MATING-TYPE-ASSOCIATED VEGETATIVE INCOMPATIBILITY IN *NEUROSPORA CRASSA*

by

PATRICK KA TAI SHIU

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Department of Botany

The University of British Columbia
Vancouver, Canada

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Abstract

The mating-type locus in *Neurospora crassa* controls mating and sexual development. The fusion of reproductive structures of opposite mating-type, *A* and *a*, is required to initiate sexual reproduction. However, the fusion of hyphae of opposite mating-type during vegetative growth results in growth inhibition and cell death, a process that is mediated by the *tol* locus. Mutations in *tol* are recessive and suppress mating-type associated heterokaryon incompatibility; heterokaryon incompatibility is a mechanism that prevents the formation of vigorous heterokaryons between genetically dissimilar individuals. In this study, the functional domain for heterokaryon incompatibility in the *A* mating-type protein, specifically MAT A-1, is mapped to a leucine-rich repeat.

The *tol* gene encodes a putative 1011-amino acid polypeptide with a coiled-coil domain and a leucine-rich repeat (both essential for heterokaryon incompatibility). It contains a region of similarity with two *het* genes (HET-6 and HET-E). Repeat-induced point mutations in *tol* result in mutants that are wild-type during vegetative growth and sexual reproduction, but which allow opposite mating-type individuals to form a vigorous heterokaryon. Transcript analyses show that *tol* mRNA is present during vegetative growth but absent during a cross. These data suggest that *tol* transcription could be repressed in order to allow the co-existence of opposite mating-type nuclei during the sexual reproductive phase. *tol* is expressed in a *mat A*, *mat a*, *A/a* partial diploid and in a mating-type deletion strain, indicating that MAT A-1 and MAT a-1 are not absolutely required for transcription or repression of *tol*. These data suggest that TOL may interact with MAT A-1 and/or MAT a-1 to form a death-triggering complex.

To understand how *tol* may mediate mating-type incompatibility, attempts have been made to isolate TOL-interacting proteins (*top*) by yeast 2-hybrid system. Five *N. crassa* mycelial cDNA-pGAL4 AD clones are shown to have plasmid-dependent interaction with TOL in yeast. One of them is a homologue of *Schizosaccharomyces pombe vip-1*, which is a p53-related protein.

The *tol* gene is apparently conserved in many different *Neurospora* species. One particular allele, tol^T from *N. tetrasperma*, is of special interest. tol^T is a natural tol^- in the regard that it does not mediate mating-type incompatibility. Evidence provided in this thesis suggests that two loci closely linked to tol^T may mediate non-allelic vegetative incompatibility and sexual incompatibility, respectively.

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Thank God it's over.

1. Overview and Introduction

1.1 THESIS OBJECTIVES

In *Neurospora crassa* Shear & Dodge, mating is controlled by a single locus with two mating types, *A* and *a*. This mating-type locus has an additional unique function during vegetative growth known as heterokaryon (or vegetative) incompatibility: if hyphae of opposite mating-types fuse during the vegetative stage, the resulting heterokaryotic cells are inhibited in their growth. Fusion of hyphae with the same mating-type shows no such reaction. An unlinked mutation *tol* suppresses the heterokaryon incompatibility but does not affect sexual function.

Sequencing of the two mating-type alleles, *A* and *a*, showed that the two have totally dissimilar sequences of 5301 and 3235 base pairs, respectively. A single open reading frame (ORF) from each mating-type idiomorph, *mat A-1* and *mat a-1*, confers both mating function and heterokaryon incompatibility. Both MAT A-1 and MAT a-1 contain conserved DNA-binding motif as shared by some other transcriptional activators and their presence/absence affect expression of many sexual developmental genes (*sdv*). The objectives of this thesis were to understand the roles of *mat A-1* and *tol* in mediating mating-type-associated vegetative incompatibility in *N. crassa*. These aims were approached by dissection of functional domains necessary for heterokaryon incompatibility of *mat A-1* and by the cloning and characterization of *tol*.

1.2 GENERAL INTRODUCTION TO NEUROSPORA CRASSA

In this section, a summary on the background of pioneering *Neurospora* research is given. The advantages that make *Neurospora crassa* (and related species) a model organism for genetic and molecular biology will also be discussed.

1.2.1 *Neurospora crassa*, a model genetic organism

French microbiologists first described Neurospora more than 150 years ago. It was discovered in Paris when bread from bakeries was spoiled by massive growth of an orange mold ("le champignon rouge du pain" – the red bread fungus). A commission was set up to investigate the event and to make recommendations. The commission's report (Payen, 1843) described the morphology of the vegetative phase of the fungus. This report and later works by A. Payen (1848, 1859) were cited by Pasteur (1862) in refuting the theory of spontaneous generation.

The next round of study on Neurospora began in Java, Indonesia during the early 1900's. Attracted by a local food called oncham (a bright orange cake consisting of Neurospora grown on soybean), F.A.F.C. Went (a Dutch plant physiologist) decided to study the orange oncham fungus. His work included using Neurospora to study the effect of various substrates on different enzymes activities (Went, 1901) and the effect of light on carotenoid production (Went, 1904).

B.O. Dodge was the first to discover heat activation of ascospores (1912). This finding made Neurospora available for genetic studies. In his first Neurospora paper (Shear & Dodge, 1927), Dodge showed that hybrid perithecia from crosses between different species developed slowly and were sterile (or poorly fertile). This innovation of differentiating species based on production of fertile offspring (biological species concept), in contrast to purely morphological criteria, was later popularized by many biologists. The orange fungus was assigned to the new genus *Neurospora* (name based on the nerve-looking grooves on the ascospores); previously, the vegetative stage was called *Oidium aurantiacum*, *Penicillium sitophilum* and *Monilia sitophila.* Dodge also carried out the first tetrad analysis with *Neurospora crassa* (a heterothallic species), demonstrating that the eight ascospores of an ascus display a perfect Mendelian ratio (4:4) in mating-type segregation (Dodge, 1927). This finding fascinated a graduate student at a lecture

by Dodge given at Cornell University in 1930. This graduate student, G.W. Beadle, later teamed up with E.L. Tatum in working on Neurospora and obtained the first biochemical mutants.

Another Neurospora research pioneer influenced by Dodge was C.C. Lindegren. As a junior associate of T.H. Morgan (founder of the genetic map unit; leader of a Drosophila group and the head of the Biology division at CalTech), Lindegren followed Dodge's advice to work on Neurospora. Lindegren (1936) worked out the first linkage maps using mating-type, centromeres, and morphological mutants. His work established the genetics of Neurospora on a sound foundation and made the orange mold an ideal choice for obtaining the first auxotrophic mutants in experiments by B.O. Beadle and E.L. Tatum.

The first advocacy of inherent defects in metabolism can be dated back to the early 1900's (Garrod, 1909). At that time, most geneticists still resisted accepting the direct relationship between genes and proteins, because they thought that development was too complex to be explained by a simple theory such as gene action (Sturtevant, 1965). It was not until Beadle and Tatum, in the early 1940's, began using *Neurospora crassa* as an experimental organism for biochemical genetics that the theory became testable. Neurospora was an ideal choice for obtaining auxotrophic mutants because of its rapid life cycle and its simple nutritional requirements (Butler *et al.*, 1941). In the Beadle-Tatum paper (1941), they described three X-ray-induced mutants that grew on "complete medium", but failed to grow on "minimal medium." Their interpretation was that the mutations in these isolates affected genes required for the production of growth-essential compounds present in complete, but not minimal, medium. The three growth-essential substances were found to be pyridoxine, thiamine and *p*-aminobenzoic acid. Other mutants that became the focus for Beadle and Tatum's later work were arginine auxotrophs. They were of specific value because the mutations were mapped to three loci on separate chromosomes, and the three mutants had different growth responses to supplements (arginine and two related compounds, ornithine and citrulline). Beadle and Tatum proposed that

biochemical reactions *in vivo* occur as a series of discrete, stepwise reactions. Each reaction is specifically catalyzed by a single enzyme, which is specified by a single gene. This model, now known as the one-gene-one-enzyme hypothesis (Beadle, 1945), soon gained support from similar studies of other biosynthetic pathways and achieved general acceptance.

1.2.2 Advantages of Neurospora

Neurospora is blessed with advantages for genetic and molecular research. It has over a thousand loci identified and mapped to linkage groups, 25% of which have been molecularly characterized (Perkins *et al.*, 2000). It is a stable haploid (with a diploid zygote), and all four products of individual meioses can be recovered (Perkins & Barry, 1977). Centromeres can be mapped easily. It has low DNA content (43 megabases; 90% of the genome is of unique sequence) and the seven chromosomes can be resolved by pulse-field gel electrophoresis. In fact, the smallest chromosome of Neurospora has DNA content less than that of the *Escherichia coli* sequence. It has a short generation time, a fast growth rate, and large numbers of sexually derived progeny; even rare recombination events can be detected. It has relatively simple nutritional requirements and easy cultivation and preservation (Perkins, 1986). The chromosome cytology of *N. crassa* resembles that of higher plants and animals (Perkins, 1979). It also forms heterokaryons, which are very useful in studies of dominance and complementation. Tetrad analysis is also a great asset in studying meiosis, chromosomal rearrangements and recombinations. The Neurospora genome project is ongoing (with linkage groups II and V near completion) and numerous sequences and physical maps are available through the world wide web (http://www.mips.biochem.mpg.de/proj/ neurospora/; http://biology.unm.edu/~ngp/home.html; http://fungus.genetics.uga.edu:5080).

1.3 FUNGAL MATING-TYPES

Some parts from this section are published in the review by Shiu & Glass (2000).

Sexual development in fungi is initiated by the fusion of haploid individuals. However, fusion between two haploid cells only occurs if they contain non-identical mating-types. The existence of mating types was first demonstrated in 1904 when two sexually compatible cultures (+ and -) of Mucorinae (zygomycetes) were isolated (Blakeslee, 1904). It was later discovered in Ascobolus and Neurospora that mating-types are differentiated by different alleles at a single locus (Dodge, 1920; Shear & Dodge, 1927). The mating-type locus not only governs the fusion event between individuals but also influences directly or indirectly the expression of developmentally regulated genes. In many cases, the mating-type locus contains a gene(s) that encodes a protein with a characteristic DNA binding domain, *i.e.*, it resembles a transcription factor. Many mating-type genes from different fungal species have been cloned and sequenced; a striking difference is generally found between alternative mating-type sequences. The term "idiomorph" was coined to describe these dissimilar genes occupying homologous positions, *i.e.* the mating-type locus. In this section, I will review recent information on mating-type loci organization and the regulation of sexual development in different fungal species, including unicellular yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, mycelial ascomycetes *Neurospora crassa*, *Podospora anserina*, *Cochliobolus heterostrophus* and *Sordaria macrospora*, heterobasidomycetes *Ustilago maydis*, and homobasidiomycete *Coprinus cinereus* and *Schizophyllum commune*.

1.3.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae Meyer ex Hausen is a unicellular yeast that grows vegetatively by budding of haploid cells (Herskowitz, 1988). Individual haploid cells are of either **a** or α mating-type. The different **a** and α mating-types of haploid cells are determined by genes at the *MAT* locus (Mortimer & Hawthorne, 1969; Herskowitz & Oshima, 1981). A cell signals its presence to another cell of the opposite mating-type by producing mating-type-specific pheromones (Fields, 1990). An **a** cell produces **a**-factor (12-aa peptide; Betz *et al.*, 1987) which is secreted across the cell membrane and binds to a specific **a**-factor receptor (STE3 protein; Hagen *et al.*, 1986) on the surface of α cells. Similarly, α cell secretes α-factor (13-aa peptide; Stotzler *et al.*, 1976) that binds to the α-factor-specific receptor (STE2 protein; Blumer *et al.*, 1988) on **a** cells. Binding of the pheromones to their specific receptors facilitates mating by inducing a series of signal transduction events (Marsh *et al.*, 1991) that lead to (i) the transient arrest of the cell cycle in G1 (so that only a single copy of chromosomes is present; Bucking-Throm *et al.*, 1973) and (ii) the production of glycoproteins and agglutinins that enhance conjugation of opposite mating-type cells (Lipke & Kurjan, 1992; Leberer *et al.*, 1997). The two cells fuse together into a single cell (cytogamy), followed immediately by nuclear fusion (karyogamy) to produce a diploid cell. The diploid stage is the prominent form of *S. cerevisiae*, which reproduces vegetatively by budding. The mating functions in the **a**/α diploid cells are shut off -- diploid cells do not produce or respond to pheromone and are unable to switch mating type (Schmidt & Gutz, 1994). Upon starvation, the diploid cell stops vegetative growth and proceeds through meiosis (Klein *et al.*, 1994). Meiosis is followed by sporulation to form an ascus containing four haploid cells, two of each mating-type (Herskowitz, 1988).

Organization and function of the MAT locus

The mating-type locus *MAT* of *S. cerevisiae* is located near the centromere of chromosome III (Mortimer & Hawthorne, 1969). The mating-type of a haploid individual is

determined by the sequence encoded in the Y region (Astell *et al.*, 1981). For **a** cells, this region contains the Y**a** sequences (642 bp). On the other hand, α cells contain the totally dissimilar Yα sequence (747 bp) in the *MAT* Y region. Flanking the Y region are the X (704 bp) and the Z1 (239 bp) region. X and Z1 are also found flanking two silent copies of the mating-type determining sequence (sometimes referred as the Y cassette) at the *HML* and the *HMR* locus (Klar *et al.*, 1981). *HML* and *HMR* provide intact but unexpressed copies of mating-type genes that allow mating-type switching in yeast (see below). Although located on chromosome III, these two loci are not closely linked to *MAT*. Two additional homologous regions, W (732 bp) and Z2 (88 bp), are present in *MAT* and *HML*.

Two divergent transcripts, *MATα1* and *MATα2*, were identified in the *MATα* region (Tatchell *et al.*, 1981). MATα1 (175-aa) is a positive transcriptional factor that activates the expression of α-specific genes, such as *MFα1/MFα2* (encoding α-factor precursor; Kurjan & Herskowitz, 1982) and *STE3* (encodes **a**-factor receptor; Sprague *et al.*, 1983). MATα1 forms a complex with a transcriptional factor MCM1 (Passmore *et al.*, 1988). This complex binds to upstream regulatory regions of α-specific genes and activates their expression (Ammerer, 1990). Although MCM1 can bind to the upstream promoter regions by itself, it cannot activate gene expression without MATα1 (Tan & Richmond, 1990). It has been suggested that the STE12 transcriptional factor may play a role (by interacting with MATα1) in the activation of α-specific genes (Sengupta & Cochran, 1991). Although MATα1 is required for mating and α–specific phenotype, it is clearly not required for meiosis (Strathern *et al.*, 1981).

MCM1 is capable of binding to the upstream promoter region of **a**-specific genes, such as *MFa1/MFa2* (encoding **a**-factor precursor) and *STE2* (α-factor receptor), and activate transcription (Tan & Richmond, 1990). Since expression of **a**-specific genes does not require specific activation, these genes must be switched off in α cells. This is the function of the

MATα2 protein (Herskowitz, 1989). MATα2 contains a characteristic DNA binding motif, the homeodomain (Stepherd *et al.*, 1984). MATα2 forms homodimer in α cells and acts in conjunction with MCM1 and two general transcriptional repressors, SSN6 and TUP1, to repress **a**-specific genes (Mukai *et al.*, 1991; Keleher *et al.*, 1992). *MATα2* mutants display some **a**-specific phenotype and are able to mate (weakly) with α cells (Strathern *et al.*, 1981). The DNA binding and protein interaction characteristics of MATα2 has been determined both functionally and structurally (Hiscock & Kües, 1999).

Two divergent transcripts, *MAT**a**1* and *MAT**a**2*, have also been identified in the *MAT**a*** region (Tatchell *et al.*, 1981). *MAT**a**2* has no known function in conjugation or sporulation, and is apparently dispensable (Herskowitz, 1988). MAT**a**1, together with MATα2, is important in identity in **a**/α diploid cells. As mentioned above, diploid cells do not produce or respond to pheromone and are unable to switch mating type. However, diploid cells can express genes required for meiosis and sporulation, such as *IME-1*, *SPO12* and *SPO13* (Herskowitz, 1988, 1989). All these characteristics of diploid cells are controlled by the MATα2 homodimer (which represses **a**-specific genes) and the **a**1/α2 heterodimer (Goutte & Johnson, 1988). MAT**a**1-MATα2 heterodimer (with SSN6/TUP1) represses the transcription of *MATα1* (hence repressing α-specific genes) and other haploid-specific genes (Dranginis, 1990), such as *HO* (involved in mating-type switching; Schmidt & Gutz, 1994) and *RME1* (repressor of meiosis; Covitz *et al.*, 1991).

Mating-type silencing and switching in Saccharomyces cerevisiae

As mentioned above, *HML* and *HMR* regions also contain mating-type cassette Y (most strains are *HMLα HMR**a***), in addition to that found in the *MAT* locus. However, the mating-type cassettes in *HML* and *HMR* are silent. Repression of the silent mating-type cassettes in *HML* and *HMR* is mediated by many genes. Among these are *trans*-acting SIR1 to SIR4 proteins, each of

which is required for repression of *HML* and *HMR* (Ivy *et al.*, 1986; Rine & Herskowitz, 1987; Chien *et al.*, 1993; Triolo & Sternglanz, 1996; Hecht *et al.*, 1996; Strahl-Bolsinger, 1997). Two *cis*-acting sites required for HM silencing, E and I (called the silencers), flank *HML* and *HMR* but not *MAT* (Feldman *et al.*, 1984; Mahoney & Broach, 1989; Loo & Rine, 1994). These *cis*-acting elements, together with histone proteins, SIR1-4, RAP1 and ORC (origin recognition complex), form a short region of highly ordered heterochromatin-like structure resulting in the repression of *HML* and *HMR* (Loo & Rine, 1995; Weiss & Simpson, 1998; Haber, 1998).
It has been suggested that DNA replication may play a role in maintenance of silencing, which is supported by the following evidence: (i) E and I contain binding sites for the transcriptional factors RAP1 and ABF1, (ii) E and I exhibit ARS activity and (iii) repression at both *HML* and *HMR* requires passage through S phase of the cell cycle (Miller & Nasmyth, 1984). However, recent experiments have provided evidence that the S phase requirement may be due to enzymes specific for S phase rather than replication (Bi & Broach, 1997; Fox *et al.*, 1997).

Mating-type switching in yeast has been observed for decades (Oeser, 1962; Strathern & Herskowitz, 1979). In the process of mating-type interconversion, a copy of the mating-type cassette (Y**a** or Yα) is transposed from *HMR* or *HML* into *MAT*. The switch is initiated by a double-strand break (DBS) in the Z1 region, 3 bp to the right of Y (Raveh *et al.*, 1989). The DBS is introduced by an endonuclease (encoded by *HO*; defects in *HO* lead to heterothallism) during G1 phase of the cell cycle (Kostriken *et al.*, 1983; Herskowitz, 1989). A symmetric priming model has been proposed to explain the switching mechanism (which is related to gene conversion) and how the DNA originally present in *MAT* is degraded (Klar *et al.*, 1984a). The phase specificity of DBS can be explained by the fact that the transcription of *HO* is restricted to the G1 phase by several regulatory genes, including 6 *SWI* genes and 3 *SIN* genes (Herskowitz *et al.*, 1992). Other genes required for completion of switching include those involved in DNA damage repair and recombination, such as the *RAD* genes (Game, 1983; Weeda *et al.*, 1993; Schmidt & Gutz, 1994). DSBs at *MAT* are lethal if they are not healed by a switching event, *i.e.*,

S. cerevisiae cannot repair *HO*-induced DSBs without mating-type switching (Klar *et al.*, 1984a, b).

In *S. cerevisiae*, only experienced cells, *i.e.*, those cells which have budded off at least one daughter cell, can produce switched progeny (Strathern & Herskowitz, 1979). Both progeny of a cell (the mother and the daughter) are switched whenever a switch occurs (Klar, 1989). Switching in *S. cerevisiae* is very efficient, *i.e.*, a cassette is preferentially replaced by a cassette with opposite mating-type. A recombination enhancer (RE) is the molecular basis for this directionality (Wu & Haber, 1996). In *MAT**a*** cells, the RE is activated, thus allowing the left arm of chromosome III (*HML*α) to be the preferred donor. In *MAT*α cells, RE activity is repressed (by the binding of MATα2-MCM1; Wu *et al.*, 1998a) and the entire left arm (and part of right arm) is "cold" and thus ***HMRa*** becomes the preferred donor.

Pheromone response and signal transduction

The α-factor of *S. cerevisiae* is encoded by two unlinked genes, *MF*α*1* and *MF*α*2* (Kurjan & Herskowitz, 1982). Each gene contains 6 tandem copies of the α-factor precursor sequence: WHWLQ(N)LK(R)PGQMY (5 perfect repeats and 1 repeat with conservative substitutions as shown in parentheses; Singh *et al.*, 1983). The prepropheromone is transported to the endoplasmic reticulum and is subject to multiple rounds of proteolytic actions. The mature α-factor is exported by the typical secretory pathway via the golgi apparatus (Julius *et al.*, 1984). Similarly, the **a**-factor precursor is encoded by two unlinked structural genes, *MFa1* and *MFa2* (Brake *et al.*, 1985). Both precursor polypeptides contain a single copy of **a**-factor near the carboxy-terminus (Betz *et al.*, 1987), which terminates in a CAAX motif (C = cysteine, A = aliphatic amino acid, and X = any amino acid). CAAX motifs have been identified as a consensus signal for isoprenylation of cysteine (Powers *et al.*, 1986). Maturation of **a**-factor requires multiple post-translational modification steps. Unlike α-factor, the export of **a**-factor does not depend on the typical secretory pathway (Sterne & Thorner, 1987). The secretion of **a**-

factor requires the *STE6* gene, which encodes a membrane-bound, ATP-dependent transport protein (Kuchler *et al.*, 1989). These observations suggest that the **a**-factor is exported by a novel secretory pathway.

As mentioned above, binding of the pheromones to their specific receptors facilitates mating by inducing a series of signal transduction events that lead to the transient arrest of the cell cycle in G1 and the induction of conjugation-specific genes. The pheromone signal transduction pathway is initiated by binding of pheromone to its receptor in the cytoplasmic membrane (Marsh *et al.*, 1991; Duntze *et al.*, 1994). The signal transduction machinery appears to be the same for both α- and **a**-factor after this step. The pheromone-binding signal, via coupling of a heterotrimeric G-protein, in which the β (STE4) and γ (STE18) subunits are believed to be the active signal transmitter, activates a cascade of MAP kinase phosphorylation in this order: STE11 → STE7 → KSS1/FUS3 (Peter *et al.*, 1993; Cook *et al.*, 1997). The FUS3 and KSS1 (in the absence of FUS3) kinase phosphorylates the transcriptional factor STE12 (Madhani *et al.*, 1997), which in phosphorylated form binds and activates the transcription of many conjugation-specific genes, including *AGA1* and *AGα1* (cell agglutination), *KAR3* (nuclear fusion), *STE2* and *STE3* (receptors for α- and **a**-factor), *SCG1/STE4/STE18* (structural genes of G-protein), and *MFα1* and *MFa1* (structural genes of the pheromone precursors) (Marsh *et al.*, 1991; Kurjan, 1993). The activation of STE12 is more complicated than once thought and may involve its dissociation from two inhibitors (RST1 and RST2; Cook *et al.*, 1996; Tedford *et al.*, 1997). On the other hand, the FUS3 kinase phosphorylates FAR1, thus enabling it to inhibit the CDC28-CLN2 kinase complex, probably by causing degradation of CLN2 (Peter *et al.*, 1993). The inhibition of the CDC28-CLN2, which controls cell cycle progression in *S. cerevisiae*, is believed to cause arrest of cells in G1 (Futcher, 1991). The mechanism for activation of this MAPK cascade is unknown, however, it may involve action of STE5 scaffold protein and STE20 serine/threonine protein kinase (Banuett, 1998).

If a cell cannot find a partner after pheromone induction, it can recover from pheromone action and resume growth by the following mechanisms: (i) internalization/degradation of occupied receptor (Chvatchko *et al.*, 1986; Jeness & Spatrick, 1986), (ii) phosphorylation of the C-terminus of the receptor protein, which interferes with the ability of the receptor to transmit the signal (Konopka *et al.*, 1988; Reneke *et al.*, 1988), (iii) degradation of α- and **a**-factor (MacKay *et al.*, 1988; Marcus *et al.*, 1991), (iv) de-sensitization of the cell to pheromone by the RGS (regulator of G-protein signalling) protein, SST2, through regulation of Gα GTPase activity (Dohlman *et al.*, 1996; Apanovitch *et al.*, 1998).

1.3.2 *Schizosaccharomyces pombe*

Schizosaccharomyces pombe Lindner is a unicellular yeast that grows by tip extension and divides by medial fission (Egel, 1989). There are two mating-types in *S. pombe* haploid cells: *P* (plus) and *M* (minus) (Leupold, 1958). Under starvation, cells of opposite mating-type elongate to each other through the actions of pheromones (Leupold, 1987). The cells arrest in G1 and agglutinate. Unlike *S. cerevisiae*, meiosis occurs immediately after cell fusion and karyogamy, results in the formation of a four-spored ascus. Two of the spores are of each mating-type.

Mating-type locus and sexual cycle in S. pombe

The mating-type locus organization in *S. pombe* is similar to that of *S. cerevisiae* (Kelly *et al.*, 1988). The two different mating-types are determined by genes at a single locus on the right arm of chromosome II, *mat-1* (*mat1-P* for *P* cells and *mat1-M* for *M* cells). The *P* and *M* mating-type alleles contain dissimilar DNA sequences of 1104 and 1128 bp respectively. Two additional loci that contain silent copies of mating-type sequence, *mat-2* and *mat-3*, are also present on chromosome II. The three loci are linked, with a 15-kb sequence separating each neighboring locus. Surrounding the *mat-1*, *mat-2* and *mat-3* loci are short regions of homology, H1 (59 bp) and H2 (135 bp). An additional sequence, H3 (57 bp), is present at *mat-2* and *mat-3*.

Two transcripts, *mat1-Pc* and *mat1-Mc* (c = conjugation), one from each mating-type, are detectable during vegetative stage (Kelly *et al.*, 1988). Mutations in these genes lead to defects in conjugation. The predicted polypeptides of MAT1-Pc (118 aa) and MAT1-Mc (181 aa) are basic, a characteristic of DNA binding protein. MAT1-Pc encodes a α1-domain protein (Coppin *et al.*, 1997). MAT1-Mc protein contains a DNA binding domain known as HMG box (Ner, 1992), which is found in the SRY protein that determines maleness in humans and mice (Sinclair *et al.*, 1990). The function of MAT1-Pc and MAT1-Mc is proposed to be analogous to that of MATα1, a transcriptional factor in *S. cerevisiae* that controls the expression of conjugation-specific genes, including those encoding pheromones and pheromone receptors. MAT1-Mc activates *M*-specific genes by recruiting the ubiquitous HMG transcription factor STE11 to binding sites (Kjaerulff *et al.*, 1997). MAT1-Pc, in association with a MCM1-like transcriptional factor (MAP1), controls expression of *P*-specific genes (Nielson *et al.*, 1996). Unlike MATα1, MAT1-Pc and MAT1-Mc are required for conjugation as well as sporulation (Kelly *et al.*, 1988; Willer *et al.*, 1995; Hiscock & Kües, 1999). Expression of *mat1-Pc* and *mat1-Mc* is induced by nitrogen limitation (which in turn lowers the level of intracellular cAMP; Mochizuki & Yamamoto, 1992).

Two additional mating-type transcripts, *mat1-Pm* (from *mat1-P*; m = meiosis) and *mat1-Mm* (from *mat1-M*) are detectable under starvation. Both transcripts are required for meiosis and sporulation, but not conjugation. MAT1-Mm (42 aa) bears no striking amino acid resemblance with other mating-type proteins, although the *mat1-Mm* gene contains DNA sequence similarity (at its 3' end) with that of the *mat1-Pc* gene. The homologous region does not affect amino acid similarity between the two proteins because they are out of frame (Kelly *et al.*, 1988). MAT1-Pm contains homology with the homeodomain of MATa1 and MATα2, suggesting that it may have similar DNA-binding properties. MAT-Pm is a transcriptional factor required for the expression of a meiosis-specific genes, *mei3* (McLeod *et al.*, 1987; Van Heeckeren *et al.*, 1998).

MEI3 binds to and inhibits PAT1, a protein kinase that serves as a negative regulator of meiosis (also known as RAN1) (see below; McLeod & Beach, 1988).

The sexual cycle in *S. pombe* is thought to be regulated by a 3-step progressive inactivation of the PAT1 (Nielson & Egel, 1990). Nitrogen starvation triggers the first step of inactivation and results in the expression of STE11, a HMG-type transcriptional factor required to express the *mat1-Pc* and *mat1-Mc* genes (Sugimoto *et al.*, 1991). In *P* cells, *mat1-Pc* and the uncharacterized *map1* genes are required for the pheromone (P-factor) response pathway. The binding of M-factor (pheromone produced by *M* cells) to *P* cells triggers the second stage of the PAT1 inactivation (as mediated by STE6-induced RAS protein; Hughes *et al.*, 1990), and results in the expression of *mat1-Pm* and other conjugation-specific genes, *e.g.*, *fus1* (Nielson & Egel, 1990). The regulatory pathway in *M* cells is believed to be similar to that of *P* cells. However, expression of *mat1-Mm* is independent of *mat1-Mc* and is not pheromone induced. There is evidence that *mat1-Mc* regulates the pheromone receptor gene, *mam2* (Kitamura & Shimoda, 1991).

The M-factor pheromone is a modified nanopeptide: YTPKVPYMC (with S-farnesylation and carboxy-methylation at the cysteine rsidue) (Davey, 1991, 1992). The C-terminus contains a CAAX box, which is also found in **a**-factor of *S. cerevisiae*. M-factor is secreted through an ABC transporter (MAM1; Christensen *et al.*, 1997). The P-factor is a 23-aa unmodified polypeptide, which is a KEX2-cleavage product of its four-repeat precursor (Davey *et al.*, 1994; Imai & Yamamoto, 1994). The M-factor is encoded by 3 redundant genes, *MFm1* to *MFm3* (Davey, 1992; Kjaerulff *et al.*, 1994) while P-factor is encoded by *map2* (Imai & Yamamoto, 1994). The M-factor receptor and the P-factor receptor are encoded by *mam2* (Kitamura & Shimoda, 1991) and *map3*, respectively (Tanaka *et al.*, 1993). Both receptors contain seven transmembrane domains, which characterize them as the rhodopsin/adrenergic type receptors (Dohlmann *et al.*, 1991). MAM2 is homologous to **a**-factor receptor of *S.*

cerevisiae (STE2) and pheromone receptors of *U. maydis* (PRA1 and PRA2). All these receptors recognize farnesylated mating factors (Schafer & Rine, 1992). MAP3 is homologous to α-factor receptor of *S. cerevisiae* (STE3). The pheromone binding signal in *S. pombe* is transmitted by G-protein, with the G_α subunit playing the active role in the MAP kinase signal transduction (Obara *et al.*, 1991; Banuett, 1998). Unlike *S. cerevisiae*, the mating response pathway in *S. pombe* is dependent upon *ras* and nitrogen starvation (Hiscock & Kües, 1999).

Mating-type silencing and switching in S. pombe

In *S. pombe*, the mating-type cassettes in *mat-2* and *mat-3* are silent. Mutants were found in which the silent *mat-2* and *mat-3* cassettes are expressed. These include *clr1* to *clr4*, *swi6* and *rik1* (Thon & Klar, 1992; Lorentz *et al.*, 1992; Ekwall & Ruusala, 1994). Meiotic recombination in the K region between *mat-2* and *mat-3* (which normally does not occur; Grewal & Klar, 1997) is permitted in some of these mutants (Egel *et al.*, 1989). The silencing of *mat-2* and *mat-3* is believed to be mediated by heterochromatin formation in these loci (Lorentz *et al.*, 1994; Allshire *et al.*, 1995; Ekwall *et al.*, 1996). SWI6 contains a chromatin organization modifier domain and might represent the structural component of the chromatin. *Cis*-acting sequences flanking *mat-2* are required for effective blockage of transcription (Ekwall *et al.*, 1991).

Switching of mating-type in *S. pombe* involves the transfer of a mating-type cassette from *mat-2* or *mat-3* to the *mat-1* locus. The transfer is initiated by a double-strand break (DSB) in the H1 homology box of *mat-1* at the border to the mating-type-specific sequence (Nielsen & Egel, 1989). Binding of SAP1 protein at SAS1 (see below) is necessary for generation of DSB (Arcangioli & Klar, 1991). DSBs are detectable during all stages of the cell cycle (*c.f.* DSBs detectable only in G1 in *S. cerevisiae*). In *S. pombe*, only daughter cells show a converted mating-type (Egel, 1984; Klar 1992a, b; *c.f.* both progeny show switching in *S. cerevisiae*). This imprinting is due to strand-specific DNA modification at the *mat1* locus (Dalgaard & Klar, 1999).

There are three classes of switching-defective mutations that define *tran*-active genes required for recombination (Egel *et al.*, 1984; Klar, 1992b): (i) Class Ia (*swi1*, *swi3*, *swi7*) represent the genes required for making a DSB at *mat-1*, (ii) Class Ib (*swi2*, *swi5*, *swi6*) represent the genes involved in the utilization of the DSB for recombination, and (iii) Class II (*swi4*, *swi8*, *swi9*, *swi10*, *rad 22*) represent the genes required for general recombination and repair. In addition, at least two *cis*-acting sites, SAS1 and SAS2 (which span 150 bp on the left of H1 at the *mat-1* locus), are required for the generation of DSB (Klar *et al.*, 1991). Switching directionality (*i.e.* donor is preferentially selected from the locus containing the opposite mating-type cassette) is observed in *S. pombe*. One explanation for this effect is that the chromatin structure of the donor loci is altered between *P* and *M* cells, therefore promoting preferential selection of the opposite mating-type cassette (Klar, 1992b).

1.3.3 *Neurospora crassa*

The life cycle of *Neurospora crassa* Shear & Dodge begins when a mature ascospore, upon heat activation, germinates and produces a multinucleate mycelium (Fincham *et al.*, 1979; Figure 1-1). Under nitrogen starvation, the female reproductive structures (protoperithecia) are formed (Johnson, 1978). The outer layer of the protoperithecium is a wall of hyphae. Inside is a coiled hypha, the ascogonium, from which projects female receptive hyphae called trichogynes. Mating occurs between these trichogynes and a target cell (conidium, microconidium, or hypha) of the opposite mating-type (either *A* or *a*) which is destined to function as the male. Pheromone emitted by the male cell directs the growth of trichogynes (of opposite mating-type) toward them (Bistis, 1981). Once fertilized, the protoperithecium develops into the perithecium.

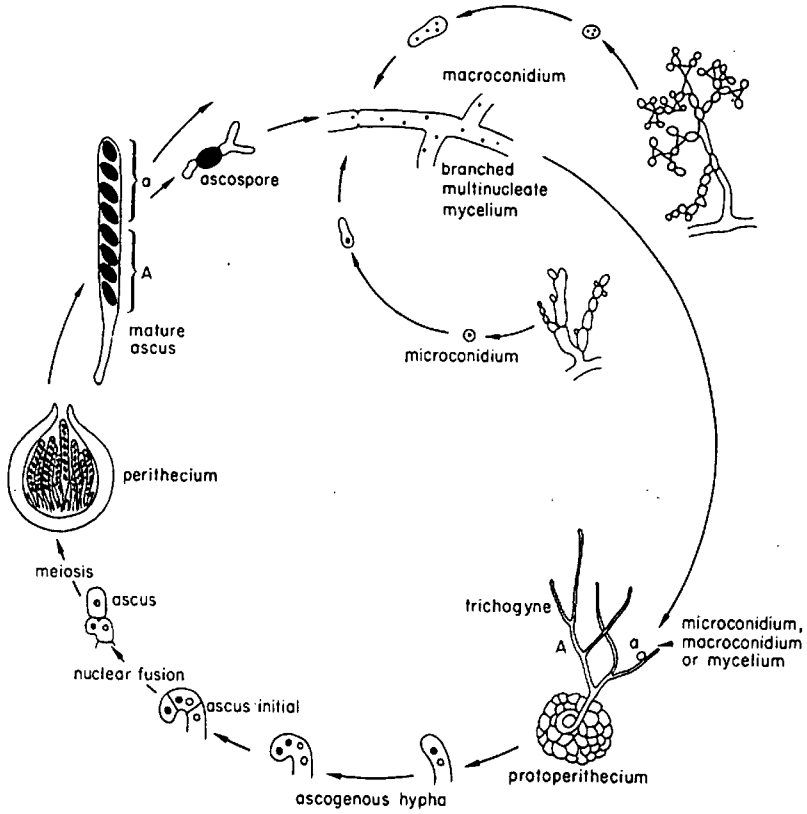


Figure 1-1. Life cycle of *Neurospora crassa.* Reprinted from Fincham *et al.* (1979).

The male nuclei, presumably under their own genetic control (Vigfusson *et al.*, 1971), travel down the trichogyne, where one nucleus enters the ascogonium and becomes associated with the female nucleus. A series of synchronous nuclear divisions, accompanied by the darkening and the enlargement of the perithecium, gives rise to a cluster of dikaryotic ascogenous hyphae. After 8-10 divisions, karyogamy occurs in the penultimate cells of the ascogenous hyphae, followed by two meiotic divisions and a postmeiotic mitosis. The final products are perithecia containing many mature asci, each housing eight ellipsoidal ascospores that are shot through an opening in the perithecial beak (Fincham *et al.*, 1979).

Mating-type locus organization in Neurospora crassa

In *N. crassa*, mating is controlled by a single locus (*mat*) with two mating-types, *A and a* (Figure 1-2; Shear & Dodge, 1927; Perkins *et al.*, 1976). The *mat* locus, located on the left arm of linkage group I, has an additional unique function in vegetative growth known as heterokaryon incompatibility. Heterokaryon incompatibility, a process that eliminates heterokaryon formation between fungi with genetically distinct nuclei, is controlled by *mat* and at least 10 other *het* loci in *N. crassa* (Saupe, 2000; Glass *et al.*, 2000; see section 1.4). An allelic difference at the *mat* locus leads to death and compartmentation of cells along the line of fusion between hyphae of opposite mating-types (Beadle & Coonradt, 1944). Early attempts to separate the heterokaryon incompatibility function and the mating function of *A* and *a* idiomorphs by genetic recombination were unsuccessful (Newmeyer *et al.*, 1973), indicating that either the two functions are conferred by the same gene, or they are encoded by separate genes that are closely linked. An unlinked locus *tol* was discovered that suppressed the vegetative incompatibility without affecting mating function (Newmeyer, 1970).

A cosmid containing the *N. crassa A* mating-type locus (*mat A*) was first isolated by complementation of *un-3* (closely linked to *mat*) activity (Glass *et al.*, 1988). The 5.3 kb *mat A*

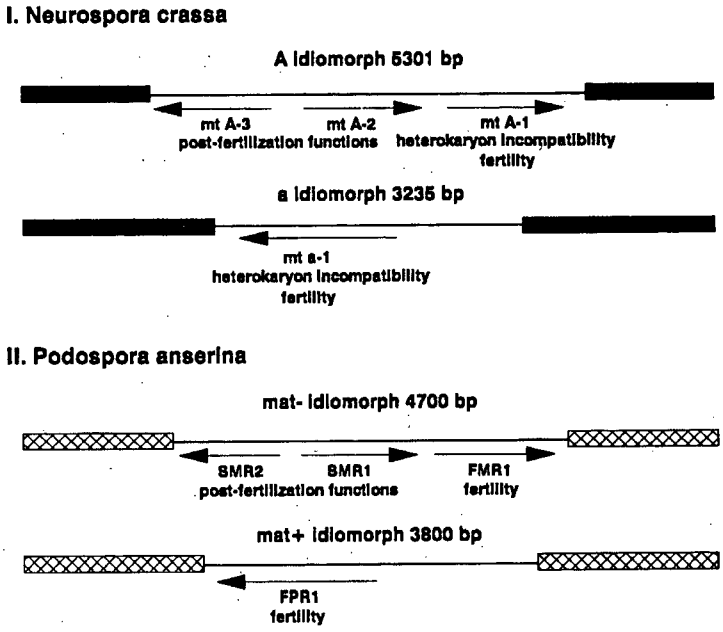


Figure 1-2. Mating type locus organization in *Neurospora crassa* and in *Podospora anserina*. Reprinted from Glass & Nelson (1994).

locus, which contains three ORFs: *mat A-1*, *A-2* and *A-3*. The *mat A-1* gene is both necessary and sufficient to confer mating specificity of *A* and trigger heterokaryon incompatibility with *a* (see below for heterokaryon incompatibility) (Glass *et al.*, 1990). MAT A-1 protein is believed to be a transcriptional factor (although direct transcriptional activity has not been shown). It contains an α1 (sometimes called α-) domain that is shared by other ascomycetes mating-type proteins, namely MATα1 (*S. cerevisiae*) (Astell *et al.*, 1981), MAT1-Pc (*S. pombe*) (Kelly *et al.*, 1988; Coppin *et al.*, 1997), FMR-1 (*P. anserina*) (Debuchy & Coppin, 1992), and MAT-1 (*C. heterostrophus*) (Turgeon *et al.*, 1993). The α1 domain of MATα1 protein is proposed to interact with MCM1 in *S. cerevisiae* to recognize promoters of α-specific genes (Grayhack, 1992; Yuan *et al.*, 1993). The first 85 amino acids of MAT A-1 (includes the α1 domain) is sufficient for low female fertility but the entire polypeptide is required for male and full female fertility (Saupe *et al.*, 1996b).

MAT A-1 also plays a role in the post-fertilization functions. When a functional *mat A-1* copy is transformed to an ectopic site in a *mat A-1* mutant, the transformants gain mating activity as *A* but only produce barren perithecia after a cross (Glass *et al.*, 1990; Saupe *et al.*, 1996b; Vellani, 1988). This observation shows two important phenomena: (i) MAT A-1 is required for sexual development after mating. MAT A-1 protein could be required for nuclear identity for conjugate mitotic divisions of opposite mating-type nuclei or nuclear transport into the ascogonium. (ii) The *mat A-1* gene has to be located at the *mat* locus for completion of the sexual cycle. The mechanism for physical location requirement for *mat A-1* is unknown (note: the ectopic restriction also applies to *mat a-1*). It is possible that the *mat* locus gives a strict expression pattern for *mat A-1* to function during sexual development. Alternatively, mating-type genes may be required to pair during the sexual cycle. Transvection (regulation dependent on pairing of alleles; Wu & Morris, 1999) between alleles of the *Asm-1* locus has been reported to affect meiosis (Aramayo & Metzenberg, 1996).

Two more ORFs were identified within the *A* idiomorph (Glass & Lee, 1992). Mutations in *mat A-2* and *mat A-3* do not affect mating or heterokaryon incompatibility, but affect ascosporogenesis; a *mat A-2* and *mat A-3* double mutant makes few asci with ascospores (the development of the perithecia is arrested 3-4 days after fertilization). Hence, the two ORFs may govern ascogenous hyphal development. Interestingly, the *mat A-2* and *mat A-3* single mutants give abundant biparental asci when crossed with *a*, indicating that MAT A-2 and MAT A-3 could be redundant and may function in the same pathway (Ferreira *et al.*, 1998). Since *mat A-2/mat A-3* double mutants give rare asci that are biparental (instead of uniparental "selfish" asci; see section 1.3.4), it has been suggested MAT A-2 and A-3 are involved in the synchronous dikaryon proliferation in the ascogenous hyphae, and not in the migration of opposite mating-type nuclei to the crozier (Ferreira *et al.*, 1998).

MAT A-2 is not similar to any known protein except the *P. anserina* SMR1 mating-type protein (see section 1.3.4) (Ferreira *et al.*, 1996). MAT A-2 and SMR1 contain a highly conserved amphipathic α-helical motif that may specify a new DNA binding motif (Debuchy *et al.*, 1993). MAT A-3 contains a HMG (high mobility group) domain (Bianchi & Beltrame, 1998). HMG domain proteins bind to the minor groove and introduce bends in DNA (Bewley *et al.*, 1998). MAT A-3 binds to the same DNA fragments *in vitro* as MAT a-1 does (see below) (Kronstad & Staben, 1997). Although there is no evidence for direct regulation by these mating-type proteins of downstream genes, it has been shown that expression of mating pheromones (D. Ebbole, personal communication) and at least two sexual developmental (*sdv*) genes (Nelson & Metzenberg, 1992; Nelson *et al.*, 1997; Ferreira *et al.*, 1998) are under the control of *N. crassa* mating-type polypeptides. The size and expression pattern of transcripts adjacent to the MAT locus is also found to be mating type-specific (Randall & Metzenberg, 1995, 1998).

The *a* idiomorph contains two ORFs – *mat a-1* (Staben & Yanofsky, 1990) and *mat a-2* (Pöggeler & Kück, 2000). Griffiths and DeLange (1978) obtained 25 *mat a* mutants. All but one

of the mutants had lost both mating and heterokaryon incompatibility functions. The exceptional a^{m33} mutant lost only the heterokaryon incompatibility function. MAT a-1 is necessary and sufficient for all mating type functions for *a* (mating, post-fertilization, heterokaryon incompatibility), since replacement of the *A* idiomorph with a construct containing only *mat a-1* can switch mating-type (Chang & Staben, 1994). *mat a-2* was identified by transciptional analysis and may represent a non-essential gene or a non-translated pseudogene (Pöggeler & Kück, 2000). MAT a-1 has an HMG box and binds to DNA in vitro centering on the sequence CAAAG in common with binding sites of other HMG proteins (Philley & Staben, 1994; Kronstad & Staben, 1997). Mating function of MAT a-1 correlates with DNA binding activity; deletion within the HMG box eliminated both DNA binding *in vitro* and mating *in vivo*, but did not affect heterokaryon incompatibility (Philley & Staben, 1994). Unlike MAT A-1, MAT a-1 is apparently required after karyogamy, since RIP (Selker, 1990) mutants in *mat a-1* cannot be isolated (Kronstad & Staben, 1997). MAT a-1 is also believed to play a role in dikaryon proliferation since strains bearing duplication in *mat a-1* always resolve after a cross (Chang & Staben, 1994). Preliminary yeast two-hybrid data showed that MAT a-1 interacts with MAT A-1 (Badgett & Staben, 1999). In yeast, the MAT**a**1-α2 heterodimerization of the mating type proteins is important in the repression of haploid mating-specific genes and de-repression of meiotic genes (Johnson, 1995; Johnson *et al.*, 1998). However, heterodimerization of MAT A-1 and MAT a-1 may be relevant to heterokaryon incompatibility rather than to sexual development; a mutant MAT a-1 protein lacking interaction activity with MAT A-1 affected heterokaryon incompatibility but not sexual function (C. Staben, personal communication). In *P. anserina*, mating-type-associated heterokaryon incompatibility does not exist; a yeast two-hybrid experiment failed to demonstrate interaction between FMR1 and FPR1 (homologs of MAT A-1 and MAT a-1, respectively) (Coppin *et al.*, 1997). These data suggested that an **a**1/α2-type heteromeric complex may not be required for meiosis function in filamentous ascomycetes.

