lankala Koderu-1</td>
</tr>
<tr>
<td>FCL(B)</td>
<td>5’CGAGCATCTGAGTGAGATTAAC 5’AAATCCTTGTGATGCTTA</td>
<td>Lankala Koderu-1</td>
</tr>
<tr>
<td>CUL(B)</td>
<td>5’TCTGAGTTTTTGTGGAGACACGA 5’TGACTTCTCGAACCAGAGAC</td>
<td>Lankala Koderu-1</td>
</tr>
<tr>
<td>FCR(B)</td>
<td>5’CGACTACAACCTCCTCATTAC 5’TAGCTTGTGTGGCTGAAAA</td>
<td>Lankala Koderu-1</td>
</tr>
<tr>
<td>CUR(B)</td>
<td>5’GAAGCGTTCCTCTCTTGGTGAATAG 5’CCTTCTTCCCTCATTTTTGCTT</td>
<td>Lankala Koderu-1</td>
</tr>
<tr>
<td>ylo-l</td>
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<td>Bichpuri-1</td>
</tr>
<tr>
<td>FCL(I)</td>
<td>5’TCCTCCGATGAGCTATTCTAACA 5’TGATGTGTGGTACTTTGGTGG</td>
<td>Bichpuri-1</td>
</tr>
<tr>
<td>CUL(I)</td>
<td>5’CAAGAGATTTGTTGGAGAGATGA 5’TACCTCTGTGAAAGGTAAGACA</td>
<td>Mughalsarai-2</td>
</tr>
</tbody>
</table>

48D
FCR(I) 5’AGTTTCTACGTTGTGTCAGA Mughalsarai-2 EcoRV
5’GCACAGACACACATCAGACTCTCTCTC
CUR(I) 5’CTTCAGCTCTTCCCTACAGTTA Mughalsarai-2 EcoRV
5’GGATCAAGCGACACTCAATAATC
trp-2 5’ACAGCTGAGTACTCGTTCCTCCTC Franklin (4490) EcoRV
5’AGTACTTCTCAGCCAGTTGCATG
FCL(O) 5’GAAAAAGGAGTTGATCCTCTCTCACC Bichpuri-1 BamHI
5’AATGCCACCTCCTTTTCGTAATA
CUL(O) 5’CCACTGATTGTATGTGGGGATAG Mughalsarai-2 EcoRI
5’GGTGTCTACTTCGTGATGTGA
FCR(O) 5’GACCGACTACGCCGTTTATACTA Lankala Koderu BamHI
5’ACACGTACCCTCCCTACATAAT
CUR(O) 5’CCTAACGTCTAGGATAATTTAATTGCMughalsarai-2 EcoRI
5’CGCAGTAATCCTTAAAAATTTATATCG

1FC and CU stand for the probes that detect the farthest covered and closest uncovered RFLP markers, respectively. L and R refer to markers on the left or right; A, B, I and O refer to the duplications AR17, B362i, IBj5 and OY329, respectively.

2The enzyme listed is the one used to obtain the RFLP between the alleles in the indicated wild-isolated strain and the relevant translocation strain.
Table 3.5: Minimum and maximum size of different chromosome segment large duplications

<table>
<thead>
<tr>
<th>Duplication</th>
<th>Initial marker</th>
<th>D</th>
<th>$A^L$</th>
<th>$B^L$</th>
<th>$A^R$</th>
<th>$B^R$</th>
<th>CL</th>
<th>CR</th>
<th>AL+D+AR</th>
<th>BL+D+BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Dp(AR17)$</td>
<td>$dow$</td>
<td>4920</td>
<td>246322</td>
<td>248861</td>
<td>100216</td>
<td>103453</td>
<td>2539</td>
<td>3237</td>
<td>351458</td>
<td>357234</td>
</tr>
<tr>
<td>$Dp(B362i)$</td>
<td>$met-1$</td>
<td>1755</td>
<td>5086</td>
<td>46411</td>
<td>69974</td>
<td>71168</td>
<td>1325</td>
<td>1194</td>
<td>116815</td>
<td>119334</td>
</tr>
<tr>
<td>$Dp(IBj5)$</td>
<td>$ylo-1$</td>
<td>1686</td>
<td>280551</td>
<td>282726</td>
<td>120636</td>
<td>121377</td>
<td>2175</td>
<td>741</td>
<td>402873</td>
<td>405789</td>
</tr>
<tr>
<td>$Dp(OY329)$</td>
<td>$trp-2$</td>
<td>1826</td>
<td>193690</td>
<td>195849</td>
<td>508009</td>
<td>509279</td>
<td>2159</td>
<td>1270</td>
<td>703525</td>
<td>706954</td>
</tr>
</tbody>
</table>
*Dp(AR17)* covers the *dow* locus therefore, *dow* was chosen as the initial marker D for sizing *Dp(AR17)*. A 1.4 kb fragment of the *dow* gene on contig 7.17 was used as a probe for Southern analysis using genomic DNA digested with *BamH1* (Table 3.4). The *Dp(AR17)* strains showed evidence for coverage of the *dow* associated RFLPs (Fig 3.8A). (Fig 3.8B – E). As shown in Fig 3.7 and Table 3.5, the minimum and the maximum size of the duplication was then determined to be 351458 bp and 357234 bp respectively. The sizes of the duplications *Dp(B362i), Dp(IBj5) and Dp(OY329)* were also determined using the same strategy (Fig 3.9 – 3.11). The results are summarized in Table 3.5.

### 3.2.3 Translocation breakpoints and their use in identifying duplications in the Oak Ridge background.

Further localization and confirmation of the breakpoint of the specific duplications was done by PCR analysis using a combination of forward and reverse primers that amplified products flanking the probable breakpoint (Table 3.6 A,B). At the region spanning the breakpoint, a product was obtained only from DNA of the normal sequence strain and not from that of the translocation strain. The control primers that did not bracket the breakpoint could amplify products from both, the translocation and the normal sequence strain. Using this approach, the breakpoints of *T(AR17)* were localized to 20 bp and 700 bp segments and that in *T(OY329)* to 2.3 and 3 kb segments. The right breakpoint in *T(IBj5)* is known to lie within 100 bp of the promoter for the *cpc-1* gene (Paluh *et al.*, 1990). I was able to localize the other breakpoint to a ~2 kb segment.

