On Nonself Recognition in *Neurospora crassa*: Macroscopic and Genetic Studies

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of

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by

Oana Cristina Micali

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submitted by
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In partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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“It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to Heaven, we were all going direct the other way.”

Charles Dickens, A Tale of Two Cities

ABSTRACT

On Nonself Recognition in *Neurospora crassa*: Macroscopic and Genetic Studies

Nonself recognition is a universal biological process that entails the detection of genetically different individuals. In filamentous fungi, nonself recognition limits cellular interactions such as hyphal fusions, to individuals that are genetically similar. The nonself recognition response is apparent at the macroscopic scale, by the presence of lines of demarcation (barrages) at the interface between genetically different individuals. Nonself recognition is also characterized in fungi by heterokaryon incompatibility as governed by *het* genes. Fusions between cells with genetic differences at any *het* locus produce unstable fusion products (heterokaryons) that disintegrate into their component homokaryons, display inhibited growth rates or die.

This thesis explores nonself recognition phenomena in the filamentous fungus *Neurospora crassa* by two different approaches. First, in Chapter 1, nonself recognition at the macroscopic scale was analyzed by studying the appearance and genetic basis of barrage formation. Phenotypically diverse barrages were observed to form between genetically different isolates under limiting nutrient growth conditions. The appearance of barrages in *N. crassa* is similar to barrages observed in other fungal species. I provide evidence suggesting that barrage formation is under complex genetic control, that is independent from that of heterokaryon incompatibility.

The genetic basis of heterokaryon incompatibility in *N. crassa* was explored in Chapters 2 and 3 of the thesis. Genetic differences at *un-24* and *het-6* trigger strong

incompatibility reactions leading to cell death. The genes exist as two allelic variants Oak Ridge (OR) and Panama (PA) and are found in linkage disequilibrium in the world population. Only OR/OR and PA/PA combinations at *un-24* and *het-6* are found in nature. The thesis explores structural and functional factors responsible for linkage disequilibrium at *un-24* and *het-6*. I provide evidence that the two loci are situated close to the centromere-proximal breakpoint of a novel paracentric inversion *In(III)1131 het-6*. The decreased occurrence of recombination events near the inversion breakpoints may be responsible for linkage disequilibrium at *un-24* and *het-6*. Inversion-associated polymorphisms appear to be maintained by balancing selection acting at *un-24* and *het-6*.

Whether linkage disequilibrium at *un-24* and *het-6* is maintained because OR/OR and PA/PA combinations at the two loci operate optimally together (the supergene hypothesis) was explored. The isolation and characterization of functional PA alleles at *un-24* and *het-6* revealed that incompatibility reactions associated with the transformation of PA alleles into OR cells are weaker than when the OR alleles are introduced into PA cells. However, when combined, the effects of PA alleles at *un-24* and *het-6* result in a strong incompatibility reaction leading to cell death. Selection for strong, rather than weak incompatibility reactions may have led to selection for inversion polymorphisms at *In(III)1131 het-6* and ultimately to linkage disequilibrium at *un-24* and *het-6*.

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I dedicate this thesis to my parents, Oana and Radutu and to the memory of my grandparents, Maria, Mircea and Ion. This work would not have been possible without their love and encouragement.

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LIST OF ABBREVIATIONS

λ	Lambda
μ l	Microliters
μ M	Micromolar
Arg	Arginine
b ₁	Back-cross progeny first generation
bp	Basepairs
cm	Centimeters
<i>cot-5</i>	Colonial temperature sensitive-5
dN	Non-synonymous substitutions at non-synonymous sites
<i>cys-3</i>	Cysteine-3
<i>Dp</i>	Partial diploid
dS	Synonymous substitutions at synonymous sites
EDTA	Ethylenediamine tetraacetic acid
f ₁	Filial progeny first generation
f ₂	Filial progeny second generation
FGSC	Fungal Genetics Stock Center
g/l	Grams per liter
Glu	Glutamine
<i>het</i>	Heterokaryon incompatibility locus
HLA	Human leukocyte antigen
<i>hsp-1</i>	Heat shock protein-1
kbp	Kilobasepairs
IS	Insertion sequence
Lys	Lysine
<i>mat</i>	Mating-type locus
Met	Methionine
MHC	Major histocompatibility complex
mm	Millimeters
ng	Nanograms
°C	Degrees Celsius
O	Other
OR	Oak Ridge
PA	Panama
PCR	Polymerase chain reaction
<i>pyr-4</i>	Pyrimidine-4
RFLP	Restriction fragment length polymorphism
RIP	Repeat Induced Point mutation
RNR	Ribonucleotide reductase
<i>RNR-1</i>	Ribonucleotide reductase large subunit
rpm	Revolutions per minute
RS	Repeated sequence
SDS	Sodium Dodecyl Sulphate
SI	Self-incompatibility

<i>thr-2</i>	Threonine-2
Tyr	Tyrosine
<i>un-24</i>	Unknown temperature sensitive mutation-24
V/cm	Volts per centimeter

GENERAL INTRODUCTION AND LITERATURE REVIEW

Nothing is more common than uniqueness.

(anonymous)

Nonsel self recognition: Classification and occurrence across kingdoms

Self-nonsel self recognition appears to be ubiquitous across kingdoms in bacteria, protists, fungi, plants and animals. Although the mechanisms governing it may vary, the common result of self-nonsel self recognition is the limitation or regulation of cell-cell interactions between individuals. Several types of self-nonsel self recognition may occur both at the inter-species and intra-species levels. This thesis is concerned specifically with the study ofonsel self recognition that occurs between individuals of the same species.

Incompatibility systems within species are divided into two major subgroups; vegetative or somatic incompatibility, and sexual incompatibility. These two subgroups have also been referred to as heterogenic and homogenic incompatibility, respectively (Esser and Blaich, 1973). Vegetative incompatibility serves to limit the cellular interaction to individuals of very similar genotype and as such does not promote genetic variability. In contrast, sexual incompatibility promotes out-breeding by encouraging cell interactions between different genotypes.

From an evolutionary standpoint, the two types of incompatibility are stabilizing during different times in the life of the individual. During the vegetative stage, when nutrients are readily available, it is beneficial for an individual adapted to the environment to maintain a stable genome. In such a case, vegetative incompatibility may protect the genome from invasion by disruptive genetic elements (Buss, 1982). For example,onsel self recognition may prevent the transmission of viruses, hypovirulence dsRNA molecules or senescence plasmids between individuals (Debets *et al.*, 1994; Coenen *et al.*, 1997). Moreover, vegetative incompatibility may prevent the exploitation

of a successful group of individuals by unfit genomes which may use the established colony for their own propagation (Buss, 1982).

On the other hand, environmental stress such as drought or limited nutrient supplies may encourage the onset of events that lead to genetic variation. Genotypic variation is achieved primarily through sexual reproduction, wherein sexual incompatibility will restrict mating to genetically different individuals. The recombination and shuffling of different genetic traits into new combinations, through sexual mating and meiosis, will possibly result in offspring that can occupy new niches (De Boer, 1995).

Some of the most studied systems for sexual reproduction are from the flowering plants. It is perhaps, thus, not surprising that self-nonsel self recognition during the sexual phase has also been extensively studied in plants. In hermaphroditic plants, “self-incompatibility” (SI) is thought to prevent inbreeding by preventing selfing (Nasrallah, 2002). Mechanisms of SI are diverse, suggesting that they have independent evolutionary origins. Self-fertilization can be prevented by inhibition of pollen hydration and germination (*Brassica* spp.), through active destruction of the pollen tube (poppy) and through self-pollen RNA degradation (e.g. *Petunia* spp.) (Kao and McCubbin, 1996; reviewed in Nasrallah, 2002). Sexual self-incompatibility systems also exist in fungi, invertebrates and have been hypothesized for vertebrates. In the hermaphroditic ascidian *Ciona intestinalis*, self-fertilization is prevented by highly polymorphic receptor-ligand interactions between sperm and oocyte (Marino *et al.*, 1998). Interaction of self ligands and receptors on the surface of the two gametes, render the oocyte refractive to fertilization. In contrast, failure of nonself ligand/receptor to bind to one another leaves

the oocyte fertile. In humans, homozygosity at some HLA loci (human nomenclature for MHC class I and II subfamilies) mother and embryo has been linked to recurrent spontaneous abortions (Coulam *et al.*, 1987). Based on this observation, it has been suggested that the MHC may play a role in sexual self-recognition in addition to its known role in somatic nonself recognition. In somatic cells, MHC is responsible for recognizing nonself molecules and presenting them to T lymphocytes for subsequent destruction of nonself entities (Nei *et al.*, 1997). This duality between sexual and vegetative nonself recognition is not unique to the human MHC. In some fungal species, both sexual and somatic (i.e. vegetative) nonself recognition functions are encoded by the same gene, as described below.

In addition to sexual self-nonself recognition, somatic or vegetative nonself recognition is widespread in protists, animals, plants and fungi and appears to be a necessary feature of living cells (De Boer, 1995). In support of this hypothesis, it is interesting to note that all organisms capable of somatic cell fusion have been found to possess some form of nonself recognition or somatic tissue compatibility (Crampton and Hurst, 1994). In addition, most organisms undergoing somatic cell fusion events also show evidence of somatic cell parasitism. Prevention of cell exploitation by another genome has been evoked to justify the existence of nonself recognition. For example, in the cellular slime mold *Dictyostelium mucoroides*, hybrid fruiting bodies formed between wild type and certain mutant cell types result in a majority of wild type cells in the stalk and a majority of mutant cells in the head structure that is responsible for germ-line cell formation. The end result is a preponderant reproduction of the mutant genotype to the detriment of the wild type genotype (Crampton and Hurst, 1994). In this species, the

fusion of amoebae to form the fruiting body depends on their genetic identity (Buss, 1982). Genetically different amoebae fail to aggregate and fuse. The limitation of cell fusion events to genetically similar individuals may protect *Dictyostelium* from invasion by parasitic nuclear variant. Similar examples of nuclear parasitism exist in the myxomycete *Physarum polycephalum*. Fusion between “sensitive” wild type and “killer” mutant strains results in the complete elimination of the sensitive nucleus (Lane and Carlile, 1979). In this species, fusion between plasmodia is controlled by multiple polymorphic loci. Genetic identity at all fusion loci is required for somatic compatibility. Similar examples exist among sponges (*Ephydatia fluviatilis*), hydroids (*Hydractinia echinata*), bryozoa and ascidians (*Botryllus primigenus*) (Scofield *et al.*, 1982; Crampton and Hurst, 1994). In all cited examples of somatic incompatibility, somatic cell parasitism typically results in the elimination of the susceptible nucleus and the overrepresentation of the parasitic nucleus in the offspring. Nonspecific recognition mechanisms in these species operate by restricting somatic cell fusion to genetically identical or nearly identical individuals.

The examples of self-nonspecific recognition presented above constitute a small fraction of the diversity of mechanisms found in nature. A review of these mechanisms is beyond the scope of this introduction (see Esser and Blaich, 1973 and Crampton and Hurst, 1994). To narrow the focus of this study, I use the remainder of the introduction to discuss phenomena of nonspecific recognition in the fungi. A diversity of nonspecific recognition mechanisms occurring during both the sexual and vegetative phases also exists in this kingdom. Nonspecific recognition has been identified in all fungal species where it has been

sought, indicating that in fungi, like in other organisms the identification and protection of self is of biological importance to the organism.

General characteristics of nonself recognition systems in fungi

Fungi are among the best-studied models for nonself recognition. From a practical point of view, fungi have relatively short generation times and their genetic makeup and biology are well understood in some respects thanks to more than 150 years of study (Esser and Blaich, 1994). The growth requirements and life cycles of many fungal species have been investigated, and specifically derived strains are available for laboratory studies. More importantly, individuals belonging to this kingdom generally display a variety of nonself incompatibility phenomena both during the asexual and the sexual phases of the life cycle.

Nonself recognition during the mitotic (asexual) growth phase was first described in fungi by Reinhardt more than 100 years ago as the “repulsion” or “aversion” between fungal mycelia growing on the same substratum. Forty years later, the term “barrage” (French word meaning barrier or dam) was introduced by Vanderdries to describe the aversion reaction between different fungal mycelia (Esser and Blaich, 1994). Since then, barrage, the formation of a line of demarcation or aversion between different fungi has been used as a marker of vegetative nonself recognition (review in Esser and Blaich, 1994; Figure I-1), to establish vegetative compatibility groups and to identify species.

Figure I.1. Example of barrage formation in *N. crassa*. The Standard Mating Reaction occurs between fungal isolates that are different at the mating-type locus (Griffiths and Rieck, 1981). The reaction results in the formation of two rows of perithecia surrounding a hypothetical line of fusion between the two mycelia. The clear zone between the two lines of perithecia presumably indicates a zone of hyphal lysis and regeneration due to mating-type-associated vegetative incompatibility. Fluffy strains FGSC4317 *A* and FGSC4347 *a* were inoculated in alternate quadrants of the petri plate on synthetic crossing medium.

The sexual incompatibility system

In the sexual incompatibility system, mating-type genes control fusion between gametes and the subsequent stages leading to successful meiosis. The mating type is defined by one or more genetic loci each with one or more alleles (Glass and Kulda, 1992). In the simplest system, two different forms of the mating-type locus control the sexual stage in that individual. The ascomycete fungus *N. crassa*, for example, possesses two idiomorphs *mat a* and *mat A* at the mating-type locus (Glass *et al.*, 1988). Idiomorphs are genes with different DNA sequences present at the same genetic location. Similarly, *Podospora anserina*, *Puccinia* sp. and *Mucor* sp. have sexual phases regulated by what is called two-factor heterothallism (Moore-Landecker, 1996). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* individuals can switch from one mating type to another (Glass and Lorimer, 1991). In *S. cerevisiae* it has been proposed that mating-type switching occurs through a mechanism similar to directional transposition involving the protein complex used in recombination (Lewin, 1994). This allows cells from the same lineage to mate.

Similarly, in the bipolar system found in some Basidiomycetes, a compatible mating reaction will occur only if both individuals have different alleles at their mating-type locus. Alternatively, tetrapolar heterothallism is governed by two mating-type loci located at different regions in the genome. Multiple alleles can occur at either mating-type locus and gametes must carry different alleles at both mating-type loci in order for a compatible mating reaction to occur. It is estimated that roughly 65% of all

Basidiomycete species possess this type of mating system (Moore-Landecker, 1996).

Examples include the smut fungus *Ustilago maydis*, *Armillaria* species and *Schizophyllum commune*. The control of the sexual compatibility system in heterothallic ascomycetes is becoming well understood and has been recently reviewed (Casselton, 2002). It is interesting to note that in some fungal species, mating-type genes also regulate vegetative incompatibility, suggesting that the two forms of recognition may share a common mechanism of action. This feature of *N. crassa*, *Ascolobus stercorarius* and *Aspergillus heterothallicus* (Beadle and Coonradt, 1944; Glass and Kulda, 1992) may prove useful in elucidating mechanisms underlying nonself recognition during the vegetative phase.

Vegetative incompatibility

Vegetative incompatibility and heterokaryon incompatibility are not always equivalent terms. In vegetative incompatibility, fusion and subsequent growth of a heterokaryon is controlled by *het* (heterokaryon incompatibility) and/or *vic* (vegetative incompatibility) genes (Perkins and Turner, 1988). Heterokaryon incompatibility genes control the formation and proliferation of heterokaryons, cells containing genetically different nuclei. Vegetative incompatibility genes may in some cases control heterokaryon formation but have primarily been shown to control barrage formation. In *P. anserina*, there is a strong correlation between barrage formation and genetic differences at *het* loci (Rizet, 1952). Barrages were, therefore, used to identify and clone *het* genes and their modifiers in *P. anserina*. However, this correlation is not a common feature of all fungal species. In *S. sclerotiorum* and in *N. crassa*, for example, barrage

formation is not always the result of differences at *het* genes and differences at *het* genes do not always result in barrage formation (Ford *et al.*, 1995; Chapter 1). This observation suggests that vegetative incompatibility encompasses a variety of phenomena that are distinct from heterokaryon incompatibility, and that factors in addition to *het* genes may be involved in vegetative incompatibility (Chapter 1). Nonetheless, the current understanding of the phenomenon of nonself recognition in fungi is primarily the result of studies of heterokaryon incompatibility and mating-type genes. In the remainder of this introduction, the function of heterokaryon incompatibility genes will be discussed and used to draw conclusions about fungal vegetative incompatibility in general.

Heterokaryon incompatibility has been classified into two types, allelic and non-allelic, based on the interactions involved in the recognition mechanism. In allelic incompatibility, different alleles at the same locus encode proteins that interact in a negative manner to produce the incompatibility reaction. *N. crassa* possesses at least 11 *het* loci (including mating-type) involved in allelic incompatibility reactions. In addition, allelic interactions have been shown for some heterokaryon incompatibility genes in *P. anserina*, *C. parasitica*, and *Aspergillus nidulans* (Glass *et al.*, 2000). Non-allelic incompatibility involves the interaction of alleles at different genetic loci. In the ascomycete *P. anserina*, for example, negative interactions between the products of genes *het-c*, *het-e* and *het-d* can lead to lysis of heterokaryons. Such dramatic incompatibility reactions could interfere with proper mating between genetically different individuals should they occur in the sexual structure. How can mating between genetically different individuals occur in the context of *het*-associated incompatibility?

In order to prevent vegetative incompatibility from interfering with the sexual stage, it is thought that *het* genes and related gene functions are downregulated during mating. In *P. anserina*, for example, the expression of certain *het* genes is thought to be locally suppressed in the sexual structures but is active in the surrounding vegetative tissue. During the sexual phase, the *het-c* and *het-v* genes under the regulatory control of *mod-A* and *mod-B*, initiate an incompatibility-like killing reaction which serves to disintegrate the vegetative tissue into macromolecular sub-units. Material from the surrounding mycelium is then thought to be redistributed to the growth of the sexual structures (Bernet, 1992). In contrast, in *N. crassa*, the protein *TOL* controls the mating-type associated vegetative incompatibility response by interacting with *mat a* and *A* gene products (Shiu and Glass, 1999). The transcription of *tol* is suppressed in sexual structures (Shiu and Glass, 1999). Ultraviolet-induced mutations within *tol* will alleviate the incompatibility reaction associated with allelic differences at the mating-type locus during the vegetative phase (Newmeyer, 1970; Vellani *et al.*, 1993). Interestingly, in homothallic (self-fertile) *Neurospora* species such as *N. tetrasperma*, *tol* is naturally mutated and vegetative incompatibility due to mating type is constitutively suppressed. Introgression of *N. crassa tol* into *N. tetrasperma* triggers a vegetative incompatibility response in individuals heteroallelic at *mat* (Jacobson, 1992). Thus, suppression of vegetative incompatibility during the sexual phase may occur through suppression of *het* gene expression or suppression/modification of other genes involved in the incompatibility response. The discovery that mutations within modifier genes could suppress the heterokaryon incompatibility reaction associated with several *het* genes

(Barreau *et al.*, 1998) suggested that heterokaryon incompatibility may follow a unified, common pathway of nonself recognition.

It has been hypothesized that heterokaryon incompatibility has an adaptive function to the organism. As described in the previous section, the adaptive function may be in preventing the spread of infectious elements. The alternative hypothesis is that heterokaryon incompatibility is due to accidental deleterious interactions between different protein variants, forming a “poison” complex (Bégueret *et al.*, 1994), and may have no adaptive value to the organism. The genetic dissection of heterokaryon incompatibility in fungi is expected to provide answers to the evolutionary dilemma concerning whether or not heterokaryon incompatibility has an adaptive function.

het gene products and heterokaryon incompatibility reactions

Two lines of evidence favour the hypothesis that *het* gene function is non-adaptive. First, products of *het* genes have disparate structures and functions that are difficult to conceive in a common pathway (Table I-1). Second, some *het* gene products have other essential roles, for example, in sexual structure development (*het-e*, *het-d*, *het-c*; Barreau *et al.*, 1998) and DNA precursor synthesis (*un-24*, Smith *et al.*, 2000a), suggesting that incompatibility associated with heteroallelism at these loci is an accident and does not have an adaptive role.

Three lines of evidence suggest that heterokaryon incompatibility has an adaptive function in fungi. First, different *het* gene products have been shown to have sequence similarity within a novel protein domain termed the “death domain”. *HET-6* (*N. crassa*), *TOL* (*N. crassa*), *HET-E* (*P. anserina*) and *HET-D* (*P. anserina*) share three conserved

Table I.1. *het* genes from *N. crassa* and *P. anserina* and their known suppressors and modifiers of heterokaryon incompatibility

Species and gene	<i>het</i> protein features	Suppressor gene	Suppressor protein features	References
<i>N. crassa</i>				
<i>mat-A1</i>	α domain	<i>tol</i>	coiled-coil domain,	Glass <i>et al.</i> , 1990, Shiu and Glass, 1999.
<i>mat-a1</i>	HMG box	<i>tol</i>	leucine-rich	Phillely and Staben, 1994
<i>het-c</i>	glycine-rich	<i>vib-1</i>	acid phosphatase	Saupe <i>et al.</i> , 1996, Xiang and Glass, 2002
<i>un-24</i>	signal peptide	-		Smith <i>et al.</i> , 2000a
	ribonucleotide			
	reductase			
<i>het-6</i>	similarity to <i>tol</i> , <i>het-e</i> and <i>het-d</i>	-		Smith <i>et al.</i> , 2000b
<i>P. anserina</i>				
<i>het-s</i>	prion protein	-		Turcq <i>et al.</i> , 1990
<i>het-c</i>	glycolipid transfer protein	<i>mod-D</i>	α subunit G protein	Saupe <i>et al.</i> , 1994, Barreau <i>et al.</i> , 1998
		<i>mod-A</i>	SH3-binding domain	Loubradou <i>et al.</i> , 1999
<i>het-e</i>	GTP binding domain, WD40 repeats	<i>mod-D</i>		Saupe <i>et al.</i> , 1995
		<i>mod-A</i>		
<i>het-d</i>	GTP binding domain, WD40 repeats	<i>mod-D</i>		Espagne <i>et al.</i> , 2002
<i>het-R</i>	unknown	<i>mod-E</i>	Hsp90 heat shock protein	Loubradou <i>et al.</i> , 1997
<i>het-V</i>	unknown	<i>mod-E</i>		

amino acid blocks (Smith *et al.*, 2000b). Although the function of the conserved domain and its possible role in the incompatibility reaction are unclear, it is unlikely that these protein domains have appeared independently by accident in four distinct incompatibility factors. Interestingly, since the publication of the complete *N. crassa* genome, nine additional putative proteins carrying the same death domain are evident (Chapter 2).

It is possible that the death domain was recruited at the several genetic locations in the *N. crassa* genome through duplication. It is, however, unlikely that all nine putative proteins are paralogs of *het-6*, as was proposed for *het-e* and *het-d* in *P. anserina* (Espagne *et al.*, 2002). Unlike the *P. anserina* proteins which are overall 53% identical (71% similar) at the amino-acid level, the *N. crassa* putative peptides only share similarity to *HET-6* in the death domain. The nine *N. crassa* peptides may have other unknown functional domains in addition to the death domain. It has been proposed that the common death domain may be important for nonself specificity, heterocomplex formation or activity regulation during the sexual phase (Smith *et al.*, 2000b). The presence of additional functional domains such as the WD40 repeat and GTP-binding P-loop domains within HET-E and HET-D of *P. anserina* (Espagne *et al.*, 2002) and the coiled coil and leucine rich domains within *TOL* (Shiu and Glass, 1999) suggest that *het* genes may interact with other proteins to trigger the incompatibility response.

A second observation that goes against the accident hypothesis is that certain *het* genes are under balancing selection (Wu *et al.*, 1998; Mir-Rashed *et al.*, 2000). In particular, *het-c* in *N. crassa*, has an unusually high ratio of nonsynonymous to synonymous nucleotide substitutions within regions coding for nonself specificity. A

high non-synonymous/synonymous substitution ratio suggests that positive Darwinian selection is acting to maintain sequence diversity. Based on sequence analysis of species related to *N. crassa*, polymorphisms at *het-c* have been maintained for approximately 36 million years, consistent with selection for allelic diversity at this locus. Such trans-species polymorphisms are typical of self-nonself recognition genes in plants and animals (Richman, 2000). Similarly, *het-s* and *het-c* (unrelated to the *het-c* gene from *N. crassa*) in *P. anserina* have higher nonsynonymous compared to synonymous nucleotide substitutions (Glass *et al.*, 2000). In *N. crassa*, alleles at *un-24* and *het-6* are maintained at balanced frequencies in the population, further suggesting selection for polymorphisms at these loci (Mir-Rashed *et al.*, 2000). Balancing selection has not been tested at all *het* genes. However, it seems that all *het* genes tested so far, have high levels of nonsynonymous compared to synonymous substitutions within coding regions, consistent with the diversifying selection hypothesis.

Finally, in addition to their common protein domains, *HET-E*, *HET-D* and *HET-6* trigger phenotypically similar incompatibility reactions. These reactions are characterized by septal plug formation, hyphal compartmentalization, organelle degradation, cell vacuolization and death (Jacobson *et al.*, 1998; Bégueret *et al.*, 1994). DNA fragmentation has also been detected in dying cells (Glass *et al.*, 2000). Steps of the incompatibility response occur in an organized, concerted manner analogous to programmed cell death reactions occurring in plants and animals (Jacobson *et al.*, 1998; Biella *et al.*, 2002). The similarity between incompatibility reactions and steps leading to cellular disassembly, further suggest that heterokaryon incompatibility occurs according to a pathway and is not consistent with the accident hypothesis.

Thus, common domains associated with vegetative incompatibility, common incompatibility reactions and common selective forces acting on *het* genes support the hypothesis that heterokaryon incompatibility is not an accident but an important biological process that is under selection. However, some *het* genes have unusual ways of triggering the incompatibility reaction, not consistent with a unified pathway. For example, *het-s* in *P. anserina* is a prion analog (Coustou *et al.*, 1997). In *het-s* homokaryons, *HET-s* forms protein aggregates analogous to amyloid plaques described in scrapie and other transmissible spongiform encephalopathies (TSE). The cellular role of these aggregates is unknown, since *het-s* deletion mutants do not appear affected in their normal functions (Saupe *et al.*, 2000). Interactions between *HET-S* and *HET-s* protein types may inhibit further protein aggregation of *HET-s*. In this case, failure of protein aggregation may lead to cell death associated with the *het-s* incompatibility reaction in *P. anserina*. In contrast, protein aggregation by formation of *HET-C*^{PA}/*HET-C*^{OR} heterocomplexes is proposed to be responsible for cell death in *N. crassa*. The mechanism of cell death is unclear. Two possibilities, representative of the “unified pathway” and the “poison complex” hypotheses have been proposed (Sarkar *et al.*, 2002). Protein aggregation may be a trigger for a pathway leading to cell death. Alternatively, protein aggregates themselves could trigger cell death in a non-organized fashion, by destabilizing the plasma membrane. In addition, not all incompatibility reactions lead to cell death. Some heterokaryons are able to sustain very limited growth of distorted hyphae until the individual either loses one of its nuclear types or “escapes” self-incompatibility apparently through deletion events (Smith *et al.*, 1996). In *N. crassa*, the *het-I* locus controls the ability of nuclei to divide. When the ratio of “i” to “I” nuclei is

lower than 70%, “i” nuclear division decreases until the nucleus is lost and the cell becomes homokaryotic for het-I nuclei (Pittenger and Brawner, 1961). Such an incompatibility reaction would be difficult to reconcile with the unified pathway of heterokaryon incompatibility.

It is possible that some of the heterokaryon incompatibility genes are part of a unified pathway, whereas others trigger heterokaryon incompatibility function in addition to encoding other cellular functions. As in the plant self-incompatibility system, heterokaryon incompatibility genes may have different evolutionary origins. In any case, the ultimate function in nonself recognition appears to have been selected for over time.

Importance of the study of heterokaryon incompatibility

As more information emerges from the study of heterokaryon incompatibility, new connections between this phenomenon and other essential cellular mechanisms become apparent. In *P. anserina*, a link has been drawn between developmental processes and vegetative incompatibility (Bernet, 1992). Similarly, in *N. crassa*, vegetative incompatibility appears to share common features with pathways of hyphal growth and conidiation (Xiang *et al.*, 2002). The similarity between cell death during the incompatibility reaction and apoptosis/programmed cell death in mammalian and plant cells suggests a common function. The observation that some *het* genes limit the spread of infectious elements and are under balancing selection further draws a parallel between nonself recognition in fungi and in other organisms such as animals and plants. The study of this phenomenon in fungi can thus provide insight into classic fields of study such as programmed cell death and nonself recognition, from a different perspective.

Beyond the obvious applications of nonself recognition study in the understanding of the evolution of individuality and speciation, heterokaryon incompatibility study has direct applications in the taxonomy and population biology of fungi. In fungi in which species identification is difficult, identification of a particular compatibility group may help in taxonomic determination. In pathogenic fungi in particular, vegetative compatibility groups are correlated to the range of hosts affected and the virulence of the pathogen (Puhalla, 1985). The variability in the number of incompatibility groups and the types of incompatibility can be used to trace the population dynamics of the pathogen. For example, based on vegetative compatibility group studies, new strains of virulent *Fusarium oxysporum* were shown to be introduced to North America from Europe and the Middle East (Jacobson and Gordon, 1990).

At the same time, *het* gene activity has been responsible for the failure of some attempts to control some plant pathogenic fungi such as *Cryphonectria parasitica* and *Ophiostoma novo-ulmi*. In Europe, the chestnut blight disease caused by *Cryphonectria parasitica* could be partially controlled by the introduction of a double stranded (ds) RNA element which induced hypovirulence in this fungus (Nuss, 1992). The dsRNA molecule is spread through cytoplasmic contact during heterokaryosis. In North America, the presence of a larger number of *het*-gene alleles may have prevented the spread of the dsRNA factor. Better knowledge of this system, may therefore help to understand and devise alternative biological control measures.

In the long term, the study of *het* genes may also help develop new anti-fungal drugs which exploit the incompatibility mechanisms of fungi. The emergence of fungi as infectious agents of humans and economically important plants and animals, as well as

the increased resistance of fungal pathogens to currently used antifungal drugs further reinforces the need for new antifungal treatments. The understanding of nonself recognition may help harness the killing potential of the nonself incompatibility biochemical pathway towards antifungal therapy design.

The *het-6* region in *N. crassa*

Neurospora has one of the best studied heterokaryon incompatibility systems. The extensive knowledge of its genetics and the existence of near isogenic strains have also facilitated the study of this organism. The discovery and assay of *het* loci in *N. crassa* has primarily been performed by two different methods. The first involves the synthesis of forced heterokaryons by growing auxotrophic strains on a minimal medium. Their growth requirements can thus only be complemented through heterokaryosis. Incompatible heterokaryons show poor growth or no growth at all. Four *het* genes namely, *het-c*, *het-d*, *het-e* and *het-i* were discovered using the “heterokaryon incompatibility test” (Smith *et al.*, 1996). This particular method may be inconvenient due to the requirement of near isogenic strains. An alternative method involves the use of partial diploid strains (Perkins, 1975). During its vegetative growth, *N. crassa* is a haploid organism. It is however possible to create partially diploid individuals by crossing a wild-type strain with a translocation strain that has a region of one chromosome moved to another chromosome. One third of the viable progeny obtained through such a cross are partially diploid in the region of the translocation. Using this method, Mylyk (1975) identified six additional *het* loci namely *het-5*, 6, 7, 8, 9, and 10. The partial diploids bearing two different alleles at the same *het* locus exhibit an inhibited growth phenotype termed “self-incompatibility”.

More recently, an additional method of studying the action of *het* genes has been DNA transformation. The method involves transforming a linear or cloned DNA fragment into fungal spheroplasts (cells lacking a large portion of their cell wall) bearing either one or the other allele at that *het* locus. The transforming DNA also carries along a selectable marker such as a gene encoding resistance to hygromycin B. At the molecular level, the transforming DNA inserts into the fungal genome and replicates with it. If the transforming DNA carries an active *het* gene and is transformed into spheroplasts of different genotype, its product will interact in a negative manner with the products of the endogenous *het* gene and will trigger an incompatibility reaction. However, if it is transformed into a similar genotypic background, the DNA will have no negative effect on the spheroplasts. Incompatibility activity is recorded as a decrease in the number of recoverable transformants of opposite genotype compared to a control DNA carrying the hygromycin resistance gene alone. The transformation assay is similar to a partial diploid analysis, in which the duplicated region is small and introduced via transformation rather than by mating. Unlike in the transformation assay, in the partial diploid assay the location of the ectopic duplicated region is constant and known (Smith *et al.*, 1996).

One of the *het* loci discovered through partial diploid analysis was *het-6* (Mylyk, 1975), mapping to the left arm of linkage group II (Figure I.2). The locus was discovered by crossing a wild type strain to the *T(II→III)AR18* translocation strain. Subsequent studies of escape from *het-6* incompatibility localized the gene more precisely (Smith *et al.*, 2000b). Two different genotypes at *het-6* have been identified which interact in a negative manner in heterokaryon tests and transformation assays. The two genotypic backgrounds are called Oak Ridge (OR) and Panama (PA). In self-incompatible partial

Figure I.2. Genetic location of *un-24* and *het-6* genes on the left arm of linkage group II. This map was constructed based on OR genomic sequence and cosmid mapping (M. L. Smith). Dotted lines represent the extent of two chromosomal translocations that were used for mapping the location of *un-24* and *het-6* genes.

diploids, the growth inhibition can be by-passed through deletion of one or the other of the duplicated regions (Smith *et al.*, 1996). Usually it is the ectopic segment that is the target of deletion. Studies of strains that escaped from *het-6*-associated incompatibility identified a DNA segment that was necessarily deleted during escape. This region was identified by a chromosomal walk to the cosmid G8:G:1 and G8:G:1 was shown to have *het-6*^{OR} incompatibility activity through transformation assays. Subcloning of the cosmid identified two genes with putative *het* activity. The genes were termed *het-6* and *un-24*; *het-6* comprises a putative open reading frame of ~ 400 amino acids and *un-24* encodes the 680 amino acid large subunit of ribonucleotide reductase.

Purpose and Objectives

The aim of this thesis is to investigate heterokaryon incompatibility in *N. crassa* by analyzing the function and structure of *un-24*^{PA} and *het-6*^{PA}. The study of heterokaryon incompatibility in *N. crassa* has been approached from several different aspects. First, the behaviour of incompatible *N. crassa* colonies was studied at the macroscopic level. In Chapter 1, I describe nutrient requirements for the detection of barrages in *N. crassa*, the appearance of barrages in this fungus and I initiate the dissection of the genetic basis for barrage formation and its relationship to heterokaryon incompatibility. The material presented in Chapter 1 was published in Fungal Genetics and Biology (Micali and Smith, 2003). Chapters 2 and 3 focus more specifically on *het* genes *un-24* and *het-6*. In Chapter 2, I compare the physical structure of the *un-24/het-6* region in OR and PA *N. crassa* from different parts of the world. In Chapter 3, I isolate

and characterize the sequences associated with PA incompatibility activity at *un-24* and *het-6* and investigate mechanisms of escape from *un-24* self-incompatibility.

Chapter 1: Barrage formation and vegetative incompatibility

Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

Albert von Szent-Györgyi, The Scientist Speculates.

INTRODUCTION

The ability to recognize self from nonself is ubiquitous across kingdoms. Nonself recognition among conspecifics occurs in filamentous fungi during both the sexual and asexual phases of the life cycle. Mating occurs only between individuals that carry different determinants at the mating-type locus or loci (reviews in: Kronstad and Staben, 1997; Coppin *et al.*, 1997; Shiu and Glass, 2000). Nonself recognition during the asexual phase is referred to as vegetative, heterokaryon or somatic incompatibility (reviews in: Worrall, 1997; Glass *et al.*, 2000; Saupe, 2000). This vegetative incompatibility system may act before, during or after hyphal fusion (Leslie, 1993) to prevent the transmission, and protect individuals from invasion by parasitic genetic elements (Buss, 1982; Debets *et al.*, 1994; Debets and Griffiths, 1998; Cortesi *et al.*, 2001).

Fungal strains can be classified into distinct vegetative incompatibility groups by three different methods: the barrage test, the heterokaryon incompatibility test and by partial-diploid analysis. Barrage formation is evident as an “aversive” reaction that occurs at, or near, the point of contact between two incompatible mycelia. Barrage reactions may be apparent as dark lines, clearing zones or other forms of demarcation. The phenomenon has been described in a variety of basidiomycetes (review in: Worrall, 1997) and ascomycetes including *Podospora anserina* (Bernet, 1965), *Cryphonectria parasitica* (Anagnostakis, 1977, 1982), *Ophiostoma novo-ulmi* (Brasier, 1984; Milgroom and Brasier, 1997), *Sclerotinia sclerotiorum* (Kohn *et al.*, 1990) and *Neurospora crassa* (Griffiths, 1979; Griffiths and Rieck, 1981).

Heterokaryon incompatibility is tested by a complementation assay with paired strains that have different genetic markers. For example, in *N. crassa* heterokaryon tests are done by co-inoculating two strains with complementary auxotrophic markers on a medium that will not support the vigorous growth of either strain separately. Compatibility of the two strains is evident when a heterokaryon with near wild-type growth rate results, whereas aberrant growth characteristics, or failure to grow, indicate that the two strains are heterokaryon incompatible and have different alleles at one or more heterokaryon incompatibility (*het*) loci (Perkins, 1988). Similarly, in *Fusarium oxysporum*, heterokaryon formation can be recognized by pairing *nit* mutants (unable to reduce nitrate) on a minimal medium with nitrate as the sole nitrogen source (Puhalla, 1985; Correll *et al.*, 1987). *nit* mutants grow poorly on this medium but vigorous growth where the two strains make contact represents *nit* complementation and the formation of a compatible heterokaryon. Heterokaryon compatibility tests may also utilize the autonomous expression of complementating color mutations in uninucleate conidia of *Aspergillus nidulans* (Grindle, 1963).