Although the pheromone response pathway of *N. crassa* has not been characterized, proteins similar to components of the yeast MAP kinase pheromone pathway have been identified, such as mating pheromones (MFA and MFa; D. Ebbole & D. Bell-Pedersen, unpublished data), a heterotrimeric G proteins (Turner & Borkovich, 1993, unpublished data), a STE11 homolog (NRC-1; Kothe & Free, 1998), a FUS3 homolog (MAK-2; P. Bobrowicz & D. Ebbole, unpublished data) and a STE12 homolog (Neurospora Genome Project; http://biology.unm.edu/~ngp/home.html).

1.3.4 *Podospora anserina*

Podospora anserina (cas.) Niessl is in the same family (Sordariaecae) as *N. crassa*, although it displays a different sexual life style (Esser, 1974). *P. anserina* is a pseudohomothallic ascomycete, with opposite mating-type nuclei (*mat+* and *mat-*) compartmentalized within a single ascospore, yielding functional self-fertility. The mating-type locus organization of *P. anserina* is very similar to that of *N. crassa*. The *mat-* and *mat+* idiomorphs are composed of 4.7 kb and 3.8 kb of dissimilar DNA sequence, respectively (Debuchy & Coppin, 1992). Deletion of the *mat* locus (*mat0*) results in sterility, but has no effect on vegetative growth or formation of female reproductive organs (Coppin *et al.*, 1993). Transformation of the entire *mat* locus into ectopic locations in a *mat0* strain results in full complementation of mating and ascospore formation (Coppin *et al.*, 1993). Hence the positional effect observed in *N. crassa* (ectopic mating-type genes cannot complement *mat* mutants in post-fertilization functions) does not apply to *P. anserina*.

Mating-type genes of *P. anserina* (Debuchy & Coppin, 1992; Debuchy *et al.*, 1993) are homologous to those of *N. crassa* (Glass *et al.*, 1990; Ferreira *et al.*, 1996) (Figure 1-2). The *mat-* mating type was identified by heterologous hybridization using *N. crassa mat A* sequences (Picard *et al.*, 1991). FPR1 is a homolog of MAT a-1, while FMR1, SMR1 and SMR2 are homologs of MAT A-1, MAT A-2 and MAT A-3, respectively. Mating-type genes from *P.*

anserina and *N. crassa* are interchangeable for mating function; *FMR1* confers *A* mating activity in *N. crassa while mat A-1* confers *mat-* mating activity in *P. anserina* (Arnaise *et al.*, 1993). However, functional conservation for heterokaryon incompatibility and post-fertilization events are not observed; when FMR1 and FPR1 were introduced into a *N. crassa* background, an incompatibility reaction was not induced (Arnaise *et al.*, 1993). *FMR1* (*mat-*) and *FPR1* (*mat+*) are involved in mating function much like MAT A-1 and MAT a-1, while *SMR1* and *SMR2* are involved in fruiting bodies maturation and ascospore formation (Debuchy *et al.*, 1993).

Internuclear complementation shows that SMR1 is not a *bona fide* mating type protein, since *SMR1* expression is not nucleus limited and that it can function in a *mat-*, *mat+* or both nuclei (Arnaise *et al.*, 1997). Since mutations in *SMR1* lead to barren perithecia with no ascogenous hyphae, SMR1 is likely to be responsible for initial development of biparental ascogenous hyphae, after the nuclear recognition step (Arnaise *et al.*, 1997). There is evidence SMR1 does so by lifting the growth arrest of biparental ascogenous hyphae after *mat+* and *mat-* have paired and thus allow proliferation. This hypothesis was based on the observation that *mat+* ascospores carrying constitutively transcribed *FMR1* and *SMR2* are unable to germinate, but ascospore lethality can be suppressed by the over-expression of *SMR1* (Coppin & Debuchy, 2000).

The *bona fide* mating type proteins in *mat-*, FMR1 and SMR2, physically interact with each other in a yeast two-hybrid system (cited in Coppin *et al.*, 1997). The two proteins are possibly involved in nuclear recognition since they are required to ensure biparental dikaryotic state after fertilization (Zickler *et al.*, 1995). Loss-of-function mutations in *FMR1* or *SMR2* lead to the formation of monokaryotic progeny and uniparental dikaryotic progeny (Zickler *et al.*, 1995). Since FMR1 is not likely to be nuclear limited while SMR2 is, it was proposed that SMR2 acts as a carrier protein that directs FMR1 to the *mat-* nucleus (Arnaise *et al.*, 1997). FPR1 is apparently limited to the *mat+* nucleus (Arnaise *et al.*, 1997). Debuchy (1999) has

proposed a model that explains the roles of mating-type proteins in nuclear recognition during formation of dikaryotic ascogenous hyphae. Internuclear recognition and nuclear positioning is proposed to be controlled by nucleus-limited expression of mating-type gene products: each nucleus controls the expression and localization of mating-type-specific pheromone/receptors in its cell wall and plasmalemma (Debuchy, 1999). Spatial cues are generated when the two opposite *mat* nuclei are close enough to superimpose their specific expression domains and therefore allowing the binding of pheromones to their respective receptors. This cue in turn triggers the migration of the opposite *mat* nuclei into the ascogenous hyphae, probably by reorganization of the cytoskeleton and secretory apparatus as described in yeast (Drubin & Nelson, 1996; Thompson-Coffe & Zickler, 1994; Berteaux-Lecellier *et al.*, 1998; Inoue *et al.*, 1998). The uniparental progeny observed in a *SMR2* mutant can be explained with the model that the SMR2/FMR1 nuclear recognition system is no longer functional, and that it allows contaminating *mat*+ pheromones to trigger a spatial cue for *mat*- nuclei to migrate to the ascogenous hyphae (Debuchy, 1999).

1.3.5 *Cochliobolus heterostrophus*

The *C. heterostrophus* (Drechsler) Drechsler mating-type idiomorphs are much smaller than that of *N. crassa* and *P. anserina*. Each idiomorph only contains one mating-type gene, *MAT-1* (1297 bp) or *MAT-2* (1171 bp) (Turgeon *et al.*, 1993). The *MAT-1* gene was isolated based on the dual-mating phenotype it produces when transformed into a *MAT-2* strain. MAT-1 contains the α1 domain that is found in some other fungal mating-type proteins (Coppin *et al.*, 1997). MAT-2 contains an HMG DNA binding domain that is shared by other fungal mating type proteins, MAT a-1 (Staben & Yanofsky, 1990), MAT A-3 (Ferreira *et al.*, 1996), FPR1 (Debuchy & Coppin, 1992), SMR2 (Debuchy *et al.*, 1993) and MAT1-Mc (Kelly *et al.*, 1988). Interestingly, the position of the intron is conserved in the above-mentioned α1 and HMG families (Coppin *et al.*, 1997). MAT-1 and MAT-2 have a small island of nucleotide sequence similarity, suggesting they may share a common origin (Coppin *et al.*, 1997; Turgeon, 1998).

Although the mating type sequences of *C. heterostrophus* (a loculoascomycete) do not share extensive similarity with those of pyrenomycetes (*N. crassa* and *P. anserina*), introduction of either *MAT-1* or *MAT-2* into a *P. anserina* mating-type deletion strain confers mating activity (Coppin *et al.*, 1997), showing that the function of mating-type genes are conserved between these diverse fungi. Homologs of MAT A-2/ SMR1 and MAT A-3/SMR2 were not found in *C. heterostrophus*. Genes flanking the *MAT* locus are not involved in the mating process and MAT-1 and MAT-2 alone confer full mating and post-fertilization functions (Wirsel *et al.*, 1998).

Transcript analysis reveals special features of *C. heterostrophus* mating type system. Unlike the other filamentous ascomycetes, the *MAT-1/MAT-2* idiomorphs encode transcripts which start 5' and end 3' within sequences common to both mating types (Leubner-Metzger *et al.*, 1997). *MAT* transcripts are controlled and can be detected in cultures grown in minimal medium but not in complete medium (Leubner-Metzger *et al.*, 1997). Transcriptional and translational controls may also play a role in *MAT* gene expression during sexual development – (i) optional splicing of the two 5' untranslated introns and at least two transcription start sites result in three types of transcripts (although deletion of the 5' end does not interfere with crossing ability) (Wirsel *et al.*, 1998; Leubner-Metzger *et al.*, 1997); (ii) 160 bp of 3' UTR is required for ascospore production (Wirsel *et al.*, 1998). The 3' *MAT* UTR region can potentially provide localized expression of *MAT* genes so that the two opposite mating type nuclei can distinguish one and another (Wirsel *et al.*, 1998). Unlike *N. crassa* (Chang & Staben, 1994), expression of *MAT* genes specifically at the *MAT* locus is not required for ascospore formation in *C. heterostrophus* (Wirsel *et al.*, 1996). However, interference by ectopic *MAT* transgenes with the resident *MAT* gene function (at the *MAT* locus) is observed in *N. crassa*, *P. anserina* and *C. heterostrophus* (Chang & Staben, 1994; Coppin *et al.*, 1993; Wirsel *et al.*, 1996).

MAT-2-like genes have been isolated from several *Cochliobolus* spp. (and related loculoascomycete genera) using primers matching *C. heterostrophus* sequences (Arie *et al.*, 1996; Turgeon *et al.*, 1995; Sharon *et al.*, 1996). Despite the possession of a *MAT-2* homolog (which is functional as a transgene in *C. heterostrophus*), *Bipolaris sacchari* (sugarcane pathogen related to *Cochliobolus* spp.) remains an asexual fungus (Sharon *et al.*, 1996). The transformation of *C. heterostrophus MAT* genes to *B. sacchari* does not introduce selfing or crossing ability with other *B. sacchari* strains, but transformants can initiate (but not complete) sexual development with *C. heterostrophus*. The asexual nature of *B. sacchari* therefore lies in the mutation of some non-mating-type gene(s), and there is a potential for an asexual fungus to gain sexual development. A mating type-like locus with functional mat genes has also been reported in another asexual fungus, *Candida albicans* (Hull & Johnson, 1999).

Although transformation of an asexual fungus to a sexual fungus has not been achieved, the conversion of a heterothallic *C. heterostrophus MAT*-deletion strain into a self-fertile homothallic strain has been achieved by the expression of a natural MAT-1/MAT-2 fusion gene from the homothallic *C. luttrellii* (Yun *et al.*, 1999). This conversion plus the analysis of the recombination points in the fusion sequence provides evidence that a homothallic species may arise from an uneven crossover in a heterothallic species. This gives support to the speculation that heterothallic species are ancestral to homothallic species and that a single recombination event between the two mating types can lead to this transition (Yun *et al.*, 1999).

1.3.6 *Sordaria macrospora*

S. macrospora (a pyrenomycete) is closely related to *N. crassa* and *P. anserina*. However, it is a true homothallic species – a single strain of *S. macrospora* containing one type of nucleus can enter the sexual pathway without a partner. The mating type locus of *S. macrospora* contains 4 ORFs, *Smta-1*, *SmtA-1*, *SmtA-2* and *SmtA-3*, which were named according to their homology with the *N. crassa* mating type genes (Pöggeler *et al.*, 1997). These

mating type genes are present in a single-copy and are transcriptionally expressed. In contrast, the presence of all four mating type genes is not demonstrated in other homothallic Sordariaceae (Glass *et al.*, 1988; Cisar *et al.*, 1994; Glass *et al.*, 1994; Beatty *et al.*, 1994; Glass & Smith, 1994). *S. macrospora* mating type proteins have strong sequence similarity to the counterparts found in *N. crassa* (Glass *et al.*, 1990; Ferreira *et al.*, 1996) and *P. anserina* (Debuchy & Coppin, 1992; Debuchy *et al.*, 1993) with the exception of *smta-1* and *smtA-3*. Although a conserved HMG box is found, SMTa-1 lacks 100 amino acids at the C-terminus. The *Smta-3* gene contains sequence similarity to both *N. crassa A* and *a* idiomorphs. In SMTA-3, the HMG box is missing and a high degree of identity with MAT A-3 can only be found in the first 91 amino acids. Functional conservation between *P. anserina* and *S. macrospora* mating-type proteins can be demonstrated by the fact when the entire *Smt* locus was transformed into a *mat+* or *mat-* nucleus, self-mating was observed (Pöggeler *et al.*, 1997). However, the perithecia produced by the transformants do not contain any asci or ascospores. The formation of barren perithecia in the transformants is dependent on the *Smt-a1* and *Smt-A1* genes, since each alone can induce the mating reaction in *mat-* and *mat+* strain respectively; fruiting bodies cannot be detected in either *mat+* or *mat-* strains after transformation of plasmid containing *SmtA-2* and *SmtA-3* only.

Although it is not necessary for a nuclear recognition system to function in *S. macrospora*, the mating nuclei are still differentially recognized (Esser & Straub, 1958; Heslot, 1958). It is not clear how differentiation can be accomplished in a population of a single type of nuclei, but mechanisms involving differential expression of mating-types and/or imprinting through male/female origin have been suggested (Coppin *et al.*, 1997).

1.3.7 *Ustilago maydis*

Ustilago maydis (DC.) Corda (or corn smut fungus) belongs to the basidiomycetes, a class of fungi that include many common mushrooms and plant pathogens, such as smuts and rusts. *U. maydis* is dimorphic: the non-pathogenic, unicellular haploid form and the pathogenic

filamentous dikaryotic form (Banuett, 1992). The haploid, yeast-like sporidia grow vegetatively by budding. When sporidia of opposite mating-type are mixed, the formation of irregularly shaped conjugation tubes is induced. The conjugation tubes then fuse and result in the formation of a dikaryon, which is characterized by its long regularly shaped filament. The growth of the pathogenic dikaryon is strictly dependent on the corn plant. The hyphal cells grow within the plant and cause the formation of tumors. The fungal hyphae differentiate within these tumors, where karyogamy takes place, resulting in the formation of diploid spores, teliospores. Upon release from the tumors, the teliospores are spread by the wind. The teliospores germinate on a plant or on a rich medium and produce a short filament, into which the diploid nucleus migrates and undergoes meiosis. A short septated filament (probasidium) that consists of 4 haploid cells is formed (*c.f.* unicellular basidium in homobasidiomycetes). Each of the haploid cells can produce numerous sporidia by successive budding.

Mating-type locus organization in Ustilago maydis

The life cycle of *U. maydis* is governed by two mating-type loci, *a* and *b* (Bölker *et al.*, 1992; Gillissen *et al.*, 1992; Kämper *et al.*, 1994). For the formation of the pathogenic filamentous dikaryon, the haploid mating partners must have different alleles at both loci. The *a* locus controls fusion events (Trueheart & Herskowitz, 1992) and has two alleles, *a1* and *a2*. The *a* locus encodes two structural components of the pheromone response pathway: a pheromone and a pheromone receptor (Bölker *et al.*, 1992; Snetselaar *et al.*, 1996). Two putative pheromone structural genes found in the *a* locus, *mfa1* (from *a1*) and *mfa2* (from *a2*), encode short polypeptides (of 40 and 38 aa, respectively) which contain a CAAX motif (a motif found in other fungal pheromones; C = cysteine, A = aliphatic amino acid, and X = any amino acid) at the C-terminus. Two other genes that resemble pheromone receptors, *pra1* and *pra2*, are found in *a1* and *a2*, respectively. These two genes encode protein with several putative trans-membrane domains that show similarity to the **a**-factor receptor (STE3) of *S. cerevisiae*. The *a2* locus contains two additional genes, *lga2* and *rga2*; although they have unknown functions, their

expression is activated by the pheromone response pathway (Urban *et al.*, 1996a, b). Expression of all genes from the *a* and *b* loci is enhanced (10 to 50 fold) by the pheromone response pathway through the pheromone response element (PRE, ACAAAGGGA) and the HMG binding protein, PRF1 (Urban 1996b, Hartmann *et al.*, 1996).

The *a* locus, together with the *b* locus, controls filamentous growth of the dikaryon: a diploid strain homozygous for *a* (but heterozygous for *b*) grows yeast-like unless externally supplied pheromones of the opposite mating type are present (Banuett & Herskowitz, 1989; Spellig *et al.*, 1994; Kämper *et al.*, 1994). A model explaining linkage of the *a* locus with filamentous growth is as follow: *U. maydis* sporidia produce a low level of pheromone that is sufficient for mating. Once the pheromone response pathway is activated through receptor binding, *prf1* is induced by a post-transcriptional mechanisms (Hartmann *et al.*, 1999). PRF1 in turn induces production of bE and bW (see below), which form heterodimers and bind to genes that promote dikaryotic filamentous growth and sexual differentiation.

A Gα protein in the cAMP signaling pathway has been isolated: *gpa3* mutants fail to respond to pheromone and are mating-deficient (Regenfelder *et al.*, 1997; Krüger *et al.*, 1998). A gene homologous to yeast MCM1 (see section 1.3.1), *umc1*, is not essential for pathogenic development – it only functions to modulate (increase) the expression of several pheromone-inducible genes (Kruger *et al.*, 1997). Another gene potentially involved in the pheromone response pathway is *fuz7* (Banuett & Herskowitz, 1994; Banuett, 1995). It is a homolog of the *STE7* MAPKK of *S. cerevisiae* and is required for *a*-locus-dependent processes (conjugation tube formation, filament formation, and maintenance of filamentous growth). The signal transduction pathway for pheromone response is apparently conserved between *U. maydis* and *U. hordei* (Bakkeren & Kronstad, 1996), which is a bipolar-mating smut (Bakkeren & Kronstad, 1994; Lee *et al.*, 1999).

Development after cell fusion is controlled by the *b* locus (Kronstad & Leong, 1989, 1990; Schulz *et al.*, 1990). The *b* locus of *U. maydis* is mutliallelic: at least 25 *b* alleles exist in nature, implying that there are at least 300 different *b* combinations that trigger development while 25 combinations do not. Any combination of 2 different alleles triggers development to a similar degree. Heterozygosity at the *b* locus is required for tumor development, sexual development (meiosis and sporulation) and filamentous growth. This can be demonstrated by the observation that a diploid strain with genotype *a1a2b1b1* grows yeast-like and is non-pathogenic, while a diploid with genotype *a1a1b1b2* also grows yeast-like but is able to induce tumors and to form basidiospores. The *b* locus contains two dissimilar genes, *bE* and *bW*, which encode polypeptides of 410 and 626 aa, respectively (Gillissen *et al.*, 1992). Each *b* allele contains such a gene pair, e.g., *b1* allele contains *bE1* and *bW1* while *b2* allele contains *bE2* and *bW2*. Both genes contain a constant and variable region (Kronstad & Leong, 1990). The constant region is highly conserved (>90%) among the *b* alleles. The bE polypeptide contains a HD1 homeodomain (WFINAR) whereas the bW polypeptide contains a HD2 homeodomain (WFQNRR) (Kües & Casselton, 1992a, b). The non-self recognition can be explained by the fact that bE and bW proteins will dimerize (through the N-terminal variable domains) only if they are derived from different alleles (Kämper *et al.*, 1995). In *U. maydis*, both homeodomains are required for function of the bE/bW complex (Schlesinger *et al.*, 1997), whereas in *Schizophyllum commune* (another basidomycete; see section 1.3.8), only one of the paired HD1/HD2 complex encodes a homeodomain essential for *A*α-regulated development (Luo *et al.*, 1994). The active bE/bW complex is likely to act as a repressor of a repressor, since a mutation in *rtf1* bypasses the requirement for heterozygosity at *b* and confers pathogenicity to a mutant haploid strain (Banuett, 1991). Allele specificity of bE and bW has been demonstrated to lie within the N-terminal variable region (Dahl *et al.*, 1991; Yee & Kronstad, 1993, 1998). Interestingly, novel specificity of bE and bW can be created by chimeric construction between two *b* alleles (Yee & Kronstad, 1993, 1998). It is noteworthy that a single point mutation into the variable region of bE3 leads to a change of specificity (Kämper *et al.*, 1994, 1995).

Downstream targets directly regulated by the *b*-locus have not been identified, although a gene that is involved in filament growth and virulence has been characterized (Giasson & Kronstad, 1995; Laity *et al.*, 1995). The cAMP signaling pathway responsible for budding/filamentous growth regulation is apparently linked with the mating MAPK pathway, since mutants defective in the cAMP pathway are attenuated for the mating and/or virulence function (Gold *et al.*, 1994, 1997; Dürrenberger *et al.*, 1998; Dürrenberger & Kronstad, 1999). The two pathways share the same Gα protein (Regenfelder *et al.*, 1997; Kahmann & Basse, 1997).

1.3.8 *Coprinus cinereus and Schizophyllum commune*

Coprinus cinereus (Schaeffer:Fr.) Gray has two distinct mycelial stages: the uninucleate monokaryon with abundant oidia (uninucleate asexual spore) and the binucleate dikaryon with characteristic clamp connections between each cell (Casselton & Kües, 1994). Two multiallelic mating-type factors, *A* and *B*, determine mating compatibility in *C. cinereus*. *A* and *B* independently regulate different developmental steps. Following fusion between mating-compatible hyphal cells, there is a reciprocal exchange of nuclei (Casselton, 1978). Migration of the donor nucleus triggers the breakdown of the dolipore (septium), a structure which normally precludes passage of nuclei (Giesy & Day, 1965). Once the tip cells have two different nuclei, a clamp cell develops on the side of the tip cell, one nucleus moves into this, then both nuclei divide synchronously. New septa are laid down, cutting one nucleus off in the clamp cell and one in the subterminal cell. The clamp cell then fuses with the subterminal cell and its nucleus travels into this cell. Given the right temperature and light condition the dikaryon will differentiate into mushroom fruiting bodies, in which nuclear fusion, meiosis and sporulation take place (Tymon *et al.*, 1992). The *A* factor represses asexual sporulation and controls the formation of the clamp cell, nuclear pairing and synchronized nuclear divisions (Raper, 1966; Tymon *et al.*, 1992; Kertesz-Chaloupkova *et al.*, 1998; Kües *et al.*, 1998), whereas the *B* factor controls clamp cell fusion and all the nuclear migration steps (Raper, 1966; O'Shea *et al.*, 1998). Both *A* and *B* factors are required for maintenance of the dikaryon.

The *A* genes *C. cinereus* are divided into two functionally redundant subcomplexes, α and β, which are separated from each other by a 7.0 kb-long "homologous hole" (a non-coding region presents in all *A* factors) (Kües *et al.*, 1992; Pardo *et al.*, 1996; Luken *et al.*, 1996). Since difference in one subcomplex is sufficient for mating, the estimated number of *A* specificities are around 160 (Whitehouse, 1949; Raper, 1966). Three types of genes can be found within the *A* factor. The α-*fg* (encodes a metallo-endopeptidase) and β-*fg* (unknown function) genes are not involved in the *A* specificity. The rest are the *A*-specificity gene pairs of homeodomain proteins (*a1/a2* in *Aα*; *b1/b2* and *d1/d2* in *Aβ*; Pardo *et al.*, 1996). The gene pairs are redundant in function and a difference in the alleles of one gene pair, e.g., *a1-2* and *a2-1* (the "1" in *a1-2* means it contains a HD1 domain while the "2" in *a1-2* indicates that it is allele 2 of the *a* gene pair; similarly, *a2-1* is allele 1 of the HD2-containing *a2* gene), is sufficient to generate compatible *A* factors. The situation here is similar to that of *U. maydis* where a compatible interaction is triggered by a non-allelic HD1-HD2 protein interaction (Kües *et al.*, 1994a, c). The requirement for HD1/HD2 heterodimerization for *A* compatibility is shown in a self-compatible mutant (constitutive for clamp cell development), in which most of the *A* locus has been deleted, giving a fused HD2/HD1 chimeric protein which acts as a heterodimer (Kües *et al.*, 1994b). Because the specificity gene pairs are redundant, it is common to find that some are missing (Casselton & Kües, 1994; Casselton & Olesnicky, 1998). However, the specificity sequences (such as *b1* alleles) of the *A* locus are under tight balancing selection for allele variability (May *et al.*, 1999). Phylogenetic studies show that the N-terminal domain exhibits hypervariability (as required for non-self recognition) while recombination events have homogenized the C-terminal region to preserve functional domains (Badrane & May, 1999). These data explained how *b1* alleles have evolved high levels of variation while at the same time conserved function.

Two coiled-coil dimerization domains are found in HD1 proteins (Gieser & May, 1994; Banham *et al.*, 1995). The fact that the relative positions of the dimerization domains are

different among *a*, *b* and *d* HD proteins gives an explanation why proteins from different sub-families do not cross-react (Padro *et al.*, 1996). The dimerization of HD1/HD2 proteins is important because the HD1 protein provides the nuclear localization and activation domain while HD2 protein provides the homeodomain required for DNA-binding (Asante-Owusu *et al.*, 1996; Spit *et al.*, 1998).

The *B* mating type (required for nuclear migration during somatic and clamp cell fusion) of *C. cinereus* is defined by a single locus, with three sets of functionally redundant, yet independent sub-family of genes (O'Shea *et al.*, 1998). Each sub-set contains a 7-transmembrane helix receptor gene (*rcb*) and two CAAX-modified pheromone precursor genes (*phb*). Like the *A* locus, only one subfamily within the *B* locus has to be different for mating compatibility. *C. cinereus* pheromones genes from a particular sub-family that can only react with receptors from a different allele of the same sub-family. The mechanism for such pheromone-recognition discrimination is still unknown, although the secondary and tertiary structures of the receptors may play a role in the recognition process (Casselton & Olesnicky, 1998). What is known is that the receptor protein is G-protein-coupled; a single amino acid change in the transmembrane domain VI of one receptor is constitutively activating and confers a self-compatible mating phenotype (Olesnicky *et al.*, 1999). So like *U. maydis*, a pheromone response pathway is required for G-protein-mediated signal transduction. However, unlike *U. maydis*, mating-type gene expression is not dependent on pheromone signaling (Richardson *et al.*, 1993). Although pheromone signaling is also not required for mate attraction (c.f. *U. maydis*; Snetselaar *et al.*, 1996), *B* mating-type genes are required for clamp cell fusion, a process mimicking fusion of haploid sporidia (Casselton & Olesnicky, 1998). Components downstream of the mating cascade remain to be identified in *C. cinereus*, however, an HMG-box encoding gene affecting sexual morphogenesis (which is regulated by both *A* and *B* genes), *pcc1*, has been characterized (Murata *et al.*, 1998).

The *A* mating-type system of *Schizophyllum commune* Fr. is similar to that of *C. cinereus* (Casselton & Kües, 1994) except that the $A\alpha$ and $A\beta$ complex is further apart (8 map units; Novotny *et al.*, 1991). Analysis of 114 isolates identified 96 *A* alleles and 56 *B* alleles (Raper *et al.*, 1958). Genes required for fruiting are regulated by the *A* and *B* factors (Wessels, 1992; Schuren *et al.*, 1993). Two genes encoding homeodomain proteins, *y* and *z*, are found in the $A\alpha$ locus (Stankis *et al.*, 1992; Specht *e al.*, 1992). Active Y-Z heterodimers can only be formed between alleles from different mating-types (Magae *et al.*, 1995), which in turns act as a transcriptional factor for $A\alpha$-regulated developmental genes (with the Y homeobox as the active motif; Luo *et al.*, 1994). The $A\beta$ locus, which is functionally redundant to the $A\alpha$ locus, is partially characterized and encodes a gene product (V) with a homeodomain similar to those found in Y and Z (Shen *et al.*, 1996).

The *B* mating-type genes are defined by two discrete loci, $B\alpha$ and $B\beta$ (Parag & Koltin, 1971; Specht, 1995). The two loci contain independent but redundant genes (recombination between two loci does not lead to self-compatibility). The $B\alpha$ and $B\beta$ loci (Wendland *et al.*, 1995; Vaillancourt *et al.*, 1997) contains independent subfamilies of gene cassettes, with one receptor (*bar* or *bbr*) and three pheromone genes (*bap(1-3)* or *bbp(1-3)*). The pheromone genes contain farnesylation signal CAAX as found in yeast **a**-factor. The pheromone receptors have sequence similarity to other pheromone receptors such as *STE3* from yeast and *pra1/pra2* from *U. maydis*. A single pheromone can activate more than one receptor and one receptor can recognize more than one pheromone (Wendland *et al.*, 1995). Nine $B\alpha$ and nine $B\beta$ alleles have been found in nature and eight of the pheromone genes have been analyzed (Kothe, 1999; Hegner *et al.*, 1999). With nine $A\alpha$ and 32 $A\beta$ alleles, 23,328 different mating-types are possible (Kothe, 1999). The pheromone/receptor system may give rise to nuclear identity (by nuclear-limited expression) that facilitates the process of dikaryon proliferation and induction/ continuation of nuclear migration throughout sexual development (Schuurs *et al.*, 1999;

Raudaskoski, 1998). A gene that is controlled by the *B*-regulated pheromone response system and another gene induced by dikaryon proliferation have been isolated (Lengeler & Kothe, 1999a, b). The pheromone response pathway of *S. commune* is similar to those from yeast, since the pheromones/receptors of *Schizophyllum* can induce the yeast pheromone response pathway through the yeast G protein (Fowler *et al.*, 1999).

1.3.9 Other filamentous ascomycetes

Magnaporthe grisea

The rice blast pyrenomycete *Magnaporthe grisea* contains idiomorphs of 2.5 kb (*MAT1-1*) and 3.5 kb (*MAT1-2*). A *MAT1-1* clone was isolated by subtractive hybridization. The introduction of the *MAT1-1* clone into *MAT1-2* recipients results in self- and dual-maters (Kang *et al.*, 1994). Dual-maters containing both mating-type genes are unstable and dual-mating ability is easily lost through a cross or even vegetative growth. *M. grisea* field isolates are poor maters (generally female-sterile and poorly male-fertile) (Valent *et al.*, 1986), suggesting that mating is not important in pathogenesis. However, mating behaviour has been linked with appressorium (infection structure) by G-proteins (Liu & Dean, 1997). Why *M. grisea* mate poorly in the field is still unknown, but it is unlikely to be due to a limitation in pheromone precursor expression (Shen *et al.*, 1999).

Pyrenopeziza brassicae

In the *mat A*-type idiomorph from heterothallic discomycete *Pyrenopeziza brassicae*, a metallothionein-like protein is encoded by the mating-type locus in addition to *mat A-1* and *mat A-3*-like proteins (metallothioneins sequester metal and may function in oxygen detoxification and sulphur metabolism); a *mat A-2* homolog is not present (Singh & Ashby, 1998). It is not clear how a metal-sequestering protein may be involved in mating, but it was suggested that the metallothionein could be an environmental sensor for metal ion concentration in plant and subsequently trigger sexual morphogenesis. Alternatively, it could provide a nuclear recognition

mechanism since it is only found in *MAT 1-2* and metallothionein mRNAs are shown to localize in the perinuclear cytoplasm and cytoskeletal-bound polysomes (cited in Singh & Ashby, 1998). PCR and hybridization data showed intra-specific conservation of mating-type genes (including the metallothionein gene) between *P. brassicae* and closely related *Tapesia yallundae* (Singh *et al.*, 1999).

Glomerella cingulata

Most heterothallic ascomycetes contain a single mating-type locus with two alternate alleles (so-called "bipolar or biallelic mating system"). By contrast, most basidiomycetes have a "tetrapolar mating system" in which multiple alleles exist for each of two mating-type loci (Casselton & Olesnicky, 1998). Multiple mating-types in filamentous ascomycetes is only reported in the pyrenomycete *Glomerella cingulata*, where there are at least three mating-type groups (*MATA-1* to *MATA-3*) that are self-sterile but cross-fertile (Cisar & TeBeest, 1999).

Chromocrea spinulosa

Unlike yeast (Haber, 1998), mating-type in filamentous ascomycetes is believed to be stable and mating type switching (transposition of a silent cassette of mating-type alleles into the active mating type locus) does not happen frequently. However, evidence for unidirectional switching of mating-type has been reported in filamentous ascomycetes (Perkins, 1987), such as *Chromocrea spinulosa* (Mathieson, 1952), *Sclerotinia trifolium* (Uhm & Fujii, 1983), *Botrytinia fuckeliana* (Faretra & Pollastro, 1996), *Ceratocystis coerulescens* (Harrington & McNew, 1997) and *Glomerella cingulata* (Wheeler, 1950). *C. spinulosa* is apparently self-fertile, but in its asci, four progeny are self-fertile and four are self-sterile. The self-fertile progeny always produce four self-sterile and four self-fertile progeny; the self-sterile isolates will cross with self-fertile isolates. The self-fertile progeny contain sequences for *MAT-1* (α-domain protein) and *MAT-2* (HMG protein) homologs, but the self-sterile progeny contain sequences only for MAT-1 protein (G. Turgeon, personal communication). How a single culture produces

progeny with different *mat* gene organization is unclear, but a programmed genomic rearrangement comparable to mating type switching in yeast is possible.

1.4 HETEROKARYON INCOMPATIBILITY IN FUNGI

1.4.1 Introduction to heterokaryon incompatibility

When a spore of a filamentous fungus germinates, hyphae (filament-shaped multinucleate cells) grow by hyphal tip extension and branching. Hyphal tip fusions are continuously formed (a process called anastomosis), giving a network of hyphae called a mycelium, in which the fungus grows and propagates. Occasionally, when two fungal individuals meet, they may undergo hyphal fusion and form a heterokaryon (an individual that contains genetically distinct nuclei within the same cytoplasm). However, a viable, stable heterokaryon between two isolates can only be formed if they contain compatible alleles at genetic loci that restrict heterokaryon viability. These loci are called *het* (*het*erokaryon) or *vic* (*v*egetative *i*ncompatiblity) loci. Genetic differences at any of these *het* loci can result in inhibited growth of the heterokaryotic cells and compartmentalized cell death by a lytic process (Garnjobst & Wilson, 1956). This growth inhibition/cell death phenomenon is termed heterokaryon incompatibility (and sometimes referred to as heterogenic, vegetative, or somatic incompatibility). In ascomycetes such as *N. crassa*, heterokaryon incompatibility is strictly a vegetative feature; differences in one or more *het* loci do not result in an incompatibility reaction between mating nuclei during the sexual cycle and do not impair fertility (Bégueret *et al.*, 1994).

Heterokaryon incompatibility is thought to be a universal phenomenon among filamentous ascomycetes and basidiomycetes (reviewed by Glass & Kuldau, 1992; Leslie, 1993; Esser & Blaich, 1994; Bégueret *et al.*, 1994; Worrall, 1997; Glass *et al.*, 2000). In *N. crassa*, heterokaryon formation by hyphal fusion is virtually excluded in nature by the action of *het* genes (Mylyk, 1976a; Perkins & Turner, 1988; Pandit & Maheshwari, 1996). The phenomenon

of heterokaryon incompatibility is thought to limit the spread of deleterious organelles and infectious elements in fungal populations (Caten, 1972). Experimental data supports this theory - cytoplasmic transfer of virus and detrimental plasmids within fungal populations is reduced or completely blocked by *het* actions (Anagnostakis, 1982a; Debets *et al.*, 1994; Van Diepeningen *et al.*, 1997, 1998). Heterokaryon incompatibility may provide a selective advantage for favorable mutations by preventing exploitation of heterokaryons by non-adaptive nuclei (Hartl *et al.*, 1975), and by protecting unfertilized cultures from looting of maternal resources during sexual reproduction (Debets & Griffiths, 1998). Others have proposed that the presence of *het* genes would limit out-breeding and thus favoring evolution of a single group leading to speciation (Esser & Blaich, 1973, 1994).

The phenomenon of heterokaryon incompatibility can be demonstrated in several ways. For example, (i) the failure for two strains to form a stable heterokaryon with continuous growth (Garnjobst, 1953), (ii) microscopic (light and electronic) features of protoplasmic killing (Garnjobst & Wilson, 1956; Jacobson *et al.*, 1998) and (iii) the formation of a barrage at the point of confrontation of incompatible strains (Reinhardt, 1892). The phenotype of the barrage varies between different fungal species, from a pigmented line of dying hyphae to a clear zone of self-lysis (Esser & Blaich, 1994). Heterokaryon incompatibility loci can be identified using forced heterokaryon formation between near isogenic strains (Garnjobst & Wilson, 1956; Pittinger & Browner, 1961). Alternatively, they can be identified by abnormal growth and morphology of fungal strains with partial diploid heterozygous for a *het* gene (Newmeyer & Taylor, 1967). Most of the *het* loci in *Neurospora* have been identified using partial aneuploids or disomic methods, in which progeny with partial diploids are generated by crossing a translocation strain with a wild-type strain (Perkins, 1975; Mylyk, 1975).

Genetic studies in several ascomycetes show that there are a number of *het* loci in each species, for examples, there are at least eleven in *N. crassa*, nine in *P. anserina*, eight *in*

Aspergillus nidulans, and six in *Cryphonectria parasitica* (Bégueret *et al.*, 1994). Several *het* genes and suppressors of incompatibility have been cloned from *N. crassa* and *P. anserina*, while heterokaryon incompatibility in *A. nidulans* (Dales & Croft, 1990; Anwar *et al.*, 1993) and in *C. parasitica* (Cortesi & Milgroom, 1998; Milgroom & Cortesi, 1999) has been only studied genetically. In this chapter, the characteristics and nature of *het* loci and their suppressors/ modifier genes in *N. crassa* and *P. anserina* are discussed.

1.4.2 *N. crassa mat* locus

(The mating function of the *N. crassa mat* locus is summarized in section 1.3.3)

Entry into the sexual cycle in filamentous ascomycetes requires the fusion of reproductive structures of opposite mating-types. The *mat A* (*A-1*, *A-2* and *A-3*) and *mat a* gene (*a-1*) genes regulate fusion and post-fertilization events during sexual development (see section 1.3 for review of mating-type). In *N. crassa* (but not in all filamentous ascomycetes), the *mat* locus also functions as a *het* locus (Beadle & Coonradt, 1944; Garnjobst, 1953). The fusion of *mat A* and *mat a* hyphae during vegetative growth results in growth inhibition, hyphal compartmentation and death (Glass & Kuldau, 1992); the mating-type incompatible heterokaryon is aconidiating, slow-growing (10-20 fold slower than a compatible heterokaryon) and contains compartmentalized cell death events similar to those observed in *het-c* and *het-6* incompatibility reactions (see section 1.4.3). In most cases, the incompatibility function of *mat* was found to be inseparable from its sexual function, indicating that either the two functions are conferred by the same gene, or they are encoded by separate genes that are closely linked (Newmeyer *et al.*, 1973; Griffiths, 1982). An exceptional *a* mutant, a^{m33}, is fertile but heterokaryon compatible with *A* strains, suggesting the mating and incompatibility functions are separable (Griffiths & DeLange, 1978). The mating-type associated heterokaryon incompatibility is due to the molecular action of MAT A-1 and MAT a-1, since mutations that affect both mating and incompatibility functions are mapped in *mat A-1* and *mat a-1* genes (Glass *et al.*, 1990; Staben & Yanofsky, 1990). Moreover, introduction of *mat A-1* into *a* recipients (or the introduction of *mat a-1* into *A*

recipients) is sufficient to trigger heterokaryon incompatibility (Glass *et al.*, 1990; Staben & Yanofsky, 1990). Additional *mat A-1* and *mat a-1* mutants that separate mating and heterokaryon incompatibility have been obtained, indicating that the two functions lie in separate functional domains of the protein and are not always mutually inclusive (Staben & Yanofsky, 1990; Philley & Staben, 1994; Saupe *et al.*, 1996b).

Partial diploid strains that contain a duplication for mating type show inhibited growth initially (a phenotype called Dark Agar; Newmeyer, 1968) and escape to a wild-type growth rate after a few days; duplication of mating-type can be obtained by crosses heterozygous for inversion *In(ILR)H4250* (Newmeyer & Taylor, 1967). The escape strains usually contain a mutation in either the *A* or *a* locus. However, a mutation unlinked to the mating-type locus, *tol* (*tol*erant), suppresses mating-type associated heterokaryon incompatibility (Newmeyer, 1970). Attempts to isolate additional suppressors of *mat*-mediated incompatibility results only in the isolation of additional *tol* alleles (Vellani *et al.*, 1994).

Mutations in *tol* are recessive such that *tol A* and *tol a* strains form a vigorous heterokaryon (DeLange & Griffiths, 1975). *tol* maps close to *trp-4* in linkage group IV and does not suppress other heterokaryon incompatibility systems (Newmeyer, 1970; Leslie & Yamashiro, 1997). Other than the ability to suppress *mat*-mediated incompatibility, *tol* mutants show a wild-type phenotype during vegetative growth. Mutation in *tol* does not affect mating, however, the *tol* mutation (when homozygous) has been shown to restore fertility in the *fmf-1* (*f*emale and *m*ale *f*ertility) mutant (Johnson, 1979), suggesting *tol* may play a role during sexual cycle.

Why MAT A-1 and MAT a-1 exhibit such diametrically opposed functions in the sexual and vegetative phases in *N. crassa* and their relationship to each other is still an unsolved enigma of mating type function. The focus of this thesis is to study the heterokaryon incompatibility as

mediated by *mat*. Extensive reviews on mating-type-associated incompatibility are provided in later chapters.

1.4.3 *N. crassa het-c* locus

The *het-c* locus is the best-studied *het* locus in *N. crassa*. The *het-c* locus was first identified in forced heterokaryons of near isogenic strains that differed in *het-c* (Garnjobst, 1953) and later characterized using translocation strains duplicated at *het-c* locus (Perkins, 1975). Although not as strong as the *het-6* and *mat* incompatibility reactions, the incompatibile *het-c* heterokaryon displays a distinctive phenotype: aconidiating, slow-growing mycelium with curly hyphae. The *het-c*^{OR} allele was isolated by a chromosome walk from a closely linked gene, *pyr-4* (Saupe *et al.*, 1996a). The *het-c*^{OR} activity was localized in overlapping regions of two cosmids: introduction of either cosmid into a *het-c*^{PA} strain led to growth-inhibited transformants resembling *het-c* incompatible heterokaryons or partial diploids.

The 966-amino acid HET-C^{OR} polypeptide contains a putative signal peptide, a coiled coil domain and a C-terminal glycine-rich domain (Saupe *et al.*, 1996a). The presence of sequences similar to a signal peptide suggests that HET-C might enter the secretory pathway. The 5-heptad coiled-coil domain could provide a dimerization motif for protein-protein interactions. Glycine-rich domains are found in a large number of proteins such as extracellular or cell envelope proteins, RNA-binding proteins and keratin. These domains are glycine and serine-rich and contain regularly spaced aromatic residues that are proposed to allow tension-adaptable protein-protein interactions that provide extensibility and elasticity (Steinert *et al.*, 1990). Several *het-c* mutants have been obtained via RIP (repeat-induced point) mutation (Saupe *et al.*, 1996a). They no longer mediate *het-c* vegetative incompatibility (i.e., they are heterokaryon compatible with all *het-c* alleles) but are otherwise normal in vegetative growth and sexual reproduction.

Genetic and molecular characterization of the *het-c* gene from two other *N. crassa* strains (*het-c*^{PA} and *het-c*^{GR}) demonstrated that the *het-c* locus is functionally multi-allelic (Saupe & Glass, 1997). Transformation results agree with previous genetic data that *het-c* incompatibility is mediated by at least three alleles (Howlett *et al.*, 1993). Multi-allelism is rare in allelic incompatibility systems and has only been reported in one other case (Dales *et al.*, 1993). DNA sequence analysis of the three *het-c* alleles revealed a highly variable domain of 34-48 amino acids (position 247 to 284 in HET-c^{OR}; Saupe & Glass, 1997). This highly variable domain, which includes substitutions and insertion/deletions (including a 5-aa HET-c^{OR}-specific and a 15-aa HET-c^{PA}-specific insertion), determines *het-c* allelic specificity. Swapping of this highly variable specificity domain effectively changes the specificity of a *het-c* construct as tested by transformation assays (Saupe & Glass, 1997). Recent experiments on artificial *het-c* constructs show that novel specificity can be obtained by changing deletion/insertion pattern of the variable domain (J. Wu & N.L. Glass, unpublished results). These data show that the spacing (and not so much amino-acids composition) within the *het-c* variable domain is crucial in determining *het-c* specificity.

Evidence for balancing selection at the *het-c* locus was shown by studies of the evolutionary pattern of *het-c* in related species and genera to *N. crassa* (Wu *et al.*, 1998b). The HET-c specificity region of 40 different isolates, representing 11 different species and three genera within the Sordariaceae (Neurospora, Gelasinospora and Sordaria), was shown to exhibit trans-species polymorphisms, i.e. DNA sequences of the *het-c* specificity region fell into groups by *het-c* allelic type rather than according to species. These data show that allelic specificities at *het-c* were generated in an ancestral species and have been maintained over 35 million years and during multiple speciation events. Moreover, the specificity domain of *het-c* also exhibits an excess of nonsynonymous substitution over synonymous substitutions, suggesting a selection to maintain diversity at this locus (Wu *et al.*, 1998b). The *het-c* locus thus shares the evolutionary characteristics with other non-self recognition systems such as the MHC (major

histocompatibility complex; Ayala *et al.*, 1994) loci in mammals and the S (self-incompatibility; Ioerger *et al.*, 1990) locus in plants. The *het-c* locus was found to be polymorphic with all three alleles showing near equal frequency in a population isolated from a 5-hectare sugarcane field in Louisiana (Wu *et al.*, 1998b). These data suggest that the non-self recognition function mediated by difference at *het-c* is of biological significance in *N. crassa.*

Early studies by Wilson and co-workers (1961) showed that micro-injection of cytoplasm from a donor cell can kill a recipient of an incompatible *het-c* type. The killing factor in the cytoplasmic extracts can be inactivated by heat (60°C) or proteases (but not by nucleases), suggesting the incompatibility factor involved was a protein (Wilson *et al.*, 1961; Williams & Wilson, 1966). The incompatible component transferred is likely to be HET-C itself. Recent co-immunoprecipitation experiments using GFP (green fluorescent protein) and HA (hemagglutinin) tagging show that HET-c^{OR} and HET-c^{PA} can be co-precipitated while homodimerization of HET-c^{OR} was not detected (J. Wu & N.L. Glass, unpublished results). These data showed that molecular recognition mediated by *het-c* is due to dimerization of unlike HET-C proteins.

Attempts to isolate suppressors for *het-c* incompatibility has been made by Arganoza and co-workers (1994). However, the spontaneous and insertional inactivation mutants that tolerate *het-c* difference have not been further investigated. Another suppressor (*vib-1*) of *het-c* heterokaryon incompatibility has been isolated (Q. Xiang & N.L. Glass, unpublished results). Other than the ability to tolerate *het-c* differences, the phenotype of *vib-1* (*v*egetative *i*ncompatibility *b*locked) include poor aerial hyphae growth, female-sterility and hyphal fusion defects.

Microscopic and ultrastructural features associated with *het-c* (and *het-6*) incompatibility have been examined (Jacobson *et al.*, 1998). Morphological features of partial diploids

heterozygous at the *het-c* locus, as revealed by transmission electron microscopy, include nucleolar release, cell shrinkage, extensive organelle degradation and plasmolysis. These ultrastructural changes in dying cells are similar to the features of apoptosis (programmed cell death; Konopleva *et al.*, 1999) in higher eukaryotes. DNA fragmentation, a hallmark feature of early apoptosis as mediated by Ca^{2+}-dependent endoclease cleavage of nucleosomes, is also observed by TUNEL (terminal deoxyribonucleotidyl transferase) assay on *het-c*-incompatible hyphal fusion cells (R. Bostock & S. Marek, unpublished results). These microscopic and biochemical experiments suggest that fungal vegetative incompatibility and apoptosis in plants and animals may share common biochemical and genetic mechanisms. It is noteworthy that an apoptosis-like form of programmed cell death has been reported in filamentous fungi (Roze & Linz, 1998).