The breakpoint region can be used to identify duplications derived from translocation by normal sequence crosses in the Oak Ridge background. In case of *Dp(IBj5), Dp(AR17)* and *Dp(B362i)*, the duplications were confirmed by genomic Southern analysis using the localized translocation breakpoints. A fragment that overlaps the breakpoint region shows an RFLP between the translocation and the normal sequence strain as shown in Fig. 3.12 A. These RFLP patterns enable duplications strains to be identified in the OR background (Fig. 3.12B).
Figure 3.8: Genomic Southern data for coverage of markers for the duplication Dp(AR17). T- T(AR17) ; Dp- Dp(AR17)

A - Initial marker already known to be covered by the duplication. In this case it was the dow gene for Dp(AR17).
B- Farthest covered marker on the left breakpoint (FCL(A))
C- Closest uncovered marker on the left breakpoint (CUL(A))
D- Farthest covered marker on the right (FCR(A)).
E- Closest uncovered marker on the right breakpoint (CUR(A))
Figure 3.9: Genomic Southern data for coverage of markers for the duplication \( Dp(B362i) \). T- \( T(B362i) \); Dp- \( Dp(B362i) \)

A - Initial marker already known to be covered by the duplication. In this case it was the \( met-1 \) gene for \( Dp(B362i) \).

B- Farthest covered marker on the left breakpoint (FCL(B))

C- Closest uncovered marker on the left breakpoint (CUL(B))

D- Farthest covered marker on the right (FCR(B)).

E- Closest uncovered marker on the right breakpoint (CUR(B))
Figure 3.10: Genomic Southern data for coverage of markers for the duplication $Dp(IBj5)$. T- $T(IBj5)$; Dp- $Dp(IBj5)$.

A - Initial marker already known to be covered by the duplication.
   In this case it was the $ylo$ gene for $Dp(IBj5)$.
B- Farthest covered marker on the left breakpoint (FCL(I))
C- Closest uncovered marker on the left breakpoint (CUL(I))
D- Farthest covered marker on the right (FCR(I)).
E- Closest uncovered marker on the right breakpoint (CUR(I))
Figure 3.11: Genomic Southern data for coverage of markers for the duplication Dp(OY329). T- T(OY329) ; Dp- Dp(OY329)
A - Initial marker already known to be covered by the duplication. In this case it was the trp-2 gene for Dp(OY329).
B- Farthest covered marker on the left breakpoint (FCL(O))
C- Closest uncovered marker on the left breakpoint (CUL(O))
D- Farthest covered marker on the right (FCR(O)).
E- Closest uncovered marker on the right breakpoint (CUR(O))
### Table 3.6A: Further localization of the breakpoints

**T(AR17)**

<table>
<thead>
<tr>
<th>Proximal side</th>
<th>Amplification</th>
<th>Normal sequence strain</th>
<th>Translocation strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer pair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17MO2F+D17MO2R</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>D17MO7F+D17MO7R</td>
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<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>D17MO6F+D17MO6R</td>
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<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>D17MO2R+D17MO7F</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

Reverse complement of D17MO6R 5' agaaaggctcttacacaaggtag
Reverse complement of D17MO2F 5' gtgcgaagtcacaatcatgaac

![Diagram showing amplification sites on proximal and distal sides.](image)

<table>
<thead>
<tr>
<th>Distal side</th>
<th>Amplification</th>
<th>Normal sequence strain</th>
<th>Translocation strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer pair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17BRF+D17BRR</td>
<td>yes</td>
<td></td>
<td>yes</td>
</tr>
<tr>
<td>D17ZF+D17ZR</td>
<td>yes</td>
<td></td>
<td>yes</td>
</tr>
<tr>
<td>D17BR1.2F+D17ZR</td>
<td>yes</td>
<td></td>
<td>no</td>
</tr>
</tbody>
</table>
Reverse complement of D17BR1.2 R 5' caagtgaagcaagattggt

Reverse complement of D17ZF 5' attcttcacaactccatcttga

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17MO6F</td>
<td>5'gctgggatctttgaaatagga</td>
<td>3347</td>
</tr>
<tr>
<td>D17MO6R</td>
<td>5'ctaccttgctgaagaacgttctttct</td>
<td></td>
</tr>
<tr>
<td>D17MO7F</td>
<td>5'aacaaagcgttctacattagg</td>
<td>923</td>
</tr>
<tr>
<td>D17MO7R</td>
<td>5'atttatccaaacaaacagcaagt</td>
<td></td>
</tr>
<tr>
<td>D17MO2F</td>
<td>5'gttcatgattttgacctcgtac</td>
<td>1524</td>
</tr>
<tr>
<td>D17MO2R</td>
<td>5'tcccatcagagaaatctgtga</td>
<td></td>
</tr>
<tr>
<td>D17MO7F+D17MO2R</td>
<td></td>
<td>3480</td>
</tr>
<tr>
<td>D17BRF</td>
<td>5'tctctcggcaactgatattggt</td>
<td>1360</td>
</tr>
<tr>
<td>D17BRR</td>
<td>5'aacagaatgtcaagcaggatgt</td>
<td></td>
</tr>
<tr>
<td>D17BR1.2F</td>
<td>5'catctcttgcaacatcctgtta</td>
<td>1258</td>
</tr>
<tr>
<td>D17BR1.2R</td>
<td>5'accaactttttgcttttcacttg</td>
<td></td>
</tr>
<tr>
<td>D17ZF</td>
<td>5'tcaaggatgggtggtggtgaagaat</td>
<td>1472</td>
</tr>
<tr>
<td>D17ZR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17BR1.2F+D17ZR</td>
<td></td>
<td>2752</td>
</tr>
</tbody>
</table>
**Table 3.6 B:** Proximal breakpoint of $T(IBj5)$

CPC-1 gene sequence  ATG  -718020

GGA - 715321

<table>
<thead>
<tr>
<th>Primer</th>
<th>Start nucleotide on contig 7.4</th>
<th>Amplification</th>
<th>Normal sequence</th>
<th>Translocation strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBj13F</td>
<td>714114</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IBj13R</td>
<td>715193</td>
<td>(1080)</td>
<td>(1080)</td>
<td></td>
</tr>
<tr>
<td>CPC1F</td>
<td>715934</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CPC1R</td>
<td>717518</td>
<td>(1585)</td>
<td>(1585)</td>
<td></td>
</tr>
<tr>
<td>IBj13F + CPC1R</td>
<td></td>
<td>(3404)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate the product size.
Figure 3.12 A: RFLP between the translocation and the OR strains at the breakpoint region. OR and T- the translocation strains i. (TAR17), ii. T(IBj5) and iii. T(B362i) digested with different enzymes and probed with junction fragments to identify RFLP. B-BamH1; E1-EcoRI; EV-EcoRV; H-HindIII; Xb-XbaI; Xh-XhoI; P-PstI.