Finally, analysis of specific *het* gene differences can be done by partial-diploid analysis (Mylyk, 1975; Perkins, 1975). For this, a strain with a duplication-generating chromosome rearrangement is crossed to a strain of unknown *het* gene constitution. Homokaryotic, partial diploid progeny from such a cross that are heterozygous for a given *het* gene within the duplicated segment can be recognized as self-incompatible by phenotypic characteristics that include slow growth, production of brown pigment on an appropriate medium and aberrant colony morphology. Partial diploids that are homozygous at the relevant *het* locus are wild-type in appearance.

Despite differences in these tests, it is plausible that the same genetic factors and biochemical processes underlie barrage formation, heterokaryon incompatibility and self-incompatible partial diploids in a given species. Indeed, the latter two methods yield congruent incompatibility phenotypes and map locations (Perkins, 1975), and thus provide complementary approaches to the cloning of *het* genes in *N. crassa* (Smith *et al.*, 2000). Likewise, in *C. parasitica* and *P. anserina* there is a correlation between vegetative incompatibility, expressed phenotypically as a barrage, and heterokaryon incompatibility as assayed through complementation (Huber, 1996; Rizet, 1952). In *S. sclerotiorum*, however, barrage formation is not necessarily an indication of heterokaryon incompatibility (Ford *et al.*, 1995). This observation may not be surprising considering that a barrage reaction could occur between hyphae separated by some distance through chemical signaling, during cell-cell contact through surface molecules, or after cell fusion through heterokaryon incompatibility mechanisms. Therefore, in some species, *het* genes, as recognized through heterokaryon incompatibility testing and partial-diploid analysis, may represent a subset of all factors involved in vegetative incompatibility.

In this study we examined barrage formation in the context of the heterokaryon incompatibility system in *N. crassa*. Initial observations of the similarity between the interaction zones during mating reactions of *N. crassa* strains and barrage formation in *P. anserina*, *C. parasitica* and *S. sclerotiorum* prompted our study. Although barrages are used as a diagnostic for *het* gene differences in many fungi, few studies on barrages in *N. crassa* have been undertaken.

Previous studies on barrages in *N. crassa* described the “Standard Mating Reaction” as a barrage type that occurs when aconidial (fluffy) mutants of opposite

mating type are confronted on synthetic crossing medium (Griffiths, 1979; Griffiths and Rieck, 1981). Two dense lines of perithecia bordering a clear zone that contains relatively fewer hyphae than surrounding areas, but no perithecia, are the major features of this reaction. Perithecia on either side of the clear zone are derived from resident protoperithecia that have been fertilized by nuclei from the strain located on the opposing side of the clear zone (Perkins, 1988). The clear zone also sometimes contains a band of orange pigment. Formation of the Standard Mating Reaction barrage may be correlated to heterokaryon incompatibility associated with allelic differences at *mat* and *het-c* (review in Perkins, 1988). This association is based on observations that the Standard Mating Reaction does not occur in strains of opposite mating type which are homoallelic for *tol*, a suppressor of mating-type-associated heterokaryon incompatibility. Likewise, confrontations of *mat-A* strains to those bearing *mat-a^{ml}*, which lacks *mat*- associated heterokaryon incompatibility function, do not form the Standard Mating Reaction barrage. The introduction of different alleles at *het-c* in these pairings restores the barrage. The Standard Mating Reaction does not occur with all strains of opposite mating types, has not been investigated beyond laboratory strains and its genetic basis is complex (Griffiths and Rieck, 1981).

On the other hand, the genetic basis of heterokaryon incompatibility in *N. crassa* is well understood. At least ten specific *het* loci and the mating-type (*mat*) locus govern heterokaryon incompatibility in *N. crassa* (reviews in: Perkins, 1988; Saupe, 2000). A difference at any one of these loci prevents the formation, or proliferation, of heterokaryotic cells during asexual growth. To link barrage formation to this well-defined heterokaryon incompatibility system would help unify our understanding of

vegetative incompatibility in *N. crassa*, and fungi generally, and provide alternative approaches to the study of *N. crassa* population structure based on nonself recognition factors.

The objectives of this study were, first, to describe the types of barrages that occur in *N. crassa*, and second, to determine whether or not the genetic basis of barrage formation could be correlated to *het* gene and mating-type differences. We used strains with differences at *het-6*, *het-c* and *mat* to test whether or not these loci are associated with barrage formation in addition to having heterokaryon incompatibility functions. Finally, we wanted to find out whether barrage formation could be used to identify different genotypes of field isolates of *N. crassa*.

The term barrage has been used extensively in different fungal species to describe a variety of reactions occurring at the interface between genetically different isolates. Although in *Neurospora* the term barrage has been previously used to describe only the Standard Mating Reaction, the present study follows the broad definition of the term barrage applied to other fungi. We use the term barrage to collectively refer to dark-line, clear zone and hyphal aggregate interactions in *N. crassa*. Our analysis suggests that, although commonly called barrage, such interactions are under distinct, complex genetic controls that may require that they are treated as independent phenomena.

MATERIALS AND METHODS

Strains and growth conditions

Laboratory and wild strains used in this study are given in Table 1.1. Wild isolates used represent a population sample from Louisiana (Mir-Rashed *et al.*, 2000) and

strains collected in west Africa. Cultures were maintained on 1x Vogel's medium (Vogel, 1964) with 1.5% sucrose, 1.5% agar and the appropriate additives as required (Davis and de Serres, 1970).

Mycelial confrontations

Conidia were suspended in sterile water and about 2 μ L of this suspension was used to inoculate petri dishes containing synthetic crossing medium with 1.5% sucrose, 1.5% agar, (Westergaard and Mitchell, 1947) and 0.1x supplements where required. About 20 ml of medium was used per petri plate (8.5 cm diameter), which corresponded to an agar thickness of ~4 mm. Strains were confronted in pairs; each strain was inoculated twice on each plate, 2.5 cm apart in alternate quadrants (Figure 1.1). Confrontations were incubated at 25 °C for one to two weeks, in alternating dark/natural light before examining for the presence of barrages. At least four types of barrages were observed in the confrontations, denoted as dark line, raised hyphal aggregate, and two distinct types of clear zone. The previously defined clear zone associated with the Standard Mating Reaction (Griffiths and Rieck, 1981) is denoted as a "type A" clear zone. A distinct, second "type B" clear zone was detected in some confrontations that interferes with mating reactions.

Heterokaryon incompatibility and mating type determination

Heterokaryon tests were used to verify alleles at *het* and *mat* loci as described in Smith *et al.* (1996) by overlaying aqueous conidial suspensions on a nutrient-limited Vogel's medium that would not support growth of either strain. After incubation at 30° C

Table 1.1. Strains of *N. crassa* used in barrage confrontations

Strain	Genotype	Origin
<u>Laboratory</u>		
<u>strains¹</u>		
C2(2)-1	<i>het-6^{PA} het-c^{OR} thr-2 a</i>	FGSC 8236
C2(2)-5	<i>het-6^{PA} het-c^{PA} thr-2 a</i>	Smith <i>et al.</i> , 1996
C2(2)-9	<i>het-6^{PA} het-c^{OR} thr-2 A</i>	FGSC 8237
C2(3)-25	<i>het-6^{PA} het-c^{PA} thr-2 A</i>	M. L. Smith
C9-2	<i>het-6^{OR} het-c^{PA} thr-2 a</i>	Smith <i>et al.</i> , 2000
6-13	<i>arg-1 ad-3B; het-6^{PA} het-c^{PA} pyr-4</i> <i>A</i>	Smith <i>et al.</i> , 1996
6-49	<i>arg-1 ad-3B; het-6^{PA} het-c^{OR} pyr-</i> <i>4; inl a</i>	M. L. Smith
RLM58-18	<i>het-6^{PA} het-c^{PA} pyr-4; inl a</i>	Smith <i>et al.</i> , 1996
FGSC 987	74-OR23-1 <i>A</i> , Oak Ridge wild type	FGSC
FGSC 988	OR8-1 <i>a</i> , Oak Ridge wild type	FGSC
FGSC 1946	<i>tol a</i>	FGSC
FGSC 2336	<i>tol trp-4 A</i>	FGSC
FGSC 2337	<i>tol trp-4 a</i>	FGSC
FGSC 2489	74-OR23-1V <i>A</i> , Oak Ridge wild type	FGSC
FGSC 4317	<i>fl A</i>	FGSC

[Table 1.1 continued]

FGSC 4347	<i>fl a</i>	FGSC
FGSC 4564	<i>ad-3B cyh-1 a^{m1}</i>	FGSC
FGSC 5383	<i>arg-3 a^{m33}</i>	Griffiths and DeLange, 1978
<u>Wild-collected strains²</u>		
P4449	<i>het-6^{PA} A</i>	FGSC, Mir-Rashed <i>et al.</i> 2000
P4451	<i>het-6^{OR} het-c^{PA} A</i>	"
P4452	<i>het-6^{PA} het-c^{OR} A</i>	"
P4453	<i>het-6^{OR} het-c^{PA} A</i>	"
P4454	<i>het-6^{OR} het-c^{OR} A</i>	"
P4455	<i>het-6^{OR} het-c^{OR} A</i>	"
P4456	<i>het-6^{OR} het-c^{OR} A</i>	"
P4457	<i>het-6^{OR} het-c^{PA} A</i>	"
P4459	<i>het-6^{OR} A</i>	"
FGSC 4819 – 4822		Congo
FGSC 4825, 4828 – 4834		Ivory Coast

¹Laboratory strains carry the OR allele at all undesignated *het* loci.

²Wild-collected strains have unknown alleles at all undesignated *het* loci, *het-6* alleles based on partial -diploid analyses (Mir-Rashed *et al.*, 2000), *het-c* alleles based on RFLP analysis (Wu *et al.*, 1998; Glass N.L., pers. comm.)

for two days, heterokaryon formation was evident as wild-type growth rates, abundant aerial hyphae and conidiation. Heterokaryon incompatibility due to differences at *het-c* and *mat* were evident as slow growing, “spidery” shaped colonies (Saupe *et al.*, 1996; Jacobson *et al.*, 1998). Differences at *het-6* result in no appreciable heterokaryotic growth (Smith *et al.*, 1996). Mating type of progeny was determined using strains *fl A* (FGSC4317) and *fl a* (FGSC4347), or C2(2)-9A and C2(2)-1a (Table 1.1) by standard methods (Davis and de Serres, 1970).

RESULTS

Barrage characteristics in *N. crassa*

For this study, we define barrages as any form of demarcation between genetically different individuals. This definition follows that proposed by Esser and Meinhardt (1984) and encompasses the Standard Mating Reaction in *N. crassa*. We looked for barrages in over 400 unique confrontations (over 90 of which were replicated at least twice) and carried out multiple self-confrontations for all strains used. Lines of demarcation were never observed to occur when strains were confronted to themselves, only when different strains were confronted. Barrages could be divided into four categories based on the formation of a dark line, a “type B” clear zone, a hyphal aggregate or a Standard Mating Reaction in the interaction zone between mycelia. Representative barrage types observed in this study are illustrated in Figure 1.1.

Dark lines, varying in width from 0.5 to 1.5 cm, were primarily the result of localized pigment deposition (Figure 1.1A). The pigment appeared to originate in the hyphae and to diffuse into the surrounding agar medium. The color of the dark line was dependent upon the strains being confronted and ranged from green-brown to red-brown.

Figure 1.1. Types of barrage in *N. crassa*. A) Dark line between f_1 -6 *a* and C2(2)-9 *A* of *N. crassa* (see Table 1.3A). B) Presence of both dark line and type B clear zone at the interface between the parental strain 6-49 *a* and the b_1 progeny b_1 -13 *A* (see Table 1.3B). The confrontation between these two strains did not produce perithecia. C) Appearance of a type B clear zone in confrontations between two b_1 progeny (b_1 -2 and b_1 -21) from the cross between f_1 -1 and C2(2)-9 (see Table 1.3B). D) Raised hyphal aggregates (bourelet) at the interface between b_1 progeny obtained from a cross of two wild *N. crassa* isolates. Notice that the arrangement of perithecia, irregular dark areas aligned just outside the white bourelet barrage, approaches that of a Standard Mating Reaction. E) Example of a typical confrontation between a wild strain and a f_1 progeny from a cross between two wild Louisiana strains. Notice the lack of barrage and even distribution of perithecia. F) Standard Mating Reaction (type A clear zone) between two f_2 progeny strains of wild isolates.

Dark lines became evident at 7-12 days after inoculation. Wide pigmented areas were often observed between strains of opposite mating-type and were located in the region where perithecia would develop a few days later. Such dark lines became more intense after perithecial formation, apparently due to pigment originating from the perithecia.

In some strain combinations, “type B” clear zones developed in the zone of interaction. The width of these clear zones ranged from 0.2 to 1.5 cm depending on the confrontation strains (Figures 1.1B, 1.1C). Type B clear zones contained relatively fewer hyphae than the surrounding areas of mycelia outside of the interaction zone based on both macro- and microscopic observations and became evident about two weeks after inoculation or about ten days after the confrontation plate was completely covered by the mycelia. These observations suggest that type B clear zones are due to hyphal lysis rather than growth inhibition as hyphae approach one another. In rare cases, the type B clear zone became wider in certain areas and could spread in a patchy or continuous fashion into one or both mycelia. Type B clear zones and dark lines were observed to occur together in the same confrontation in some cases (Figure 1.1B). In such cases, the clear zones developed a few days after dark line formation. The occurrence of a dark line did not appear to interfere with mating reactions. However, when type B clear zones formed (n=25) no perithecia were ever observed to develop from the pairing, even when it was known that the interacting strains differed in mating type (n=16).

Hyphal aggregates (Figure 1.1D) were visible in the interaction zones as a raised hyphal mat of approximately 1 cm width. These aggregates are similar in appearance to the “bourelet” barrage in *P. anserina* (Rizet, 1952). In *N. crassa*, the hyphal aggregate

was white, in contrast to the light orange color of the surrounding mycelia. Hyphal aggregates were often observed in confrontations involving progeny derived from crosses with wild strains, but not with our laboratory strains.

In addition to these types of barrages, the distribution of perithecia relative to barrage lines varied with strain combinations, from an even distribution of perithecia over the entire plate, with or without (Figure 1.1E) barrage lines, to perithecia produced by only one of the strains. In some cases, perithecia were observed to form two more or less well defined lines adjacent to the zone of contact between cultures (Figure 1.1F). This pattern of perithecial distribution was previously described as the Standard Mating Reaction by Griffiths and Rieck (1981). We verified that the Standard Mating Reaction occurs under our culture conditions by confronting fluffy strains, *fl A* and *fl a* (FGSC4317 and FGSC4347, respectively) to each other and to selected laboratory and wild strains (Table 1.2). A double line of perithecia, separated by a 0.5 cm zone containing a relatively low density of hyphae was apparent in confrontations between fluffy strains of opposite mating type after approximately 10 days of growth. The zone with a low density of hyphae, denoted here as the “type A” clear zone, is distinct from the type B clear zone described above. Whereas the type B clear zone appeared to interfere with mating, the type A clear zone was flanked by the two rows of perithecia. In addition, type A clear zones were never observed to spread to the surrounding mycelium, as occasionally occurred with type B clear zones.

Confrontations between *tol A* (FGSC2336) and *fl a* (FGSC4347) strains produced a Standard Mating Reaction with most of the perithecia on the fluffy strain side. As in previous reports (Perkins1988), the Standard Mating Reaction was not evident in

confrontations between *tol A* (FGSC2336) and *tol a* (FGSC1946) strains, nor between fluffy strains of either mating type and the mating-type mutant α^{ml} (FGSC4564), which is deficient in *mat*-associated heterokaryon incompatibility function. These results support the view that the Standard Mating Reaction barrage is suppressed along with *mat*-associated heterokaryon incompatibility (Perkins, 1988). However, contrary to this, a Standard Mating Reaction-like barrage, in which perithecia were sparsely distributed along two lines, was observed to form between *tol* strains FGSC2336 and FGSC2337 (Table 1.2).

Influence of medium on barrage formation

We found that barrages were only evident under growth conditions that did not favor the formation of aerial hyphae and conidia as reported for the Standard Mating Reaction by Griffiths and Rieck (1981). This could be achieved by using synthetic crossing medium (SCM) that has relatively low amounts of nitrogen. Media that are rich in nitrogen and carbon, such as Vogel's medium, Malt Extract or Sabouraud Dextrose media (Difco Laboratories, Detroit, MI) did not promote barrage formation. Of the two defined media, SCM contains KNO_3 (1 g/L) as a nitrogen source whereas Vogel's minimal medium contains NH_4NO_3 (2 g/L). When the source and level of nitrogen in Vogel's medium were adjusted to that of SCM, confrontations developed barrages similar to those observed on SCM medium, however, of lower color intensity. Trace elements and biotin were also found to be important in promoting barrages. Their omission from the medium caused a 1 to 2 week delay in the formation of barrages and in the development of perithecia. It is interesting to note that, with respect to the medium,

barrage formation and mating reactions are controlled in a similar manner, both are promoted under nutrient limiting conditions.

Dark line and type B clear zone barrages are independent of *mat* and *het* genes

Barrages were never observed to occur when strains were confronted to themselves. A genetic difference between individuals is therefore required for barrage formation. In other fungi such as *P. anserina* and *C. parasitica*, barrage formation is associated with differences at heterokaryon incompatibility loci (Rizet, 1952; Barreau *et al.*, 1998; Huber, 1996). In *N. crassa*, Standard Mating Reaction is also associated with differences at the mating-type locus (Griffiths and Rieck, 1981; Perkins, 1988). Our observations suggest that in *N. crassa*, the genetic determinants of the dark line and type B clear zone barrage types are distinct from both heterokaryon incompatibility and mating type (Table 1.2). For example, strains that are heterokaryon compatible did [RLM58-18*a* + C2(2)-5*a* and 6-49*a* + C2(2)-1*a*] or did not [6-13*A* + C2(3)-25*A*] form barrages. On the other hand, the confrontations C2(2)-9*A* + C2(2)-1*a* (different at *mat*), C2(3)-25*A* + C2(2)-9*A* (*het-c* incompatible) and C9-2 + RLM58-18 (*het-6* incompatible) did not form barrages. These results indicate that although barrages may form between *het* incompatible strains, differences in the alleles present at *het-6*, *het-c* or *mat* are not responsible for dark line or type B clear zone barrages.

Genetic basis of barrage formation

We examined barrage formation in two sets of progeny. First, 19 *f*₁ progeny were analyzed from the cross, 6-49*a* x C2(2)-9*A*. These two parental strains are identical at all

Table 1.2. Barrages among laboratory strains of *N. crassa* with known heterokaryon

incompatibility genotypes. All confrontations between strains with different mating types produced perithecia. Note that all strains were also confronted to themselves and produced no barrages.

<i>het</i> differences	Confrontation pair	Type of barrage
none	6-13 <i>A</i> + C2(3)-25 <i>A</i>	None
none	RLM58-18 <i>a</i> + C2(2)-5 <i>a</i>	Dark line
none	6-49 <i>a</i> + C2(2)-1 <i>a</i>	Dark line
none	4347 <i>a</i> + 4564 <i>a</i> ^{m1}	None
none	4317 <i>A</i> + 2336 <i>A</i>	None
none	4347 <i>a</i> + 1946 <i>a</i>	None
none ¹	4317 <i>A</i> + 4564 <i>a</i> ^{m1}	None
none ¹	2336 <i>A</i> + 5383 <i>a</i> ^{m33}	None
<i>mat</i> ²	2336 <i>A</i> + 2337 <i>a</i>	SMR-like ³
<i>mat</i> ²	2336 <i>A</i> + 1946 <i>a</i>	None
<i>mat</i>	4317 <i>A</i> + 4347 <i>a</i>	SMR ³
<i>mat</i>	4347 <i>a</i> + 2336 <i>A</i>	SMR
<i>mat</i>	4317 <i>A</i> + 1946 <i>a</i>	SMR
<i>mat</i>	4347 <i>a</i> + 2489 <i>A</i>	SMR, Dark line
<i>mat</i>	4347 <i>a</i> + 987 <i>A</i>	SMR, Dark line
<i>mat</i>	4317 <i>A</i> + 988 <i>a</i>	SMR, Dark line
<i>mat</i>	2489 <i>A</i> + 988 <i>a</i>	SMR

[Table 1.2 continued]

<i>mat</i>	987 <i>A</i> + 988 <i>a</i>	SMR
<i>mat</i>	6-13 <i>A</i> + RLM58-18 <i>a</i>	None
<i>mat</i>	6-13 <i>A</i> + C2(2)-5 <i>a</i>	None
<i>mat</i>	C2(3)-25 <i>A</i> + RLM58-18 <i>a</i>	Dark line
<i>mat</i>	C2(2)-9 <i>A</i> + C2(2)-1 <i>a</i>	None
<i>mat</i>	C2(3)-25 <i>A</i> + C2(2)-5 <i>a</i>	SMR-like ³
<i>mat</i>	6-49 <i>a</i> + C2(2)-9 <i>A</i>	Dark line
<i>het-c</i>	6-13 <i>A</i> + C2(2)-9 <i>A</i>	None
<i>het-c</i>	C2(3)-25 <i>A</i> + C2(2)-9 <i>A</i>	None
<i>het-c</i>	6-49 <i>a</i> + RLM58-18 <i>a</i>	Dark line
<i>het-c</i>	6-49 <i>a</i> + C2(2)-5 <i>a</i>	Dark line
<i>het-c</i>	C2(2)-5 <i>a</i> + C2(2)-1 <i>a</i>	Dark line
<i>het-c</i>	C9-2 <i>a</i> + 4564 <i>a</i> ^{m1}	Dark line
<i>het-c</i>	6-13 <i>A</i> + C2(2)-9 <i>A</i>	None
<i>het-c</i>	RLM58-18 <i>a</i> + C2(2)-1 <i>a</i>	None
<i>het-6</i>	C9-2 <i>a</i> + RLM58-18 <i>a</i>	None
<i>het-6</i>	4564 <i>a</i> ^{m1} + 6-49 <i>a</i>	None
<i>het-6</i>	4564 <i>a</i> ^{m1} + C2(2)-1 <i>a</i>	None
<i>het-6</i>	C9-2 <i>a</i> + C2(2)-5 <i>a</i>	Dark line
<i>het-c</i> , <i>het-6</i>	C9-2 <i>a</i> + C2(2)-1 <i>a</i>	Dark line
<i>mat</i> , <i>het-c</i>	6-49 <i>a</i> + 6-13 <i>A</i>	Dark line

[Table 1.2 continued]

<i>mat, het-c</i>	C2(3)-25 <i>A</i> + C2(2)-1 <i>a</i>	SMR-like ³
<i>mat, het-c</i>	C2(2)-5 <i>a</i> + C2(2)-9 <i>A</i>	SMR-like ³
<i>mat, het-c</i>	RLM58-18 <i>a</i> + C2(2)-9 <i>A</i>	Dark line
<i>mat, het-c</i>	6-49 <i>a</i> + C2(3)-25 <i>A</i>	Dark line
<i>mat, het-c, het-6</i>	C9-2 <i>a</i> + C2(2)-9 <i>A</i>	Dark line

¹ Strain FGSC4564 carries the a^{m1} mutant allele, which has neither *mat*-associated heterokaryon incompatibility or mating-type functions. Strain FGSC5383 carries the a^{m33} allele and lacks heterokaryon incompatibility function but has sexual mating-type function (Griffiths and De Lange, 1978).

² Confrontations FGSC2336 + FGSC1946 and FGSC2336 + FGSC2337 are both between opposite mating-type strains that carry *tol*, the recessive suppressor of *mat*-associated heterokaryon incompatibility (Newmeyer, 1970).

³ SMR = Standard Mating Reaction, or SMR-like when double line of perithecia are less well developed (diffuse) than in Standard Mating Reaction.

het loci, but will not form a stable heterokaryon due to differences at *mat*. When confronted on SCM, 6-49 and C2(2)-9 produced a single, well-defined dark line (Table 1.3A). The 21 progeny were scored for their ability to form barrages with the parental strains. Most of the progeny (17/19 where mating type could be determined) formed a dark line with the parent of opposite mating type. Two of the progeny (f_1 -1*a* and f_1 -4*a*) formed a dark line with both parents and produced perithecia with C2(2)-9*A*.

From these observations on the f_1 progeny, we built a genetic model of the dark line formation where two loci (one of which could be *mat*) control dark line formation and genetic differences at either of the two loci result in a dark line barrage. We assumed that both loci are closely linked to *mat* and that C2(2)-9*A* and 6-49*a* differ at both barrage loci. This model explains why most of the f_1 progeny form barrages with the parent of opposite mating type. However, a cross-over between the two loci could give rise to progeny such as f_1 -1 and f_1 -4 that form dark lines with both parents. In order to test the model, f_1 -1*a* was back-crossed to C2(2)-9*A* to obtain b_1 progeny and these were scored for barrage type when confronted with each of C2(2)-9*A*, 6-49*a* and f_1 -1*a*.

Based on our model, we expected most of the progeny from our second cross to be similar in barrage characteristics to either parent strain, f_1 -1*a* or C2(2)-9*A*. In addition, we expected that all b_1 progeny would form barrages with 6-49 irrespective of their mating type. Thirty-four b_1 progeny were analyzed and the results of the confrontations are presented in Table 1.3B. Fifteen progeny produced barrages like either C2(2)-9 (10/34) or f_1 -1 (5/34) parental type. Of the remaining 19 non-parental progeny, 16 did not form barrages with 6-49 and three formed a barrage with 6-49 but either did (b_1 -16), or did not (b_1 -32 and b_1 -37), form barrages with both parents. Our two-gene model

proposed to explain barrage formation in the original C2(2)-9 + 6-49 pair of confronting strains was not supported by these data.

Barrage formation in *N. crassa* appears to be under complex genetic control. Some of the confrontations involving the f_1 and b_1 progeny listed in Table 1.3 produced different barrage types from the original parental strain confrontations. For example, type B clear zones were evident in seven confrontations involving f_1 progeny [f_1 -8, -10, -11, -13 all gave type B clear zones with 6-49; f_1 -15 with C2(2)-9; f_1 -14 with both 6-49 and C2(2)-9], and seven involving b_1 progeny [b_1 -20 with f_1 -1; b_1 -13 with C2(2)-9; b_1 -6, -7, -37 all with 6-49; b_1 -8 with both f_1 -1 and 6-49]. Type B clear zones were not evident in confrontations between the parental strains of these progeny. In addition, 2/19 f_1 , and 9/34 b_1 progeny failed to produce perithecia during barrage tests when confronted with strains of opposite mating type. The mating type of these strains could be determined by using conidial suspensions to fertilize *mat-a* and *mat-A* tester strains. Infertility in these cases could be due to homozygosity at genes affecting mating capability, ascospore development or perithecium development.

Of 76 distinct pairings in Table 1.3 that produced a dark line, 62 involved strains that differed at mating type. The association between a difference at mating type and dark line formation, although not perfect, was significant (χ^2 , $P < 0.001$), and led us to explore mutations at *tol*, a recessive suppressor of *mat*-associated heterokaryon incompatibility but not sexual compatibility (Newmeyer, 1970; Shiu and Glass, 1999). To evaluate the effect of homoallelism at *tol* on dark line formation, we collected *tol* progeny from the crosses 6-49 x FGSC2336 and C2(2)-1 x FGSC2336 and set up confrontations between these progeny and the *tol* strains FGSC2336 *A* and FGSC2337 *a*.

A dark line was observed in one of 14 confrontations between *tol A* and *tol a* strains compared to four confrontations that gave dark lines from 20 *A/a* pairings between *tol⁺* strains. The difference between these two groups was not significant (χ^2 of arcsin-transformed proportions, $P > 0.8$). Regardless of mating type, there was no significant difference (χ^2 of arcsin-transformed proportions, $P > 0.4$) between the number of confrontations that produced dark lines where both strains were *tol* (5 in 29 total) compared to where both strains were *tol⁺* (13 in 40 total). However, it should be pointed out that, unlike in Table 1.3, there was no difference between the number of dark lines formed in *A/A* and *a/a* pairings compared to *A/a* pairings among the *tol⁺* strains (χ^2 , $P > 0.16$) in confrontations with this progeny set. Therefore, the dark line factor in these progeny appears to operate independent of *mat*.

Barrage frequencies in wild isolate confrontations and inbred progeny

Barrage formation was also investigated by pairing, in all combinations, nine wild isolates from a population sample of *N. crassa* collected in Louisiana. Surprisingly, none of these 36 pairings produced barrages. However, as outlined below, increased inbreeding appears to contribute to the development of barrages in these strains, suggesting that suppressors of barrage formation may be present in the wild strains, that are not found in laboratory strains.

When Louisiana isolates were confronted to *fl a* and *fl A* strains, a single faint dark line was apparent in one confrontation between P4457 and *fl A*. There were no cases of Standard Mating Reaction in these confrontations. This absence of barrages could be due to a lack of genetic variability among the Louisiana population sample or to the presence of a dominant suppressor(s) of barrage formation. The former possibility is

Table 1.3. Analysis of barrages in parental, f_1 and f_2 progeny confrontations.

A) Progeny were from a cross between C2(2)-9*A* and 6-49*a*, two strains that carry no *het* differences. The progeny were confronted back to the parental strains and dark line barrages were noted. B) Progeny from the back-cross of f_1 -1 x C2(2)-9 were confronted with C2(2)-9, 6-49 and f_1 -1 and dark line barrages were noted. Asterisk indicates where no perithecia were formed in barrage tests, mating type was identified by perithecial formation following fertilization of mating-type tester strains. Pairings that produced clear zone barrages are given in the text and strains showing this behavior are indicated with a superscript C.

A				
Total	Mating type	Dark line formation		Strains
number		C2(2)-9 <i>A</i>	6-49 <i>a</i>	
of	<i>A</i>	-	+	C2(2)-9
progeny	<i>a</i>	+	-	6-49
7	<i>A</i>	-	+	f_1 -7, f_1 -12, f_1 -13* ^C , f_1 -14* ^C , f_1 -15 ^C , f_1 -17, f_1 -18
2	ND	-	+	f_1 -13, f_1 -14
10	<i>a</i>	+	-	f_1 -3, f_1 -6, f_1 -8 ^C , f_1 -9, f_1 -10 ^C , f_1 -11 ^C , f_1 -16, f_1 -19, f_1 -21, f_1 -23
2	<i>a</i>	+	+	f_1 -1, f_1 -4

[Table 1.3 continued]

B

Total	Mating type	Dark line formation			Strains
number		f ₁ -1 <i>a</i>	C2(2)-9 <i>A</i>	6-49 <i>a</i>	
of	<i>A</i>	+	-	+	C2(2)-9
progeny	<i>a</i>	-	+	+	f ₁ -1
10	<i>A</i>	+	-	+	b ₁ -1, b ₁ -3, b ₁ -6* ^C , b ₁ -7* ^C , b ₁ -8* ^C , b ₁ -9, b ₁ -13* ^C , b ₁ -15*, b ₁ -21, b ₁ -38
5	<i>a</i>	-	+	+	b ₁ -2, b ₁ -4, b ₁ -12, b ₁ -14, b ₁ -29
1	<i>A</i>	+	+	-	b ₁ -20 ^C
2	<i>a</i>	+	+	-	b ₁ -24, b ₁ -34
9	<i>a</i>	-	+	-	b ₁ -5, b ₁ -10, b ₁ -18, b ₁ -22, b ₁ -25, b ₁ -26, b ₁ -33, b ₁ -35, b ₁ -36
3	<i>A</i>	+	-	-	b ₁ -19, b ₁ -30, b ₁ -31*
2	<i>A</i>	-	-	+	b ₁ -32*, b ₁ -37* ^C
1	<i>a</i>	+	+	+	b ₁ -16*
1	<i>a</i>	-	-	-	b ₁ -11

unlikely since barrages were also not evident in confrontations between these wild isolates and laboratory strains. Furthermore, genetic variability in this population sample is evident from analyses of markers at *mat*, *het-c* and *het-6* (Mir-Rashed *et al.*, 2000; Wu *et al.*, 1998). Finally, we performed a similar survey by pairing 12 wild-collected strains from west Africa in all combinations. Again, none of the 66 confrontations produced barrages (including no Standard Mating Reactions).

To examine the possibility of a dominant suppressor of barrage in the Louisiana population sample, we determined the frequencies of barrage formation in four inbreeding series. Confrontations between progeny within each series were scored for the presence or absence of barrages. Series 1 and 2 were initiated with crosses between wild strains (P4449 + P4455 and P4453 + P4451, respectively). Two of nine (series 1) and two of twenty (series 2) confrontations between the f_1 progeny produced a brown line and the remainder produced no barrages. f_2 progeny from one randomly selected cross in each of series 1 and 2 were confronted and yielded 21 barrages in 44 confrontations for series 1 and 14 in 45 confrontations for series 2. The barrages that formed between these f_2 progeny were of the brown line and/or hyphal aggregate type. The distribution of perithecia in the fertile combinations of f_1 progeny was generally more orderly in appearance and, in confrontations among f_2 progeny, often approached that of a Standard Mating Reaction (Figure 1.1F), in comparison to a scattered distribution of perithecia in confrontations among the wild isolates. There was no evidence of type B clear zones in any of the confrontations involving wild isolates or their f_1 or f_2 progeny.

Series 3 and 4 were initiated with wild x laboratory strain crosses P4455 x C2(3)-25 and P4455 x C2(2)-9, respectively. In each series, one randomly selected f_1 progeny

was back-crossed to the appropriate parental laboratory strain to obtain b_1 progeny. Confrontations were set up between all progeny from a given generation for each series. f_1 progeny from series 3 developed eight barrages in 36 confrontations and 9/35 in b_1 confrontations. Series 4 yielded 5/21 and 21/40 in f_1 and b_1 confrontations, respectively. Again, barrages in series 3 and 4 were more defined than those of series 1 and 2, especially from series 4 where barrage intensity was comparable to our barrages in laboratory strain pairings. Barrages in series 3 and 4 were mostly of the dark line type, although hyphal aggregates were also evident in the b_1 confrontations, especially in series 3. In addition, one type B clear zone barrage was observed in a confrontation between b_1 progeny of series 3.

In Figure 1.2 the barrage frequencies from series 1 and 2 were pooled, as were those from series 3 and 4, and presented together with barrage frequencies for confrontations among our laboratory strains. From this figure it is apparent that the frequency of barrages increases with inbreeding so that by the f_2 (series 1 and 2) or b_1 (series 3 and 4) generations the frequency of barrage occurrence is comparable to that for our laboratory strains. This observation suggests that barrage formation requires that the confronted strains are homoallelic at two or more (suppressor) loci, and is consistent with the hypothesis that suppressors of barrage occur in this wild population of *N. crassa*.

DISCUSSION

The main objectives of this study were to describe the different types of barrages observed in *N. crassa*, to identify whether there is a correlation between heterokaryon incompatibility and barrage formation and to investigate barrages in wild *N. crassa*

Figure 1.2. Barrage frequency (# confrontations with dark line, clear zone or hyphal aggregate barrages / # total nonself confrontations) among wild isolates (P) and among f_1 , f_2 or b_1 progeny of selected crosses (see text). W+W = confrontations among progeny from inbreeding series 1 and 2. W+L = among progeny from back-cross series 3 and 4. L+L = barrage frequency among laboratory strains (Table 1.2). Note that Standard Mating Reaction-like barrages are excluded from frequency calculations but that confrontations in which the distribution of perithecia approached that of a Standard Mating Reaction also became more common with inbreeding strains.

Our results challenge the link between barrage formation and heterokaryon incompatibility. As in other fungal species, barrages in *N. crassa* form at the interface between genetically different individuals. The common requirement for barrage formation in *N. crassa* was a low nitrogen medium. It is noteworthy that the same nutritional conditions are required for mating in *N. crassa* (Ricci *et al.*, 1991). This may indicate that genes involved in barrage formation and mating are under a common regulatory pathway. In contrast, we found that heterokaryon incompatibility associated with differences at *het-c*, *het-6* and *mat* occurs on both low and high nitrogen-containing media.

In addition to the Standard Mating Reaction previously described by Griffiths and Rieck (1981), we observed three types of barrages. Dark lines, clear zones and hyphal aggregates all have counterparts in other fungi. Dark lines were described in *C. parasitica* confrontations (Newhouse and MacDonald, 1991) and are common in mycelial interactions involving basidiomycetes (Worrall, 1997). Dark lines in *N. crassa* are due to the production of pigment(s) that accumulates in the hyphae and diffuses into the medium near the interaction zone. Melanin-like pigments are produced by a variety of fungal species and are often associated with perennating structures and/or environmental stress. Such pigments appear to protect fungal cells from lysis (Bloomfield and Alexander, 1967) and microbial and chemical degradation. Pigmented lines were previously described in the interaction zone between two *N. crassa* strains of the same mating type (Kuwana, 1958). The pigments were presumed to be melanin, or melanin-like compounds, and were correlated to hyphal lysis and to an increase in tyrosinase activity within the interaction zone.

Clear zones in *N. crassa* appear to be of two types. The first type (type A), is associated with the Standard Mating Reaction barrage, is evident in some confrontations with strains of opposite mating type and is under complex genetic control (Griffiths and Rieck, 1981). The type A clear zone contains hyphae, but at a lower density than in the surrounding areas. Conversely, the “type B” clear zone was observed here in confrontations between strains of similar or dissimilar mating type, appeared to completely block mating and occurred between some heterokaryon compatible strains (e.g. in Table 1.3). Type B clear zones were apparently nearly devoid of hyphae and became evident a few days after colonies grew together, suggesting that, as in *P. anserina*, *C. parasitica* and *S. sclerotiorum*, these clear zones result from hyphal fusion coupled with degradation of the fusion products (Bernet, 1965; Anagnostakis, 1977; Kohn *et al.*, 1990). Both types of clear zone barrages may also be present in *P. anserina* (Esser and Blaich, 1994). Clear zones that can interfere with mating in *P. anserina* result from non-allelic interactions between *het* genes *a*, *b*, *c* and *v* [note that genes follow Esser and Blaich (1994) nomenclature designation and that the Bernet (1965) equivalents are *e*, *c*, *r*, *v*]. Clear zone barrages that are the result of allelic interactions at *het* genes *t* and *u*, however, do not interfere with mating in *P. anserina*.