Homologs of *het-c* have been found in *P. anserina* and *A. nidulans*. The *Podospora* het-c homolog (*hch*) has high sequence similarity to *N. crassa het-c*, although the specificity region is divergent (Saupe *et al.*, 2000). Population studies show that *hch* is apparently non-polymorphic in *P. anserina* and therefore may not act as a *het* locus. It is possible that the occurrence of *het-c* polymorphism and the acquisition of *het-c* as a *het* locus occurred after speciation between *Podospora* and *Neurospora*, 75 millions years ago. However, *hch* appears to be potentially active and could act as a *het* gene in *P. anserina* if polymorphism exists – heterologous expression of any of the three *N. crassa het-c* alleles in *P. anserina* led to growth defect and cell death that mirrored *N. crassa het-c* incompatibility (Saupe *et al.*, 2000). The *Aspergillus het-c* homolog (*nipC*) was isolated from a yeast 2-hybrid screen using *nim-A* as bait. NIMA (*n*ever *i*n *m*eiosis) is a cell cycle regulator that is responsible for the transition from interphase to prophase (G2-M) (Osmani *et al.*, 1988). NIMA, a SER/THR protein kinase, is proposed to target G2/M checkpoint controls by promoting nuclear localization of CDC1/cyclin B (Wu *et al.*, 1998c). Over-expression of NIPC causes cell cycle arrest at interphase, suggesting that the *het-c* homolog may act as a negative regulator of cell cycle (S. Osmani, unpublished results). It is not clear if

nipC is polymorphic in nature and acts as a *het* gene in *A. nidulans*. Polymorphism is found at *het-c* in a pseudohomothallic species of *Neurospora*, *N. tetrasperma* (Powell *et al.*, 2000). These data suggested the self-fertile fungal species, thought to be non-outcrossing in nature, does outcross. The fact that diversity is found at *het-c* locus while other genes are highly homozygous (Merino *et al.*, 1996) illustrates that non-self recognition may also be selected for in a fungal species that is effectively homothallic (Powell *et al.*, 2000).

1.4.4 *N. crassa het-6* locus

het-6 was first identified by partial diploid analysis – strains carrying partial diploid at the *het-6* locus are inhibited in their growth (Mylyk, 1975). The region of translocated (duplicated) DNA, *T(IIL→IIIR)AR18* defines the *het-6* locus. The *het-6* locus was later mapped to a 35 kb region within the translocated fragment, which is the amount of deletion at one of the *het-6* loci required for the partial diploid strain to "escape" from the incompatible phenotype (Smith *et al.*, 1996). The incompatibility phenotype of *het-6* is among the most severe – the heterokaryon has a 100-fold reduced growth rate (Perkins, 1975; Jacobson *et al.*, 1998).

Cosmids spanning the *het-6* translocation region were isolated by probing the genomic library with LG II DNA from wild type and *T(IIL→IIIR)AR18* DNA (Smith & Glass, 1996; Smith *et al.*, 1996). Four of the six cosmids isolated by this method were used to initiate a chromosome walk, resulting in a near-contiguous segment across the *het-6* translocated region (Smith *et al.*, 1996). Two of the cosmids gave a dramatically reduced number of transformants (20 fold less) when introduced into strains that have a different *het-6* haplotype, suggesting that *het-6* function is within the overlapping region (Smith *et al.*, 2000b). Two genes (14 kb apart) identified within this region, named *het-6* and *un-24*, exhibited incompatibility activity as tested by the transformation reduction assay and were further characterized (Smith *et al.*, 2000b).

The *un-24* gene encodes the large subunit of ribonucleotide reductase (RNR) (Smith *et al.*, 2000a). The class I ribonuclease reductase is essential for de novo synthesis of DNA, replacing the C-2' hydroxyl of ribonucleoside diphosphate (NDP) with a hydrogen to give deoxyribonucleoside diphosphate (dNDP) (Jordan & Reichard, 1998). A temperature-sensitive mutant (*un-24*^{TS}) grows at a wild-type growth rate below 30°C but does not grow when the temperature exceeds 34°C (Smith *et al.*, 2000a). The *un-24*^{TS} allele has three GC→AT transition mutations, one of which changes a conserved amino-acid (C26→Y) located within the allosteric activity site where ATP (activator) or dATP (inhibitor) binds. The mutations in *un-24*^{TS}, which may affect proper folding of the allosteric activity site at elevated temperature, can be suppressed by osmotic remediation (which reduce water content and change the conformation of enzymes). Ribonucleotide reductase activity is apparently separable from incompatibility activity within *un-24*. When DNA from the *un-24*^{TS} allele (derived from wild-type OR background) is transformed into spheroplasts of alternate haplotype (PA background), a 20-fold reduction in transformation frequency is observed even at restrictive temperature (37°C) (Smith *et al.*, 2000b). A C-terminal domain, which is variable between UN-24^{PA} and UN-24^{OR} and does not exist in other RNRs, may be responsible for the incompatibility.

The *het-6* gene encodes a 680-aa polypeptide (Smith *et al.*, 2000b). Three regions of sequence similarity are found between HET-6^{OR} and HET-E, which mediates non-allelic incompatibility (with HET-C) in *P. anserina* (Saupe *et al.*, 1995a). The three regions of similarity are distinct from the GTP-binding domain and the β-transducin-like repeats in HET-E and may thus represent a novel incompatibility domain. The alternative *het-6* allele encodes a protein (HET-6^{PA}) that is only 68% identical to HET-6^{OR} with polymorphic positions scattered throughout the ORFs.

Transformation results suggest that *un-24* and *het-6* genes do not act as allelic incompatibility loci. Although a transformation reduction is observed when *un-24*^{OR} and *het-6*^{OR}

DNA were introduced into PA recipients, no incompatibility reaction is triggered when *un-24*^{PA} or *het-6*^{PA} alleles are transformed into OR spheroplasts (Smith *et al.*, 2000b). This result suggests that there are additional factor(s) within the *het-6* region (that span both *het-6* and *un-24* genes) that mediate incompatibility and that incompatibility may be of non-allelic nature.

The *het-6* and *un-24* polymorphisms are apparently selected for in nature. In a population study of 40 wild *N. crassa* isolates from Louisiana, OR-like and PA-like alleles at *het-6* and *un-24* were equally frequent (Mir-Rashed *et al.*, 2000). This result suggests that, like *het-c*, the *het-6/un-24*-mediated non-self recognition may also be subject to balancing selection. Interestingly, all the Louisiana isolates are of either *het-6*^{OR} *un-24*^{OR} or *het-6*^{PA} *un-24*^{PA} haplotype (while all combinations of *het-6* and *het-c* were found); the *het-6*^{OR} *un-24*^{PA} or the *het-6*^{PA} *un-24*^{OR} haplotype cannot be identified in 126 wild isolates (Smith *et al.*, 2000b). These data indicate that the *het-6-un-24* region is inherited as a block. The recombination block in probably due to lack of recombination between *het-6* and *un-24*, since a low level of DNA similarity is detected in the intergenic region (Mir-Rashed *et al.*, 2000). Examples of recombination block can also be found in other Neurospora regions, such as the spore killer locus (Campbell & Turner, 1987) and the mating-type locus (Gallegos *et al.*, 2000). Taken together, these data suggest that *un-24* and *het-6* act as an incompatibility complex.

1.4.5 *P. anserina het-s* locus

The *het-s/S* system mediates allelic incompatibility in *P. anserina* and is the most studied *het* locus in that species. The incompatible *het-s* and *het-S* alleles originated from geographic strains, s and S (from Normandy, France; Rizet, 1952). The two strains were analyzed for their *het* genotype (Bernet, 1967a) and were backcrossed for 20 generations to give isogenic strains differ only at the *s* locus. Fusion between strains with differences in *het-s* leads to death of heterokaryotic cells, as shown by the formation of a barrage (a dense and unpigmented line of dying cells) in the region where the two isolates meet.

Cloning of *het-s* was made possible by the identification of a natural *het-s* allele, *het-s*^{x}, from strain H collected in Burgundy (Bernet, 1967a). The het-s^x allele is compatible with both *het-S* and *het-s* (later characterization of the *het-s*^{x} allele revealed that it has a duplication of 46 bases, leading to a frameshift mutation and a truncated version of *het-s*; Deleu *et al.*, 1993). The *het-s* gene was cloned by transformation of a s^x strain with DNA from a *s* cosmid library; transformants (from pools of cosmids) were screened for the ability to induce barrage formation when confronted with the S strain (Turcq *et al.*, 1990).

Both *het-S* and *het-s* genes encode novel polypeptides of 289-aa (Turcq *et al.*, 1991). HET-S and HET-s differ by 14 amino acids and are apparently not essential for cell viability. Strains carrying a disrupted copy of the *het-S* or *het-s* (named strains S° and s°) behave like wild type in growth, pigmentation, differentiation and fertility (Turcq *et al.*, 1991). DNA Sequence analysis of 4 natural *het-s* alleles from *P. comata* revealed that only 8 of the 14 polymorphic amino acids strictly correlate with *het-s/S* specificity. Further analysis of *het-S/s* by chimeric constructs and site-directed mutagenesis indicated that a single amino-acid change (H33P) in *het-S* allele can switch its phenotype to *het-s* (Deleu *et al.*, 1993).

There are several lines of evidence indicating that the *het-s* gene product behaves as a prion analog (Coustos *et al.*, 1997). A prion is an abnormal conformational state of a normal cellular protein capable of "infecting" and converting the normal form (PrP^C) into the altered ones (PrP^{Sc}) (Prusiner, 1998). An infectious prion is implicated in a mammalian neurodegenerative disease called spongiform encephalopathies (e.g. mad cow disease). The normal PrP^C protein resides predominantly on the surface of the neuron while the infectious, harmful form of the prion protein PrP^{Sc} is more stable (resistant to protease) and accumulates in the cytoplasm (Horwich & Weissman, 1997). The two forms of the proteins contain the same chemical structure but have alternative secondary structures that may explain their conformational differences. Infectious propagation of protein conformation has also been

reported in fungi (Silar & Daboussi, 1999; Wickner *et al.*, 1999). [URE3] (which derepresses nitrogen catabolism) and [PSI+] (which increases suppression of non-sense mutations) are two non-mendelian yeast elements. They are shown to be the inactive forms of cellular proteins (URE2 and SUP35, respectively) that are capable of converting the normal forms into the inactive forms (Wickner *et al.*, 1995, 1999).

It has been known for decades that a strain carrying the *het-s* allele can express a neutral [Het-s*] phenotype, which is compatible with both *het-s* and *het-S* strains. Such a non-reactive [Het-s*] state (formerly het-s^S) can be found in wild-type isolates, in protoplasts prepared from *het-s* mycelium (Belcour, 1976) or in progeny recovered from a cross between *het-S* and *het-s* strains (Rizet & Schecroun, 1959). Self-crosses from *het-S* and *het-s* strains yield progeny with uniform phenotypes ([Het-S] and [Het-s], respectively). When the [Het-s] phenotype is present in the male (but not in the female), the [Het-s] phenotype is not transmitted; when a *het-s* strain (expressing [Het-s] phenotype) acts as female, all *het-s* progeny have the [Het-s] phenotypes (Beisson-Schecroun, 1962). These data show that other than the *het-s* genetic composition, a cytoplamic determinant found in the [Het-s] strain is crucial for the transmission of the [Het-s] phenotype. Additionally, the [Het-s*] strain can revert to [Het-s] phenotype spontaneously during vegetative growth (at a low rate of under 10^{-7} per nucleus; Beisson-Schecroun, 1962) or through anastomoses (independent of nuclear transmission) with a *het-s* strain (Rizet & Schecroun, 1959; Beisson-Schecroun, 1962). From these results, it was hypothesized that the HET-s protein can induce (and regulate) its own expression (Beisson-Schecroun, 1962). However, modern concepts of an infectious protein capable of catalyzing its own modification led to a different interpretation of the results.

Recent observations on the [Het-s]/[Het-s*] phenomenon suggested that the HET-s protein acts as a prion (Coustos *et al.*, 1997): (i) a functional *het-s* gene is required for expression of [Het-s] phenotype and for the propagation of transformation from [Het-s*] to [Het-s]; (ii)

Over-expression of *het-s* gene led to an elevated frequency of spontaneous [Het-s*] → [Het-s] transition; (iii) Interaction between HET-s/S (S/S, s/s, S/s) proteins (monomers and multimers) were detected; (iv) HET-s and HET-s* proteins were present in similar amounts in the respective strains, with similar electrophoretic mobility under denaturing condition; (v) HET-s is more resistant to proteinase K than HET-s*; (vi) the [Het-s*] → [Het-s] transition is independent of protein translation. All of these properties support the prion model of self-propagating conformational modification.

In an effort to isolate intra- and extragenic suppressor for *het-s/S* incompatibility, a self-incompatible strain containing both antagonistic alleles (brought together by transformation) was UV-mutagenized and escape sectors recovering normal growth rate were isolated (Coustou *et al.*, 1999). Of 44 analyzed sectors, 27 resulted from mutations outside the *het-s/S* locus while 17 were intragenic suppressors (14 affected *het-S* and 3 affected *het-s*). The characterization of the intragenic suppressor mutants plus the additional functional analysis in *het-s* (by deletion constructs and site-directed mutagenesis; Coustou *et al.*, 1999) revealed the amino acid positions in HET-s/S that are crucial in determining reactivity (e.g. HET-s aa 1-112 confers full [Het-s] functionality), allele specificity (e.g. single amino acid changes at several positions lead to altered specificity), converting activity (e.g. HET-s aa1-25 allows prion propagation) and metastability (e.g. the *het-S* K201R mutation leads to a metastable [Het-S*] phenotype).

1.4.6 *P. anserina het-c/e* (*het-c/d*) system

Several loci mediate non-allelic incompatibility in *P. anserina*. In non-allelic incompatibility systems, special combinations at two separate and distinct loci result in incompatibility. In a cross between non-allelic incompatible strains, progeny with the two antagonistic genes are inhibited upon germination and subsequently destroyed in a lytic process (Bernet, 1965; Bernet *et al.*, 1973). The *het-c* locus in *P. anserina* is involved in two non-allelic incompatibility systems, *het-c/e* and *het-c/d* (Bernet 1967c, 1992). Each of these three loci are

multi-allelic: from genetic analysis of 17 wild-type isolates, 4 alleles are found at *het-c* (*c1* to *c4*) and in *het-e* (*e1* to *e4*) while 3 alleles are present in *het-d* (*d1* to *d3*) (Bernet, 1967b). Each *het-c* allele is incompatible with a specific set of *het-d* and/or *het-e* alleles. For example, *het-c2* is incompatible with *het-e1* and *het-d1* while *het-c3* is incompatible only with *het-e2*. The *het-c/d* and *het-c/e* systems could be redundant since HET-E and HET-D polypeptides are very similar (Barreau *et al.*, 1998; J. Bégueret, unpublished results).

The *het-c* gene was cloned by phenotypic expression of *het-c2* allele. Pools of cosmids from a *het-c2* library were introduced to a *het-c3* strain and transformants were screened for incompatibility with a *het-e1* strain in a barrage test (Saupe *et al.*, 1994). The 208-aa HET-C2 polypeptide displays similarity to a glycolipid transfer protein (GLTP) isolated from pig brain, which is responsible for transport of specific glycolipids (such as glycosphingolipids and glyceroglycolipids) from donor to acceptor membrane (Abe, 1990). HET-C2 contains a putative amphipathic α-helix, a functional domain that is involved in protein-protein interaction and lipid binding in some proteins. Disruption of *het-c* by gene replacement generated two mutants with distinct, recessive phenotypes (Saupe *et al.*, 1994). The *het-cΔA* (partially disrupted) strain is colonial, has higher branching of apical hyphae and is more susceptible to growth-inhibitors that target cell-wall biosynthesis (e.g. 2-deoxyglucose). The *het-cΔB* (total-knockout) mutant is not affected in vegetative growth, but is drastically impaired in ascospore production – in a *het-cΔB* x *het-cΔB* cross, over 80% of the perithecia do not contain any mature ascospores while the remaining ones have abortive asci with rare, asynchronously-developing ascospores. The heterogeneity in ascospore size could be an outcome of defects in the distribution of nuclei. The sequence similarity and the mutant phenotypes suggest that HET-C could be involved in maintaining normal membrane composition and vesicle transport.

To delineate the molecular basis of *het-c* allelic specificity, three more wild-type *het-c* alleles and two mutant alleles have been characterized (Saupe *et al.*, 1995b). Sixteen

polymorphic positions (distributed all over the sequence) have been found in the four HET-C wild-type proteins. Protein variability ranged from one (between *c1* and *c3*) to 15 (between *c1* and *c2*) amino acid differences. A single amino acid change at position 133 (between *c1* and *c3*) and 153 (between *c2* and mutant *c2-2*) can effectively change the specificity of *het-c* e.g. *het-c1* is incompatible with *het-e2* and *het-e3* while *het-c3* is incompatible only with *het-e2*. The higher the degree of sequence variation between two alleles, the more differences they display in their specificity. For example, *het-c1* and *het-c2* differ at 15 positions and they react with completely different *het-d* alleles. Some of the chimeric constructs obtained from different *het-c* alleles confer novel specificities. However, hybrid gene analysis did not reveal any limited region of HET-C that is crucial in specificity determination. *P. anserina het-c*, like *het-s* but unlike *N. crassa het-c*, does not have a limited specificity region embedded within conserved domains. However, the *P. anserina het-c* is similar to all the above-mentioned systems, in that it contains a high ratio of nonsynonymous substitution over synonymous substitutions, suggesting a selective pressure to maintain polymorphism.

The *het-e* gene was also cloned by phenotypic expression – pools of cosmids containing the *het-e1*^{A} allele (*het-e1* from strain A) were transformed into a *het-e4* (a neutral, inactive allele) strain and transformants were tested in a barrage test for incompatibility with a *het-c2* strain (Saupe *et al.*, 1995a). The 1356-aa HET-E1^{A} polypeptide contains a GTP-binding domain and a WD40-repeats region; it is the first report of these two domains being present in the same protein. The HET-E1^{A} GTP-binding domain contains the conserved P-loop, G2, G3, G4 motifs, suggesting that it belongs to the GTPase superfamily (Bourne *et al.*, 1991). This GTP-binding domain is functional and is crucial in HET-E reactivity – substitution of a conserved lysine of P-loop (K306S) involved in hydrogen-bonding with phosphates of GTP/GDP can abolish both GTP-binding activity and reactivity in *het-c/e* incompatibility (Saupe *et al.*, 1995a; Espagne *et al.*, 1997).

The WD40 repeat is a protein-protein interaction domain first identified in the β-subunit of transducin (a trimeric G-protein responsible for phototransduction in animal) and is found in all Gβ proteins (and other proteins with various functions) (Neer *et al.*, 1994). Contrary to other members of the WD40 family, the 10 WD40 repeats in HET-E1^{A} show exceptionally high conservation (with over 70% identity compared to the usual 20-30% range). The high degree of sequence conservation may provide a means for unequal crossing over that may lead to polymorphism in the number of repeats in wild-type alleles of *het-e*. It was found that the number of WD40 repeats varies from 3 to 12 in wild-type isolates (Saupe *et al.*, 1995a; Espagne *et al.*, 1997). HET-E specificity cannot be solely dependent upon the number of repeats, since there is no apparent correlation between numbers of repeats and specificity (Saupe *et al.*, 1995b). However, analysis of reactivity of wild-type and UV-mutant alleles demonstrated that the reactive *het-e* alleles contain at least ten WD40 repeats (Espagne *et al.*, 1997). Deletion of one repeat (as shown by UV mutants and *in vitro* deletion constructs) is sufficient to inactivate *het-e* incompatibility reactivity. The requirement of such a high number of WD40 repeats (the normal range is 4 to 8) for reactivity suggests that HET-E may be associated with many different proteins, which may be necessary for correct assembly and function. As HET-E contains functional domains found in both α and β-subunits of trimeric G proteins, it may define a new class of proteins involved in signal transduction. Disruption of *het-e* by gene replacement does not reveal further information about HET-E functionality; a *het-e* knock-out strain behaves as a null incompatibility allele but otherwise displays wild-type phenotype (Espagne *et al.*, 1997). However, the lack of altered phenotype (such as colonial growth and female sterility as found in *het-c* mutants) in the *het-e*-null strain could be due to the presence of *het-e*-related genes found in the genome (Espagne *et al.*, 1997).

1.4.7 *P. anserina idi* genes (*i*nduced *d*uring *i*ncompatibility)

Like the *het-c/d* and *het-c/e* systems, the *P. anserina het-r* and *het-v* loci mediate non-allelic incompatibility (Bernet, 1967b). The two loci each have two alleles (*R/r* and *V/V1*, respectively), with the *het-R het-V* alleles being the antagonistic combination. The *het-R/V* system is unique in the sense that the lethality of the incompatibility reaction is conditional – it is triggered at 26°C but not at 32°C (Labarère, 1973). This feature has allowed the biochemical characterization of the incompatibility reaction. For example, the autolytic reaction of the *het-R het-V* self-incompatibility (SI) strain at non-permissive temperature (26°C) is correlated with a decrease in RNA production (Labarère *et al.*, 1974), an appearance of new proteins (Boucherie *et al.*, 1981) and an increase in proteolytic and other enzymatic activities (Bégueret & Bernet, 1973; Boucherie & Bernet, 1978). Two-dimensional electrophoresis of *in vitro*-translated mRNA products from autolytic *het-R het-V* strain has identified at least 11 polypeptides preferentially expressed during induction of incompatibility (Bourges *et al.*, 1998). Three *idi* genes which are *i*nduced *d*uring *i*ncompatibility were isolated using a subtractive cDNA library enhanced for mRNAs which are more abundant in the *het-R het-V* SI strain 8 hours after the shift to non-permissive temperature (Bourges *et al.*, 1998).

Northern analysis verified that the expression of *idi-1*, *idi-2* and *idi-3* genes is highly induced during *het-R/V* incompatibility reaction. All three IDI polypeptides are small proteins (201, 157 and 196-aa) with putative signal peptides. The expression of two *idi* genes is strictly associated with cell-lysis mediated by non-allelic (and not allelic) incompatibility. The induction of *idi-1* and *idi-3* is observed in *het-R het-V* and *het-C het-E* strains but not in an *het-s het-S* strain. The induction is eliminated in strains that are suppressed for non-allelic incompatibility (*het-R het-V mod-A1 mod-B1* and *het-R het-V mod-C1*; see below). The expression of *idi-1* and *idi-3* is not enhanced during stress (heat shock) conditions, therefore it is likely that IDI-1 and IDI-3 are directly involved in the cell death reaction rather than merely induced as a consequence of cell death reaction. Recently, a GFP-localization study demonstrated that all three *idi* gene

products are located in the septa during incompatibility cell-lysis. Since plugging of septal pores is a hallmark feature of dying incompatible cells (Beckett *et al.*, 1974), presumably to confine the disintegrating cells and prevent the spreading of incompatible components, the *idi* genes are very likely candidates for cell disintegration and/or hyphal compartmentation during vegetative incompatibility.

1.4.8 Suppressor (*mod*ifier) genes for heterokaryon incompatibility in *P. anserina*

As mentioned above, a cross between non-allelic incompatible strains yields occasional uninucleate progeny containing the two non-allelic antagonistic genes (e.g. *het-c2* and *het-e1*). Such progeny are inhibited upon germination and are subsequently destroyed in a generalized lytic reaction (Bernet *et al.*, 1973). Some of these self-incompatible (SI) strains, however, can escape from restricted growth and emerge as wild-type sectors. Most of the escape mutations occur at *het* loci, but some occur at unlinked loci that give a modified self-incompatible (MSI) phenotype; the apical growth of the SI strain is restored (although with less dense mycelium and fewer aerial filaments) while the lytic reaction is only partially suppressed (Belcour & Bernet, 1969; Bernet *et al.*, 1973). MSI strains also exhibit cold sensitivity (failure to grow at 11°C), which can be suppressed by mutations in the *mod-B* locus (Bernet *et al.*, 1973). A mutation at *mod-C*, which specifically suppresses both growth inhibition and lysis of incompatible *het-R/V* (but not other allelic or non-allelic) incompatibility, has also been isolated (Labarère & Bernet, 1977).

Modifier mutations at one locus, *mod-A*, give such an MSI phenotype (Belcour & Bernet, 1969). The *mod-A1* mutation is recessive and only suppresses growth inhibition of non-allelic (*het-c/d*, *het-c/e* and *het-r/v*) but not allelic incompatibility systems (Becour & Bernet, 1969; Bernet *et al.*, 1973). Self-lysis in the *mod-A1* MSI strain can be suppressed by *mod-B1* – *mod-A1 mod-B1* double mutant in SI background has normal growth with no lytic defect (Bernet, 1971; Labarère, 1973). Although no altered phenotype is observed for single *mod-A1* or *mod-B1*

mutants in a compatible background, the *mod-A1 mod-B1* double mutant has a defect in protoperithecia formation and is female sterile, most noticeably in unfavorable growing conditions (such as exhaustion of nutrients) and in certain combination of *het* backgrounds (Bernet, 1971, 1992; Boucherie & Bernet, 1974, 1980).

The *mod-A* gene was cloned from a cosmid library by complementation of the *mod-A1 mod-B1* female sterility defect (Barreau *et al.*, 1998). The 687-aa MOD-A is not essential; a strain with *mod-A* gene deleted confers a typical *mod-A1* phenotype (MSI and female sterile in *mod-B1* background). MOD-A contains a proline-rich region with a potential SH3-binding domains; SH3 domains are found in all kinds of signaling molecules such as kinases and phosphatases (Cohen *et al.,* 1995). Many fungal proteins containing SH3 domain or SH3 ligand motifs are involved in morphogenesis (Barreau *et al.*, 1998). It is possible that MOD-A is involved in a signal transduction pathway that affects female organ formation during nutrient depletion. A model to explain the female sterility data is that for *P. anserina* to gather growing potential for protoperithecial formation, growth arrest (*mod-A* function) coupled with cell lysis (*mod-B* function) of hyphae surrounding the fruiting bodies are required (Barreau *et al.*, 1998). *mod-A* may also affect regulation of protease activity. Protease III and IV activities, which are responsible for most of the proteolysis in het-R het-V cells, is absent in mod-A mod-B double mutant and mod-A mutation eliminate proteases C activity in certain physiological condition (Bégueret & Bernet, 1973). Expression of the pap A gene (encoding an aspartyl protease expressed during carbon starvation) is also strongly reduced in the mod-A1 background (Paoletti et al., 1998).

Mutations at the mod-D locus have been isolated by their suppression of lytic reaction. mod-D1 suppresses the lytic phenotype of a MSI strain (het-C het-E mod-A1 mod-C1; Labarère & Bernet, 1979a), while mod-D2 and mod-D3 mutants were selected for their autolytic-inhibition in the self-lytic mod-A1 mod-B11 strain (Durrens et al., 1979). Mutations at mod-D

cannot suppress the lytic reaction by itself, but mod-D mutants have defects in aerial hyphal growth, protoperithecial formation, spore germination and secondary ramification (Durrens et al., 1979; Labarère & Bernet, 1979a, b). mod-D was cloned by complementation of both vegetative and sexual defects in a mod-D1 strain (Loubradou et al., 1999). The 354-aa MOD-D polypeptide is similar to the α-subunit of an heterotrimeric G protein. The less severe mod-D1 and mod-D3 mutations probably result in a decrease in cAMP levels since supplements of cAMP can restore vegetative growth in strains with these mutations. This result agrees with previous data that an extra copy of *PaAC* (which encodes an adenylate cyclase) can relieve the vegetative defect of *mod-D1* (Loubradou *et al.*, 1996). The involvement of *mod-D* in vegetative incompatibility was confirmed since either *mod-D1* or *mod-D2* can partially restore growth of a cold-sensitive MSI strain *(het-C het-E mod-A1)* at 11°C (Loubradou *et al.*, 1999). However, the suppressor effect of *mod-D1* is probably due to its induction of defects in a signal pathway other than the cAMP pathway since increase in cAMP concentration does not attenuate the growth restoration due to *mod-D1* mutation in the *het-C het-E mod-A1 mod-D1* strain.

The *mod-E1* mutation was selected on its ability to restore growth renewal from the quiescent stage in the *mod-D2* mutant (Durrens, 1982). It was later shown to suppress all developmental defects of the *mod-D2* mutation (Durrens & Bernet, 1985). The *Mod-E1* mutant is thermosensitive (cannot grow at 35°C), defective in glucose-dependent growth control (has excessive dry weight production and reduced life span following glucose exhaustion) and has spontaneous development of (sterile) perithecia from protoperithecia in the absence of fertilization (Durrens, 1983). The *mod-E* gene was cloned by its ability to complement growth of the *mod-E1* mutant at 35°C (Loubradou *et al.*, 1997). The 701-aa MOD-E is a member of the Hsp90 family and is responsive to heat-shock condition – *mod-E* expression is strongly induced at elevated temperature (37°C). Further characterization of *mod-E* demonstrated that it is involved in both sexual development and vegetative incompatibility – (i) a *mod-E1* x *mod-E1* cross contains fewer asci with mature spores and gives a dramatically higher frequency of

uninucleate spores (50% vs. 1-5% in a wild-type cross); (ii) *mod-E1* mutation partly restores growth of SI *het-R het-V* strain at non-permissive temperature (with similar radial growth rate as wild type but with less dense mycelium and presence of lysed cells); *mod-E1* has no effect on *het-c/d* or *het-c/e* incompatibility system. The involvement of *mod-E1* with *het-R/V* system agrees with previous finding that the *mod-C* mutations, which suppress the incompatibility reaction in a SI *het-R het-V* strain (but do not affect other incompatibility systems; Labarère & Bernet, 1977), are epistatic to *mod-E1* growth and developmental defect (Durrens & Bernet, 1985). Based on the *mod-E1* phenotypes and the fact that other Hsp90 proteins are implicated in cell cycle control, MOD-E could be involved in negative control of cell cycle (e.g. by association with a protein kinase) that affects both sexual development and vegetative incompatibility (Loubradou *et al.*, 1997).

2. Functional Analysis of *mat A-1* Gene of *Neurospora crassa*

Some of the results described in this chapter are published in Saupe *et al.* (1996b).

2.1 INTRODUCTION

Mating type in *N. crassa* exists as two alternative forms, *A* and *a*, which interact to initiate the sexual cycle (Dodge, 1935). The mating type locus also acts as a *het* locus (Beadle & Coonradt, 1944; Mylyk, 1976b); *A* and *a* hyphae cannot fuse and grow as a vigorous heterokaryon. The *A* and *a* sequences occupy the same locus but are dissimilar (Glass *et al.*, 1988) and have been termed idiomorphs (non-homologous alleles; Metzenberg & Glass, 1990). The *mat a* locus contains two ORFs, *mat a-1* and *mat a-2* (Staben & Yanofsky, 1990; Pöggeler & Kück, 2000). The *mat a-1* gene provides all functions for the *a* mating type (mating, post-fertilization and heterokaryon incompatibility) (Staben & Yanofsky, 1990; Chang & Staben, 1994). The function of *mat a-2* is unknown – it is non-essential to mating and sexual development and could represent a non-translated pseudogene (Pöggeler & Kück, 2000). The *mat A* locus of *N. crassa* contains three ORFs: *mat A-1*, *A-2* and *A-3* (Glass *et al.*, 1990; Ferreira *et al.*, 1996). MAT A-2 and MAT A-3 are responsible for post-fertilization events (ascospore formation) (Ferreira *et al.*, 1998). The *mat A-1* gene is both necessary and sufficient to confer mating specificity of *A* and trigger heterokaryon incompatibility with *a* (Glass *et al.*, 1990). Mutations in an unlinked *tol* locus suppress mating-type incompatibility such that *A tol* and *a tol* strains form a vigorous heterokaryon (Newmeyer, 1970). The mating-type-associated incompatibility is therefore mediated by the gene products of *mat A-1*, *mat a-1* and tol^{+}.

The 293-aa MAT A-1 contains a region of similarity (α-domain) with the MATα1 mating-type protein of *S. cerevisiae* (Tatchell *et al.*, 1981). The α-domain is also found in other mating-type polypeptides, such as MAT-1 (of *C. heterostrophus*; Turgeon *et al.*, 1993) and FMR-1 (of *P. anserina*; Debuchy & Coppin, 1992) (see section 1.3). MATα1 is a positive transcriptional factor that activates the expression of α specific genes (in cooperation with

MCM1; Passmore *et al.*, 1988), such as *MFα1/MFα2* (encode α-factor precursor; Kurjan & Herskowitz, 1982) and *STE3* (encodes **a**-factor receptor; Sprague *et al.*, 1983). In order to delineate the functional domains of *mat A-1*, a series of nonsense and frameshift constructs were tested for their male-mating activity and heterokaryon incompatibility.

2.2 MATERIALS AND METHODS

2.2.1 Strains, media and culturing

Culturing and crossing, using Vogel's (Vogel, 1964) and Westergaard's (Westergaard & Mitchell, 1947) media, respectively, were performed as previously described (Davis and Deserres, 1970; Perkins, 1986). Wild-type Oak Ridge *A* strain (Fungal Genetics Stock Center - FGSC 2489) and previously obtained A^m sterile mutants were used for DNA isolation; *un-3 ad-3A nic-2 cyh-1* A^{m42} (FGSC 4569), *un-3 ad-3A nic-2 cyh-1* A^{m44} (FGSC 4570), *un-3 ad-3A nic-2 cyh-1* A^{m54} (FGSC 4571), *un-3 ad-3A nic-2 cyh-1* A^{m56} (FGSC 4572) and *un-3 ad-3A* A^{m64} (FGSC 4573) originated from Griffiths (1982). The *un-3 ad-3A nic-2 cyh-1* A^{m99} strain was isolated by Saupe *et al.* (1996b) according to Griffiths (1982). The homothallic Neurospora strain *N. pannonica* (FGSC 7221) was also used. *fl a* (FGSC 4347) and *fl A* (FGSC 4317) were used for mating assays (Perkins *et al.*, 1989). C15-1 (*pan-2*; *inl*; *arg-5 a*; M.L. Smith), C15-2 (*pan-2*; *arg-5 A*; M.L. Smith), I-10-1 (*ad-3A nic-2*; *tol a*; A.J.F. Griffiths) and I-20-41 (*ad-3B arg-1*; *tol A*; A.J.F. Griffiths) were used for spheroplast preparation and transformation experiments.

2.2.2 DNA isolation and PCR amplification

Genomic DNA isolation was performed according to Stevens & Metzenberg (1982). PCR amplification (Saiki *et al.*, 1988) from genomic DNA was performed using primers that span *mat A-1* (base 3802-3826 and 4843-4867) (Glass *et al.*, 1990) in a model 480 Perkin-Elmer DNA cycler. PCR products were cloned into the PCRII vector using the TA cloning kit (Invitrogen, San Diego, CA).

2.2.3 Plasmid construction for *mat A-1* and *FMR1*

PCR-amplified *mat A-1* mutant alleles (as a 1.1 kb *Bam*H1 fragment) were subcloned from the PCRII vector into pOKE3 vector carrying the *pan-2* marker (Grotelueschen & Metzenberg, unpublished results) and into the pCB1004 vector carrying the hygromycin-resistant phosphotransferase (*hph*) gene (Carroll *et al.*, 1994). *Escherichia coli* DH5α (Hanahan, 1983) was used as bacterial host strain for plasmid amplification.

To construct a *Neurospora*-compatible plasmid containing the *P. anserina FMR1* gene, a 2.3 kb *Kpn*I/*Xho*I *FMR1* fragment (from plasmid pBPLP-0; Debuchy *et al.*, 1993) was cloned into pCB1004 vector. The final construct is named FMR1/pCB. The FMR1(Δ155-305)/pCB plasmid is constructed by deleting a 3' *Eco*RV fragment from the FMR1/pCB plasmid.

In order to build a pCB1004 plasmid containing an over-expressing *FMR1* gene, a 3.5 kb *Eco*RI/*Xba*I fragment of *FMR1* gene (from plasmid P_{GPD}::FMR1/pUC18; gift from R. Debuchy) fused with a strong promoter from *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (Punt *et al.*, 1988) was cloned into pCB1004. The construct is named P_{GPD}::FMR1/pCB.

2.2.4 Site-directed mutagenesis and chimeric construction

To introduce the L109T mutation in *mat A-1*, a 1.1 kb *Bam*H1 *mat A-1* fragment was cloned from the *mat A-1*/pCB vector into the smaller pUC19 plasmid and was subjected to site-directed mutagenesis using the U.S.E. mutagenesis kit (Pharmacia, Bale d'Urfé, PQ) following the manufacturer's protocol; oligo-based mutagenesis has poor efficiency when performed in a large plasmid. Primer LRR 5' TGAATCCAGGTTTGCAGAGT, which contains a novel *Bst*N1 restriction site for screening, was used to introduce the L109T mutation. The *Sca*I selection primer 5' CTGTGACTGGTGACGCGTCAACCAAGTC 3' (Pharmacia, Bale d'Urfé, PQ), which converts a *Sca*I site to *Mlu*I site in the *amp* gene carried in the pUC19 plasmid, was used to enhance selection of mutant plasmid. After the *mat A-1*/pUC19 single-stranded DNA was

annealed to both primers and had the second strand synthesized, the plasmids were cut by *Sca*I enzyme such that non-mutated plasmids were linearized and eliminated. The mutant plasmid was recovered after one round of selection in NM522 *mutS E. coli* cells and the site-directed *mat A-1* mutation was verified by DNA sequencing (Sanger *et al.*, 1977). The mutant allele was cloned into pOKE3 vector and was subjected to transformation assays.

Chimeric constructs were made between *mat A-1* and *FMR1*. However, since there is no convenient restriction site between the DNA sequences that encode the α-domain and the first LRR (i.e. between amino acids V_{92} and Y_{93}), an artificial *Bst*1107I site was introduced into both *mat A-1* and *FMR1* in order to facilitate construction. Primer NC92 5'CATGTGCTCGGTATA CTCGTCAATCCGG 3' and primer PA92 5' CATGTGTGCAGTATACTCTGCGATTCGC 3' were used to introduce a novel *Bst*1107I restriction site in the plasmids *mat A-1*/pUC19 and *FMR1*/pUC19, respectively. Site-directed mutagenesis was performed as described above. Mutated plasmids were recovered after NM522 *mutS* selection and their 3' *Bst*1107I-*Xba*I fragments were swapped. Two plasmids containing reciprocal chimeric constructs, the UFA92 (p**U**C19 vector containing aa 1-92 of **F**MR1 and aa 93-end of MAT **A**-1) and UAF92 (p**U**C19 vector containing aa 1-92 of MAT **A**-1 and aa 93-end of **F**MR1), were obtained. The chimeric genes were cloned into hygromycin-resistant p**C**B1004 and the plasmids were named CFA92 and CAF92, respectively.

2.2.5 DNA sequence analyses

DNA sequences were determined using the ABI automated Taq DyeDeoxy Terminator cycle method (Mississauga, ON) at the NAPS unit (Biotechnology Laboratory, University of British Columbia). Computer sequence analyses for protein and DNA were done using the GCG package available from the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

2.2.6 Transformation reduction assay for heterokaryon incompatibility

Competent spheroplast preparation and transformation were performed as previously described (Schweizer *et al.*, 1981; Vollmer & Yanofsky, 1986). The transformation reduction assay for heterokaryon incompatibility was done according to Glass *et al.* (1988). To test if the *mat A-1* mutants contain the incompatibility activity, the pOKE3 constructs were introduced to *a* (C15-1) and *A* (C15-2) spheroplasts. To test if *tol* suppresses transformation reduction activity, the pCB1004 constructs were introduced into the *tol a* (I-10-1) and *tol A* (I-40-1) spheroplasts.

2.3 RESULTS

2.3.1 PCR amplification and construction of various *mat A-1* (A^m) mutant constructs

To delineate the functional domains of *mat A-1*, a series of mutant *mat A-1* alleles were tested for their ability to confer mating and incompatibility function. Genomic DNA from wild type and various frameshift and nonsense sterile mutants of *mat A-1* (A^{m54}, A^{m64}, A^{m42}, A^{m99}, and A^{m44}) (Griffiths, 1982; Saupe *et al.*, 1996b) was isolated and subsequently PCR-amplified using two primers that bracket *mat A-1* (bases 3802-3826 and 4843-4867; Glass *et al.*, 1990). The wild type *mat A-1* allele encodes a polypeptide of 293-amino acids while the mutant alleles encode the first 163 (A^{m54}), 111 (A^{m64}), 99 (A^{m42}), 85 (A^{m99}) and 52 (A^{m44}) amino acids of MAT A-1 protein, respectively. The 1.1-kb PCR products of *mat A-1* were obtained for each strain and were subsequently cloned into the PCRII vector. To ensure that the PCR reactions amplified the *mat A-1* gene, the PCRII vectors containing *mat A-1* clones were tested for the presence of *mat A-1* gene using Southern blot analysis (Southern, 1975) with the entire *A* idiomorph (Glass *et al.*, 1988) clone as a probe. Southern blot results showed that *mat A-1* was successfully amplified (data not shown).

To obtain a *Neurospora*-compatible vector containing the various constructs, the 1.1 kb *Bam*H1 *mat A-1* fragments from wild-type and each of the five mutant alleles were subcloned

from the PCRII vector into the pOKE3 vector carrying the *pan-2* marker and into the pCB1004 vector carrying the hygromycin resistance gene.

2.3.2 Incompatibility of mutant *mat A-1* alleles in a transformation assay

To test if the *mat A-1* mutant alleles retain heterokaryon incompatibility activity, the subclones (in pOKE vector) were introduced into *a* spheroplasts. If a *mat A-1* mutant allele confers heterokaryon incompatibility activity, then the transformation frequency should be significantly reduced, because transformants containing functional *mat a-1*, *mat A-1* and *tol*$^{+}$ do not regenerate (Glass *et al.*, 1988). To demonstrate that the transformation reduction is due to mating-type incompatibility and that *tol* suppresses incompatibility activity in these mutant alleles, the pCB1004 constructs were introduced into the *tol a* and *tol A* spheroplasts. The PCR cloning and transformation strategy is summarized in Figure 2-1.

The results of the transformation experiment are shown in Fig. 2-2 and summarized in Figure 2-3. Three of the constructs, A^{m42}, A^{m99} and A^{m44}, transformed the *a* and *A* recipient strains with similar efficiencies. However, when the A^{m64} and A^{m54} constructs were introduced into the *a* recipient strain, a significantly reduced transformation frequency was obtained. In three separate replicates, an average of 30 transformants (per 10-cm petri dish) were recovered following the transformation of the A^{m64} and A^{m54} alleles into the *a* spheroplasts, as compared to an average of 300 transformants recovered when *A* spheroplasts were recipients. In contrast, an average of two transformants are recovered when the wild-type *mat A-1* allele was introduced into the *a* spheroplasts.

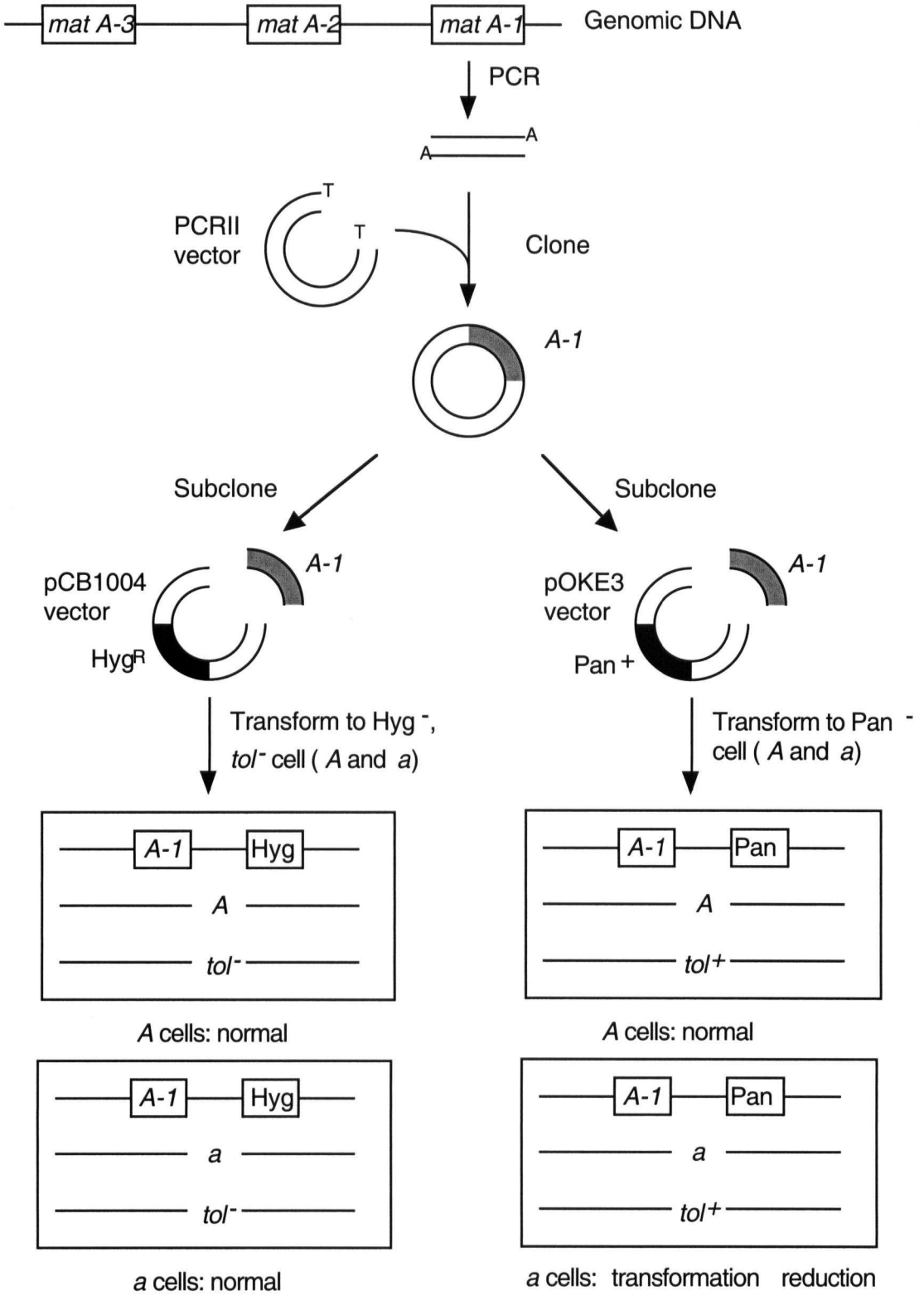


Figure 2-1. Strategy for transformation reduction assay for heterokaryon incompatibility. Wild type and various A^m alleles of the *mat A-1* gene were amplified by PCR and were later tested for their ability to trigger mating-type associated incompatibility. If a *mat A-1* construct contains incompatibility function, its introduction into *a tol*$^+$ recipient will lead to dramatic decrease in number of transformants as compared to transformation into *A tol*$^+$ background. On the other hand, the transformation reduction should not be observed in a *tol*$^-$ background since the *tol* mutation suppresses mating-type-associated incompatibility.

