Chapter 2

Materials and Methods
2.1 MATERIALS

2.1.1 Source of chemicals and materials used

Chemicals used for media preparation

Bacto-agar, Bacto-tryptone and Yeast Extract were from Difco Laboratories, USA. Calcium chloride, sorbitol, sorbose, fructose and sucrose were procured from Sigma Chemical Company, USA.

Media supplements

Amino acids, biochanin A, hygromycin, biotin, and tomatine were procured from the Sigma Chemical Company, USA.

Molecular biology reagents

Agarose, ethidium bromide, sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB), Sigma Chemical Company, USA. Sephadex G-50 was from Pharmacia Biotech, Sweden. All restriction enzymes were from New England Biolabs Inc., USA; Klenow polymerase, and \textit{Taq} DNA polymerase were from New England Biolabs Inc., USA, or Promega Corporation, USA. The high fidelity \textit{Taq} DNA polymerase and the \textit{Taq} polymerase used for long range PCR was procured from Eppendorf International. The DNA size markers were purchased either from Bangalore Genei, India or New England Biolabs Inc., USA. The DNA multiprime labelling kit was procured from BRIT, India; Qiagen DNA purification columns were from Qiagen Inc., USA. Custom-made oligonucleotides were initially synthesized in CCMB’s inhouse Oligonucleotide synthesis facility and later at the Bioserve India Ltd, Hyderabad; Hybond N+ membrane was procured from Amersham International, UK, and Whatman filter paper from Whatman International Ltd., USA. X-ray films were obtained from Konica Corporation, Japan, and radiolabeled nucleotides were procured from BRIT, India. All other chemicals were purchased from local manufacturers and were of analytical grade.
2.1.2 *Neurospora crassa* strains used in this study

74-OR23-1 *A* (FGSC #987) and *OR8-1 a* (FGSC #988)

These are standard wild-type *N. crassa* laboratory strains. These were obtained from the Fungal Genetics Stock Center (FGSC- School of Biological Sciences, SBS 404, University of Missouri, Kansas City, 5007 Rockhill Road, Kansas City, MO 64110 USA. Available at www.fgsc.net).

*dow A* (FGSC #4051) and *dow a* (FGSC #4052)

These strains are mutant in the *dow* locus which is linked to *erg-3* (10%) on IIIR. They have a characteristic soft, matty growth with no aerial hyphae. They conidiate normally, have good viability, fertility and scorability (Perkins *et al.*, 2001). These were obtained from the FGSC.

*erg-3 A* (FGSC #3439) *erg-3 a* (FGSC #2725)

These strains lack a functional sterol C-14 reductase. The nature of the mutation is unknown. They have a slower rate of growth than the wild type, have reduced conidiation and are female sterile. They were obtained from the FGSC.

*T (IIIR→[I;II]) AR17 a* (FGSC #1463, Perkins lab stock no. xx-366)

This strain is a complex insertional translocation involving IR, IIR and IIIR. One-third of the viable progeny from the cross of the translocation with normal sequence are duplicated for a segment of distal IIR, designated *Dp (IIIR→[I;II])AR17*. The duplication covers the IIR marker *dow* but not *erg-3* (Perkins, 1997). The *dow* and *erg-3* loci are separated by 10% crossover distance (Perkins *et al.*, 2001). The duplications are stably barren in crosses. This strain was a kind gift of David D. Perkins (Stanford University).

*T(IIL>IIIR) AR18 A*(FGSC #2643) and *T(IIL>IIIR) AR18 a*

It is an insertional translocation with a segment between *cys-3* and *cot -5* on IIL inserted in IIIR. Duplications *Dp(IIL>IIIR) AR18* are produced at one third the
frequency of the viable progeny and have a very slow growth initially. The crosses between duplications and non-duplication strains are stably barren. The duplication covers the het-6 locus. Heterozygosity for the het-6 locus makes the duplications heterokaryon incompatible and ascospore colonies of such progeny have a bud like appearance. These strains were obtained from FGSC.

\[ T (IV \to I) B362i A \ (FGSC \# \ 2935) \text{ and } T (IV \to I) B362i a \ (FGSC \# \ 2988) \]

It is an insertional translocation of a segment of IVR including the met-1 gene to I. TxT crosses have reduced fertility. One third of the viable progeny from a TxN cross have a barren phenotype in crosses with euploid strains. The barrenness is exceptionally stable. These strains were obtained directly from FGSC.

\[ T(VIL \to IR)IBj5 cpc-1 A \ (FGSC \# \ 4433) \text{ and } T(VIL \to IR)IBj5 cpc-1 a \ (FGSC \# \ 4434) \]

These are insertional translocation strains in which a segment of VIL, extending from cpc-1 through ylo-1 is translocated to IR. One-third of the viable progeny from a cross of the translocation with a normal sequence are duplications designated, \( Dp(VIL \to IR)IBj5 \). The duplication covers the markers, ylo-1 and vvd and is stably barren in crosses (Perkins et al., 1997; 2001). These strains were obtained from the FGSC.

\[ T(VIR \to IIIIR)OY329 A \ (FGSC \# \ 3670) \text{ and } T(VIR \to IIIIR)OY329 a \ (FGSC \# \ 3671) \]

These strains are insertional translocation strains in which a segment of LG VR is inserted in LG IIIIR. The translocated segment covers the col-18\(^+\) locus on VIR (Perkins et al., 1997). A proportion of the progeny from crosses of the translocation with normal sequence strains are duplicated for a segment of VIR. The duplication strains, designated \( Dp(VIR \to IIIIR)OY329 \), are stably barren in crosses. These strains were obtained from the FGSC.

\[ Dp(IVR \to VII)S1229 A \ (FGSC \# \ 264) \text{ and } Dp(IVR \to VII)S1229 a \ (FGSC \# \ 265) \]

These strains were obtained from the FGSC. These can also be obtained as segregants from the cross of the complex insertional translocation.
T(IVR\(\rightarrow\)VIIL;IL;IIR;IVR)S1229 arg-14 with normal sequence. The duplications are stable in crosses and segregate 1:1 in progeny from duplication crossed with normal sequence (Perkins, 1997).