Hyphal aggregates similar to those we observe in *N. crassa* were identified in *P. anserina* as “bourelet” (Rizet, 1952) and in *S. sclerotiorum* (Kohn *et al.*, 1990). This type of barrage does not interfere with mating in *P. anserina* nor, we find, in *N. crassa*. In *P. anserina*, the formation of the bourelet appears to be due to hyphal lysis coupled with hyphal regeneration and branching at the interface between the two colonies, leading

to a localized high density of hyphae. We did not observe hyphal lysis associated with aggregates in *N. crassa* in preliminary microscopical examinations.

The dark line and type B clear zone barrages described above are not dependent on differences at the major *het* genes *mat*, *het-c* and *het-6*. It is possible that differences at minor *het* genes are responsible for these barrages. Our preliminary investigation into the genetic basis of dark line barrages revealed a complex system. Dark lines form mostly between strains that are different at *mat*, occasionally when both strains carry *mat-α*, and rarely when both strains carry *mat-A* (e.g. Table 1.3). In spite of the imperfect correlation between dark line formation and mating-type differences, and in contrast to the situation with the Standard Mating Reaction, we observed a non-significant decrease in the incidence of dark line barrages in confrontations between *tol* strains compared to between *tol*⁺ strains. Our data suggest that there are multiple factors controlling dark line formation and at least some of these are not associated with *mat* differences.

That barrage formation is under complex genetic control was further suggested by the appearance of barrage types among *f*₁, *f*₂ and *b*₁ progeny confrontations that were not evident in the parental confrontations. We hypothesize that suppressors that prevent barrage formation may exist in some strains. Suppressors or modifiers of heterokaryon incompatibility function have been described in *N. crassa* (Shiu and Glass, 1999; Xiang *et al.*, 2002) and *P. anserina* (Boucherie and Bernet, 1974). Our laboratory strains, being isogenic at many loci, may have lost such suppressors and thus form barrages more frequently. By selecting random progeny from a series of inbreeding crosses involving wild isolates, we observed that the frequency of barrage formation increased from zero

among wild strains to ~40% in the f_2 and b_1 generations. This observation lends support to the suppressor hypothesis.

The collective observations reported here present a general image of barrages in *N. crassa*. Various types of demarcation lines are evident when different *N. crassa* strains are confronted on nutrient limiting medium. These lines are similar to barrages described in other fungi in regards to form (color and shape), physiology (dependent on low nutrient medium) and function (e.g. type B clear zones inhibit mating). Genetic segregation data suggest barrage formation in *N. crassa* is under complex genetic control that can be independent of heterokaryon incompatibility and mating type. Confronted with this variety of barrage types, one might wonder how to define vegetative incompatibility. Are interactions that result in barrages, but that do not noticeably restrict heterokaryosis, involved in nonself recognition? Should the definition of vegetative incompatibility factors be limited only to those that prevent heterokaryosis? Our observations suggest that barrages and heterokaryon incompatibility are largely different subsets of what might be termed “vegetative incompatibility” in *N. crassa*. The assumption that barrages and heterokaryon incompatibility are different manifestations of a single phenomenon should be carefully examined in other fungi.

Chapter 2: Structural basis of linkage disequilibrium at *un-24* and *het-6*

They are in you and in me; they created us, body and mind; and their preservation is the ultimate rationale for our existence... They go by the name of genes, and we are their survival machines.

Richard Dawkins, The Selfish Gene

INTRODUCTION

Linkage disequilibrium is the non-random association of alleles at two or more loci (Ardlie *et al.*, 2002). At its simplest, linkage disequilibrium is indicative of the physical proximity of two or more loci. The inheritance pattern of alleles at linked loci is dependent on the distance separating the two loci. Newly formed allelic variants at two closely-linked loci will tend to be inherited together through subsequent generations, resulting in linkage disequilibrium. In this context, disequilibrium between a known marker locus and a disease-causing locus was used to map and clone several human disease loci (Pritchard and Przeworski, 2001). In addition to physical linkage, linkage disequilibrium can be indicative of specific selective pressures such as epistatic selection (selection for particular interacting allelic combinations), suppression of recombination via chromosomal rearrangements or population changes such as inbreeding or massive extinction (Ardlie *et al.*, 2002).

In the context of this study, the discovery of linkage disequilibrium at two genes involved in heterokaryon incompatibility in *N. crassa* provided an opportunity to study some of the evolutionary aspects that shape nonself recognition genes in fungi. A previous study on the distribution of alleles in a *N. crassa* population from Louisiana provided evidence that *un-24* and *het-6* are in severe linkage disequilibrium (Mir-Rashed *et al.*, 2000). Only OR/OR or PA/PA haplotypes of *un-24* and *het-6* were detected in this population. The primary question that stimulated research summarized in this chapter was, “Why are *un-24* and *het-6* in linkage disequilibrium?”

Population genetic factors that could account for the observed linkage disequilibrium at *un-24* and *het-6* are numerous. First, the recent introduction of a new haplotype in a population could limit the number of recombinant genotypes in that population (Ardlie *et al.*, 2002). However, isolates from very distant regions of the globe including USA, East Africa, Costa Rica, Haiti, Malaya and India display only two of four possible allelic combinations, OR/OR or PA/PA, at *un-24* and *het-6* (Mir-Rashed, 1998), suggesting that factors affecting linkage are global and constant in time rather than local and transient within populations. Second, the apparent lack of recombinant haplotypes could be due to selection against recombinant progeny. For example, self-incompatible recombinant progeny due to non-allelic interactions could be selected against. This explanation for linkage disequilibrium is addressed in Chapter 3, where I test the hypothesis that the *un-24*^{PA} *het-6*^{OR} recombinant haplotype is deleterious. Finally, the possibility that recombination between the two haplotypes is suppressed would provide another reason for *un-24/het-6* linkage disequilibrium. This possibility is investigated in this chapter.

That there are low rates of recombination between *un-24* and *het-6* is suggested by three observations from genetic studies. First, based on sequence analyses, OR and PA alleles at both *un-24* and *het-6* have highly divergent nucleotide sequences (Smith *et al.*, 2000b). Second, low levels of cross-hybridization in Southern analyses (Mir-Rashed *et al.*, 2000) indicated that there is little sequence identity between OR and PA haplotypes in the region between *un-24* and *het-6*. Finally, restriction site maps show that the distance between *un-24* and *het-6* is ~19 kbp in OR strains and only ~5.2 kbp in PA strains (Mir-Rashed *et al.*, 2000). In this chapter I investigate structural reasons for

linkage disequilibrium through a physical study of the *un-24/het-6* region in OR and PA haplotypes.

MATERIALS AND METHODS

Fungal and bacterial strains and growth conditions

Laboratory and wild fungal strains used in this study are presented in Table 2.1. Strains were maintained on 1x Vogel's medium with 1.5% sucrose, 1.5% agar and 1x supplements as necessary (Davis and de Serres, 1970). Cloning and bacterial transformations were performed into *E. coli* DH5 α cells (GIBCO/BRL, Burlington, ON) and bacterial cultures were maintained on LB solid (1.5% agar) or liquid medium supplemented with the appropriate antibiotics as described in Sambrook *et al.* (1989).

DNA isolation

Neurospora genomic DNA was isolated by the method of Oakley *et al.* (1987). Plasmid and cosmid DNAs were isolated using the Wizard™ miniprep kit (Invitrogen, Carlsbad, CA). Lambda clone DNA was isolated by the liquid lysis method as described in Sambrook *et al.* (1989).

PCR amplification and restriction digestion

PCR reactions were set-up in a final volume of 50 μ l using approximately 60 ng template DNA and *Taq* DNA polymerase (Gibco/BRL) or Expand High Fidelity PCR system (Roche, Laval, PQ) according to the manufacturer's specifications. PCR primers (Table 2.2) were designed based on *N. crassa* genomic sequences obtained in the present

Table 2.1. Fungal strains used in this study.

Strain ¹	Genotype ²	Origin
<i>N. crassa</i>		
P4450	<i>un-24^{OR} het-6^{OR} a</i>	FGSC, Mir-Rashed <i>et al.</i> , 2000
P4493	<i>un-24^{PA} het-6^{PA} a</i>	FGSC, Mir-Rashed <i>et al.</i> , 2000
FGSC 429	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Mir-Rashed, 1998
FGSC 3212	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Mir-Rashed, 1998
FGSC 3223	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Louisiana, Mir-Rashed, 1998
FGSC 3226	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Texas, Mir-Rashed, 1998
FGSC 3885	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, California, Mir-Rashed, 1998
FGSC 4705	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Brazil, Mir-Rashed, 1998
FGSC 4707	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Haiti, Mir-Rashed, 1998
FGSC 6212	<i>un-24^{OR} het-6^{OR} A</i>	FGSC, Costa Rica, Mir-Rashed, 1998
FGSC 851	<i>un-24^{PA} het-6^{PA} A</i>	FGSC, Costa Rica, Mir-Rashed, 1998
FGSC 852	<i>un-24^{PA} het-6^{PA} A</i>	FGSC, Costa Rica, Mir-Rashed, 1998
FGSC 1133	<i>un-24^{PA} het-6^{PA} a</i>	FGSC, Panama, Mir-Rashed, 1998
FGSC 1165	<i>un-24^{PA} het-6^{PA} a</i>	FGSC, Panama, Mir-Rashed, 1998
FGSC 2222	<i>un-24^{PA} het-6^{PA} A</i>	FGSC, Iowa, Mir-Rashed, 1998
FGSC 1132	<i>un-24^{PA} het-6^{PA} a</i>	FGSC, Panama, Mir-Rashed, 1998
FGSC 2224	<i>un-24^{PA} het-6^{PA} a</i>	FGSC, Louisiana, Mir-Rashed, 1998
FGSC 2225	<i>un-24^{PA} het-6^{PA} A</i>	FGSC, Texas, Mir-Rashed, 1998
RLM 58-18	<i>un-24^{PA} het-6^{PA} a</i>	FGSC, Smith <i>et al.</i> , 1996

[Table 2.1 continued]

FGSC 2489	74-OR23-1V <i>A</i> , Oak	FGSC
	Ridge wild type	
<i>N. intermedia</i>		
FGSC 962	<i>a</i>	Nanking, China, Mir-Rashed, 1998
FGSC 1754	<i>a</i>	Nanking, China, Mir-Rashed, 1998
FGSC 1882	<i>A</i>	Queensland, Mir-Rashed, 1998
FGSC 2540	<i>a</i>	Nanking, China, Mir-Rashed, 1998
FGSC 3996	<i>A</i>	Queensland, Mir-Rashed, 1998
<i>N. tetrasperma</i>		
FGSC 350	<i>a</i>	A. Sussman, Mir-Rashed, 1998
FGSC 965	-	Liberia, Mir-Rashed, 1998
FGSC 1270	<i>A</i>	Powell <i>et al.</i> , 2001
FGSC 1271	<i>a</i>	Powell <i>et al.</i> , 2001
FGSC 2503	<i>A</i>	Welsh, Louisiana, Powell <i>et al.</i> , 2001
FGSC 2504	<i>a</i>	Welsh, Louisiana, Powell <i>et al.</i> , 2001
P4379	<i>A</i>	Welsh, Louisiana, Powell <i>et al.</i> , 2001
P4380	<i>a</i>	Welsh, Louisiana, Powell <i>et al.</i> , 2001
P4381	<i>A</i>	Welsh, Louisiana, Powell <i>et al.</i> , 2001
P4382	<i>a</i>	Welsh, Louisiana, Powell <i>et al.</i> , 2001
FGSC 2510	<i>A</i>	Hanalei, Hawaii, Powell <i>et al.</i> , 2001
FGSC 2511	<i>a</i>	Hanalei, Hawaii, Powell <i>et al.</i> , 2001

[Table 2.1 continued]

FGSC 2508	<i>A</i>	Lihue, Hawaii, Powell <i>et al.</i> , 2001
FGSC 2509	<i>a</i>	Lihue, Hawaii, Powell <i>et al.</i> , 2001
P4367	<i>A</i>	Lihue, Hawaii, Powell <i>et al.</i> , 2001
P4368	<i>a</i>	Lihue, Hawaii, Powell <i>et al.</i> , 2001
Coba 131	<i>A</i>	Borneo, Powell, 2002
Coba 131	<i>a</i>	Borneo, Powell, 2002
<u><i>N. sitophila</i></u>		
FGSC 417	<i>A</i>	Unknown
FGSC 6673	<i>a</i>	Brazil
<u><i>S. brevicollis</i></u>		
FGSC 1903	<i>A</i>	FGSC
FGSC 1904	<i>a</i>	FGSC
<u><i>S. sclerogenia</i></u>		
FGSC 2740	<i>A</i>	FGSC
<u><i>S. macrospora</i></u>		
FGSC 4818	homothallic	FGSC
FGSC 6540	homothallic	FGSC

¹FGSC: Fungal Genetics Stock Center, University of Kansas Medical Center. Numbers preceded by “P” are from the David Perkins collection, curated by the FGSC.

²(OR) indicates OR alleles at all undesignated *het* loci, where not marked, *het* alleles are not determined.

Table 2.2. Primer sequences used in this study for allele-typing.

Primer name	Primer sequence 5'→3'
Gene2-P1	TGCCTGTGCTGAACATGTG
Gene2-P2	GCAGCCAGTTACTGTCCA
Gene2-P3	TCACGTCGGATGGGCAGACTC
6J-P11	CTCCGGATGAGGTTGCCG
6J-P6	GTGCGGGCTTAACCGCTG
6J-P15	GTTGCTCAGAAAGTGGAG
Gene4-P1	ATCGATGTAAGTCACTCG
Gene4-P2	ATCTCTTCATGTCGTCTC
Gene5-P1	GGTGAAGTATCATGCGAGG
Gene5-P2	ATCGACGATTGCTGTGGA
cys3-P4	GCTGCCGTGCTTCTCCG
Gene7-P1	CTGATGCTGAACGCGCCA
Gene7-P5	CTGCGACGGACTTGTTTCGTGA
6V-P3	CGGTAACCTGTTCAGCT
6V-P5	CCCGCTAAGCCAAGGAGTCC
Gene9-P5	GGATATCGCGTGTAAGAGG
Gene9-P6	TGCGTGGAGCTGATTAGG
brk-2-P1	TCCAGACATCAAGTGTCC
brk-2-P2	ACAGGCTGTTTCAGCA
brk-2-P3	TGCTGAACACAGCCTGT
6J-inv2	TCAGCCCATCCAGTTCCTGTA

study and through the *Neurospora* sequencing program at MIPS [Munich Information Centre for Protein Sequences (MIPS <http://mips.gsf.de/>) *Neurospora crassa* database, Institute of Biochemistry, Heinrich-Heine-University, Düsseldorf]. PCR amplification products were digested by appropriate restriction enzymes (Table 2.3) and were separated by electrophoresis in 0.7% agarose, 1x TAE (40 mM Tris Acetate, 1 mM EDTA) gels. DNA was transferred to Hybond nylon membranes according to the manufacturer's recommendations (Amersham, Oakville, ON). Probe DNAs were labeled with [α - 32 P]dCTP (Amersham) using a Random Prime DNA labeling kit (Roche, Laval, PQ). PCR products were cloned into the vectors pCRIITM or TOPO-TATM (Invitrogen).

A lambda DNA library of the PA standard strain FGSC1131 was prepared (Sambrook *et al.*, 1989) with an average insert size of 19 kbp using Lambda FixII vector and packaging mix (Invitrogen). A contiguous set of OR-specific cosmids spanning the *un-24/het-6* region was obtained as previously described (Smith *et al.*, 1996) from the Orbach/Sachs genomic library obtained from the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, KS). The vectors pUC118 (Sambrook *et al.*, 1989) and pCB1004 were used for subcloning. Plasmid pCB1004 carries the *hph* gene that conveys hygromycin B resistance in transformations of *N. crassa* (Carrol *et al.*, 1994). DNA sequences were obtained by LICOR (Lincoln, NE) 4200L automated sequencing at Canadian Molecular Research Services (Ottawa, Ontario).

Table 2.3. Diagnostic markers for allele typing in the *un-24/het-6* region. DNA

fragments were amplified with marker-specific primer pairs (see Table 2.2 for primer sequence) and in some cases subjected to restriction digestion with enzymes with OR and PA specific polymorphic restriction sites. The location of markers is shown in Figure 2.3.

Genetic marker	Primer pair	Restriction enzyme	Size of diagnostic fragments (kbp)	
			OR	PA
<i>Gene 2</i>	Gene2-P1 and Gene2-P2	<i>KpnI</i>	0.7, 0.2	0.9
<i>un-24</i>	6J-P11 and 6J-P6	<i>PvuI</i>	0.75, 0.75	1.5
<i>Gene 4-1</i>	Gene4-P1 and Gene4-P2	<i>XhoI</i>	0.7, 0.7	1.4
<i>Gene 4-2</i>	Gene4-P1 and Gene4-P2	<i>KpnI</i>	1.6, 0.2	1.4, 0.2, 0.2
<i>Gene 5</i>	Gene5-P1 and Gene5-P2	<i>NcoI</i>	2.2, 1.4, 0.4, 0.3	2.2, 1.7, 0.4
<i>het-6</i>	6V-P3 and 6V-P5	<i>MboI</i>	0.5, 0.5	1
<i>Gene 9-1</i>	Gene9-P5 and Gene9-P6	<i>BglII</i>	1.9, 0.2	2.1
<i>Gene 9-2</i>	Gene9-P5 and Gene9-P6	<i>HincII</i>	0.8, 0.7, 0.5	1.3, 0.7
<i>brk 1-1</i>	6V-P5 and 6J-P11	none	-	7.5
<i>brk 1-2</i>	Gene7-P1 and cys3-P4	none	4.5	-
<i>brk 1-3</i>	Gene7-P1 and 6J-P11	none	-	3.5
<i>brk 2-1</i>	Gene2-P3 and 6J-inv2	none	1.8	-
<i>brk 2-2</i>	Gene2-P3 and Gene7-P5	none	-	2.3
<i>brk-2-3</i>	Gene2-P1 and 6J-P11	none	5.6	-

Data analysis

OR DNA sequences used for analysis were obtained from MIPS and the *Neurospora* Genome database at the Whitehead Institute Center for Genome Research (WICGR, Cambridge, Massachusetts, USA, <http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>). BLAST searches were performed at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/blast/>). DNA sequencing of PA-derived clones was carried out at the Canadian Molecular Research Services (Ottawa, ON). DNA sequence alignments were done using the software ClustalX and ClustalW (<http://clustalw.genome.ad.jp/>; Thompson *et al.*, 1997) with manual adjustments. Neighbor-joining trees were built using ClustalX and were verified by the bootstrap method using ClustalX (Felsenstein, 1989). Sliding window analyses were performed by hand with a window width of 60 bp and sliding increments of 30 bp.

The distribution of synonymous substitutions per synonymous site (dS) and non-synonymous substitutions per non-synonymous site (dN) was calculated according to the method of Nei and Gojobori (Korber, 2000; Nei and Gojobori, 1986). For all samples, intron sequences were removed as needed and pairwise alignments in all combinations were done using ClustalW (<http://clustalw.genome.ad.jp/>) and adjusted by hand. Aligned sequences in GDE format were submitted to SNAP (Synonymous/Non-synonymous Analysis Program, <http://hiv-web.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html>). Synonymous substitutions per synonymous site and

nonsynonymous substitutions per non-synonymous site were averaged for 10 codons and were plotted against nucleotide number using Microsoft Excel.

RESULTS

Identification of an inversion in the *un-24/het-6* region of *N. crassa*

The aim of this study was to investigate structural features of the *un-24/het-6* region through a comparative analysis in OR and PA haplotype strains. Cosmids from an OR genomic library spanning the *un-24*^{OR}/*het-6*^{OR} region (Smith *et al.*, 1996) and DNA sequence from contig B2A19 (MIPS at, <http://mips.gsf.de/proj/neurospora/>) were used as reference points for the OR haplotype. For the study of the *un-24*^{PA}/*het-6*^{PA} region, a genomic library of strain FGSC1131 was constructed in lambda FixII. Using *het-6*^{PA} as a starting point, a chromosomal walk of approximately 85 kbp, ~50 kbp towards and ~35 kbp away from the centromere was performed (Figure 2.1). An 18 kbp clone containing both *un-24*^{PA} and *het-6*^{PA}, hereafter referred to as λ B, was isolated and used in further studies. Inserts of λ B were subcloned in pUC118 and pCB1004 vectors and were partially sequenced. In addition to λ B, pA3 in pUC118 was used for RFLP and sequence analyses of the PA haplotype (Mir-Rashed *et al.*, 2000). The PA haplotype sequence in pA3 was derived from FGSC1131 via FGSC2190 (Smith *et al.*, 1996).

Southern hybridizations were done to compare the marker distribution and general structure of clones carrying PA or OR derived sequences. DNAs of pA3, λ B, λ TLP131 and λ 11-1 were digested with *Bam*HI and *Not*I, and DNA of G8:G:1 was digested with *Bam*HI to liberate the insert from vector sequences. Following agarose gel electrophoresis and Southern blotting, the hybridization membranes were probed sequentially with the radioactively-labeled PCR products of primer pairs 6J-P3/6J-P6

Figure 2.1. Chromosomal walk across the *un-24/het-6* region. The lambda clones were mapped with respect to the OR genetic map by hybridization to OR cosmid clones in the region.

10 kbp

(*un-24*^{OR}), 6V-PA3/6V-PA4 (*het-6*^{PA}) and *cys-3*-P1/*cys-3*-P2 (*cys-3*^{OR}) (Figure 2.2).

RFLP patterns for *un-24* and *het-6* probes are consistent with previously published restriction maps (Mir-Rashed *et al.*, 2000). The autoradiograms showed that G8:G:1, pA3 and λ B contain sequences that hybridize to the *un-24*^{OR} probe (Figure 2.2B). This is consistent with these clones carrying *un-24* and with the observed near 100% sequence identity in the 5' region of *un-24*^{OR} and *un-24*^{PA} (Smith *et al.*, 2000b). Strong hybridization signals were visible in lanes containing pA3, λ TLP131 and λ B DNA when probed with 6V-PA3/6V-PA4 PCR product containing *het-6*^{PA} (Figure 2.2C). Some hybridization was also evident to lambda DNA since the PCR-based *het-6*^{PA} probe was obtained from a lambda clone. A weak signal was obtained when OR-derived cosmid G8:G:1 was probed with 6V-PA3/6V-PA4, consistent with the low (78%) DNA sequence identity of *het-6*^{PA} and *het-6*^{OR} alleles (Smith *et al.*, 2000b). Based on hybridization to the *cys-3*^{OR} probe, neither pA3 nor λ B harbors this gene. This result was verified by PCR amplification with *cys-3* specific primers *cys-3*-P1 and *cys-3*-P2 (data not shown). A PCR product was obtained when OR cosmid G8:G:1 was used as a template whereas no product was obtained from either λ B or pA3. The *cys-3* gene was detected by PCR amplification and hybridization to *cys-3* probe in the PA-derived clone λ TLP131, which maps centromere-distal to *un-24* (Figure 2.1). Thus, we corroborate sequence data (MIPS) that marker *cys-3* is situated between *un-24* and *het-6* in the OR haplotype and we show that it is located centromere distal to *un-24* and *het-6* in the PA haplotype. These findings, in addition to the smaller distance between *un-24* and *het-6* in the PA (~5.2 kbp) compared to the OR haplotype (~19 kbp) suggested that a paracentric inversion, occurs in the region. This inversion, designated here as *In(III)1131 het-6*, for

inversion associated with *het-6*, was verified by determining the DNA sequence of the termini of pA3. In pA3 (PA haplotype) *un-24* and *het-6* are transcribed from the same strand of DNA whereas in the OR haplotype (MIPS database) the genes are on opposite strands.

Characterization of inversion breakpoints *brk-1* and *brk-2*

The location of the *In(IIL)1131 het-6* [abbreviated *In(het-6)*, hereafter] breakpoints was determined by sequence comparisons of OR and PA haplotypes. First, the approximate location of the centromere-proximal breakpoint (*brk-1*) was established. Based on the location and orientation of *un-24*^{PA} in relation to *het-6*^{PA}, and the absence of *cys-3* from the *un-24*^{PA}/*het-6*^{PA} intergenic region, we surmised that *brk-1* was located between *un-24*^{PA} and *het-6*^{PA} close to *Gene7a* or *Gene 7b* (Figure 2.3). The location of the distal breakpoint, *brk-2*, had to account for the small distance between *un-24* and *het-6* in PA relative to OR haplotypes, and was inferred to be just downstream of *un-24*^{OR} likely between *un-24*^{OR} and *Gene 2*. Based on this reasoning, hypothetical genetic maps were generated for the OR and PA haplotypes (Figure 2.3). These maps account for haplotype differences in *un-24* orientation, map location of *cys-3* and distance between *un-24* and *het-6*. We sought confirmation of these maps by examining marker orientation around the inversion breakpoints. Genomic DNAs from PA strain (RLM58-18) and OR strain 74-OR23-1V (FGSC2489) were used in PCR amplifications designed to amplify *brk-1*^{PA}, *brk-1*^{OR}, *brk-2*^{PA} and *brk-2*^{OR} as follows. Primer pairs Gene7-P1/6J-P11, Gene7-P1/*cys-3*-P4, Gene2-P3/Gene7-P5 and Gene2-P3/6J-inv2 were synthesized based on contig B2A19 sequence (MIPS) and were used to generate diagnostic PCR products to

verify the inversion breakpoint map (Table 2.3). The PCR products were present and of the predicted size as expected based on the map (Figure 2.4). No product was obtained when primers specific for the OR configuration were used with PA DNA template or vice versa. Thus marker orientation as predicted based on RFLP analysis is correct and distinct in OR and PA strains. Based on the location of the inversion breakpoints, the inversion *In(het-6)* spans a ~18.6 kbp region from approximately 4.2 kbp upstream from *het-6* to approximately 100 bp upstream from *Gene 2*. Both PA inversion breakpoints were isolated and sequenced as described below.

The entire pA3 clone was sequenced, which contained *brk-1^{PA}*. *brk-2^{PA}* was isolated by PCR from clone λ G12 (Figure 2.1) using primers cys3-P4 and Gene2-P1. The PCR product was sub-cloned into pCRII and sequenced. *brk-1^{PA}* and *brk-2^{PA}* sequences were compared to the OR genomic sequence available at MIPS. The structure of the two inversion breakpoints is summarized in Figures 2.5B and 2.5C using a sliding window analysis of the two regions. For the analysis, OR and PA sequences were aligned using ClustalW and sequence identity was calculated as number of nucleotide identities per total number of nucleotides in a window of 60 bp. The window was shifted 30 bp and the calculation was repeated. The two inversion breakpoints are characterized by regions of sequence dissimilarity between OR and PA haplotypes flanked by regions of significant sequence identity. The region near *brk-1* is composed of five distinct sub-regions as defined by sequence identity between OR and PA haplotypes (Figure 2.6).

Within region *brk-1-1* there is no identity between the OR and PA haplotypes, likely reflecting an insertion into the OR sequence relative to the PA form. The *brk-1-1* region, which is directly adjacent to *het-6*, contains unique sequence spanning 0.4 kbp

Figure 2.2. RFLP analysis of PA lambda and OR cosmid clones mapping to the *un-24/het-6* region. Ethidium bromide-stained agarose gel (A) showing lambda genomic DNA digested with *Hind*III size standard and cloned DNAs digested with *Bam*HI (G8:G:1) and *Not*I and *Bam*HI (λ B, λ TLP131, λ 11-1 and pA3) were hybridized to α^{32} P dCTP probes of *un-24*^{OR} (B), *het-6*^{PA} (C) and *cys-3*^{OR} (D). Notice the absence of hybridization of *cys-3* probe to λ B and pA3.

Figure 2.3. Hypothetical map of the *un-24/het-6* region in OR and PA haplotypes showing gene order and orientation and inversion diagnostic primers.

2 kb

Figure 2.4. Inversion diagnostic PCR products corresponding to *brk-1*^{OR}, *brk-1*^{PA}, *brk-2*^{OR} and *brk-2*^{PA} (lanes 1 to 4, respectively). The standard molecular size marker is the 1 kbp ladder.

Figure 2.5. Graphic depiction of the inversion loop associated with *In(het-6)*(A) and sliding window analysis of sequence identity between OR and PA strains near *brk-1* (B) and *brk-2* (C). Panels B and C are drawn in the PA configuration. In panel B, *brk-1* is approached from left by aligning *un-24*^{PA} and *un-24*^{OR} (blue arrow “a” in panel A) and from right by aligning *het-6*^{PA} and *het-6*^{OR} (red arrow “b” in panel A). *brk-2* (panel C) is approached from right by aligning *Gene 2* sequences (blue arrow “c” in panel A) and from left by aligning *Gene 7a* sequences (purple arrow “d” in panel A). Alignments were with ClustalX and sequence identity analysis was done by hand with a window size of 60 bp and a window shift of 30 bp.

A

B

C

and 1.3 kbp in PA and OR haplotypes, respectively. Region *brk-1-2*, is composed of a short 0.4 kbp stretch which is inverted in the PA with respect to the OR configuration and is therefore not identified as a region of 0.25 identity in the two haplotypes by the sliding window analysis (at ~10000 bp in Figure 2.5B). Region *brk-1-3* has distinct sizes and DNA sequences, specific to PA (2.3 kbp) and OR (4.6 kbp) haplotypes, and likely represents an insertion in the OR form relative to the PA form. Region *brk-1-4* spans approximately 1.8 kbp in the two haplotypes and in the PA haplotype corresponds to DNA sequence from within the 3' coding region and downstream regions of *Gene 7b*. Sequence identity values are about 0.65 in the *brk-1-4* region. Finally, region *brk-1-5*, spanning approximately 3 kbp in the PA haplotype and 1.1.kbp in the OR haplotype contains haplotype-specific DNA sequence. Thus, the structure of the *brk-1* region is complex, making the identification of the precise breakpoint location difficult. However, the fact that the relative genetic order between OR and PA haplotypes is maintained in the regions *brk-1-1* to *brk-1-4*, would indicate that neither *brk-1-3* nor *brk-1-1* represent the breakpoint of *In(het-6)*. Gene order appears to be altered past region *brk-1-5*. Whereas the region downstream from *Gene 7b* is occupied in the OR haplotype by *Gene 7a*, this place is taken by *un-24* in the PA haplotype. This suggests that the exact location of *brk-1* is within the DNA region defined by *brk-1-5*.

The structure of the *brk-2* region is less complex than that of *brk-1* (Figures 2.5 and 2.6). *brk-2* is putatively located in a region of unique DNA sequence (~40% identity between OR and PA haplotypes) spanning ~ 0.7 kbp in the PA haplotype and ~3 kbp in the OR haplotype. In the PA haplotype, *brk-2* is flanked centromere-proximally by the intergenic space between *Gene 7a* and *Gene 7b* and centromere-distally by *Gene 2*.

DNA sequence comparisons between the entire region encompassing *brk-1* and *brk-2* revealed no striking sequence identity between the two putative inversion breakpoints. In addition, OR and PA sequences corresponding to both inversion breakpoints were compared to other sequences in the databases and to the *N. crassa* genome sequence. DNA sequence corresponding to *brk-2*^{OR} did not show significant identity to any other sequences in the databases. *brk-2*^{PA} was ~85% similar over 30 to 40 bp to unknown regions within linkage group I (contig 3.153, WICGR) and linkage group III (contig 3.321, WICGR; BLAST score 66 bits, E value $4e^{-10}$). In contrast, analysis of *brk-1*^{OR} and *brk-1*^{PA} indicates that they have sequence identity to several common regions within the *N. crassa* genome. Within *brk-1-3*^{OR}, a 53 bp region has 86% identity to two regions mapping to linkage group I or VI (contig 3.213, WICGR) and to an unknown location (contig 3.400, WICGR). Interestingly, sequences from contigs 3.213 and 3.400 that are similar to *brk-1-3*^{OR} are adjacent to sequences sharing similarity to *brk-1-3*^{PA}. Thus although *brk-1-3*^{OR} and *brk-1-3*^{PA} do not share sequence identity with each other, they are similar to distinct but adjacent fragments at other locations in the genome. Specifically, within *brk-1-3*^{PA}, a 397 bp stretch of sequence shows ~75% identity to 14 different regions within the *N. crassa* genome, among them contigs 3.213 and 3.400, based on a survey of the sequence database at the Whitehead Institute Center of Genome Research (cut-off score 50 bits, E-value $2e^{-5}$). This 397 bp region contains 53 bp inverted repeats with 88% identity to one another. Three of the regions with which *brk-1*^{PA} shares identity also carry similar inverted repeats (contig 3.213, mapping to LGI or VI; contig 3.622, mapping to LGII and contig 3.400 mapping to an unknown location, Figure 2.7). The remaining 11 sequences lack the inverted repeats but otherwise share significant

sequence identity to the 397 bp sequence. Thus, this 397 bp sequence has several qualities that qualify it as a bona fide repetitive sequence (RS). It has inverted terminal repeats, short imperfect direct repeats, “CCTAACC” and “CCTACC”, and it is present in multiple (at least 14) copies in the genome of *N. crassa*. Sequence comparison between the four most conserved genomic regions with this sequence revealed several GC to AT transitions indicative of RIP. A BLAST survey indicates that neither the inverted repeats nor the internal repeated sequence match to any known mobile genetic elements or repeated sequences in the databases, and the region has no similarity to any known transposases, which suggests that this is a novel RS element. BLAST searches also revealed that there are no apparent sites in the *N. crassa* genome containing the inverted repeats but lacking the internal sequence suggesting that the sequence is not actively transposing.

Recently, several RS elements and transposons have been implicated in chromosomal rearrangements in Maize, *Drosophila*, and *Anopheles* species (Zhang and Peterson, 1999; Mathiopoulos *et al.*, 1998; Cáceres *et al.*, 1999). In a majority of cases, each inversion breakpoint was associated with a mobile genetic element, suggesting that recombination between the two homologous transposon sequences was responsible for the rearrangement. However, in some cases, a single mobile genetic element was detected at only one of the inversion breakpoints. The absence of a transposable element at the other inversion breakpoint may be explained through the subsequent loss of the element due to its inherent mobility (Mathiopoulos *et al.*, 1998). For example, the transposon may have been present at both inversion breakpoints and was lost through non-conservative transposition. Although the exact mechanism that generated the *In(het-6)* inversion is

Figure 2.6. Genetic structure of inversion breakpoints *brk-1* and *brk-2* in OR and PA haplotypes. DNA sequence identity between OR and PA inversion breakpoints were done by BLAST searches against the *N. crassa* genome sequence at WICGR. Regions shown in black are common in OR and PA haplotypes. Regions shown in red and blue correspond to unique sequences found in OR or PA haplotypes, respectively. See text for description of *brk-1* sub-regions 1 through 5.

2 kbp

Figure 2.7. ClustalW DNA sequence alignment of a 397 nucleotide sequence within *brk-1*^{PA} with significant similarity to three distinct regions on linkage group I or VI (contig 3.213), linkage group II (contig 3.622) and an unknown location (contig 3.400). Sequence numbers refer to the contig name as identified by the *Neurospora* Genome Sequence Project (WICGR). The region is bound by 53 nucleotide inverted repeats identified by arrows and by direct repeats (underlined).

3.213 CCCGAAAAGCTATCGTATTTTCTCGGGGCATCCATCTTTATGTTCCTCCCAAGAAATCAGACATAAATCCACG
3.622 TACCTCTACCTACCGCATTTCTCGGGGCATCCATCTTTAGGTTCCAGGAAATCAGGCATAAAACCCACA
brk-1 CCTAACCAAAATACCAATTTTCTCGGGGCATCCATCTTTAGGTTTCCAGGAAATCAGACATAAATCCACG
3.400 AGGGATGCAGTACCGCATTTTCTCGGGGCATCCATCTTTAGGTTCCCTGGAAATCAGACATAA-TCTGTGA

3.213 TACA-TATGCCCCAATTTCTGTGCATTTCTATGCCC-ACCAAAATAAAGTGGGGAGTTGTGT
3.622 TACA-TATGCCCCAATTTCTGTGCATTTTATGCCC-ATCAAAATAAGCGGGAGTTGTGT
brk-1 CACA-TATGCCCCAATTTCTGTGCATTTTATGCCC-ACCAAAATAAGAGGGGGAGCCGTGT
3.400 AGCATCTGGGCAAAATATGTGCTTTTATGCCCGACAAAAGAGGGGGGGGAAACAGC

3.213 GCCAC--CCCGGAGCACGTGTAATTCGATTTTATTTGGAGCTCTGCCITTTTCCCTACCTATT
3.622 GCCAC--TCCGGAGCACGTGTAATTCGATGCTCTAGTACCATGCTTTT-CCTACCCATT
brk-1 GCCAC--CCCGGAGCACGTGTAATTCGATTTCCCTCGTGCCTATATCTTT-TCTACCTATT
3.400 GTAAGAGCCCCGGAGCACGTGTAATTTCTATTCTTTCTTTGTAATATCTTT-TCTACCTATT

3.213 TCTTCAAGGGAGCTGGAGGGTGGAGACATTGAGAAATCACGGTCTTTGCATAGAAATTTTCG
3.622 TTGTCAAGGGAGTTGGAGGGTGGAGACTGAGAAATCACGGTCTTTGCATAGAAATTTTGG
brk-1 CTCCTCAAGGAGTTGGAGGGTGGGGCCATTGAAAAATCACGGTCTTTGCCGTAGAAACTTTGG
3.400 TC-TCAAGAAAGTTGGAGGGTGGAGCCATCTAGAAATCACCCGCTTTGCATACGATTTTCCAG

3.213 TGGGCATAGATATTGTAGTA-----TGCCTTATATAGTATACCTGATTTTCTGGGAACCT
3.622 TGGGCATAAATGTTGCAGTAGAGGTATGCTTTATACATCATGCTGAGTTCTCTGGGAACCT
brk-1 TGGGCATAACTATTGCAGTA-----TGCCTTATACATTTATACCTGATTTTCCAGGGAGCCT
3.400 TGGGCATAAATGTTGTAGCA-----TGCCTTACAGATTATGCTGATCTCTCTGGGAACCT

3.213 -----AAAGATGGATGCCCCCGAGAAATACGGTATACATATATAA
3.622 GTAGGTAAAGATGGATGCCCCCGAGACAATGCGGTATACATATATAA
brk-1 -----AAAGATGGATGCCCCCGAGAAATGCGGTACCTACCTAGC
3.400 AGAGGTAATGATGGGTGCGCCGAGAAATGCTGTATCAAAAGTCGG

unknown, it is plausible that the presence of a repeated sequence contributed to its occurrence.