Figure 3.12 B: Identification of duplications in OR background using RFLP at the breakpoint region. OR; T- T(AR17), T(IBj5) and T(B362i). Southern blots for identification of duplication (Dp) progeny of i. Dp(AR17), ii. Dp(IBj5) and iii. Dp(B362i). Using junction fragment RFLPs shown in Fig. 3.12 A, the duplication strains that were genotypically rid-1, Sad-1 or rid-1 Sad-1 were identified in the OR background (refer section 2.1.3 of chapter for a list of the specific primers used to amplify the junction fragments for each of the above duplications).
3.2.4 Dominant suppression of RIP in small duplications is not specific to Dp(erg-3)

To test whether dominant RIP suppression by large duplications was specific to Dp(erg-3/1), I analyzed crosses of large duplications with a gene-sized duplication for the dow gene. The dow and erg-3 genes are ~340 kb apart on LG IIIR (10-14% crossover) (Perkins et al., 2001).

Identification of the downy ORF

Earlier mapping studies placed the dow locus very close to ropy-11 (ro-11), which is known to be covered by Dp(AR17). That the dow gene might be large was inferred from the fact that the frequency of RIP-induced dow mutant progeny from crosses heterozygous for Dp(AR17) is ~ 4% (Bhat and Kasbekar, 2001). The ORF NCU8565.1 extends from nucleotide 13248 to 18168 on contig-3.520 (Release 3) and it was chosen as a candidate dow ORF because of its proximity to ro-11 and because of its large size in comparison to other ORFs in the vicinity. A 1421 bp fragment of this ORF was amplified by PCR with specific primers and cloned into the EcoRV site of the vector pCSN44, which has hygromycin-resistance as the selectable marker. If the cloned fragment was indeed from the dow gene, a duplication should target RIP to the endogenous copy of dow and produce RIP-induced dow mutant progeny. To test this, the N. crassa erg-3 a strain (FGSC #, 2725) was transformed with the pCSN44 (dow) plasmid. The hygromycin-resistant transformants were crossed with the wild type and homokaryotic progeny from this cross bearing the Dp(dow/1) transgene were crossed with OR. Segregation of dow mutants at a frequency of about 15% (N= 132) among progeny from this cross confirmed that ORF NCU8565.1 indeed represents the dow gene.

Construction of Dp(dow/1); erg-3 and Sad-1; Dp(dow/1); erg-3 strains

Screening for dow mutants requires picking large number of individual germinated ascospores to Vogel's glucose medium and scoring their morphology. This tedious screening procedure can be simplified by scoring for progeny that have inherited LGIII from the Dp(dow/1) parent. If the strain bearing Dp(dow/1) is marked by erg-3 then disregarding crossovers, RIP-induced dow mutants are expected to be obtained only in the erg-3 progeny (Fig. 3.13 A). I obtained a Dp(dow/1), erg-3 mutant strain by selecting
Figure 3.13 A: RIP induced dow mutants from wild-type by erg-3; Dp(dow/l) cross. In the cross erg-3+ x erg-3; Dp(dow), RIP induced dow mutants will segregate only among the erg-3 mutant progeny disregarding crossovers. This is because erg-3 and dow are on the same linkage group. RIP occurs only in the erg-3; Dp(dow) nucleus.
for hygromycin-resistant erg-3 mutants from a cross of the primary transformant with ORA. Of the twenty erg-3 segregants screened, eleven had the Dp(dow/1) transgene based on hygromycin-resistance and Southern hybridization. Two strains, Dp(dow/1); erg-3 a # 16 and Dp(dow/1); erg-3 A # 14 were used for crosses with large duplication strains.

Nine hygromycin-resistant erg-3 mutant progeny from the cross Dp(dow/1); erg-3 a #16 x Sad-1 A were screened for the presence of Dp(dow/1) by Southern analysis (Fig. 3.13 B). Two mat A strains had the Dp(dow/1) transgene and were non-productive in crosses with Sad-1 a suggesting that they had the Sad-1 mutant allele. One of these strains was used as Sad-1; Dp(dow/1); erg-3 A # 23.

Construction of the strains Sad-1; Dp(AR17), Sad-1; Dp(B362i) A, rid-1 Sad-1; Dp(IBj5) A and Dp(OY329) A

The translocation T(AR17), was crossed to Sad-1 A and f1 progeny that had the mating type of the Sad-1 parent were screened for the presence of Dp(AR17) by virtue of segregation of dow mutants in crosses with OR and by the ability to suppress RIP in Dp(erg-3/1). One strain Sad-1; Dp(AR17) A # 23 was used for crosses with Dp(dow/1); erg-3; a.

From the cross Dp(B362i) A # 8 x Sad-1; Dp(erg-3/1) a, one progeny, 8-5 A had the mating type of the Dp(B362i) parent and was non-productive with Sad-1 a (Sad-1 x Sad-1 crosses are infertile). This strain was confirmed to be Dp(B362i) by Southern analysis using the met-1 gene as probe and was used as the Sad-1; Dp(B362i) A strain.

Forty f1 progeny from each of the crosses T(IBj5) a x rid-1 Sad-1 A (33-10) and T(IBj5) A x rid-1 Sad-1a (33-11) were screened for their ability to suppress RIP in Dp(erg-3/1). In both cases, progeny with the mating type of the rid-1 Sad-1 parent were confirmed to be non-productive in crosses with Sad-1 of the opposite mating type and fertile in crosses with OR. One strain that had the Sad-1 mutant allele and suppressed RIP in Dp(erg-3/1) was used as the rid-1 Sad-1; Dp(IBj5) A # 47 parent.