**rid-1 Sad-1 (33-10) A and rid-1 Sad-1 (33-11) a**

These are strains that are double mutants for the sad-1 and rid-1 loci. The Sad-1 mutation is semi-dominant and rid-1 is recessive. These two strains were kind gifts from Prof. Robert L. Metzenberg (University of California, Los Angeles).

**C-2 (3)- 50 a**

This strain is a euploid strain with all markers except IIL of the Oak Ridge type. The IIL is from the Panama strain (FGSC # 1165), a wild isolate (See section 3.3). The het-6 of this strain is the PA type and is incompatible with het-6 of OR. Heterokaryons between the two types of strains display vegetative incompatibility. It was a kind gift from Prof. Louise Glass (University of California, Berkeley).

**Dp1.3\(^{\text{hph}}\) A and Dp 1.3\(^{\text{hph}}\) a/ Dp(erg-3/1) A and Dp(erg-3/1) a**

These strains, described in Prakash et al. (1999), were from our laboratory collection. They contain a 1.3 kb HindIII fragment of the erg-3 gene which is tagged with the hygromycin resistance marker hph. The ectopic erg-3 fragment does not make a functional product but serves to target RIP to the endogenous erg-3\(^{+}\) gene on LG IIIR. The 1.3 kb transgene is linked to to al-3 on LG VR (3/80) and unlinked to markers on other linkage groups. These strains when crossed with the wild-type strain produce RIP-induced erg-3 mutants, which are very easily distinguished, hence these strains are very useful for assaying RIP. These strains have been referred to as Dp(erg-3/1) A and Dp(erg-3/1) a in the thesis. **rid A and rid a**

The rid A (N1977) and rid a (N2148) strains were kindly provided by Eric U. Selker and Michael Freitag (University of Oregon) and are described in Freitag et al. (2002). The rid A strain has a hygromycin-sensitive phenotype whereas the rid a strain is hygromycin-resistant.
Sad-1 A (96-01) and Sad-1 a (96-02)

These are semi-dominant MSUD-suppressor strains in which the sad-1 gene has been disrupted by the insertion of the hygromycin resistance gene, hph. Consequently, they are hygromycin-resistant. These strains were kind gifts from Prof. Robert L. Metzenberg.

rid; Dp 1.3ecc hph A # 10 and rid; Dp1.3ecc hph a # 2

These strains were constructed in the lab. The rid; Dp 1.3ecc hph A and rid; Dp 1.3ec hph a were obtained as segregants from the crosses Dp 1.3ecc hph a x rid A and Dp 1.3ec hph A x rid a. Dp(erg-3/l) has previously been referred to as Dp 1.3ecc hph The genotypes of these segregants was confirmed by verifying that RIP-induced erg-3 mutant progeny failed to be produced (frequency <0.5%) in crosses with rid strains of the opposite mating type but were produced in crosses with 74-OR23-l A or OR8-l a. They have been designated as rid; Dp(erg-3/l) A # 10 and rid; Dp(erg-3/l) A # 2 in the thesis.

Sad-l; Dp1.3ec/hph A and Sad-1; Dp1.3ec/hph a or Sad-1; Dp(erg-3/l) A and Sad-1; Dp(erg-3/l) a

These strains were created in the lab and are a part of the lab collection. The Sad-l; Dp1.3ec/hph A and Sad-1; Dp1.3ec/hph a strains were generated as segregants from Sad-l A x Dp1.3ec/hph A and Sad-1 a x Dp1.3ec/hph A, respectively. The presence of Sad-l was confirmed by verifying their ability to suppress the barren phenotype in crosses with Dp(lBj5) and the presence of Dp1.3ec/hph was confirmed by verifying that RIP-induced erg-3 mutants were produced in crosses with 74-OR23-l A or OR8-l a. They are referred to as Sad-l; Dp(erg-3/l) A and Sad-1; Dp(erg-3/l) a.

dow a # 4 and dow A # 11

These are segregants obtained from the cross dow A (FGSC # 4051) x dow a (FGSC # 4052). Of the 15 progeny analyzed from this cross six were a and nine were A. One of each mating type was used later for crosses.
Chapter 2

\[Dp1.4^{\text{hph}} A\] and \[Dp1.4^{\text{hph}} a/ Dp(dow/1) A\] and \[Dp(dow/1) a\]

These strains, described in Vyas et al. (2006), were constructed in the lab as a part of a knockout study. These strains have a duplication of a 1.4 kb fragment of the \(dow\) gene tagged with a selectable marker \(hph\) which imparts resistance to Hygromycin B. They were constructed by transforming an \(erg-3\) mutant strain of \textit{Neurospora crassa} with a vector construct carrying a fragment of the \(dow\) gene. Like the duplication strains for \(erg-3\), the ectopic copy of the \(dow\) gene targets RIP to the endogenous copy when these strains are crossed to wild type strains. RIP induced \(dow\) mutants are produced among the progeny as a result of this. \(dow\) mutants have a distinct matty morphology and hence can be used for RIP assays. In the thesis these strains have been referred to as \(Dp(dow/1) A\) and \(Dp(dow/1) a\).