Significant sequence dissimilarity between the OR and PA forms at both inversion breakpoints may also indicate that the inversion event is ancient. At inversion breakpoints, nucleotide polymorphisms are thought to accumulate readily due to the low rates of recombination that give rise to rapidly diverging sequences (Dobzhansky, 1970). Based on the location of *un-24*^{PA} and *het-6*^{PA} close to *brk-1*, we predicted that recombination events in the region would be rare and may be the source of linkage disequilibrium at the two *het* loci. To test this hypothesis we estimated the extent of recombination within the inverted region in world isolates of *N. crassa*.

Evidence for recombination within *In(het-6)*

The position of *In(het-6)* suggests that linkage disequilibrium at *un-24* and *het-6* is due to failure to recover viable recombinant progeny. Single cross-over events between inverted/non-inverted heterozygotes form dicentric bridges and acentric chromatid fragments during meiosis. Such aberrant chromatids are lost during meiosis and result in the apparent absence of recombination within the inverted region (Perkins, 1997). However, gene conversion events and/or double cross-overs could occur within inverted regions, since neither of these forms of recombination result in transfer of the centromeric regions between chromatids. We wanted to survey the extent of genetic exchange between inverted and non-inverted haplotypes to test the hypothesis that the inversion blocks recombination in the region. In addition, we were interested in surveying the

occurrence, prevalence and correlation, if any, of inversion forms with genotype at *un-24* and *het-6*.

In order to invoke complete linkage disequilibrium at *un-24* and *het-6* due to the presence of polymorphisms at *In(het-6)* there must be a strong association between the inversion form and genotype at *un-24* and *het-6*. If both inversion forms exist in both OR and PA *het* haplotypes, then recombination between *un-24/het-6* haplotypes is possible as long as it occurs between identical inversion forms. Should this be the case, the inversion may not be a source of linkage disequilibrium at *un-24* and *het-6*. However, if a particular inversion form is only associated with a specific haplotype, it would suggest that a lack of recombination is responsible for linkage disequilibrium.

The goals for this experiment were twofold. The first goal was to establish the association, if any, of the inversion with genotype at *het-6* and *un-24*. The second goal was to search for signs of genetic recombination events within *In(het-6)* between PA and OR haplotypes. To address these points, I examined strains that were collected at widely different localities and thus probably represent individuals that have been separated by many generations. Twenty *N. crassa* strains (10 *un-24*^{OR} *het-6*^{OR} and 10 *un-24*^{PA} *het-6*^{PA}) were selected from distinct geographic origins (Table 2.1). The *het-6* and *un-24* genotype of each strain was verified using a PCR/RFLP-based assay as described previously (Mir-Rashed *et al.*, 2000). As expected, only two allelic combinations of *un-24* and *het-6* occur; *un-24*^{OR} *het-6*^{OR} or *un-24*^{PA} *het-6*^{PA}. These *N. crassa* strains were genotyped for the OR or PA forms of the inversion by using primers designed for the isolation of *brk-1* (primer pairs Gene7-P1/cys-3P4 and Gene7-P1/6J-P11; Table 2.3 and Figure 2.8). Inversion forms for strains P4450, FGSC429, P4493 and FGSC2225 were determined

using *brk-2* specific primers Gene2-P1/6J-P11 and Gene2-P1/Gene7-P5. In all strains tested, there was a perfect correlation between genotype at the two *het* loci and inversion type ($P < 0.001$). This strongly supports the idea that the *In(het-6)* is responsible for linkage disequilibrium. For now we shall refer to the inversion form associated with the PA haplotype as the PA inversion form and the inversion form associated with the OR haplotype as the OR inversion form, since it is unclear which of them is the non-inverted ancestral form.

To establish the extent of recombination within the inverted region, six additional markers, four within and two outside the inverted region, were PCR amplified and were digested with restriction enzymes producing OR or PA-specific banding patterns (Table 2.3, Figure 2.8). RFLPs at the six markers were determined for each of the twenty strains, including the two standard strains, RLM58-18 (PA) and 74-OR23-1V (OR). Within *Gene 4*, two different markers, *Gene 4-1* and *Gene 4-2* were used. For five of these markers, only two RFLP forms, either PA-like or OR-like, were detected. Within *Gene 9*, two adjacent PCR/RFLP markers identified five different variants, two corresponding to OR (O1 and OR) and three corresponding to the PA (O2, O3 and PA) tester strains (Figure 2.8). The two *Gene 9* markers were congruent and are shown in a single column in Figure 2.8.

To test whether there is a correlation between genotype at *het-6* and genotype at each of the other markers in addition to *un-24*, pair-wise allelic associations were calculated using Fisher's exact test (Figure 2.8; <http://www.physics.csbsju.edu/stats/exact.html>, Jerrold 1984). The *het-6*, *un-24* and *In(het-6)* forms are perfectly associated with no exceptions, as described previously. Other markers from within the inversion

Figure 2.8. PCR/RFLP analysis of the *un-24/het-6* region in twenty *N. crassa* strains from around the world. OR and PA specific PCR/RFLP patterns were established at eight different markers mapping within and outside *In(het-6)* using primer pairs and restriction enzymes presented in Table 2.3. Inversion type was determined by PCR with primers Gene 7-P1/cys3-P4 (OR) and Gene 7-P1/6J-P11 (PA), corresponding to *brk-1* or with Gene 2-P3/6J-P11 (OR) or Gene 2-P3/Gene 7-P5 (PA), corresponding to *brk-2*. Strains RLM58-18 and 74OR23-1V were used as reference PA and OR strains, respectively. Correlation between markers and genotype at *het-6* was calculated with the Fisher exact test.

breakpoints were significantly correlated to the allelic form of *het-6* except for marker *Gene 4-2* ($P=0.152$), which is situated in the center of the inverted region. Markers outside the inverted region were not significantly correlated to the *het-6* genotype ($P=0.328$ for *Gene 2*, $P=0.328$ for *Gene 9*). Apparently, then, polymorphisms are more frequently shared between PA- and OR-haplotype strains in the center of, and outside the inverted region. The presence of the shared polymorphisms at markers within *In(het-6)* is consistent with the view that recombination is possible within the inverted region between the OR and PA haplotypes. Gene conversion events and double cross-overs in the center of the inversion, where chromosomal pairing is more efficient than at inversion breakpoints (Chovnik, 1973), would account for these observations. As one approaches the *brk-1/het-6* region or the *brk-2/un-24* region, and homologous pairing is not possible due to divergent sequences at each breakpoint, the correlation between markers increases to unity. Thus we propose that the close linkage of the incompatibility genes to the respective inversion breakpoints and the lack of recombination at inversion breakpoints account for linkage disequilibrium of *un-24* and *het-6* in *N. crassa*.

How are inversion polymorphisms maintained at *In(het-6)* ?

That *het-6*^{OR} and *het-6*^{PA} are balanced polymorphisms can be inferred from previous studies on allelic frequencies in a Louisiana population (Mir-Rashed *et al.*, 2000). Based on estimates from 40 isolates, OR and PA alleles at *un-24* and *het-6* are present in equal frequencies in the population. An estimate of the PA and OR allelic frequencies is 1.88 to 1, respectively, based on PCR/RFLP markers at *un-24* and *het-6* from 121 strains taken from throughout the tropical and sub-tropical range of *N. crassa*

(Mir-Rashed, 1998). The strong association between OR and PA inversion forms with OR and PA haplotypes respectively, as described above, can likely be extrapolated to the *N. crassa* species as a whole, since members of the sample population used for the present study are representatives of various geographical locations. By association, both inversion types must also be present at relatively high frequencies in the species and be balanced. What is the mechanism by which inversion polymorphisms are maintained in a balanced state and what is the significance of this equilibrium for *N. crassa* ?

Although neutral selection may fix the inverted form in the population, it is unlikely that both inverted and non-inverted forms are maintained by neutral selection. More likely, balancing selection, often invoked to explain maintenance of inversion polymorphisms in *Drosophila* (Kelly, 2000), is also responsible for maintenance of *In(het-6)* polymorphisms. Balancing selection acts to maintain polymorphism in a population either by conveying an advantage to the heterozygote (heterozygote advantage or overdominant selection theory, Doherty and Zinkernagel, 1975) or by conveying a fitness advantage to rare allelic variants (frequency-dependent selection theory, Dobzhansky, 1970).

According to Dobzhansky, inversions fix favorable genetic combinations in a particular configuration called a “supergene”. In an inversion heterozygote, suppression of recombination within the inversion prevents the breakdown of the favorable genetic combination. Maintenance of both inverted and non-inverted forms, in a balanced state also maintains associated favorable genetic combinations. In Chapter 3, I discuss the possibility that PA/PA and OR/OR allelic combinations at *un-24* and *het-6* operate optimally in this combination, consistent with the supergene theory.

In addition to supergene selection, balancing selection may be acting directly on *un-24* and *het-6* and this selection may extend to the closely-linked *In(het-6)* inversion. Diversifying selection, a type of balancing selection, operates at a variety of nonself recognition loci such as MHC (in vertebrates), S (in plants) and *het-c* (in *N. crassa*), ostensibly to maintain high allelic variability. Although both supergene selection and diversifying selection at *un-24* and *het-6* may represent forms of balancing selection, and are detectable by similar markers (see below), one significant difference exists between the two forms of selection. Both supergene selection and diversifying selection result in balanced allelic frequencies at *un-24* and *het-6* in the population. In addition, polymorphisms associated with alleles under balancing selection are expected to be long-lived and can be maintained across species (Richman, 2000). However, at loci under diversifying selection, substitutions leading to amino acid changes are favored, in order to maximize functional variability in the molecule. Variability presumably allows for broader specificity of nonself recognition during protein interactions (Hughes, 1999). This is in contrast to most genes undergoing neutral evolution, where the rate of synonymous substitutions typically exceeds that of non-synonymous substitutions (Hughes, 1999). This pattern of non-synonymous nucleotide substitutions exceeding synonymous nucleotide substitutions would also not be expected for loci under supergene selection, since supergene selection acts at a larger scale on genetic structure and gene associations rather than at the single gene scale. We hypothesized that balancing selection at the tightly-linked nonself recognition loci *un-24* and *het-6* may extend to polymorphisms at *In(het-6)*.

Several observations suggest that both *un-24* and *het-6* may be candidates for balancing selection. First, balancing selection has been shown to act consistently at nonself recognition loci (Richman, 2000). Second, a survey of the global and local distribution of alleles at *un-24* and *het-6* suggests that they are in some sort of balance at the species and the population levels, consistent with balancing selection (Mir-Rashed *et al.*, 2000; Mir-Rashed, 1998). Third, a study of isolates of *N. tetrasperma*, a close relative of *N. crassa*, revealed that polymorphisms associated with OR and PA alleles at both *un-24* and *het-6* are conserved between the two species (Powell, 2002). Balanced, long-lived polymorphisms that transcend the species barrier are a hallmark of balancing selection (Richman, 2000). To further test for balancing selection at *un-24* and *het-6*, I estimated the amount of synonymous and non-synonymous substitutions between OR and PA alleles in two isolates of *N. crassa* and two isolates of *N. intermedia*.

For analysis of *un-24* and *het-6* genes in *N. crassa*, DNA sequences corresponding to the entire coding region of both OR and PA alleles were obtained from GenBank (accession numbers AF206700, AF208542, AF171697 and AY027867). For *N. intermedia*, strains FGSC1754 and FGSC1882 carrying OR and PA alleles, respectively, at *un-24* and *het-6* were used for the analysis. Sequences corresponding to the specificity regions for *un-24* and *het-6* for the two *N. intermedia* strains were isolated by PCR amplification from genomic DNA using primers 6J-P11/6J-P6 and 6V-P3/6V-P5, respectively. The products were cloned into vector TOPO-TA and sequenced. For all samples, intron sequences were removed from *un-24* sequences and pairwise alignments in all combinations were done using ClustalW (<http://clustalw.genome.ad.jp/>) and adjusted by hand. Aligned sequences in GDE format were submitted to SNAP

(Synonymous/Non-synonymous Analysis Program, <http://hiv-web.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html>). The distribution of synonymous substitutions per synonymous site (dS) and non-synonymous substitutions per non-synonymous site (dN) was calculated according to the method of Nei and Gojobori (Korber, 2000; Nei and Gojobori, 1986). A distribution of average dS and dN per 10 codons for *un-24* and *het-6* in *N. crassa* and *N. intermedia* is presented in Figures 2.9A through 2.9D.

In *N. crassa*, *het-6* shows a high frequency of non-synonymous substitutions compared to synonymous ones throughout the coding region (Figure 2.9A). Several islands with higher synonymous than non-synonymous substitutions are also visible. The largest ones are in regions bound by codons 30-90, 340-400 and 610-690. Interestingly, a sub-region of one of these islands encompassed by amino acids 50 to 60 appears to be conserved among at least two other nonself recognition genes: *tol* from *N. crassa* and *het-e* from *P. anserina* (Smith *et al.*, 2000b). However, the other two islands that display larger synonymous than non-synonymous substitution rates do not correspond to conserved regions in *het-6*, *tol* and *het-e*. Similarly, in *N. intermedia*, the level of non-synonymous substitutions across *het-6* exceeds that of synonymous ones for the most part throughout the region analyzed (codons 23 to 344) with the exception of a region bound by codons 23 to 99, where dN is not consistently larger than dS (Figure 2.9C). The pattern of nucleotide substitutions at *un-24* is distinct from that observed for *het-6*. In *N. crassa*, *un-24* displays two distinct regions with different patterns of nucleotide variability (Figure 2.9B). The two allelic forms are highly conserved in the 5' region of *un-24*, which encompasses 87% of the open reading frame. Only two of the 22 nucleotide substitutions

Figure 2.9. Average synonymous substitutions per synonymous site (dS, black bars) and non-synonymous substitution per non-synonymous site (dN, orange bars) distribution for PA and OR comparisons at *het-6* (A and C) and *un-24* (B and D) in *N. crassa* (A and B) and *N. intermedia* (C and D). Average dS and dN frequencies were calculated for non-overlapping regions of 10 codons using SNAP (Synonymous/Non-synonymous Analysis Program). For *N. crassa*, the entire coding region was used for the analysis of both genes. For *N. intermedia*, sub-regions correlated to nonself specificity at *un-24* (codons 447 to 930) and *het-6* (codons 23 to 344) were amplified and sequenced (see text for details).

in the first 816 codons lead to non-synonymous changes. This 5' region of *un-24* encodes ribonucleotide reductase function and is highly conserved in amino acid sequence across taxa (Smith *et al.*, 2000a). The 3' region, corresponding to codon 817 to the end of the peptide (930) shows a significant bias towards non-synonymous substitutions. This carboxy terminal region appears to be unique to *Neurospora* species and is correlated to specificity of *un-24*-associated heterokaryon incompatibility function in *N. crassa* (Smith *et al.*, 2000b). Based on partial sequences of *un-24* alleles from *N. intermedia*, there are also two regions with distinct patterns of nucleotide substitution (Figure 2.9D). The 3' region of *un-24* in *N. intermedia*, corresponding to the incompatibility specificity domain of *N. crassa*, carries significantly more non-synonymous than synonymous substitutions ($dS/dN=0.44$ for region from codon 820 to 920). The remainder of the sequence displays predominantly synonymous substitutions ($dS/dN=1.16$). Overall, the level of non-synonymous substitutions exceeds that of synonymous substitutions at both *het-6* (average $dS/dN=0.58$) and the specificity region of *un-24* (average $dS/dN=0.56$), supporting the hypothesis of balancing selection acting at both *un-24* and *het-6*.

In further support of the balancing selection hypothesis is the observation that alleles at *un-24* segregate into two distinct clades based on incompatibility specificity rather than based on species of origin (Figure 2.10A). The effect of different evolutionary pressures acting on genetic variability at *un-24* is evident from Figures 2.9B and 2.9D; the region of *un-24* responsible for ribonucleotide reductase activity has accumulated relatively few mutations and primarily of the synonymous type, compared to the nonspecificity region which displays a larger number of non-synonymous substitutions. A

phylogenetic tree constructed based on the “non-specificity” region alone indicates that *N. crassa un-24*^{OR} and *un-24*^{PA} alleles are more closely related to each other than to the *N. intermedia* variants (Figure 2.10A). A phylogenetic tree based on the *un-24* specificity region indicates that the PA allele of *N. crassa* is more closely related to the PA allele of *N. intermedia* than to the OR allele of *N. crassa*. Likewise, the corresponding OR alleles are more similar in the specificity region between the two species (Figure 2.10B). Similarly, a phylogenetic tree based on the *het-6* coding region spanned by primers 6V-P3/6V-P5 indicates that PA alleles from *N. crassa* and *N. intermedia* are more closely related to each other than to their alternate OR alleles within the same species (Figure 2.10C). The same is true for *het-6*^{OR} alleles in the two species. For each tree, branch points are fully supported by bootstrap values of 1000 out of 1000 trials. The trees in Figure 2.10 show that polymorphisms at *un-24* and *het-6* originated before *N. crassa* and *N. intermedia* species divergence and were maintained after speciation. Thus, the polymorphisms associated with OR and PA alleles at *un-24* and *het-6* are trans-species polymorphisms.

Together, the observations of trans-species polymorphisms and the significantly larger non-synonymous to synonymous substitution rates at *un-24* and *het-6* provide compelling evidence for balancing selection acting on both *het-6* and within the specificity region of *un-24*. Furthermore, the clear allelic association between *un-24* and *het-6*, as well as with *In(het-6)*, suggests that this unit functions as a supergene in *N. crassa*.

Figure 2.10. Neighbor-joining trees of *un-24* and *het-6* genes in *N. crassa* (Nc: OR, 74-OR23-1 V, PA, RLM58-18), and *N. intermedia* strains (Nt: OR, FGSC1754 and PA, FGSC1882). In panel A, the tree was based on codons 447 to 887 corresponding to the non-specificity region of *un-24*. The tree presented in panel B is based on aligned sequences corresponding to the nonself specificity region of *un-24* (codons 887 to 929). Panel C shows a tree based on sequence alignments corresponding to *het-6* (codons 23 to 344). Sequences were aligned with ClustalX and adjusted by hand. Trees were built in ClustalX, based on sequence alignments. Numbers appearing at branchpoints represent bootstrap values from 1000 trees. Note that the scale of branch lengths may vary between panels.

Trans-species polymorphisms at *In(het-6)*

As pointed out previously, DNA sequence comparisons of *brk-1*^{OR} and *brk-1*^{PA} haplotypes revealed that they are too divergent to determine whether they originally corresponded to homologous regions (Figure 2.5B). The same holds for *brk-2* (Figure 2.5C). Based on the significant sequence divergence (~40% sequence identity) between PA and OR sequences over the regions corresponding to *brk-1* and *brk-2*, the inversion may likely be very old. If so, inversion polymorphisms may exist in the closely related species *N. tetrasperma* and *N. intermedia*. Based on preliminary observations of linkage disequilibrium at *un-24* and *het-6*, in *N. tetrasperma* (Powell, 2002), we predicted that inversion polymorphisms should be detectable and perfectly associated with genotype at *un-24* and *het-6*, as they were shown to be in *N. crassa*. To test this hypothesis, I initiated a PCR-based search for inversion polymorphisms at *In(het-6)* in a variety of *N. crassa* related species. As described below, the use of PCR was a poor choice as a tool for the isolation of such potentially divergent sequences as inversion breakpoints. Instead, alternative methods such as partial genomic library construction should be considered.

I screened six *N. tetrasperma* strains of known *un-24* and *het-6* genotype for inversion polymorphisms using *brk-2*-based PCR primers Gene2-P3, 6J-inv2 and Gene7-P5 as described above. Strains known to carry the PA alleles at both *un-24* and *het-6* (*N. tetrasperma* Coba 131 a, P4368 and FGSC2511) and strains carrying OR alleles at the two loci (*N. tetrasperma* FGSC1271, FGSC2504 and P4380) were used for this study (Table 2.1). Representative PCR products obtained were sequenced and found to correspond to sequences on linkage group V, of *N. crassa* that were not indicative of

inversion haplotype. A second attempt was made at determining the inversion form of *N. tetrasperma* strains by PCR analysis using primer pairs corresponding to *brk-1*^{OR} (Gene7-P1/cys-3P4) and *brk-1*^{PA} (6V-P5/6J-P11, Table 2.3). In *N. crassa*, PCR products corresponding to markers *brk-1*^{OR} and *brk-1*^{PA} are 4.5 kbp and 7.5 kbp, respectively. DNA from eight representative OR strains and eight PA strains of *N. tetrasperma* were amplified using both OR- and PA-specific *brk-1* primer pairs. PCR reactions from each strain resulted in multiple products of various sizes that were hybridized to *het-6*^{PA} (6V-P3/6V-P5 PCR product) and *brk-1*^{OR} (Gene7-P1/cys3-P4 PCR product) probes. Results are presented in Table 2.4. None of the PCR products except for the OR positive control hybridized to the *brk-1*^{OR} probe. Also, none of the Gene7-P1/cys-3P4 PCR products (i.e. *brk-1*^{OR}-specific) hybridized to the *het-6*^{PA} probe. Of the 16 strains tested, only eight (4 OR and 4 PA) produced PCR products with *brk-1*^{PA} specific primers that hybridized positively to the *het-6*^{PA} probe. Of these, all strains carrying the OR *un-24/het-6* haplotype had a unique 6.2 kbp fragment and all strains carrying the PA *un-24/het-6* haplotype displayed 4.5 kbp and 1.2 kbp fragments. The sizes of the hybridizing fragments obtained from *N. tetrasperma* strains do not correspond to those observed when *N. crassa* PA DNA was used as template for PCR. It is possible that the hybridizing fragments obtained represent non-specific amplification products. However, the apparent correlation between their size and the genotype at *un-24* and *het-6* suggest that they may be indicative of inversion type in *N. tetrasperma*. The absence of hybridizing OR-specific PCR products would suggest that this inversion form is more difficult to detect than its PA counterpart or that it is absent in *N. tetrasperma*. Difficulties in isolating inversion-specific PCR products are likely the result of the highly divergent sequence around the

inversion breakpoints. Based on this analysis, it is difficult to conclude whether or not *In(het-6)* occurs in *N. tetrasperma*. An alternative method to PCR may be the screening of a *N. tetrasperma* genomic library and a characterization of the inverted regions as was done for *N. crassa*.

To pursue the survey of trans-species polymorphisms associated with *In(het-6)*, eleven *Neurospora* and *Sordaria* strains were analyzed. Polymorphisms were assayed using four different PCR-based markers. In a first attempt, DNA from strains FGSC6673 (*N. sitophila*), FGSC1754, FGSC962 (*N. intermedia*) and FGSC965 (*N. tetrasperma*) were used in PCR reactions with primer pairs designed to amplify *N. crassa brk-1^{OR}* (Gene7-P1/cys3-P4) or *brk-1^{PA}* (Gene7-P1/6J-P11). All three primers were used in the same PCR reaction mix. Based on PCR reactions with *N. crassa* strains, the expected sizes for OR- and PA-specific PCR products are 4.5 kbp and 3.5 kbp, respectively. PCR products were hybridized to probes corresponding to *cys-3^{OR}* (cys3-P1/cys3-P2) and *un-24^{OR}* (6J-P11/6J-P6) and results are presented in Table 2.5. All PCR reactions produced numerous fragments. However, none of these hybridized to the *cys-3^{OR}* probe, with the exception of the positive control (74-OR23-1V). As in the previous experiment with the *N. tetrasperma* strains, the lack of positively hybridizing OR-specific PCR product may indicate that the OR inversion form does not exist in this sample or that the experimental technique was unable to detect it. When the PCR products corresponding to *brk-1^{PA}* were hybridized with the *un-24^{OR}* probe, all strains produced a 3.2 kbp fragment. With the exception of FGSC1754, all isolates also displayed a 0.5 kbp fragment. The 3.2 kbp fragment is close in size to the 3.5 kbp fragment corresponding to *brk-1^{PA}* in *N. crassa* strains, suggesting that all four strains carry the PA inversion form. This prediction was

Table 2.4. Diagnostic PCR/hybridization analysis of *brk-1* inversion polymorphisms in *N. tetrasperma* isolates. PCR products were obtained with primers corresponding to *brk-1*^{PA} (6V-P5/6J-P11) and *brk-1*^{OR} (Gene7-P1/cys3-P4). Products were hybridized to probes corresponding to *het-6*^{PA} (6V-P3/6V-P5) and *brk-1*^{OR} (Gene7-P1/cys3-P4). *N. crassa* OR (74-OR23-1V) and PA (RLM58-18) standard strains are included as references.

¹ Genotype at	Strain	Size of hybridizing PCR fragments (kbp)		
		<i>brk-1</i> ^{OR} PCR		
		<i>brk-1</i> ^{PA} PCR products		
		products		
		<i>brk-1</i> ^{OR} probe	<i>het-6</i> ^{PA} probe	<i>brk-1</i> ^{OR} probe
<i>un-24</i> ^{OR} <i>het-6</i> ^{OR}	FGSC 1270, <i>A</i>	-	6.2	-
	FGSC 1271, <i>a</i>	-	6.2	-
	P4381, <i>A</i>	-	6.2	-
	P4382, <i>a</i>	-	6.2	-
	FGSC 2503, <i>A</i>	-	-	-
	FGSC 2504, <i>a</i>	-	-	-
	P4379, <i>A</i>	-	-	-
	P4380, <i>a</i>	-	-	-
	74-OR23-1V	4.5	-	-
<i>un-24</i> ^{PA} <i>het-6</i> ^{PA}	Coba131, <i>a</i>	-	4.5, 1.2	-
	P4368, <i>a</i>	-	4.5, 1.2	-

[Table 2.4 continued]

FGSC 2508, <i>A</i>	-	4.5, 1.2	-
FGSC 2509, <i>a</i>	-	4.5, 1.2	-
P4367, <i>A</i>	-	-	-
FGSC 2510, <i>A</i>	-	-	-
FGSC 2511, <i>a</i>	-	-	-
Coba 131, <i>A</i>	-	-	-
RLM 58-18	-	7.5	-

¹Genotypes for *N. tetrasperma* strains were determined by A. J. Powell (2002) by sequencing *un-24* and *het-6* alleles.

Table 2.5. Survey of inversion polymorphisms in *Neurospora* species using PCR

diagnostic primer pairs corresponding to *brk-1*^{OR} (Gene7-P1/cys3-P4) and *brk-1*^{PA} (Gene7-P1/6J-P11). PCR reactions were set up with all three diagnostic primers and PCR products were hybridized to probes derived from *un-24*^{OR} (6J-P11/6J-P6) and *cys-3*^{OR} (cys3-P1/cys3-P2) PCR products.

Species	Strain	Size of hybridizing PCR fragments (kbp)	
		<i>un-24</i> ^{OR} probe	<i>cys-3</i> ^{OR} probe
<i>N. intermedia</i>	FGSC962	3.2, 0.5	-
<i>N. intermedia</i>	FGSC1754	3.2	-
<i>N. tetrasperma</i>	FGSC965	3.2, 0.5	-
<i>N. sitophila</i>	FGSC6673	3.2, 0.5	-
<i>N. crassa</i> (PA)	RLM58-18	3.5	-
<i>N. crassa</i> (OR)	74-OR23-1V	-	4.5

verified by partial sequencing of the 3.2 kbp fragment obtained from strain FGSC962 (see Appendix 1 for DNA sequence). That the remaining three strains display a similar hybridization pattern to FGSC962 suggested that these strains were also likely to have the PA inversion form. Attempts to use this PCR diagnostic to identify inversion polymorphisms for the additional eight strains were unsuccessful.

Two additional PCR markers were used to study the distribution of *In(het-6)* in species related to *N. crassa*. Primers pairs 6V-P5/6J-P11 (*brk-1^{PA}*-specific) and Gene2-P1/6J-P11 (*brk-2^{OR}*-specific) were used in separate PCR reactions. PCR products were hybridized sequentially to probes *het-6^{OR}* (6V-P3/6V-P5) and *un-24^{OR}* (6J-P3/6J-P6). PCR products corresponding to the PA inversion form in *N. crassa* (7.5 kbp) were expected to hybridize to *het-6^{OR}* and *un-24^{OR}* probes whereas PCR products corresponding to the OR inversion form in *N. crassa* (5.5 kbp) were expected to hybridize only to the *un-24^{OR}* probe. A summary of the hybridization patterns obtained is presented in Table 2.6.

Again, none of the PCR fragments obtained with *brk-2^{OR}* specific primers hybridized to the *un-24^{OR}* probe, with the exception of the *N. crassa brk-2^{OR}* positive control (74-OR23-1V). This suggests that the OR inversion form does not exist or is difficult to isolate under these experimental conditions. The sizes of the *brk-1^{PA}* PCR products were variable. Based on the hybridization patterns of the *brk-1^{PA}* PCR products to *un-24^{OR}* and *het-6^{OR}* probes, isolates could be divided into three categories based on whether the *brk-1^{PA}* PCR products isolated from them hybridized to *het-6^{OR}*, *un-24^{OR}* or both probes. All four *N. intermedia* strains in Table 2.6 displayed a 7.5 kbp fragment (as expected for *brk-1^{PA}*) that hybridized to the *het-6^{OR}* probe. The second category is represented by strain

Table 2.6. Diagnostic PCR/hybridization analysis of inversion polymorphisms in *Neurospora* and *Sordaria* species. Diagnostic PCR products corresponding to *brk-2*^{OR} (Gene2-P1/6J-P11) and *brk-1*^{PA} (6V-P5/6J-P11) were hybridized to probes derived from *het-6*^{OR} (6V-P3/6V-P5) and *un-24*^{OR} (6J-P3/6J-P6) PCR products.

Species	Strain	Size of hybridizing PCR fragments (kbp)		
		<i>brk-2</i> ^{OR} PCR	<i>brk-1</i> ^{PA} PCR products	
		products	<i>un-24</i> ^{OR} probe	<i>het-6</i> ^{OR} probe
<i>N. intermedia</i>	FGSC962	-	4.6, 3	7.5
<i>N. intermedia</i>	FGSC1754	-	4.8, 3.2	7.5, 1
<i>N. intermedia</i>	FGSC1882	-	3.2	7.5
<i>N. intermedia</i>	FGSC2540	-	3.2	7.5
<i>N. sitophila</i>	FGSC417	-	6.2, 4.5	6.2
<i>N. sitophila</i>	FGSC6673	-	6.2, 4.5	-
<i>S. brevicollis</i>	FGSC1903	-	6.2, 4.5	-
<i>S. macrospora</i>	FGSC4818	-	6.2, 4.5	-
<i>S. macrospora</i>	FGSC6540	-	4.5	-
<i>S. brevicollis</i>	FGSC1904	-	6.2	-
<i>S. sclerogenia</i>	FGSC2740	-	6.2	-
<i>N. crassa</i>	RLM58-18	-	7.5	7.5
<i>N. crassa</i>	74-OR23-1V	5.6	-	-

FGSC417 (*N. sitophila*) that produced a 6.2 kbp fragment hybridizing to both probes.

Finally, in the largest category, composed of six strains representing four different species, one or two fragments of 6.2 and/or 4.5 kbp are detectable that hybridize only to the *het-6*^{OR} probe. Note that fragments of 6.2 and 4.5 kbp were also observed in a previous experiment using the same *brk-1*^{PA} specific primers to type *N. tetrasperma* strains.

The consistent detection of the same fragments corresponding to the *brk-1*^{PA} PCR diagnostic in several species from the Sordariaceae suggests that these strains carry the PA form of the *In(het-6)* inversion. This was verified for one *N. intermedia* strain, FGSC962, by sequencing part of *brk-1*^{PA}. It is therefore likely that the other three *N. intermedia* strains also carry the PA inversion form since they display a similar PCR hybridization pattern. This prediction could be verified, in a future experiment, by cloning and sequencing the 7.5 kbp fragment. In other *Neurospora* and *Sordaria* strains surveyed, a similar pattern of hybridization is observed, suggesting that they also carry the PA inversion form. The fact that the PCR product does not always hybridize to both *un-24* and *het-6* probes may be due to sequence differences between probe and target. For example, in *N. crassa*, *het-6*^{PA} and *het-6*^{OR} alleles are only 78% similar at the DNA level (Smith *et al.*, 2000b). Sequence dissimilarity between OR and PA alleles results in very little hybridization under standard conditions (see Figure 2.2, cosmid G8:G:1 containing *het-6*^{OR} does not hybridize to *het-6*^{PA} probe). Similarly within *un-24*, a large number of nucleotide differences between OR and PA alleles have accumulated in the 3' end of the gene encoding nonself recognition specificity. In my experiment, the 3' region of *un-24* is specifically targeted by the *un-24*^{OR} probe. Allelic differences between the *un-*

24^{OR} probe and target (e.g. if the strain tested carries the *un-24*^{PA} allele) could affect hybridization intensity.

Overall, 28 strains representative of three *Neurospora* and three *Sordaria* species were surveyed for the presence of inversion polymorphisms associated with *In(het-6)*. The lack of positively hybridizing PCR products corresponding to the OR inversion configuration in any of the isolates tested indicates that the OR inversion form is either absent or rare in these other species in contrast to *N. crassa* where it is common. The absence of the OR inversion form in *N. tetrasperma* would be particularly surprising, since alleles at *un-24* and *het-6* are in linkage disequilibrium in this species. It is possible that linkage disequilibrium is maintained by another mechanism such as selection against recombinant haplotypes or intergenic sequence differences. Alternatively, but seemingly less likely, the OR inversion form may be present but difficult to isolate using PCR-based methods due to sequence divergence at inversion breakpoints between *N. crassa* and related species. However, the consistent detection of positively hybridizing fragments using PA-specific primers suggests that the PA inversion form is present and may be more prevalent than the OR inversion form. If this hypothesis is correct it would indicate that the PA inversion form is either the only form or is more prevalent than the OR form in these other species. This would further suggest that the PA inversion form is ancestral to the OR form, a discovery with interesting implications for the understanding of the evolution of this genetic region. For example, a mechanism for the generation of the inversion can more easily be envisioned when the ancestral and derived forms of the inversion are known. Possible mechanisms of inversion generation are discussed in the Discussion section.

An estimate of the age of the inversion polymorphism is important in understanding the evolutionary pressures acting on this genetic structure. For example, sequence divergence at OR and PA inversion breakpoints suggested that the inversion is very old. However, if the OR form of *In(het-6)* only exist in *N. crassa* it would suggest that the inversion event is more recent and that strong selective forces act to cause rapid sequence divergence between the two inversion forms. A comparison of the age of the inversion polymorphisms and the age of alleles at *un-24* and *het-6* may help understand the history of this genetic association. Did nonself recognition function at *un-24* and *het-6* evolve before or after the inversion event? Has the inversion had any impact on the development of nonself recognition function at *un-24* or *het-6* by driving sequence divergence between different alleles at the two loci? Clues to these questions can be provided by a further study of the *un-24/het-6* region in species related to *N. crassa*.

DISCUSSION

The aim of this study was to investigate structural causes of linkage disequilibrium at *un-24* and *het-6* in *N. crassa*. The characterization of the *un-24/het-6* region in OR and PA strains of *N. crassa* suggests that linkage disequilibrium at *un-24* and *het-6* is due to the presence of a paracentric inversion, *In(het-6)*, spanning approximately ~18.6 kbp. Here I discuss possible mechanisms responsible for inversion generation, selection and maintenance in *N. crassa* and the association of the inversion with nonself recognition genes *un-24* and *het-6*.

Inversion *In(het-6)* was identified in this study by restriction site mapping of OR and PA regions and sequence comparisons between OR and PA forms of the *un-24/het-6* region. *In(het-6)* encompasses six putative open reading frames; among them *un-24* and *cys-3* have been previously shown to correspond to expressed genes (Smith *et al.*, 2000a; Fu *et al.*, 1989). The discovery of *In(het-6)* provided a clue for the observed linkage disequilibrium at *un-24* and *het-6*. Recombination frequencies in paracentric inverted regions are known to be lower than in non-inverted regions due to improper pairing of chromatids (Chovnik, 1973) and to generation of acentric and dicentric chromatids that are lost during cell division. We postulated that decreased recombination events between inversion forms was responsible for linkage disequilibrium. A PCR/RFLP survey of twenty *N. crassa* strains from around the world for eight markers within and outside the inversion indicated that markers close to inversion breakpoints such as *un-24* and *het-6* are perfectly correlated with the inversion form. Correlation with inversion form decreases for markers located in the center of the inversion where gene conversion events

likely occur. Thus, lack of viable recombinant products between OR and PA inversion forms at inversion breakpoints has probably led to linkage disequilibrium at *un-24* and *het-6* in *N. crassa*.