Of the twenty f1 segregants screened for barren phenotype from the cross T(OY329) a x ORA, four barren segregants were obtained. These strains suppressed RIP
Figure 3.13 B: Southern blot of hygromycin-resistant *erg-3* mutant progeny from the cross *Sad-1 A x erg-3; Dp(dow/1)* a. The enzyme *BamH1* used was to digest the genomic DNA and a fragment of the *dow* gene was used as the probe. Progeny #3, 4, 17, 18, 23 and 29 have the *Dp(dow/1)* transgene.
in $Dp(\text{erg-3/1})$ and hence were used as $Dp(OY329)$ strains. The $Dp(S1229)\ A$ or $a$ (FGSC \# 264 and FGSC \# 265) strain was obtained from FGSC.

Large duplications suppress RIP in $Dp(dow/1)$.

In the crosses of $\text{erg-3; } Dp(dow/1)$ strains with the chromosome segment duplications, recovery of RIP-induced $dow$ mutants among the $\text{erg-3}$ progeny occurred at a frequency $<0.5\%$. This suggests that dominant suppression of RIP was effective on $Dp(dow/1)$ as well (Table 3.7). The control cross of $\text{erg-3; } Dp(dow/1)\ A \times ORa$ produced $dow$ mutants at a frequency of $11/200$. Surprisingly, $Dp(B362i)$ which had failed to suppress RIP in $Dp(\text{erg-3/1})$, could suppress RIP in $Dp(dow/1)$. Of the ninety-four $\text{erg-3}$ mutant progeny examined from the cross $\text{Sad-1; } Dp(B362i)8/5\ A \times Dp(dow/1);\ \text{erg-3}a$ none were $dow$ mutants. This discrepant finding was investigated further and is described in Chapter 4.

$RIP$ in $Dp(dow/1)$, in the cross $Dp(AR17)\ dow^+/dow^+ \times \text{erg-3; } Dp(dow/1)$

As a special case where two duplications involved the same locus, I screened $\sim 100$ progeny of each kind ($\text{erg-3}^+$ and $\text{erg-3}$) from the cross $\text{Sad-1; } Dp(AR17)\ A \times \text{erg-3; } Dp(dow/1)$ (Fig. 3.14) and found that RIP was suppressed in the $Dp(dow/1)$ nucleus. As expected from the fact that $dow$ is covered by $Dp(AR17)$, RIP-induced $dow$ mutants were obtained among the $\text{erg}^+$ segregants at a frequency of $4/98$, whereas no $dow$ mutants were recovered among the $\text{erg-3}$ mutant segregants. That is, no $dow$ mutants were recovered among eighty-six $\text{erg-3}$ mutant progeny screened. (Table 3.7).

3.3 Discussion

Small, gene-sized duplications as well as large chromosome segment duplications are acted upon by RIP (Perkins et al. 1997). It is likely that in a cross parented by two duplications of different size, there could be a preference for the larger duplication as RIP target. Results with additional duplications like $Dp(AR18)$ and $Dp(B362i)$ studied in this work further strengthen the observation of Bhat and Kasbekar (2004) that large duplications dominantly suppress RIP in small gene sized duplications. The size of
Table 3.7: RIP frequencies in crosses with Dp(dow/1); erg-3 strains.

<table>
<thead>
<tr>
<th>Cross</th>
<th>dow erg-3/ erg-3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. OR A x Dp1.5dowec hph; erg-3 a</td>
<td>9/68</td>
</tr>
<tr>
<td>2. OR a x Dp1.5dowec hph; erg-3 A</td>
<td>11/200</td>
</tr>
<tr>
<td>3. Sad-1 rid; Dp(IBj5) A x Dp1.5dowec hph; erg-3 a</td>
<td>0/105</td>
</tr>
<tr>
<td>4. Sad-1; Dp(AR17) A x Dp1.5dowec hph; erg-3 a</td>
<td>0/86 (erg-3 progeny)</td>
</tr>
</tbody>
</table>
|                                                                       | 4/98 (erg+ progeny)
| 5. Sad-1; erg-3; Dp1.5dowec hph A x Dp(OY329) a                       | 0/125             |
| 6. Sad-1; erg-3; Dp1.5dowec hph A x Dp(S1229) a                       | 0/119             |
| 7. Sad-1; Dp(B362i)8/5 A x Dp1.5dowec hph; erg-3 a                     | 0/94              |

- dow mutants generated by RIP in the Dp1.5dowec hph; erg-3 nucleus are linked to erg-3. Thus the frequency of dow erg-3/erg-3 progeny provides a measure of RIP efficiency in Dp1.5dowec hph. The erg-3 segregants can be recognized by their colony morphology on Vogel's-sorbose medium.

1 dow mutants amongst the erg+ progeny are induced by RIP in Dp(AR17).
Figure 3.14: dow mutants from Dp(AR17) by erg-3; Dp(dow/1) cross. This figure shows a strain bearing Dp(AR17) which covers the dow gene, crossed to Dp(dow/1) a small gene sized duplication of the dow gene. Dp(AR17) suppresses RIP in Dp(dow) therefore the occurrence of dow mutants in the erg-3 mutant background is expected to be <0.5% whereas the frequency of dow mutants in the erg-3+ background is ~4-5%.
Dp(AR18) has been estimated to be 270±10 kb (Smith et al., 1996). Dp(S1229), includes the gene mtr and it has a breakpoint that is inseparable from an arginine-14 (arg-14) mutation (Perkins 1997). There is a gap of unknown size between the supercontigs that bear these two genes. Taking into account the known sequence between the two genes, the size of Dp(S1229) is >270 kb. Dp(AR17), Dp(IBj5) and Dp(OY329) are >350 kb and they all behave as dominant suppressors of RIP. This suggests that large duplications, typically >270 kb, can suppress RIP in gene-sized duplications. Dp(B362i) at 117 kb does not suppress RIP in Dp(erg-3/I) suggesting that it may lie below the threshold window that determines the ability of a large segmental duplication to dominantly suppress RIP in gene-sized duplications.

We also tried an alternative approach to estimate the duplication size that is required for dominant suppression of RIP. We attempted to determine the size of partial diploids obtained from the unstable, Dp(AR18) het-6°R/het-6°P4 progeny generated from the cross T(AR18) het-6°R, x C2(3)-50 het-6°P4 thr (Smith et al., 1996). Our inability to distinguish deletions from gene conversions prevented us from fully exploring this approach.