\[Dp(dow/1); \ erg-3 \ A \ # \ 14 \] and \[Dp(dow/1); \ erg-3 \ a \ # \ 16\]

These strains were created by selecting \(erg-3\) mutants that had inherited the \(Dp(dow)\) transgene among progeny from the cross of a transformed \(erg-3\) mutant bearing the \(Dp(dow/1)\) transgene with wild type strain.

\[dow; \ Dp(erg-3/l) \ a \ # \ 1 \] and \[dow; \ Dp(erg-3/l) \ a \ # \ 7\]

These strains were obtained as a hygromycin-resistant \(dow\) mutants from the cross \(Dp(erg-3/l) \ a \times dow \ A \ # \ 11\).

\[Sad-1; \ Dp(dow/l) \ A \] and \[Sad-1; \ Dp(dow/l) \ a\]

These strains were obtained as segregants from the cross \(Sad-1 \ A \times Dp(dow/l) \ a\).

\[Sad-1; \ Dp(dow/l) \ erg-3 \ A \ /23\]

This segregant was obtained from the cross \(Sad-1 \ A \times erg-3; \ Dp(dow) \ a\). It was non-productive in a cross with \(Sad-1 \ a\). Presence of the \(Dp(dow/l)\) transgene was confirmed by Southern analysis (Fig. 3.13 B in Chapter 3).
Dp(dow/1) Dp(erg-3/1) A # 1, Dp(dow/1) Dp(erg-3/1) A # 16 and Dp(dow/1) Dp(erg-
3/1) A # 14

These strains were obtained as segregants from the cross Dp(erg-3/1) x Dp(dow/1). The presence of both the duplications was confirmed by Southern analysis (Fig. 4.1 A-B in Chapter 4).

Dp(erg-3/2) a, Dp(erg-3/3) a, Dp(erg-3/4) a, Dp(erg-3/5) a, Dp(erg-3/6) a, Dp(erg-3/7)
a, Dp(erg-3/8) a.

These are the different transgenic strains that have the Dp(erg-3) transgene. They were selected as independent transformants obtained as homokaryons from crosses with the wild-type strain.

Sad-1; Dp(erg-3/7) a # 11

This strain was obtained as one of the two mat a, hygromycin-resistant strains from the Dp(erg-3/7) x Sad-1 a. It was non-productive in a cross with Sad-1 A and produced erg-3 mutants in a cross with ORA

Dp(dow/2) A, Dp(dow/3) A and Dp(dow/4) A

These were independent transformants for the Dp(dow) transgene. They were obtained as homokaryotic transgenic strain after crossing the primary transformants with the wild type strain.

Dp(AR17) a # 23 and Dp(AR17) A # 17

These were segregants obtained from the cross T(AR17) A x ORa. These were barren in crosses with ORA/a and segregated dow mutants in crosses with Sad-1 A/a.

Sad-1; Dp(AR17) dow+/dow− A and Sad-1; Dp(AR17) dow+/dow−

These are Sad-1; Dp(AR17) dow+/dow− described by Bhat and Kasbekar (2004) generated from the cross of Dp(AR17) a with Sad-1; dow A # 2. These were fertile and segregated dow− progeny in crosses with 74-OR23-1 A or OR8-1 a.
These are \textit{rid}; \textit{Dp(AR17)} \textit{dow\textsuperscript{+}/dow\textsuperscript{-}} strains described by Bhat and Kasbekar (2004). The presence of \textit{Dp(AR17)} in the strains was confirmed by the segregation of \textit{dow} segregants from a cross with 74-OR23-1 \textit{A} or OR8-1 \textit{a}. Crosses of \textit{downy} segregants of the same mating type as the \textit{Dp(AR17)} containing parent with a \textit{rid}; \textit{Dp1.3\textsuperscript{ehp}} strain failed to produce RIP-induced \textit{erg-3} mutant progeny, whereas crosses of \textit{dow} segregants of the opposite mating type did. This confirmed the presence of the \textit{rid} mutation.

\textit{rid-1 Sad-1; Dp(AR17) A/23}

This was a segregant obtained from the cross \textit{rid-1 Sad-1A x Dp(AR17) a}. It was non-productive in a cross with \textit{Sad-1 a} the \textit{rid-1} phenotype was confirmed by crossing the \textit{f\textsubscript{1}} progeny from the cross \textit{rid-1 Sad-1; Dp(AR17)/23 A x ORa} with \textit{rid-1; Dp(erg-3/1)}. \textit{Dp(AR17)} was also confirmed by Southern hybridization analysis (Fig. 6.4 in Chapter 6).

\textit{Dp(B362i) a/2} and \textit{Dp(B362i) A/9}

These were the \textit{Dp(B362i)} segregants obtained from the crosses \textit{T(B362i) a x ORA} and \textit{T(B362i) A x ORa} respectively. Presence of \textit{Dp(B362i)} was confirmed by barren phenotype and Southern analysis (Fig. 3.4 B in Chapter 3).

\textit{Dp(IV \rightarrow I) B362i(1110 \# 3), Dp(IV \rightarrow I) B362i(1110 \# 8), Dp(IV \rightarrow I) B362i(1110 \# 10)} or \textit{Dp(B362i) A \# 3, \# 8 and \# 10}.