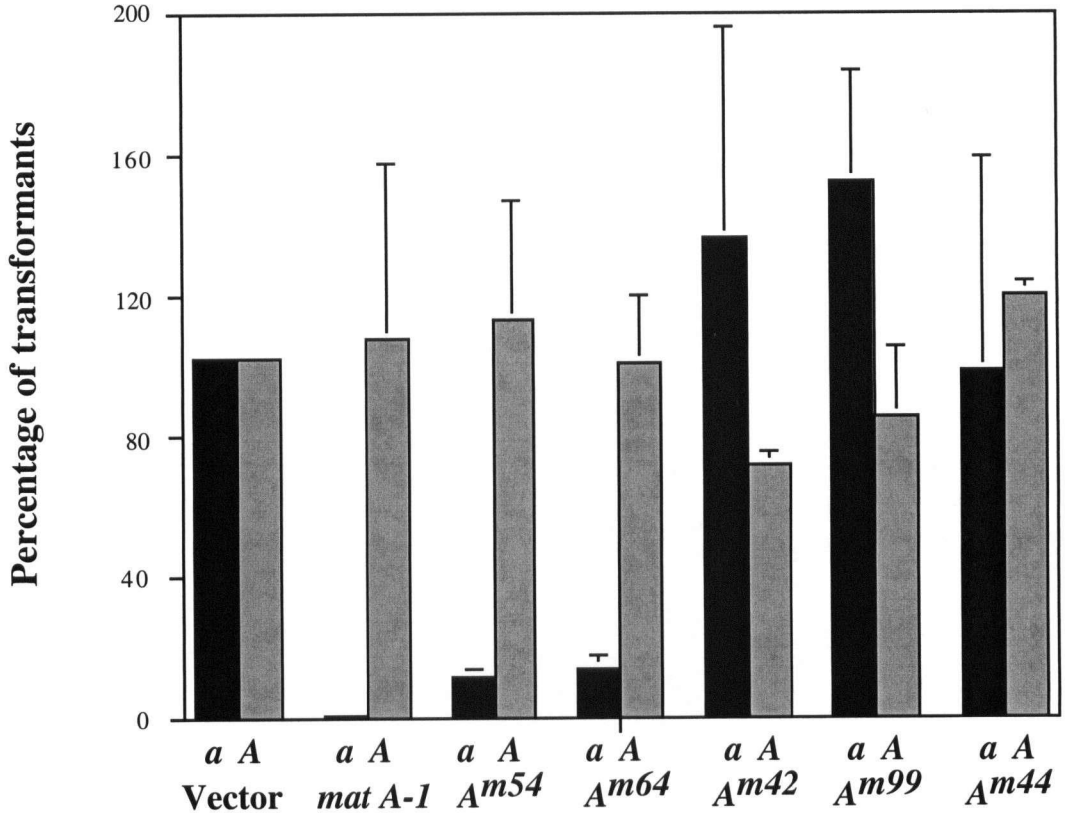


Figure 2-2. Results of the introduction of mutant *mat A-1* alleles (A^m) into *a* and *A* recipients strains. Results correspond to the mean of three independent experiments. The transformation frequency for each transformation test is illustrated as a percentage of the transformation efficiency of vector (no insert) control. Transformation frequency of a hundred percents correspond to the number of transformants can be obtained when the vector control gives 100 transformants per 200 ng of DNA (introduced into 4 x 10^6 spheroplasts) in a transformation test. For the vector control, the typical number of transformants per plate (500 ng of DNA into 10^7 spheroplasts) is roughly 250.

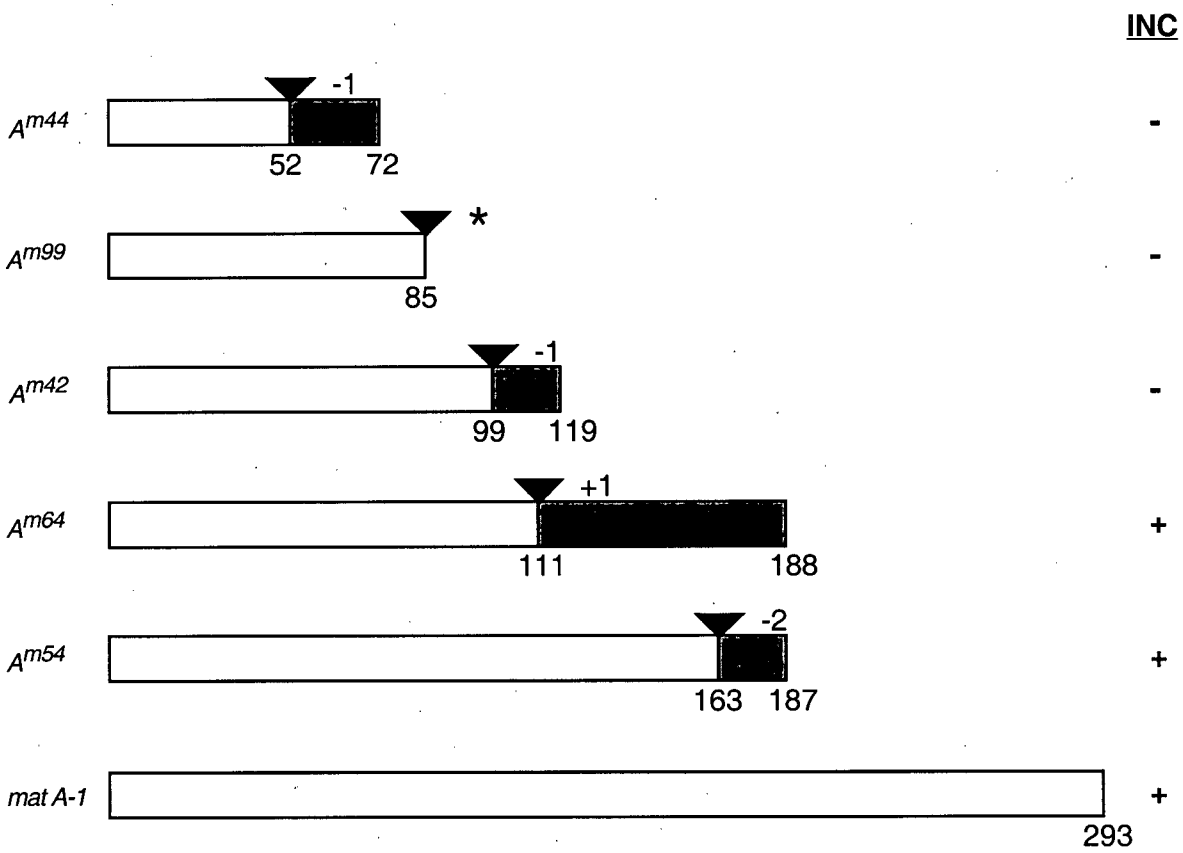


Figure 2-3. Mutant and truncated *mat A-1* alleles: topography of presumptive translational products and their properties. The position of the A^m mutations in the *mat A-1* ORF is indicated by arrowheads together with the nature of the mutation, frame shift (-2, +1, etc) or stop codon (*). The position of the last amino acid corresponding to the original *mat A-1* sequence is given. The solid area represents the regions of the open reading frame translated out of frame before the novel stop codon. On the right, the results heterokaryon incompatibility (INC) assays are given. Incompatibility was assayed by introducing cloned mutant alleles into an *a* recipient. A construct is determined to confer *mat A-1* heterokaryon incompatibility function (+) if its introduction into *a* spheroplasts lead to a reduction in transformation efficiency.

Based on the results of the transformation assay, an ectopic *mat A-1* containing only the first 111 amino acids is sufficient to elicit the mating-type incompatibility function when the *A* and *a* sequences reside in the same nucleus. D1, D2, D3 and D4, four *in vitro* deletion constructs truncated at amino acid positions 288, 227, 184, and 163 respectively, also confer incompatibility functions as tested by transformation assay (Saupe *et al.*, 1996b). These data are in agreement with the A^m mutant phenotypes such that *mat A-1* constructs containing at least the first 111 amino acids retain incompatibility function.

The *tol* mutation suppresses the mating-type incompatibility in both partial diploids for mating-type and mixed mating-type heterokaryons (Newmeyer, 1970). The A^m mutant alleles and the wild-type *mat A-1* were introduced into *tol a* and *tol A* strains to determine if the *tol* mutation can suppress the incompatibility as assayed by transformation experiments. Transformation efficiencies into *tol a* and *tol A* recipients were comparable for all A^m mutant alleles and the wild-type *mat A-1* (averaging 200-300 transformants per plate), demonstrating that the reduction in transformation frequency as observed for the case of *mat A-1*, A^{m64} and A^{m54} was due to triggering of mating-type-associated incompatibility.

By examining the amino acids sequence flanking 100 to 111, a putative leucine-rich repeat (LRR) was identified (a second one was found further downstream; Figure 2-4A). LRRs, which have been found in numerous proteins -- nuclear, cytoplasmic, and secreted, and are thought to mediate protein-protein interactions (Kobe & Deisenhofer, 1994). Each LRR contributes an exposed β-sheet that could participate in interactions with other proteins. Two LRRs are also found in MAT a-1 (Figure 2-4B). A 5-amino acid deletion within the first LRR of *mat a-1* led to the loss of heterokaryon incompatibility without affecting mating activity and DNA binding *in vitro* (Philley & Staben, 1994). If the sequences depicted in Figure 2-4C are functional LRRs, then they may represent important protein-protein interaction domains required for the heterokaryon incompatibility reaction.

A.

INC

```
                          100       110        150         160         170
                           |         |         |           |           |
CYR1 LRR                  PXXaXX LXXLXX LXaXXXX aXXa  PXXaXX LXXLXXLXaXXXXaXX a
N. crassa                 YSSIRTYLEQEKVTLQLWIHYAVGHL  PL VQHNLQPMNGLCLLTKLLESGL
N. terricola              YSSIRTYLEQEKVTLQLWIHYAVGHL  PL VQHNLQPMNGLCLLTRCLESGL
N. africana               YSSIRTYLEQEKVTLQLWIHYRVRHL  PL VQHNLQPMNGLCLFTKCLESGL
N. pannonica              YSAIRTYLEEEKVNLQLWNHYSVGHL  PL VQHNPPPMDGLCLLTKLLESGL
P. anserina               YSAIRDQLAEQNVTLQTWIQFAVTPL  PD IRYHLQPMNGLGLFLSCLNGGL
```

B.

INC

```
                      200       210                 370         380
                       |         |                   |           |
CYR1 LRR          PXXaXX LXX LXXLXaXXXXaXX a   PXXaXXLXXLXXLXaXXXXaXX a
MAT a-1           RRRVSPYLKIKLLNYDVNGNLLWGTV   PLRLLSLR LW LMIPLTQLSFPL
```

C.

```
MAT A-1   93 YSSIRT LEQEKVTLQLWIHYAV GHL 118
         150 PL VQHNLQPMNG LCLLTKL LESGL 173
MAT a-1  194 RRRVSPYLKIKLLNYDVNGNL LWGTV 219
         361 PLRLLS LR  LW LMIPLTQ LSFPL 382

Consensus    P--a---L---L--L-a-a---a---a
```

Figure 2-4. Leucine-rich repeats in mating-type proteins. **A.** Leucine-rich repeats (LRRs) found in *Neurospora crassa* MAT A-1 and its homologs in other fungal species. Numbers indicate the amino acid position according to *N. crassa* MAT A-1 protein. The top sequence (CYR1 LRR) depicted the consensus sequence found in yeast adenylate cyclase, where "a" represents V, L or I and "X" represents any amino acid (Kataoka *et al.*, 1985). *Podospora anserina* FMR1 is the only protein listed that does not confer heterokaryon incompatibility function. Position 100 to 111 is required for incompatibility (INC) function in MAT A-1. The α-domain of MAT A-1 is found at position 42 to 89 (not shown), 4 amino acids upstream of the first LRR. **B.** LRRs identified in *Neurospora crassa* MAT a-1. Deletion of amino acids from 216 to 220 or a R258S substitution (not shown) in MAT a-1 abolishes heterokaryon incompatibility (INC) but does not affect mating nor DNA binding *in vitro* (Philley & Staben, 1994). The HMG box is found at position 115 to 185 (not shown), 9 amino acids upstream of the first LRR. **C.** Leucine-rich repeats (LRR) within the mating-type protein of *N. crassa* are implicated in the heterokaryon incompatibility function. Two LRRs from each mating-type gene and their consensus (bottom line) are depicted. Deletion or change of the underlined residues abolishes incompatibility but does not affect mating function.

2.3.3 Construction of chimerics between *mat A-1* and *FMR1*

The *P. anserina FMR1* (fertilization minus regulator) gene confers mating identity for the *mat-* strains and is a homolog of *N. crassa mat A-1* (Debuchy & Coppin, 1992) (see section 1.3.4). The amino terminal portions of the two proteins are very similar (Figure 2-5). The two mating-type proteins are functional homologs since the introduction of *P. anserina FMR1* confers mating in *N. crassa* and the introduction of *N. crassa mat A-1* confers mating in *P. anserina* (Arnaise *et al.*, 1993). In *P. anserina*, a pseudohomothallic ascomycete, a germinating spore contains both opposite mating type nuclei (*mat+* and *mat-*). The two nuclear types grow as a heterokaryon and therefore do not exhibit mating-type-associated incompatibility, although incompatibility mediated by other *het* loci is observed *P. anserina*.

In order to test if the *FMR1* gene confers heterokaryon incompatibility in *N. crassa* in a transformation reduction assay, the *FMR1* gene was cloned into a *Neurospora*-compatible plasmid, pCB1004 (containing a hygromycin resistance gene) (Carroll *et al.*, 1994). The FMR1/pCB construct was then subjected to the transformation assay as described above. Transformation results showed that the FMR1/pCB plasmid has comparable transformation frequencies when introduced into *A* and *a* spheroplasts (Figure 2-6). These data confirmed that the *FMR1* gene does not confer heterokaryon incompatibility (with *mat a-1*) in *N. crassa*, although it confers mating with *N. crassa a* strain. To ensure that the failure of *FMR1* to confer heterokaryon incompatibility is due to the alteration in amino acid sequence and not because of the low expression of *FMR1* in *N. crassa*, or the masking of the incompatibility domain by carboxyl terminus (which is different than that of MAT A-1; see Figure 2-5) through steric hindrance, two additional *FMR1* constructs were tested in the transformation reduction assay. The first construct is made from a gene fusion of *FMR1* with a strong promoter from the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (Punt *et al.*, 1988). The second construct was made by deleting a 3' fragment from the *FMR1* construct so that the carboxyl

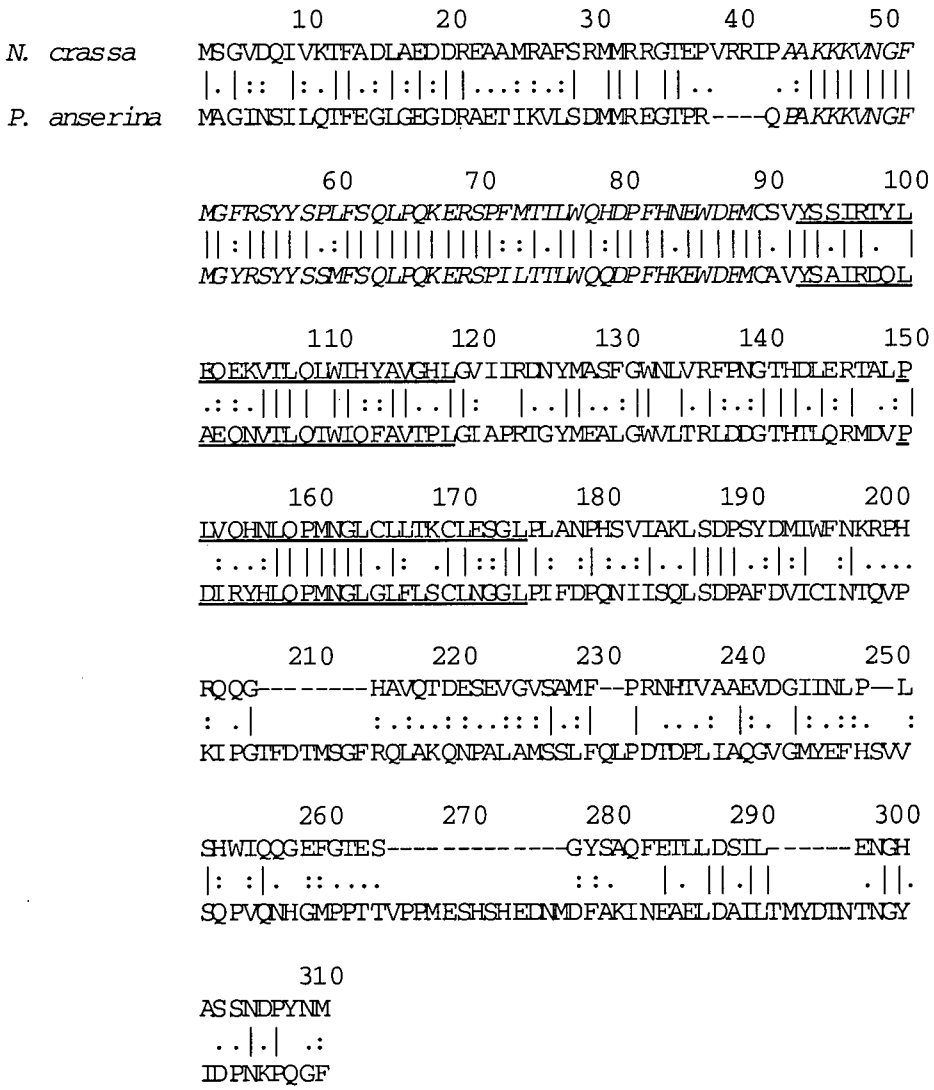


Figure 2-5. Mating-type amino acid comparison between *Neurospora crassa* MAT A-1 and *Podospora anserina* FMR1 (Glass *et al.*, 1990; Debuchy & Coppin, 1992). The leucine-rich repeats (LRRs) within the two proteins are underlined (positions 93-118 and positions 150-173). The α-domain is found in position 42 to 89 and is italicized. "|" indicates an identical amino acid. ":" indicates a conservative change. "." indicates a semi-conservative change.

terminal portion of the protein (from H_{155} to the end) was eliminated. Transformation reduction assays showed that neither of the FMR1(Δ155-305)/pCB nor the PGPD::FMR1/pCB constructs conferred transformation reduction when introduced into N. crassa a spheroplasts. These data agreed with previous results by Arnaise *et al.* (1993), which have shown that when *FMR1* (and *FPR1*) were introduced into *N. crassa*, incompatibility reaction was not elicited.

Since *FMR1* confers mating activity in *Neurospora* as *mat A* but does not confer heterokaryon incompatibility, it can be viewed as a *mat A-1* mutant in the heterokaryon incompatibility function. However, as shown in Figure 2-5, although *mat A-1* and *FMR1* are similar, there are many amino-acids differences between the two polypeptides. It was impossible to identify differences between the two polypeptides that can correlate the presence of *het* function in *mat A-1* but the lack of it in *FMR1*. Chimeric proteins between MAT A-1 and FMR1 were therefore the next logical approach to pinpoint the heterokaryon incompatibility domain.

From the transformation test of the A^m strains, a LRR-like sequence in MAT A-1 is absolutely required for mediating heterokaryon incompatibility. To examine if the LRR sequence is functionally important in mediating incompatibility (and not merely enhancing the folding of α-domain or increasing mRNA stability in the A^{m64} and A^{M54} mutants), chimerics between MAT A-1 and FMR1 with a fusion point at the beginning of the first LRR (i.e. between amino acids V_{92} and Y_{93}) were constructed. Since there is no convenient restriction site, an artificial *Bst*1107I site was introduced into both *mat A-1* and *FMR1* clones in order to facilitate chimeric construction. Two plasmids containing reciprocal chimeric genes, the CFA92 (p**C**B1004 vector containing aa 1-92 of **F**MR1 and aa 93-end of MAT **A**-1) and CAF92 (p**C**B1004 vector containing aa 1-92 of MAT **A**-1 and aa 93-end of **F**MR1), were constructed and used in the transformation reduction assay.

When introduced into *N. crassa* competent cells, the CAF92 plasmid exhibited similar transformation efficiencies in *A* and *a* spheroplasts, wheras the CFA92 plasmid exhibited

considerably lower transformation frequency (20 fold) in the *a* recipient (Figure 2-6). These data indicated that the CFA92 chimeric (containing the MAT A-1 LRR) confers heterokaryon incompatibility in *N. crassa* whereas CAF92 (containing the FMR1 LRR) does not. Furthermore, when CFA92 and CAF92 were introduced into a *tol a* recipient (I-10-1), CFA92- and CAF92- transformants conferring dual mating activity can be recovered (*i.e.* they initiated peritherial development with *A* and *a* tester strains). These data demonstrated that functions of mating and incompatibility in MAT A-1 can be separated and that the absence of incompatibility function in CAF92 is due to lack of an incompatibility domain and not due to global mis-folding of the protein. The position of the breakpoint in the two chimeric constructs and their transformation/mating results shows that the difference in the LRR sequence could be responsible for the lack of incompatibility function in *FMR1* gene.

2.3.4 Site-directed mutagenesis of LRR in *mat A-1*

Results from the transformation assay of chimeric *mat A-1/FMR1* genes show that the difference in the LRR sequence between MAT A-1 and FMR1 could be responsible for the lack of the incompatibility function in FMR1. One potential candidate found in the first LRR that may be responsible for the functional difference is the L109T (Figure 2-4A). L_{109} is a conserved leucine among different *Neurospora* MAT A-1 homologs in the first LRR. This leucine residue at position 109 is replaced by a threonine in the FMR1 polypeptide.

To test if the L109T is responsible for the loss of heterokaryon incompatibility in FMR1, the same amino acid substitution was introduced to the wild type *mat A-1* gene. The *mat A-1* gene was cloned into pUC19 and was subjected to site-directed mutagenesis. The mutated *mat A-1* clone was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) and was subject to transformation assay as described above. Results of the transformation assay indicated that the site-directed mutant *mat A-1* L109T has the same degree of transformation reduction as wild type (averaging 4 transformants in *a* recipient versus 200+ transformants in *A* recipient)

(Figure 4-6). Therefore, the L109T substitution is not solely responsible for the absence of heterokaryon incompatibility function in FMR1.

2.3.5 Heterokaryon incompatibility function of other mating-type protein

The L109T site-directed mutagenesis did not reveal a disruption of incompatibility function in MAT A-1. However, there are other candidates within the LRR that could explain the "compatible" phenotype of FMR1. As shown in Figure 2-4A, there are many other changes between FMR1 and the *Neurospora* MAT A-1 consensus within the LRR, namely S95A, T98D, Y99Q, E101A, Q102E, E103Q, K104N, H112Q, Y113F, G116T and H117P. One of the residues mentioned here is of special interest - S_{95} is the only potential protein kinase phosphorylation site (S/T-X-R/K) in the MAT A-1 protein. It is possible that the S95A change in FMR1 leads to the absence of phosphorylation at that position and therefore no longer allows FMR1 to mediate incompatibility.

To test if the S95A substitution is responsible for the loss of incompatibility function in FMR1 is possible even without the use of site-directed mutagenesis, since another *Neurospora* species, *N. pannonica* (a homothallic species; Krug & Khan, 1991), contains the same S95A substitution (see Figure 2-4A). The *N. pannonica mat A-1* gene was amplified, cloned and subject to transformation assay as described in sections 2.3.1 and 2.3.2. Transformation results indicated that the *N. pannonica mat A-1* gene confers heterokaryon incompatibility in *N. crassa* (transformation reduction is as dramatic as that of *N. crassa mat A-1*) (Figure 2-6). Therefore the S95A substitution is not solely responsible for the loss of incompatibility function in the FMR1 polypeptide.

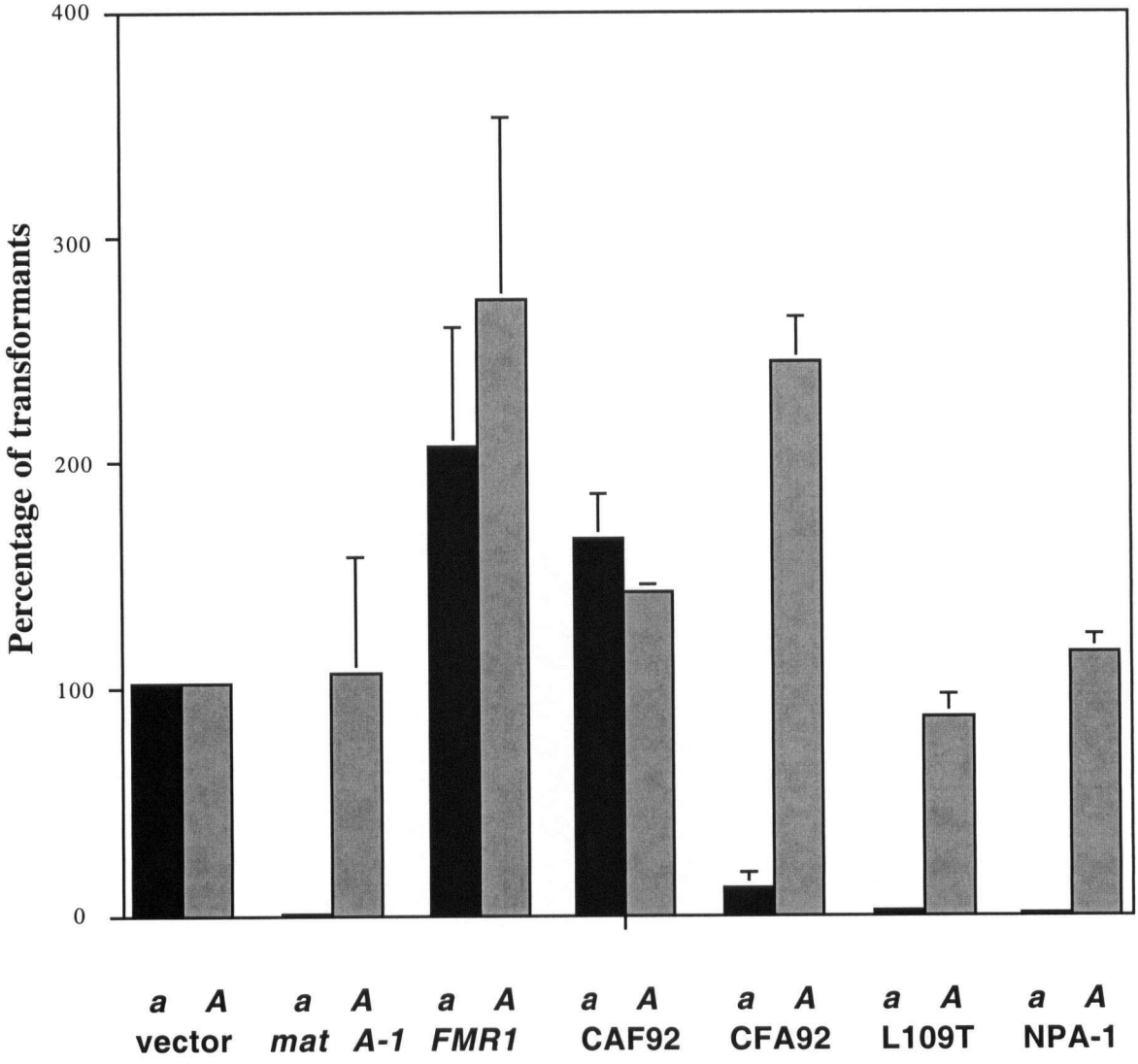


Figure 2-6. Results of the introduction of various constructs into *a* and *A* recipient strains. CAF92 and CFA92 are chimerics constructed with *N. crassa mat A-1* and *P. anserina FMR1* gene. CAF92 encodes amino acids 1-92 of MAT A-1 and 93-end of FMR1. CFA92 encodes amino acids 1-92 of FMR1 and 93-end of MAT A-1 (including first LRR of MAT A-1). L109T represents a site-directed mutant of *mat A-1*. NPA-1 represents the *mat A-1* gene from *N. pannonica*. The transformation frequency for each transformation test is illustrated as a percentage of the transformation efficiency of the vector (no insert) control. Transformation frequency of a hundred percents correspond to the number of transformants can be obtained when the vector control gives 100 transformants per 200 ng of DNA (introduced into 4 x 10^6 spheroplasts) in a transformation test. For the vector control, the typical number of transformants per plate (500 ng of DNA into 10^7 spheroplasts) is roughly 250.

2.4 DISCUSSION

In heterothallic and some pseudohomothallic species, fusion of opposite mating-type reproductive structures initiates sexual reproduction. In *Neurospora crassa*, the mating type locus is unique in that it controls both sexual and heterokaryon compatibility (Beadle and Coonradt, 1944). Hence, although the two mating types, *A* and *a*, must fuse to go through the sexual cycle, they cannot fuse somatically and grow as a vigorous heterokaryon.

Genetic studies have provided some insights on this unique dual function. Newmeyer (1970) showed that the unlinked mutation, *tol* (tolerance), suppresses the mating type-associated heterokaryon incompatibility function but does not affect sexual function, thus pointing to a distinct delineation of these two roles of mating type at the cellular level. Griffiths and Delange (1978) and Griffiths (1982) obtained mutations of both *A* and *a* alleles (A^m and a^m) by selecting for heterokaryon formation in mixtures of cells of both mating types. Most mutants were found to have lost both sexual and incompatibility functions, but one mutant, a^{m33}, had lost the incompatibility function and retained full fertility as a protoperithecial (maternal) and a conidial (paternal) parent. These data showed that, for the *a* allele, the two mating-type functions are structurally separable.

The A^m and a^m sterile mutants produce protoperithecia with trichogynes, showing that the mating-type genes do not code for structural components of the female reproductive structures. However, in a mating-type pheromone bioassay (Bistis 1981, 1983), the A^m and a^m mating-type mutants do not produce pheromone, nor do they respond to the pheromone of the opposite mating type (G. Bistis, personal communication). In *S. cerevisiae*, the mating identity of cells is determined by a pheromone and receptor system; a cell of a particular mating-type expresses only one pheromone and a receptor for the pheromone produced by the opposite mating-type (Banuett, 1998) (see Chapter 1.3). Binding of the pheromone to its cognate receptor facilitates

mating by inducing a signal transduction event that leads to the transient arrest of the cell cycle and the induction of conjugation-specific genes. It is likely that the mating type proteins in *N. crassa* also control expression of a pheromone/receptor system such that cells with alternate mating-type alleles can recognize each other as compatible mating partner. In support of this hypothesis, the pheromone precursor genes found in *N. crassa* are present in all cell types but are expressed in a mating-type specific manner (D. Ebbole and D. Bell-Pedersen, personal communication).

The *mat A-1* gene in *A* cells and *mat a-1* gene in *a* cells are responsible for both mating and heterokaryon incompatibility; all previous A^m and a^m mutations are found in these two ORFs. Two additional genes found in the *A* locus, *mat A-2* and *mat A-3*, are required for post-fertilization events only (Ferreira *et al.*, 1996, 1998). The purpose of the present study was to delineate functional regions in the *mat A-1* encoded polypeptide. Figure 2-7 summarizes the analyses of incompatibility function in various mutant and chimeric *mat A-1* constructs.

Several mutant *mat A-1* (A^m) alleles (Griffiths, 1982; Saupe *et al.*, 1996b) were tested for their ability to trigger heterokaryon incompatibility in a transformation assay. All the A^m mutants, as generated by UV and HAP (6-N-hydroxylaminopurine) treatment, were selected for the ability to form heterokaryons with an *a* reference strain. All but one of the initial A^m mutants (Griffiths, 1982) were found to have frameshift mutations and have lost both male and female fertility. The exception is the A^{m99} mutant (with a nonsense mutation after aa position 85), which is completely male sterile but showed partial female fertility (Saupe *et al.*, 1996b). When mated as female, A^{m99} produces 20-50 fertile, beaked perithecia on a 10-cm petri dish (as compared to hundreds of perithecia found in wild-type), has slower development of mature asci with ascospores (takes 15 days for asci development compared to 7 days for wild-type) and smaller number of mature asci per perithecium. The A^{m99} mutant contains all of the α-domain, which is a region of homology found among various mating-type proteins, including MATα1 of *S.*

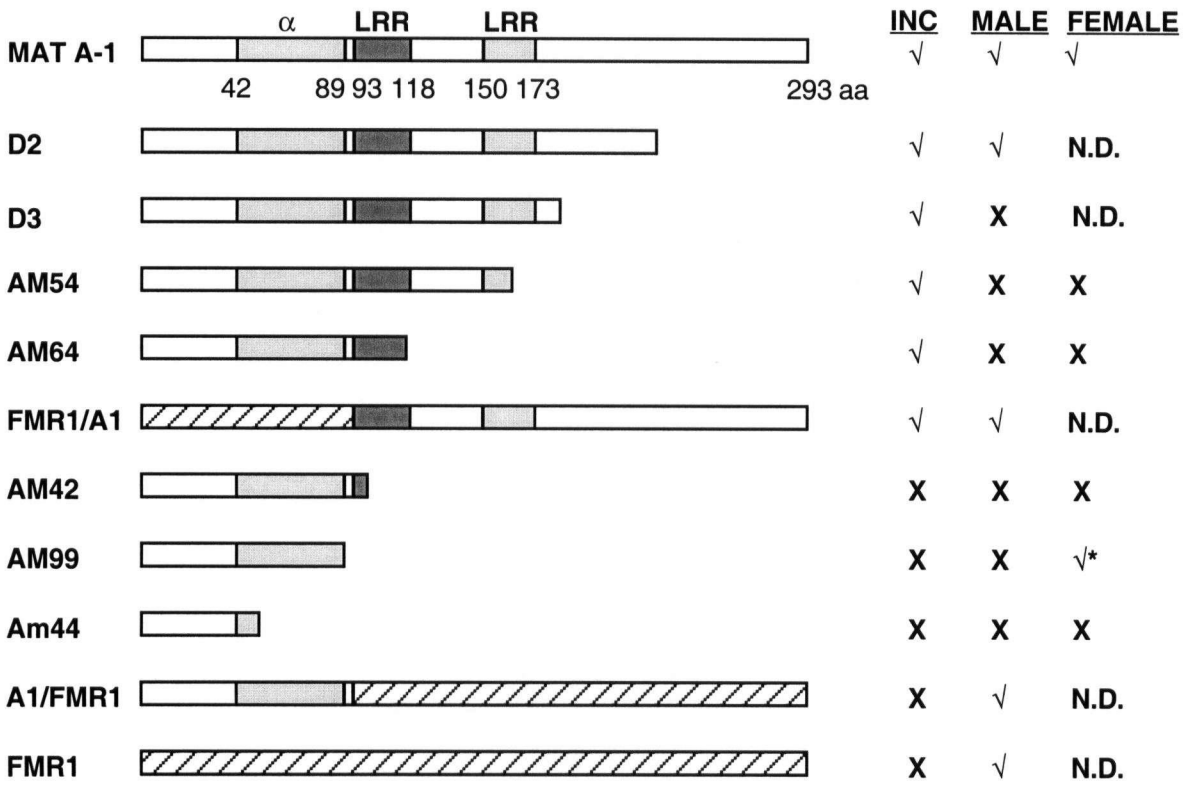


Figure 2-7. *mat A-1* constructs and their functional analyses. Assayed functions include mating-type incompatibility (INC), male mating (MALE) and female mating (FEMALE) activity (N.D. – not determined). Detailed topography of mutations and translational products of A^m mutants are shown in Figure 2-3. D2 and D3 are deletion constructs truncated at positions 227 and 184, respectively. FMR1/A1 and A1/FMR1 are chimeric proteins constructed from MAT A-1 and FMR1. * A^{m99} mutant confers partial female mating activity – it forms few perithecia and its asci and ascospore development is delayed. √ = presence of activity. X = absence of activity.

cerevisiae (Tatchell *et al.*, 1981) (see section 1.3). MATα-1, in cooperation with the MCM1 protein, is a transcription activator that recognize the promoter region of many α-specific genes (Grayhack, 1992). A short region within the α-domain (which has the greatest similarity among fungal mating-type proteins) is proposed to interact with MCM1 (Hagen *et al.*, 1993; Yuan *et al.*, 1993). Even though the region of amino acids 1-85 is intact in A^{m54}, A^{m64} and A^{m42}, these mutants are female sterile (Figure 2-7). It is possible that their out-of-frame C-terminal tails affect female fertility through steric hindrance or causes these fusion products to be rapidly targeted to proteolytic degradation (although heterokaryon incompatibility function is intact in these mutants). Alternatively, the premature stop codon in A^{m99} may be ignored during translation in the developing perithecia such that a small amount of full length MAT A-1 is produced.

Results from the transformation of A^m constructs suggest that position 100-111 in MAT A-1 is important for heterokaryon incompatibility, since the A^{m54} and A^{m64} alleles confer transformation reduction in an *a* recipient while A^{m42}, A^{m99} and A^{m44} do not (Figure 2-3). The results from transformation of A^m mutants agree with the phenotype of *in vitro* deletion constructs of *mat A-1*, namely D1 (truncated at position 288), D2 (227-aa), D3 (184-aa) and D4 (163-aa), which confer incompatibility function as tested by transformation assay (Saupe *et al.*, 1996b). However, since all the A^m mutants were selected for their ability to form a stable heterokaryon with an *a* strain (Griffiths, 1982), the A^{m54} and A^{m64} strains have conflicting phenotypes of incompatibility function. The same mutant *mat A-1* allele has lost the incompatibility function in forced heterokaryon but is still capable of triggering the incompatibility in a transformation assay. Different hypotheses can be proposed to explain this difference. Heterokaryon compatibility of the A^{m54} and A^{m64} mutants could be a consequence of the instability of the chimeric MAT A-1 polypeptide or of the mRNA bearing premature stop codons. Nonsense codon-mediated mRNA degradation has been reported in yeast (Peltz *et al.*, 1993, 1994; Hagan *et al.*, 1995). On the other hand, integration of transforming DNA often occurs in transcriptionally active regions of the genome and in multiple copies. This difference

could result in a higher expression level of the mutated alleles as transgenes, thus compensating for the instability of A^{m64} and A^{m54} mRNA. Moreover, in heterokaryons, *mat A-1* and *mat a-1* are expressed from distinct nuclei whereas in transformants the transgenic *mat A-1* allele is expressed from the same nucleus as the resident *mat a-1*. Considering that *mat a-1* and *mat A-1* are putative transcriptional regulators, it is possible that in mixed mating type heterokaryons these products are preferentially targeted back to the nucleus they originated from, thus limiting their interaction when expressed from different nuclei.

The transformation assay suggests that the incompatibility function is mildly affected in A^{m54} and A^{m64}. The reduction of transformation efficiency is 10 to 12 fold for *mat A-1* alleles from A^{m54} and A^{m64} whereas the reduction is at least 50 fold for the wild type *mat A-1* allele. Thus, the amino acids from164 to 293 (including the second LRR) appear to contain sequences enhancing the incompatibility response although they do not appear to be essential. Alternatively, this region might not be involved in the incompatibility function at all; the transformation frequencies observed in experiments with A^{m54} and A^{m64} constructs could be explained by mRNA instability as described above.

Amino acids from position 100 to 111, which are required for heterokaryon incompatibility, are within a putative leucine-rich repeat (LRR) (Figure 2-4A). LRRs are thought to mediate protein-protein interactions (Kobe & Deisenhofer, 1994). A second LRR is found at positions 150-173 in MAT A-1 while two putative LRRs are found in MAT a-1 (Figure 2-4B, C). A 5-amino acid deletion within the first LRR of MAT a-1 led to the loss of heterokaryon incompatibility without affecting mating activity and DNA binding *in vitro* (Philley & Staben, 1994). These data demonstrated that these putative LRRs may be functionally important for heterokaryon incompatibility.

To investigate the importance of the LRR in mediating mating-type-associated incompatibility, chimeric constructs with a fusion point at the start of the first LRR were made from the *mat A-1* and *FMR1* genes. FMR1 specifies mating identity for *mat-* nuclei in *P. anserina* and has strong homology with MAT A-1 (Debuchy & Coppin, 1992). The two mating-type proteins are true functional homologs since they confer mating activity as heterologous transgenes (Arnaise *et al.*, 1993). FMR1, however, does not confer incompatibility in *N. crassa* as assayed by transformation (see section 2.3.3). Thus, FMR1 is an ideal candidate for chimeric construction in determining incompatibility domain in MAT A-1. The two chimeric genes, FMR1/A1 (with aa 1-92 of FMR1 and aa 93-end of MAT A-1) and A1/FMR1 (with aa 1-92 of MAT A-1 and aa 93-end of FMR1), were used in the transformation reduction assay. Results (Figure 2-7) show that whereas FMR1/A1 (which contains the MAT A-1 first LRR) confers incompatibility with *a*, A1/FMR1 (which contains the FMR1 first LRR) does not. Since both chimeric genes confer male mating activity in *N. crassa*, the lack of incompatibility in A1/FMR1 is unlikely to be due to global misfolding of the polypeptide. A reasonable explanation for the data is that the first LRR in MAT A-1 is functionally important in mediating mating-type incompatibility. As shown in Figure 2-7, the incompatibility function of MAT A-1, as tested in various deletion, frameshift and chimeric constructs, is correlated with the presence of its first LRR.

Site-directed mutagenesis of the LRR and testing of another *Neurospora mat A-1* gene did not yield further information on which amino acid residue within the LRR is required for mediating incompatibility (see section 2.3.4 and 2.3.5). For example, the L109T (as shown in the site-directed mutant) and S95A (as shown in the *N. pannonica mat A-1*) change cannot be solely responsible for the lack of incompatibility function in the FMR1 protein. Since there are many differences between MAT A-1 and FMR1 polypeptide within the first LRR region, further chimeric construction and site-directed mutagenesis are required to delineate the amino acids required for the incompatibility function. It is noteworthy to point out that the first LRR in MAT

A-1 is predicted to form a β-sheet (Chou & Fasman, 1978), whereas in FMR1, only the last three-quarters of the LRR is predicted to form the same secondary structure. The amino-acid difference in the first-quarter of the LRR (position 89-97; Figure 2-4A) may account for the difference in incompatibility activity of the two proteins.

N. pannonica is a homothallic species (Krug & Khan, 1991); all four mating type genes are present and appear to be linked (L. Wheeler & N. L. Glass, personal communication). Since both *mat A-1* and *mat a-1* are present in the vegetative cells, the presence of *mat A-1* gene containing mating-type incompatibility suggested there may be a *tol*-like suppression in *N. pannonica* such that mating-type incompatibility is tolerated (see Chapter 4).

The MAT A-1 protein has different functions in *N. crassa* and the results from this study suggest that they are mediated by different domains and probably by different biochemical mechanisms. MAT A-1 is required for the mating event. The first 227 amino acids are required for male mating while the first 85 amino acids are required for female mating (see Figure 2-7). In comparison with the yeast system, the MAT A-1 protein is probably a transcriptional regulator that controls mating-type specific expression of pheromone and receptors. MAT A-1 (probably in cooperation with other transcriptional activator) may bind to the promoter of the *A* pheromone and the *a* pheromone-receptor genes and activate their expression. This activation activity is suggested to be mediated by the α-domain (Hagen *et al.*, 1993; Yuan *et al.*, 1993) (see section 1.3).

From the result of this study, the incompatibility function of MAT A-1 is likely to be mediated by its first LRR. LRR is a protein-protein interaction domain (Kobe & Deisenhofer, 1994). It is possible that this motif may mediate protein-protein interaction between MAT A-1 and MAT a-1 or between MAT A-1 and TOL (or its downstream products). Recent yeast 2-hybrid data showed MAT A-1 and MAT a-1 physically interact with each other (C. Staben,

unpublished results). Moreover, the *mat a-1* Δ216-220 mutation (a 5-amino acid deletion within the first LRR of MAT a-1), which affects heterokaryon incompatibility without affecting mating activity and DNA binding *in vitro* (Philley & Staben, 1994), also destroys MAT a-1 ability to interact with MAT A-1 (C. Staben, unpublished results). From the above data, the biochemical basis of mating-type incompatibility can be explained by physical interaction of MAT A-1 and MAT a-1 and this interaction is mediated by LRRs. In *P. anserina*, a pseudohomothallic species, opposite mating-type nuclei exist within the same cytoplasm upon germination and mating-type incompatibility does not exist. Interestingly, an interaction between FMR1 (MAT A-1 homolog) and FPR1 (MAT a-1 homolog) has not been detected via yeast two-hybrid system (Coppin *et al.*, 1997). The MAT A-1/MAT a-1 dimerization model, however, does not explain how incompatibility is triggered. It is possible that the heterodimer binds to the promoter region of *tol* and regulates its expression; expression of *tol* would then trigger the incompatibility response, possibly through a signal transduction pathway. Alternatively, the two mating-type proteins may form multimers with TOL and that complex may in turn trigger the incompatibility reaction. These models cannot be fully tested until the components mediating incompatibility such as TOL have been characterized.

The MAT A-1 and MAT a-1 proteins are multi-functional. Other than conferring mating and heterokaryon incompatibility function, MAT A-1 and MAT a-1 are required for post-fertilization events. The a^{m1} mutant (with frameshift mutation in *mat a-1*) does not enter the ascogonium or participate in meiosis (Perkins 1984; Raju, 1992b). Ectopic *mat A-1* copies can restore mating activity but not ascospore formation (Glass *et al.*, 1988). In heterothallic and even some homothallic species, alternate mating-type nuclei are differentially recognized and paired during migration of nuclei into ascogenous dikaryotic hyphae (Shiu & Glass, 2000). Nuclear recognition is thought to be mediated by mating-type proteins (see section 1.3.4). The C-terminal part of both MAT A-1 and FMR-1 is relatively acidic. In FMR-1, the C-terminal portion, which interacts with SMR2, is not required for mating but is essential for post-

fertilization function (R. Debuchy, personal communication). By analogy, MAT A-1 may interact with MAT A-3 through its C-terminal tail, possibly to mediate its proposed post-fertilization function (although the yeast two-hybrid system has failed to detect interaction between the two proteins; C. Staben, personal communication). Future experiments (e.g. through chimeric construction and mutagenesis) will give a finer delineation of the molecular basis of how the *mat A-1* gene mediates mating, incompatibility and post-fertilization functions.

3. Molecular Cloning and Sequencing of *tol* (*tol*erant)

Some results from this chapter are published in Shiu & Glass (1999).

3.1 INTRODUCTION

In addition to its role during the sexual cycle, the mating-type locus in *N. crassa* has a function during vegetative growth. Although fusion of opposite mating-type reproductive structures is required for entry into the sexual cycle, fusion of opposite mating-type hyphae during vegetative growth results in growth inhibition and cell death (Beadle and Coonradt, 1944; Garnjobst, 1953). At least 10 additional genetic determinants called *het* (for *het*erokaryon incompatibility) loci also restrict formation of vegetative heterokaryons; genetic differences at these loci are not required for fertilization nor do they interfere with sexual reproduction (For review see Glass & Kuldau, 1992; Leslie, 1993).

The *mat A* and *mat a* sequences have been characterized and are composed of dissimilar sequences (Glass *et al.,* 1988). The *mat A-1 and mat a-1* genes are responsible for mating and heterokaryon incompatibility while the *mat A-2* and *mat A-3* genes are required for post-fertilization functions (Glass *et al.,* 1990; Staben & Yanofsky, 1990; Ferreira *et al.*, 1996, 1998). A recessive mutation unlinked to the mating-type locus, *tol* (for *tol*erant), suppresses mating-type associated heterokaryon incompatibility such that *tol A* and *tol a* strains form a vigorous heterokaryon (Newmeyer, 1970). Mating activity of these strains is unaffected and *tol* strains show normal fertility in crosses with an opposite mating-type strain. Attempts to identify additional suppressors of mating-type associated incompatibility resulted only in the isolation of additional *tol* alleles (Vellani *et al.*, 1994). The *tol* mutation apparently does not suppress incompatibility due to differences at other *het* loci (Newmeyer, 1970; Leslie and Yamashiro, 1997).