A comparison of OR and PA inversion types indicated that both inversion breakpoints *brk-1* and *brk-2* display 40% to 50% sequence identity and span 3 kbp and 0.7 kbp in the PA haplotype and 1.1 kbp and 3 kbp in the OR haplotype, respectively. Further comparison of OR and PA inversion breakpoints identified a novel repetitive sequence associated with *brk-1*^{PA}. This sequence spans 397 bp, is flanked by 53 bp inverted repeats and is reminiscent of IS elements of bacteria and RS elements of maize and rice. Fourteen copies of this RS element were detected in the *N. crassa* genome based on BLAST searches. The result has yet to be verified by hybridization analysis against genomic *N. crassa* DNA. Based on previous studies on RS elements in rice, BLAST estimates of the number of copies of RS elements are typically an under-representation of the actual copy number based on hybridization analysis (Nagano *et al.*, 2000). Mobile genetic elements and RS elements are often associated with genome instability and rearrangement (Kazazian and Goodier, 2002). A variety of transposons are associated with inversion breakpoints in *Drosophila* (transposon *Galileo*, Cáceres *et al.*, 1999; transposon *hobo*, Lyttle and Haymer, 1993), mosquito (transposon *Odysseus*, Mathiopoulos *et al.*, 1998), Maize (transposon *Ac*, Zhang and Peterson, 1999). Inversions can occur by homologous recombination between two similar elements situated on the same chromosome or may be the secondary consequence of a double stranded break associated with a transposition event. On the other hand, the physical association between a transposable element and a chromosomal rearrangement does not necessarily imply that

one is the cause of the other. There is evidence that transposable elements tend to accumulate in chromosomal regions displaying low recombination frequencies, such as inversions (Lyttle and Haymer, 1993). Therefore, it is difficult to establish whether the RS element is responsible for the generation of the *In(het-6)* inversion.

Although the RS element associated with *In(het-6)* was only identified at the proximal breakpoint, another copy of the element may have been present at the distal breakpoint and subsequently lost, or its sequence may have rapidly diverged through the accumulation of mutations. In *N. crassa*, repeat induced point mutation (RIP) is responsible for mutation and inactivation of repeated and mobile sequences (Anderson *et al.*, 2001). Sequence comparison of the RS element situated within *In(het-6)* to two of its homologues indicates that approximately 53% of nucleotide differences are GC to AT transitions indicative of RIP. It is plausible that similar mutagenic effects associated with RIP may be responsible for sequence divergence at *brk-2* that may have altered the RS sequence beyond recognition. It is interesting that the RS is only present in the non-inverted PA haplotype and is absent from the OR haplotype. This suggests that the element may have been lost during the inversion event. To capitulate on this subject one would have to analyze inversion breakpoints of several independent *N. crassa* isolates. Absence of correlation between the presence of the RS element and inversion type, would suggest that the RS element is not involved in the generation of the inversion. In contrast, a correlation between the presence of the RS element and inversion type would support the hypothesis that this element is involved in generating the *In(het-6)* inversion.

A priori, repetitive sequences and transposons are considered to be “junk DNA” and deleterious, causing rearrangements that may be responsible for severe detrimental

effects. In humans, such chromosomal rearrangements are responsible for ten to fifteen contiguous gene syndromes (Kazazian and Goodier, 2002). On the other hand, chromosomal rearrangements generate novel genetic combinations that may provide a selective advantage to the individual. In *A. gambiae*, different inversion mechanisms are responsible for cytologically identical chromosomal inversions, suggesting that this rearrangement is adaptive (Caccone *et al.*, 1998). In *N. crassa*, inversion *In(het-6)* OR appears to have been maintained in *N. crassa* populations in some sort of equilibrium with the non-inverted PA form, suggesting that like in *A. gambiae*, the inversion polymorphism may provide a fitness advantage.

Inversion polymorphisms were first proposed to be adaptive in *Drosophila* by Dobzhansky in his “Supergene” hypothesis (1970). In *Drosophila*, a variety of inversion polymorphisms are thought to be maintained because they protect favorable associations between different genetic variants from being broken down by recombination. The suppression of recombination between inversion heterozygotes maintains the favorable genetic combination, i.e. the “supergene”, intact. Thus, selection for the supergene also selects for maintenance of inversion polymorphism. In the case of *un-24* and *het-6*, we argue that certain allelic combinations may provide an advantage to the cell by making nonself recognition more efficient and argue in favor of the supergene hypothesis (see Chapter 3). A strong incompatibility reaction leading to death of the heterokaryon is triggered in *N. crassa* by *un-24^{OR} het-6^{OR}* and *un-24^{PA} het-6^{PA}* genetic combinations. Selection for strong incompatibility reactions may in turn select for “supergene” haplotypes *un-24^{OR} het-6^{OR}* and *un-24^{PA} het-6^{PA}* and their associated inversion forms. This mechanism of selection may account for the increase in the frequency of the

inverted haplotype. In addition, balancing selection for nonself recognition function, associated with PA and OR alleles at *un-24* and *het-6* may explain why both inverted and non-inverted forms are maintained in the population.

That *un-24* and *het-6* are both under balancing selection is strongly suggested by several observations. First alleles at the two *het* loci are present at balanced frequencies in the world population of *N. crassa* (Mir-Rashed, 1998). Second, polymorphisms associated with allelic specificities at *un-24* and *het-6* appear to be conserved in at least two other species *N. tetrasperma* (Powell, 2002) and *N. intermedia* (this study). Third, a comparison of OR and PA alleles at *un-24* and *het-6* in *N. crassa* and *N. intermedia* indicates that nucleotide substitutions leading to non-synonymous amino-acid changes outnumber substitutions leading to synonymous changes, a phenomenon consistent with diversifying selection. The observation that PA and OR-specific haplotypes of *un-24* and *het-6* are maintained across different species has prompted us to investigate whether inversion polymorphisms also exist in these other species. Trans-species polymorphism associated with *In(het-6)* would further support the hypothesis that balancing selection is acting on the inversion polymorphisms.

The extensive sequence divergence at inversion breakpoints suggested that the *In(het-6)* inversion may be old. In most recent inversions, sequence identity at inversion breakpoints drops within a limited area around the actual breakpoint. In *A. gambiae*, for example, sequence identity is maintained throughout the breakpoint junctions with the exception of a 7 bp sequence of unknown origin (Mathiopoulos *et al.*, 1998). In contrast, in *N. crassa*, the extent of sequence divergence at *In(het-6)* breakpoints ranges from 0.7 to 3 kbp. A survey of 28 isolates representative of *N. intermedia*, *N. tetrasperma*, *N.*

sitophila, *S. brevicollis*, *S. sclerogenia* and *S. macrospora* species failed to detect evidence of the OR inversion form. The PCR/hybridization based assay may not be appropriate for use with highly divergent sequences likely to occur at inversion breakpoints and other techniques of isolation will be required to verify this finding. However, the detection of the putative PA inversion form in all strains we were able to test (20) suggests that the PA form is the ancestral, non-inverted form.

The general picture emerging from this study has important implications for the understanding of nonself recognition in *N. crassa* and for the evolutionary forces acting on this genetic complex. It is becoming evident that linkage disequilibrium at *un-24* and *het-6* is not coincidental. It is more consistent with the view that OR or PA combinations at *un-24* and *het-6* are beneficial to the organism. This linkage disequilibrium apparently arose as a consequence of paracentric inversion *In(het-6)* that changed the configuration of *un-24* and *het-6* genes from the PA to the OR form. PA and OR polymorphisms at *In(het-6)* appear to be maintained in the population by balancing selection.

Clearly the study of the *un-24/het-6* gene complex not only provides insight into the evolution and function of nonself recognition but also provides a novel paradigm for the study and testing of hypotheses on supergene evolution and selection. Is the inversion polymorphism associated with *In(het-6)* a trans-species polymorphism? Preliminary studies indicate that it is not, although a more thorough survey of other *Neurospora* species is needed. The lack of inversion polymorphisms in *N. tetrasperma* is particularly disconcerting since linkage disequilibrium has previously been reported at *un-24* and *het-6* in this species (Powell, 2002). If the inversion is absent, how is linkage disequilibrium maintained in this species? Is epistatic selection for two closely-linked

loci strong enough to explain linkage disequilibrium in *N. tetrasperma* or is there another mechanism at work? Since *N. tetrasperma* is a pseudohomothallic species (self-fertile) it is likely to undergo selfing more frequently than it out-crosses, although there is evidence for out-crossing in nature (Powell *et al.*, 2001). In the event of out-crossing, epistatic selection may eliminate recombinant progeny that carry OR/PA or PA/OR combinations at *un-24* and *het-6*. How old is the polymorphism associated with *un-24* and *het-6* nonself specificity ? Studies on alleles at *het-c* estimate that polymorphisms associated with this nonself recognition gene are more than 36 millions of years old (Wu *et al.*, 1998). Finally, it may be of interest to determine whether longevity is a common feature of *het* genes.

CHAPTER 3: Incompatibility function of PA alleles at *un-24* and *het-6*

No virtue can be thought to have priority over the endeavors to preserve one's being.

Spinoza, Ethica IV-20

INTRODUCTION

The process of nonself recognition is found in many biological systems. Examples of nonself recognition have been reviewed for fungi (heterokaryon incompatibility, Saupe, 2000), invertebrates, (histocompatibility system controlling colony fusion, Grosberg and Quinn, 1986), and mammals (the Major Histocompatibility Complex, Hughes and Yeager, 1998). The diversity of recognition mechanisms and the pervasiveness of the recognition phenomenon across taxa suggest that nonself recognition has emerged independently several times in evolutionary history and is of biological significance with respect to maintenance of genetic individuality and protection against the spread of infectious elements such as viruses.

In fungi, the widespread occurrence of heterokaryon incompatibility also indicates an adaptive function. During the vegetative phase, frequent somatic cell fusions between different individuals may facilitate transfer of viruses, invasions by selfish genes or entire genomes. Heterokaryon incompatibility may decrease the spread of invasive elements by limiting cell fusions to individuals with very similar genotypes, such as siblings (Caten 1972; Hartl *et al.*, 1975). For example, heterokaryon incompatibility diminishes the transfer frequency of the Kalilo senescence plasmid in *N. crassa* (Debets *et al.*, 1994). In *C. parasitica*, Programmed Cell Death (PCD) associated with vegetative incompatibility is negatively correlated with virus transmission (Biella *et al.*, 2002). However, in both *N. crassa* and *C. parasitica*, protection conveyed by vegetative incompatibility is not complete since genetic elements can be transferred between some incompatible strains (Debets *et al.*, 1994; Biella *et al.*, 2002). In *C. parasitica*, the rate of virus transfer appears to be inversely proportional to the intensity of the incompatibility

reaction. Rapid cell death as a result of the heterokaryon incompatibility reaction results in lower virus transmission rates. If heterokaryon incompatibility has indeed been selected to restrict the spread of deleterious genetic elements, one might predict that genetic interactions leading to stronger incompatibility reactions and to rapid cell death may be favored. According to this hypothesis, such reactions may lead to increased protection against the spread of deleterious genetic elements.

An alternative hypothesis to the deterministic evolution of nonself recognition in fungi proposes that *het* gene involvement in heterokaryon incompatibility is a coincidence or a mistake (Saupe, 2000). In this view, heterokaryon incompatibility function was secondarily acquired by genes with other biological functions. This “secondary function hypothesis” has been cited to explain the variety and apparent dissimilarity in structure and function of *het* gene products. For example, in *P. anserina*, *het-c* encodes a protein with similarity to a glycolipid transfer protein from pig brain. When inactivated, *het-c* causes defects in ascospore maturation, suggesting that the gene has a biological function besides vegetative incompatibility (Saupe, 2000). In *N. crassa*, *un-24* provides another example of a gene with incompatibility function that has an essential metabolic role. *un-24* encodes the large subunit of ribonucleotide reductase, an essential enzyme that converts RNA precursors into DNA precursors (Smith *et al.*, 2000b). Homologues of *un-24* are found across kingdoms whereas nonself recognition function of *un-24* is apparently unique to *N. crassa*. This suggests that the heterokaryon incompatibility specificity in these genes was acquired secondarily. In the case of *un-24*, heterokaryon incompatibility function is localized to a unique region at the 3' end of the gene (Smith *et al.*, 2000b). This 3' region has a significantly greater non-

synonymous/synonymous nucleotide substitution ratio than does the 5' region of the gene, suggesting that there are different evolutionary pressures acting on the two regions of the same gene (Chapter 2).

In contrast, some *het* genes do not appear to carry any function other than heterokaryon incompatibility. *het-6* from *N. crassa* and *het-e* and *het-d* from *P. anserina* encode unique amino-acid sequences, with similarity only to other *het* proteins and do not appear to affect other cellular functions when inactivated (Smith ML, personal communication; Espagne *et al.*, 1997). It is therefore apparent that *het* genes such these have only heterokaryon incompatibility functions while others, such as *un-24*, carry out specific cellular functions in addition to heterokaryon incompatibility.

One way to test whether incompatibility function is of biological significance is to examine these genes for traces of positive selection. Positive selection acts to maintain genetic variants that have an adaptive value to the organism. Balancing selection is a form of positive selection (Hughes, 1999) by which functionally different alleles at a locus are maintained in a frequency equilibrium. Balancing selection is a hallmark of nonself and self recognition systems such as MHC and self-incompatibility systems of plants, respectively. Maintenance of an optimal number of different alleles at these loci presumably allows for efficient recognition of self or nonself. Alleles at *het-c* in *N. crassa* were also shown to be under balancing selection (Wu *et al.*, 1998); they are maintained at balanced frequencies in the population, their polymorphisms are conserved across different species and non-synonymous substitution rates in coding regions exceed synonymous substitution rates. These observations are consistent with the concept that nonself recognition genes, including *het* genes, have an adaptive function.

In further support of the adaptive function hypothesis is the finding that in *N. crassa* *un-24* and *het-6* may also be under balancing selection. PA and OR alleles at *un-24* and *het-6* are found at approximately equal frequencies in populations (Mir-Rashed *et al.*, 2000). A comparison of OR and PA alleles at the two loci indicates that non-synonymous nucleotide substitutions exceed synonymous nucleotide substitutions (Chapter 2). Furthermore, polymorphisms associated with the *N. crassa* PA and OR alleles are conserved in other *Neurospora* species (Chapter 2). These characteristics indicate that balancing selection is acting at *un-24* and *het-6*.

In this chapter I characterize heterokaryon incompatibility function associated with *un-24*^{PA} and *het-6*^{PA}. The study achieves several goals. First, the discovery of PA heterokaryon incompatibility activity at *un-24* and *het-6* suggests that each gene interacts through an allelic mechanism to trigger the incompatibility reaction. Second, the discovery that strains can escape from *un-24* self-incompatibility allowed the isolation and study of novel allelic combinations at *un-24* and *het-6*. Third, the finding that incompatibility reactions associated with PA alleles at *un-24* and *het-6* are weaker than the ones associated with OR alleles may provide an explanation for the observed linkage disequilibrium at *un-24* and *het-6*. Selection for a strong incompatibility reaction is consistent with selection for increased protection against the spread of infectious elements (Biella *et al.*, 2002). This idea is based on the hypothesis that heterokaryon incompatibility has a primary function in the protection of genetic individuality from invasion by selfish genes.

MATERIALS AND METHODS

Fungal and bacterial strains and growth conditions

N. crassa strains used in this study are given in Table 3.1. Strains were grown at 30°C on 1x Vogel's medium (1.5% sucrose, 1.5% agar) with supplements as required (Vogel, 1964). Heterokaryon incompatibility tests were done as described previously (Smith *et al.*, 2000b). Crosses were performed on synthetic crossing medium with 0.1x supplements at 25°C (Davis and de Serres, 1970). To monitor germination, ascospores were harvested with a sterile loop, suspended in sterile water and incubated at room temperature overnight. Subsequently, ascospores were placed at 60°C for 1 hour, plated onto 1xVogel's solid medium with appropriate supplements, and allowed to germinate at room temperature for 16 hours before germination was assessed under a dissecting microscope.

Plasmid-based cloning was into *E. coli* DH5 α cells (GIBCO/BRL, Burlington ON). Cells were maintained in liquid or solid (1.5% agar) LB medium supplemented with appropriate antibiotics. Sub-cloning was done with vectors pUC118 (Sambrook *et al.*, 1989) and pCB1004, which carries a hygromycin resistance marker (Carrol *et al.*, 1994).

Table 3.1. *N. crassa* strains used in this study.

Strain	Genotype ¹	Origin
C2(2)-1	<i>het-6</i> ^{PA} <i>het-c</i> ^{OR} <i>thr-2 a</i> (OR)	Smith <i>et al.</i> , 1996
C2(2)-5	<i>het-6</i> ^{PA} <i>het-c</i> ^{PA} <i>thr-2 a</i> (OR)	Smith <i>et al.</i> , 1996
C2(2)-9	<i>het-6</i> ^{PA} <i>het-c</i> ^{OR} <i>thr-2 A</i> (OR)	Smith <i>et al.</i> , 1996
C2(3)-25	<i>het-6</i> ^{PA} <i>het-c</i> ^{PA} <i>thr-2 A</i> (OR)	M. L. Smith
C9-2	<i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i> (OR)	Smith <i>et al.</i> , 2000b
C9-15	<i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 A</i> (OR)	M. L. Smith
6-13	<i>arg-1 ad-3B; het-6</i> ^{PA} <i>het-c</i> ^{PA} <i>pyr-4 A</i> (OR)	Smith <i>et al.</i> , 1996
6-49	<i>arg-1 ad-3B; het-6</i> ^{PA} <i>het-c</i> ^{OR} <i>pyr-4; inl a</i> (OR)	M. L. Smith
RLM58-18	<i>het-6</i> ^{PA} <i>het-c</i> ^{PA} <i>pyr-4, inl a</i> (OR)	Smith <i>et al.</i> , 1996
RLM57-30	<i>cyh; pyr-4 A</i> (OR)	Smith <i>et al.</i> , 1996
C8c-32	<i>het-6</i> ^{OR} <i>het-c</i> ^{PA} (OR)	Smith <i>et al.</i> , 2000b
C8c-164	<i>un-24 het-6</i> ^{OR} <i>trp, inl a</i> (OR)	Smith <i>et al.</i> , 2000a
FGSC4564	<i>het-6</i> ^{OR} <i>het-c</i> ^{OR} , <i>ad-3B cyh-1 a</i> ^{m1} (OR)	FGSC
FGSC1131	<i>het-6</i> ^{PA} <i>het-c</i> ^{PA} <i>A</i>	FGSC

¹ (OR) indicates that the strain carries Oak Ridge alleles at all undesignated *het* loci.

Where not indicated, *het* constitution at undesignated loci is unknown.

Transformation-based incompatibility assays

Transformation into fungal spheroplasts was as described by Royer and Yamashiro (1992). Heterokaryon incompatibility function encoded by DNA fragments cloned into pCB1004 was assayed following transformation into both OR and PA spheroplasts. Control transformations included pCB1004 (hygromycin resistance vector) and water as positive and negative controls, respectively. In addition, plasmids that carry *het-6^{OR}* or *un-24^{OR}* genes were used as controls in all transformation experiments. When introduced into PA cells, either of these constructs will trigger a heterokaryon incompatibility response resulting in 90% to 100% decrease in the number of recovered PA colonies compared to the pCB1004 vector control. Incompatibility in transformation-based assays was evident after 3 to 5 days of incubation at 30° or 37° C as a significant decrease in the number of recovered transformants or as an inhibited growth phenotype when compared to control transformations using pCB1004 vector alone. Activity of a construct was assessed based on at least three independent transformation assays (Appendix 2).

Isolates obtained as a result of transformation of *un-24^{PA}* into OR spheroplasts carry an ectopic copy of *un-24^{PA}*. The recommended nomenclature for these strains is *un-24^{OR} het-6^{OR} het-c^{PA} thr-2; inl; un-24^{PA}(EC)*. Throughout this chapter I abbreviate the name of these strains to *un-24^{OR}/un-24^{PA}*. Note that the *un-24* genotype of the endogenous copy is given first.

DNA extraction

For *N. crassa* genomic DNA extractions, mycelia were grown in liquid 1x Vogel's medium for approximately one week. Mycelial mats were harvested by vacuum filtration through 3MM Whatman filter paper, washed with 0.9% NaCl and dried under vacuum. Dried mycelium was pulverized in corex tubes using a glass rod and DNA was extracted as described by Oakley *et al.* (1987). Plasmid DNA extractions were performed on 1 to 3 mls of overnight bacterial culture grown in LB broth with appropriate antibiotics. Cells were harvested by centrifugation and plasmid DNA was extracted by the alkaline lysis miniprep method (Sambrook *et al.*, 1989) with or without purification using WizardTM resin (Promega, Madison, WI). Lambda DNA was extracted using the liquid lysis method (Sambrook *et al.*, 1989).

DNA hybridization

DNA was digested with appropriate restriction enzymes (New England Biolabs, Mississauga ON; Invitrogen, Carlsbad, CA) and subjected to electrophoresis in a 0.7 % agarose gel in 1x TAE (40 mM Tris Acetate, 1 mM EDTA) at ~2 V/cm for approximately 12-14 hours (*Neurospora* genomic DNA) or 30-45 minutes at 10 V/cm (plasmid DNA). Southern blots were with nylon membranes according to the manufacturer's recommendations (Amersham, Oakville ON) and as described in Sambrook *et al.* (1989). DNA blots were hybridized overnight at 58°C in hybridization solution (5x Denhardt's, 5x SSPE, 0.5% SDS), unless otherwise specified. DNA probes were synthesized using [α -³²P] dCTP (Amersham) and the random primer labeling method (Sambrook *et al.*, 1989).

Lambda library construction

Genomic DNA from *N. crassa* strain FGSC1131 was partially digested with *Mbo*I and separated on a 10% to 40% sucrose gradient by ultracentrifugation (26 000 rpm, for 42 hours) in a Beckman swinging bucket SW27 rotor. Fragments between 10 and 20 kbp in length were ligated into lambda FixII arms and packaged into lambda phage heads according to the manufacturer's recommendation (Stratagene, LaJolla, CA). Lambda clones were recovered following infection into *E. coli* XL-1 Blue cells.

PCR amplification, DNA sequencing and restriction digestion

Sequences of PCR primers used in this study are presented in Table 3.2. PCR amplifications were done in 25 µl or 50 µl reaction volumes using approximately 60 ng of template DNA and *Taq* (Gibco/BRL, Burlington ON) or ExpandTM (Roche, Laval PQ) polymerases according to the manufacturer's recommendations. PCR products were cloned into vectors pCRII or TOPO-TA (Invitrogen, Carlsbad CA). DNA sequences were obtained using a LI-COR (Lincoln, NE) 4200L automated sequencer at the Canadian Molecular Research Services (Ottawa, ON).

RESULTS

Isolation of PA incompatibility activity at *un-24* and *het-6*

Previous studies based on partial diploid analysis localized the *het-6* locus to the *T(IIL→IIIR)AR18* translocated region (Mylyk, 1975). Colonies with heterozygous duplications within the translocated *T(IIL→IIIR)AR18* region [*Dp(AR18)*] were inhibited

Table 3.2. List and sequences of PCR primers used in this study.

Primer	Sequence 5'→3'	Target gene
6V-P1	TCCCTCCGATATCCTTCG	<i>het-6</i>
6V-P2	AAATCCTCCCTCTCCGCC	<i>het-6</i>
6V-P3	CGGTAACCTGTTCAGCT	<i>het-6</i>
6V-P5	CCCGCTAAGCCAAGGAGTCC	<i>het-6</i>
6V-PA3*	CGGCGCACAGACCACCAG	<i>het-6</i>
6V-PA4*	CCCTAAACATGCAACTTCC	<i>het-6</i>
6J-P3	GGTGACACGGCGCTGTG	<i>un-24</i>
6J-P6	GTGCGGGCTTAACCGCTG	<i>un-24</i>
6J-P7	CGAGGGTCTGAAGCCG	<i>un-24</i>
6J-P11	CTCCGGATGAGGTTGCCG	<i>un-24</i>
6J-P15	GTTGCTCAGAAAGTGGAG	<i>un-24</i>
6J-P34	GGGCTCCCAGTACTCC	<i>un-24</i>

* Panama-specific primers

in their growth and produced a brown pigment, indicative of heterokaryon self-incompatibility. Escape from self-incompatibility of *Dp(AR18)* colonies was shown to occur by deletion of 70 to 270 kbp of either OR or PA duplicated DNA (Smith *et al.*, 1996). The minimum deleted region required for escape corresponded to a ~35 kbp stretch of DNA. In the OR haplotype, this region was later found to encompass both *un-24^{OR}* and *het-6^{OR}* incompatibility genes (Smith *et al.*, 2000b). That deletion of the same region in both PA and OR haplotypes led to escape from self-incompatibility suggested that PA activity in this region was due to the same genetic factors as the OR activity. However, PCR products obtained from PA strains with primer pairs 6V-P1/6V-P2 and 6V-PA3/6V-PA4 (*het-6^{PA}*) and 6J-P3/6J-P6 (*un-24^{PA}*) (Figure 3.1) did not trigger incompatibility reactions when transformed into OR spheroplasts (Smith *et al.*, 2000b). Transformations of equivalent OR PCR products into PA cells resulted in strong incompatibility reactions. This suggested that incompatibility activity at *un-24* and *het-6* occur through a non-allelic interaction between the two *het* loci and/or other closely-linked loci. These other loci would have to be situated in the region deleted during escape from self-incompatibility in partial diploids.

To isolate PA sequences that carry incompatibility activity in the *het-6* region, I constructed a lambda library of the PA strain FGSC1131 in lambda FixII. PA genotypes at *un-24* and *het-6* were first verified in FGSC1131 by PCR amplification with 6J-P11/6J-P6 and 6V-P3/6V-P5 and digestion with *PvuI* and *MboI*, respectively, as described previously (Mir-Rashed *et al.*, 2000). Next, a lambda library of FGSC1131 was constructed and clones carrying *un-24^{PA}* and/or *het-6^{PA}* were identified by hybridization to probes corresponding to *het-6^{OR}*, *het-6^{PA}* and *un-24^{OR}*. A

Figure 3.1. Map of selected restriction sites, incompatibility and catalytic activities of subclones of pλB. Position and orientation of *un-24*^{PA} and *het-6*^{PA} are indicated by arrows. Restriction sites are designated as B= *Bam*HI, C= *Cl*aI, H= *Hind*III, N= *Not*I, Nr= *Nar*I, P= *Pst*I, X= *Xmn*I. Incompatibility activity for each construct is given at right and was assayed by transformation and comparison into OR [C9-2] and PA [C2(2)-1] spheroplasts. Incompatibility activity was defined as strong (++), weak (+), weak with a star phenotype of transformants (+*, see text for details) or absent (-). Ribonucleotide reductase (RNR) function by UN-24 is designated as “+” or “-” (far right), based upon whether or not the construct was able to complement the *un-24* temperature-sensitive mutation in C8c-164 spheroplasts.

2 kb

Activity
incompatibility/
RNR function

Construct

pλB	_____	++	-
pλBB	_____	+	-
pλBB/H	_____	+	-
pλBC/P	_____	++	+
pλBX/C	_____	++	-
pλBN/P	_____	-	-
pλBC/N	_____	+	nt
6VPA3/PA4	_____	-	nt
pλBB/A	_____	+	nt

chromosomal walk was initiated from *het-6* both towards and away from the centromere. From this walk lambda clone λ B, which hybridized to both *un-24* and *het-6*, was chosen for further study. λ B was sub-cloned using the restriction enzyme *NotI* into pCB1004 as p λ B (Figure 3.1), which was transformed into C9-2 (*het-6*^{OR}) and C2(2)-1 (*het-6*^{PA}) spheroplasts (Figure 3.2). p λ B caused a 82% (+/- 17%, n=4) decrease in the number of OR transformants recovered as compared to the hygromycin vector control. There was no such decrease when the construct was transformed into PA cells. This indicated that p λ B carries PA-specific incompatibility function.

To test whether one or both of *un-24* and *het-6* have incompatibility function, the p λ B construct was further sub-cloned as p λ BC/P (*un-24*^{PA}) and p λ BC/N (*het-6*^{PA}) (Figure 3.1). Each construct triggered distinct but less dramatic incompatibility reactions when transformed into OR spheroplasts, as described below (Figure 3.3).

Characterization of *un-24*^{PA} activity

Construct p λ BC/P contains the entire *un-24*^{PA} coding region along with 550 bp upstream and 380 bp downstream regions (Figure 3.1). When the construct was introduced into PA spheroplasts there was no significant decrease in the number of PA transformant colonies recovered in comparison to the vector control. When p λ BC/P was transformed into OR spheroplasts there were on average 73% (+/- 30%, n=3) fewer colonies compared to the control (Figure 3.3). Of the regenerated colonies, ~63% were star-like in appearance on the original transformation medium (Figure 3.4). The remainder of the colonies (~37%) were cloud-like in appearance, similar to self-compatible transformant colonies. The

Figure 3.2. Transformation-based incompatibility assay. Spheroplasts with the *un-24^{PA}het-6^{PA}* [strain C2(2)-1] and *un-24^{OR}het-6^{OR}* [strain C9-2] haplotype were transformed with constructs pCB1004 (*hyg^R* vector positive control), pCOR-1 (*het-6^{OR}* in *hyg^R* vector), pλB (*un-24^{PA}het-6^{PA}* in *hyg^R* vector) or water (negative control). Incompatibility was identified as a significant decrease of the number of transformants recovered in comparison to the positive control.

C9-2(OR)

C2(2)-1(PA)

hyg.B vector control
pCB1004

pCOR-1
(*het-6*^{OR})

pλB
(*un-24*^{PA} *het-6*^{PA})

water control

Figure 3.3. Detection of incompatibility function of *un-24*^{PA} and *het-6*^{PA} constructs by DNA transformation assays. Spheroplasts with the *un-24*^{PA}*het-6*^{PA} [strain C2(2)-1] and *un-24*^{OR}*het-6*^{OR} [strain C9-2] haplotype were transformed with constructs pCB1004 (hyg^R vector positive control, not shown), pCOR-1 (*het-6*^{OR} in hyg^R vector), pλB (*un-24*^{PA}*het-6*^{PA} in hyg^R vector), pλBC/P (*un-24*^{PA} in hygromycin vector) and pλBC/N (*het-6*^{PA} in hyg^R vector).

C9-2 (OR)

C2(2)-1 (PA)

pCOR-1
(*het-6^{OR}*)

p λ .B
(*un-24^{VA} het-6^{VA}*)

p λ .BC/P
(*un-24^{VA}*)

p λ .BC/N
(*het-6^{VA}*)

Figure 3.4. Appearance of C9-2 (*un-24^{OR}*) colonies on sorbose medium with hygromycin B, four days after transformation with *un-24^{PA}* in the *hyg^R* vector construct, p λ BC/P. Star colonies (2) are approximately 1/4 the size of wild-type (self-compatible) colonies (1) and have irregular edges.

transformation medium contains 20 g/l sorbose to promote colonial growth of transformants. When transformants were transferred to 1x Vogel's medium containing no sorbose, star colonies had a lower growth rate than wild-type colonies and displayed a spidery morphology. A similar spidery morphology is typical of self-incompatible strains due to heteroallelism at *mat* and *het-c* (Saupe *et al.*, 1996; Beadle and Coonradt, 1944). Thus, incompatibility activity is evident when *un-24*^{PA} DNA is transformed into *un-24*^{OR} spheroplasts as a reduction in the number of transformants, aberrant colony morphology and lower growth rates of some regenerating colonies.

In addition to incompatibility function, I assayed catalytic activity of ribonucleotide reductase encoded by *un-24*^{PA}, by testing for *un-24* complementation. Strain C8c-164 grows at near wild-type growth rates at 30°C but is unable to grow at 37°C or above, due to a mutation (designated *un-24*) in the 5' end of the coding region of the gene encoding the large subunit of ribonucleotide reductase (Smith *et al.*, 2000a). The *un-24* mutation was derived from a *un-24*^{OR} strain and still displays the OR heterokaryon incompatibility specificity. Transformation with *un-24*^{OR} DNA, enables *un-24* strains to grow at 37°C. However, when the *un-24* mutation is not complemented, few or no colonies regenerate at 37°C. C8c-164-derived star colonies were recovered after 4 to 5 days of incubation at 37°C, following transformation with *un-24*^{PA}. This indicated that *un-24*^{PA} was capable of complementing the *un-24* temperature-sensitive mutation, in spite of the PA-specific incompatibility activity. Therefore, *un-24*^{PA} carries both incompatibility and UN-24 catalytic functions, as does *un-24*^{OR} (Smith *et al.*, 2000b).

Four subclones were constructed to examine the region of DNA required for *un-24*^{PA}-encoded catalytic activity and incompatibility function (Figure 3.1). Subclone

pλBN/P has a 30 bp deletion in the 3' end of the *un-24*^{PA} coding region and displays neither catalytic activity nor incompatibility function based on transformation assays.

pλBX/C has a 532 bp deletion of the 5' coding region and promoter region of *un-24*^{PA}, yet it maintains both heterokaryon incompatibility and UN-24 function. Constructs pλBB and pλBB/H, derived from pλB, carry a 1143 bp deletion in the 5' end of *un-24*.

Compared to construct pλBB/H, construct pλBB carries an additional 1200 bp of centromere-proximal DNA, and thus carries a full copy of *het-6*^{PA} (Figure 3.1). Both pλBB and pλBB/H exhibited incompatibility function when transformed into OR strains. Transformation of C9-2 spheroplasts with either pλBB or pλBB/H resulted in a smaller number of transformants and proportionately fewer star-like (inhibited) colonies than when pλBX/C is used. The average number of star/cloud colonies of C9-2 colonies obtained after transformation with pλBB and pλBB/H was 4/12 and 10/29, respectively (n=3). In contrast, the average number of star and cloud colonies obtained after transformation with pλBX/C was 105 and 40, respectively (n=3). The observed incompatibility activity associated with construct pλBB could be due to *het-6*^{PA} alone or to a combination of *un-24*^{PA} and *het-6*^{PA}. However, pλBB/H lacks a portion of the 3' end of *het-6*^{PA} and likely does not have *het-6*^{PA}-associated activity, since constructs such as 6V-PA3/6V-PA4 that lack the 3' terminal region of *het-6*^{PA} are inactive based on transformation assays (Figure 3.1). Thus incompatibility activity conveyed by the pλBB/H construct must be due to *un-24*^{PA}. By extrapolation, construct pλBB must also carry *un-24*^{PA}, in addition to *het-6*^{PA} activities. This explains the enhanced incompatibility activity of pλBB compared to pλB/H. In further support of this is the observation that

following transformation of OR spheroplasts with constructs pλBB or pλB, a similar number of transformants is obtained, suggesting that the two constructs have equivalent incompatibility activities.

Based on these transformation assays, a 1772 bp region of the 3' end of *un-24*^{PA} is required and sufficient for vegetative incompatibility function. Overall, the *un-24*^{PA} and *un-24*^{OR} DNA sequences are 95% identical, however, they differ significantly at the 3' end wherein the specificity region for nonself recognition is likely encoded (Smith *et al.*, 2000b). This region, although lacking ribonucleotide reductase function, still triggers the incompatibility response, suggesting that the incompatibility reaction occurs through a mechanism that is unrelated to ribonucleotide reductase function.

Characterization of *het-6*^{PA} activity

Construct pλBC/N carries a full-length copy of *het-6*^{PA} and 3030 bp of 5' upstream and 880 bp of 3' downstream non coding region (Figure 3.1). Transformation of pλBC/N into OR cells resulted in an average 82% (+/- 9%) decrease in the number of OR transformants compared to the hygromycin B vector control but did not cause a significant decrease in the number of transformants when PA cells were used as recipients (Figure 3.3). None of the colonies that regenerated following transformations with this construct were observed to have a phenotype distinct from that of hygromycin B control transformants. Based on these transformation assays, pλBC/N was considered to carry incompatibility activity associated with *het-6*^{PA}. It is notable that this *het-6*^{PA}-associated incompatibility activity is less pronounced than that of *het-6*^{OR}. A smaller subclone was constructed using PCR primers 6V-PA3/6V-PA4 and pλB as template. This

construct encompasses the entire *het-6*^{PA} open reading frame as well as 340 bp upstream and 110 bp downstream from the open reading frame. The transformation of a similar construct carrying *het-6*^{OR} resulted in death of all PA-background spheroplasts; this is defined as a strong incompatibility reaction (Smith *et al.*, 2000b). In contrast, the 6V-PA3/6V-PA4 construct was not active based on transformation assays (no reduction in the number of OR-genetic background transformants, n=1). Compared to construct 6V-PA3/6V-PA4, construct pλBB/A, has an additional 760 bp in the upstream 5' region of *het-6*^{PA} and triggers an incompatibility reaction when transformed into OR-genetic background spheroplasts (98% decrease in the number of OR-genetic background colonies, compared to the hygromycin resistance vector, n=3). This suggests that *het-6*^{PA} activity, unlike *het-6*^{OR} activity, requires additional 3' downstream and 5' upstream regions. The *het-6*^{PA} sequence was reported previously (Smith *et al.*, 2000b). The gene putatively encodes a 680 amino acid peptide with three regions of identity to at least nine putative *N. crassa* proteins of unknown function (Figure 3.5). The common regions correspond to three domains of unknown function that are 10, 27 and 21 amino acids in length. In addition, HET-E (*P. anserina*) and TOL (*N. crassa*) share the last two domains with HET-6 (Smith *et al.*, 2000b). The positioning of the three domains within the peptides is not conserved, but the inter-domain spacing, in number of residues, and order of domains is well conserved in most of the sequences surveyed. These domains were not detected in other sequence databases, including the complete *Saccharomyces cerevisiae* genome sequence, suggesting that they are restricted to filamentous fungi.