Presence of large chromosome segment duplications might overwhelm the limiting amounts of the RIP machinery, since the structure formed by large duplications when paired might be more easily detected. Therefore the RIP machinery may be titrated by the large duplication. The titration hypothesis will be falsified if any duplication that fails to dominantly suppress RIP in small duplications turns out to be greater that 270 kb. My colleague Mr. Parmit Kumar Singh is screening other duplications and his study will further test the titration hypothesis. Translocation breakpoints of the four duplications used in this study have been localized to <5 kb segments. This information can be used to identify the exact location of any translocated segment. Comparing the breakpoint regions may provide clues about sequences that are potential sites for breaks and help characterize other translocations and predict their breakpoints.

The results of experiments using Dp(dow/I) showed that the RIP-suppression ability of large duplications is not specific to Dp(erg-3/I). In the cross Dp(AR17) erg-3° x erg-3; Dp(dow/I), dow mutants were obtained only among the erg-3° progeny i.e. due to RIP in Dp(AR17). Recovery of dow mutants only among the erg-3° progeny
strengthens the titration model. Strains harboring large duplications may serve as niches wherein small duplications escape RIP and may in the long evolve into new genes.
Chapter 4

Duplication Dp(B362i) elicits differential RIP-sensitivity of transgenes
4.1 Introduction

The RIP-susceptibility of a duplicated sequence depends on factors like the minimum size required for detection by the RIP machinery and whether there are large chromosome segment duplications present in cis or trans in the cross. A duplication that has a high AT content is likely to be less susceptible to RIP. As shown in Chapter 3 of the thesis, large chromosome segment duplications behave as dominant suppressors of RIP in small duplications (Bhat and Kasbekar, 2001; Vyas et al., 2006). We hypothesize that this happens because the large duplication titrates out the RIP machinery. Of the six large duplications studied, five were in the size range of 270 – 700 kb and they suppressed RIP in the probe duplications Dp(erg-3/1) and Dp(dow/1). The sixth duplication Dp(B362i), was only 117 kb in size and it failed to suppress RIP in Dp(erg-3/1) but not in Dp(dow/1).

This unusual behavior of Dp(B362i) has been investigated using additional transgenes that are similar to Dp(erg-3/1) and Dp(dow/1). Our results suggest that Dp(erg-3/1) and Dp(dow/1) are rendered differentially sensitive to RIP by Dp(B362i). In the course of this study we also found that RIP can target two different duplications in the same nucleus and that the occurrence of RIP on any one of the two (or more) duplications is not independent of the other.

4.2 Results

4.2.1 Dp(B362i) suppresses RIP in Dp(dow/1) but not in Dp(erg-3/1).

A cross was performed between the strains Sad-1; Dp(B362i)8/5 A (construction of this strain is described in section 3.2.4 of chapter 3) and Dp(dow/1); erg-3 a and the frequency of dow mutants was determined among the erg-3 progeny. Of the ninety-four erg-3 mutants examined, none were mutant for dow. The control cross Dp(dow/1); erg-3 a x OR A produced dow mutants among the erg-3 progeny at frequencies > 8 %. Another control cross; Sad-1; Dp(B362i)8/5 A x Dp(erg-3/1) a, produced erg-3 mutants at a frequency of 5.6% (see Chapter 3, section 3.2.4). These
results allowed us to conclude that $Dp(B362i)$ shows differential behavior towards $Dp(erg-3/1)$ and $Dp(dow/1)$ with regard to RIP suppression.

$rid-1 Sad-1; Dp(B362i) a \times Dp(dow); Dp(erg-3) A$

We also performed crosses in which both the duplications, $Dp(erg-3/1)$ and $Dp(dow/1)$ were present in the same nucleus. We analyzed eighty f$_1$ progeny from the cross $Dp(B362i) A \times rid-1 Sad-1 a$ and identified one mat $a$ strain which contained $Sad-1$ (the strain was not productive in a cross with $Sad-1 A$ strain; $rid-1$ mutation is of not consequence in this cross) and also had the $Dp(B362i)$ duplication based on Southern hybridization. This strain was thus genotypically $rid-1 Sad-1; Dp(B362i)$. The $Dp(erg-3/1); Dp(dow/1)$ strain was constructed by screening among the hygromycin-resistant, $erg-3^+$ progeny from $Dp(erg-3/1) a \times Dp(dow/1); erg-3 A$ by Southern analysis for the presence of ectopic fragments of $dow$ and $erg-3$ genes. Two progeny that were double duplications for $Dp(erg-3/1)$ and $Dp(dow/1)$ were designated as $Dp(dow/1) A \#14$ and $Dp(erg-3/1) A \#36$ (Fig 4.1 A and 4.1B). Frequency of $dow$ mutants among $erg-3^+$ and $erg-3$ segregants from the cross $rid-1 Sad-1; Dp(B362i) a \times Dp(dow/1); Dp(erg-3/1) A$ was determined. RIP suppression was observed in $Dp(dow/1)$ but not in $Dp(erg-3/1)$ (Table 4.1). The control cross of $Dp(dow/1); Dp(erg-3/1) A \times ORa$ produced $dow$ mutant progeny.

### 4.2.2 RIP-suppression ability of $Dp(B362i)$ on specific $Dp(erg-3)$-like transgenes

The differential sensitivity of $Dp(dow/1)$ and $Dp(erg-3/1)$ to RIP suppression by $Dp(B362i)$ could be due to the sequence or the position of the transgene in the genome, or it could be due to differential RIP-sensitivity of the target genes. I analyzed additional $Dp(erg-3)$-like transgenes, which would be insertions at different positions in the genome. An $erg-3$ mutant strain of $N. crassa$ was transformed with the plasmid construct $pSS17$ that has an insert of a 1.3 kb $Kpn1$ fragment of the $erg-3$ gene tagged with the $hph$ gene for hygromycin-resistance (Prakash et al. 1999). Hygromycin-resistant primary transformants were crossed with $OR A$ and homokaryotic segregants were obtained bearing the transgene.

Seven strains, homokaryotic for different transgenes were obtained from crosses with the primary transformants. These strains, designated as $Dp(erg-3/2) – Dp(erg-3/8)$
Figure 4.1: Confirmation of the presence of Dp(erg-3/1) and Dp(dow/1) transgenes by Southern analysis.