This chapter describes the molecular cloning and sequencing of the *tol* gene of *N. crassa*. The cloning of *tol* was made possible by the use of genetic mapping, chromosome walking and a functional assay.

3.2 MATERIALS AND METHODS

3.2.1 Strains, media and culturing methods

The *N. crassa* strains used in the study are listed in Table 3-1. Culturing and crossing, using Vogel's (Vogel, 1964) and Westergaard's (Westergaard & Mitchell, 1947) media respectively, were performed as previously described, with modification (Davis and Deserres, 1970; Perkins, 1986). For heterokaryon tests, 2 µl conidial suspensions (10^7/ml) of two strains containing different auxotrophic markers were co-inoculated onto vegetative growth media (Vogel, 1964). A compatible heterokaryon forms a vigorously conidiating culture after 3 days of incubation with a mean growth rate of 7 cm/day. Mating-type incompatible heterokaryons are usually aconidial and have a growth rate of approximately 0.7 cm/day (Vellani *et al.*, 1994).

3.2.2 Strain construction

In order to introgress part of Mauriceville linkage group (LG) IV into *T(IL→IIR) 39311 ser-3 A* (Perkins & Barry, 1977; RLM 04-08, Table 3-1), RLM 04-08 was crossed with FGSC (Fungal Genetics Stock Center, Kansas City, KS) strain 2226 (Mauriceville wild type *a*). Numerous restriction fragment length polymorphisms (RFLP) have been observed between Mauriceville strains (FGSC 2225 and 2226; Table 3-1) and the Oak Ridge background of standard laboratory strains. Genomic DNA from *ser-3 A* progeny were screened for Mauriceville LG IV by hybridization to cosmid G4:A9 (which contains *trp-4*, a LG IV locus closely linked to *tol*). A progeny, R5-28 (Table 3-1) had RFLP patterns identical to the FGSC 2226 parent when probed with G4:A9.

Strain	Genotype	Origin
-	*his-5 A*	FGSC 456
R1-09	*un-3 a*	R.L. Metzenberg
-	*tol trp-4 a*	FGSC 2337
-	*tol trp-4 pan-1 A*	FGSC 4158
NE-1	*his-5 tol trp-4 A*	This study
MV 1c-a	Mauriceville WT *a*	FGSC 2226
MV 1 c-A	Mauriceville WT *A*	FGSC 2225
RLM04-08	*T(IL→IIR)39311 ser-3 A*	R.L. Metzenberg
R5-28	*T(IL→IIR)39311 ser-3 A* (Mauriceville background on LG IV)	This study
R5-27	*his-5 tol trp-4 un-3; a*	This study
12-21-388	*ad-3B al-2; cot-1; pan-2 A*	A.J.F. Griffiths
R4-71	*his-5 tol trp-4; pan-2 a*	This study
R4-72	*ad-3B; trp-4 cot-1; pan-2 A*	This study
74-OR23-1VA	Oak Ridge WT *A*	FGSC 2489
I-20-41	*ade-3B arg-1; tol A*	A.J.F. Griffiths
I-20-26	*ade-3B arg-1 A*	A.J.F. Griffiths
I-10-1	*ade-3A nic-2; tol a*	A.J.F. Griffiths
I-1-51	*ade-3A nic-2 a*	A.J.F. Griffiths
OR8-1a	Oak Ridge WT *a*	FGSC 988

Table 3-1. List of *Neurospora crassa* strains used in this study. All strains are *N. crassa* Oak Ridge compatible (unless otherwise stated) and all *tol* alleles are N83. (FGSC = Fungal Genetic Stock Center. WT = Wild type.)

The strain NE-1 (*his-5 tol trp-4 A*) was constructed by crossing *his-5 A* (FGSC 456) with *tol trp-4 a* (FGSC 2337); His$^-$ and Trp$^-$ progeny were selected. Crossing of NE-1 to R1-09 (*un-3 a*) gave R5-27 (*un-3; his-5 tol trp-4 a*). The presence of the *tol*$^-$ allele (N83) in R5-27 was confirmed by crossing with translocation strain RLM 04-08 (Table 3-1); approximately half of the *A/a* partial diploid progeny displayed normal growth rates (see Figure 3-2). The strain R5-27 was subsequently crossed with a *pan-2* containing strain (12-21-388) to give *pan-2* strains R4-71 and R4-72 (Table 3-1), which were used for transformation assays.

3.2.3 Chromosome walk and physical mapping

A *trp-4*-containing cosmid was identified from the Orbach/Sachs pMOcosX genomic library (Orbach, 1994; available from the FGSC) using a *trp-4* clone (Vollmer & Yanofsky, 1986) as a probe. A chromosome walk was performed in both directions from *trp-4* using end fragments as probes for each step. Selected cosmids were physically mapped by restriction fragment length polymorphism (RFLP) analysis (Metzenberg *et al.*, 1985).

3.2.4 Transformation assay and subcloning of *tol*

Spheroplast preparation and transformation were performed as previously described (Schweizer *et al.*, 1981; Vollmer & Yanofsky, 1986) with the exception of selecting for resistance to hygromycin B (250 units/ml; Calbiochem). Cosmids in the walk were assayed for Tol$^+$ activity by co-transforming the cosmids (conferring hygromycin resistance) with a *mat A-1*-containing pOKE103 construct (pOKE103 has a *pan-2* selectable marker; J. Grotelueschen and R.L. Metzenberg, unpublished results) into strain R4-71 (*his-5 tol trp-4; pan-2 a*) and strain R4-72 (*ade-3B; trp-4 cot-1; pan-2 A*) spheroplasts. The *tol* gene was further subcloned into the hygromycin-resistant vector (pCB1004; Carroll *et al.*, 1994). Homokaryotic transformants were isolated according to Ebbole & Sachs (1990).

3.2.5 Nucleic acid isolation and hybridization

Standard molecular biology procedures were used throughout (Sambrook *et al.*, 1989). Genomic DNA isolation from *Neurospora* was adapted from Oakley *et al.*, (1987). Gel electrophoresis and nucleic acids transfer to Nylon filters (Schleicher and Schuell, Keene, NH) was performed according to manufacturer's specifications. [α-^{32}P] dCTP (Amersham, Oakville, ON) labeled probes were generated from digested DNA using the T7 QuickPrime Kit (Pharmacia, Baie d'Urfe, PQ). The *tol*$^-$ mutant allele (N83) was amplified from strain R5-27 DNA using primers tol ATG1 (5'CCTGGGCTCACCTATGC 3'; base -50 to -34) and tol 3'-end (5' CGGCGGGATCTCTTTCTG 3'; base 2894 to 2877). The *tol* cDNA and *tol*$^-$ (N83) PCR products were cloned into the PCRII vector using the TA cloning kit (Invitrogen, San Diego, CA) and subjected to DNA sequencing. The DNA sequence of two different *tol*$^-$ (N83) clones was determined and the mutation point was confirmed from three additional subclones.

3.2.6 DNA sequence analyses

A 6.9-kb *Eco*RI *tol*$^+$ containing construct was subcloned into an overlapping fragment suitable for DNA sequencing. DNA sequences were determined for both strands using the ABI automated Taq DyeDeoxy Terminator cycle method (Mississauga, ON) at the NAPS unit, Biotechnology Laboratory, University of British Columbia. Computer sequence analyses for protein and DNA were done using the MacVector/AssemblyLIGN software (International Biotechnologies, New Haven, CT) and GCG package available from the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

3.3 RESULTS

3.3.1 Isolation of a cosmid spanning the *tol* locus

The *tol* locus is on the right arm of linkage group (LG) IV and is flanked by *trp-4* (~ 1 map unit) and *his-5* (2-6 map units) (Figure 3-1). A cosmid containing *trp-4* (G4:A9), was identified from a genomic library using the *trp-4* gene (Vollmer & Yanofsky, 1986) as a probe. A chromosome walk was initiated from G4:A9 in both directions. Twelve overlapping cosmids spanning a region of 350 kb around the *trp-4* locus were isolated (Figure 3-1). The physical location of selected cosmids was determined using RFLP mapping (Metzenberg *et al.*, 1985) and all mapped to *trp-4* (Table 3-2).

To determine the orientation of the walk from *trp-4*, progeny that contained crossovers between *his-5*, *tol* and *trp-4* from a cross between R5-27 (*un-3; his-5 tol trp-4 a*; Oak Ridge background) and MV1c-*A* (FGSC 2225; Mauriceville background) were analyzed. Genomic DNA was isolated from His^+ Trp^- progeny and probed with cosmids shown in Figure 3-1. Recombination points in two *his-5*$^+$ *tol*$^+$ *trp-4* progeny (43-9 and 43-31) were identified when cosmid X14:C2 was used as a probe (Figure 3-2A). These results indicated that cosmids directed toward G13:C8 were oriented towards the *tol* locus, and that the *tol* locus was centromere proximal to X14:C2 (Figure 3-1).

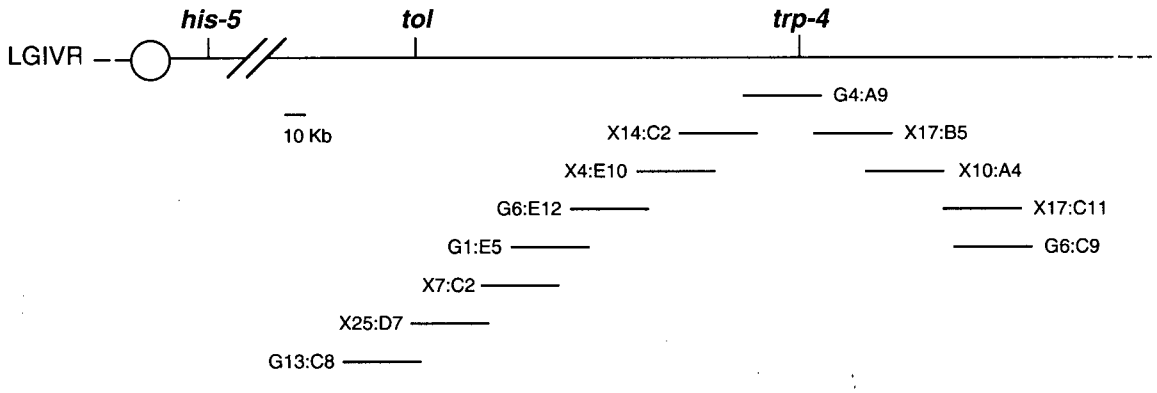


Figure 3-1. Contiguous cosmids isolated from a chromosome walk around *trp-4* on the right arm of linkage group IV. The *his-5* locus is centromere proximal (2-6 map units) to both the *tol* and *trp-4* loci. A chromosome walk was initiated in both direction using the G4:A9 cosmid, which contains the *trp-4* gene. Orientation of the walk was determined by detection of genetic crossovers using cosmids as hybridization probe (Figure 3-2).

	A	B	C	D	E	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
	14	67	14	57	13	57	13	14	57	68	14	14	14	58	23	24	14	24	14
G6:C9	mo	mo	mm	oo	mm	oo	oo	mm	om	oo	mo	mm	mm	mm	mo	om	mo	mo	mm
X17:C11	mo	mo	mm	oo	mm	oo	oo	mm	om	oo	mo	mm	mm	mm	-o	om	mo	mo	mm
X10:A4	mo	mo	mm	oo	mm	oo	oo	mm	om	oo	mo	mm	mm	mm	mo	om	mo	mo	mm
trp-4	mo	mo	mm	oo	mm	oo	oo	mm	om	oo	mo	mm	mm	mm	mo	om	mo	mo	mm
X4:E10	mo	mo	mm	oo	mm	oo	oo	mm	om	oo	mo	mm	mm	mm	mo	om	mo	mo	mm
G6:E12	mo	mo	mm	oo	mm	oo	oo	mm	om	oo	mo	mm	mm	mm	mo	om	mo	mo	mm
X25:D7	mo	mo	mm	oo	m-	oo	oo	mm	om	oo	mo	mm	mm	mm	mo	oo	mo	mo	mm
G13:C8	mo	mo	mm	oo	m-	oo	oo	mm	om	ho	mo	mm	mm	mm	mo	oo	mo	mo	mm

Table 3-2. Physical (RFLP) mapping of the *trp-4* gene (in plasmid pSV50-31:2G) and cosmids identified in the chromosome walk. Segregation of RFLP for 38 ordered progeny is detected by each clone in the cross multicent-2a (Oak Ridge background or o, FGSC 4488) X MV 1c-A (Mauriceville background or m, FGSC 2225). Progeny can be of either parental RFLP pattern (m or o) or be a hybrid (h). All cosmids identified in the chromosome walk hybridization experiment (for the *tol* locus) is mapped closely to *trp-4* in LG IV. (- = not scored)

3.3.2 Bracketing of *tol* by genetic crossovers and RFLP analysis

To bracket the *tol* locus within the cosmid walk, it was necessary to detect recombination points that were centromere proximal to *tol*, i.e., between *tol* and *his-5*. A cross was performed between a R5-28 (*T(IL→IIR) 39311 ser-3 A*; MV LG IV) x R5-27 (*un-3; his-5 tol trp-4 a*) (Figure 3-2B). The R5-28 strain contains an insertional translocation in which the left arm of LG I (which includes *ser-3, un-3* and *mat*) was inserted into the right arm of linkage group II (Perkins & Barry, 1977). Progeny were selected for their ability to grow at wild-type rate at 30^0C in medium lacking serine (selection for *A/a tol* partial diploid progeny; Figure 3-2B) and histidine but containing tryptophan (selection for progeny with recombination between *his-5* and *tol*). Genomic DNA from sixty-eight *A/a tol trp-4* progeny were screened for RFLP differences from the R5-27 parent when probed with the cosmids centromere proximal to G4:A9. Fifty-nine progeny contained RFLPs that were identical to the R5-27 parent, indicating that in these progeny the recombination point between *his-5* and *tol* lay centromere proximal to the cosmids identified in the walk. In genomic DNA from eight progeny, a R5-28 pattern was identified when G13:C8 was used as a probe, but a R5-27 pattern was observed when probed with X25:D7 (Figure 3-2B; Appendix 8.1). These data indicated that the recombination point between *his-5* and *tol* in these progeny occurred between G13:C8 and X25:D7 and that *tol* must be centromere distal to the crossover point. When genomic DNA from progeny T11 was probed with G13:C8, a hybrid RFLP pattern was identified, consistent with the interpretation that the *tol* locus resided between G13:C8 and X14:C2.

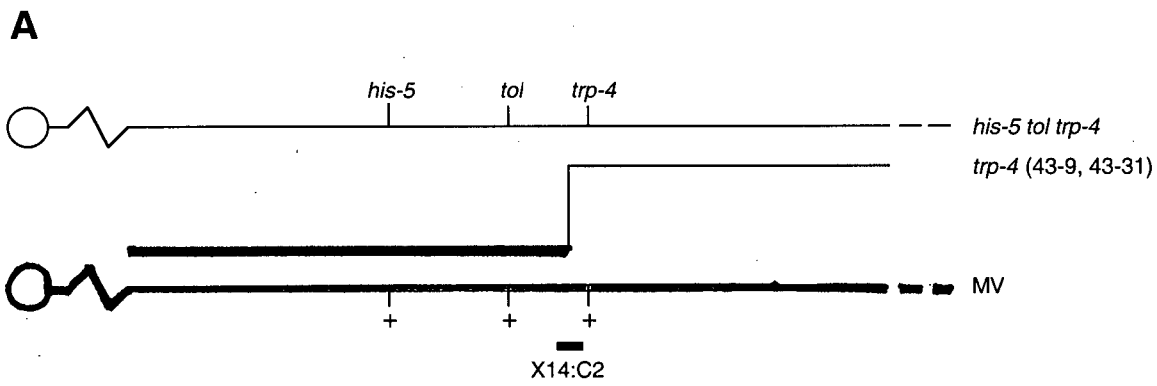


Figure 3-2. The *tol* locus was bracketed by genetic crossovers and restriction fragment length polymorphism (RFLP) analysis of progeny. (A) Crossovers between *tol* and *trp-4* were detected in the progeny 43-9 and 43-31 (*his-5*$^{+}$ *tol*$^{+}$ *trp-4*) which displayed hybrid RFLP patterns that differed from both parental strains when probed with X14:C2.

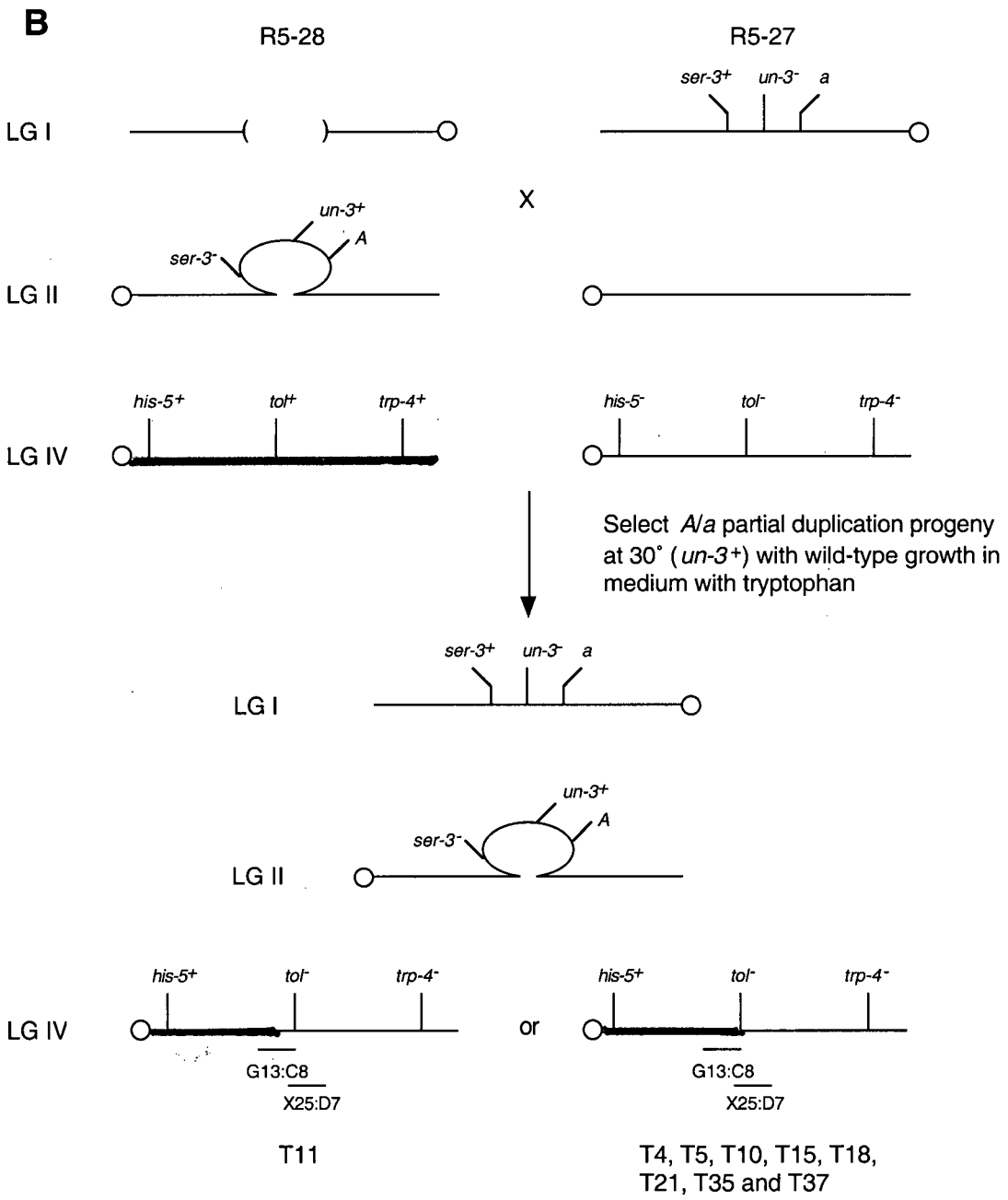


Figure 3-2. The *tol* locus was bracketed by genetic crossovers and restriction fragment length polymorphism (RFLP) analysis of progeny. (B) Recombination between *his-5* and *tol* was selected for by isolating *A/a* partial duplication progeny that were *tol*, *trp-4* and *his-5*$^{+}$. RFLP analysis of genomic DNA from *A/a tol trp-4* progeny identified crossover points between cosmids G13:C8 and X25:D7. When probed with G13:C8, many progeny displayed the 39311 (MV LG IV) (T4, T5, T10, T15, T18, T21, T35 and T37) or hybrid pattern (T11) (see Figure 8-1), indicating that the *tol* locus resided between G13:C8 and X14:C2 in the cosmid walk.

3.3.3 Transformation reduction assay for Tol$^+$ activity

The Tol$^+$ activity of contiguous cosmids between G13:C8 and G4:A9 was assayed by co-transforming each cosmid with pOKE*mat A-1* into R4-71 (*his-5 tol trp-4; pan-2 a*) and R4-72 (*ad-3B; trp-4 cot-1; pan-2 A*) spheroplasts. Transformants were selected for both hygromycin resistance (cosmid marker) and for growth in the absence of panthothenic acid (pOKE*mat A-1* marker). Transformants fail to regenerate following the induction of mating-type associated incompatibility (Glass *et al.* 1990) and therefore a cosmid with Tol$^+$ activity should exhibit significantly lower transformation frequencies when introduced with *mat A-1* into *tol a* spheroplasts as compared to *A* spheroplasts. Only cosmid X25:D7 (plus *mat A-1*) exhibited a significant reduction in transformation frequency in a *tol a* background. Further subcloning identified a 6.9 kb *Eco*RI fragment from X25:D7 that caused a 20 - 30 fold reduction in transformation frequencies when introduced into *tol a* spheroplasts as compared to *A* spheroplasts (See Appendix 8.2).

3.3.4 Molecular characterization of *tol* sequence

DNA sequence determination of a 4.2 kb *Sal*I-*Nsi*I sub-fragment from the 6.9 kb *Eco*RI fragment revealed an open reading frame (ORF) of 3127 bp interrupted by a putative intron of 94 bp. The 5' and 3' and internal sites of the *tol* intron fit intron splicing consensus sequences (Bruchez *et al.*, 1993; Figure 3-3). The sequence of a *tol* cDNA spanning the intron site was characterized and confirmed the presence of the intron in the genomic *tol* sequence. DNA sequences surrounding the *tol* ATG start codon were in good agreement with the consensus for *N. crassa* (Edelman & Staben, 1994). A sequence matching the transcription initiation consensus for *N. crassa* was found at position -324 (TCATCANC; Bruchez *et al.*, 1993) and a CAAT motif (Bucher, 1990; Chen & Kinsey 1995) was identified at position -413, 85 bp away from the proposed transcription start site (Figure 3-3). Three pairs of short perfect repeats (CGCCGCCCA, TTTGTTG, and GAGAAGTTCA) were found 5' to the proposed CAAT box.

Translation of the *tol* ORF identified a 1011 amino acid (aa) polypeptide (Figure 3-3) with a calculated M_r of 113712. TOL has a predicted isoelectric point of 4.67 and is made up of 40% non-polar and 26% charged residues. The carboxyl-terminal portion of TOL (amino-acids position 837 to 1011) is rather hydrophilic, composed of 30% non-polar and 30% charged residues (see Hydrophobicity Plot in Appendix 8.3). BLAST (Altschul *et al.*, 1990) and FASTA algorithm (Pearson & Lipman, 1988) searches did not reveal significant similarity between TOL and any other protein sequence present in protein databases. However, a heptad repeat structure was identified from amino acid position 177-211 (Figure 3-4A and B), as predicted by the COILS program (Lupas *et al.*, 1991). A coiled-coil is an α-helical bundle that is thought to wind into a superhelix, with the hydrophobic residues (**a** and **d**) forming the hydrophobic packing interface (for review see Lupas, 1996). Coiled-coil domains play structural roles in numerous fibrous proteins (Pauling & Corey, 1951; Crick, 1953) and are also a dimerization motif in the leucine-zipper class of transcription factors (Landschulz *et al.*, 1988). The putative coiled-coil domain in TOL had five heptad repeats and contained mostly hydrophobic residues in position **a** and **d** with some charged residues in position **b**, **c**, **e**, **f**, and **g**. Occasional polar residues in the core (**a** and **d**) (such as the case in TOL) favors dimerization over trimer- or tetramerization, since they can still be partly solvated (Harbury *et al.*, 1993; Lumb & Kim, 1995).

A putative leucine-rich repeat (LRR) was found in the carboxyl-terminal portion of TOL (position 804-823; Figure 3-4A). LRRs, which have been found in numerous proteins, are thought to mediate protein-protein interactions (For reviews, see Buchanan & Gay, 1996; Kobe & Deisenhofer, 1994). Each LRR contributes an exposed β-sheet that could participate in strong interactions with other proteins. The LRR in TOL is predicted to form a β-sheet by both the Chou-Fasman (Chou and Fasman, 1978) and the Robson-Garnier algorithms (Garnier *et al.*, 1978). Although LRRs are usually present in tandem arrays, at least three proteins with a single LRR have been reported; Rev (HIV-1 nuclear regulatory protein; Malim *et al.*, 1989), GPIbβ (Human platelet glycoprotein Ib β subunit; Lopez *et al.*, 1988) and GPIX (Human platelet

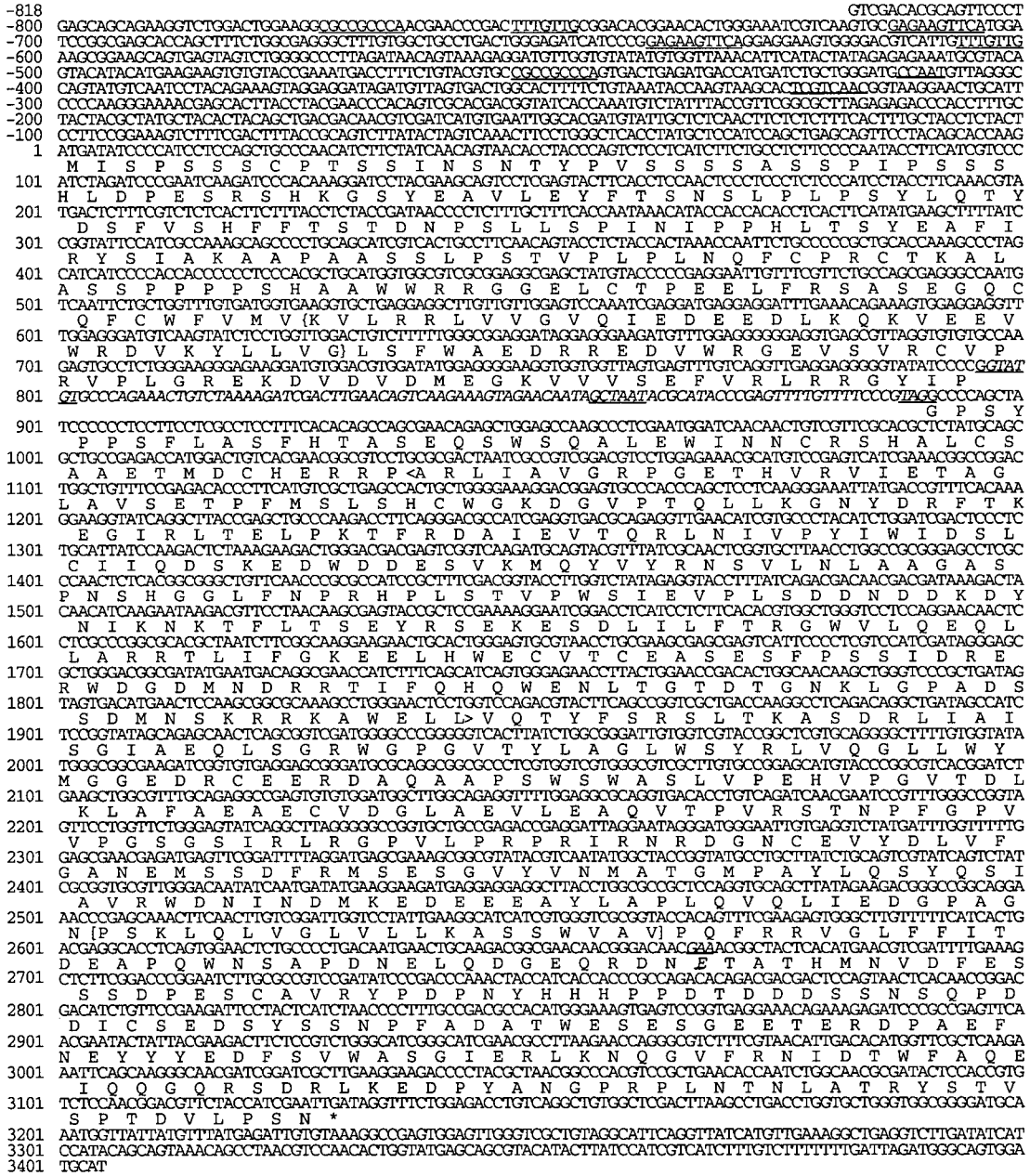


Figure 3-3. Nucleotide and amino acid sequence of the *tol*$^{+}$ gene of *N. crassa*. The nucleotide sequence (4.2-kb *Sal*I-*Nsi*I fragment) and the predicted TOL protein sequence are shown. The intron sequence is italicized and the 5', 3' and internal splicing consensus sequences are underlined. Sequences matching the consensus for a transcriptional start site and CAAT box are underlined in bold. Short repetitive sequences (3 pairs) in the promoter region are underlined. The putative coiled-coil domain, the leucine-rich repeat (LRR) and the SET domain (a region of homology between HET-SIX, HET-E and TOL) are bracketed with {}, [] and <>, respectively. The original *tol* mutation, E858 (GAA) → Stop (TAA) is underlined in bold and italicized. The GenBank accession number for this sequence is AF085183.

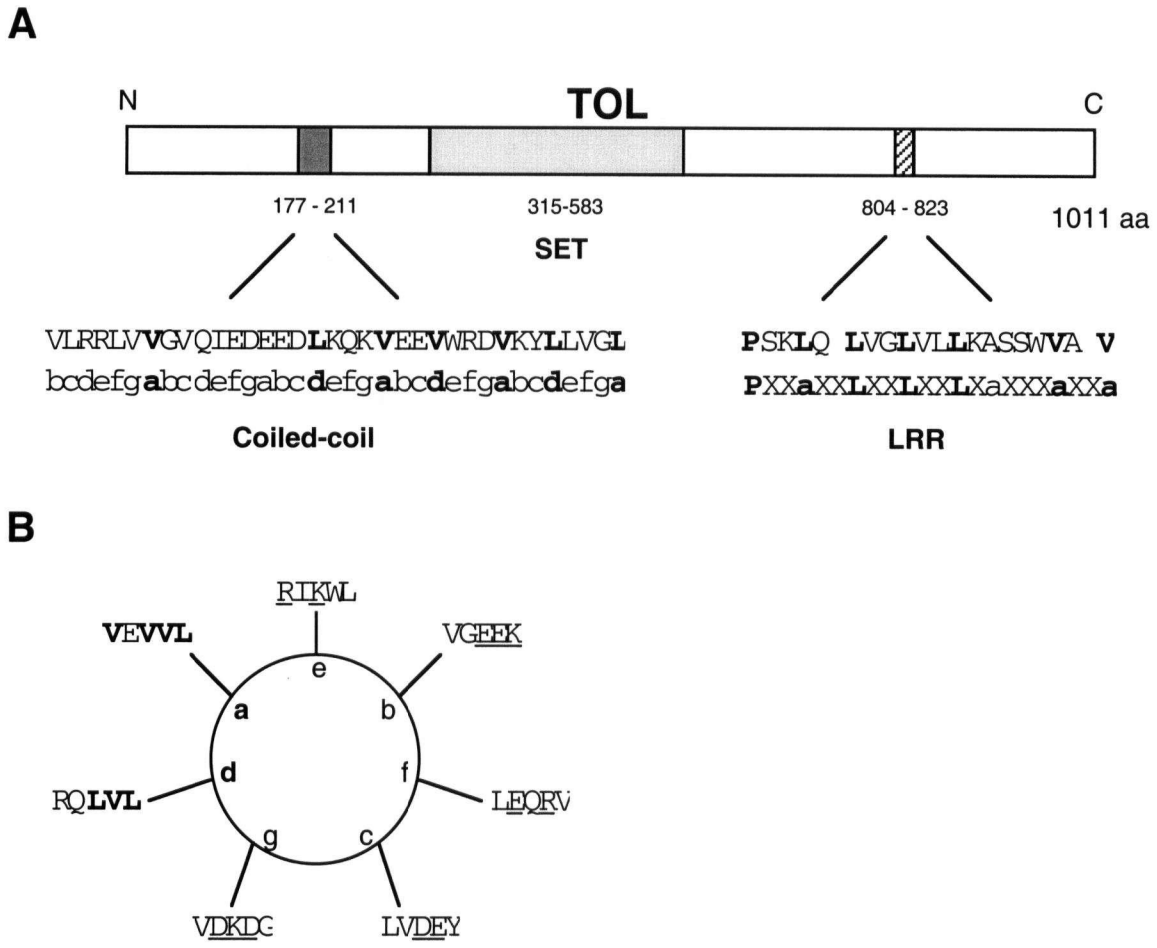


Figure 3-4. Structural features identified in TOL. (A) The amino acid sequence of the coiled-coil domain (position 177-211) in TOL is shown with a schematic heptad position a-g underneath. The leucine-rich repeat (LRR; amino-acid position 804-823) is shown and the consensus for *Arabidopsis thaliana RPS2* LRR (Mindrinos *et al.*, 1994) is shown underneath. For the LRR, an "X" stands for an arbitrary amino acid while an "a" stands for an aliphatic amino acid. The SET domain (position 315-583) represents a region of homology between HET-SIX, HET-E and TOL (see figure 3-5). (B) A helical wheel representation of the coiled-coil domain in TOL. The hydrophobic residues of the heptad repeats are in bold while charged residues are underlined.

glycoprotein IX; Hickey *et al.*, 1989). These single LRRs are thought to be functionally important (Malim *et al.*, 1989; Noris *et al.*, 1997).

A region of similarity has been detected between TOL and the predicted products of the *het-6* locus of *N. crassa* and *het-e* locus in *P. anserina* (Smith *et al.*, 2000b) (Figure 3-5). This region, hereafter referred to as the SET domain (HET-SIX, HET-E and TOL), corresponds to several conserved stretches of amino acids that span 269-aa in TOL (from position 315 to 583). This region is distinct from the GTP-binding domain and the β-transducin-like WD40 repeats in HET-E and the LRR and coiled-coil domains in TOL (see section 1.4), and thus may represent a domain necessary for vegetative incompatibility that is in common between all three *het* interactions.

A search on the database in the Neurospora Genome Project (Munich Information Center for Protein Sequences; http://www.mips.biochem.mpg.de/) revealed two ORFs located on LG II that encode predicted polypeptides with similarity to TOL. Predicted polypeptide from clone B3E4 contains sequence simlarity with TOL from amino-acid position 310 to 658 (p-value = 3×10^{-25}), which spans the SET domain. Predicted polypeptide from clone B14D6 has similarity with TOL from position 119 to 635 (p-value = 1.4×10^{-22}), which spans the coiled-coil domain and the SET domain; the coiled-coil domain is not conserved in clone B14D6. The two clones do not have significant DNA identity with *tol*.

3.3.5 Complementation of the *tol* mutant and DNA sequence analysis of the *tol*$^-$ allele

The original *tol* mutation was identified in an *A/a* partial diploid strain that had escaped from inhibited to wild-type growth rates (Newmeyer, 1970). The mutation was subsequently mapped to LGIV, near *trp-4*. To confirm that the transformation reduction result is not an artifact and that the *tol* gene was cloned, complementation experiments with the *tol*$^-$ mutant was performed and the DNA sequence of the *tol*$^-$ allele was determined (N83).

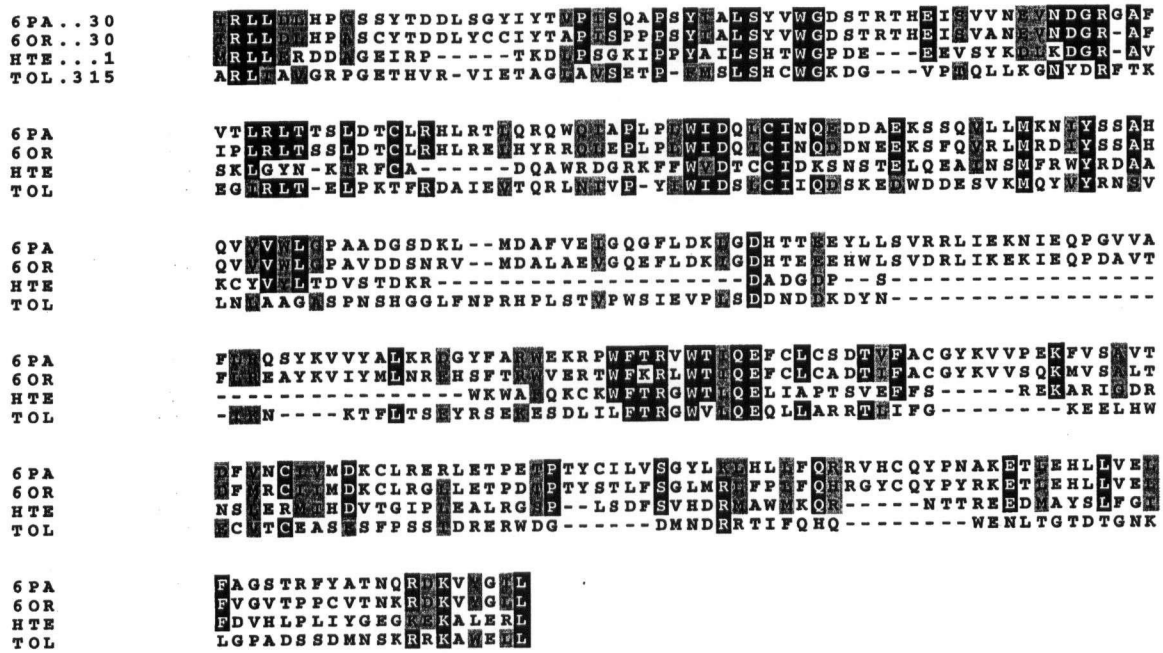


Figure 3-5. Sequence similarity among TOL and the predicted products of two *het* genes, *het-6* of *N. crassa* (denoted by 6OR and 6PA) and *het-e* of *P. anserina* (denoted by HTE). Black boxed illustrate amino-acids identity while grey boxes represent amino-acids similarity. This region of homology will be referred to as the SET domain (HET-SIX, HET-E, and TOL). Reprinted from Saupe (2000).

A pOKE103-*tol*$^+$ construct containing the 6.9 Kb *Eco*RI-*Eco*RI fragment (pOKE-EE6.9) (Figure 3-5) and a vector control (pOKE103) were transformed into *tol a* spheroplasts (strain R4-71). Homokaryotic *pan-2*$^+$ *tol a* (pOKE103 and pOKE-EE6.9) transformants were isolated and subjected to heterokaryon tests with *tol*$^+$ *A* (I-20-26) and *tol A* (I-20-41) testers. As expected, homokaryotic *pan-2*$^+$ *tol a* transformants containing only the vector (pOKE103) formed a vigorous heterokaryon with the *tol A* (I-20-41) strain, but formed an incompatible heterokaryon with the *tol*$^+$ *A* tester (I-20-26). In contrast, the *pan-2*$^+$ *tol a* (pOKE-EE6.9) transformants exhibited typical mating type associated incompatibility with both *tol A* and *tol*$^+$ *A* testers. These data indicated the original *tol* mutant phenotype was reverted to wild-type (Tol$^+$) by the introduction of the pOKE-EE6.9 clone. To confirm further that *tol* was cloned, the DNA sequence of the *tol*$^-$ mutant allele was determined. The *tol*$^-$ allele (N83) contained a transversion mutation at position 2666 (G→T) that resulted in the change of a glutamic-acid codon (GAA) into a stop codon (TAA) at amino acid 858 (Figure 3-3).

3.4 DISCUSSION

This chapter describes the molecular cloning and DNA sequence analysis of *tol*, a mediator of mating-type associated incompatibility in *N. crassa.* The *tol* locus is the only molecularly characterized mediator of allelic incompatibility from fungi. Extragenic suppressors for allelic incompatibility loci such as *N. crassa het-c* (N. L. Glass, unpublished data) and *P. anserina het-s* (cited in Coustou *et al.*, 1999) have been isolated but they have not been molecularly characterized (see section 1.4). Non-allelic suppressors of incompatibility have been characterized in *P. anserina* and encode proteins thought to be involved in developmental processes and signal transduction (Loubradou *et al.*, 1997, 1999; Barreau *et al.*, 1998) (see section 1.4).

Since heterokaryon incompatibility is a phenomenon leading to hyphal compartmentation and death, it is impossible to isolate components of the *het* system by virtue of positive screening using functional complementation. A combination of genetic mapping, chromosome walking and functional assays was utilized to clone the *tol* locus. The *het-c* and *het-6* loci of *N. crassa* were cloned using similar methods (Saupe *et al.*, 1996a; Smith *et al.*, 2000b), whereas *het-c*, *het-e* and *het-s* of *P. anserina* were isolated using a combination of SIB selection and phenotypic expression (Saupe *et al.*, 1994; Saupe *et al.*, 1995a; Turcq *et al.*, 1990) (see section 1.4). Mutants in *mod*ifier (*mod*) genes of *P. anserina* have phenotypes (sexual and/or vegetative) other than compatibility which allows their cloning by complementation (Loubradou *et al.*, 1997, 1999; Barreau *et al.*, 1998).

The Tol^+ activity (and hence the *tol* ORF) was identified by a transformation reduction assay utilizing the fact that co-transforming the *mat A-1* and tol^+ genes into a strain with *tol a* background induces cell death and hence reduces transformation frequencies. The successful cloning of the *tol* gene was confirmed by complementation of the tol^- mutant and the sequencing of the tol^- alleles. The predicted TOL polypeptide was not identified as a functional homolog of any other proteins found in the genome database. However, it contains two protein-protein interaction domains, the leucine-rich repeat (LRR) and coiled-coil domain. A LRR is implicated in the mediation of incompatibility in MAT A-1 (see Chapter 2). Therefore, protein-protein interaction between TOL and other protein(s), such as MAT A-1, could be an important factor in mating-type incompatibility. A region of homology is found among TOL, HET-6 of *N. crassa* and HET-E of *P. anserina* (the SET domain; Figure 3-5). HET-6 and HET-E are both *het* genes involved in mediating non-allelic incompatibility (Smith *et al.*, 2000b; Saupe *et al.*, 1995a). The HET-E polypeptide contain a GTP-binding domain and a WD40-repeats (as found in all Gβ proteins) whereas HET-6 does not contain any identifiable functional motif. The GTP-binding activity and the number of WD40 repeats are crucial for HET-E function (Saupe *et al.*, 1995a; Espagne *et al.*, 1997). The region of similarity (a span of 269-aa in the case for TOL) is distinct

from the LRR, WD40, coiled-coil and GTP-binding domain. This is the first time that sequence similarity between gene products of different *het* systems has been reported. Saupe (2000) has raised the possibility that this region of homology may represent some sort of "death domain controlling common downstream targets". Although it remains to be determined whether this region may represent a functional domain of any importance, the similarity provides encouraging evidence that different *het* systems may share a common biochemical pathway.

Two ORFs that encode a predicted polypeptides similar to TOL have been discovered on LG II. It is not clear if the two ORFs are expressed and what their function might be. It is possible that they are related to *tol* and that they may be of the same gene family. Future study of the two *tol*-like ORFs may give us hints on the evolution of *tol* and how *tol* mediates mating-type-associated incompatibility. Since the region of similarity between TOL and the two ORFs spans the SET domain, they may confer function for heterokaryon incompatibility, or even represent unidentified *het* loci in *N. crassa*. Further investigation is required to determine if these *tol*-like sequences have any role in heterokaryon incompatibility.

Some *het* genes have cellular functions other than to limit heterokaryosis (For review, see section 1.4). The original *tol* mutant shows normal vegetative growth and sexual reproduction. However, the *tol* mutation has been shown to suppress the phenotype of a *fmf-1* mutant (Johnson, 1979), which is sterile as both a male and a female in crosses to a wild-type partner. Fertility is restored when a *fmf-1 tol* strain is crossed to a *fmf-1*$^{+}$ *tol* strain. It is not clear how mutations in *tol*, which do not affect sexual development, can suppress the fertility defect of *fmf-1*.

Some species within the genus *Neurospora* do not exhibit mating-type-associated incompatibility. This fact is presumably due to the presence of *tol* mutations within these species that suppress mating-type-associated incompatibility. In the heterothallic species *N. sitophila*, isogenic strains that differ only in mating-type will form a vigorous heterokaryon. However,

when *mat A* and *mat a* from *N. sitophila* were introgressed into *N. crassa,* mating-type associated incompatibility was observed (Perkins, 1977). In the pseudohomothallic species, *N. tetrasperma*, opposite-mating type nuclei normally reside in a common cytoplasm. Similar to the results observed with *N. sitophila*, *mat A* and *mat a* from *N. tetrasperma* displayed mating-type incompatibility when introgressed into *N. crassa* (Metzenberg and Ahlgren, 1973). The introgression of *tol*$^{+}$ from *N. crassa* into *N. tetrasperma* induced mating-type associated incompatibility and disrupted the pseudohomothallic nature of the species (Jacobson, 1992). All of these data, and the fact that all suppressors for mating-type incompatibility that have been isolated are allelic to *tol* (Vellani *et al.*, 1994), argue that *tol* is the major mediator of mating-type associated incompatibility. The evolutionary history and selection for or against mating-type associated incompatibility in the different species within the genus *Neurospora* remains an enigma. The molecular characterization of *tol* from *N. crassa* and related species will provide the necessary tools to address this question.

It is believed that heterokaryon incompatibility is a universal phenomenon among filamentous ascomycetes and basidiomycetes (Glass & Kuldau, 1992; see section 1.4). It has been proposed that vegetative incompatibility may protect an individual from the transfer of deleterious cytoplasmic factors (Caten, 1972). Cytoplasmic transfer of hypovirulence-associated dsRNA virus in *Cryphonectria parasitica* and detrimental KalDNA plasmids in *N. crassa* are reduced by vegetative incompatibility (Anagnostakis, 1982a; Debets *et al.*, 1994). In black *Aspergilli*, vegetative incompatibility completely blocks transfer of mycoviruses (Van Diepeningen *et al.*, 1997). Vegetative incompatibility mediated by mating-type could be selected for by two mechanisms. The first mechanism is that incompatibility mediated by mating type could promote outbreeding by eliminating the possibility of heterokaryon formation with opposite mating-type siblings. However, in a outbreeding population, the formation of vegetative heterokaryons is also excluded by differences at any of 10 additional *het* loci in *N. crassa*; *N. crassa* populations are highly polymorphic for *het* loci (Mylyk, 1976a). A second

mechanism is that *mat A-1* and *mat a-1* evolved solely for sexual reproduction, but that molecular divergence of *tol* resulted in mating-type associated incompatibility during vegetative growth. Since the mating-type locus is always polymorphic in populations in a heterothallic species like *N. crassa*, mating-type associated incompatibility would be an efficient way to restrict heterokaryon formation with 50% of the population, even in the absence of polymorphisms at other *het* loci.