The OR and PA alleles at *het-6* share 78% identity at the nucleotide level and only 68% identity at the amino-acid level. The nucleotide sequence differences are

spread over the entire length of the open reading frame with no significant blocks of amino-acid sequence conservation. As is the case for *het-6*^{OR} (Smith *et al.*, 2000b), no intron consensus sequences occur in *het-6*^{PA} (Bruschez *et al.*, 1993; Edelmann and Staben, 1994).

Escape from *un-24* self-incompatibility

When *un-24*^{PA} is introduced into OR cells, and transformants are recovered on sorbose medium, some transformants display the star-like colony morphology described previously (Figure 3.4). When transferred to 1.5% sucrose (1x Vogel's) medium, star colonies grow slowly and with an aberrant, "spidery morphology". This inhibited phenotype is typical of partially diploid strains carrying incompatible alleles at *het* genes such as *mat* and *het-c* (Beadle and Coonradt, 1944; Saupe *et al.*, 1996) and further corroborates our hypothesis that *un-24*^{PA} acts as a heterokaryon incompatibility gene. Often, within a few days, fast-growing sectors were observed to emerge from these self-incompatible colonies (Figure 3.6). This behaviour resembles the phenomenon of "escape" in *N. crassa*, as was previously described in heteroallelic partial diploids at *het-c* (Xiang *et al.*, 2000), *mat* (Newmeyer, 1970), the region encompassing *het-6* and *un-24* (Smith *et al.*, 1996) as well as in *mat*-incompatible heterokaryons (DeLange and Griffiths, 1975).

One of the characteristics of escape is a rapid transition from the inhibited growth form to a near-wild-type form. Escape has been ascribed to suppressor mutations (Newmeyer, 1970; Xiang *et al.*, 2002) and mutation or deletion of one the duplicated incompatible gene copies (DeLange and Griffiths, 1975; Smith *et al.*, 1996). In the case

Figure 3.5. Conserved protein domains among nine putative proteins with similarity to HET-6. Amino acid sequences preceded by “NC” and “PIR” are from *N. crassa* and were obtained from LGII and LGV sequences through MIPS (<http://mips.gsf.de/proj/neurospora/>). Protein sequences HET-E1 (Swissprot Q00808) and HET-D2Y (AF323585.1) are from *P. anserina*. Sequences were aligned using BCM multiple alignment tools at (<http://searchlauncher.bcm.tmc.edu>). The alignment was used to create a BLOCKS motif. For each sequence, width of the conserved block is given above and the number of amino acids between each block is provided. Identical residues are highlighted in yellow, similar residues are highlighted in gray.

	10 residues	27 residues	21 residues
het-6VOR	VIALSVVWGD.....49.....	EWIDQICINQDDNEEKS FQVRIMRDIY.....76.....	WFKRLWTIQEFCLCADTIFAC
het-6VPA	VIALSVVWGD.....50.....	EWIDQLCINQEDDAEKSSQVLLMKNIY76.....	WFTRVWTIQEFCLCSDTVFAC
NCB208_230	YSALSYEWGL.....48.....	EWVDVAVCINQDDVEKGHQVQMMGQIY90.....	YWRRVWIVQEYFVMARDCLLLC
NCB12N19.040	YQALSYEWGL.....90.....	EWIDALCINQDDAEKSQOVREMGKIR47.....	YWKRIWIIQELFLAKRLVPMC
NCB208_240	YKALSYEWGS.....46.....	EWVDALCINQDDWEKGHQVQLMKTIIY45.....	YWRRVWILQEYFVLARDYVVLC
NCB8L21_1	FFALS YVWGK.....50.....	EWVDQFCIDQDDPDDKHAQIGNMAAIF47.....	YNRRQLTYQSDTLKAITGLLS
PIR.T5105B12F1.90	YVTL S YWGO.....41.....	EWIDRYCIPQNDSPVKHLQIRKMDRIY44.....	PVTRLNGQPETMYERAHLQH
NCB23H20_7	YLTLSHCWGP.....44.....	EWIDSLCIIQDDEQDWAYEAAIMAKIY72.....	LC'RAWTLQESRLSRRVIYFA
tol	FMSLSHCWGK.....45.....	EWIDSLCIIQDSKEDWDDESVMQYVY48.....	LFTRGWVLEQQLLATTLLIFG
het-d2Y	XAILSHTWGD.....37.....	FWVDTCCIDKSDSTEVQRAINSMFQWY39.....	WFTRGWTLQEAHSPVIVEFFS
het-e1	XAILSHTWGP.....39.....	FWVDTCCIDKSNSTELQEAINSMFRWY36.....	WFTRGWTLQELIAPTSEVEFFS

Figure 3.6. Fast-growing sectors (1) emerge from self-incompatible *un-24^{OR}* / *un-24^{PA}* colonies (arrowheads) 3 to 10 days after transfer to 1.5% sucrose 1xVogels' medium.

of self-incompatibility strains derived by transformation, escape could also occur through nuclear segregation or a combination of any of the above mechanisms

In determining the basis of escape from *un-24*-associated self-incompatibility in our system, we predicted distinct genetic and phenotypic signatures for each of these possible mechanisms. The *het* genotype of the self-incompatible C9-2 transformants is presumed to be *un-24*^{OR} / *un-24*^{PA} *het-6*^{OR} *het-c*^{PA} *mat a*. All strains used in these studies carry the OR allele at all other *het* loci.

If a dominant suppressor mutation was responsible for escape, escape isolates would be heterokaryon compatible with *un-24*^{OR} *het-6*^{OR} *het-c*^{PA} *mat a* tester strains. If a recessive suppressor is involved, escape strains would be incompatible with the *un-24*^{OR} *het-6*^{OR} tester strain due to heteroallelism at *un-24*. In either case, escape strains that carry a suppressor mutation would still carry functional copies of both *un-24*^{OR} and *un-24*^{PA}.

Another possible mechanism of escape is through nuclear segregation and loss of the transformed nucleus. Spheroplasts obtained from *N. crassa* macroconidia germlings are typically multinucleate. Transformation events rarely occur in all nuclei and thus transformants typically represent heterokaryons in which some of the nuclei are transformed and some are not (Grotelueschen and Metzenberg, 1995; Dev and Maheshwari, 2002). Under non-selective conditions, partially diploid nuclei may be lost from the heterokaryon after multiple cellular divisions and may be replaced by non-transformed nuclei. Such escapes are expected to be indistinguishable from the original, untransformed C9-2 spheroplasts.

Escape could also occur through local modifications such as point mutations, methylation or other forms of gene silencing that inactivate one copy of *un-24*, or by deletion of either the ectopic or endogenous of *un-24* copy. Escape strains arising through such local modifications, would be either *un-24*^{OR}*het-6*^{OR}*het-c*^{PA}-compatible or incompatible, depending on whether the ectopic or the endogenous copy of *un-24* was silenced by modification. Repeat-induced point mutations (RIP) is one well-characterized form of modification associated with genetic duplications in *N. crassa* (Selker, 1997). RIP occurs in duplicated sequences prior to meiosis and results mainly in GC to AT transitions (Selker, 1997). In the present case, escape occurred within 3 to 10 days of vegetative growth, which is not consistent with RIP. Two additional possible mechanisms of escape are quelling and DNA methylation. Both are rapid processes that are induced upon introduction of DNA into the cell and are known to occur during the vegetative phase in *N. crassa* (Cogoni and Machino, 1997; Tamaru and Selker, 2002). Both mechanisms can influence gene expression levels, either at the transcriptional level (methylation) or at the post-transcriptional level (quelling). Quelling is a form of transgene silencing during which mature transcripts produced both by endogenous and ectopic gene copies are degraded (Cogoni and Macino, 1997; Pickford *et al.*, 2002). Therefore, it is unlikely that quelling is responsible for the selective inactivation of only one or the other gene as in the case of escape from *un-24* self-incompatibility. In addition, destruction of transcripts encoding an essential protein such as ribonucleotide reductase would presumably result in growth inhibition, not growth rate increase as is observed following escape from *un-24* self-incompatibility. On the other hand, DNA methylation at CpG dinucleotides could selectively suppress gene transcription by, for

example, interfering with binding of transcription factors at either the endogenous or ectopic copy (Pickford *et al.*, 2002).

Escape from self-incompatibility could also be due to deletion of one or the other of the duplicated copies. Previous studies on self-incompatible heterokaryons and partial diploids heterozygous at *het* loci suggested that, in some cases, escape may occur through deletion of one or the other duplicated locus in *N. crassa* (Newmeyer, 1970; Vellani *et al.*, 1993). Notably, escape via deletion within a large duplicated region bearing both *un-24* and *het-6* was shown to occur in *N. crassa* (Smith *et al.*, 1996). Although usually the ectopic copy was deleted, the endogenous copy could also be deleted. The average time of escape from self-incompatibility associated with this ~270 kbp duplication was significantly larger (20 days) than the time of escape from *un-24*-self incompatibility observed in my study.

To begin the analysis of mechanisms of escape from *un-24* self-incompatibility, 34 and 33 self-incompatible star colonies from a transformation of C9-2 strain with pλBC/P were obtained as described above and were allowed to escape on hygromycin B and hygromycin B-free medium, respectively. These isolates presumably carry the genotype *un-24*^{OR} / *un-24*^{PA} *het-6*^{OR} *het-c*^{PA} and carry OR alleles at all other *het* loci. Average growth rates (+/- SD) on 1.5% sucrose 1x Vogel's before escape were 1.14 +/- 0.57 mm/day and 2.47 +/- 1.15 mm/day for isolates cultured on hygromycin and hygromycin-free medium, respectively (see Appendix 3). After escape, isolates on hygromycin and hygromycin-free medium had average growth rates of 26.84 +/- 10.67 mm/day and 46.66 +/- 17.02 mm/day, respectively. The inclusion of hygromycin B in the growth medium causes a statistically significant reduction in growth rate (χ^2 ,

$P<0.001$) both before and after escape. Hygromycin B inhibits protein synthesis. In *Neurospora*, resistance conferred by the hygromycin resistance marker is by expression of an *E.coli*-derived protein kinase that phosphorylates and inactivates hygromycin B (Staben *et al.*, 1986).

Heterokaryon incompatibility status was assessed by performing heterokaryon incompatibility tests with standard OR (C8c-32 *un-24*^{OR} *het-6*^{OR} *het-c*^{PA}) and PA (RLM58-18 *un-24*^{PA} *het-6*^{PA} *het-c*^{PA}) strains. All strains, irrespective of their *un-24* compatibility status, were expected to be incompatible with RLM58-18 due to differences at *het-6*. Of escapes selected on hygromycin-free medium, 17/33 were OR-compatible and 16 were OR-incompatible. Of isolates selected on medium with hygromycin B, 26/34 were OR-incompatible and eight were OR-compatible. The difference in the ratios of OR compatible to OR incompatible isolates in the two experiments is significant (χ^2 , $P<0.005$). Escape on hygromycin B medium resulted in 3.25 OR-incompatible colonies for each OR-compatible colony, whereas escape on hygromycin B-free medium results in 1.06 OR-incompatible for each OR-compatible colony. Evidently, selection for the hygromycin resistance gene also selects for maintenance of the adjacent sequences of *un-24*^{PA}. Under non-selective conditions, escape results in nearly equal frequencies of OR-compatible and incompatible isolates. It can be inferred from these observations that most escape events did not occur through suppressor mutations elsewhere in the genome. If escape had occurred through a suppressor mutation, the presence/absence of hygromycin B in the medium should not have affected the relative ratio of OR-compatible/OR-incompatible escape strains. Both nuclear sorting and modification or deletion of one or the other duplicated *un-24* copies are escape mechanisms consistent with the apparent

correlation between hygromycin resistance and heterokaryon compatibility type in the escape strains. Thus, in the course of the escape process, some of the strains restored the heterokaryon compatibility status prior to transformation while others gained a new compatibility status. These novel strains are incompatible with both *un-24^{PA} het-6^{PA}* and *un-24^{OR} het-6^{OR}* strains. They can be thought of as being functionally *un-24^{PA} het-6^{OR}*.

The isolation of escape strains that appeared to be functionally *un-24^{PA} het-6^{OR}* provided an opportunity to test the ability of these strains to form heterokaryons with similar *un-24^{PA} het-6^{OR}* strains. For this purpose, *un-24^{PA} het-6^{OR}* tester strains with auxotrophic markers different from the original C9-2 strain were constructed. Strain *col(4)-2 un-24 het-6^{OR} het-c^{PA} pyr-4 a* was derived from the cross between C8c-164 *un-24 het-6^{OR} het-c^{OR} trp-1; inl a x 6-13 arg-1 ad 3B¹¹⁴; het-6^{PA} un-24^{PA} het-c^{PA} pyr-4 A*. Progeny that were temperature-sensitive (*un-24*) and required uridine (*pyr-4*) were selected and verified to be *un-24^{OR} het-6^{OR} het-c^{PA} pyr-4 a* and OR at all other *het* genes by heterokaryon compatibility testing against C9-2. Spheroplasts derived from *col(4)-2* were transformed with p λ BC/P (*un-24^{PA}*) and self-incompatible star-colonies were allowed to escape at 37°C. Escape to a wild-type growth rate at 37°C presumably occurs through modification or silencing of the endogenous *un-24* copy. Three “*col-PA*” escapes were obtained, *col-PA1*, *col-PA4* and *col-PA5* and used in heterokaryon compatibility tests with 30 C9-2-derived escape strains characterized previously, as well as a C9-2 non-transformed control (Table 3.3). Control heterokaryon incompatibility tests were also performed between C9-2 escapes and the untransformed *col(4)-2* strain. It was expected that all C9-2 escape strains would be compatible with either the *col(4)-2* tester strain or all three *col-PA* escape strains.

Table 3.3. Summary of heterokaryon compatibility of 30 C9-2 escapes with col(4)-2 and three col-PA escapes. Tests were performed on minimal 1x Vogel's medium with 1.5% sucrose as described in materials and methods. Compatible heterokaryon formation is indicated with "+". Incompatible heterokaryons are indicated as "-".

Escape strain	Compatibility based on heterokaryon tests			
	col(4)-2 <i>un-24 het-6^{OR} het-c^{PA}</i>	col-PA1 <i>un-24^{PA} het- 6^{OR} het-c^{PA}</i>	col-PA4 <i>un-24^{PA} het- 6^{OR} het-c^{PA}</i>	col-PA5 <i>un-24^{PA} het- 6^{OR} het-c^{PA}</i>
C9-2	+	-	-	-
e10, e12, e13, e14, e16, e20, e23, e26, e28, e34, e38, e41, e44	+	-	-	-
e21, e22, e25, e30, e31, e32, e33, e48	-	-	-	-
e36	-	-	-	+
e9, e39	-	-	+	-
e17	-	-	+	+
e24, e45	-	+	-	+
e18, e40, e42	-	+	+	+

Heterokaryon incompatibility due to allelic differences at *un-24* between C9-2 and the *col-1*-PA escape strains was evident as spidery morphology and slow growth of forced heterokaryons. These incompatible heterokaryons were similar in appearance to C9-2 *un-24*^{OR} / *un-24*^{PA} and *col-1(4)-2 un-24*^{OR} / *un-24*^{PA} strains prior to escape. After 3 days at 30°C, these heterokaryons measured approximately 40 to 60 mm in diameter and produced few or no conidia in comparison to *col-1(4)-2* + C9-2 heterokaryons, which covered the plate with heavily conidiating mycelium within 3 days (Figure 3.7). Twenty-one of the 30 C9-2 escape strains tested were incompatible with all three *col-1*-PA escape strains (Table 3.3 and Figure 3.8). The nine remaining C9-2 escape strains were compatible with one, two, or three *col-1*-PA escapes. All OR-compatible C9-2 escapes were incompatible with the *col-1*-PA escapes. Among 16 OR-incompatible C9-2 escapes, half were compatible with at least one *col-1*-PA escape. Strains e18, e40 and e42 were compatible with all three *col-1*-PA escapes.

Two observations on *un-24* escape strains are particularly interesting. First, it appears that the heterokaryon compatibility specificity displayed by some *un-24*^{OR} / *un-24*^{PA} escape strains are diverse. Second, some of the escapes have incompatibility phenotypes that are different from heterokaryon compatibility phenotypes previously described in nature. These strains, of which e18, e40 and e42 as well as the *col-1*-PA escapes are examples, are heterokaryon incompatible with *un-24*^{OR}*het-6*^{OR}, *un-24*^{PA}*het-6*^{PA} and may or may not be compatible with other *un-24*^{PA}*het-6*^{OR} escapes. Incompatibility with *un-24*^{OR}*het-6*^{OR} strains occurs presumably because of differences at *un-24*, while incompatibility with *un-24*^{PA}*het-6*^{PA} strains is due to differences at *het-6*. Incompatibility with only some but not all *un-24*^{PA}*het-6*^{OR} strains is more difficult to

Figure 3.7. Novel $un-24^{PA}het-6^{OR}$ heterokaryon incompatibility type of $col(4)-2$ and C9-2 derived escape strains. Self-incompatible $col(4)-2\ un-24^{OR} / un-24^{PA}het-6^{OR}het-c^{PA}pyr-4a$ strains were allowed to “escape” at 37°C. C9-2 $un-24^{OR} / un-24^{PA}het-6^{OR}het-c^{PA}thr-2a$ escape strains that were $un-24^{OR}$ incompatible were selected and confronted to $col-PA$ escape strains at 37°C on 1xVogels, 1.5% sucrose minimal medium. Compatibility of $col-PA$ and C9-2 escape strains demonstrates the novel heterokaryon incompatibility type of the $un-24^{PA}het-6^{OR}$ allelic combination.

Figure 3.8. Summary of proposed mechanisms of escape based on heterokaryon incompatibility tests, *Bam*HI RFLP, 6J-P11/6J-P6 *Pvu*I PCR/ RFLP, methylation and sequencing analysis (see text for details). The designation C9-2-like and C9-2-unlike indicates that strains displayed the same RFLP pattern as C9-2 or the same pattern as C9-2 plus additional bands, respectively. For the PCR analysis the symbol OR indicates that only the OR PCR/RFLP pattern was obtained when 6J-P11/6J-P6 PCR products were digested with *Pvu*I. The symbol OR+ indicates that both PA and OR patterns were detected in these strains. A time line of the assays performed is presented below. Note that when strains were tested for heterokaryon compatibility type one year after the escape event, seven strains (shown in blue) had switched from being OR incompatible to being OR-compatible or from being OR-compatible to OR-incompatible, based on *het* tests. Point mutations were detected by sequencing in underlined strains (see text for details).

explain. One possibility is that different recessive suppressors may be present in col-PA and C9-2 escape strains. When homozygous for the same suppressor mutation, col-PA and C9-2 escape strains can form a compatible heterokaryon. It is also possible that escape has generated new alleles at *un-24* that may have different nonself recognition specificities. Novel specificities of *het-c* alleles were detected in *N. crassa* following modifications within the hypervariable domain (Wu and Glass, 2001). It is possible that in our study col-PA and C9-2 escapes carry novel allelic variants at *un-24* or modifiers that affect specificity of the *un-24* alleles.

To characterize the genotype of escape strains, I further examined *un-24* RFLPs in the 33 C9-2 escape strains from hygromycin B-free medium. DNAs were isolated and were digested with *Bam*HI which cuts once within the endogenous *un-24*^{OR} copy to give two fragments of 2 kbp and 9 kbp (Figure 3.9). Escapes were of two types: 13 strains were similar to the original C9-2 strain, showing the 2 and 9 kbp fragments expected for the endogenous *un-24*^{OR} gene (e.g. e31, Figure 3.9). Twenty strains had the 2 and 9 kbp bands as well as additional hybridizing fragments that likely corresponded to ectopically inserted single copies or tandem repeats of *un-24*^{PA} (e.g. e48 in Figure 3.9).

Of the 17 OR-compatible escapes 10 had the C9-2-like RFLP pattern. These 10 OR-compatible strains could have arisen either through nuclear sorting or deletion of the ectopic *un-24*^{PA} copy. The remaining 7 OR-compatible escapes carried additional ectopically inserted *un-24*^{PA} fragments and could have escaped by suppressor mutations or mutations within the ectopic *un-24*^{PA} copy. Among the 16 OR-incompatible strains, 13 had the C9-2-unlike RFLP. Escape in these strains could have occurred through mutations within the endogenous *un-24*^{OR} copy or at other suppressor loci. The

remaining 3 OR-incompatible strains showed the C9-2-like RFLP pattern and could have resulted from gene replacement at *un-24* or an alteration in characteristics (e.g. secondary mutation(s) or nuclear sorting) between day 9 and day 27 to 52 (Figure 3.8). Gene replacement is rare in *N. crassa* but occurs at relatively high frequencies at some loci. Usually, integration of transformed DNA is within non-homologous regions of the genome (Fincham, 1989). The simplest explanation for C9-2 RFLP patterns in OR-compatible strains is that they arose through loss of the transformed nucleus (nuclear sorting). Some of these strains may have also come about by exact deletions of ectopic copies of *un-24*^{PA}. The presence of *un-24*^{PA} copies in some of both OR-compatible and OR-incompatible escapes suggests a mechanism involving either suppressor mutations or local modifications to inactivate the endogenous OR copy (OR-incompatible escapes) or the ectopic copy(s) (OR-compatible escapes).

To complement the RFLP study, a PCR/RFLP analysis was designed, that discriminates between OR and PA *un-24* alleles. PCR primers 6J-P11 and 6J-P6, were used to amplify a 1552 bp 3' region of *un-24* that is associated with heterokaryon incompatibility allelic specificity (Smith *et al.*, 2000b). Digestion of the PCR product from strains that carry *un-24*^{OR} with *PvuI* generates two fragments of 768 and 784 bp. The restriction site is absent from the PA allele, thus strains that carry *un-24*^{PA} display a fragment of 1.5 kbp (Figure 3.10).

Overall, RFLP and PCR/RFLP analyses are not congruent (see Figure 3.8). First, in 5 out of 13 strains with C9-2-like RFLP pattern, PCR analysis indicated the presence of additional *un-24*^{PA} copies that were not detected by the RFLP analysis. This is not surprising given the greater sensitivity of the PCR-based assay compared to that of DNA

Figure 3.9. *Bam*HI restriction fragments that hybridize to *un-24*^{PA} in DNAs extracted from representative C9-2 escape strains. 6J-P3/6J-P6 PCR product obtained from PA strain RLM58-18 (*un-24*^{PA}) was digested with *Bam*HI and run alongside C9-2 non-transformed (OR) DNA and escape strain samples. DNA was probed with *un-24*^{PA} 6J-P3/6J-P6. The molecular weight standard (std.) is *Hind*III digested lambda genomic DNA.

Figure 3.10. PCR-based detection of *un-24*^{PA} copies within C9-2 escape strains.

6J-P11/6J-P6 PCR products corresponding to the nonself specificity region of *un-24* were amplified from genomic DNA of escape strains and digested with *PvuI*. Digestion of the OR allele generates two fragments of 768 bp and 784 bp which co-migrate as a single band under these electrophoresis conditions. The *un-24*^{PA} allele lacks the *PvuI* restriction site and is evident as a ~1.5 kbp fragment. Note that all escapes maintained the endogenous *un-24*^{OR} copy.

hybridization. However, this suggests that transformed nuclei represent a minority of the total nuclei in escape strains. In these strains, escape may have occurred through changes in nuclear ratios, mutations or methylation-based silencing within the ectopic or endogenous *un-24* copy or mutations at other suppressor loci. Second, in 5 out of 20 escapes that were C9-2-unlike by RFLP analysis, the PCR did not detect *un-24*^{PA} copies that had previously been identified by RFLP analysis. This may be attributed to local mutations or rearrangements that altered the priming site(s) required for PCR and may be indicative of local small-scale modifications within *un-24*^{PA}. Based on this analysis, strains that are OR-compatible and have no evidence of ectopic *un-24* sequences either by RFLP or PCR (e.g. e13), may have escaped through a precise deletion of *un-24*^{PA} or loss of the transformed nucleus. Strains that are OR-incompatible and carry both endogenous and ectopic copies of *un-24* (e.g. e18) may have escaped through point mutations, small-scale deletions or methylation of the endogenous *un-24*^{OR} allele, or could be due to a suppressor mutation elsewhere. Strains e12, e38 and e43 have several copies of *un-24* and are OR-compatible so, they could have escaped by modification or silencing of the *un-24*^{PA} copies.

To test whether methylation-associated gene silencing is involved in escape, I examined RFLP patterns in representative strains using methylation sensitive (*HpaII*) and insensitive (*MspI*) isochizomers. Both inherent and induced DNA methylation was observed in some but not all of the escape isolates tested, suggesting a role for methylation in the escape process. The exact function of methylation as a marker or a trigger of escape remains to be established. For this study of methylation patterns, strains were selected with distinct hygromycin B resistance and heterokaryon compatibility

types. Strains e31 and e34 were OR-compatible and hygromycin-sensitive, strains e39 and e48 were OR-incompatible and hygromycin-sensitive, strains e42 and e45 and e36 were OR-incompatible and hygromycin-resistant and strain e12 was OR-compatible and hygromycin-resistant. Hygromycin resistance was chosen as an indicator of the presence of ectopic *un-24^{PA}* copies within escape strains. Digests were probed with full-length *un-24^{PA}* DNA (Figure 3.11). In the absence of methylation, *HpaII* and *MspI* digestion should generate eight internal fragments of 300, 680, 590, 280, 360 and 90 bp from the endogenous *un-24^{OR}* copy (Figure 3.12). Two fragments were predicted to differ between the endogenous OR copy and the ectopic PA copy. First, a ~ 1.8 kbp fragment is associated with the *un-24^{OR}* allele but not with the *un-24^{PA}* allele. The fragment is composed of ~ 1.3 kbp within the 5' upstream sequence of *un-24^{OR}* and ~ 0.5 kbp within the coding region up to restriction site H-1 (Figure 3.12). The corresponding *un-24^{PA}* fragment is ~960 bp in length and is generated by digestion at *HpaII/MspI* sites within the multiple cloning site of the pCB1004 vector and at the H-1 site within *un-24^{PA}*. A second difference is due to an additional *HpaII/MspI* site that occurs in the OR form. Digestion at this site generates fragments of 256 and 24 bp in the OR form in place of a 280 bp fragment in the PA form. This difference was not detectable using our experimental protocol.

With the electrophoresis conditions used in this experiment, restriction fragments from the untransformed C9-2 strains occur as three distinct hybridizing groups (Figure 3.11). Fragments of 280, 300 and 360 bp make up the first group, fragments of 590 and 680 bp form the second group, and the final group is made up of the 1.8 kbp fragment. In the C9-2 control, and in escape strains e31, e34 and e39, this restriction pattern is slightly

different in *HpaII* and *MspI* digests. The differences in these strains are noticeable as additional diffuse bands of variable intensity above the 1.8 kbp and 0.58kbp to 0.68 kbp fragments. The relatively large size (~2 kbp) of the new restriction fragment observed above the 1.8 kbp fragment could have resulted from multiple methylation events at different *Hpa II* sites [for example, H-2, H-3, H-4, and H-5 (2.1 kbp) or H-3, H-4, H-5, H-6 (1.9 kbp)] or a unique methylation event at H-1. Methylation at the unique H-1 site appears to be the simplest mechanism. The second shadow fragment appearing above the 0.56 to 0.68 group of fragments could be explained by methylation at H-5 (280 bp+360 bp= 640 bp). These additional bands likely represent a “normal” level of methylation at the endogenous *un-24^{OR}* copy, since they were detected within the untransformed C9-2 strain as well as escapes e31, e34 and e39 that do not carry additional copies of *un-24^{PA}*. This suggests that strains e31, e34 and e39 and C9-2 carry a variable level of endogenous, non-induced, methylation within *un-24^{OR}*. Endogenous methylation without prior introduction of DNA by transformation is known to occur in maize, soybean and *Arabidopsis* (Jeddeloh *et al.*, 1998) and is present in *N. crassa* within duplicated regions that have been the subject of RIP (Selker *et al.*, 1993).

The remaining escape strains analyzed were different from the C9-2 control and had restriction digestion patterns of two types. Some differences between these escapes and C9-2 were detectable with both *HpaII* and *MspI* treatments, while other differences were between methylation-sensitive and methylation-insensitive digests. Based on both RFLP and PCR diagnostic patterns (Figure 3.8), strains e48, e42, e45, e12 and e36 have at least one copy of *un-24^{PA}* in addition to the endogenous *un-24^{OR}* copy. The additional PA copy is evident in both *MspI* and *HpaII* digests by the 960 bp fragment that is not

Figure 3.11. *un-24* hybridization patterns of representative escape strains after methylation-sensitive and insensitive restriction enzyme digests. Genomic DNA from selected escape strains was digested with methylation sensitive *HpaII* and insensitive *MspI* enzymes, and the DNA was probed with the 6J-P3/6J-P6 PCR product containing the *un-24*^{OR} gene. Selected fragment sizes are given at right in kbp.

C9-2	e31	e34	e39	e48	e42	e45	e12	e36
M H	M H	M H	M H	M H	M H	M H	M H	M H

1.8 kbp
0.96 kbp
0.59-0.68 kbp
0.28-0.36 kbp

Figure 3.12. *Hpa*II restriction map of *un-24* showing common (H) and polymorphic (HP) restriction sites. The 890 bp fragment at far left is specific to the cloned *un-24*^{PA}, the leftmost *Hpa*II site being within the vector. Polymorphic fragments corresponding to OR (O) and PA (P) strains are indicated below the map. All undesignated fragments are common between the OR and PA strains.both.

bp) or multiple methylation events at several sites either within *un-24^{OR}* or *un-24^{PA}*.

Thus, there is a correlation between the presence of duplicated *un-24* copies and signatures of de novo methylation, but it is unknown whether methylation triggers escape or whether it is a consequence of escape.

To further investigate the possible role of methylation in the mechanism of escape, I assessed the time at which escape occurs under growth conditions which inhibit DNA methylation. If DNA methylation precedes and is necessary for escape, then inhibition of methylation should interfere with or delay escape. I used treatments with 5-azacytidine at 60 μ M and 200 μ M concentrations in growth medium to compare escape times in self-incompatible C9-2 *un-24^{OR}* / *un-24^{PA}* strains. 5-azacytidine differs from cytidine by the presence of a nitrogen atom at the 5 position of the pyrimidine ring (Jones, 1984). 5-azacytidine is phosphorylated by uridine-cytidine kinase and incorporated into RNA. Once reduced by ribonucleotide reductase, it can also be incorporated into DNA. In DNA, it inhibits DNA methyltransferase by binding irreversibly and sequestering the enzyme. DNA methyltransferase becomes unavailable for methylation of other target DNA sequences.

To assess the role of methylation in escape from self-incompatibility, each of 20 self-incompatible *un-24^{OR}* / *un-24^{PA}* star colonies was divided into three inocula that were subsequently transferred to separate Petri dishes containing 1x Vogel's, 1.5% sucrose and

Figure 3.13. Escape times of *un-24^{OR}* / *un-24^{PA}* self-incompatible C9-2 in response to 5-azacytidine (5AC). Twenty star colonies were sub-divided and sub-cultured on 1x Vogel's 1.5% sucrose medium with 0 μ M, 60 μ M and 200 μ M 5-azacytidine and were incubated at 30°C in the dark. Colonies were viewed daily and time of escape was recorded as the day on which fast-growing sectors were seen emerging from the inhibited colony. either 0 μ M, 60 μ M or 200 μ M 5-azacytidine. Cultures were incubated at 30°C in the dark to reduce the rate of light-mediated degradation of 5-azacytidine. Time of escape on 5-azacytidine was determined by examining colonies every day for fast-growing sectors emerging from the inhibited colony.

found in C9-2, e31, e34 or e39. In addition, two of the strains carrying multiple *un-24* copies, e42 and e45 have a well-defined fragment of 1100 kbp in the *HpaII* lane only. This novel fragment appears to be due to de-novo methylation at a specific site. This fragment could be the result of single methylation events at H-1 (300 bp+890 bp=1190). Times of escape at 0 μ M, 60 μ M or 200 μ M 5-azacytidine for 20 strains are presented in Figure 3.13. From this figure it is evident that the presence of 5-azacytidine in the growth medium is associated with a delay in the time of escape. The first escape strains appear at day 2 on medium without 5-azacytidine. At concentrations of 60 μ M and 200 μ M, the first escapes appear one day later than in the control treatment. The maximum number of escapes was on day 4 for untreated samples, day 5 for the 60 μ M treatment and day 6 for the 200 μ M 5-azacytidine treatment. Treatment with 5-azacytidine causes a significant delay in the time of escape in both 60 μ M and 200 μ M treated isolates compared to untreated ones ($P < 0.001$, two-tailed, paired t-test). During the experiment it was observed, however, that the addition of 5-azacytidine to the medium reduces the growth rates of escape strains. Selker and Stevens also found that 5-azacytidine at a concentration of 48 μ M causes a 30% growth rate reduction in *N. crassa* (1985). In addition to inhibition of DNA methylation, other physiological effects of 5-azacytidine may influence the time of escape. For example, at a concentration of 300 μ M, 5-azacytidine causes a 50% decrease in the rate of incorporation of uridine into rRNA in vitro (Vesely, 1985). Also, 5-azacytidine interferes with protein synthesis in the liver of mice, by decreasing the ability of tRNA to become charged with amino acids (Vesely, 1985). These inhibitory effects of 5-azacytidine may increase the time of escape. For

example, a majority of self-incompatible strains grown at 12° C (which causes a 92% reduction in growth rate compared to strains grown at 30°C) had not escaped 14 days after transfer to 1.5% sucrose, 1xVogel's medium. The maximum number of escape events was reached in these strains five days later than strains grown at 30° C. Thus, lowered growth rates associated with 5-azacytidine in the growth medium may also have affected time of escape in our experiment. Nonetheless, these preliminary tests with 5-azacytidine at concentrations that do not significantly affect growth rate (60 µM) suggest that methylation plays a role in the escape process.

In *N. crassa*, methylation frequently occurs at sequences that have been previously mutated by RIP (Selker *et al.*, 1993). In the case of RIP, then, methylation is a marker and not a trigger of mutation. It is also possible that methylation is a primary signal which marks duplicated sequences in *N. crassa* and targets them for permanent modification. In support of this latter hypothesis is the observation that methylation at *un-24* appears to be transient. When the methylation status of escapes e42 and e45 was reassessed approximately one year after the first analysis, it was found that the *HpaII* RFLP pattern was indistinguishable from the *MspI* RFLP pattern, indicating that methylation at *un-24* had been lost. However, phenotypic changes associated with the escape event, such as fast growth rate, wild-type appearance and conidiation, and OR-compatibility status were unchanged. One possibility is that methylation-associated silencing of incompatibility function is transient and that gene silencing is fixed through mutation. To test this hypothesis four escape strains, one OR-compatible (e10) and three OR-incompatible (e18, e42 and e45), that still carried *un-24^{PA}* in addition to the endogenous *un-24^{OR}* sequences were selected and screened for mutations within a 1.1 kbp

region encompassing the promoter region. Multiple mutations were identified in the putatively silenced and active *un-24* copies in all strains tested, suggesting the involvement of a mutation inducing mechanism in the process of escape from self-incompatibility.

For this sequencing study, a 1.1 kbp region was PCR-amplified corresponding to 530 bp in the upstream promoter region and 580 bp of the 5' coding sequence of the *un-24*^{OR} from e18 and e42, and *un-24*^{PA} from e10 and e45. Primer pairs 6J-P34/6J-P7 and M13reverse/6J-P7 were used to amplify *un-24*^{OR} and *un-24*^{PA} sequences, respectively. PCR products were sequenced and compared to the standard *un-24*^{OR} and *un-24*^{PA} sequences (Figure 3.14). Whenever the same nucleotide(s) change was detected in both *un-24*^{OR} sequences, the change was assumed to reflect polymorphisms existing between the C9-2 strain and the standard OR strain sequence used for comparison. Such changes were ignored for the analysis. It was predicted that OR-compatible strains would carry mutations within the ectopic *un-24*^{PA} copy, whereas OR-incompatible strains would have mutations in the endogenous *un-24*^{OR} copy. As predicted, within OR-compatible strain e10 mutations were found in the *un-24*^{PA} copy. The *un-24*^{PA} 5' coding region isolated from e10 carried two, single base-substitutions predicted to cause non-synonymous amino acid substitutions: A→T (Lys₈₈→Met) and G→T (Val₁₂₄→Phe). In addition, a single nucleotide deletion within codon 136 would cause a frameshift and presumably inactivate this *un-24*^{PA} allele in e10. The *un-24*^{OR} sequence isolated from escape e18, an OR-incompatible strain, displayed multiple base substitutions and one insertion within the 5' upstream region. These mutations could interfere with the initiation or regulation of transcription or transcript stability. In addition, the insertion of an adenine within the

third codon would result in a frameshift in *un-24*^{OR} from e18. The *un-24*^{OR} sequence isolated from e42 has a T→C transition mutation upstream from the translation start site that could affect transcript function. Within the coding region, three nucleotide substitutions leading to non-synonymous amino-acid substitutions were detected: A→G (Lys₈₈→Arg), A→G (Lys₉₁→Arg), A→G (Tyr₁₃₇→Cys). Finally, the *un-24*^{PA} fragment isolated from e45 was predicted to be free of mutations, since the e45 escape strain is OR-compatible. Surprisingly, two single base-pair deletion events within codons 10 and 136 as well as a base substitution A→G at codon 149 (Lys→Glu) were detected. The frameshift mutation may inactivate the incompatibility function of this *un-24*^{PA} allele within e45, unless another basepair deletion downstream restored the open reading frame of *un-24*. Based on transformation assays, incompatibility function associated with *un-24* does not require the first 1140 kbp of the open reading frame. If a downstream single basepair deletion occurred, *un-24*^{PA} activity could be restored. Alternatively, it is possible that several copies of *un-24*^{PA}, some of which may be active and some inactive, exist in the genome of strain e45. The discovery of mutations in one of these copies may not reflect the status of all *un-24*^{PA} copies present in the genome. In contrast, the analysis of mutations within the endogenous *un-24*^{OR} allele isolated from these strains should more accurately reflect the compatibility status of the strains, since *un-24*^{OR} is presumably present in a single copy in the genome.