A - Progeny from cross Dp(dow) x Dp(erg-3/1) screened for the Dp(erg-3/1) transgene. PstI was used for the digestion and a fragment of the erg-3 gene was used as the probe. Segregant # 1, 13, 14, 17 and 36 are duplicated for erg-3 fragment.

B - The same set of segregants was also screened for the presence of the Dp(dow) transgene. The genomic DNA of the segregants was digested with BamHI and a fragment of the dow gene was used as a probe. Segregant #1, 14, 32 and 36 have the Dp(dow) transgene. Progeny No. 1, 14 and 36 have both the transgenes.
Table 4.1: Frequency of *erg-3* and *dow* mutants from *Dp(dow/1); Dp(erg-3/1) A x Sad-1; rid-1; Dp(B362i) a* crosses.

<table>
<thead>
<tr>
<th>Cross type</th>
<th>erg-3 % (N)</th>
<th>dow % (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dp(dow/1); Dp(erg-3/1) A/14 x ORa</em></td>
<td>14.0 (200)</td>
<td>8.2 (121)</td>
</tr>
<tr>
<td><em>Dp(dow/1); Dp(erg-3/1) A/36 x ORa</em></td>
<td>24.0 (449)</td>
<td>5.1 (117)</td>
</tr>
<tr>
<td><em>Dp(dow/1); Dp(erg-3/1) A/14 x Sad-1; rid-1; Dp(B362i) a</em></td>
<td>4.3 (449)</td>
<td>&lt;0.9 (110)</td>
</tr>
<tr>
<td><em>Dp(dow/1); Dp(erg-3/1) A/36 x Sad-1; rid-1; Dp(B362i) a</em></td>
<td>4.2 (425)</td>
<td>&lt;0.9 (109)</td>
</tr>
</tbody>
</table>
were crossed with ORA, ORa, Sad-1; Dp(B362i) A and rid-1Sad-1; Dp(B362i) a strains and the progeny were screened for the segregation of RIP induced erg-3 mutants. Of the seven Dp(erg-3)-like transgenes analyzed, Dp(B362i) appeared to suppress RIP in two; Dp(erg-3/4) and Dp(erg-3/7). The frequency of erg-3 mutants in crosses of these two transgenes with Sad-1; Dp(B362i) A # 8/5 was < 0.5%. Crosses with other transgenic strains produced erg-3 mutants at frequencies > 2% (Table 4.2). The results suggest that the sequence of the target genes is not responsible for the differential sensitivity of Dp(dow/1) and Dp(erg-3/1) to RIP.

4.2.3 RIP – suppression ability of Dp(B362i) on specific Dp(dow)-like transgenes

Additional Dp(dow)-like transgenes were tested for the RIP-suppression ability of Dp(B362i). An erg-3 strain of N. crassa was transformed with a plasmid construct having a 1.4 kb insert of the dow gene and transformants were selected in the manner mentioned in the previous section. Homokaryotic progeny bearing the Dp(dow) transgene were obtained in erg-3 mutant background. Three different transgenes were analyzed for segregation of dow mutation among the erg-3 mutant progeny in crosses with Sad-1; Dp(B362i) A and rid-1Sad-1; Dp(B362i) a strains. RIP frequencies obtained for all three Dp(dow) transgenes suggest that Dp(B362i) could dominantly suppress RIP in all of them (Table 4.3).

4.2.4 Dp(B362i) can differentiate between two different Dp(erg-3) transgenes present in the same cross.

Since Dp(B362i) was able to suppress RIP in two other Dp(erg-3)-like transgenes, we examined whether this difference was maintained when both the Dp(erg-3)-like transgenes were present in the same cross. For this, we need to distinguish between the RIP induced erg-3 mutants produced from the two types of Dp(erg-3)-like trangenes, one in which RIP is suppressed by Dp(B362i) and the other in which it is not suppressed. erg-3 mutant progeny were analyzed from the cross, Dp(B362i); dow; Dp(erg-3/1) x Sad-1; Dp(erg-3/7) (see chapter 2 for details on Dp(erg-3/1)) (Fig 4.2- construction of the strain). In this cross the dow mutation allows one to differentiate between the erg-3 mutants that result from RIP in Dp(erg-3/1) or Dp(erg-3/7).
Table 4.2: RIP-induced *erg-3* mutants from crosses heterozygous for novel *Dp(erg-3)*-like transgenes. These transgenes have been used to determine whether *Dp(B362i)* can suppress RIP in them.

<table>
<thead>
<tr>
<th><em>mat a</em> parent</th>
<th>RIP frequency [% (N)] in crosses with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>OR A</em></td>
</tr>
<tr>
<td><em>Dp(erg-3/2) a</em></td>
<td>12.0 (296)</td>
</tr>
<tr>
<td><em>Dp(erg-3/3) a</em></td>
<td>9.0 (204)</td>
</tr>
<tr>
<td><em>Dp(erg-3/4) a</em></td>
<td>7.3 (342)</td>
</tr>
<tr>
<td><em>Dp(erg-3/5) a</em></td>
<td>5.5 (198)</td>
</tr>
<tr>
<td><em>Dp(erg-3/6) a</em></td>
<td>8.3 (227)</td>
</tr>
<tr>
<td><em>Dp(erg-3/7) a</em></td>
<td>6.5 (259)</td>
</tr>
<tr>
<td><em>Dp(erg-3/8) a</em></td>
<td>11.2 (322)</td>
</tr>
</tbody>
</table>

Table 4.3: RIP-induced *dow* mutants among *erg-3* progeny from crosses heterozygous for novel *Dp(dow)*-like transgenes.