Mating-type-associated incompatibility has been reported in other fungal species, such as in *Ascobolus stercorarius* (Bistis, 1994), *Aspergillus heterothallicus* (Kwon & Raper, 1967) and *Sordaria brevicollis* (J. Bond, personal communication). It is possible that the phenomenon of mating-type associated incompatibility is even more widespread in filamentous ascomycetes, but cannot be assessed because of the lack of isogenic strains that differ only at the mating-type locus. The characterization of the three major components of mating-type associated incompatibility, *mat A–1*, *mat a-1* and *tol*, will provide tools to analyze the molecular mechanism of mating-type associated incompatibility in filamentous fungi and will facilitate the challenge of delineating the molecular mechanism of growth inhibition and cell death that is a characteristic feature of this phenomenon.

4. Characterization of *tol* and Isolation of *top* (TOL-interacting Protein) Genes

Some results from this chapter are published in Shiu & Glass (1999).

4.1 INTRODUCTION

The cloning and sequencing of *tol* is described in chapter 3 of this thesis. The *tol* gene encodes a putative 1011-amino acid polypeptide with a coiled-coil domain and a leucine-rich repeat. Characterization of mutant alleles in chapter 2 demonstrates that a leucine-rich repeat is required for incompatibility function in *mat A-1*. Attempts were made in this study to identify region(s) of the TOL protein required for mediating mating-type-associated incompatibility.

Since the mutation in the original *tol*$^-$ strain (allele N83) occurred at the 3' end of *tol* (near the carboxyl-terminus of TOL), it is possible that the N83 mutant is not a *tol*-null mutant and may retain partial function. Attempts were made to obtain additional *tol* mutants by repeat induced point (RIP) mutation (Selker, 1990) and to analyze their phenotypes. RIP is a mechanism in *Neurospora* that causes duplicated sequences to undergo multiple G-C to A-T transition mutations during sexual reproduction. The RIP phenomenon is utilized as a means to introduce mutations in a gene *in vivo*. A given gene can be mutagenized by transformation of a copy of that gene into a recipient and taking the transformant through a cross. Before karyogamy, but during mitotic divisions within ascogenous hyphae, the resident as well as the introduced ectopic copy undergo GC→AT transition mutations. The frequency of the mutations range from 10-70%, depending on the gene used, the insertion site, and the size of the fragment introduced (Irelan *et al.*, 1994).

One enigma of mating-type associated incompatibility is the nature of relationship between mating-type proteins and TOL. One possibility is that *tol* is a downstream effector of the incompatibility pathway whose expression is regulated by the mating-type proteins. Expression patterns of *tol* were analyzed in this study in order to shed light on plausible mechanism of interactions between mating-type proteins and *tol*.

The mating-type genes have seemingly opposite functions in different stages of development. Although the mating-type of two isolates must differ for sexual development to occur, the alternative alleles trigger incompatibility during vegetative growth. Since mating-type proteins are required after fertilization, presumably for nuclear recognition during proliferation and partitioning of opposite mating-type nuclei in the crozier (Shiu & Glass, 2000), the incompatibility reaction between antagonistic *mat A-1* and *mat a-1* genes must be suppressed during sexual development. The possibility of differential regulation of *tol* during sexual development was also examined in this study.

Vegetative incompatibility between opposite mating-types is due to the molecular actions of MAT A-1, MAT a-1 and TOL. The MAT A-1 and MAT a-1 gene products are transcriptional regulators while the molecular function of TOL is not apparent, although it contains putative protein-protein interaction domains. To understand the role of TOL in mating-type heterokaryon incompatibility and to identify other proteins involved in this process, several putative TOL-interacting proteins (*top*) were identified using the yeast 2-hybrid system. The yeast 2-hybrid system detects protein-protein interactions *in vivo* (Fields & Song, 1989). It utilizes the property of eukaryotic transcriptional activators, which consist of two separable domains - the DNA-binding domain (BD) and the transcriptional activation domain (AD). When a protein X, which is fused with BD, interacts with a protein Y, which is fused with AD, the transcriptional activator is reconstituted and transcription of reporter genes is therefore activated.

Many *Neurospora* species do not exhibit mating-type incompatibility. Examples are the heterothallic *N. sitophila* and pseudohomothallic *N. tetrasperma* (Dodge, 1935; Mishra, 1971). However, the mating-type genes of these two species are capable of inducing mating-type incompatibility when introgressed into *N. crassa* background (Metzenberg & Ahlgren, 1973; Perkins, 1977). In the case for *N. tetrasperma*, the suppression of mating-type incompatibility is due to the presence of the inactive tol^T locus (Jacobson, 1992). When tol^C is introgressed *from N. crassa* into *N. tetrasperma*, the heterokaryotic nature of that species breaks down upon germination (Jacobson, 1992; see chapter 5). It is possible that the absence of Tol^+ activity is also responsible for the compatibility in other heterothallic and homothallic species. It is important to determine if *tol* is present in these species and what activity it might have.

4.2 MATERIALS AND METHODS

4.2.1 Strains, media and culturing methods

The *N. crassa* strains used in the study are listed in Table 4-1. Construction of strains R5-27 and R5-28 was described in section 3.2.2. The A/a partial diploid strain (D25) was constructed by selecting dual-mating (*A* and *a*) progeny in the cross R5-27 X R5-28. Culturing and crossing, using Vogel's (Vogel, 1964) and Westergaard's (Westergaard & Mitchell, 1947) media respectively, were performed as previously described, with modification (Davis & Deserres, 1970; Perkins, 1986). Mating tests were performed using *fl a* (FGSC 4347) and *fl A* (FGSC 4317) tester strains (Perkins *et al.*, 1989).

For perithecial RNA extraction, crosses were performed in petri dishes containing Westergaard medium layered with Miracloth membrane (Calbiochem, La Jolla, CA). Perithecia were scraped from the Miracloth and RNA was extracted as described below.

Strain	Genotype	Origin
R4-71	*his-5 tol trp-4; pan-2 a*	This study
R4-72	*ad-3B; trp-4 cot-1; pan-2 A*	This study
-	*his-5 A*	FGSC 456
A2	*ade-3A nic-2 cyh-1 a*	A.J.F. Griffiths
I-20-41	*ade-3B arg-1; tol A*	A.J.F. Griffiths
I-20-26	*ade-3B arg-1 A*	A.J.F. Griffiths
I-10-1	*ade-3A nic-2; tol a*	A.J.F. Griffiths
74-OR23-1VA	Oak Ridge *A*	FGSC 2489
I-1-51	*ade-3A nic-2 a*	A.J.F. Griffiths
OR8-1a	Oak Ridge *a*	FGSC 988
RLM44-02	*thi-4; lys-1* Δ *mat A*	FGSC 8292
R5-28	*T(IL→IIR)39311 ser-3 A* (Mauriceville background on LG IV)	This study
R5-27	*un-3; his-5 tol trp-4 a*	This study
D25	*his-5 tol A/a* partial duplication	This study
R2-11	*nic-2 al-1; pan-1* a^{m33}	N.L.G. Glass
-	*fl a*	FGSC 4347
-	*fl A*	FGSC 4317
P405	*N. intermedia a*	FGSC1940
Kirbyville 8127	*N. discreta a*	FGSC 4378
SK-1^{S} *a*	*N. sitophila a*	FGSC 5941
85A	*N. tetrasperma A*	FGSC 1270
WFS 5000	*N. terricola* (homothallic)	FGSC 1889
PR300	*N. dogei* (homothallic)	FGSC 1692
G349	*N. galapagosensis* (homothallic)	FGSC 4628
A-236	*N. linolata* (homothallic)	FGSC 1910
TRTC 51327	*N. pannonica* (homothallic)	FGSC 7221
N200	*N. africana* (homothallic)	FGSC 1740

Table 4-1. List of *Neurospora crassa* strains used in this study. All strains are *N. crassa* Oak Ridge compatible (unless otherwise stated) and all *tol* alleles are N83. FGSC = Fungal Genetic Stock Center.

4.2.2 *tol* plasmid construction and transformation assay

The 6.9 kb *tol* fragment was subcloned into pCB1004 (a hygromycin resistant vector; Carroll *et al.*, 1994) from cosmids X25:D7, which contains Tol^+ activity. C-terminal, N-terminal, and internal deletion constructs of *tol* were made by using various restriction endonucleases. A frameshift mutation of *tol* was constructed by cutting the 6.9-kb *tol*/pCB plasmid with *Xho*I restriction endonuclease. The linearized plasmid (with the *Xho*I overhang) was then end-filled by Klenow fragment of *Escherichia coli* DNA polymerase I and religated. The result is a +1 frameshift mutation in *tol* after amino-acid position 50; the frameshift mutation was verified by DNA sequencing. *E. coli* DH5α (Hanahan, 1983) was used as bacterial host strain for all plasmid amplification.

Spheroplast preparation and transformation were performed as previously described (Schweizer *et al.*, 1981; Vollmer & Yanofsky, 1986) with the exception of selecting for resistance to hygromycin B (250 units/ml; Calbiochem). Constructs of *tol* were assayed for Tol^+ activity by co-transforming the cosmids (conferring hygromycin resistance) with a *mat A-1*-containing pOKE103 construct (pOKE103 has a *pan-2* selectable marker; J. Grotelueschen and R.L. Metzenberg, unpublished results) into strain R4-71 (*his-5 tol trp-4; pan-2 a*) and strain R4-72 (*ade-3B; trp-4 cot-1; pan-2 A*) spheroplasts (see section 3.2.2).

4.2.3 Isolation of *tol*-RIP mutants

In *N. crassa*, duplicated sequences undergo methylation and GC→AT mutation during mitotic divisions that precede karyogamy (Selker, 1990). To order to obtain *tol* mutants, an internal *tol* copy (2.7 kb *Eco*RI fragment; DNA position 3-2735) was transformed into strain A2 (*ad-3A nic-2 cyh-1 a*) and the transformant was crossed with a *his-5 A* strain. Since *N. crassa* transformants are heterokaryotic and contain both transformed and untransformed nuclei, a homokaryotic strain (4-9) with only transformed nuclei was isolated according to Ebbole & Sachs (1990). *his-5*$^+$ progeny (which contain the potentially mutated *tol* locus from parent 4-9)

were isolated and assayed for Tol^+ activity in a heterokaryon test. For heterokaryon tests, 2 µl conidial suspensions (10^7/ml) of two strains containing different auxotrophic markers were co-inoculated onto vegetative growth media (Vogel, 1964). A compatible heterokaryon forms a vigorously conidiating culture after 3 days of incubation with a mean growth rate of 7 cm/day. Mating-type incompatible heterokaryons are usually aconidial and have a growth rate of approximately 0.7 cm/day (Vellani *et al.* 1994). Progeny without Tol^+ activity were subjected to southern and RFLP analysis. The presence of altered restriction patterns in the RIP progeny is indicative of the GC→AT mutations that occur during the RIP process.

4.2.4 Nucleic acid isolation/hybridization and Reverse Transcriptase PCR analysis

Standard molecular biology procedures were used throughout (Sambrook *et al.*, 1989). Genomic DNA isolation from *Neurospora* was adapted from Oakley *et al.*, (1987). Total RNA was extracted according to Logemann *et al.* (1987) and enriched for poly(A)+ using the OligoTex mRNA kit (Qiagen, Chatsworth, CA). Gel electrophoresis and nucleic acids transfer to Nylon filters (Schleicher and Schuell, Keene, NH) and Zeta membranes (Bio-Rad, Hercules, CA) were performed according to manufacturer's specifications. [α-^{32}P] dCTP (Amersham, Oakville, ON) labeled probes were generated from digested DNA using the T7 QuickPrime Kit (Pharmacia, Baie d'Urfe, PQ). For the hybridization experiment on various *Neurospora* species, 3 µg of each DNA was digested with *Eco*RI restriction enzymes before they were loaded into a 0.7 % agarose gel. For reverse transcriptase-PCR (RT-PCR), cDNAs were synthesized by the Not I-d$(T)_6$ primer using the procedure from First Strand cDNA Synthesis Kit (Pharmacia). PCR of DNA and cDNA were performed using the tol 13 (5' GGGCGGAGGATAGGAGG 3'; bases 641 to 657) and tol 11 (5' CCAGCAGTGGCTCAGC 3'; bases 1144 to 1129) primers.

4.2.5 Yeast 2-hybrid system and screening of a cDNA/pAD-GAL4 library

A 2-hybrid library for *N. crassa* was obtained from the Fungal Genetic Stock Center (FGSC) as aliquots of a phage suspension. It was constructed from mycelial cDNA (from conidia of FGSC 2489 grown in 1X Vogel's and 2% sucrose for 24 hours at 25°C) uni-directionally ligated into the HybriZAP-2.1 vector (Stratagene, La Jolla, CA). Preparation of bacterial host strains (XL1-Blue MRF' and lambda resistant XLOLR) and f1 helper phage (ExAssist) as well as amplification, titer determination and *in vivo* excision of the pAD-GAL4/mycelial cDNA phagemid (from the hybridZAP vector) were performed according to the instructional manual from the "HybridZAP-2.1 Two Hybrid Predigested Vector Kit" (hereafter referred to as the HybridZap System) (Stratagene, La Jolla, CA). The titer of the phagemid and helper phage were determined to be 3.5×10^8 and 3×10^{11} pfu/ml, respectively. Mass library *in vivo* excision of phagemid was performed using 4×10^9 XL1-Blue MRF' cells, 3.5×10^8 pfu of amplified library phagemid (which should give at least 100 fold coverage of *N. crassa* primary transcripts) and 3.5×10^9 pfu of the helper phage.

The DNA binding domain vector used as bait in the 2-hybrid library screen was named tol/pGBT9. It is a fusion protein between TOL cDNA and the GAL4 DNA binding domain. A partial *tol* cDNA was obtained from an RT-PCR reaction using primers tol ATG1 (5'CCTGGGCTCACCTATGC 3'; base -50 to -34) and tol 11 (5' CCAGCAGTGGCTCAGC 3'; bases 1144 to 1129) from FGSC 2489 mRNA; the primer set brackets the only intron found in the *tol* ORF. After cloning into a PCRII vector using the TA cloning kit (Invitrogen, San Diego, CA), the partial cDNA fragment was swapped with its genomic counterpart from the vector tol/pGEM in order to create the full-length *tol* cDNA construct (named tol cDNA/pGEM). The complete *tol* cDNA (a *Nsi*I-*Nsi*I fragemt) was then introduced into the *Pst*I site of pGBT9 (Clontech, Palo Alto, CA) to create an in-frame fusion TOL/GAL4-BD construct (named tol/pGBT9). The in-frame fusion of tol/pGBT9 was confirmed by DNA sequencing.

The pAD-GAL4/mycelial cDNA target plasmid library and the tol/pGBT9 bait plasmid were transformed into yeast and were screened for protein-protein interactions between bait and target proteins by transcriptional activation of reporter genes. The reporter genes in yeast in this case were *his3* and *lacZ*, and therefore the positive interactors should give a His^+ and blue color colony. The yeast YRG-2 strain (*Matα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538* LYS::UAS_{GAL1}-$TATA_{GAL1}$-HIS3 URA3::$UAS_{GAL4\ 17mers(x3)}$-$TATA_{CYC1}$-*lacZ*) was used as host for transformation. Transformation of bait and target plasmids into yeast was performed according to the manual from the "YRG-2 Yeast Competent Cell Kits" (Stratagene, La Jolla, CA); 10 μg of each plasmid was used to transform 1 ml of yeast competent cells (with an efficiency of 4 x 10^5 colonies/ml). The transformed yeast cells were plated in solid SD (synthetic minimal) media (1 ml per 150-mm plate) containing supplements and 5 mM 3AT (3-aminotriazole, a histidine antimetabolite) in order to select for positives with strong interactions. Cells grown in 3AT SD media without supplement of histidine were replicated, and were tested for $LacZ^+$ activity in a filter lift blue/white assay (according to procedure from HybridZap System); their tolerance for different concentration of 3AT (from 5 mM to 25 mM) was also tested.

Isolation of plasmid DNA from yeast was performed according to the HybridZap System manual. The presence and size of inserts in pAD-GAL4 plasmid in the 2-hybrid positives were determined by PCR (according to protocol from the "GAL4 Vector Recombinant Screening Kit"; Stratagene) using the 5'AD (5' AGGGATGTTTAATACCACTAC 3'; nt 745-765) and 3'AD (5' GCACAGTTGAAGTGAACTTGC 3'; nt 929-949) primers. Partial sequence of the insert was determined by one run of DNA sequencing from the 5' end of the gel-purified PCR products. The pAD-GAL4 plasmids were transformed into MAX Efficiency DH5α competent cells (Life Technologies, Rockville, MD) according to manufacturer's specification. The recovered plasmids were retransformed into yeast and the transformants were subject to the filter lift

blue/white assay for verification of interactions. Positive and negative controls for the assay were used according to the HybridZap System manual.

4.2.6 DNA sequence analyses

DNA sequences were determined using the ABI automated Taq DyeDeoxy Terminator cycle method (Mississauga, ON) at the NAPS unit, Biotechnology Laboratory, University of British Columbia. Computer sequence analyses for protein and DNA were done using the MacVector/AssemblyLIGN software (International Biotechnologies, New Haven, CT) and GCG package available from the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

4.3 RESULTS

4.3.1 Functional analysis of *tol* constructs

Several *tol* deletion and frameshift constructs were obtained to determine what portions of TOL are important for function (Figure 4-1). These constructs were subcloned into a hygromycin-resistant vector and were assayed for Tol^+ activity. In the transformation assay, constructs of *tol* were co-transformed with *mat A-1* into a *tol a* strain and an *A* strain. If a construct retained Tol^+ activity, it should trigger mating-type incompatibility in the *tol a* recipient and reduce transformation frequencies.

The results from the co-transformation experiments showed that a +1 frame-shift mutation at the *Xho*I site (aa position 50) abolished Tol^+ activity, confirming that the 1011-aa ORF encoded TOL. Deletion of the portion including LRR and/or the SET domain (a region of homology between HET-SIX, HET-E and TOL) also abolished Tol^+ activity. However, an internal deletion of 139 amino acids in the region before the LRR did not affect TOL function. N- terminal deletion constructs indicated that a region between aa position 98 and 531, which includes the coiled-coil domain and part of the SET domain, was also essential.

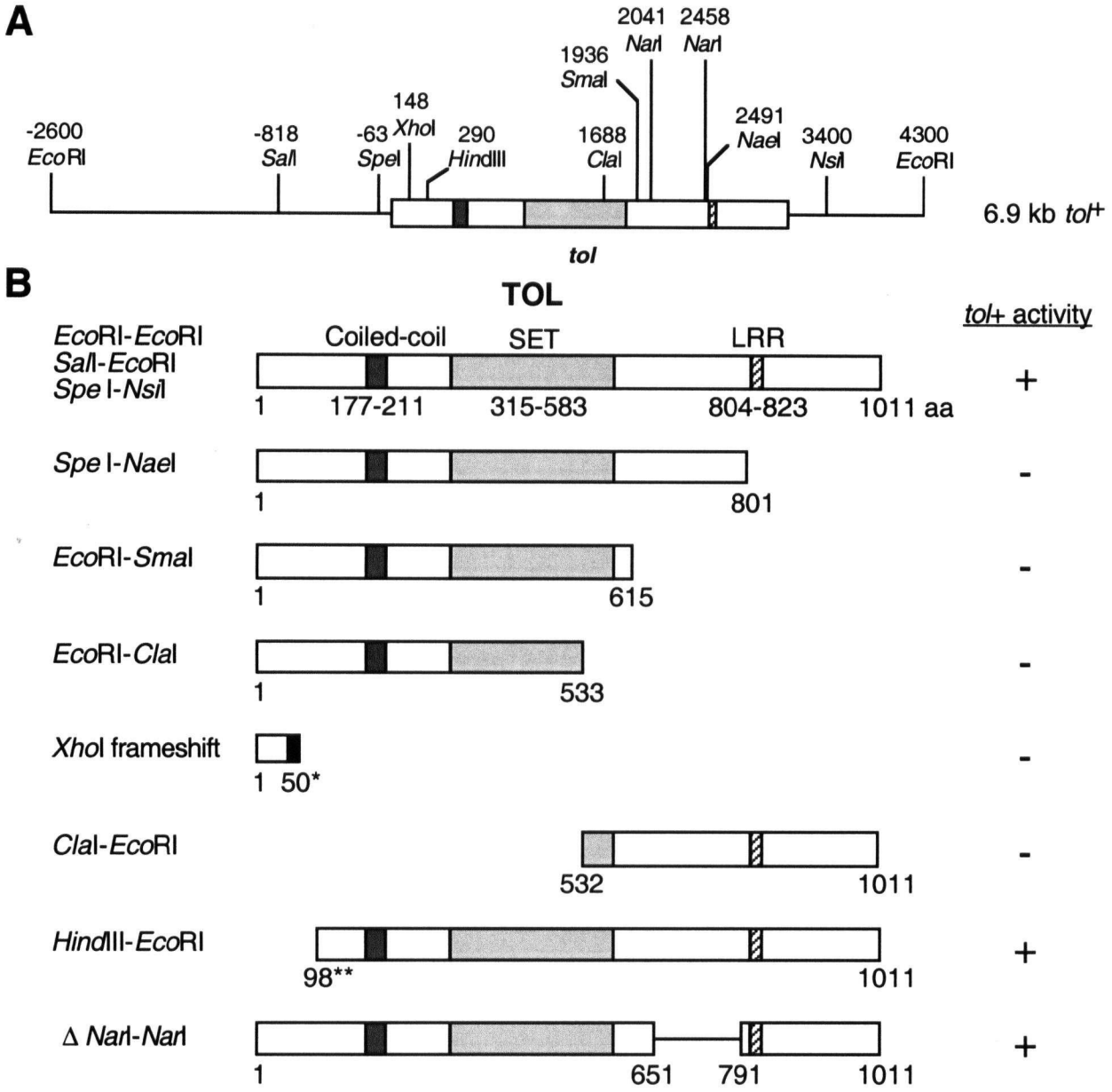


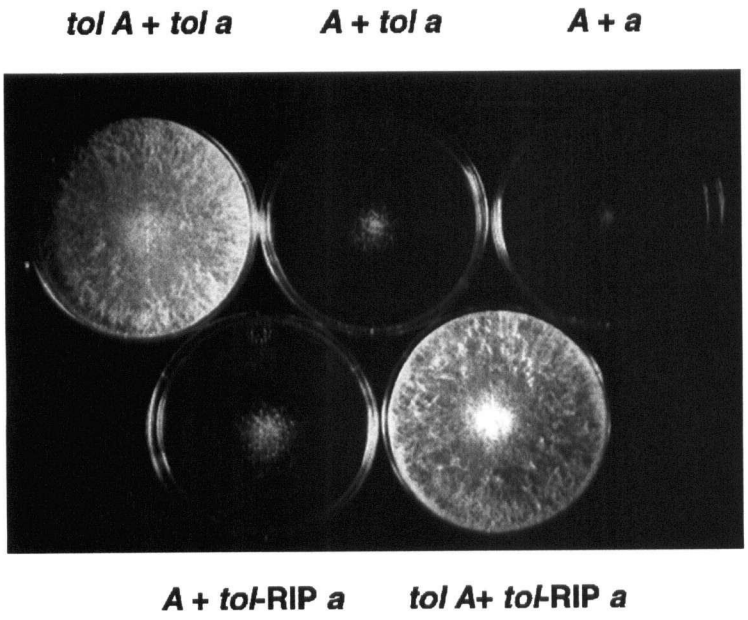
Figure 4-1. Functional analysis of *tol* deletion and frame-shift constructs. (A) Schematic representation of the 6.9 kb *tol* DNA sequence including relevant restriction sites. DNA position is given according to Figure 3-3. (B) Schematic diagram of various *tol* constructs used to examine functional domains of TOL. The three putative functional motifs in TOL are the coiled-coil domain, the LRR (leucine-rich repeat) and the SET domain (a region of homology between HET-SIX, HET-E, and TOL; see Figure 3-5). Amino acid position is according to Figure 3-3. A *tol* construct is considered to have Tol^+ activity if it results in a reduction in transformation frequency (at least 20-fold) when co-transformed with *mat A-1* into *tol a* spheroplasts, as compared to frequencies when co-transformed into *A* spheroplasts. **Xho*I frameshift contains 50 functional amino acids and terminates at position 68. ** The *Hin*dIII-*Eco*RI construct could use a vector ATG as start codon. On the other hand, it could use an internal alternative in-frame ATG site (at amino-acid position M174) to initiate translation. Alternative in-frame initiation of translation is also inferred to occur with *het-6* (Smith *et al.*, 2000b).

4.3.2 Isolation of *tol*-RIP mutants

The mutation in the original *tol* allele (N83) occurred at the 3' end of the gene (near the carboxyl-terminus of TOL), and therefore it is possible that TOL retains partial function. Attempts were made to obtain additional *tol* mutants by repeat induced point (RIP) mutation (Selker, 1990). A 2.7 kb *Eco*RV fragment (cloned into pCB1004) that contained an internal portion of *tol* (position 3 to 2735, Figure 3-3) was transformed into strain A2 (*ad-3A nic-2 cyh-1 a;* Table 4-1). A hygromycin-resistant homokaryotic transformant (#4-9) was obtained and crossed with FGSC 456 (*his-5 A*). Forty-nine *his-5*$^+$ *ade-3A nic-2 a* progeny were analyzed for Tol$^+$ activity in heterokaryon tests. Five *his-5*$^+$ *ade-3A nic-2 a* progeny formed vigorous heterokaryons with the *tol A* tester (I-20-41), indicating that the *tol* allele in these strains was not functioning to confer mating type associated incompatibility (Figure 4-2A). Heterokaryons between these same five *his-5*$^+$ *ad-3A nic-2 a* progeny and the *tol*$^+$ *A* tester (I-20-26) displayed typical heterokaryon incompatibility (Figure 4-2A), indicating that the *tol* mutation in these strains was recessive. The *tol*-RIP strains were phenotypically normal during vegetative growth and mated as either a male or a female, with similar fertility to their parental strains. Thus, these five progeny displayed a phenotype identical to the original *tol* mutant and were thus designated as *tol*-RIP strains (*tol-43*, *tol-83*, *tol-95*, *tol-106* and *tol-135*).

To confirm that the suppressor phenotype in the *tol*-RIP mutants segregated with the *tol* locus, *tol-43 a* (as a male) and *tol-83 a* (as a female) were crossed separately to *his-5 A*. Of the 30 His$^+$ progeny tested from the 2 crosses, all but one formed vigorous heterokaryons with both *tol a* (I-10-1) and *tol A* (I-20-41) strains (3.3% recombination), indicating that the new mutations in *tol-43* and *tol-83* were closely linked to the *his-5* (and hence *tol*) locus. By Southern blot and RFLP analysis, it was determined that the *tol* sequence in the *tol*-RIP strains showed restriction site changes (Figure 4-2B), consistent with the presence of transition mutations that are characteristic of sequences that have undergone RIP (Selker, 1990).

A



B

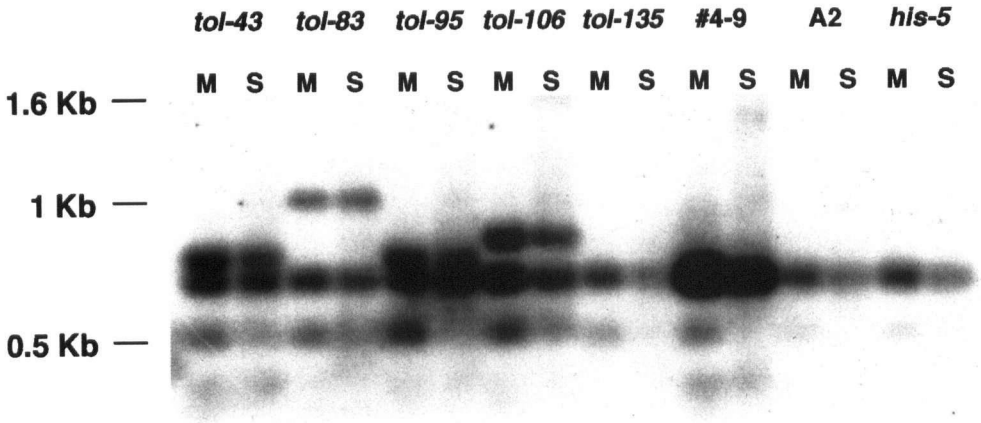


Figure 4-2. Phenotypic and genotypic analysis of *tol*-RIP mutants. (A) The top three plates are controls showing growth characteristics of compatible (*tol A* + *tol a*) and incompatible (*A* + *tol a* and *A* + *a*) heterokaryons. The bottom plates show the phenotype of RIP strain *tol-43*. The *tol-43* strain was compatible with a *tol A* strain, but incompatible with an *A* strain. Strains I-20-41 (*tol A*), I-20-26 (*A*), I-10-1 (*tol a*), I-1-51 (*a*) and R5-52 (*tol-43 a*) (Table 4-1) were used in heterokaryon tests. (B) RFLP and cytosine methylation analysis of *tol* sequences in the *tol*-RIP strains. Genomic DNA from parental strains (*his-5* and transformant #4-9) plus the transformation recipient strain (A2; Table 4-1) and each *tol*-RIP strain was digested with *Mbo*I (M; cytosine methylation-insensitive) and *Sau*3AI (S; cytosine methylation-sensitive) and probed with a 3.5 kb fragment containing *tol*. The presence of altered restriction patterns in the RIP progeny as compared to their parental controls is indicative of GC→AT transition mutations that occur during the RIP process (Selker, 1990).

4.3.3 Expression analysis of *tol* by RT-PCR

The exact role of *tol* in mediation of mating-type-associated incompatibility is unclear. One possibility is that *tol* is a downstream effector of incompatibility whose expression is regulated by the mating-type proteins. Transcripts of *tol* were not detectable by RNA hybridization analysis (P.K.T. Shiu, unpublished results); therefore the expression of *tol* was analyzed by reverse transcriptase (RT)-PCR. Primers bracketing the intron (tol 11 and tol 13) were used to amplify *tol* mRNA from growing vegetative cultures. Figure 4-3 shows that a *tol* cDNA could be detected in the *A*, *a* and a mating-type deletion strains grown in vegetative (V) and crossing (C) media (V and C; Figure 4-3). These data indicated that neither mating type constitution nor growth conditions materially affected the expression of *tol.* A *tol* cDNA could also be detected in an *A/a tol* partial diploid strain, indicating that the presence of both *mat A* and *mat a* in the same nucleus did not significantly alter activation or represssion of *tol.* Expression of *tol* was also detectable in both an incompatible (*A* + *a* inc.) and compatible heterokaryon (*A* + a^{m33}); the a^{m33} strain forms compatible heterokaryons with *A* strains, but sexual function is not affected (Griffiths & Delange, 1978).

Following fertilization, opposite mating type nuclei co-exist and divide within a common cytoplasm. Therefore, mating-type associated incompatibility mediated by *tol* must be suppressed during sexual reproduction. To determine if *tol* is transcriptionally repressed during a cross, thus allowing the co-existence of opposite mating type nuclei, the presence of the *tol* cDNA was analyzed by RT-PCR in mRNA prepared from perithecia at 3, 5, 6, and 9 days post-fertilization. Although a cDNA for *mat A-2* could easily be detected by RT-PCR from perithecial RNA preparations for all time points, a *tol* cDNA could not be detected at any post-fertilization time point (cross; Figure 4-3).

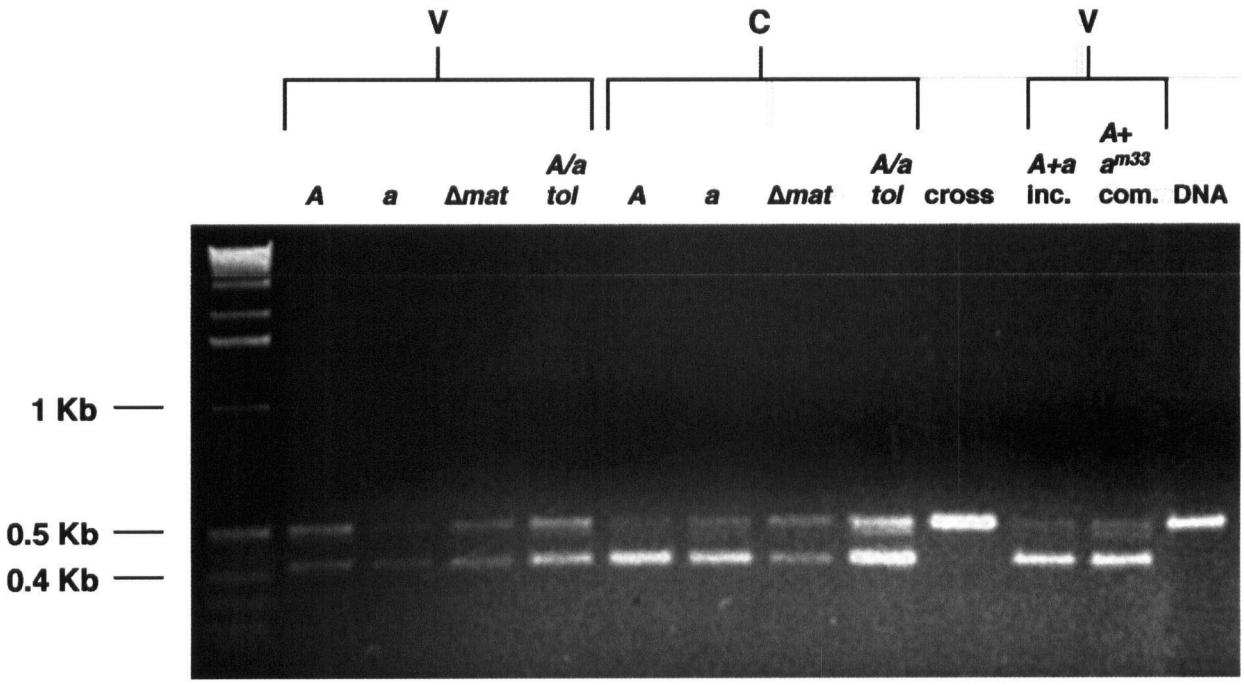


Figure 4-3. Expression of *tol* as analyzed by RT-PCR. Primers bracketing the intron (tol 11 and tol 13) were used to amplify *tol* cDNA from different mRNA preparations. The size of the genomic *tol* product is 504 bp with these primers, whereas the *tol* cDNA product is 410 bp. Strains used are as follow: 74-OR23-1VA (*A*), OR8-1a (*a*), RLM44-02 (Δ *matA*), D25 (*A/a tol*), I-20-26/I-1-51 (*A+a*) and I-20-26/R2-11 ($A+a^{m33}$). *tol* mRMA was isolated from the above cultures grown either in vegetative medium (Vogels, 1964; **V**) or crossing medium (Westergaard and Mitchell 1947; **C**) or from perithecia (**cross**). A *tol* cDNA could be detected in mRNA from all strains, with the exception of from perithecial mRNA. The quality of the perithecial mRNA samples was checked by the amplification of a *mat A-2* cDNA (using primers rI.1 and 2423-2406; Ferreira *et al.*, 1996); *mat A-2* is expressed throughout sexual development (data not shown). DNA size marker (1 kb ladder) is given on the left.

4.3.4 Phenotypic studies of *tol*⁻ mutant as complemented by different *tol* constructs

Efforts were made to study the sexual and vegetative phenotypes of Tol$^+$ transformants under native and constitutive promoters. Five different constructs of *tol* (with different promoters) were made and they were subcloned into a Pan-2$^+$ vector (pOKE103) (Table 4-2). They are tol6.9 (with the 2.6 kb native promoter), tol5.1 (with the 0.8 kb native promoter), tol4.3 (with the 63 bp native promoter), ptrpC-tol (with the *trp*C promoter fused to *tol*), and pcrp2-tol (with the *crp-2* promoter fused to *tol*). The *trp*C promoter is a constitutive promoter from the *A. nidulans trp*C gene (Mullaney *et al.*, 1985) whereas the *crp-2* is a constitutive promoter from the *N. crassa* ribosomal gene (Cujec & Tyler, 1996). A *tol*⁻ *pan-2*⁻ strain (R4-71) was transformed with the above five constructs and several homokaryotic transformants were obtained. The Pan-2$^+$ homokaryotic transformants were then tested for Tol$^+$ activity in a complementation test.

As expected, homokaryotic transformants conferring Tol$^+$ phenotype can be obtained from each of the five transformation experiments; these transformants failed to form vigorous heterokaryons with the opposite mating-type, showing that they can complement the original *tol*⁻ mutation, ectopically. They do not have a vegetative phenotype different from that of a wild-type strain. When used as males in a cross, fertility was unaffected; the number of perithecia formed, the time required for perithecia development and the maturation of spores was normal. However, Northern analysis (during vegetative growth) demonstrated that Tol$^+$ transformants from ptrpC-tol- and pcrp2-tol-mediated transformation (D and I series; Table 4-2) do not contain over-expressing *tol* transcripts (data not shown). The failure in obtaining over-expressing *tol* constructs can be due a phenomenon called quelling (Cogoni *et al*, 1996). In *N. crassa*, a transgene-induced silencing mechanism occurs that represses (reduces) expression of a transgene. It is thought to be mediated by RNA-RNA interaction between resident gene and transgene (Cogoni & Macino, 1999). Alternatively, the transgenic *tol* copy could be introduced into an ectopic site that does not allow the proper transcriptional control.

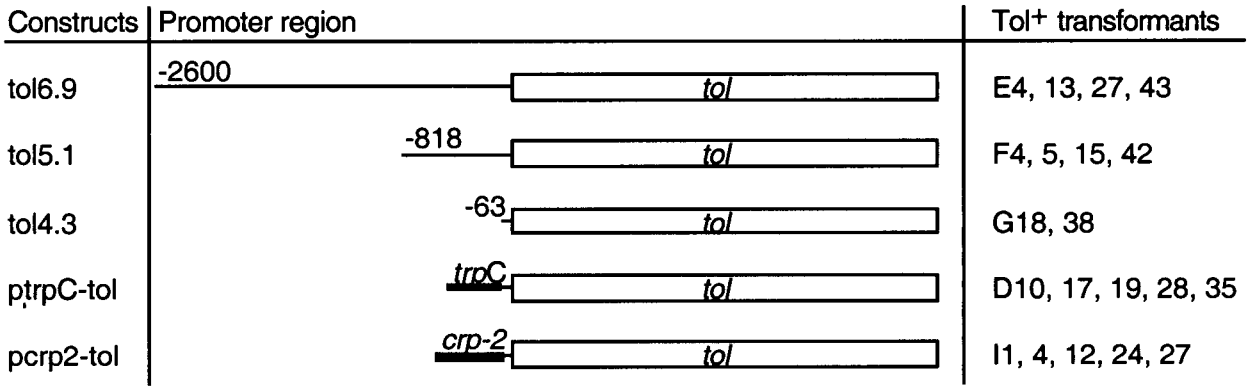


Table 4-2. Constructs used in complementation of *tol* mutant. Five constructs of *tol*, under native and constitutive promoters, are used in complementation experiment. tol6.9 (*Eco*RI-*Eco*RI fragment; see Figure 4-1), tol5.1 (*Sal*I-*Eco*RI), and tol4.3 (*Spe*I-*Nsi*I) are constructs of *tol* gene under native promoters of 2.6 Kb, 818 bp and 63 bp, respectively. ptrpC-tol was constructed by fusing the promoter region (-357 to –2) of the *trp*C gene from *A. nidulans* (Mullaney *et al.*, 1985) to construct tol4.3. pcrp2-tol was constructed by fusing the promoter region (-438 to –1) of the *crp-2* gene from *N. crassa* (Cujec & Tyler, 1996) to construction tol4.3. The five constructions were then transformed into a *tol*$^-$ strain (R4-71). Homokaryotic transformants conferring Tol$^+$ phenotype can be obtained for each transformation and they were listed on the rightmost column. The Tol$^+$ transformants were then tested for their sexual phenotype as males. Results showed that none of the transformants are affected in their fertility.

4.3.5 Detection of *tol* sequences in other *Neurospora* species

A 3.5 kb *Spe*I-*Nsi*I fragment (position -63 to 3407, Figure 3-3) was used as a probe in Southern analysis using genomic DNA from *N. crassa* (both the wild type and the *tol* mutant) and ten other *Neurospora* species. Three μg of DNA was loaded into each lane and quality and quantity of genomic DNA were confirmed by hybridizing the blot with a β-tubulin gene probe (Orbach *et al.*, 1986). Under high stringency of hybridization (60°C), only one copy of *tol* was detected in *N. crassa* (Figure 4-4); although sequences that encode TOL-like gene product are present in *N. crassa* genome, they do not have significant DNA sequence similarity with *tol* (see section 3.3.4). The *tol* mutant has the same size band as the wild type, confirming that the *tol*$^-$ phenotype is due to a point mutation, rather than due to a large DNA deletion as observed in *het-6* escape events (Smith *et al.*, 1996).

Sequences that hybridized to *tol* were also detected under high stringency in many different *Neurospora* species, including all the heterothallic and pseudohomothallic species, and four of the six homothallic species tested (Figure 4-4). These data show that *tol* may be evolutionarily conserved and present among other *Neurospora* species. *N. tetrasperma*, the only other species listed in Figure 4-4 with mating-type-associated incompatibility, has a similar size band as *N. crassa*. Interestingly, in DNA from homothallic strains which contain sequences of both *mat A* and *mat a* (i.e. *N. terricola and N. pannonica*), *tol*-like sequences cannot be detected. It has been shown that *mat A-1* from *N. pannonica* confers mating-type-associated incompatibility in *N. crassa* and that mating-type compatibility in *N. pannonica* is probably due to a *tol*-like suppression (see section 2.3.5).

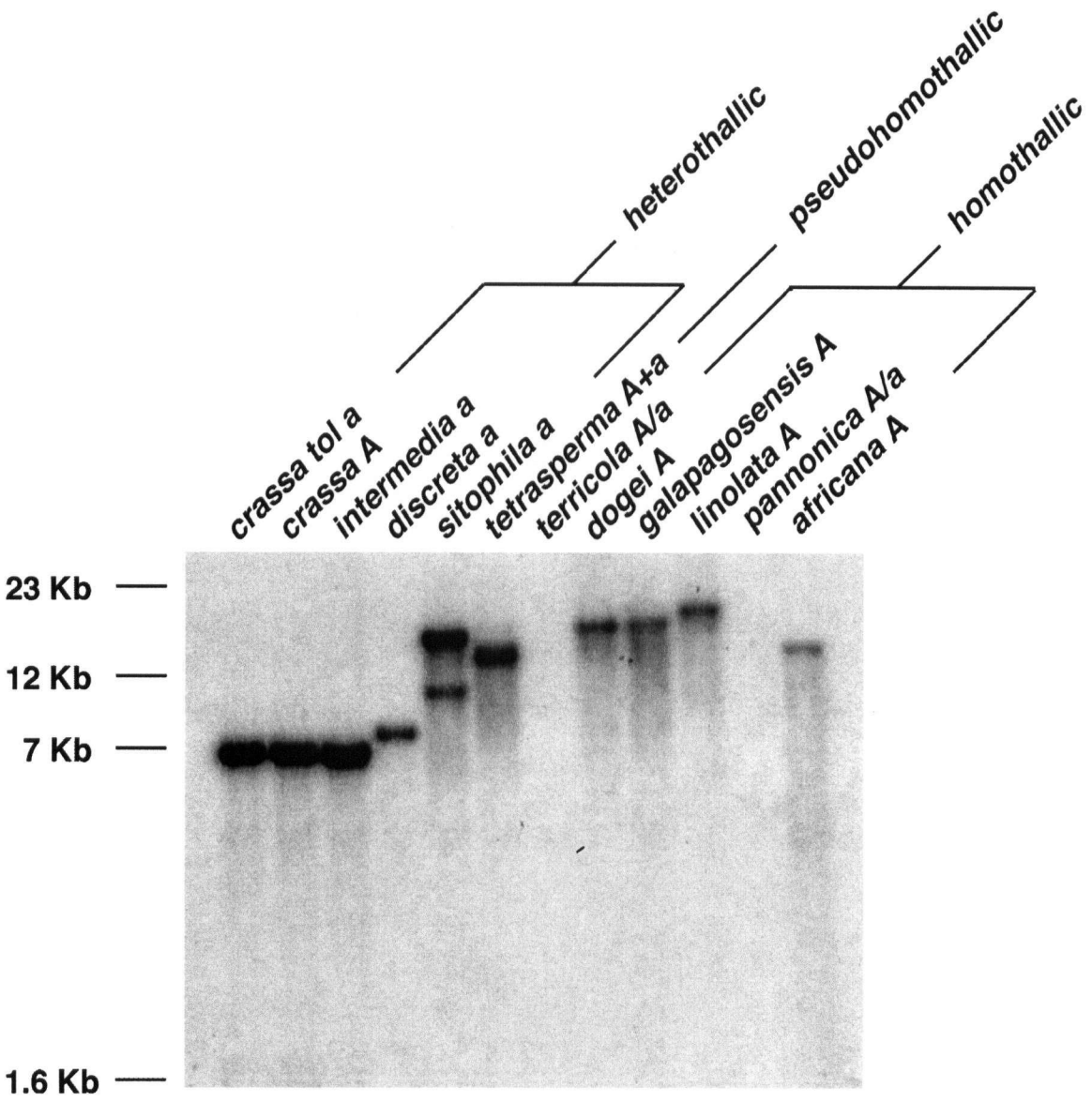


Figure 4-4. Detection of *tol* sequences in various *Neurospora* species. Genomic DNA was digested with *Eco*RI and probed with the *tol* 3.5-kb *Spe*I-*Nsi*I fragment. Species names and the presence of mating-type sequences are given (*A+a* = contains both *mat* nuclei; *A/a* = homokaryon with both *mat* genes) (Pöggeler, 1999). Among the *Neurospora* species listed here, *N. crassa* and *N. intermedia* are the only species that confer mating-type-associated incompatibility. *N. sitophila* and *N. tetrasperma* are mating-type compatible species with a *tol*-like suppression (Perkins, 1977; Jacobson, 1992). The *crassa* strains used are #43 (*tol*) and 74-OR23-IVA (wild type). Other species used are listed in Table 4-1.

4.3.6 Isolation of *top* (TOL-interacting protein) genes

Efforts were made to isolate TOL-interacting proteins using yeast 2-hybrid system. The yeast 2-hybrid system is used to detect protein-protein interaction *in vivo* (Fields & Song, 1989). When a protein X, which is fused with the GAL4 DNA binding domain, interacts with a protein Y, which is fused with the GAL4 activation domain, the GAL4 transcriptional activator is reconstituted and transcription of reporter genes are therefore activated.

A complete *tol* cDNA sequence was constructed using RT-PCR and was fused with the DNA binding domain of the yeast GAL4 gene (see Materials and Methods). This fusion construct, tol/pGBT9, was used in screening a *N. crassa* mycelial cDNA library (fused with GAL4 activation domain). The reporter genes in yeast in this case are *his3* and *lacZ*, so the positives should give a His^+ and blue color colony.