In summary, multiple mutations were detected in the putatively silenced and active *un-24* alleles in four escape strains. Frameshift mutations detected in e10, e18 and e45 strains are predicted to inactivate incompatibility and ribonucleotide reductase activity associated with the affected *un-24* allele. Base substitutions may also serve to

inactivate one or the other allele. In particular, the loss of Lys₈₈ in both e42 *un-24*^{OR} and e10 *un-24*^{PA} is expected to negatively affect UN-24 function, since this residue is conserved and necessary for allosteric activity regulation in all type I ribonucleotide reductases (Smith *et al.*, 2000a). Unlike methylation, which appears to be transient in escape strains, the observed sequence modifications (nucleotide substitutions, deletions and insertions) would have permanent effects on incompatibility function associated with *un-24*. The next step in this experiment would be to verify that the predicted active copy (e.g. the *un-24*^{OR} allele in e10 and the *un-24*^{PA} allele in e18 , e42 and e45) does not carry any mutations that affect incompatibility activity. This step could be carried out by sequencing the entire allele or by determining whether these genes complement the *un-24* temperature-sensitive mutation in a transformation assay. In light of the findings presented above, a more accurate method to measure incompatibility activity, would be the isolation, in all escape strains of the *un-24*^{OR} allele and its activity assay by transformation into OR and PA spheroplasts.

Thus, the process of escape from self-incompatibility may involve a methylation step that marks the duplicated sequences and initiates the silencing process. In further support of this hypothesis are two observations. First, in some strains, hygromycin resistance lost during escape could be recovered in f₁ progeny. Strains e11 and e29 (both hygromycin-sensitive) were separately crossed to C9-15 and progeny were isolated. From crosses e11 × C9-15 and e29 × C9-15, 4/25 and 3/38 progeny tested were hygromycin-resistant, respectively (data not shown). This suggests that loss of hygromycin resistance, at least in some escapes, was through a reversible modification of the *hph* gene. The second observation is that some escape strains switched incompatibility type during the

Figure 3.14. Sequence analysis of representative upstream promoter and coding regions within *un-24^{OR}* and *un-24^{PA}* copies of OR-compatible (e10) and OR-incompatible (e18, e42 and e45) escape strains. DNA sequences corresponding to *un-24^{OR}* (primers 6J-P34/6J-P7) for e18 and e42, or *un-24^{PA}* (primers M13reverse/6J-P7) for e10 and e45 were obtained by PCR. PCR products were sequenced and compared to the standard *un-24^{OR}* and *un-24^{PA}* sequences. Mismatches between sequences are highlighted in yellow, the start codon is highlighted in green and intron sequences are underlined. Mutations that cause frameshifts or amino acid changes are presented on the right hand-side. Deletions are indicated by “-“. Insertions are indicated by “+”.

un-240R	GCCGTGGGTGACGGCGCTTTTGACGGCGGACCCATAGAGGTCCCGCATTTGGCACTGGCAGT	G→A; G→A
e18contig	GCCGTGGGTGACGGCGCTTTTAAACGGCCGACCCATAGAAGTCCCGCATTTGGCACTGGCAGT	
e42	GCCGTGGGTGACGGCGCTTTTGACGGCCGACCCATAGAGGTCCCGCATTTGGCACTGGCAGT	
e10contig	GCCGTGGGTGACGGCGCTTTTAAACGGCCGACCCATAGAGGTCCCGCATTTGGCACTGGCAGT	G→A
e45	GCCGTGGGTGACGGCGCTTTTGACGGCCGACCCATAGAGGTCCCGCATTTGGCACTGGCAGT	
un-24PA	GCCGTGGGTGACGGCGCTTTTGACGGCCGACCCATAGAGGTCCCGCATTTGGCACTGGCAGT	
un-240R	TTCCAGTCCAAGACGAGCGCTAGAAAAAGAGTGGACGGCGCAATCTCGAAAAACCATCCTTT	
e18contig	TTCCAGTCCAAGACGAGCGCTAGAAAAAAGTGGACGGCGCAATCTCGAAAAACCATCCTTT	G→A
e42	TTCCAGTCCAAGACGAGCGCTAGAAAAAGAGTGGACGGCGCAATCTCGAAAAACCATCCTTT	
e10contig	TTCCAGTCCAAGACGAGCGCTAGAAAAAGAGTGGACGGCGCAATCTCGAAAAACCATCCTTT	
e45	TTCCAGTCCAAGACGAGCGCTAGAAAAAGAGTGGACGGCGCAATCTCGAAAAACCATCCTTT	
un-24PA	TTCCAGTCCAAGACGAGCGCTAGAAAAAGAGTGGACGGCGCAATCTCGAAAAACCATCCTTT	
un-240R	CTCGCTGCCCTTCTCCTTCGATCCGTCCTTT-CGTTCTCTCCCTTCCCTTCTCTCTCCCGT	
e18contig	CTCGCTGCCCTTCTCCTTCGGATACGTCCTTTACGTTCTCTCCCTTCCCTTCTCTCTCCCGT	C→A; +A
e42	CTCGCTGCCCTTCTCCTTCGGATCCGTCCTTT-CGTTCTCTCCCTTCCCTTCTCTCTCCCGT	
e10contig	CTCGCTGCCCTTCTCCTTTC-----CATCTCTT-CTCTCCCTTCCACTTCTCTC-CTCCCGT	
e45	CTCGCTGCCCTTCTCCTTTC-----CATCTCTT-CTCTCCCTTCCACTTCTCTC-CTCCCGT	
un-24PA	CTCGCTGCCCTTCTCCTTTC-----CATCTCTT-CTCTCCCTTCCACTTCTCTC-CTCCCGT	
un-240R	CTCTTCCGTGACTGTGACTACTTCAAGTTACTGTCTCTCCCTTCCCTTCTCTCTCTCCAT	
e18contig	ATCTACCGTAACGTGACTACTTCAAGTAACGTGTCTCTCCCTTCCCTTCTCTCTCTCCAT	C→A; T→A; G→A; T→A
e42	CTCTTCCGTGACTGTGACTACTTCAAGTTACTGTCTCTCCCTTCCCTTCTCTCTCTCCAT	
e10contig	CTCTTCCGTGACTGTAACTACTTCAAGTTACTGTCTCTCCCTTCCCTTCTCTCTCTCCAT	A→G
e45	CTCTTCCGTGACTGTGACTACTTCAAGTTACTGTCTCTCCCTTCCCTTCTCTCTCTCCAT	
un-24PA	CTCTTCCGTGACTGTGACTACTTCAAGTTACTGTCTCTCCCTTCCCTTCTCTCTCTCCAT	
un-240R	CCTATACCCCAATTTCCCCCAAGACAACCTTCGAGATTGCTTGGGCTATCTTGGTGTAGT	
e18contig	CCTATACCCCAATTTCCCCCTAGACAACCTTCGAGATTGCTTGGGCTATCTTGGTGTAGT	A→T
e42	CCTATACCCCAATTTCCCCCAAGACAACCTTCGAGATTGCTTGGGCTATCTTGGTGTAGT	
e10contig	CCTATACCCCAATTTCCCCCAAGACAACCTTCGAGATTGCTTGGGCTATCTTGGTGTAGT	
e45	CCTATACCCCAATTTCCCCCAAGACAACCTTCGAGATTGCTTGGGCTATCTTGGTGTAGT	
un-24PA	CCTATACCCCAATTTCCCCCAAGACAACCTTCGAGATTGCTTGGGCTATCTTGGTGTAGT	

un-24OR	CGTCTTCC	TGTCGTGCCATTTGT	CAGCGTAGT	CGAATCCTGTCT	ACGGCCCC	CAGCAGCAG
e18contig	CGTCTTCC	TGTCGTGCCATTTGT	CAGCGTAGT	CGAATCCTGTCT	ACGGCC---	CAGCAG
e42	CGTCTTCC	TGTCGTGCCATTTGT	CAGCGTAGT	CGAATCCTGTCT	ACGGCC---	CAGCAG
e10contig	CGTCTTCC	TGTCGTGCCATTTGT	CAGCGTAGT	CGAATCCTGTCT	ACGGCCCC	CAGCAGCAG
e45	CGTCTTCC	TGTCGTGCCATTTGT	CAGCGTAGT	CGAATCCTGTCT	ACGGCCCC	CAGCAGCAG
un-24PA	CGTCTTCC	TGTCGTGCCATTTGT	CAGCGTAGT	CGAATCCTGTCT	ACGGCCCC	CAGCAGCAG
un-24OR	CAGCAGCACTT	CAAGTTTTCGTGTTCTTCT	GTTTTTGT	CCCCCTCCATTTT	TGGTTGT	CAATA
e18contig	CAGCAGCACTT	CAAGTTCAAGAGTTCTTCT	GTTTTTGT	CCCCCTCCATTTT	TGGTTGT	CAATA
e42	CAGCAGCACTT	CAAGTTCAAGAGTTCTTCT	GTTTTTGT	CCCCCTCCATTTT	TGGTTGT	CAATA
e10contig	CAGCAGCACTT	CAAGTTCAAGAGTTCTTCT	GTTTTTGT	CCCCCTCCATTTT	TGGTTGT	CAATA
e45	CAGCAGCACTT	CAAGTTCAAGAGTTCTTCT	GTTTTTGT	CCCCCTCCATTTT	TGGTTGT	CAATA
un-24PA	CAGCAGCACTT	CAAGTTCAAGAGTTCTTCT	GTTTTTGT	CCCCCTCCATTTT	TGGTTGT	CAATA
un-24OR	ACTGTACTT---	GTTCTTGTCCCTCTTCTT	CACCTTCT	CCCCCACACAT	CAGACATT	
e18contig	ACTGTACTT---	GTTCTTGTCCCTCTTCTT	CACCTTCT	CCCCCACACAT	CAGACATT	
e42	ACTGTACTT---	GTTCTTGTCCCTCTTCTT	CACCTTCT	CCCCCACACAT	CAGACATT	
e10contig	ACTGTACTT	CCCTGTTGTTCCCTCTTCTT	CACCTTCT	CCCCCACACAT	CAGACATT	
e45	ACTGTACTT	CCCTGTTGTTCCCTCTTCTT	CACCTTCT	CCCCCACACAT	CAGACATT	
un-24PA	ACTGTACTT	CCCTGTTGTTCCCTCTTCTT	CACCTTCT	CCCCCACACAT	CAGACATT	
un-24OR	TATGTCAA-	GAAGCGGGTATGTT	CACGACTCT	CCCTTCC	TACCCCAAT	ACCATCATC
e18contig	TATGTCAA	GAAGAAAGCGGGTATGTT	CACGACTCT	CCCTTCC	TACCCCAAT	ACCATCATC
e42	TATGTCAA	GAAGCGGGTATGTT	CACGACTCT	CCCTTCC	TACCCCAAT	ACCATCATC
e10contig	TATGTCAA	GAAGCGGGTATGTT	CACGACTCT	CCCTTCC	TACCCCAAT	ACCATCATC
e45	TATGTCAA	GAAGCGGGTATGTT	CACGACTCT	CCCTTCC	TACT--	AATACCATCATC
un-24PA	TATGTCAA	GAAGCGGGTATGTT	CACGACTCT	CCCTTCC	TACT--	AATACCATCATC
un-24OR	ACGCCCA--	ATGCTAACTCTCT	CTCATAGAT	GGCCGCC	CAGGAGCGGT	CCAGTTCGAC
e18contig	ACGCCCA--	ATGCTAACTCTCT	CTCATAGAT	GGCCGCC	CAGGAGCGGT	CCAGTTCGAC
e42	ACGCCCA--	ATGCTAACTCTCT	CTCATAGAT	GGCCGCC	CAGGAGCGGT	CCAGTTCGAC
e10contig	ACGCCCA	CAATGCTAACTCTCT	CTCATAGAT	GGCCGCC	CAGGAGCGGT	CCAGTTCGAC
e45	ACGCCCA	CAATGCTAACTCTCT	CTCATAGAT	GGCCGCC	CAGGAGCGGT	CCAGTTCGAC
un-24PA	ACGCCCA	CAATGCTAACTCTCT	CTCATAGAT	GGCCGCC	CAGGAGCGGT	CCAGTTCGAC

un-24OR	AAGATCACAGCTCGTGTTCGAGGCTCTGTATGGCCTCGACATGAACCATGTTGATCCT	TTT→ACA, Val ₂₂ →Asp, Ser ₂₃ →Thr;
e18contig	AAGATCACAGCTCGTGACACGAGGCTCTGATATGGCTCGACATGATCCATGTTGATCCT	T→A, Cys ₂₆ →Stop; A→T, Asn ₃₂ →Ile
e42	AAGATCACAGCTCGTGTTCGAGGCTCTGTATGGCTCGACATGAACCATGTTGATCCT	
e10contig	AAGATCACAGCTCGTGTTCGAGGCTCTGTATGGCTCGACATGAACCATGTTGATCCT	
e45	AAGATCACAGCTCGTGTTCGAGGCTCTGTATGGCTCGACATGAACCATGTTGATCCT	
un-24PA	AAGATCACAGCTCGTGTTCGAGGCTCTGTATGGCTCGACATGAACCATGTTGATCCT	
un-24OR	GTAGCCATTACACAAAAGGTCACTCTGGCGTCTACGGTGGCGTCAACACAGCAAACTA	T→A
e18contig	GAAGCCATTACACAAAAGGTCACTCTGGCGTCTACGGTGGCGTCAACACAGCAAACTA	Val ₃₇ →Glu
e42	GTAGCCATTACACAAAAGGTCACTCTGGCGTCTACGGTGGCGTCAACACAGCAAACTA	
e10contig	GTAGCCATTACACAAAAGGTCACTCTGGCGTCTACGGTGGCGTCAACACAGCAAACTA	
e45	GTAGCCATTACACAAAAGGTCACTCTGGCGTCTACGGTGGCGTCAACACAGCAAACTA	
un-24PA	GTAGCCATTACACAAAAGGTCACTCTGGCGTCTACGGTGGCGTCAACACAGCAAACTA	
un-24OR	GACGATCTGGTAAGCACCTTCTCCCCAGTCCCTCTCCT---TGTACCGATTATAAATCAC	
e18contig	GACGATCTGGTAAGCACCTTCTCCCCAGTCCCTCTCCT---TGTACCGATTATAAATCAC	
e42	GACGATCTGGTAAGCACCTTCTCCCCAGTCCCTCTCCT---TGTACCGATTATAAATCAC	
e10contig	GACGATCTGGTAAGCACCTTCTCCCCAGTCCCTCTCCTTGTACCGATTATAAATCAC	
e45	GACGATCTGGTAAGCACCTTCTCCCCAGTCCCTCTCCTTGTACCGATTATAAATCAC	
un-24PA	GACGATCTGGTAAGCACCTTCTCCCCAGTCCCTCTCCTTGTACCGATTATAAATCAC	
un-24OR	GAGCTAACACATAATCTTTCCC-TAGGCTGCCGAGACTGCCGCCTACATGACCGTCACCC	+A
e18contig	GAGCTAACACATAATCTTTCCC-TAGGCTGCCGAGACTGCCGCCTACATGACCGTCACCC	
e42	GAGCTAACACATAATCTTTCCC-TAGGCTGCCGAGACTGCCGCCTACATGACCGTCACCC	
e10contig	GAGCTAACACATAATCTTTCCC-TAGGCTGCCGAGACTGCCGCCTACATGACCGTCACCC	
e45	GAGCTAACACATAATCTTTCCC-TAGGCTGCCGAGACTGCCGCCTACATGACCGTCACCC	
un-24PA	GAGCTAACACATAATCTTTCCC-TAGGCTGCCGAGACTGCCGCCTACATGACCGTCACCC	
un-24OR	ACCCCGACTATGCCATCCTCGCCGCCCGTATCGCCGTGTCCAACCTCCACAAGCAGACCA	
e18contig	ACCCCGACTATGCCATCCTCGCCGCCCGTATCGCCGTGTCCAACCTCCACAAGCAGACCA	
e42	ACCCCGACTATGCCATCCTCGCCGCCCGTATCGCCGTGTCCAACCTCCACAAGCAGACCA	A→G, Lys ₈₈ →Arg
e10contig	ACCCCGACTATGCCATCCTCGCCGCCCGTATCGCCGTGTCCAACCTCCACAAGCAGACCA	A→T, Lys ₈₈ →Met
e45	ACCCCGACTATGCCATCCTCGCCGCCCGTATCGCCGTGTCCAACCTCCACAAGCAGACCA	
un-24PA	ACCCCGACTATGCCATCCTCGCCGCCCGTATCGCCGTGTCCAACCTCCACAAGCAGACCA	

un-24OR	AGAAGCAATGGTCGCTCGTTATATCAGCGAGCTGTACAACATATGTCAACCCCCAGGACCCGGAA	
e18contig	AGATGCAATGGTCGCTCGTTATCAGCGAGCTGTACAACATATGTCAACCCCCAGGACCCGGAA	A→T, Lys ₉₁ →Met
e42	AGAGGCAATGGTCGCTCGTTATCAGCGAGCTGTACAACATATGTCAACCCCCAGGACCCGGAA	A→G, Lys ₉₁ →Arg
e10contig	AGAAGCAATGGTCGCTCGTCATCAGCGAGCTGTACAACATATGTCAACCCCCAGGACCCGGAA	
e45	AGAAGCAATGGTCGCTCGTCATCAGCGAGCTGTACAACATATGTCAACCCCCAGGACCCGGAA	
un-24PA	AGAAGCAATGGTCGCTCGTCATCAGCGAGCTGTACAACATATGTCAACCCCCAGGACCCGGAA	
un-24OR	AGCACTCCCCCATGATCGCCAAAGGACGTATACGAGTGTGTATGCGGCCACAAGGACCGAGT	
e18contig	AGCACTCCCCCATGATCGCCAAAGGACGTATACGAGTGTGTATGCGGCCACAAGGACCGAGT	
e42	AGCACTCCCCCATGATCGCCAAAGGACGTATACGAGTGTGTATGCGGCCACAAGGACCGAGT	
e10contig	AGCACTCCCCCATGATCGCCAAAGGACGTATACGAGTGTGTATGCGGCCACAAGGACCGAGT	G→T, Val ₁₂₄ →Phe
e45	AGCACTCCCCCATGATCGCCAAAGGACGTATACGAGTGTGTATGCGGCCACAAGGACCGAGT	
un-24PA	AGCACTCCCCCATGATCGCCAAAGGACGTATACGAGTGTGTATGCGGCCACAAGGACCGAGT	
un-24OR	TCGACTCGGGCAATTGTCTACGACCCGTGACTTCAACTACCAATACTTCGGCTTCAAGACCC	
e18contig	TCGACTCGGGCAATTG-CTACGACCCGTGACTACAACACCAATACTTCGGCTTCGAGACCC	-T;T→A, Phe ₁₄₁ →Tyr
e42	TCGACTCGGGCAATTGTCTGCGACCCGTGACTTCAACTACCAATACTTCGGCTTCGAGACCC	A→G, Tyr ₁₃₇ →Cys
e10contig	TCGACTCTGCAATT-TCTACGACCCGTGACTTCAACTACCAATACTTCGGCTTCGAGACCC	-G
e45	TCGACTCTGCAATT-TCTACGACCCGTGACTTCAACTACCAATACTTCGGCTTCGAGACCC	-G
un-24PA	TCGACTCTGCAATTGTCTACGACCCGTGACTTCAACTACCAATACTTCGGCTTCAAGACCC	
un-24OR	TCG	
e18contig	TCG	
e42	TCG	
e10contig	TCG	
e45	TCG	
un-24PA	TCG	

course of the experiments. For example, during the course of a one year analysis of C9-2 escape strains, seven of the 33 escape strains had changed heterokaryon compatibility type (Figure 3.8) . Four OR-compatible strains had become OR-incompatible and three OR-incompatible strains became OR-compatible (Figure 3.8). This observation does not affect the comparative genetic analysis of strains (based on RFLP, PCR/RFLP and methylation tests), since a unique source of DNA was used for all genetic tests. However, it does affect the overall analysis of mechanisms of escape since the phenotypic tests (heterokaryon incompatibility tests and germination experiments) were performed over a one year period. Apparently, the process of escape from self-incompatibility does not cease at the moment when the strain resumes near wild-type growth rate. Genetic changes may continue to occur during an unknown period of time can complicate comparison between different analyses taken at different times.

Nonetheless, based on the cumulative analysis of the escape strain data described above it was possible to assign escape strains to five major categories depending on the apparent mechanism of escape (Figure 3.8). Note that some strains could be placed in more than one category. Escape events due to loss of the transformed nucleus were predicted to carry exclusively the *un-24^{OR}* allele and behave in heterokaryon compatibility tests like the original C9-2 strain. OR-compatible strains with no detectable *un-24^{PA}* DNA by either RFLP or PCR/RFLP analysis were placed in this category (e.g. e13, e20). Loss of the *un-24^{PA}* ectopic copy in such strains could have occurred by deletion or by nuclear sorting and associated loss of transformed nuclei. Although we are unable to differentiate between the two possible mechanisms, nuclear sorting would appear to be the simpler and faster mechanism. Strains that escaped through a suppressor

mutation would carry functional, unmodified *un-24^{PA}* and *un-24^{OR}* copies and be OR-compatible, if the mutation is dominant, or OR-incompatible, if the mutation is recessive. Strains where additional copies of *un-24^{PA}* were detected based on both RFLP and PCR/RFLP analyses and that showed no evidence of mutation by sequencing analysis were placed in this category. Note that all OR-compatible strains in this category were also incompatible with col-PA escape strains, suggesting that a recessive suppressor may be involved in the escape process. In OR-compatible strains, if *un-24^{PA}* DNA was detected by RFLP but not by PCR methods, local modifications within the *un-24^{PA}* copy could be considered a likely mechanism of escape. In the case of e10, mutations within *un-24^{PA}* were verified by sequencing. Alternatively, a low ratio of transformed to untransformed nuclei may also explain why additional *un-24^{PA}* copies were detected by PCR but not by RFLP analysis. All OR-incompatible as well as OR-compatible escape strains in which *un-24^{PA}* copies were only detectable by PCR could be placed in this category. Similarly, OR-incompatible strains that carried *un-24^{PA}* copies and were col-PA compatible were assumed to have modifications within *un-24^{OR}*. Mutations within *un-24^{OR}* were verified for escape strains e18 and e42. Finally, escape strains e31 and e39 are OR-incompatible and do not appear to carry *un-24^{PA}* sequences as detected by RFLP or PCR/RFLP analyses. Partial gene replacement, within regions not spanned by the PCR primers, could theoretically cause a change in incompatibility specificity, that would translate into complete incompatibility with OR and col-PA tester strains. Integration of transforming DNA in *N. crassa* is typically ectopic (Fincham, 1989) but, examples of gene replacements exist (Miao *et al.*, 1995). However, gene replacement would presumably occur quickly after introduction of the DNA into the cell and is not consistent

with the initial inhibited phenotype of self-incompatible strains. Nonetheless, to test the hypothesis of gene replacement, one would have to identify the allelic specificity of the incompatibility activity associated with *un-24* in these strains. The *un-24* gene could be isolated by PCR and transformed into OR and PA spheroplasts. If gene replacement has occurred, then transformation of the PCR product into OR cells should cause an inhibited, star-like phenotype in transformants. Alternatively, alterations at other modifier of incompatibility loci could also be responsible for the phenotype observed within these strains.

Investigating the *un-24* and *het-6* non-allelic interaction hypothesis

Despite the potential of four distinct genotype combinations at the two *het* loci, only two; *un-24*^{PA} *het-6*^{PA} and *un-24*^{OR} *het-6*^{OR}, exist in natural populations (Mir-Rashed *et al.*, 2000). Why limit variation to two of four possible genetic combinations? One explanation is that a physical block in recombination between the two genes prevents genetic exchange and shuffling of the two allelic combinations. In Chapter 2, it was shown that a paracentric inversion is present in OR strains, in which the centromere-proximal breakpoint is situated between *un-24* and *het-6*. The inversion itself and the significant sequence divergence evident over more than 1 kbp around this breakpoint are likely responsible for the observed absence of *un-24*^{PA} *het-6*^{OR} and *un-24*^{OR} *het-6*^{PA} haplotypes in *N. crassa*. However, in addition to structural impediments to recombination, non-allelic interactions between products of *un-24* and *het-6* may play a role in generation of linkage disequilibrium. For example, selection against PA/OR and OR/PA combinations of *un-24* and *het-6* may be further responsible for linkage

disequilibrium. Based on supergene evolution models associated with inversions (Dobzhansky, 1970), one might envision co-evolution of *un-24* and *het-6* gene functions.

Thus, deleterious non-allelic interactions between *un-24*^{PA} and *het-6*^{OR} could interfere with proper cellular functions during the vegetative phase (e.g. growth, sporulation, nonself recognition) or the sexual phase (protoperithecial development, fertilization and spore germination). However, the analysis of functionally *un-24*^{PA}*het-6*^{OR} strains among the C9-2 and col(4)-2 escapes indicates that they do not appear to have aberrant growth rates or morphology. Among escape strains investigated in this study, at least some of the strains with the novel *un-24*^{PA}*het-6*^{OR} genotype appear to have nonself recognition function; they are able to discriminate between different *het* genotypes (i.e. they are incompatible with *un-24*^{OR}*het-6*^{OR} and *un-24*^{PA}*het-6*^{PA} strains) and in some cases display novel *het* genotypes. Therefore, during the vegetative phase, cells that co-express UN-24^{PA} and HET-6^{OR} do not appear to be compromised for growth characteristics or incompatibility activity due to a non-allelic *un-24-het-6* interaction.

In addition to the vegetative phase, proper regulation of *het* gene expression is essential for completing the sexual phase. *het* genes must be locally inactivated in ascogenous hyphae to allow for heterokaryosis and karyogamy to occur prior to meiosis. The best characterized *het* regulatory system in *N. crassa* is that controlled by *tol*, which mediates the expression of *mat*-associated heterokaryon incompatibility (Newmeyer, 1970). Although it is the interaction between *mat A* and *mat a* that triggers heterokaryon incompatibility, the presence of the TOL protein is necessary for *mat*-associated heterokaryon incompatibility. *tol* is apparently not expressed in sexual structures and this in turn suppresses the incompatibility reaction associated with heteroallelism at *mat* (Shiu

and Glass, 2000). HET-6 shares significant amino acid identity with TOL in three distinct “death domains” (Smith *et al.*, 2000b; Figure 3.5). It is, therefore, possible that *het-6* has a regulatory role similar to *tol* during the sexual phase and may influence *un-24*-associated incompatibility.

To test the hypothesis that the *un-24*^{PA} *het-6*^{OR} allelic combination has a negative effect on fertility, escapes presented in Figure 3.8 as well as the original non-transformed C9-2 strain were crossed to strains C9-15, 6-13, C2(3)-25 and C2(2)-9. It was hypothesized that escape strains that are functionally *un-24*^{PA} *het-6*^{OR} would have impaired mating or ascospore development due to a failure to coordinate this *un-24*^{PA} / *het-6*^{OR} genetic combination. The null hypothesis is that mating ability is not affected by *un-24* and *het-6* allelic combinations.

In summary of the study outlined below, strains bearing the novel *un-24*^{PA} *het-6*^{OR} haplotype do not appear to display any obvious deleterious features during the vegetative phase. *un-24*^{PA} *het-6*^{OR} strains are able to grow at wild type rates and conidiate, and can exhibit an efficient heterokaryon incompatibility reaction. Experiments investigating the effect of the *un-24*^{PA} *het-6*^{OR} haplotype on fertility are inconclusive. Signs of aberrant sexual function in some escape strains may be due to the *un-24*^{PA} *het-6*^{OR} haplotype or to other modifications that occurred during the escape process. If verified, impaired fertility associated with this novel *un-24*^{PA} *het-6*^{OR} genetic combination would constitute a functional reason for maintenance of linkage disequilibrium at *un-24* and *het-6* in *N. crassa*.

Fertility was measured for all C9-2 escapes presented in Figure 3.8 as the presence or absence of perithecia. In addition, ascospore germination frequency was

determined for crosses of 12 representative escape strains (Table 3.4). Twenty of the 127 matings performed did not produce any perithecia. Of these, 18 were with tester strain 6-13. The impaired fertility of these crosses appears to be due to properties of the 6-13 mating strain and not the escape strains tested, since crosses involving the same escape strains but different mating tester strains produced perithecia. Overall, there was no significant difference in the production of perithecia between OR-compatible and OR-incompatible strains. Similarly, there was no significant difference in the germination frequency of progeny spores of OR-compatible and OR-incompatible escape strains. This was true for both crosses involving *un-24* homozygous (n=2) and *un-24* heteroallelic (n=10) escape strains and suggests that the co-existence of *un-24*^{PA} and *het-6*^{OR} alleles within the same cell does not have significant effects on mating ability. However, a significant decrease in germination frequency was noted in progeny of escape strains that are heteroallelic at *un-24* and that are compatible with *col-PA* escapes compared to progeny from similar heteroallelic strains that were not compatible with *col-PA* strains (n=9, t-test $P < 0.003$). In particular, escape strains e18, e40 and e42 that are heterokaryon compatible with all three *col-PA* strains, have the lowest germination frequencies among all strains tested. Lack of perithecial production and barrenness could in theory be due to the presence of duplicated *un-24* sequences. In *N. crassa*, DNA fragments which are either duplicated or located at ectopic positions in the genome, are subject to “meiotic silencing by unpaired DNA” (MSUD). This defense mechanism against foreign DNA occurs prior to meiosis and often results in silencing of duplicated copies and a barren phenotype in partially diploid strains (Shiu *et al.*, 2001). MSUD could occur in escapes whether they carry homozygous or heterozygous duplications at *un-24*. Low germination

frequencies could also be the result of RIP within duplicated *un-24* sequences. In this case however, low germination frequencies are likely not the result of the *un-24* duplication, since escape strains such as e14 that carry *un-24* duplications were fertile. Alternatively, reduced spore germination may be associated with factors controlling co1-PA compatibility in these strains. Recall that one of the hypotheses explaining full compatibility between C9-2 escape strains e18, e40 and e42 and co1-PA escape strains is that they share a common recessive suppressor mutation. Such a suppressor mutation may affect progression through the sexual phase and fertility. In *P. anserina*, mutations within modifier genes that suppress non-allelic incompatibility reactions during the vegetative phase also affect female organ formation. Strains carrying mutations within *Mod-D* and *Mod-A/Mod-B* are female-sterile (Barreau *et al.*, 1998; Loubradou *et al.*, 1999). In *N. crassa*, a large deletion within *ham-2* (hyphal anastomosis) suppresses *het-c* associated vegetative incompatibility and has dominant negative effects on fertility and spore viability (Xiang *et al.*, 2002). Thus, it is possible that suppressor mutations that affect *un-24*-associated self-incompatibility may also have negative effects on the production and germination of ascospores. If low germination frequency observed in strains e18, e40 and e42 is indeed associated with a common suppressor mutation, one would expect to see a similar decreased germination frequency in crosses involving strains co1-PA1, co1-PA4 and co1-PA5. This prediction can be tested by scoring germination frequency of ascospore progeny derived from crosses of co1-PA escapes with C9-15, 6-13, C2(3)-25 and C2(2)-9. At this point, it is difficult to reach any conclusions about the effect that the *un-24*^{PA} *het-6*^{OR} allelic combination has on progression through the sexual cycle. This difficulty arises primarily from the fact that

the genotype of escape strains has a diversity of phenotypes including various degrees of fertility. The genetic combination *un-24^{PA} het-6^{OR}* does not in itself have a negative effect on fertility since strains e17 and e9 can sometimes produce abundant ascospores that germinate at high frequency. However, strains that are functionally *un-24^{PA} het-6^{OR}* (i.e. strains that are OR-incompatible and fully co1-PA compatible) appear to be impaired in their mating ability. These strains (e18, e40 and e42; Table 3.3) produce ascospores that germinate at low frequencies. This impaired mating may reflect a deleterious combination of functionalities at *un-24^{PA}* and *het-6^{OR}* or may reflect a common feature of these strains unrelated to *un-24^{PA} het-6^{OR}*, such as a common suppressor mutation that affects fertility. To distinguish between these two possibilities, further experiments would have to be performed. For example, if the suppressor was responsible for the low germination frequency in ascospores, then the suppressor and decreased germination phenotype should co-segregate and be independent from the *un-24^{PA} het-6^{OR}* haplotype in a cross. In contrast, if the *un-24^{PA} het-6^{OR}* haplotype itself was responsible for impaired mating, the low fertility phenotype should always segregate with the *un-24^{PA} het-6^{OR}* haplotype in crosses.

In the following Discussion section I will discuss possible reasons for maintenance of linkage disequilibrium at *un-24* and *het-6* by using information gathered in the course of this study on the relative intensity of incompatibility reactions triggered by PA and OR alleles at *un-24* and *het-6* and on the relative effects of the *un-24^{PA} het-6^{OR}* haplotype on the vegetative and sexual life of *N. crassa*.

Table 3.4. Fertility of OR-compatible (A) and OR-incompatible (B) C9-2 escape

strains to five *mat A* tester strains. Fertility was assayed as presence (+) or absence (-) of perithecia and spores as well as percentage of germinating spores (presented in parentheses) for selected crosses. Escape strains with multiple *un-24* copies determined by RFLP and/or PCR analyses are highlighted in gray.

A

Escape strain	Presence of perithecia (% germination)			
	C9-15	6-13	C2(3)-25	C2(2)-9
	<i>un-24^{OR}het-6^{OR}</i>	<i>un-24^{PA}het-6^{PA}</i>	<i>un-24^{PA}het-6^{PA}</i>	<i>un-24^{PA}het-6^{PA}</i>
e47	+	+	+	+
e34	+(78.6)	+(59.5)	+(71)	+
e20	+	-	+	+
e26	+	+	+	+
e41	+	+	+	+
e13	+	-	+	+
e28	+	-	+	+
e14	+(50.7)	-	+(26)	+(67.2)
e38	+(no spores)	+	+	+
e12	+	+	+	+
e23	+	+	+	+
e10	+	-	+	+
e16	+	-	+	+
e43	+	+(no spores)	+	nt
e44	+	-	+	+

[Table 3.4 continued]

B

Escape strain	Presence of perithecia (% germination)			
	C9-15	6-13	C2(3)-25	C2(2)-9
	<i>un-24^{OR}het-6^{OR}</i>	<i>un-24^{PA}het-6^{PA}</i>	<i>un-24^{PA}het-6^{PA}</i>	<i>un-24^{PA}het-6^{PA}</i>
E11	+	-	+	+
E39	+(0.8)	-	+(0.2)	+(84.4)
E31	+	+	+	+
E45	+(23.7)	-	+(2.8)	+(69.8)
E32	+(79.6)	+(69.8)	+(76.2)	+
E30	-	+	+(14.4)	+(91.2)
E24	+(38)	+	+(4)	+
E9	+(0)	+	+(1.7)	+(65.2)
E17	+(4.77)	+(30.8)	+(11.4)	+(78)
E42	+(0)	-	+(1)	nt
E18	+(1.7)	+(no spores)	+(1.4)	+(26.4)
E40	+(1.7)	-	+(3.4)	+(22)
E36	+	-	+	+
E48	+	-	+	+
E22	+	+	+	+
E33	+(no spores)	+(no spores)	+	+
E29	+	-	+	+
E37	+	-	+	nt
E21	+	-	+	+
E25	+	-	+	+
C9-2	+	+	+	+

DISCUSSION

This study outlines the isolation and characterization of heterokaryon incompatibility function associated with *un-24*^{PA} and *het-6*^{PA}. In the course of the study partially diploid *un-24*^{OR} / *un-24*^{PA} strains were observed to escape from self-incompatibility. The study of mechanisms of escape from self-incompatibility as well as the characterization of escape strains has contributed new information on two broad subjects of study in fungal genetics. First, the study has contributed to the understanding of mechanisms of defense against introduced DNA sequences and genetic instability. In addition, the self-compatible strains generated through escape may give insight into mechanisms of regulation or control of the incompatibility reaction associated with *un-24* and with other *het* genes. Furthermore, the generation through escape of novel allelic combinations at *un-24* and *het-6* provides an opportunity to study these combinations in the lab and may provide answers as to why these combinations are not found in nature.

The major objective of this study has been the isolation and characterization of incompatibility activities at *un-24*^{PA} and *het-6*^{PA}. Heterokaryon incompatibility function associated with *het-6* and *un-24* was previously characterized by studying OR alleles at the two loci (Smith *et al.*, 2000b). Studies on self-incompatible *T(AR18)* partial diploids showed that deletion of a region encompassing *un-24*^{PA} and *het-6*^{PA} was correlated to escape from incompatibility (Smith *et al.*, 1996). However, isolation of PA alleles at *un-24* and *het-6* by PCR amplification and incompatibility assays by transformation into OR spheroplasts indicated that PA alleles did not trigger incompatibility reactions. This suggested either that another gene(s) in the *un-24*^{PA}/*het-6*^{PA} region has incompatibility

activity or that *un-24^{PA}* and *het-6^{PA}* products interact in a non-allelic mechanism to trigger incompatibility. The non-allelic interaction hypothesis was favored given that only PA/PA and OR/OR allelic combinations at *un-24* and *het-6* are detected in nature (Mir-Rashed *et al.*, 2000).