These are the duplication strains obtained from the cross of \textit{Lankala Koderu} (FGSC \#1110) \textit{a x T(B362i) A}. They are barren in crosses with euploid strains and show duplication for \textit{met-1} gene on a Southern. They have been designated as \textit{Dp(B362i) A \# 3, \# 8 and \# 10} (Fig. 3.3 B in Chapter 3).

\textit{Sad-1; Dp(B362i) A \# 8-5}

This strain was obtained from the cross \textit{Dp(B362i) A \# 8 x Sad-1; Dp(erg-3/1)}. Presence of \textit{Sad-1} was confirmed by crossing with \textit{Sad-1 a} and \textit{Dp(B362i)} was confirmed by Southern hybridization (Fig. 3.5 C in Chapter 3).
**rid-1 Sad-1; Dp(B362i) a 3/10**

This segregant was obtained from the cross *rid-1 Sad-1a x Dp(B362i) # 3 A*. The genotype was confirmed in the same manner as above (Fig. 6.11 in Chapter 6).

**T(B362i); dow A # 11**

This was the only translocation type *dow* mutant out of 14 others obtained from the cross *T(B362i) A x dow a # 4*. The translocation was confirmed by Southern analysis (Fig. 4.3 in Chapter 4).

**Dp(B362i); dow; Dp(erg-3/1) A # 1-7, 1-12, 1-19 and 7-28**

These *dow* segregants were obtained from the crosses *T(B362i); dow # 11 A x dow; Dp(erg-3/1) # 1 a* and *T(B362i); dow A # 11 x dow; Dp(erg-3/1) a # 7*. Presence of *Dp(B362i)* and *Dp(erg-3/1)* was confirmed by Southern analysis (Fig. 4.4 in Chapter 4).

**Dp(IBj5) a # 8 and Dp(IBj5) A # 1**

These segregants were obtained from the crosses of *T(IBj5) a x ORA* and *T(IBj5) A x ORa*. They were barren in crosses with *ORA/a* and suppressed RIP in *Dp(erg-3/1)* in crosses with *Dp(erg-3/1) A/a*.

**rid-1; Dp(IBj5) A /4 and rid-1; Dp(IBj5) a /23**

These strains were obtained from the crosses, *rid-1 a x T(IBj5) A* and *rid-1 A x T(IBj5) a* respectively. Presence of *Dp(IBj5)* was confirmed by Southern analysis and *rid-1* was confirmed by analyzing the fertile segregants inheriting the mating type of the *Dp(IBj5)* parent from the crosses *rid-1; Dp(IBj5)/11 a x Sad-1 A* and *rid-1; Dp(IBj5)/4 A x Sad-1 a* in crosses with *rid-1; Dp(erg-3/1)*. The segregants failed to produce RIP induced *erg-3* mutants in these crosses confirming the presence of *rid-1* mutation in the two strains.

**Sad-1; Dp(IBj5) A # 5**

This segregant was obtained from a cross of *Dp(IBj5) a* with *Sad-1*. It suppressed RIP in *Dp(erg-3/1)* and was non-productive in crosses with *Sad-1 a*. 
rid-1 Sad-1; Dp(IBj5) A /47

This segregant was obtained from the cross rid-1 Sad-1A x T(IBj5) a. The rid-1 Sad-1 genotype was confirmed in a manner similar to the one described for the rid-1 Sad-1; Dp(AR17)/23 A strain (Fig. 6.6 in Chapter 6).

rid-1; erg-3

This strain was obtained from the cross rid-1 A (N1977) x erg-3 (FGSC 2725) a. The rid-1 mutation was confirmed by crossing mat a, erg-3+ f1 progeny from rid-1; erg-3 a x ORA with rid-1; Dp(erg-3/1) A. They did not produce any erg-3 mutants because the cross was recessively defective for RIP.

FGSC #8752, FGSC #8754, FGSC #8756, FGSC #8758 and FGSC #8760

These are pan-2 a strains that contain, respectively, functional copies of the meiosis-essential genes, act+ (actin), hH3hH4-1+ (histones H3 and H4-1), Bml8 (β-tubulin), pma-1+ (plasma membrane ATPase), and mei-3+ (the Neurospora RecA/Rad 51 ortholog, meiotic-3), inserted into the his-3 locus (Shiu et al., 2001). In heterozygous crosses, the induction of MSUD silences these genes and thereby causes the cross to become barren (Shiu et al. 2001). These strains were kind gifts from Robert L. Metzenberg.

2.1.3 Oligonucleotide primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG3F1</td>
<td>GCTCTCCGTGTGGTGCCTCTAC</td>
</tr>
<tr>
<td>ERG3R1</td>
<td>GCCGAAGAGGATTGGGCTGACTG</td>
</tr>
<tr>
<td>DOWF</td>
<td>CATTCAAGTCGACAGGACA</td>
</tr>
<tr>
<td>DOWR</td>
<td>CTGCCGGATATCTTCTTCAGC</td>
</tr>
<tr>
<td>NCRIDF</td>
<td>ATGCAATTTAATTTGGCTAGG</td>
</tr>
<tr>
<td>NCRIDR</td>
<td>TGGAAAAAGAAATTGGTAGCTCA</td>
</tr>
<tr>
<td>MGRIDF</td>
<td>TTTGTCGAAGTCTTCTCTCAGAAG</td>
</tr>
<tr>
<td>MGRIDR</td>
<td>TTGATCGAGCAGAAAGATAAG</td>
</tr>
</tbody>
</table>
Primer list of breakpoint sets that do not amplify from translocation strains