Thirty positives were isolated from the 2-hybrid screening (Table 4-3). Out of the 30 positives, only 5 were shown to be in-frame with GAL4 activation domain and have plasmid-dependent interaction with TOL. Four cDNAs encode novel genes (clones 7A5, 7A10, 7D3 and 7C10). The most interesting positive is clone 7D8, which is similar to *vip1* (p = 9e-39) from fission yeast (Figure 4-5). *vip1* was isolated from a *S. pombe* expression library using human p53 antibody (P. Wagner, unpublished results). P53 is a cancer suppressor in humans and is involved in cell cycle regulation and apoptosis (Sheikh & Fornace, 2000). A *vip1* homolog is apparently present in other *Neurospora* species (including species that do not mediate mating-type-associated incompatibility) (see Appendix 8.4)

Positive	3AT tolerance	Clone Size	Protein/DNA similarity	AA Size	Comment
2A3	5 mM	550 bp	= *cia35* (assembly of mitochondrial complex 1) - *Neurospora crassa*	-	3' UTR
6B3	5 mm	500 bp	XylS/AraC family txn. regulator - *Listeria monocytogenes* (0.9998)	2	
7A1	5 mM	1100 bp	None in frame. No DNA similarity	15	
7A4	5 mM	500 bp	Endothelin-1 receptor (ET-A) – mouse (0.96)	12	
7A5*	20 mM	700 bp	Flavohemoprotein - *Alcaligenes eutrophus* (0.94)	>46	
7A6	5 mM	1000 bp	None in frame. DNA similarity with a human clone (0.28)	64	
7A7	5 mM	850 bp	= *acr-2* (acriflavine resistance) – *Neurospora crassa*	-	Out of frame
7A8	5 mM	500 bp	= *rco3* (carbon catabolite repression) – *Neurospora crassa*	-	Out of frame
7A9	5 mM	600 bp	None. Strong DNA similarity (1.0e-5) with a human clone	17	
7A10*	10 mM	800 bp	mucin - rat (medium similarity, 0.015)	>106	
7A11	5 mM	1100 bp	= *tef-1* (translation elongation factor 1 alpha) - *Neurospora crassa*	-	Out of frame
7B1	5 mM	1200 bp	None in frame. DNA similarity with *Haemonchis contortus* mRNA (0.85)	21	
7B2	5 mM	1300 bp	None in frame. DNA similarity with an *Arabidopsos* clone (0.76)	67	
7B3	5 mM	900 bp	= *sod1* (Cu/Zn superoxide dismutase) – *Neurospora crassa*	-	Out of frame
7B4	5 mM	500 bp	None in frame. No DNA similarity	9	
7B5	5 mM	1200 bp	None in frame. No DNA similarity		
7B6	5 mM	650 bp			Seq. failed
7B8	5 mM	2000 bp	None in frame. No DNA similarity	30	
7B10	5 mM	800 bp	= *grg-1* (glucose repressed gene, morning specific) - *Neurospora crassa*	-	Out of frame
7B11	5 mM	900 bp	None in frame. DNA similarity with a Bovine herpesvirus 1 gene (0.0096)	11	
7B12	5 mM	900 bp	None in frame. DNA similarity with a human clone (0.997)	69	
7C1	20 mM	700 bp	= *grg-1* (glucose repressed gene, morning specific) - *Neurospora crassa*	-	Out of frame
7C5	5 mM	750 bp	None in frame. DNA similarity with Xenopus laevis XL-INCENP (0.52)	6	
7C9	5 mM	500 bp	= *grg-1* (glucose repressed gene, morning specific) - *Neurospora crassa*	14	In frame
7C10*	5 mM	950 bp	None in frame. DNA similarity with chicken delta-1,2 (0.93)	>201	
7C11	5 mM	750 bp	None in frame. DNA similarity with a *Caenorhabditis elegans* cosmid (0.998)	11	
7D1	5 mM	950 bp	None in frame. DNA similarity with Arabidopsis clone (0.996)	48	
7D3*	5 mM	1400 bp	None in frame. Weak DNA similarity with a human clone (0.23)	>103	
7D6	5 mM	500 bp	None in frame. No DNA similarity	>126	
7D8*	5 mM	1350 bp	*vip1* (a p53 related protein) – *Schizosaccharomyces pombe* (9e-39)	283	In frame

Table 4-3. Positive clones identified from 2-hybrid library screen using TOL as bait protein and mycelial cDNA products as target proteins. Clone size refers to the size of cDNA insert in the pAD-GAL4 phagemid while AA size refers to number of amino acids encoded in-frame in the cDNA. Similarity with specific protein/DNA and their organisms of origin are given with p-value in bracket.
* In-frame positives with plasmid-dependent interaction with tol/pGBT9 bait construct.

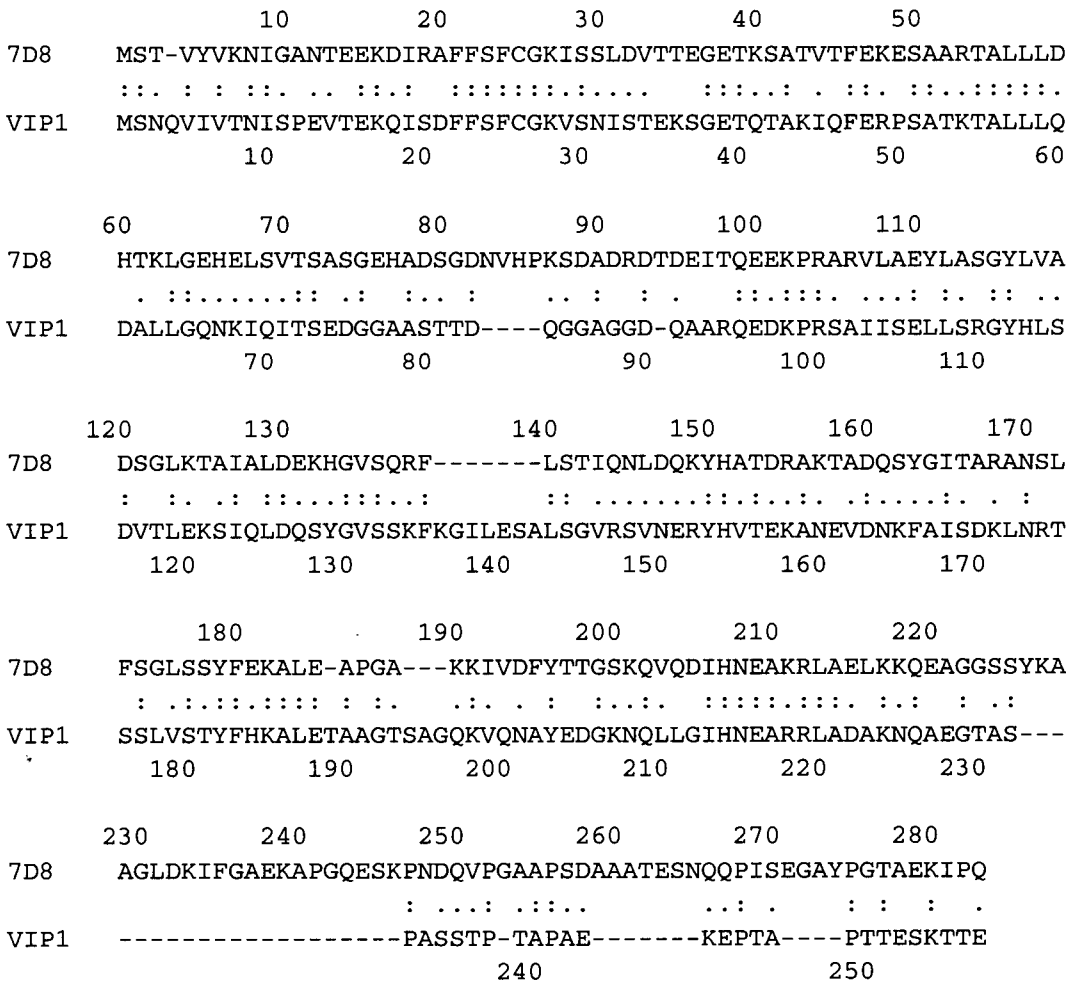


Figure 4-5. Similarity between the predicted amino-acid sequences from a *top* gene (7D8) and VIP1. Top strand: amino-acid sequence for 7D8 (283-aa). Bottom strand: amino-acid sequence for VIP1 (257-aa). *vip1* is a p53-related gene isolated from *S. pombe*. The two proteins have 33.9% identity and a p-value of 9×10^{-39} for similarity. ":" denotes identity whereas "." denotes conservative change of amino-acid (Devereux *et al.*, 1984).

4.4 DISCUSSION

This chapter describes the characterization and expression pattern of *tol*, a mediator of mating-type associated incompatibility in *N. crassa*, as well as the isolation of additional *tol* mutants and potential genes (*top*) that encode TOL-interacting proteins. Functional analysis of various deletion and frameshift constructions confirm that the *tol* ORF identified indeed confers Tol^+ function as tested in a transformation reduction assay. Furthermore, the functional analysis identified regions of TOL that are important and regions of TOL that are dispensable for proper function. For example, regions from amino-acid 1 to 97 and from amino-acid 652 to 790 are nonessential for Tol^+ function while regions from amino-acid 802-1011 and from amino-acid 99 to 521 are absolutely required. The required regions include the coiled-coil domain and the LRR motif. It is possible that the two protein-protein interaction domains are essential for the Tol^+ activity. If this is the case, then TOL probably mediates mating-type incompatibility through protein-protein interactions.

Since the original tol^- allele (N83) may retain partial activity, five additional *tol* mutants were isolated through RIP (repeat-induced point) mutagenesis (Selker, 1990; Singer & Selker, 1995). The *tol*-RIP mutants isolated (*tol-43*, *tol-83*, *tol-95*, *tol-106*, and *tol-135*) behave like the original *tol* mutant – they were phenotypically normal during vegetative phase and can mate as either a male or a female, with similar fertility to wild-type strains. On the other hand, mutations in *mod* genes, the mediators of non-allelic incompatibility in *P. anserina*, yield phenotypes (especially during sexual development) other than vegetative compatibility (see section 1.4.8). The *mod-A1 mod-B1* double mutant, which suppresses growth and lytic defect in self-incompatible (SI) strains, has a defect in protoperithecia formation and is female sterile (Barreau *et al.*, 1998). Mutations in the *mod-D* gene (Loubradou *et al.*, 1999), which mediate lytic action in certain incompatible strains, have defects in aerial hyphal growth, protoperithecial formation, spore germination and secondary ramifications (Durrens *et al.*, 1979; Labarère & Bernet, 1979a,

b). Mutation in the *mod E* gene is responsible for both partial restoration of the growth of a SI *het-R het-V* strain and a sexual dysfunction (less asci with mature spores and higher frequency of uninucleate spores) (Loubradou *et al.*, 1997). It has been proposed that *het* and *mod* genes were involved in perithecial formation (Bernet, 1992). In order to direct all growing potential toward the formation of perithecia, growth arrest and partial lysis of hyphal cells surrounding the developing perithecia is initiated through a pathway controlled by the *het* and *mod* genes. However, mutations in *tol* do not affect protoperithecial or sexual development.

In *N. crassa*, it is not clear how *tol, mat a-1* and *mat A-1* function to trigger incompatibility during vegetative growth. One hypothesis is that, in an *A* + *a* heterokaryon or *A/a* partial diploid, MAT a-1 and MAT A-1 form a heterodimer and this complex acts as a transcriptional regulator to activate or repress the expression of *tol* (Glass & Staben, 1990), which would then trigger incompatibility. Our studies show that the expression of *tol* occurs in *A*, *a* and even *Δmat* strains. These data indicate that MAT A-1 and MAT a-1 are not required for either repression or activation of *tol,* although we cannot rule out the possibility that the mating-type polypeptides may modulate *tol* expression. The expression of *tol* was also detected in a *A/a tol* partial diploid and an *A* + *a* incompatible heterokaryon, indicating that if a MAT A-1/MAT a-1 heterodimer is formed, it does not materially affect the expression of *tol.*

Based on the data presented in this study, a second more plausible possibility for the mechanism of mating-type associated incompatibility can be formulated. In our current hypothesis, TOL physically interacts with the MAT A-1/MAT a-1 complex or their downstream products to trigger incompatibility. The 1011-aa TOL protein possesses a putative coiled-coil domain and a LRR, both of which are thought to mediate protein-protein interactions. Putative LRRs are found in both MAT A-1 and MAT a-1 (see Chapter 2) and alteration of these motifs affect the capacity of MAT A-1 or MAT a-1 to induce incompatibility. In support for the model of a TOL/MAT A-1/MAT a-1 multimerization, physical interaction has been detected between

MAT A-1 and TOL and between MAT a-1 and MAT A-1 via the yeast two-hybrid system (C. Staben, T. Badgett & P.K.T. Shiu, unpublished data). A deletion within the first LRR of MAT a-1 eliminates interaction with MAT A-1 in the yeast two-hybrid system and abolishes mating-type incompatibility but does not affect mating function. For future experiments, interaction between TOL, MAT A-1 and MAT a-1 should be confirmed by immuno-precipitation and various mutants that eliminate incompatibility (such as *tol*$^-$ and other *mat A* mutants) should be assessed for their ability to mediate protein-protein interaction.

As mentioned in chapter 3, the *tol* mutation has been shown to suppress the phenotype of a *fmf-1* mutant (Johnson, 1979), which is sterile as both a male and a female in crosses to a wild-type partner. Fertility is restored when a *fmf-1 tol* strain is crossed to a *fmf-1*$^+$ *tol* strain. Our expression analysis of *tol* is consistent with the hypothesis that *tol* activity is suppressed during sexual development to allow the co-existence of opposite mating-type nuclei in the ascogenous hyphae. It is possible that FMF-1 regulates *tol* either directly or indirectly, and thus may play a role in the repression of *tol* transcription; mutation in *fmf-1* may allow expression of *tol* during sexual development and hence trigger mating-type-associated incompatibility. The presence of short repetitive sequences in the promoter of *tol* may provide putative binding sites for such regulators. Presumably, the mis-expression of *tol* during the sexual cycle would result in mating-type-associated incompatibility and block further sexual development. Attempts have been made to create constructs that over-express *tol* during sexual development. However, the constructions containing constitutive promoters (from *crp-2* and *trp*C genes) do not yield over-expressing *tol* transcripts. It is possible that these promoters may not work as expected in ectopic locations in the transformants or that the distance between these promoters and *tol* ORF may not be optimal. Alternatively, there may be sequences in *tol* mRNA that target it for rapid degradation. Until a consistent constitutive or inducible expression system during sexual development is established in *N. crassa*, we cannot unambiguously determine the effect of over-expressing *tol* during sexual development. Inducible expression systems (e.g. *ars-1* promoter; Paietta, 1989 and unpublished

results) and genes that are highly expressed in sexual tissue (e.g. *poi-1* and *poi-2*; M.A. Nelson, unpublished results) have been described. Further development of these systems may provide useful expression tools during sexual development in *N. crassa*.

Sequences similarity with *tol* are detected in many different *Neurospora* species (Figure 4-5), including those from heterothallic (nonself-mating, single nuclei), pseudohomothallic (self-mating with opposite mating-type nuclei), and homothallic groups (self-mating, single nuclei). These data show that the *tol* gene may be present in some of those species. The *tol* locus apparently plays a crucial role in the life style of mixed mating-type pseudohomothallic and some homothallic species. For example, the inactive tol^T allele evidently allows co-existence of incompatible mating-type nuclei in a common cytoplasm in the pseudohomothallic species *N. tetrasperma*. When the tol^C gene is introgressed from *N. crassa* to *N. tetrasperma*, the otherwise heterokaryotic species broke down into homokaryons of opposite mating-type (Jacobson, 1992). In some homothallic species, like *N. terricola* and *N. pannonica*, both mating-types genes are present in the same nucleus and yet an incompatible reaction is not triggered. The fact that a *tol*-like sequence is not detectable in those species may explain why mating-type incompatibility is not present (Figure 4-5). It is possible that the natural adaptation of *tol* may play an important role in the evolution of homothallic and pseudohomothallic species since mating-type incompatibility may present a problem in species that contain both mating-type genes. A *tol*-like suppression is also likely to be present in some heterothallic species. For example, mating-type incompatibility is observed in *N. intermedia* isolates, but not in *N. sitophila* or *N. discreta* isolates. When mating-type genes from *N. sitophila* were introgressed into *N. crassa*, it was found that these genes confer mating-type incompatibility and therefore suppression of incompatibility must be due to a tol^--like gene (Metzenberg & Ahlgren, 1973). The molecular characterization of *tol* in these species will provide great insight into the functions of *tol* as well as its relation with the evolution of reproductive life-styles in these species. It may provide

answers to interesting questions such as why *tol*-like sequences are retained in species that do not mediate mating-type-associated incompatibility.

To understand the role of TOL in mating-type heterokaryon incompatibility and to identify other proteins involved in the process, several potential TOL-interacting proteins were identified using the yeast 2-hybrid system. Five cDNAs encoding potential TOL-interacting protein (*top*) were identified, with four of them encode novel sequences. One potential *top* gene encodes a protein with similarity to the predicted products of the *vip1* gene from fission yeast. *vip1* was originally isolated from a *S. pombe* expression library using the human p53 antibody. P53 is a cancer suppressor in human and it is involved in cell cycle regulation and apoptosis (Sheikh & Fornace, 2000). Over-expression of a mutant form of *vip1* causes growth arrest in *S. pombe* (P. Wagner, personal communication). It is possible that the *N. crassa vip1* homolog is involved in cell cycle/cell death pathway downstream of antagonistic reactions between mating-type (and even other *het* loci) gene products. Interaction between VIP1 and TOL may trigger the growth arrest pathway through signal transduction. If VIP1 is indeed involved in the function of TOL, its function may not be limited in mediating mating-type incompatibility, since *vip-1* sequences can be detected in *Neurospora* species that do not confer mating-type incompatibility. RIP (repeat-induced point) mutational analyses of these *top* genes are under way (M.D. Hiltz & N.L. Glass, unpublished data) and the phenotype of the mutants will reveal their functions, as well as their role in mediating mating-type-associated heterokaryon incompatibility.

5. Analysis of *tol*^{T} and its relation to non-allelic and sexual incompatibility

5.1 INTRODUCTION

N. tetrasperma, a pseudohomothallic species, is a naturally occurring self-fertile *A* + *a* heterokaryon; four rather than eight ascospores are produced in each ascus, and both opposite mating-type nuclei are packaged in the same ascospore (Sansome, 1946; Raju, 1992a). Because of its heterokaryotic life-style, the mating-type incompatibility must be suppressed in this species. The mating-type genes of *N. tetrasperma* are not responsible for the suppression of incompatibility because *mat A* and *mat a* from *N. tetrasperma* displayed mating-type incompatibility when introgressed into *N. crassa* (Metzenberg & Ahlgren, 1973). The suppression is apparently due to the absence of Tol$^+$ activity. This can be shown by the introgression of *tol*^{C} (wild-type *tol* from *N. crassa*) into *N. tetrasperma*, which induced mating-type-associated incompatibility and disrupted the pseudohomothallic nature of the species. Upon germination, the opposite mating-type nuclei that were together in each ascospore disassociate, resulting in a mixed culture of segregated *A* and *a* mycelia instead of an *A* + *a* heterokaryon (Jacobson, 1992). Furthermore, the *tol*^{T} allele (wild-type *tol from N. tetrasperma*) acts as a recessive suppressor of mating-type incompatibility when introgressed from *N. tetrasperma* to *N. crassa* (Jacobson, 1992). The *tol*^{T} allele can therefore be considered a natural *tol*$^-$ mutant.

Complications occurred during the introgression of the *tol*^{T} allele into *N. crassa* and provided evidence that *tol*^{T}, or a locus closely linked to *trp-4* and *tol*^{T}, may function to reduce fertility and to mediate non-allelic incompatibility with an unlinked locus (Jacobson, 1992). The complications encountered were the observation of unanticipated reduced fertility during the backcrosses (of *tol*^{T} from *N. tetrasperma* into *N. crassa*) and the occurrence of a class of progeny with a deleterious growth phenotype.

Complication 1:

Reduced fertility was observed when the *tol*^{T} *trp-4*$^{+}$ progeny (from the *tol*^{T} to *N. crassa* introgression backcrosses: *tol*^{T} *trp-4*$^{+}$ x *N. crassa tol*^{C} *trp-4*$^{-}$) were crossed with *N. crassa*. A higher proportion of perithecia arrested early in development with delayed development of perithecia and ascospores (Figure 5-3B). The reduction in fertility occurs whether a *tol*^{C} or *tol*^{T} strain is used as the female parent. The *tol*^{C} *trp-4*$^{-}$ siblings do not show such phenotypes when backcrossed to a *N. crassa* strain.

Further characterization of the phenomenon provided evidence that the reduced fertility observed was mediated by a locus closely linked to *tol* and *trp-4* (D.J. Jacobson, unpublished results). Interestingly, the sexual incompatibility can be suppressed by a mutation in mating-type gene *mat a-1*. The fertility pattern of the *tol*^{T}-introgressed strains can be summarized as follow:

Strain 1	Strain 2	mating-type-associated vegetative incompatibility	Fertility
A; *tol*^{C}	*a*; *tol*^{T}	incompatible	Low
A; *tol*^{T}	*a*; *tol*^{C}	incompatible	Low
A; *tol*^{C}	*a*; *tol*^{C}	incompatible	Normal
A; *tol*^{T}	*a*; *tol*^{T}	compatible	Normal
A; *tol*^{T}	*a*^{m33}; *tol*^{C}	compatible	Normal
A; *tol*^{C}	*a*^{m33}; *tol*^{T}	compatible	Low

From the above data, it can be interpreted that *tol*^{T}, or a locus introduced with *tol*^{T} during its introgression to *N. crassa* (i.e. a locus closely linked to *tol*^{T} and *trp-4*) mediates the reduction in fertility. The lowered fertility is observed when the two mating partners have heterologous alleles (one from *N. crassa* and one from *N. tetrasperma*) at *tol* or at a closely linked locus. This fertility reduction can by suppressed by the *a*^{m33} allele, which has a R258S mutation in the *mat a-*

1 gene that suppresses mating-type incompatibility but does not affect mating function (Griffiths & DeLange, 1978; Staben & Yanofsky, 1990). However, the suppression is only observed when the a^{m33} mutation is present in the same nucleus as the tol^C allele.

One hypothesis to explain the above observation is that the tol^T/tol^C combination somehow triggers heterokaryon incompatibility between alternative mating-types during fertilization; normally, mating-type incompatibility is suppressed during mating. The tol^T-tol^C interaction may act to nullify the suppression by an unknown mechanism. Since the a^{m33} allele is unable to mediate incompatibility between opposite mating-types, its presence thus prevents the occurrence of incompatibility and allows normal fertility between a tol^T and a tol^C strain. This model, however, does not give any explanation (or contradiction) for the observation that a^{m33} must be present with tol^C in the same nucleus for normal fertility to occur.

Although recombination has not been observed between *tol* and the sexual incompatibility locus, there is no direct proof that *tol* is responsible for the sexual incompatible phenotype. Therefore, the newly discovered sexual incompatibility locus will be referred to as the *isi* (intergenus sexual incompatibility) locus, with isi^T and isi^C being the sexually incompatible alleles.

Complication 2:

Although a cross between a tol^C and tol^T-introgressed strain shows reduced fertility (see above), a few progeny can still be recovered. In the ninth backcross of tol^T from *N. tetrasperma* into *N. crassa* (b_8 tol^T *trp-4*$^+$; *leu-3 a* x *N. crassa* tol^C *trp-4*; *arg-1 A*), inhibited progeny and skewed ratio of markers were recovered (Jacobson, 1992). Among the ascospore progeny, 1/4 show inhibited growth. The inhibited progeny class show phenotypes resembling the "dark agar" seen in strains with a partial duplication heterozygous for a *het* gene (Mylyk, 1975). In the viable ascospore progeny, all the tol^T progeny are heterokaryon compatible with their tol^T parent,

while the tol^C progeny are split into two groups: half are compatible with the tol^C parent and the other half are incompatible. One hypothesis to explain the presence of a deleterious class and the skewed ratio is that a non-allelic incompatibility reaction is mediated by 2 unlinked loci (with one of the loci linked to *tol*). The observed mutually exclusive incompatibility group can be explained as the following, with the newly discovered incompatibility loci named *ili-1* and *ili-2* (inter-locus incompatibility):

	Progeny Groups				
	tol^T parental	tol^C parental	tol^C non-parental	Inhibited progeny	total
b9 progeny	37	34	35	36	142
Genotypes	$ili\text{-}1^T\ ili\text{-}2^T$	$ili\text{-}1^C\ ili\text{-}2^C$	$ili\text{-}1^C\ ili\text{-}2^T$	$ili\text{-}1^T\ ili\text{-}2^C$	

The deleterious class in this case contains the antagonistic $ili\text{-}1^T$ (from *N. tetrasperma*) and $ili\text{-}2^C$ (from *N. crassa*) alleles. The non-parental group in the progeny is $ili\text{-}1^C\ ili\text{-}2^T$, which is incompatible with its tol^C parent ($ili\text{-}1^C\ ili\text{-}2^C$). Non-allelic incompatibility has been well documented in *P. anserina* (Bernet, 1965; Bernet *et al.*, 1973; for review, see chapter 1.4), but it has not been widely characterized in *N. crassa*. Recently, Smith *et al* (2000b) has proposed that the *het-6*-mediated incompatibility in *N. crassa*, once thought to be mediated by a single locus, is mediated by two linked genes (see section 1.4.4).

$ili\text{-}2^C$ is closely linked to $het\text{-}c^{OR}$ (no recombination is observed in 500+ progeny) and *pyr-4* (D.J. Jacobson, unpublished data). *het-c* is a heterokaryon incompatibility locus that mediates allelic incompatibility in *N. crassa* (Saupe *et al.*, 1996a; see Chapter 1.4 for review). On the other hand, $ili\text{-}1^T$ is closely linked to tol^T and *trp-4*. Recombination between $ili\text{-}1^T$ and tol^T has not been detected in 400+ progeny; only two recombinants between $ili\text{-}1^T$ and *trp-4* were recovered (D.J. Jacobson, unpublished data). Judging from the linkage of the phenotypes, it is

possible that tol^T and $het\text{-}c^C$ may be identical to the two *ili* loci and that they could mediate non-allelic incompatibility.

The focus of this study is to determine (a) if a single locus in *N. tetrasperma* is responsible for the $ili\text{-}1^T$ non-allelic incompatible phenotype and the isi^T fertility reduction with the *N. crassa* wild-type Oak-Ridge strain, and (b) if either of the two loci is identical to tol^T. Mutagenesis of the tol^T alleles was used as the first approach to tackle this dilemma.

5.2 MATERIALS AND METHODS

5.2.1 Strains, media and culturing methods

The *N. crassa* strains used in the study are listed in Table 5-1. Strains 912-4, 912-10 and 912-72 contain $het\text{-}c^T$ and tol^T loci introgressed from *N. tetrasperma*. They were obtained with the use of the following strains (Jacobson, 1992): *N. crassa trp-4* (FGSC 4059 and 4060), *arg-1* (FGSC 324) and *leu-3* (R156 from D.D. Perkins); *N. tetrasperma* (FGSC 1270 and 1271); *N. tetrasperma*/*N. crassa* hybrid C4,T4 (FGSC 1778; Metzenberg & Ahlgren, 1969). Culturing and crossing, using Vogel's (Vogel, 1964) and Westergaard's (Westergaard & Mitchell, 1947) media respectively, were performed as previously described, with modification (Davis & Deserres, 1970; Perkins, 1986). Mating tests were performed using *fl a* (FGSC 4347) and *fl A* (FGSC 4317) tester strains (Perkins *et al.*, 1989). In a heterokaryon compatibility test, conidial suspensions from two strains (with complementary auxotrophic markers) are spotted on Vogel's minimal agar medium. Strains forming vigorous conidiating cultures after 3 days at 30°C are considered compatible.

Strain	Genotype	Origin
912-4	*het-c^{T} (ili-2^{T}); arg-1; tol^{T} (ili-1^{T} isi^{T}) A*	D.J. Jacobson
912-10	*het-c^{T} (ili-2^{T}); leu-3; tol^{T} (ili-1^{T} isi^{T}) a*	D.J. Jacobson
912-72	*het-c^{T} (ili-2^{T}); tol^{C} (ili-1^{C} isi^{C}) trp-4; leu-3 a*	D.J. Jacobson
85A	*N. tetrasperma A*	FGSC 1270
85a	*N. tetrasperma a*	FGSC 1271
R4-71	*his-5 tol trp-4; pan-2 a*	This study
R4-72	*ade-3B; trp-4 cot-1; pan-2 A*	This study
-	*fl a*	FGSC 4347
-	*fl A*	FGSC 4317
Y2	*thr-2 het-c^{OR} het-6^{PA} hyg^{R} a*	N.L. Glass
RT1-36	*arg-5; het-c^{PA} (ili-2^{C}) a*	N.L. Glass
RT1-40	*arg-5; het-c^{PA} (ili-2^{C}) a*	N.L. Glass

Table 5-1. List of *Neurospora* strains used in this study. (FGSC = Fungal Genetics Stock Center). All strains are *N. crassa* or *N. crassa* introgressed strains except *N. tetrasperma* 85A and 85a. A superscript *C* or *T* specifies the wild-type allele origin from *N. crassa* or from *N. tetrasperma*, respectively. All strains are of *N. crassa* Oak Ridge compatible background, with the exception of introgressed strains (912-4, 912-10 and 912-72), which contain a *mostly N. crassa* background with introgressed *N. tetrasperma* loci (Jacobson, 1992). Strains designated with *tol* contain the original *tol*$^{-}$ allele (allele N83; Newmeyer, 1970) while strains that do not have specification for *tol* allelism contain the wild-type *tol*$^{+}$ allele (*tol*^{C}) from *N. crassa*. *N. crassa* strains that do not have designation for *ili-1*, *ili-2*, *isi* and *het-c* loci contain alleles *ili-1*^{C}, *ili-2*^{C}, *isi*^{C} and *het-c*^{OR}, respectively. *N. crassa het-c* alleles (*het-c*^{C}) used in this study include *het-c*^{OR} and *het-c*^{PA}. In heterokaryon test, *het-c*^{OR} and *het-c*^{PA} strain show incompatibility, but *het-c*^{T} is compatible with *het-c*^{PA} but incompatible with *het-c*^{OR}, and therefore displays *het-c*^{PA}-specificity.

5.2.2 Repeat-induced point (RIP) mutation

In *N. crassa*, duplicated sequences undergo methylation and GC→AT mutation during mitotic divisions that precede karyogamy (Selker, 1990). In order to determine if the tol^T locus is responsible for the sexual and non-allelic incompatibility phenotype, attempts were made to introduce RIP mutation at the tol^T locus. An internal wild-type *tol* fragment from *N. crassa* (a 2.7 kb *Eco*RI fragment from DNA position 3-2735; Figure 3-3) was subcloned into a hygromycin-resistant vector (pCB1004; Carroll *et al.*, 1994) and transformed into strain 912-4 *het-c*^{T} (*ili-2*^{T}) *arg-1 tol*^{T} (*ili-1*^{T} *isi*^{T}) *A*. Since *N. crassa* transformants are heterokaryotic and contain both transformed and untransformed nuclei, a homokaryotic strain with only transformed nuclei was isolated from initial transformants according to Ebbole & Sachs (1990). The hygromycin-resistant homokaryotic transformants were then crossed to strain 912-71 *het-c*^{T} (*ili-2*^{T}) *tol*^{C} (*ili-1*^{C} *isi*^{C}) *trp-4 leu-3 a* and Trp$^+$ progeny were isolated. Progeny that gain sexual compatibility with either *fl A* or *fl a* tester strain (both wild-type *N. crassa* strains of Oak-ridge background) were subjected to Southern blot and RFLP analysis.

5.2.3 Nucleic acid isolation and hybridization

Standard molecular biology procedures were used throughout (Sambrook *et al.*, 1989). Genomic DNA isolation from *Neurospora* was adapted from Oakley *et al.* (1987). In the Southern blot analysis, three μg of DNA were digested with restriction enzymes and loaded in each lane; gel electrophoresis and nucleic acids transfer to Nylon filters (Schleicher and Schuell, Keene, NH) were performed according to manufacturer's specifications. [α-^{32}P] dCTP (Amersham, Oakville, ON) labeled probes were generated from digested DNA using the T7 QuickPrime Kit (Pharmacia, Baie d'Urfe, PQ).

5.2.4 *tol*^{T} isolation, plasmid construction and transformation

Initially, *tol*^{C}-specific primers were used to isolate 5' and 3' fragments of *tol*^{T} in a PCR reaction from FGSC 1270 DNA. The primers used were tol ATG1 (5' CCTGGGCTCACCTAT GC 3'; base -50 to -34) and tol 11 (5' CCAGCAGTGGCTCAGC 3'; bases 1144 to 1129) for 5' fragment amplification and primers tol 17 (5' TATATGGGCGGCGAAGATC 3'; Bases 1998 to 2016) and tol 21 (5' TGGATGATATCAAGACCTCAGC 3'; Bases 3317 to 3286) for the 3' fragment. The PCR amplification was performed in a model 480 Perkin-Elmer DNA cycler; PCR products were cloned into the PCRII vector using the TA cloning kit (Invitrogen, San Diego, CA) and subjected to DNA sequencing. The *tol*^{T} sequence revealed from the cloned 5' and 3' fragments were used to design *tol*^{T}-specific primers to amplify internal regions. Many attempts failed to amplify the *tol*^{T} sequences corresponding to regions between bases 1144 and 1998 could not be amplified. However, in a PCR reaction involving primer tol 27 (5' GTCCTGCGCGACTAATCGCCGT 3'; Bases 1032 to 1053), mis-priming on the antisense strand occurred resulting in a tol 27-tol 27 amplification. The cloning and sequencing of the tol 27-tol 27 fragment revealed the missing portion of entire *tol*^{T} open reading frame.

Spheroplast preparation and transformation were performed as previously described (Schweizer *et al.*, 1981; Vollmer & Yanofsky, 1986) with the exception of selecting for resistance to hygromycin B (250 units/ml; Calbiochem). Constructs of *tol*^{T} (see below) were assayed for Tol^{+} activity by co-transforming the cosmids (conferring hygromycin resistance) with a *mat A-1*-containing pOKE103 construct (pOKE103 has a *pan-2* selectable marker; J. Grotelueschen and R.L. Metzenberg, unpublished results) into strain R4-71 (*his-5 tol trp-4; pan-2 a*) and strain R4-72 (*ade-3B; trp-4 cot-1; pan-2 A*) spheroplasts.

To obtain a clone containing the entire *tol*^{T} open reading frame, primers tol ATG1 (5' CCTGGGCTCACCTATGC 3'; base -50 to -34) and tol 35T (5' CCCAACGCTCCCTATTGAT

GGA 3'; Bases 1770 to 1749 according to tol^C sequence) were used for PCR amplification. PCR-amplified product was cloned into the PCRII vector using the TA cloning kit (Invitrogen, San Diego, CA) and subsequently subcloned into pCB1004 vector which contains the hygromycin-resistant gene (Carroll *et al.*, 1994) to create tol^T/pCB1004. Vector tol^T/pCB1004 was used to transform strain R4-71 in order to obtain transformants containing an introduced tol^T copy. Homokaryotic strains with only transformed nuclei were isolated from the initial transformant according to Ebbole & Sachs (1990). The tol^T/pCB1004 to R4-71 transformants were tested for their ability to trigger sexual incompatibility with a tol^C isi^C strain (*fl A*); tol^C/pCB1004 to R4-71 (containing 3.5 kb *Spe*I-*Nsi*I tol^+ fragment; position –63 to 3407) and pCB1004 to R4-71 transformants were used as controls.

5.2.5 DNA sequence analyses

DNA sequences were determined using the ABI automated Taq DyeDeoxy Terminator cycle method (Mississauga, ON) at the NAPS unit, Biotechnology Laboratory, University of British Columbia and at the DNA Sequencing Facility, University of California, Berkeley. Computer sequence analyses for protein and DNA were done using the MacVector/ AssemblyLIGN software (International Biotechnologies, New Haven, CT) and GCG package available from the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

5.3 RESULTS

5.3.1 RIP (repeat-induced point) mutation of tol^T

Two loci closely linked to tol^T introduced an unexpected phenotype when introgressed from *N. tetrasperma* to *N. crassa* (Jacobson, 1992). The $ili\text{-}1^T$ locus mediates non-allelic incompatibility with $ili\text{-}2^C$ while the isi^T locus has allelic sexual incompatibility with isi^C. To determine if tol^T is equivalent to $ili\text{-}1^T$ and/or isi^T, I attempted to mutate the tol^T allele by RIP and ask if the two phenomena are still observed in tol^T mutants.

A *tol*^{C} internal DNA construct was transformed into strain 912-4 *het-c*^{T} (*ili-2*^{T}) *arg-1 tol*^{T} (*ili-1*^{T} *isi*^{T}) *A*. Since *N. crassa* transformants are usually heterokaryotic and contain both transformed and untransformed nuclei, homokaryotic strains with only transformed nuclei were isolated from initial transformants (Ebbole & Sachs, 1990). Two homokaryotic transformants (912-431 and 912-451) were then crossed with strain 912-72 *het-c*^{T} (*ili-2*^{T}) *tol*^{C} (*ili-1*^{C} *isi*^{C}) *trp-4 leu-3 a* separately (912-451 Female x 912-72 Male; 912-431 Male x 912-72 Female). Trp^{+} progeny were selected so that the potentially RIP-mutated, resident *tol*^{T} locus was preferentially recovered (*trp-4*$^{+}$ is closely linked to *tol*^{T} while *trp-4*$^{-}$ is linked to *tol*^{C}). Both crosses had a drastically lower number of mature perithecia, which is characteristic of a cross involving *tol*^{T} (*isi*^{T}) and *tol*^{C} (*isi*^{C}).

Fifty Trp^{+} progeny were isolated from the two crosses (V1-1 to V1-25 from 912-451 x 912-72 and V2-1 to V2-25 from 912-72 x 912-431). Out of the 50 *trp-4*$^{+}$ progeny selected, 20 of them gained full fertility with either *A tol*^{C} (*isi*^{C}) or *a tol*^{C} (*isi*^{C}) partner (fertility of these 50 progeny, hereafter, is referring to their fertility with a *tol*^{C} *isi*^{C} partner). Table 5-2 summarizes the genotypes of the 50 progeny. Since *trp-4*$^{+}$ is closely linked with the *tol*^{T} locus in the 912-431/912-451 parents, these progeny therefore represent potential *tol*^{T}-RIP mutants.

To test if these potential *tol*^{T}-RIP mutants also lose their ability to mediate non-allelic incompatibility with *ili-2*^{C}, some of the fully fertile progeny were tested in a heterokaryon compatibility test or in a progeny inhibition test (when forcing markers were not available) (D.J. Jacobson, unpublished results). In a progeny inhibition test, strain V1-6 *het-c*^{T} (*ili-2*^{T}) *arg-1 tol*$^{T\text{-}RIP?}$ (*ili-1*^{T} *isi*^{T}) *A*, which is fertile with the *tol*^{C} (*isi*^{C}) *fl a* tester strain, was crossed with Y2 (which contains *ili-2*^{C}). The V1-6 (*tol*$^{T\text{-}RIP?}$) x Y2 (*ili-2*^{C}) cross did not produce any inhibited progeny (all 48 germinated spores have wild type growth). This is in contrast with the control cross 912-4 (*ili-1*^{T}) x Y2 (*ili-2*^{C}), in which 1/4 of the progeny (10 out of 43 germinated spore) exhibit

Strains	Markers	*mat*	Strains	Markers	*mat*
V1-1		*a*	V2-1		*a* poorly
V1-2	*hyg*^{R}	?	V2-2		*A* poorly
V1-3	*hyg*^{R}	?	V2-3		*a*
V1-4	*arg-1*	*A* poorly	V2-4	*leu-3 hyg*^{R}	*a* poorly
V1-5	*hyg*^{R}	*a*	V2-5		*a*
V1-6	*arg-1*	*A*	V2-6	*arg-1*	*A* poorly
V1-7		*a* poorly	V2-7	*arg-1 hyg*^{R}	*A* poorly
V1-8	*arg-1*	*A*	V2-8	*arg-1 hyg*^{R}	*A* poorly
V1-9	*hyg*^{R}	*a*	V2-9		?
V1-10	*arg-1 hyg*^{R}	*A* poorly	V2-10	*leu-3*	*a*
V1-11	*arg-1*	?	V2-11	*arg-1*	*A* poorly
V1-12	*arg-1*	*A*	V2-12	*arg-1*	*A* poorly
V1-13	*hyg*^{R}	?	V2-13	*leu-3 hyg*^{R}	?
V1-14	*arg-1*	*a* poorly	V2-14	*arg-1*	*A* poorly
V1-15	*hyg*^{R}	?	V2-15		*a*
V1-16	*hyg*^{R}	*A*	V2-16		*a*
V1-17	*hyg*^{R}	*a*	V2-17	*leu-3*	?
V1-18	*hyg*^{R}	*a*	V2-18	*leu-3 hyg*^{R}	?
V1-19		*a*	V2-19		*A* poorly
V1-20		*a*	V2-20	*arg-1*	*A* poorly
V1-21	*hyg*^{R}	*a*	V2-21	*arg-1*	*A* poorly
V1-22		*a* poorly	V2-22	*leu-3*	?
V1-23	*arg-1 hyg*^{R}	*a*	V2-23	*leu-3 hyg*^{R}	?
V1-24		*a*	V2-24	*leu-3*	*a* poorly
V1-25	*arg-1 hyg*^{R}	*A*	V2-25	*arg-1 hyg*^{R}	*A* poorly

Table 5-2. Genotypes of 50 progeny from the *tol*^{T}-RIP cross. Progeny from the RIP cross were tested for their auxotrophic markers, hygromycin-resistance and mating identity. A *tol*^{C} internal DNA construction was introduced into strain 912-4 *het-c*^{T} (*ili-2*^{T}) *arg-1 tol*^{T} (*ili-1*^{T} *isi*^{T}) *A* to obtain transformants 912-451 and 912-431. The two hygromycin-resistant (*hyg*^{R}) transformants were then crossed to 912-72 *het-c*^{T} (*ili-2*^{T}) *tol*^{C} (*ili-1*^{C} *isi*^{C}) *trp-4 leu-3 a* separately to obtain V1 and V2 progeny, respectively. All the progeny were selected on a Vogel's (1964) medium lacking tryptophan (hence selecting for *typ-4*$^+$ progeny, which is linked to *tol*^{T}). Twenty *trp-4*$^+$ progeny confer full fertility when mated with *tol*^{C} (*isi*^{C}) testers. The rest show poor fertility with the tester strains; only a few (and sometimes none; as denoted by "?") mature perithecia can be observed in a 1-cm diameter mating-spot.

inhibited growth. It was concluded in the V1-6 strain, activities for non-allelic incompatibility and for sexual incompatibility were absent. It is possible that RIP mutation of tol^{T} affects both $ili\text{-}1^{T}$ and isi^{T} function.

Two fully fertile progeny, V2-10 and V1-23 (presumably $tol^{T\text{-}RIP}$), were subjected to incompatibility tests (D.J. Jacobson, unpublished results). Results show that V2-10 formed a compatible heterokaryon with two $ili\text{-}2^{C}$ tester strains (RT1-36 and RT1-40). In contrast, the sterile control strain V2-17 (presumably not RIP-mutated) did not form stable heterokaryon with the two $ili\text{-}2^{C}$ tester strains. Therefore, like V1-6, both non-allelic incompatibility and sexual incompatibility are absent in the V2-10 progeny. However, in a heterokaryon compatibility test, fertile V1-23 progeny behaved like V2-17, which did not form a vigorous heterokaryon with the two $ili\text{-}2^{C}$ tester strains. This is the first time that the non-allelic incompatibility activity ($ili\text{-}1^{T}$-$ili\text{-}2^{C}$ interaction) is segregated from the sexual incompatibility activity (isi^{T}-isi^{C} interaction) in the locus spanning $ili\text{-}1^{T}$ and isi^{T}. The interpretation is that in V1-23, isi^{T} activity is disrupted while the $ili\text{-}1^{T}$ activity is intact.

So far, the experimental data suggested that in some of the $tol^{T\text{-}RIP}$ progeny (V1-6 and V2-10), both the $ili\text{-}1^{T}$ and isi^{T} activities were disrupted, whereas in another $tol^{T\text{-}RIP}$ progeny (V1-23), only isi^{T} activity was affected. A tempting hypothesis to explain the above observation is that tol^{T} is equivalent to both $ili\text{-}1^{T}$ and isi^{T} locus and that it contains both non-allelic and sexual incompatibility activity. In the case of V1-23, only the functional domain responsible for isi^{T} activity is disrupted, therefore making it fertile with an isi^{C} strain but incompatible with an $ili\text{-}2^{C}$ strain. However, it is possible that tol^{T} represents the isi^{T} locus only, and that the extension of the RIP-mutation process beyond duplicated sequences (Selker, 1991) may introduce mutations to the closely linked $ili\text{-}1^{T}$ locus in strains V1-6 and V2-10.

However, complicating results occurred when V1-6 and V2-10 (both lost sexual and non-allelic incompatibility; presumably $tol^{T\text{-}RIP}$) were tested for mating-type incompatibility. Results (data not shown) demonstrated that V1-6 and V2-10 can only form heterokaryons with the same but not with the opposite mating-type, i.e. the potential $tol^{T\text{-}RIP}$ strains behave like a wild-type tol^{C} allele in a heterokaryon incompatibility test. A normal tol^{T} allele behaves like a tol^{-}, which, when present in both partners, allows stable heterokaryon formation between strains with opposite mating-types.

To understand the nature of the tol^{T} locus in the RIP-cross progeny, the 50 progeny and their parents were subjected for Southern blotting and RFLP analysis using tol^{C} sequences as a probe. As expected, RFLP analyses showed that all the poorly fertile progeny have the same restriction pattern as their tol^{T} parent (912-72) (Figure 5-1A). However, for the fully fertile progeny, instead of having a tol^{T} RIP pattern, as expected, all progeny have a restriction pattern resembling their tol^{C} (912-72) parent (Figure 5-1A).

Since the *trp-4*$^{+}$ marker is closely linked to the tol^{T} allele (*trp-4* is about 1 map unit from *tol* in *N. crassa*), it is possible that in these fully fertile *trp-4*$^{+}$ progeny, either conversion (i.e. tol^{T} is converted to tol^{C}) or a high frequency of recombination between tol^{T} ($ili\text{-}1^{T}$ isi^{T}) and *trp-4*$^{+}$ had occurred. The presence of a tol^{C}-like RFLP pattern in the fully fertile progeny suggested the presence of tol^{C} allele in those strains, which could explain why fully fertile strains such as V1-6 and V2-10 behave like a tol^{C} strains when tested in a mating-type heterokaryon incompatibility test.