<table>
<thead>
<tr>
<th><em>mat a</em> parent</th>
<th><em>dow</em> progeny [% (N)] from crosses with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>OR A</em></td>
</tr>
<tr>
<td><em>erg-3; Dp(dow/2) A</em></td>
<td>30.0 (20)</td>
</tr>
<tr>
<td><em>erg-3; Dp(dow/3) A</em></td>
<td>20.0 (20)</td>
</tr>
<tr>
<td><em>erg-3; Dp(dow/4) A</em></td>
<td>10.0 (20)</td>
</tr>
</tbody>
</table>

In the above case *dow* mutants were screened only among the *erg-3* mutant progeny. Since *dow* and *erg-3* are present on the same linkage group *dow* mutants are expected only among the *erg-3* progeny. The frequencies estimated for N= 100 would be equivalent to N= 200 because progeny representing only one nucleus are being considered.
Figure 4.2: Construction of the $Dp(B362i); dow; Dp(erg-3/1)$ strain. Strain # 2 is marked with $dow$. A $dow$ mutant (2) was crossed to a $T(B362i)$ (1) strain to obtain a $T(B362i); dow$ segregant (4). The same $dow$ mutant (2) was crossed to a $Dp(erg-3/1)$ (3) strain to obtain a $dow; Dp(erg-3/1)$ segregant (5). A cross between strain # 4 and # 5 produced segregants that were $Dp(B362i) dow; Dp(erg-3/1)$ (6).
Construction of $Dp(B362i)\,;\,dow;\,Dp(erg-3/1)$

To construct the $Dp(B362i)\,;\,dow;\,Dp(erg-3/1)$ strain, fourteen $dow$ mutant progeny from the cross $T(B362i)\,A\,x\,dow\,a$ were analyzed by Southern hybridization to identify one that was the translocation type (Fig 4.3). This strain was designated as $T(B362i)\,;\,dow\,A\,#\,11$. In parallel, ten $dow$ mutant progeny from the cross $Dp(erg-3/1)\,A\,x\,dow\,a\,#\,4$ were screened for hygromycin-resistance to identify five that had inherited the $Dp(erg-3/1)$ transgene. Of these, two that were $mat\,a\,(\#1\,and\,7)$ were used in crosses with $T(B362i)\,;\,dow\,A\,#\,11$.

Of a total of forty eight segregants from the crosses of $T(B362i)\,;\,dow\,A\,#\,11$ with $dow;\,Dp(erg-3/1)\,a\,#\,1\,and\,7$, nine $mat\,A$, hygromycin-resistant, $dow$ mutants were analyzed by Southern hybridization to identify four $Dp(B362i)$ and two translocation type progeny which also contained $dow$ and $Dp(erg-3/1)$ (Fig. 4.4). These $Dp(B362i)\,;\,dow;\,Dp(erg-3/1)$ segregants were used in crosses with a $Sad-1;\,Dp(erg-3/7)\,a\,#\,11$ strain obtained from the cross $Dp(erg-3/7)\,x\,Sad-1\,a$.

Differential sensitivity of $Dp(erg-3/1)$ and $Dp(erg-3/7)$ to RIP

$erg-3$ mutant progeny from the crosses of $Dp(B362i)\,;\,dow;\,Dp(erg-3/1)$ strains with $Sad-1;\,Dp(erg-3/7)\,#\,11$ were analyzed for the segregation of the $dow$ mutants. If RIP occurs predominantly in the $Dp(erg-3/1)$ transgene, then RIP-induced $erg-3$ mutants are expected to segregate only among the $dow$ mutants which represent the $Dp(erg-3/1)$ transgene. Of the 32 $erg-3$ mutants examined from these crosses, 25 were $dow$ and 7 were $dow^+$. This is a significant deviation from 1:1 segregation based on the $\chi^2$-square table provided by Perkins (1994). The $erg^+$ progeny segregated $dow$ and $dow^+$ at the frequency 23:17 which was not different from 1:1 segregation (Table 4.4). The control cross $T(B362i)\,;\,dow;\,Dp(erg-3/1)\,A\,x\,Sad-1;\,Dp(erg-3/7)\,a$ segregated $dow$ and $dow^+$ segregants among $erg-3$ and $erg-3^+$ progeny in the ratios 24:16 and 16:27 respectively. These ratios also show 1:1 segregation. Together these results suggest that the $Dp(B362i)$ elicits the differences between the sensitivity of $Dp(erg-3/1)$ and $Dp(erg-3/7)$ to RIP.
**Figure 4.3:** Southern analysis of dow mutants from the cross $T(B362i) A \times dow \# 4 a$

Genomic DNA was digested with $XhoI$ and analyzed to identify $T(B362i)$ translocation progeny. $T$ is the $T(B362i)$ parent. Among the progeny, only progeny $\# 11$ showed a translocation type pattern. The probe used is a junction fragment of the breakpoint region.

**Figure 4.4:** Southern blot analysis of hygromycin-resistant progeny from the cross $T(B362i); dow dow; Dp(erg-3/1)$. $XhoI$ was used for digestion of genomic DNA and the probe used was a junction fragment of the translocation breakpoint. Progeny $\# 1/7, 1/12, 1/19, 7/23$ and $7/28$ are duplications. progeny $\# 1/3$ and $7/20$ are translocation type.
Table 4.4: *erg-3* mutants obtained among *dow* progeny for the crosses of *Dp(B362i); dow; Dp(erg-3/1)* strains with *Sad-1; Dp(erg-3/7).*

<table>
<thead>
<tr>
<th>Cross</th>
<th>erg-3^+</th>
<th>dow</th>
<th>erg-3</th>
<th>dow</th>
</tr>
</thead>
<tbody>
<tr>
<td>dow; <em>Dp(B362i)</em>; <em>Dp(erg-3/1)</em> A 1-7 x <em>Sad-1; Dp(erg-3/7)</em></td>
<td>21</td>
<td>16</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>dow; <em>Dp(B362i)</em>; <em>Dp(erg-3/1)</em> A 1-12 x <em>Sad-1; Dp(erg-3/7)</em></td>
<td>22</td>
<td>14</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>dow; <em>Dp(B362i)</em>; <em>Dp(erg-3/1)</em> A 1-19 x <em>Sad-1; Dp(erg-3/7)</em></td>
<td>17</td>
<td>20</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>dow; <em>Dp(B362i)</em>; <em>Dp(erg-3/1)</em> A 7-28 x <em>Sad-1; Dp(erg-3/7)</em></td>
<td>17</td>
<td>23</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>dow; <em>T(B362i)</em>; <em>Dp(erg-3/1)</em> A 1-3 x <em>Sad-1; Dp(erg-3/7)</em></td>
<td>18</td>
<td>15</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>dow; <em>T(B362i)</em>; <em>Dp(erg-3/1)</em> A 7-20 x <em>Sad-1; Dp(erg-3/7)</em></td>
<td>27</td>
<td>16</td>
<td>16</td>
<td>24</td>
</tr>
</tbody>
</table>