*Dp(AR17)*

<table>
<thead>
<tr>
<th>Primer Forward</th>
<th>Primer Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17BR1.2F</td>
<td>CATCCTGCTTGAACATTCTGTTA</td>
</tr>
<tr>
<td>D17ZR</td>
<td>GGGACAAAAGATCAAGAGGTCT</td>
</tr>
</tbody>
</table>

*Dp(IBj5)*

<table>
<thead>
<tr>
<th>Primer Forward</th>
<th>Primer Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBJ13F</td>
<td>TCCCGCTTTGTTTAATAAAAAT</td>
</tr>
<tr>
<td>CPC1R</td>
<td>GGATCAAGCGACACTCAATAATC</td>
</tr>
</tbody>
</table>

*Dp(B362i)*

<table>
<thead>
<tr>
<th>Primer Forward</th>
<th>Primer Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET1F</td>
<td>CAAGCATCAGTCTTATGTACAC</td>
</tr>
<tr>
<td>MET1R</td>
<td>GGAAGAGACAGGTCTTACAGAG</td>
</tr>
<tr>
<td>TB6IKF</td>
<td>TTCTGGACCAGTATTGTTCGTT</td>
</tr>
<tr>
<td>TB6IKR</td>
<td>ACATAACCATGGAGAGCAGAGT</td>
</tr>
</tbody>
</table>

### 2.1.4 Plasmid vectors used in this study

**pEC86-Hph**

This plasmid contains the *erg-3*\(^+\) gene together with the bacterial hygromycin-resistance gene, *hph* (Prakash and Kasbekar, 2002a). This plasmid can complement the *erg-3* mutation of Neurospora.

**pCSN44**

The pCSN44 (Staben et al., 1989) vector carries the bacterial *hph* gene that encodes the enzyme hygromycin B phosphotransferase and can thereby confer resistance to the antibiotic hygromycin B. This allows for the selection of *N. crassa* transformants on hygromycin medium.

**pSS17**

This plasmid construct is described by Papavinasasundaram and Kasbekar (1994). A *HindIII* fragment of the *erg-3* gene, comprised of nucleotides 532±1911 in the numbering scheme of Papavinasasundaram and Kasbekar (1994), was ligated into the *HindIII* site of
the plasmid *pCSN44* (Staben *et al.*, 1989) and the resulting plasmid was designated *pSS17* (Prakash *et al.*, 1999). The start and stop codons of the *erg-3* ORF are at positions 390 and 1946, respectively; thus the 1±3 kb *HindIII* insert in *pSS17* lacks the promoter and coding sequences for the N- and C-terminal amino acid residues and therefore does not complement the *erg-3* mutation.

**Construction of the Mgrid plasmid construct and the Ncrid plasmid construct.**

A 5.1 kb full length *rid-1* gene fragment from the *N. crassa* genomic DNA was amplified by PCR using the primers NCRIDF and NCRIDR (See section). The *rid-1* homolog of *M. griseae* was amplified from a cosmid MGBAc163 using the primers MGRIDF and MGRIDR (see section 2.1.3). The PCR amplified fragments were gel purified using the Qiagen gel purification kit and cloned into the *EcoRV* site of the plasmid vector *pCSN44*. Plasmid DNA of these clones was used to transform the *rid-1* mutant strain of *N. crassa*.

**2.1.5 Bacterial media, antibiotics and commonly used solutions**

1. LB (Luria Bertani) broth: 1% bacto-tryptone, 1% sodium chloride, 0.5% bacto-yeast extract, pH 7.0.
2. LB agar: LB solidified with 1.5% agar
3. Ampicillin: A 1000X stock solution of 100 mg/ml ampicillin was made in sterile double-distilled water and stored in aliquots of 150 μl at −20°C.
4. α-tomatine: A stock solution of 25 mg/ml was prepared in dimethyl formamide (DMF) and stored at −20°C.
5. Hygromycin B: A stock solution of 100 mg/ml was made in sterile water and stored at −20°C.
6. 1X TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA
7. 1X TBE: 89 mM Tris-Borate (pH 8.3), 2 mM EDTA
8. 1X SSC: 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)
9. Phenol:Chloroform:Isoamyl mixture: Phenol was prepared by the equilibration of liquefied and distilled phenol containing 0.1% hydroxyquinoline with 0.1 M Tris.HCl pH
8.0. It was mixed in equal volume with chloroform: isoamyl alcohol (24:1) and stored in a dark bottle, at 4°C.

10. Lysis buffer for Neurospora genomic DNA isolation: 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 10 mM EDTA, 1% SDS and 1% CTAB.

_Solutions for growth, maintenance and crossing of Neurospora strains_

Media for culturing Neurospora are described in Davis and De Serres (1970).

**Trace element solution**

Trace element solution was prepared by adding the following compounds successively, with stirring to 95 ml of distilled water.

- Citric acid.1H2O, Fe (NH3)2(SO4)_2. 7H2O 5.00 g
- CuSO4.5H2O 5.00 g
- MnSO4.H2O 1.00 g
- H3BO3 0.05 g
- Na2MoO4.2H2O 0.05 g

The final volume was adjusted to 100 ml. 1 ml of chloroform was added as a preservative. The solution was stored at room temperature.