For the conversion theory, we can hypothesize that sometime before the fertilization, a tol^{T} to tol^{C} gene conversion event has occurred in some of the transformed nuclei, using the integrated tol^{C} internal fragment as the donor DNA. Since the converted nuclei have a tol^{C}

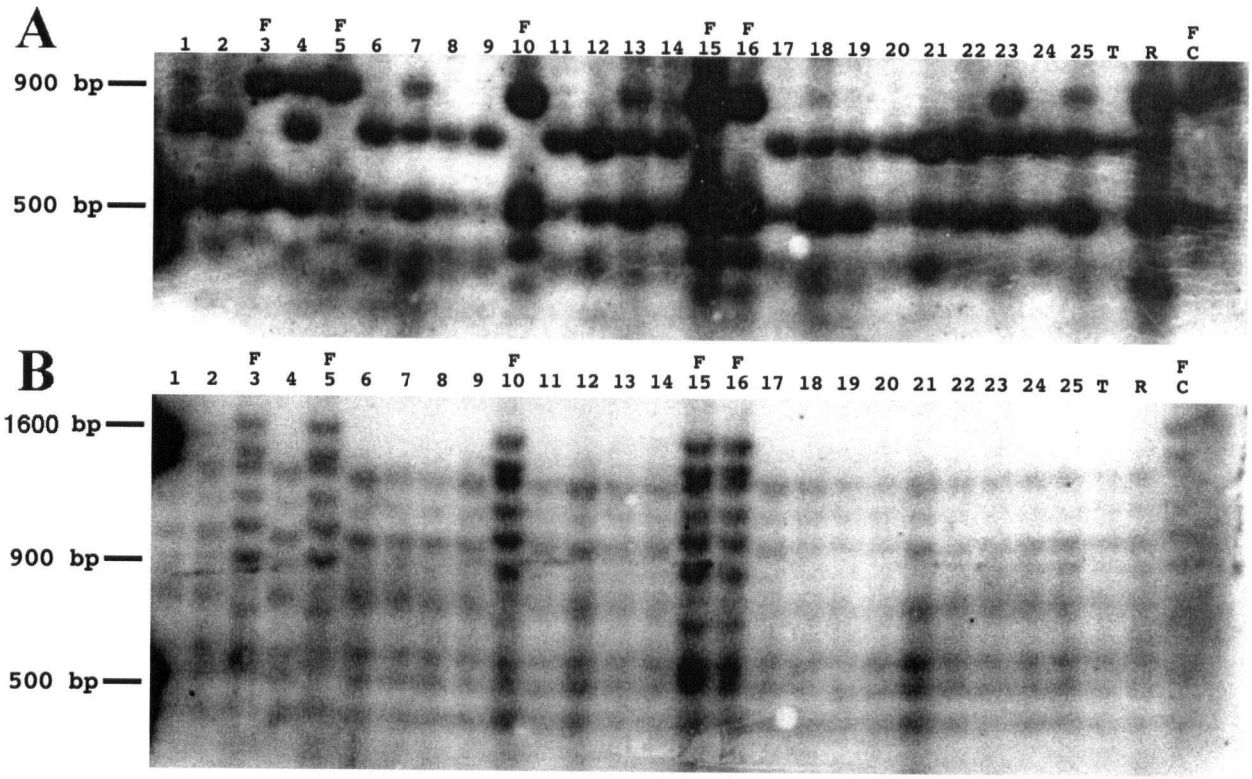


Figure 5-1. RFLP analysis of *tol* sequences in the progeny from the tol^T-RIP cross. **A.** The fertile (F) progeny isolated from the tol^T-RIP cross contain RFLP pattern similar to the tol^C allele (C) rather than to the tol^T allele (T). Number 1 to 25 referred to strains V2-1 to V2-25, which are progeny from the cross 912-431 (R) x 912-72 (C). Strain 912-431 (R) was constructed by introduction of a tol^C copy into strain 912-4 (T), which is of tol^T background. Three μg of each DNA was digested with restriction enzyme *Hpa*II and was used for Southern analysis. The blot was probed with a 5.1 kb *Sal*I-*Eco*RI *tol*/pCB1004 plasmid (see Figure 4-1). **B.** Same as panel A, except the hybridization blot was probed with pMOcosX cosmid X7:C2, which is mapped between *tol* and *trp-4* (Figure 3-1). The fertile progeny show a *N. crassa* pattern (C).

sequence instead of *tol*^{T} (assuming that *tol*^{T} locus is equivalent to *isi*^{T}), they will have a much higher fertility with an *isi*^{C} strain and therefore are much more representative in the progeny.

For the recombination theory, it is hard to explain why the introduction of the *tol*^{C} fragment would trigger such a high recombination rate between *trp-4*$^+$ and *tol*^{T} (*ili-1*^{T} *isi*^{T}) as observed in the RIP cross. One hypothesis is that the introduced *tol*^{C} fragment, through an unknown mechanism, creates a recombination hot spot between *trp-4*$^+$ and *tol*^{T} (*ili-1*^{T} *isi*^{T}). An alternative explanation is that somehow the *trp-4*$^+$ *tol*^{C} progeny have a selective advantage. For example, *trp-4*$^+$ *tol*^{C} progeny may have a faster germination rate than *trp-4*$^+$ *tol*^{T} progeny and are preferentially picked during ascospore germination.

To test if crossovers between *trp-4* and *tol* actually has occurred in the fertile progeny, DNA from the progeny were probed with cosmid X7:C2, which maps between *trp-4* and *tol* (X7:C2 contains an overlapping region with X25:D7, a cosmid that contains *tol*; Figure 3-1). Like the previous hybridization result, the fertile progeny show a RFLP pattern resembling their *tol*^{C} (912-72) parent when probed with X7:C2 (Figure 5-1B). This result agreed with the recombination theory showing that crossovers have occurred between *trp-4* and X7:C2 in the fertile progeny.

The analyses of the progeny did not give us further details on the *isi* locus, except to confirm that *tol*^{C} is closely linked to *isi*^{C} phenotype. We cannot conclude or exclude the notion that *tol*^{C} is equivalent to *isi*^{C}. However, the V1-23 phenotype (which has a *tol*^{C} pattern and is incompatible with *ili-2*^{C}) suggests it is unlikely that the *tol*^{T} locus is responsible for the *ili-1*^{T} activity.

5.3.2 Molecular cloning and sequencing of *tol*^{T}

Since the *tol*^{T}-RIP experiment does not provide unambiguous answers concerning the nature of the relationship between *tol*^{T}, *ili-1*^{C} and *isi*^{C}, the molecular nature of *tol*^{T} needed to be determined. If we know the sequence of *tol*^{T}, it would be feasible for us to determine if the fully fertile progeny contained a converted sequence or not. Moreover, the cloning of *tol*^{T} would allow us to directly test (in a transformation and complementation assay) if *tol*^{T} mediates sexual and non-allelic incompatibility.

Primers specific for *tol*^{C} were used to amplify fragments of the *tol*^{T} allele from the *N. tetrasperma* strain; primers specific for *tol*^{T} were later designed from the partial sequences and were used to amplify the *tol*^{T} ORF (see Materials and Methods). The *tol*^{T} allele contains a deletion of G at base 1244 (position according to *tol*^{C} sequence) as compared to *tol*^{C}; the single-base deletion leads to a –1 frame-shift at amino acid position 384, changing the amino acid 384A to 384P-385S-386R-387 premature stop (amino acids position according to *tol*^{C}; the entire TOL^T contains 384 amino acids, whereas TOL^C contains 1011 amino acids; see Figure 3-3). The truncated TOL^T contains the coiled-coil domain (position 177-211) but lacks the majority of the SET domain (position 315-583) and the entire leucine-rich repeat (position 804-823). All three domains are required for TOL^C function, i.e. the ability to mediate vegetative incompatibility between opposite mating-types (see Figure 4-1). The amino-acid comparison between TOL^T and the amino terminal portion of TOL^C is shown in Figure 5-2.

Since the molecular difference between *tol*^{T} and *tol*^{C} is revealed, it is possible for us to determine the molecular characteristics of the fully fertile progeny from the RIP cross. Characterization of progeny such as V2-10 (loss of both sexual and non-allelic incompatibility) and V1-23 (loss of sexual but retention of non-allelic incompatibility) would allow us to determine if a *tol*^{T} $\rightarrow$ *tol*^{C} sequence is converted as predicted by the conversion model (in the

```
               10        20        30        40        50        60
TOL-C  MISPSSSCPTSSINSNTYPVSSSSASSPIPSSSHLDPESRSHKGSYEAVLEYFTSNSLPL
       ::::::.:::::::::::: :::::::::::::::::.:::::::::::::::::::::::
Tol-T  MISPSSGCPTSSINSNTYTVSSSSASSPIPSSSHLDPDSRSHKGSYEAVLEYFTSNSLPL
               10        20        30        40        50        60

               70        80        90       100       110       120
TOL-C  PSYLQTYDSFVSHFFTSTDNPSLLSPINIPPHLTSYEAFIRYSIAKAAPAASSLPSTVPL
       ::::::::::::.::::::: :::::::::::::::::.:::.::::::: :: ::::
Tol-T  PSYLQTYDSFVSRFFTSTDNPSSLSPINIPPHLTSYEAFIQYSITKAAPAAS-LPPTVPL
               70        80        90       100       110

              130       140       150       160       170       180
TOL-C  PLNQFCPRCTKALASSPPPPSHAAWWRRGGELCTPEELFRSASEGQCQFCWFVMVKVLRR
       :::::::::::::::: :: ::::::::::::::::::.:::::::::::::::::::::
Tol-T  PLNQFCPRCTKALASSAPPRSHAAWWRRGGELCTPEELFHSASEGQCQFCWFVMVKVLRR
     120       130       140       150       160       170

              190       200       210       220       230       240
TOL-C  LVVGVQIEDEEDLKQKVEEVWRDVKYLLVGLSFWAEDRREDVWRGEVSVRCVPRVPLGRE
       ::::::.:::::::::::.:::.::: :.: ::::::::::::::::::::::::::::
Tol-T  LVVGVQVEDEEDLKQKVEKVWRDIKYLGVALFFWAEDRREDVWRGEVSVRCVPRVPLGRE
     180       190       200       210       220       230

              250       260       270       280       290       300
TOL-C  KDVDVDMEGKVVVSEFVRLRRGYIPGPSYPPSFLASFHTASEQSWSQALEWINNCRSHAL
       :::::::::::::::::: ::::::::: :::::::::::::.:::::::::::::::::
Tol-T  KDVDVDMEGKVVVSEFVRLR-GYIPGPSYPSSFLASFHTASEQSWNQALEWINNCRSHAL
     240       250       260       270       280       290

              310       320       330       340       350       360
TOL-C  CSAAETMDCHERRPARLIAVGRPGETHVRVIETAGLAVSETPFMSLSHCWGKDGVPTQLL
       ::::::::::.:::::::::::::::::::::::::::::::::::::::::::::::::
Tol-T  CSAAETMDCHDRRPARLIAVGRPGETHVRVIETAGLAVSETPFMSLSHCWGKDGVPTQLL
      300       310       320       330       340       350

              370       380
TOL-C  KGNYDRFTKEGIRLTELPKTFRDAIE
       : ::::::::::.:::::::::::  .
Tol-T  KENYDRFTKEGIKLTELPKTFRDPSR
      360       370       380
```

Figure 5-2. Amino acid alignment between TOL^T (*N. tetrasperma*) and TOL^C (*N. crassa*). The 384-aa TOL^T was compared with the first 366-aa of TOL^C; tol^T has a single base deletion as compared to tol^C (at tol^C DNA position 1244 G) leading to a frameshift mutation (after TOL^T aa position 382) and a premature stop codon. The two polypeptides are 93% identical over the TOL^T ORF. ":" indicates a conserved amino acid, whereas "." indicates a conservative change. The coiled-coil domain (position 177-211 according to TOL^C) was underlined.

case of V1-23, a partially converted sequence) or if they contain complete *tol*^{C} sequence, which is a result of recombination.

The *tol* alleles from V2-10 and V1-23 were amplified and subjected to DNA sequence analysis. The results showed that the *tol* alleles from these two strains contain the entire *tol*^{C} sequences. The *tol*^{C} sequences from these two alleles cannot be a product of *tol*^{T} → *tol*^{C} conversion, since both of them contain the *tol*^{C} promoter region (instead of a *tol*^{T} promoter region, which contains a 3-bp deletion before the ATG start codon), and that the introduced *tol*^{C} copy is composed of internal *tol*^{C} region and does not contain any *tol*^{C} promoter sequences. These results agreed with data from the hybridization experiments (Figure 5-1) and support the model that the fully fertile progeny as generated in the RIP cross (see section 5.3.1) are products of recombination between *tol*^{T} and *trp-4* and not due to a *tol*^{T} → *tol*^{C} conversion event. Since V1-23 contained *tol*^{C} (and not *tol*^{T}) sequence and yet it retains non-allelic incompatibility with *ili-2*^{C}, the hypothesis that *tol*^{T} is responsible for the *ili-1*^{T} phenotype is refuted.

5.3.3 Transformation assay for *tol*^{T} non-allelic and mating-type incompatibility activity

The *tol*^{T} allele (containing the entire *tol*^{T} ORF) was PCR-amplified, cloned and subcloned into a *Neurospora* vector conferring hygromycin-resistance (pCB1004) to obtain *tol*^{T}/pCB1004. When the *tol*^{T}/pCB1004 vector was transformed into recipient containing *ili-2*^{C} background (strain R4-71), transformation frequency is similar to control pCB1004 vector (both have >500 transformants per plate), i.e. transformation reduction is not observed. The transformants from the *tol*^{T}/pCB1004 transformation do not show an inhibited phenotype (such as aconidiating and slow growing) as observed in partial diploids or transformants containing both antagonistic *het* genes (such as *ili-1*^{T}/*ili-2*^{C} progeny). The transformation experiment therefore does not support the premise that *tol*^{T} is responsible for the *ili-1*^{T} phenotypes (i.e. incompatible with *ili-2*^{C} locus) and that the two components (*tol*^{T} and *ili-1*^{T}) may represent two linked but separate loci. This

result is in agreement with the phenotype of V1-23, which does not contain the *tol*^{T} allele (it contains *tol*^{C} instead) but still retains non-allelic incompatibility.

The *tol*^{T} allele was also tested for its ability to trigger mating-type-associated incompatibility. The *tol*^{T}/pCB1004 vector was co-transformed with a construct containing *mat A-1* into *tol a* spheroplasts. Transformation results showed that when co-transformed *with mat A-1*, *tol*^{T}/pCB1004 does not trigger transformation reduction (88±15 transformants per plate); its transformation frequency is comparable with an experiment using pCB1004 and *mat A-1* as transforming DNA (92±4 transformants per plate). The positive control (using *tol*^{C}/pCB1004 and *mat A-1* as DNA), on the other hand, induced at least a 20-fold reduction in transformation frequency when introduced into recipients containing *tol a* background (1±1 transformant per plate). These results suggested that the *tol*^{T} allele does not mediate incompatibility between *mat A-1* and *mat a-1*. This is in agreement with a previous experiment by Jacobson (1992), in which he demonstrated that when *tol*^{T} is introgressed into the *N. crassa* background, opposite mating-type no longer mediates heterokaryon incompatibility. More over, it has been shown (see chapter 4) that all three functional domains (coiled-coil domain, SET domain and leucine-rich repeat) are required for Tol^{+} function; truncation of TOL^{C} similar to the length of TOL^{T} also does not mediate mating-type incompatibility. The fact that TOL^{T} contains only the coiled-coil domain and not the entire SET domain and the leucine-rich repeat (which is implicated in the incompatibility function in MAT A-1, MAT a-1 and TOL) may explain the lack of Tol^{+} function in *N. tetrasperma*. A presence of Tol^{+} function in *N. tetrasperma* will unavoidably affect its pseudohomothallic lifestyle (*N. tetrasperma* is a self-fertile, natural heterokaryotic species). As shown by Jacobson (1992), introgression of *tol*^{C} into *N. tetrasperma* leads to a mixed culture of segregated *A* and *a* mycelia instead of an *A* + *a* heterokaryon.

5.3.4 Testing of sexual incompatibility function in *tol*^{T}

Although the phenotype of V1-23 and the transformation assay does not support the premise that *tol*^{T} and *ili-1*^{T} are an identical locus, there is still no further data on the relationship between *tol*^{T} and *isi*^{T}. So far the two loci have not been segregated by genetic recombination. As a matter of fact, all the fully fertile progeny from the RIP cross also contain *tol*^{C} RFLPs, suggesting that the two loci are closely linked.

To determine if the *tol*^{T} allele is responsible for sexual incompatibility between *isi*^{T} and *isi*^{C}, *tol*^{T} was introduced into a *tol*$^-$ background and the sexual phenotype of the transformants was observed. If *tol*^{T} is responsible for the *isi*^{T} activity, the transformants should gain sexual incompatibility with an *isi*^{C} strain. Vector *tol*^{T}/pCB1004 was used to transform a *N. crassa tol*$^-$ strain (R4-71) in order to obtain transformants containing an introduced *tol*^{T} copy. The *tol*^{T}/pCB1004 transformants were then tested for their ability to trigger sexual incompatibility with a *tol*^{C} *isi*^{C} strain. Transformants contain *tol*^{C}/pCB1004 DNA and transformants with vector only (pCB1004) DNA were also used as controls.

Twenty *tol*^{T}/pCB1004 homokaryotic transformants (from 11 individual transformants) were isolated. For controls, 10 *tol*^{C}/pCB1004 and 16 pCB1004 homokaryotic transformants (from 4 and 8 individual transformants, respectively) were isolated. Fifty µl of conidial suspension (roughly 10^7/ml) from each transformant was spotted on the *fl A* (*tol*^{C} *isi*^{C} background) tester strain (5 spots per plates). The *ili*^{C} *tol*$^-$ recipient strain (R4-71) was used as a fully-fertile control while an *isi*^{T} strain (912-10) was used as a poorly-fertile control. Results from the mating assay showed that all the transformants showed normal mating reaction with full fertility comparable to the fully-fertile control (R4-71). On the other hand, the poorly-fertile control (912-10) shows a typical poorly-fertile phenotype (roughly 20-fold less formation of mature perithecia) as expected from a reaction involving *isi*^{C} and *isi*^{T} as mating partners (see figure 5-3B for pictures of fertile and poorly-fertile crosses).

A

Strain	Number of mature perithecia per 1 cm-diameter field
L2-3 (*tol*^{C} transformant)	47 ± 28.5
L11-2 (*tol*^{C} transformant)	41.8 ± 4.4
L15-1 (*tol*^{C} transformant)	58.6 ± 10.2
M3-2 (*tol*^{T} transformant)	73.6 ± 20.1
M5-2 (*tol*^{T} transformant)	83.4 ± 12.3
M17-1 (*tol*^{T} transformant)	65.8 ± 27.2
M20-1 (*tol*^{T} transformant)	64.6 ± 8.5
N2-2 (vector transformant)	49.2 ± 24.5
N5-1 (vector transformant)	70 ± 50.8
R4-71 (*isi*^{C} control)	45 ± 15.1
912-10 (*isi*^{T} control)	2.4 ± 1.7

B

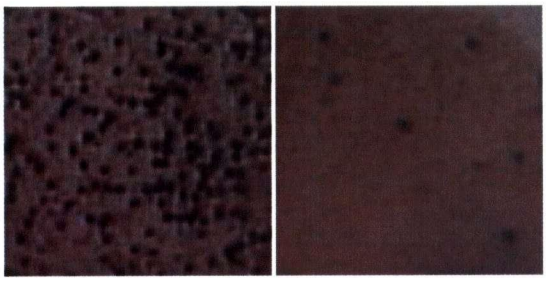


Figure 5-3. Mating assay for *tol*^{T} transformants. (A) DNA from constructs *tol*^{T}/pCB1004, *tol*^{C}/pCB1004, and pCB1004 (vector) were used to transform spheroplasts (strain R4-71) with background *tol*$^{-}$ *isi*^{C}. Equal amount of conidia (1.25 x 10^{7}) from each transformant were used to fertilize the tester strain *fl A* (which has an *ili*^{C} background) on individual 8.5 cm petri dishes. R4-71 (*isi*^{C}) was used as a fully-fertile control while 912-10 (*isi*^{T}) was used as a poorly-fertile control. A typical mating reaction between *isi*^{T} and *isi*^{C} contain roughly 20-fold less mature perithecia. For the counting of mature perithecia, five 1-cm diameter fields was randomly chosen from each plate (for the 912-10 strain, 10 fields were chosen) and the number of perithecia within the fields were counted. The right column represents the mean number of mature perithecia (with standard deviation) as seen in a field. (B) Pictures demonstrating mature perithecial production in a typical *isi*^{C} x *isi*^{C} (left panel) and a typical *isi*^{T} x *isi*^{C} (right panel) mating reaction (fields of 1 cm x 1 cm are depicted).

For a more quantitative assessment of the sexual phenotype of the transformants, equal amounts of conidia from each selective homokaryotic transformants were used in a mating test, and the number of mature perithecia was recorded. The results (Figure 5-3) showed that when mated with a isi^C partner, all the experimental transformants and the fully-fertile control (R4-71) had a roughly 20-fold margin of fertility over the isi^T control (912-10). These data showed that the transformation of the tol^T allele into an isi^C strain does not effectively convert it to a strain conferring an isi^T phenotype. Therefore, it is unlikely that the tol^T gene is responsible for the isi^T sexual incompatibility activity and that the two most probably represent two separate loci.

5.4 DISCUSSION

The mating-type genes in *N. crassa* provide seemingly opposite functions during sexual and vegetative phase. The MAT A-1 and MAT a-1 are important for conferring mating identity during cell-cell recognition in the sexual fusion event. Yet, during vegetative phase, the two proteins restrict heterokaryon formation between individuals of alternative mating-types. One key player in the restriction of heterokaryosis is the *tol* locus – a wild-type *tol* gene product is required for recognition, and eventually, rejection of opposite mating-type nuclei in a common cytoplasm.

N. tetrasperma, a pseudohomothallic species, is effectively self-mating; however, unlike homothallic species, it contains two distinct types of nuclei, *A* and *a*. After fertilization, four spores are produced in each ascus, in which both mating-types are packaged in the same spore. *N. tetrasperma* is therefore a natural *A* + *a* heterokaryon upon germination. The co-existence of the opposite mating-types during the vegetative phase is due to the inactive tol^T allele. In a series of introgression experiments, Jacobson (1992) showed that tol^T can suppress mating-type incompatibility in *N. crassa* whereas introduction of tol^C into *N. tetrasperma* leads to a breakdown of the *A* + *a* heterokaryon.

During the introgression of *tol*^{T} into *N.crassa*, Jacobson (1992) encountered two unexpected observations. Firstly, the *tol*^{T} *trp-4*$^{+}$ introgression progeny consistently show reduced fertility (less mature perithecia and slower development) as compared to *tol*^{C} *trp-4* siblings when crossed to various *tol*^{C} *trp-4*$^{+}$ strains. This fertility reduction can by suppressed by a mutation in *mat a-1* (a^{m33}), which suppresses mating-type incompatibility but does not affect mating function (Griffiths & DeLange, 1978; Staben & Yanofsky, 1990) (D.J. Jacobson, unpublished data). Since the sexual incompatibility factor (tentatively named *isi*) maps closely to *tol* and its effect can be eliminated by a suppressor of mating-type incompatibility, there is a possibility that the *tol* locus may in fact mediate the phenomenon. It is possible that the *tol*^{T}-*tol*^{C} interaction somehow prevents the switch off of mating-type incompatibility, which is normally turned off during sexual development. Alternatively, *tol* may play an active role in the sexual phase and that the *tol*^{T}-*tol*^{C} interaction may interfere with its sexual function.

The second complication observed during *tol*^{T} introgression is the presence of a class of inhibited progeny showing phenotypes resembling strains with a partial duplication heterozygous for a *het* gene. The presence of inhibited progeny is commonly found in *P. anserina* when the two mating partners contain antagonistic alleles of non-allelic *het* loci (see section 1.4). Further mapping for the antagonistic activity showed that the incompatibility is linked to *tol*^{T} and *het-c*^{C} (see section 1.4 for *het-c* locus); the two loci were named *ili-1*^{T} and *ili-2*^{C} (D.J. Jacobson, unpublished data). Since both *tol* and *het-c* are involved in mediating heterokaryon incompatibility, the newly found non-allelic interaction may indicate that an unusual interaction between the two may lead to the triggering of incompatibility. These observations have an implication that *tol* and *het-c* may be involved in the same pathway.

Mutagenesis of *tol*^{T} using the RIP mechanism was used as a first approach to determine whether *tol*^{T} represents *ili-1*^{T} and *isi*^{T} activity. A copy of *tol*^{C} allele was introduced into a *tol*^{T}

strain, in an effort to introduce RIP mutations to that allele. Although some progeny lost both *isi*^{T}-mediated sexual incompatibility and *ili-1*^{T}-mediated incompatibility, genetic characterization, RLFP analysis and sequencing of some of the *tol* alleles suggested that these progeny do not represent *tol*^{T}-RIP mutants, but rather are genetic recombinants between the selected *trp-4*$^+$ marker and the *tol*^{T} *ili-1*^{T} *isi*^{T} loci. The failure to recover *tol*^{T}*-RIP* mutants may be due to the fact that the *tol*^{T} allele is not homologous enough to trigger RIP mutagenesis. Alternatively, since the frequency of RIP is largely dependent on the gene and the location of the resident copy (Irelan *et al.*, 1994), it is possible that *tol*^{T}-RIP mutants can be recovered if more progeny are screened; only a 10 % success rate was observed when the same *tol*^{C} fragment was used to RIP-mutate the *tol*^{C} gene (see Chapter 4).

The recovery of such a high frequency of recombinants (40 %) between *trp-4*$^+$ and *tol*^{T}*/ili-1*^{T}*/isi*^{T} was unexpected. Previous genetic crossing experiments suggested a close linkage between *trp-4* and *tol/ili-1/isi* (see section 5.1). Since progeny were germinated on a medium lacking tryptophan, it is possible that progeny with the *trp-4*$^+$-linked region from the *tol*^{T} parent coupled with the *tol*^{C}-linked regions from the *tol*^{C} parent have an unknown advantage and germinated better. Alternatively, the procedure of making spheroplasts coupled with the introduction of a *tol*^{C} fragment may have somehow increased recombination around the *tol*^{T} region through an unknown mechanism.

Although *tol*^{T}-RIP progeny were not isolated, a recombinant (V1-23) progeny containing *tol*^{C} *isi*^{C} *ili-1*^{T} background was recovered. This is the first time that *ili-1* has been separated from the *tol* and *isi* locus. Since V1-23 contains the *tol*^{C} allele, we can therefore eliminate the notion that *tol*^{T} is responsible for the *ili-1*^{T} activity and conclude that *tol* and *ili-1* represent two distinct loci. Since V1-23 contains a cross-over between *ili-1*^{T} and *tol/trp-4*, this strain will be useful in the future for mapping the precise location of the *ili-1* gene using the cosmid walk that spans *trp-*

4 and *tol* (see Chapter 3) as probes. Preliminary data suggests that *ili-1* maps between *trp-4* and cosmid X7:C2 (see Figure 3-1).

The tol^T allele was cloned in order to determine if it contains isi^T activity when transformed into a non-isi^T background. As shown by the mating assay, all tol^T-transformants remained sexually compatible with a partner containing isi^C background. These results do not support the notion that tol^T is involved in the isi^T-isi^C sexual incompatibility reaction. Moreover, when an isi^T strain was crossed with various tol^C-RIP strains, reduction in fertility was still observed, demonstrating that tol^C is also unlikely to be involved in the sexual incompatibility reaction (D.J. Jacobson, unpublished results). Therefore, although *tol* and *isi* are closely linked, they are likely to be two separate loci. Nonetheless, we cannot discount the possibility of tol^T containing isi^T activity with absolute certainty. For example, it is possible that the introduced tol^T allele did not function properly as a transgene, hence explaining the absence of isi^T activity in the tol^T transformants. Mechanisms that interfere with expression of transgenes have been described in *N. crassa* (Cogoni & Macino, 1999).

Originally, it was thought that the sexual incompatibility between tol^T $trp\text{-}4^+$ strains and tol^C *trp-4* strains is due to the allelic interaction between tol^T and tol^C. However, the above results have forced us to renew our model to better suit all the data collected. One possible scenario is that the *isi* gene product(s) may have an active role in the mating process of *N. crassa*. The fact that a mutation in the *mat a-1* gene can suppress the sexual incompatibility between alternative *isi* alleles hints that the *isi* gene product may interact with the mating-type proteins.

Mating-type genes seem to be conserved among different *Neurospora* (and even related ascomycetes such as *P. anserina*) species and can function to confer mating activity in heterologous systems (see section 1.3; Shiu & Glass, 2000). However, despite conservation of

mating-type gene function, interspecies mating between closely related *Neurospora* species are either impossible or highly infertile. It is possible that the divergence of mating-modifiers such as the *isi* locus may act to restrict interspecies mating and contribute to speciation. If the *isi* locus does have an active role in mating and participates in speciation, one prediction is that the *isi* locus should have a strict separation between *Neurospora* species that have more divergent mating strategies. Until we have fully characterized the nature and the function of the *isi* locus, we cannot assess whether it has a significant biological role in preventing interspecies mating.

This study provides evidence that *ili-1*^{T}, a locus involved in non-allelic incompatibility, is a distinct locus from *tol*^{T}. Non-allelic heterokaryon incompatibility has been implicated in fungi such as *P. anserina* and *C. parasitica* (Begueret *et al.*, 1994; Anagnostakis, 1982b). The antagonistic alleles for the *het-c/het-e* system in *P. anserina* have been characterized; they encode a glycolipid transfer protein and a GTP-binding protein, respectively (Saupe *et al.*, 1994; Saupe *et al.*, 1995a; see section 1.4). Since glycolipids have been implicated in the stress response during cell death (both apoptotic and non-apoptotic), it has been proposed that *het-c* and *het-e* may act as components of a signaling pathway that triggers incompatibility reaction (inhibition of growth, cell death, etc.) (Saupe, 2000). Interestingly, two other genes involved in non-allelic incompatibility in *P. anserina*, *het-d* (mediates *het-c/d* interaction) and *het-v* (mediates *het-r/v* interaction), are highly homologous to the *het-e* gene (J. Bégueret, personal communication). Moreover, modifier mutations (*mod-A mod-B*) that suppress all three non-allelic incompatibility systems in *P. anserina* have been described (see section 1.4.8). These data suggest a close relationship between the different non-allelic incompatibility systems in *P. anserina*. Molecular characterization of *ili-1* and *ili-2* will show whether these two genes have sequence (and functional) homology to the non-allelic *het* genes in *P. anserina*.

Although allelic heterokaryon incompatibility systems have been well characterized in *N.crassa* (see section 1.4), non-allelic incompatibility has not been broadly studied; *het-6-*

associated incompatibility is the only *het* system in *N. crassa* that is speculated to be mediated by two distinct loci (Smith *et al.*, 2000b). In *Neurospora*, abortive and inviable progeny are commonly observed in crosses involving natural (non-laboratory) strains. It is possible that the low viability of progeny from these crosses is due to the effect of non-allelic incompatibility systems that have been overlooked. If non-allelic incompatibility does exist in *N. crassa*, it will affect the success rate of outbreeding in a species that is obligatory non-selfing. Alternatively, it is possible that the non-allelic incompatibility observed in the *ili-1/ili-2* system is strictly between different species (*N. crassa and N. tetrasperma* in this case) and not within a species. Extensive out-breeding and selective preference for viable progeny in *N. crassa* may effectively eliminate polymorphisms at non-allelic incompatibility loci; many allelic incompatibility systems already exist in *N. crassa* to serve the function of nonself recognition. Characterization of *ili-1* and *ili-2* from natural *N. crassa* strains will definitely shed light on this dilemma.

6. Concluding Remarks

Although the two mating-type genes in *N. crassa*, *mat A-1* and *mat a-1*, confer mating identity for compatible sexual fusion, they mediate heterokaryon incompatibility during the vegetative phase. This aim of this study was to delineate the mechanism involved in mating-type mediated heterokaryon incompatibility in *N. crassa*. Two separate but connected approaches were conducted to tackle the dilemma. Firstly, the mating-type gene *mat A-1* was functionally dissected for its heterokaryon incompatibility and mating ability. Secondly, cloning and characterization of *tol* was performed in order to understand its role in mediating mating-type incompatibility.

Dissection of the *mat A-1* functional domain indicated that mating and incompatibility can be spatially separated (Chapter 2). Mating function of *mat A-1* (and *mat a-1*) seem to be related to their roles in transcriptional regulation. This can be shown by the importance of α-(MAT A-1) and HMG (*mat a-1*) domains in mating function. However, data from this study on *mat A-1* (and previous studies on *mat a-1*) suggest that protein-protein interaction domains (i.e. leucine-rich repeat, LRR) are important in the mediating mating-type incompatibility. In support of this theory, preliminary yeast 2-hybrid data from C. Staben (University of Kentucky) showed that the MAT A-1 and MAT a-1 mating-type proteins physically interact. The interaction between the two mating-type proteins appears to be important for heterokaryon incompatibility but not for mating. A mutation in *mat a-1* (a deletion in the LRR region), which abolishes mating-type incompatibility but does not affect mating function, eliminated physical interaction between MAT A-1 and MAT a-1. It is possible that the MAT A-1 leucine-rich repeat identified in this study may facilitate the physical interaction of the two mating-type proteins, which in turn represents the molecular basis of recognition between individuals containing antagonistic *mat* genes.

Although now that we have a model for recognition of alternative *mat* alleles, little is known about the pathway or biological actions that the recognition may trigger. One way to study the mechanism involved in the incompatibility reaction is to delineate its mediator(s). The *tol* locus was first identified as a suppressor mutation for the mating-type incompatibility. In this study, the *tol* gene was cloned and characterized (chapter 3 and 4). The *tol* gene is not a homolog to any known genes. It contains two protein-protein interaction domains (coiled coil and LRR) and a region of similarity to two *het* genes (*het-6* and *het-e*). The discovery of a common domain shared by different genes involved in the incompatibility reaction is encouraging. It may hint that although different *het* systems encode functionally diverse proteins (chapter 1), they may share a common biochemical pathway (cell death pathway). Microscopic studies have shown that all the *het* systems tested so far show similar responses during an incompatibility reaction (Chapter 1). How *tol* mediates the incompatibility between alternate mating-type proteins is still a dilemma. However, data presented here hint that a direct interaction between the mating-type proteins and the *tol* gene product is possible (Chapter 3). In support for the model for a TOL/A-1/a-1 multimerization, a weak physical interaction has been detected between TOL and MAT A-1 (but not between TOL and MAT a-1) by yeast two-hybrid system (C. Staben, T. Badgett and P. Shiu, unpublished data).

Since the *tol* gene product does not contain any identifiable domains as shared by other proteins with known biochemical function, we have little clues for the biochemical mechanism that mediate incompatibility. However, the presence of two protein-protein interaction domains suggested that TOL may act as an adapter protein for formation of a larger protein complex. Attempts have been made to isolate proteins that interact with TOL (Chapter 4). One of the *top* genes (TOL-interacting protein) identified encodes a protein that could be involved in cell cycle control (VIP1). It is possible that multimerization between VIP1 and the TOL/MAT A-1/MAT a-1 complex may trigger a pathway similar to apoptosis (programmed cell death).

Additional genetic studies around the *tol* locus have identified an additional locus (*isi*) that is involved in mating, and possibly mating-type associated incompatibility (Chapter 5). What we know so far for the newly identified locus is that its alternative alleles mediate sexual incompatibility and that incompatibility can be suppressed by mutations that suppress mating-type-associated heterokaryon incompatibility. Sexual incompatibility as mediated by the *isi* locus may represent an extension of heterokaryon incompatibility to the sexual phase. Its association with *tol* is still unclear, and there is no evidence that the two gene products act in the same pathway. Although it is possible that previously identified suppressors for mating-type incompatibility, which map closely to *tol*, may in fact be due to mutation in the *isi* locus ; we cannot rule out the possibility of a gene cluster that controls both mating functions and mating-type incompatibility.

This study has provided clues about the mechanism involved in the mating-type-associated incompatibility. The model we have is premature, but the data collected so far has opened the opportunity for future experiments to address the questions that we may have generated in the course of this study, such as the biological significances of the *top* genes, the biochemical function of the SET domain, the localization of TOL and the mating-type proteins, the mechanism of *tol* repression during sexual cycle, the molecular characterization of *tol* alleles in various species and their relations to their sexual strategy and the mechanism of recognition and triggering of cell death. Further characterization of the key players identified (*tol*, *mat A-1*, *mat a-1*, *top* and *isi*) will contribute to our understanding of the possible pathways, regulation and mechanisms involved in this fascinating biological phenomenon.

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8. Appendices

8.1 Genetic crossover between *his-5* and *tol* can be detected by cosmid G13:C8

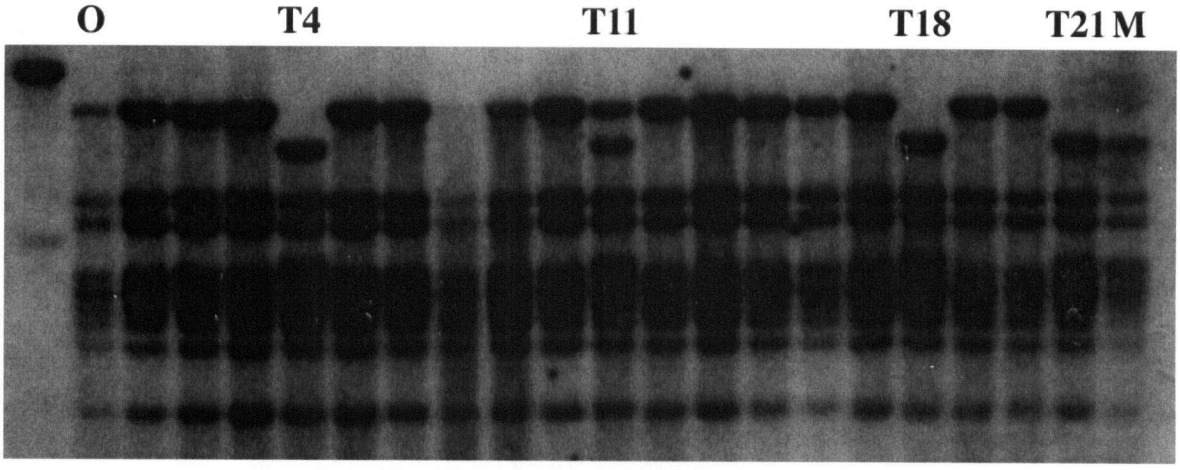

Figure 8-1. Genetic crossover between *his-5* and *tol* as detected by G13:C8. Progeny containing genetic crossover between *his-5* and *tol* was probed with probed with DNA from cosmid G13:C8 (see Figure 3-2B). Since the parental strains contain different RFLP pattern on LG IV, it is possible to detect a crossover point if a particular cosmid is located between *his-5* and *tol*. When probed with cosmid G13:C8, progeny T4, T18 and T21 show a RFLP pattern of the Mauriceville parent (M) (strain R5-28), demonstrating that the recombination point must occurred between G13:C8 and the last cosmid walk (X25:D7; which detect only Oak Ridge pattern in all the progeny). Progeny T11 shows a hybrid pattern resembling both the Mauriceville (M) (R5-28) and the Oak Ridge (O) (R5-27) parents, demonstrating that the crossover point in this progeny must be within the cosmid G13:C8.

8.2 physical map of cosmids containing the *tol* gene

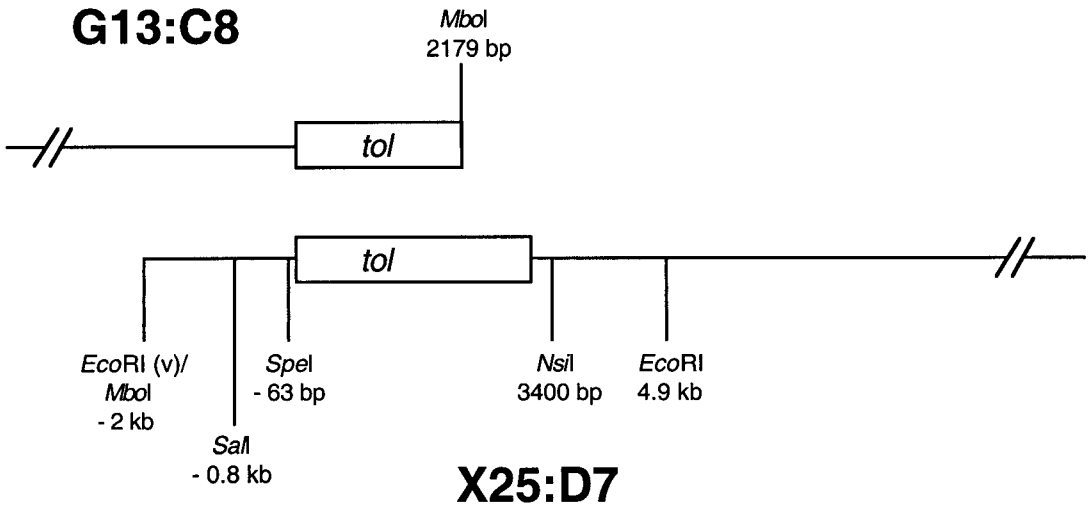

Figure 8-2. Physical map for cosmids X25:D7 and G13:18. A 6.9-Kb *Eco*RI-*Eco*RI fragment from cosmid X25:D7 was determined to contain Tol^+ activity in a co-transformation assay (see section 3.2.4). A 4.2 kb *Sal*I-*Nsi*I fragment was later subjected to DNA sequencing. DNA analysis also shows that G13:C8 contain partial sequence for the *tol* ORF. *Eco*RI (v) denotes a restriction site present in the multi-cloning site in the pMOcosX cosmid (Orbach, 1994).

8.3 Hydrophobicity Plot for TOL

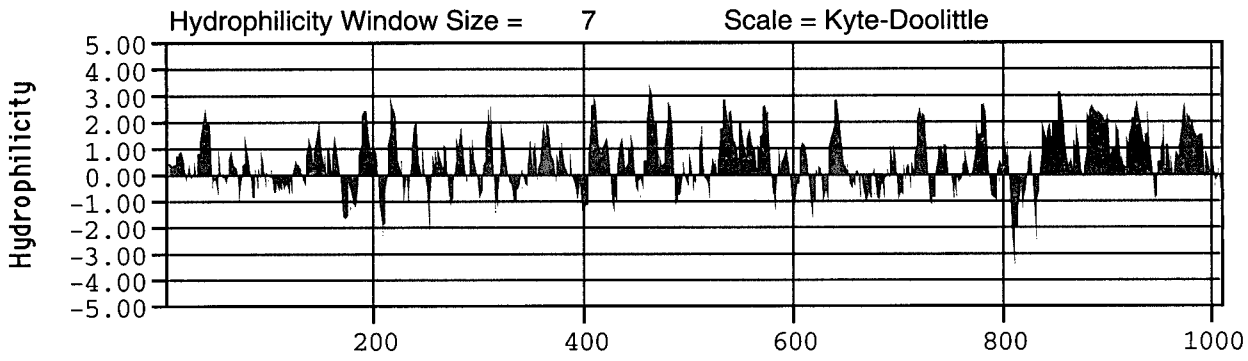

Figure 8-3. Hydropathy plot of TOL as analyzed by the method of Kyte and Doolittle (1982) with a 7-residue window. Hydrophilic regions correspond to the positive index numbers. TOL has a predicted isoelectric point of 4.67 and is made up of 40% non-polar and 26% charged residues. The carboxyl-terminal portion of TOL (amino-acids position 837 to 1011) is relatively hydrophilic, composed of 30% non-polar and 30% charged residues.

8.4 Detection of *vip1* sequence in various *Neurospora* species

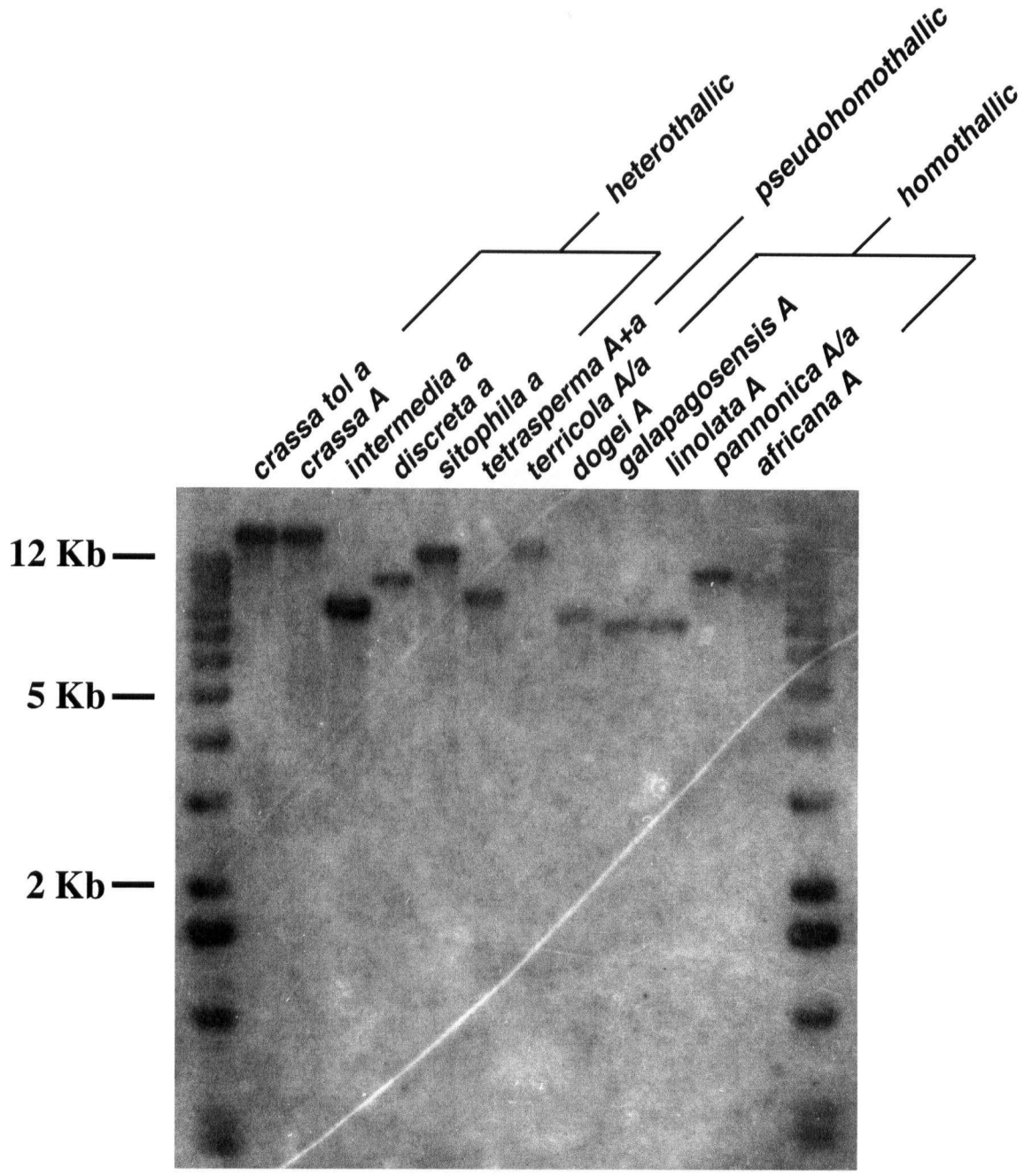

Figure 8-4. Detection of *vip1* sequences in various *Neurospora* species. Genomic DNA was digested with *Eco*RI and probed with clone 7D8 (*N. crassa vip1* homolog). Species names and the presence of mating-type sequences are given (*A+a* = contains both *mat* nuclei; Pöggeler, 1999); *A/a* = homokaryon with both *mat* genes). Among the *Neurospora* species listed here, *N. crassa* and *N. intermedia* are the only species that confer mating-type-associated incompatibility. *N. sitophila* and *N. tetrasperma* are mating-type compatible species with a *tol*-like suppression (Perkins, 1977; Jacobson, 1992). The *crassa* strains used are #43 (*tol*) and 74-OR23-IVA (wild type). Other species used are listed in Table 4-1.