Frequencies of RIP in this case have been compared for the two transgenes *Dp(erg-3/1)* and *Dp(erg-3/7)* in the presence of *Dp(B362i).* *Dp(B362i)* is expected to suppress RIP in *Dp(erg-3/7)* and not in *Dp(erg-3/1).* *erg-3* mutants from the two transgenes can be differentiated by the *dow* mutation as the marker.
4.2.5 RIP in one of two (or more) duplications present in the same nucleus

I also examined the efficiency of RIP on two gene-sized duplications Dp(erg-3/1) and Dp(dow/1) when they were present in cis. If RIP in the two duplications is independent, the frequency of dow mutants among the erg-3 and erg-3+ progeny from the cross Dp(dow/1) Dp(erg-3/1) x ORA should be equal. To test this, erg-3+ and the RIP-induced erg-3 progeny were examined for RIP-induced dow mutants from the crosses of the double duplication Dp(dow/1) Dp(erg-3/1) A strains #1, #14 and #36 with OR (Table 4.5A and 4.5B). The erg-3 mutation frequencies for these crosses were scored for ascospores harvested at different time-points and they were not different from that of the control cross of Dp(erg-3/1) with OR. This suggested that the presence of Dp(dow/1) does not affect RIP in Dp(erg-3/1). The results in the table show that RIP-induced dow mutants are found predominantly among the RIP-induced erg-3 mutants in these crosses. This suggests that the occurrence of RIP in the two duplications is not independent.

4.3 Discussion

Results of the Dp(B362i) crosses with Dp(erg-3/1) and Dp(dow/1) suggested that Dp(B362i) can dominantly suppress RIP in Dp(dow/1) but not in Dp(erg-3/1). Reproducibility of the <1.0% frequency of dow mutants and >1.0% frequency of erg-3 mutants among the progeny from the cross Dp(dow/1); Dp(erg-3/1) x Sad-1; Dp(B362i) suggests that the RIP efficiency was not intrinsically low in the erg-3; Dp(dow/1) x Sad-1 Dp(B362i) cross. Dp(B362i) may be below the size threshold required to suppress RIP Dp(erg-3/1) but not in Dp(dow/1). Duplications that are >270 kb are probably larger than the threshold for RIP suppression in probe duplications which may differ with respect to their "titration endpoints". These results also indicate that additional factors apart from titration might influence dominant RIP suppression by large duplications that are ~117 kb in size. The fact that Dp(B362i) can suppress RIP in two other Dp(erg-3)-like transgenes suggests that the differential sensitivity is not due to sequence difference between the target genes. Dp(B362i) was able to differentiate between Dp(erg-3/1) and Dp(erg-3/7) when the two transgenes were present in the same cross indicating that the difference in the RIP-susceptibility of the two transgenes was elicited in presence of Dp(B362i).
Table 4.5 A: Frequency of erg-3 mutant progeny from $Dp(dow/1); Dp(erg-3/1)$ $A \times OR$ a at specific days of harvest. This is a control to show that the cross is proficient for RIP. The erg-3 mutants would be produced because the transgene $Dp(erg-3/1)$ targets RIP to the endogenous erg-3 locus.

<table>
<thead>
<tr>
<th>Parent</th>
<th>23 days</th>
<th>26 days</th>
<th>31 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.5 (336)</td>
<td>28.9 (138)</td>
<td>16.0 (179)</td>
</tr>
<tr>
<td>14</td>
<td>14.0 (264)</td>
<td>20.4 (210)</td>
<td>10.5 (105)</td>
</tr>
<tr>
<td>36</td>
<td>20.0 (249)</td>
<td>22.0 (210)</td>
<td>20.0 (149)</td>
</tr>
</tbody>
</table>

Table 4.5 B: Frequency of dow mutants among erg-3 ($e^-$) and erg+ ($e^+$) progeny from $Dp(dow/1); Dp(erg-3/1)$ $A \times OR$ a. The dow mutants are produced in this case because the $Dp(dow)$ targets RIP to the endogenous dow locus.

<table>
<thead>
<tr>
<th>Parent</th>
<th>23 days</th>
<th>26 days</th>
<th>31 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$e^+$</td>
<td>$e^-$</td>
<td>$e^+$</td>
</tr>
<tr>
<td>1</td>
<td>4.8 (103)</td>
<td>14.4 (90)</td>
<td>13.1 (114)</td>
</tr>
<tr>
<td>14</td>
<td>2.5 (117)</td>
<td>34.4 (90)</td>
<td>8.2 (121)</td>
</tr>
<tr>
<td>36</td>
<td>1.5 (65)</td>
<td>16.2 (62)</td>
<td>5.1 (117)</td>
</tr>
</tbody>
</table>

Frequencies take into account both the nuclei, independent events of RIP on $Dp(erg-3/1)$ and $Dp(dow/1)$ would give equal number of dow mutants among the RIP induced erg-3 and the erg-3+ progeny. dow mutants were predominantly seen among the RIP-induced erg-3 mutants. Frequencies were obtained from harvests of different days to account for lack of synchrony in the sexual phase of the different strains.
Factors pertaining to the insertion site of a transgene might be responsible for such differences. Such factors may include proximity of the transgenes to the NOR, which is known to be immune to RIP (Galagan et al., 2003). Transgenes residing in such regions may have a greater chance of escaping RIP. Similarly, chromosomal territories may also play a role in making certain regions more accessible to RIP. Therefore duplications like $Dp(B362i)$ can serve as reagents to study aspects of RIP that remain as yet unexplored. However it is not clear whether this unusual behavior of $Dp(B362i)$ is an intrinsic property of the duplication. Investigating more chromosome segment duplications of size comparable with $Dp(B362i)$ for such a behavior may provide clues. Certain mutations, which slow down the growth rate, like the genes involved in nuclear distribution, may increase, RIP efficiency by enhancing detection of the duplications (Bouhouche et al., 2004). If such mutations are used in the $Dp(B362i)$ crosses with the $Dp(erg-3)$ and $Dp(dow)$ transgenes, there might be a change in the RIP-sensitivity of the transgenes.

Analysis of the $Dp(dow/1); Dp(erg-3/1) \times OR$ cross suggests that RIP events in individual duplications residing in the same nucleus are not independent of each other. The rate-limiting factor for this might be assembly of the RIP machinery. This characteristic may be helpful in generation of RIP-induced mutants.