_Biotin solution_

5 mg of biotin was dissolved in 100 ml of 50% (v/v) ethanol and stored in refrigerator.

_Vogel’s Medium N (50X)_

To 750 ml of distilled water, the following compounds were added, dissolving each one prior to the addition of the next.

- Na3Citrate.5H2O 150 g
- KH2PO4 250 g
- NH4NO3 100 g
- MgSO4.7H2O 10 g
- CaCl2.2H2O 5 g
- Biotin Solution 5 ml
Trace element solution 5 ml
The volume of the solution was adjusted to 1 litre and 3 ml of chloroform was added as a preservative.

*Vogel's-glucose medium*

Vogel's medium N 1X
Glucose 1.5%

*Vogel's-glucose agar medium*

Vogel's medium N 1X
Glucose 1.5%
Agar 2.0%

*4X Synthetic crossing medium (SCM)*

4X strength synthetic crossing medium was prepared by adding the following compounds with stirring to 500 ml of water.

KNO₃ 2.0 g
K₂HPO₄ 1.4 g
KH₂PO₄ 1.0 g
MgSO₄·7H₂O 1.0 g
CaCl₂ 0.2 g
NaCl 0.2 g
Biotin solution 0.2 ml
Trace element solution 0.2 ml

*SCM agar*

SCM 1X
Glucose 1.5%
Agar 2.0%
10X FGS ("Sorbose")

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Concentration (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbose</td>
<td>20%</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.5%</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Vogel’s-sorbose agar medium

- FGS: 1X
- Vogel’s medium N: 1X
- Agar: 2.0%

Sterilization

All glassware and plastic ware were sterilized by autoclaving at a steam pressure of 15 psi at 120°C for 20 min. Solutions were prepared in double-distilled water and generally sterilized by autoclaving. Heat-sensitive solutions were sterilized by filtering through a sterile 0.45μm nitrocellulose filter (Millipore).

2.2 METHODS

2.2.1 Neurospora culturing and handling

Growth conditions

Growth and maintenance of the Neurospora strains on Vogel’s medium N supplemented with glucose or FGS was essentially as described in Davis and De Serres (1970).

Scoring for antibiotic resistance

Antibiotic resistance was scored by streaking conidia onto 1.5% agar plates containing Vogel’s-sorbose medium and supplemented with the antibiotic. The antibiotics used were α-tomatine (90 μg/ml for *N. crassa*) and hygromycin B (220 μg/ml).
**Crossing and ascospore collection**

Crosses were performed by confrontation between mycelia inoculated as plugs on synthetic crossing medium in petri dishes. Generally ascospores began to be shot within 16-18 days in *N. crassa* crosses. Ascospores were harvested by washing the lids with ~1 ml of sterile water.

The frequency of erg-3 mutant progeny has been used as a measure of RIP efficiency. It is known in *N. crassa* that the frequency of RIP increase with the age of the cross i.e., spores from the early harvest show less RIP frequency than the spores from later harvests (Singer et al., 1995b). Therefore, to minimize variation in RIP frequencies among crosses because of their age, all *N. crassa* crosses were harvested at 31 days.

### 2.2.2 Isolation of sterols and analysis by UV spectrophotometry

The Neurospora strain of interest was grown in liquid Vogel’s-glucose medium at 30°C for 3 to 5 days. The mycelial mass was harvested by vacuum filtration and lyophilized. The dried mycelia were ground with glass beads (0.2 mm in diameter) using a mortar and a pestle to a fine powder. About 50 mg of the powdered mycelia was taken in a 1.5 ml microfuge tube and 750 ml each of methanol and chloroform was added to the tube and kept on a rotary shaker overnight. The mycelial mass was removed by centrifugation at 12,000 rpm for 10 minutes. The chloroform-methanol extract which contains the lipids was washed once with 0.9 % NaCl and twice with 2M KCl to remove the saponifiable lipids. The aqueous and organic phases were separated by centrifugation and the bottom organic phase was transferred to a fresh tube. This step separates the saponifiable lipids from non-saponifiable lipids like sterols. The organic phase, which contains the sterols was air-dried and the sterols were dissolved in about 20 ml of chloroform. This sample was diluted 1: 200 in ethanol and its UV absorption spectrum (200 to 300 nm) was recorded in a Hitachi spectrophotometer.

### 2.2.3 Small scale isolation of plasmid DNA

Small scale preparation of plasmid DNA was made using the Qiaprep Spin Miniprep Kit (Qiagen) according to the instructions provided by the manufacturer.
### 2.2.4 Neurospora genomic DNA isolation

The strain of interest was grown in liquid Vogel's-glucose medium at 30°C for 3 to 5 days. The mycelial mass was harvested by vacuum filtration and lyophilized. The dried mycelia were ground with glass beads (0.2 μm in diameter) using a mortar and a pestle to a fine powder. Approximately 150 mg of the powdered mycelia was taken in a 1.5 ml microfuge tube and 1 ml of lysis buffer added to it. Complete mixing of the mycelia and lysis buffer was achieved using a pipette tip. The tube was incubated at 65°C for 30 min., followed by centrifugation at 15,000 rpm for 10 min. All centrifugations were carried out at 25°C. The supernatant was taken in a fresh microfuge tube and 500 μl of phenol:chloroform:isoamyl alcohol mixture was added to it. The tube was rotated in a cell mixer for 15 min. and centrifuged at 15,000 rpm for 10 min. The aqueous phase was carefully removed and the phenol:chloroform:isoamyl alcohol treatment repeated. The aqueous phase was taken in a fresh microfuge tube and washed with 600 μl of chloroform to remove the last traces of phenol. The aqueous phase was taken in a fresh tube and genomic DNA precipitated by adding 1.5 volumes of absolute ethanol. The tube was gently inverted a few times and the genomic DNA pelleted by centrifuging the tube briefly at 15,000 rpm. The supernatant was discarded and the pellet was washed with 70% ethanol. The tube was centrifuged for 2 min. at 15,000 rpm and the supernatant was completely removed. The genomic DNA pellet was allowed to dry at room temperature for 15 min. and finally dissolved in about 80 μl of Tris EDTA.

### 2.2.5 Digestion of DNA with restriction endonuclease

An aliquot of 500 ng to 2 μg of DNA was digested with 5 units of restriction endonuclease per μg of DNA in a final volume of 30 μl. The reaction was carried out for 3 hrs using suitable buffers and assay conditions specified by the manufacturers. The enzyme was heat-inactivated by heating the digested samples at 65°C for 10 min. The digested DNA fragments were analyzed by agarose gel electrophoresis. Digestion with two enzymes was done after consulting the enzyme compatibility chart present in the New England Biolabs catalogue. Restriction digestion of genomic DNA was done overnight to allow for complete digestion.
2.2.6 Agarose gel electrophoresis

The DNA samples were suspended in water containing one-sixth the volume of 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). Depending upon the size of the fragments to be resolved, the samples were loaded on 0.7% to 1.5% agarose gels cast on 1X TAE containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in 1X TAE at 5 V/cm. Standard DNA size markers were run alongside for estimation of DNA fragment sizes. The ethidium bromide stained DNA samples were visualized on a UV transilluminator and photographed.

2.2.7 Purification of DNA fragments from agarose gels

PCR-amplified DNA required for the preparation of probes or for transformation were purified from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen). The sample containing DNA was resolved on a 1% high purity agarose gel. The DNA bands were visualized on a UV transilluminator and the DNA band to be eluted was excised and transferred to a weighed 1.5 ml centrifuge tube. DNA from the gel slice was eluted according to the instructions provided by the manufacturer.

2.2.8 Quantitation of nucleic acids

The concentration of nucleic acids was estimated by measuring the OD at 260 nm (Maniatis et al. 1982). The following empirical relationships were used to calculate the concentrations. OD of 1 corresponds to approximately 50 μg/ml of double-stranded DNA, 40 μg/ml of RNA and 33 μg/ml of single-stranded oligos. The purity of nucleic acids was estimated by calculating the OD260: OD280 ratio. Pure DNA has OD260/OD280 of around 1.8.

2.2.9 Preparation of radiolabeled DNA probes by random primer labeling

Double-stranded DNA was radiolabeled using random primers as described by Feinberg and Vogelstein (1983) using a multiprime labelling kit. About 50-100 ng of double-stranded DNA was denatured in a volume of 20 μl by boiling for 5 min and quick chilling on ice. This was followed by the sequential addition of 5 μl of random primers solution, 5 μl of 10X reaction buffer, 4 μl each of dCTP, dGTP, dTTP, 40 μCi of α-
[32P]-dATP and 2 units of Klenow enzyme. The reaction volume was made upto 50 µl and the reaction was carried out at 37°C for 2 hrs. The enzyme was inactivated at 75°C for 10 min and the probe was separated from the unincorporated nucleotides by Sephadex G-50 spun column chromatography (described below), denatured in boiling water for 5 min and rapidly chilled on ice.

### 2.2.10 Sephadex G-50 column chromatography for purification of radiolabeled probe

Sephadex gel filtration column chromatography was employed to separate out unincorporated radionucleotides from DNA solutions. A sterile 1 ml disposable plastic syringe was plugged with sterile glass wool and filled with the Sephadex G-50 slurry previously equilibrated with TE pH 8.0. The column was packed by centrifugation at 1500 rpm for 5 min in a Sorvall HB-4 rotor at room temperature. To minimize the loss in void volume, 50 µl of TE was added to the reaction mixture and then the mixture was loaded onto the column and centrifuged at 1500 rpm for 5 min at room temperature to elute the purified DNA.

### 2.2.11 Southern hybridization

The DNA samples to be hybridized were digested with appropriate restriction enzymes and resolved on 1.0 % to 1.5% agarose gels alongside DNA markers. The gel was stained with ethidium bromide, photographed and the DNA was transferred to Hybond N+ membrane by vacuum blotting in a vacuum blotter apparatus (made by the CCMB in-house facility) for 15 min in .25 N HCl and then for 1.5 hrs in 0.4N NaOH. The blot was then rinsed in 2X SSC, and air-dried. Prehybridization was carried out at 65 °C for 2 hrs in 20ml of 0.5 M sodium phosphate and 7% SDS. The blot was hybridized at 65°C for 12 hrs by the addition of denatured radiolabeled probe (prepared by random primer labeling as described above). The blot was washed in 100 ml of 2X SSC, 0.5% SDS for 20 min. at room temperature, in 1X SSC, 0.5% SDS for 20 min at 65°C and 0.5X SSC, 0.5% SDS for 20 min at 65°C. The hybridized blot was exposed to a phospho imaging plate for 1-2 hrs and visualized in the scanner.
2.2.12 Colony hybridization for colony lifts to identify bacterial clones

Transformed bacterial culture was plated out on LB plates supplemented with the appropriate antibiotic. These plates were incubated at 37°C for about 12-13 hours. Nylon membrane cut to the size of the plate was marked asymmetrically and wetted on a blank plate. This was placed on the transformation plates to lift up the colonies. The asymmetric markings on the nylon membrane were replicated on the master plate correctly and the membrane was lifted off the plate and placed in a tray with the colony side up. In a glass dish, Whattman #3 paper was saturated with denaturing solution and the colony blots were placed on it and incubated for seven minutes with the colony side facing up. The blot was transferred to another Whatmann paper saturated with the neutralization solution and incubated for three minutes. This step was repeated for another three minutes on a fresh whatmann paper saturated with neutralization solution. The blots were rinsed in 2x SSC and air-dried. They were UV treated for 2 minutes at 2 kjoules. The blots were scrubbed lightly with a cotton swab soaked in 2x SSC and air-dried. Hybridization was done for this blot and the positive clones were picked up and streaked purified. Single isolated colonies colonies were inoculated and cultured for plasmid isolation.

2.2.13 Transformation of Neurospora by electroporation

This protocol is based on the method described by Turner et al. (1997). The Neurospora erg-3 mutant strain (FGSC # 2725) was grown in Vogel's medium N supplemented with 2% glucose, for a week at 30°C. The conidia were harvested in sterile water and separated from the mycelium by passing the conidial suspension through cheesecloth attached to a 500 ml conical flask (sterilized). The conidial suspension was centrifuged at 4000 rpm for 10' in a Sorvall SS34 rotor. The conidial mass was washed twice in 30 ml sterile water and finally resuspended at a concentration of $3 \times 10^9$/ml. About 40 μl of the conidial suspension was mixed with 300 ng of the of DNA and the mixture was placed in a 0.2 cm BTX eletroporation cuvette. The conditions for electroporation were 1.5 KV and 186 Ω. The time constant varied from 10-13 msec. Immediately after the pulse, the 960 μl of 1 M sorbitol was added to the transformant mix. Around 300ul of the transformation mix was added to 8ml of melted and cooled top
agar (with “sorbose”) and the resulting mixture was poured on to a Vogel’s medium plate supplemented with sorbose and hygromycin at a concentration of 220 μg/ml. Usually about 10-25 transformants were obtained and were pickable under a dissection microscope after 3-5 days. A mock transformation without adding DNA was performed to eliminate the possibility of any contamination.

2.2.14 PCR amplification

Polymerase chain reactions were performed using oligonucleotide primers synthesized either at the CCMB oligonucleotide synthesis facility or the at the Bioserve India limited. The reaction conditions used were a 4 min denaturation at 94° followed by 30 cycles of 1 min denaturation at 94°, 1 min annealing at 55° and 1 min elongation at 72°. Fragments that were > 1.6 kb were amplified by changing the elongation time to 1.2-1.5 min. All the PCR reactions were done using the same conditions unless otherwise mentioned. The rid-1 gene fragment was amplified from the 

*Magnaporthe griseae*

cosmids using the primers MGRID F, corresponding to nucleotides 347-367. The entire rid-1 gene of *N. crassa* was amplified using the primers NCRIDF and NCRIDR of the *N. crassa* erg-3 gene sequence (Accession number X77955) and MGRIDR, complementary to nucleotides 964-987. The resulting 0.64 kb product was purified by gel electrophoresis and used for automated DNA sequencing with the same primers, the Big Dye Terminator Ready Reaction kit (Perkin Elmer) and an ABI Prism 3700 DNA analyzer.

2.2.15 Long range and high fidelity PCR

Fragments >5.0 kb were amplified using the eppendorf triple master PCR systems. Fragments requiring high fidelity and proof reading activity were also amplified using the same kit. The procedure mentioned by the manufacturer was followed as it is.

2.2.16 Cloning

Cloning of PCR fragments was done by following the Amersham pMOS Blunt end cloning kit procedure. Modifications were done depending on the size of the fragment to be cloned. In most cases the pMOS vector was replaced with the pCSN43/
pCSN44 vector. Transformation was done by following the alkali lysis method mentioned in Maniatis protocols (Maniatis et al., 1982). The presence of the insert in most cases was confirmed by southern hybridization of the plasmid DNA.

2.3 Computer softwares used for sequence analysis

**BLAST**

This was used for aligning nucleotide and amino acid sequences and for searching sequences databases. This is available at [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/).

**CLUSTALW**

This software was used for multiple alignments of DNA and protein sequences. It is available at [http://clustalw.genome.ad.jp/](http://clustalw.genome.ad.jp/).

**EXPASY TRANSLATE TOOL**

This was used for translating DNA sequences to protein sequences. It is available at [http://us.expasy.org/tools/dna.html](http://us.expasy.org/tools/dna.html).

**WEBCUTTER 2.0**

This was used for restriction analysis of DNA sequences. It is available at [http://www.firstmarket.com/cutter/cut2.html](http://www.firstmarket.com/cutter/cut2.html).

**PROMOTER PREDICT**

This was used for predicting the rid-1 promoter sequence from the *Magnaporthea grisea* sequence. It can be accessed at [http://www.fruitfly.org/seq_tools/promoter.html](http://www.fruitfly.org/seq_tools/promoter.html).

**ORF FINDER**

This was used to identify promoter regions in a sequence of DNA. It is available at [http://www.ncbi.nlm.nih.gov/gorf/gorf.html](http://www.ncbi.nlm.nih.gov/gorf/gorf.html).