In this study, both *un-24^{PA}* and *het-6^{PA}* incompatibility activities were identified. Two reasons account for the apparent absence of incompatibility activity in earlier studies (Smith *et al.*, 2000b). First, the PCR constructs used to characterize activity in previous studies were not large enough to encompass the regions responsible for PA incompatibility function at either locus. For *un-24^{PA}*, a region of approximately 1 kbp downstream from the stop codon is required for incompatibility function. This is in contrast to the *un-24^{OR}* gene, which carries full incompatibility activity in the fragment that ends at the stop codon. In a similar manner, although primer pairs 6V-PA3 and 6V-PA4 encompass the entire *het-6^{PA}* coding region, additional segments upstream (~1100 bp) and downstream (~130 bp) from the coding region are required for incompatibility function. In contrast, incompatibility activity associated with *het-6^{OR}* is encompassed by a region that is equivalent to the 6V-PA3/6V-PA4 PCR product, that spans from 260 bp upstream of the translational start to 120 bp downstream of the stop codon. The minimum region required for activity of *un-24^{PA}* and *het-6^{PA}* remains to be established but, clearly non-coding regions are necessary for PA activity that are not required for OR activity at both loci. Second, the incompatibility reaction triggered when either of *un-24^{PA}* or *het-6^{PA}* alleles are introduced separately into OR cells is significantly weaker than when the OR allele of either gene is transformed into PA cells. Therefore, the distinct incompatibility activity of either *un-24^{PA}* or *het-6^{PA}* may have been overlooked in

previous studies because both alleles are unlike the OR counterparts both in sequence extent and incompatibility intensity. In the current study, incompatibility activity associated with both *un-24*^{PA} and *het-6*^{PA} genes was evident based on transformation experiments.

het-6^{PA} encodes heterokaryon incompatibility as detected in transformation assays by a drop in the number of recovered OR background spheroplasts compared to the *hyg*^R vector positive control. However, the decrease in the number of transformants is significantly less pronounced than that obtained when *het-6*^{OR} is introduced into PA spheroplasts. At least nine putative proteins with common amino acid sequences to HET-6 were identified in *N. crassa*, suggesting that this putative protein may belong to a novel protein family.

un-24^{PA} encodes both incompatibility activity and ribonucleotide reductase function. Incompatibility activity was detected as an inhibited star phenotype when *un-24*^{PA} is introduced into either temperature-sensitive (*un-24*) or wild-type OR cells. Similarly to its OR counterpart, incompatibility function associated with *un-24*^{PA} corresponds to ~590 of the total 929 amino acids of the protein. In contrast, the entire coding region is required for ribonucleotide reductase function in both OR and PA backgrounds. This suggests that incompatibility function associated with *un-24* is independent from ribonucleotide reductase function. In transformation assays, the intensity of the reaction triggered by *un-24*^{PA} is significantly weaker than that triggered by *un-24*^{OR}. Whereas transformation of *un-24*^{OR} causes death in nearly all PA background spheroplasts, *un-24*^{PA} causes an inhibited spidery phenotype and a significant reduction in transformant numbers. The relatively weaker incompatibility reaction and the higher

Figure 3.15. Summary of mechanisms of escape from *un-24* self-incompatibility.

growth rate of self-incompatible strains obtained when *un-24*^{PA} is transformed into OR cells facilitated the recovery of strains that escape from *un-24* self-incompatibility. The characterization of escape strains from *un-24* self-incompatibility revealed a variety of mechanisms of escape (Figure 3.15).

Escape from *un-24* self-incompatibility shares common features with other escape phenomena in fungi and also has novel characteristics. Escape from *un-24* self-incompatibility occurred rapidly in nearly all transformants. It was detected as a switch from slow growth, inhibited phenotype to wild-type growth rate and appearance. Within ten days, nearly all of 67 self-incompatible strains had escaped. This is in contrast to a previous report in which only approximately 25% of self-incompatible *Dp(AR18)* strains escaped, after ~ 20 days at 30°C (Smith *et al.*, 1996). Escape from *un-24* self-incompatibility may occur through several genetic mechanisms. It may be the result of nuclear segregation (6 escape strains) or other changes in the ratio of transformed/untransformed nuclei (7 strains) suppressor mutations (12 escape strains), deletion or mutation of the duplicated genes (18 strains) or partial gene replacement (2 strains, Figure 3.8). All these mechanisms of escape, with the exception of gene replacement have been previously reported (Smith *et al.*, 1996; Newmeyer and Galeazzi, 1977; Newmeyer, 1970; Xiang *et al.*, 2002). Mechanisms of escape appear to depend on the nature of the duplication, the size of the duplication and the number of *het* genes responsible for self-incompatibility. For example, if self-incompatibility is due to duplication of a *het* gene within a subset of nuclei, as would occur in transformation assays, the simplest mechanism of escape may be through a decrease in the number or complete loss of the transformed nucleus. In this study, at least six escape events are

likely to have occurred through this mechanism. However this mechanism could not have occurred in self-incompatible strains obtained from crosses between translocated and wild-type strains (Smith *et al.*, 1996). In the study by Smith *et al.* (1996), partially diploid nuclei for *un-24* and *het-6* were obtained from a cross and thus all nuclei carried the *het* gene duplication. Consequently, escape could not have occurred through nuclear loss. In addition, the size of the duplication may play a role in the escape mechanism. In cases where self incompatibility is due to large duplications [270 kbp +/- 90 kbp for *T(AR18)*], escape appears to occur predominantly through deletion of one or the other duplicated regions, with a bias for the ectopic copy (Newmeyer and Galeazzi, 1977; Smith *et al.*, 1996). In the case of escape from mating type-associated incompatibility characterized by Newmeyer and Galeazzi (1977), the heterozygous duplications were due to a paracentric inversion situated near the end of the chromosome, in a region of repetitive satellite sequence. Escape from self-incompatibility was postulated to be due to mitotic recombination between the satellite sequence flanking the inverted region. It is possible that large chromosomal rearrangements are more easily detectable and more easily eliminated through deletion than small duplications.

Finally, the number of *het* genes involved in the incompatibility reaction may also play a role in the mechanism of escape. In the experiment reported by Smith *et al.* (1996), self-incompatibility was due to heterozygosity at two *het* genes, *un-24* and *het-6*. Escape from self-incompatibility through mutation or small-scale deletion would require local modifications at two distinct loci. In contrast, a large-scale deletion could eliminate heterozygosity at both *het* loci in a single step. Similarly, two independent mutations at suppressors of self-incompatibility would be required to trigger escape from self

incompatibility associated with both *un-24* and *het-6*. That a unique mutation or deletion in one or the other *het* locus was not sufficient to trigger escape from self-incompatibility associated with both *un-24* and *het-6* supports the hypothesis that the two *het* genes operate independently to trigger an incompatibility reaction. Thus, similarity and differences between escape mechanisms may reflect similarity and differences in the nature of the self-incompatibility.

In this study, the most frequent mechanisms of escape from *un-24* self-incompatibility are small-scale deletions and modifications of one of the duplicated *un-24* copies. These modifications were implied based on PCR and RFLP analysis and were confirmed in four escape strains through partial sequencing of the *un-24* alleles. The mutations do not appear to be due to RIP (Selker and Stevens, 1985), but may involve an intermediate methylation step. To my knowledge, this is the first example of methylation associated with the escape process during the vegetative phase. Transient methylation was detected in the endogenous *un-24*^{OR} copy prior to transformation and also in two of the eight escape strains tested. Endogenous methylation marks are known to exist within several imprinted genes in mammals such as the *Igf2R* glucose receptor and are known to influence gene expression (Bartolomei and Tilghman, 1997). Induced methylation has also been shown to be important in the process of RIP in *N. crassa*. Methylation can cause gene silencing by inducing repressive chromatin modifications such as histone methylation (Tamaru and Selker, 2002) and histone deacetylation (review in Richards and Elgin, 2002). In this study, treatment of self-incompatible *un-24* partial diploids with 5-azacytidine delayed the time of escape in a dose-dependent manner, suggesting that methylation is involved in the escape process. In this case, methylation may temporarily

silence one of the duplicated *un-24* copies and/or mark the site for subsequent mutation. Whether methylation and mutation processes are linked or whether they represent independent phenomena is still the subject of major debate. Nonetheless, the discovery of mutations within *un-24* that may or may not be associated with methylation and their correlation with escape from self-incompatibility could prove useful in the study of incompatibility activity at *un-24*.

The study of mutations within *un-24* that are responsible for escape from self-incompatibility may pinpoint precise regions within the *un-24* gene that affect incompatibility function. For example, the isolation by PCR of *un-24*^{OR} and/or *un-24*^{PA} alleles from escape strains e10, e18, e42 and e45 may allow a mutagenesis-based study of *un-24* function. Based on heterokaryon compatibility assays, mutations within *un-24*^{PA} are predicted to be responsible for escape in e10. Similarly, mutations within *un-24*^{OR} are proposed to be responsible for escape in strains e18, e42 and e45. Activity assays of putatively mutated *un-24* copies by transformation into OR and PA spheroplasts as well as sequence analysis of the mutant *un-24* copies may identify new heterokaryon specificity variants. This method could also be applied to escape strains generated through gene replacement. Gene replacement events may be responsible for chimeras with novel heterokaryon specificity function in e31 and e39. For example, e31 is incompatible with PA, OR and co1-PA escape strains. The isolation and characterization of the *un-24* copy in this strain could test the gene replacement hypothesis and possibly identify a novel functionality at *un-24*. In a similar manner the study of putative suppressor mutations within escape strains may provide insight into the regulation of heterokaryon incompatibility.

In this study, suppressor mutations may be responsible for escape from *un-24* self-incompatibility in twelve strains. Strains e12, e38 and e43 have *un-24*^{PA} copies and are OR-compatible and co1-PA incompatible. Strains e17, e36 and e40 also carry *un-24*^{PA} copies but are OR-incompatible and co1-PA compatible. Finally strains e22, e25, e29, e33, e37 and e48 have *un-24*^{PA} copies and are incompatible with both OR and co1-PA strains. All these strains may have escaped through a mutation within one of the *un-24* duplicated copies or through a recessive suppressor mutation. A mutation that blocks the incompatibility reaction associated with *un-24* or prevents its initiation could suppress self-incompatibility. Suppressors of incompatibility have been previously isolated from escape strains. For example, a mutation within *ham-2*, a gene encoding a protein with a transmembrane domain, inhibits hyphal fusion and suppresses heterokaryon incompatibility associated with *het-c* in *N. crassa* (Xiang *et al.*, 2002). The isolation of this mutant suggested a link between heterokaryon incompatibility and hyphal anastomosis pathways (Glass *et al.*, 2000). In my study, no dominant suppressors of self-incompatibility were detected. The mapping and cloning of recessive suppressors is difficult, particularly if no phenotype other than suppression of incompatibility is associated with the mutation. However, the isolation and characterization of such suppressors would contribute to the understanding of *un-24* incompatibility function regulation and its possible relationship to *het-6* function and regulation. Overall, escape from *un-24* self-incompatibility, by whatever mechanism, generated compatibility types that appear to be *un-24*^{PA} *het-6*^{OR}. These *un-24*^{PA} *het-6*^{OR} strains will provide additional insight into reasons for linkage disequilibrium at *un-24* and *het-6*.

In addition to structural barriers associated with inversion *In(het-6)* (Chapter 2), functionally deleterious interactions between products of *un-24* and *het-6* genes could result in selection against recombinant products. Escape from *un-24* self-incompatibility allowed the isolation of strains with the novel genetic combination *un-24^{PA}het-6^{OR}*. These strains could be used to test the hypothesis that deleterious functional interactions between the *un-24* and *het-6* play a role in maintenance of linkage disequilibrium at the two loci. Strains that are *un-24^{PA}het-6^{OR}* strains have a wild type appearance and growth rate. During the sexual phase, no correlation between *un-24^{PA}het-6^{OR}* genotype and low fertility or barrenness could be established. However, three strains (e18, e40 and e42) carrying this genotype that were compatible with all three *co1*-PA strains produced spores with significantly lower germination frequencies than strains that were not compatible with *co1*-PA strains. Low germination frequency could be the result of deleterious interactions between the two alleles or could be due to RIP or MSUD, mechanisms that are activated by DNA duplications and DNA mispairing prior to meiosis, respectively (Selker, 1990; Shiu *et al.*, 2001). If impaired germination is due to the *un-24^{PA}het-6^{OR}* allelic combination, then selection against this combination in order to preserve increased mating ability may result in linkage disequilibrium at *un-24* and *het-6*. However, only 3 among the 17 strains putatively carrying this genotype displayed significantly lower germination frequencies. Clearly, the fertility status of all *un-24^{PA}het-6^{OR}* escape strains needs to be further analyzed. However, assuming that these 3 strains are not representative of all *un-24^{PA}het-6^{OR}* escape strains, let us further explore mechanisms of linkage disequilibrium at *un-24* and *het-6*.

un-24^{PA}het-6^{OR} strains are not impaired in their nonself recognition function.

In fact, they display novel incompatibility function. These strains are incompatible with *un-24^{PA}het-6^{PA}* strains (e.g. RLM 58-18), and *un-24^{OR}het-6^{OR}* strains (e.g. spidery morphology in confrontations with C8c-32 and *col(4)-2*) and partially to completely compatible with independently-derived *un-24^{PA}het-6^{OR}* strains (*col-PA* escape strains). A summary of the incompatibility reactions associated with *un-24* and *het-6* is presented in Table 3.5. It is notable that *un-24^{OR}het-6^{OR}* and *un-24^{PA}het-6^{PA}* strains display “strong” incompatibility reactions in *het* tests with each other, whereas *un-24^{PA}het-6^{OR}* incompatibility reactions in confrontations with either *un-24^{OR}het-6^{OR}* or *un-24^{PA}het-6^{PA}* tester strains are “weak”. We define “strong” incompatibility reactions as those generating close to zero transformants in transformation assays and nearly no heterokaryotic growth (~0.1-0.3 mm/day). Escape from strong incompatibility reactions only occurs in approximately 25% of isolates and takes 20 or more days at 30°C (Smith *et al.*, 1996). In contrast, “weak” incompatibility reactions, obtained through transformation of *un-24^{PA}* into OR spheroplasts, may allow the growth of numerous transformants that are inhibited in their growth (~2.4 mm/day). Escape from weak incompatibility reactions is complete for all strains within ten days.

Based on this definition of incompatibility reactions, there is a notable asymmetry associated with the *un-24* and *het-6* incompatibility responses. When *un-24^{PA}* or *het-6^{PA}* is the source of transforming DNA and the recipient cell is *un-24^{OR}het-6^{OR}*, incompatibility reactions appear to be weaker than when the reciprocal experiments are performed. Observations were also made on the asymmetry of incompatibility reactions associated with *het-c* and *het-d* in *N. crassa* in microinjection studies (Wilson *et al.*,

1961). Cytoplasm was extracted and microinjected in all possible combinations into cells with similar or different genotypes at *het-c* and/or *het-d*. It was found that incompatibility reactions depended on the combination of alleles at the two loci and on whether a particular strain is a donor or recipient of cytoplasm. For example, whereas all injections of *het-C het-d* cytoplasm into *het-c het-d* cells were lethal, the reciprocal injection of *het-c het-d* cytoplasm into *het-C het-d* cells produced 100 % viable cells. However, mortality of *het-C het-D* cells injected with *het-c het-D* cytoplasm increased to 71% and mortality of *het-c het-D* cells injected with *het-C het-D* cytoplasm was 100%. The presence of *het-D* in recipients appears to enhance the intensity of the incompatibility reaction associated with *het-C/het-c*. This suggests that non-allelic interactions occur between *het-c* and *het-d* gene products. In *P. anserina*, non-allelic interactions may or may not lead to incompatibility reactions depending on which alleles are interacting. For example, HET-C1 and HET-E2 together in the same cytoplasm trigger an incompatibility reaction, whereas HET-C1 and HET-E1 are compatible in the *P. anserina* system. However, *het-e1* does not lack incompatibility function since its product triggers an incompatibility reaction when it interacts with HET-C2. We suspected that similar non-allelic interactions might be occurring at *un-24* and *het-6* in *N. crassa*. Our suspicions were based on several observations. First, preliminary studies with *un-24* and *het-6* indicated that PA alleles do not act independently to trigger the incompatibility reaction. This finding was not supported by experiments in this chapter that showed that *un-24*^{PA} and *het-6*^{PA} both have incompatibility function. Second, OR and PA forms at *un-24* and *het-6* are inherited as a block, a phenomenon often observed at epistatically interacting loci (Kelly, 2000). Third, genes with sequence identity to *het-6* (*het-e* and

Table 3.5. Summary of vegetative incompatibility reactions at *un-24* and *het-6* based on transformation assays (A) and heterokaryon incompatibility tests (B).

A.

Transforming DNA	Incompatibility in spheroplast recipient	
	<i>un-24</i> ^{OR} <i>het-6</i> ^{OR}	<i>un-24</i> ^{PA} <i>het-6</i> ^{PA}
<i>un-24</i> ^{OR}	none	Strong
<i>un-24</i> ^{PA}	weak	None
<i>het-6</i> ^{OR}	none	Strong
<i>het-6</i> ^{PA}	weak	None
<i>un-24</i> ^{OR} <i>het-6</i> ^{OR}	none	Strong
<i>un-24</i> ^{PA} <i>het-6</i> ^{PA}	strong	None

B.

Genotype	Incompatibility in heterokaryons			
	<i>un-24</i> ^{PA} <i>het-6</i> ^{OR}	<i>un-24</i> ^{OR} <i>het-6</i> ^{PA}	<i>un-24</i> ^{PA} <i>het-6</i> ^{PA}	<i>un-24</i> ^{OR} <i>het-6</i> ^{OR}
<i>un-24</i> ^{PA} <i>het-6</i> ^{OR}	none	unknown	weak	Weak
<i>un-24</i> ^{OR} <i>het-6</i> ^{PA}	unknown	unknown	unknown	Unknown
<i>un-24</i> ^{PA} <i>het-6</i> ^{PA}	strong	unknown	none	Strong
<i>un-24</i> ^{OR} <i>het-6</i> ^{OR}	weak	unknown	strong	None

het-d, in *P. anserina*) are involved in non-allelic interactions. In this study, we found no evidence of non-allelic interactions between *un-24*^{PA} and *het-6*^{OR}. However, non-allelic interactions causing incompatibility between *un-24*^{OR} and *het-6*^{PA} can not be excluded at this point since strains carrying this combination of alleles have not been examined. Nonetheless, a clear asymmetry is evident in both *un-24* and *het-6* incompatibility reactions. The effect of *het-6*^{PA} and *un-24*^{PA} in the same *un-24*^{OR} *het-6*^{OR} cell is additive and comparable in intensity to that of *un-24*^{OR} or *het-6*^{OR} introduced alone into *un-24*^{PA} *het-6*^{PA} cells. How is asymmetry achieved and how can it affect linkage disequilibrium at *un-24* and *het-6*?

An alternative to the non-allelic interaction hypothesis is that alleles at both loci have inherently different properties, such as different transcript levels or different peptide or mRNA half-lives. It is also possible that the PA alleles at *un-24* and *het-6* are transcriptionally co-regulated, such that one gene depends on cis-acting enhancer or other regulatory elements present in the other gene. The finding that *un-24*^{PA} and *het-6*^{PA} genes require for activity long upstream and downstream regions in comparison to their OR counterparts, suggests different transcript properties and regulation in OR and PA genetic backgrounds. Such features could influence the effective concentration and relative ratios of OR versus PA products in the cell and may explain the observed asymmetry in incompatibility reactions. If both *un-24*^{OR} and *het-6*^{OR} gene products are present in higher concentrations than their PA counterparts, one would expect a quantitatively stronger response when they are introduced into PA cells than when the opposite transformation is performed. Along these lines, it may be interesting to test whether the structure of the *het-6*^{PA} region plays a role in the incompatibility response.

For example, would the incompatibility reaction be the same when the PA alleles at *un-24* and *het-6* are introduced as two distinct clones as when the entire region encompassing the two genes is transformed into OR cells? One would predict that, if structural features associated with the *un-24*^{PA}*het-6*^{PA} region are not important, and the effects of the two genes are simply additive, a strong incompatibility reaction would be triggered by co-transformation into OR cells.

Whatever is the mechanism leading to asymmetry in the incompatibility reaction, that only two allelic combinations occur at the two *het* loci suggests that strong incompatibility reactions are favored. What are the advantages of triggering a strong rather than a weak incompatibility reaction? An explanation for the emergence and maintenance of heterokaryon incompatibility in fungi is that it prevents transmission of infectious genetic elements such as viruses. In a manner similar to the cytotoxic response in animals and the hypersensitive response in plants, cell death associated with vegetative incompatibility may limit the spread of infectious elements. In plants, the hypersensitive response culminates in death of plant cells infected with pathogens. Suppression of the cell death response results in increased susceptibility to the pathogen (Morel and Dangl, 1999). In *N. crassa*, differences at *het* genes decrease the frequency of transfer of senescence plasmids between incompatible strains (Debets *et al.*, 1994). Genes that trigger incompatibility reactions, such as *het-c*, effectively prevented the transfer of the Kalilo plasmid in most confrontations. However, differences at *het-i* which does not trigger cell death but rather homokaryotization through loss of one of the nuclear types, was unsuccessful in blocking the transfer of Kalilo plasmid (Debets *et al.*, 1994). The strength of the “vegetative incompatibility barrier” appears, therefore, to influence the

degree of protection against infectious particles. In *C. parasitica*, transmission of hypovirulence associated with a double-stranded RNA virus is significantly decreased between vegetatively incompatible isolates (Cortesi *et al.*, 2001). Significantly, there is a strong negative correlation between the intensity and/or speed of the incompatibility reaction and virus transmission in *C. parasitica* (Biella *et al.*, 2002). In a pair of interacting, vegetatively incompatible isolates, a strain that undergoes cell death more readily has a decreased probability of being infected by a virus than a strain where cell death is delayed. In our study, a stronger incompatibility reaction was defined as a reaction leading to fewer partially diploid transformants or to slower growing heterokaryons. In nature, fusions between strains with multiple differences at *het* loci, or with single differences at “strong” *het* genes would presumably undergo rapid cell death. As a consequence, there would be a lower infection frequency during fusions between such strains than fusions between strains with a single difference in a “weak” *het* gene. In the case of *un-24* and *het-6* we predict that transmission rates of infectious elements would be significantly higher between strains which are different at *un-24* than between strains with differences at both *un-24* and *het-6*.

Based on this prediction, we can imagine a population of individuals with haplotypes *un-24*^{OR}*het-6*^{OR} and *un-24*^{PA}*het-6*^{PA} in which a certain percentage of individuals carry an infectious genetic element. Virus transfer can only occur between individuals with the same genotype at the two *het* loci, because interactions between individuals with different genotypes at *het* loci lead to cell death (Figure 3.16). However, if the recombinant forms *un-24*^{PA}*het-6*^{OR} and *un-24*^{OR}*het-6*^{PA} are introduced into the population they are predicted to act as intermediaries, allowing the movement of

Figure 3.16. Model of infectious element transmission between different *un-24 het-6* haplotypes. Haplotypes displayed in the black boxes are found in nature, whereas those displayed in the red box are not found. The model assumes that infectious elements can be transferred between strains during vegetative fusions only when an incompatibility response is absent or delayed. Based on this model, the transfer of an infectious element is unidirectional from *un-24^{PA}het-6^{PA}* to *un-24^{OR}het-6^{OR}* and can only occur via hybrid haplotypes *un-24^{PA}het-6^{OR}* and *un-24^{OR}het-6^{PA}*.

infectious elements from $un-24^{PA}het-6^{PA}$ to $un-24^{PA}het-6^{OR}$ to $un-24^{OR}het-6^{OR}$ cells, for example. The end result may be a significantly higher rate of infection of the population in general. Individuals carrying genotype $un-24^{OR}het-6^{OR}$ would become the most susceptible, since strong incompatibility reactions could only be elicited in the presence of the $un-24^{PA}het-6^{PA}$ combination. Any other genetic combination would only elicit a weaker response and would allow passage of infectious particles. By elimination of two haplotypes, either $un-24^{OR}het-6^{OR}$ and $un-24^{PA}het-6^{PA}$ or $un-24^{OR}het-6^{PA}$ and $un-24^{PA}het-6^{OR}$, the movement of infectious elements may be most effectively impeded. Under this model, there would be a strong selective pressure to maintain an inversion in the $un-24/het-6$ region (Chapter 2) because it prevents the generation of recombinant $un-24^{PA}het-6^{OR}$ and $un-24^{OR}het-6^{PA}$ forms. The occurrence of the inversion possibly provided a structural barrier to the generation of intermediary hybrid haplotypes by blocking recombination between $un-24$ and $het-6$.

The interactions between $un-24$ and $het-6$, whether direct, by protein-protein interactions, or indirect by triggering two discrete but additive incompatibility reactions contribute to a general cell death pathway. This cell death response represents a form of nonself recognition.

In this study, evidence in favor of the adaptive hypothesis of het gene evolution, comes from the analysis of incompatibility reactions triggered by PA alleles at $un-24$ and $het-6$. Strong incompatibility reactions, leading to rapid cell death, may have been selected for in the population to reduce the spread of infectious elements. Selection for strong incompatibility reactions may have led to a selective sweep of the inversion

polymorphisms and ultimately explain why *un-24* and *het-6* are in severe linkage disequilibrium in *N. crassa*.

SUMMARY AND CONCLUSIONS

*What we call the beginning is often the end and to make an end is to make a beginning.
The end is where we start from*

T. S. Eliot. Four Quartets "Little Gidding"

This thesis investigated the phenomena of nonself recognition in the filamentous fungus *N. crassa*. The study was approached from two different fronts, a macroscopic analysis of interactions between genetically different mycelia, and a genetic analysis of *un-24* and *het-6*, two genes involved in nonself recognition in *N. crassa*.

The macroscopic analysis summarized in Chapter 1 outlines the discovery of apparently novel phenotypes associated with vegetative incompatibility in *N. crassa*. Barrages in *N. crassa* resemble in appearance barrages in other fungal species. The study revealed that the genetic control of barrage formation in *N. crassa* is complex and apparently distinct from heterokaryon incompatibility. This point has important implications for the understanding of nonself recognition in filamentous fungi. Based on early experiments with *P. anserina*, barrage formation was strongly linked to genetic controls of heterokaryon incompatibility. Thus, genes that control the co-existence of genetically different nuclei in the same cytoplasm were also assumed to be involved in the formation of barrages between genetically different mycelia. This study reveals that, at least in some fungi, the control of vegetative incompatibility may be more complex than previously anticipated and distinct from that of heterokaryon incompatibility. For example, barrages or zone lines may be the result of diffusible substances secreted into the substrate or cellular reactions that occur at the cell wall interface (Leslie, 1993). Although the genetic basis of barrage formation in filamentous fungi is still poorly understood, my study opens up a field of investigation into nonself recognition mechanisms that is not *het* gene-based. A chemical analysis of barrage zones may identify compounds that trigger, or are associated with, fungal cell lysis or cell death and

that may have interesting therapeutic applications. Findings obtained in Chapter 1 also open up new questions on the evolution of nonself recognition in fungi. The results suggest that barrages may have a different underlying genetic basis in different fungal species. This implies that nonself recognition in fungi has multiple, independent evolutionary origins. Based on Chapter 1, two distinct forms of vegetative nonself recognition exist; vegetative incompatibility (barrage formation) and heterokaryon incompatibility. What is the reason for their co-existence? It is possible that they operate at different stages of the life cycle or under different growth conditions. In *N. crassa*, like in *P. anserina*, barrage formation is apparent under nutrient limiting growth conditions, whereas heterokaryon incompatibility is detectable under both high and low nutrient growth conditions. Barrage formation could constitute a method of competition for or protection of food substrates. In this context, nonself recognition, resulting in the limitation of genetic exchange, may be a secondary function of vegetative incompatibility. In contrast, the main function of heterokaryon incompatibility appears to be the preservation of genetic individuality.

This main function of *het* genes is reinforced by findings reported in Chapters 2 and 3. In both chapters I investigate the basis for linkage disequilibrium at *het* genes *un-24* and *het-6* and present evidence for positive selection at *un-24* and *het-6*. Chapter 2 reports on structural features responsible for linkage disequilibrium. Both the current structure of the *un-24/het-6* region and the evolutionary history of the region play important roles in maintenance of linkage disequilibrium. PA and OR alleles at both *un-24* and *het-6* are associated with polymorphisms at a newly discovered paracentric inversion *In(het-6)*. Recombination events between inversion forms are drastically

decreased and result in linkage disequilibrium at *un-24* and *het-6*. The inversion polymorphisms appear to have been maintained in *N. crassa* through balancing selection at *un-24* and *het-6*. Allelic specificities at the two *het* loci were shown to be conserved in two related species, *N. intermedia* (this study) and *N. tetrasperma* (Powell, 2002), suggesting that nonself recognition function is an ancient feature of *un-24* and *het-6*. Although the inversion polymorphisms were not detected in species other than *N. crassa*, this discovery opens multiple avenues of research and investigation. The preliminary investigation of rates of recombination within the inverted region indicates that recombination can occur. With the availability of numerous wild collected strains of *N. crassa* further studies of the region could be possible. Information on the rate of evolution of the DNA sequence in the region, the rate and nature of recombination events and the limiting factors affecting recombination in the region could be studied. At the genetic locus scale, the effect of balancing selection on amino acid variability at *un-24* and *het-6* could provide information about mechanisms of nonself recognition. For example, the identification of hypervariable residues at *un-24* and *het-6* in a large population survey of *N. crassa* may pinpoint specific regions involved in nonself recognition in contrast to more conserved residues responsible for triggering cell death. At a larger scale, and expanded study of the occurrence and prevalence of inversion polymorphisms at *In(het-6)* and their association with allelic specificities at *un-24* and *het-6* in other fungal species could provide a detailed history of the evolution of the inverted region. This would provide information on whether the inversion predates or follows the emergence of nonself recognition function at *un-24* and *het-6*. Can sequence divergence at inversion breakpoints be responsible for the acquisition of nonself

recognition function? Furthermore, the association of the inversion with an apparently dispersed repetitive sequence with transposon-like features, and the observation that numerous *het-6* similar sequences exist in the *N. crassa* genome may not be coincidental. So far, fourteen copies of this novel repetitive sequence and over twenty sequences with similarity to *het-6* have been identified. It is possible that a transposon is associated with *het-6*. To test this hypothesis, a bioinformatics approach could be used to survey the position of the repetitive sequence with respect to *het-6* homologues in the *N. crassa* genome. If there is a correlation between the presence of the transposon-like sequence and the presence of *het-6* homologues, this would strongly suggest that *het-6* gene duplication is through a transposition mechanism. If this correlation is not detected, other mechanisms in addition to gene duplication (e.g. convergent evolution) could be entertained.

It appears desirable, as a result of this study, that structural and functional investigations at *un-24* and *het-6* be undertaken in parallel. Indeed, Chapters 2 and 3 have provided information on the ways in which genetic structure at *un-24* and *het-6* may affect biological function and biological function may in turn shape genetic structure in *N. crassa*. A dual approach may expedite the study of the region. For example, in Chapter 3 we provide preliminary evidence that the *un-24/het-6/In(het-6)* complex may act as a supergene. This chapter provides a possible functional basis for selection of linkage disequilibrium at *un-24* and *het-6*. The isolation and characterization of incompatibility activity associated with PA alleles at *un-24* and *het-6* indicates that these variants trigger weaker incompatibility reactions when transformed into OR cells compared to when the OR counterparts are transformed into PA cells. The ability to

trigger a strong incompatibility reaction, leading to rapid cell death may have been selected for, as a means of protecting the cell from invasion by parasitic genetic elements. In the case of PA alleles at *un-24* and *het-6*, this selection may have resulted in the co-inheritance of the two genes through selection for *In(het-6)*.

These studies further support the previously proposed theory that rapid cell death may be a universal form of defense against parasitic invaders. Thus, fungi join the ranks of plants (hypersensitive response, Morel and Dangl, 1999) and animals (apoptosis induced by natural killer T lymphocytes, Scalzo, 2002) in their cell death-based approach to defense. The similarity in the approaches that fungi, plants and animals have evolved for defense purposes further reinforces the theory that nonself recognition (regulated by *het* genes in the case of *N. crassa*) has not evolved “by mistake” but has an important biological function: the preservation of self.

Although the studies presented in Chapter 3 provide a glimpse at the complexity of the mechanisms of nonself recognition, numerous questions remain to be addressed by future research. How are self and nonself recognized at the molecular level? In the case of *un-24* and *het-6* is heterocomplex formation necessary for this recognition step? At *het-c* in *N. crassa*, heterocomplex formation is necessary and sufficient to trigger the incompatibility response (Sarkar *et al.*, 2002). Are other factors involved in nonself recognition associated with *un-24* and *het-6*? Co-immunoprecipitation studies for example could identify numerous proteins or other factors that may be associated with *un-24* and *het-6* and may play a role in nonself recognition. How is cell death triggered during the incompatibility reaction? Factors interacting with *un-24* and *het-6* may provide some clues. How do PA alleles at *un-24* and *het-6* trigger a strong incompatibility

reaction? The strong incompatibility reaction could be due to the additive effects of *un-24* and *het-6* incompatibility as has been described for other incompatibility systems in *N. crassa* and *A. nidulans*. However, the genetic background of the incompatible interaction may also prove to be very important in determining the intensity of the reaction. The study of escape strains with novel genetic combinations at *un-24* and *het-6* would be a useful tool in investigating this question. However, the use of transformants that may carry a mosaic of nuclear types makes the study complex. A more desirable system would be one in which all the nuclei have the same genetic constitution. To isolate such a strain, a cross between a *col-PA* escape strain and an *un-24* strain of OR background could be used to isolate OR background progeny that grow at 37°C. Such progeny would carry the *un-24*^{PA} *un-24*^{OR} (inactive) *het-6*^{OR} genotype. This strain could then be used to further test the *un-24*^{PA} *het-6*^{OR} genetic combination in incompatibility, fertility or other assays. In addition, the reciprocal genetic combination (*un-24*^{OR} *het-6*^{PA}) may be recovered and studied in a similar manner. In preliminary transformation assays with the *het-6*^{PA} construct into OR spheroplasts, several colonies were noted to grow on the plate after 3 to 5 days. If these colonies are the result of escape from *het-6* self-incompatibility, some of them may carry the *un-24*^{OR} *het-6*^{PA} combination. The study of such strains could contribute to the investigation of putative epistatic effects at *un-24* and *het-6*.

Thus, novel tools, some more effective than others, have been developed to study nonself recognition not only in *N. crassa* but in filamentous fungi as a whole. In addition, numerous questions (more numerous than those answered) were generated that should fuel future research in the field. In concluding this study, I can't help but be

amazed and humbled by the complexity and perfection of life as I have seen it through the study of the fungus *N. crassa*. The brief journey spent studying this organism has provided a significant amount of novel information about a variety of biological subjects ranging from developmental biology, ecology, evolution, genetics and physiology. I am confident in the belief that the amount of scientific knowledge one can gain from *Neurospora* is infinite and I am honored and thankful to have been working on one of the great model organisms of our times.

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APPENDIX 1.

DNA sequence of *brk-1* from *N. intermedia* strain FGSC 962. *brk-1* was isolated with primer pair Gene7-P1/6J-P11, cloned into vector TOPO-TA (Invitrogen) and partially sequenced. The dotted line represents unknown sequence (not sequenced) in the center of the cloned fragment. As a result of low template quality or sequencing errors, the exact identity of all nucleotides in the sequence could not be determined. Letters that do not correspond to any of the four nucleotides symbolize possible combinations of nucleotides at that particular site as follows:

K: G or A	Y: T or C
W: A or T	B: G, T or C
M: A or C	H: A, T or C.

GCCCTTGCTTGTCGACTTTGACTGCTGCGAGAACATGGCGATGCCAACCGAC
 GACACCGACGAAATGAATCTTCTCACTATCGCGGCAAGGGCTTTTGGCGGA
 ATGATCCGTACTACCCAGACCTCCAGCCAACCACACTCCCGACTCAWACCT
 CGCAGACGGACAGCTCTGGGAACTTTCAAGAAGCGATATCTCACGACGAGC
 GGGGAAATTCTGGAGAGAAAAGCGAAAGATGAAAGAGTGAAGATGTTACCA
 GGGAAGGTGATGAGTATGATTGAKAATACGAGAGAGAGGTGGAGTAAGAGT
 GTTCTGTAAGGGGAGAGAGAAAGAGAGGGAGGGAGAGAGAGAGAGAGAGA
 GAGAGTTCAGGACAGTCAGGACACAAGCCTCTATATTCCCACATGAYAGTGG
 CGGGGGCGTGCGTACCACCACCAAGCCACAACACACAAGGCAAGGGCACAC
 ACAGGGTGTGCATACAGTATCACTTTTBTWTTTTCTCAGCAGTTATCTGTMT
 TGHTATCGTTTTTCGATGGTGCGTCATCAAACCAAGGAGACCCACGAAGAGA
 C.....ACTTTGAWCTTTTGGTAGATTAGWHMAGACAGAGCTTCCGAA

CCACTATCCCACCATTCAGGTCTCCTTCCAATACATACWTWTYCGAKCG
AGTGTCTGAGGTGTATGAKGGGAGGCTATCGGWWTCCTGAGCGTTCGACT
GATCCTTCTACACAWCCGCTGGCCAAAWGTGCAATAKCAGGTACATCTTA
GCAGGGTAGGTAAACATACTCCTTGCACGCATTGGGCTCTATGCCACCCCAG
GCCTTTYCATCGTACCTAGTCAGAGGACAATCACTTATTTYGATTCATTCACA
ACGAAGWTATGCGCCTGGCAAAGCTAACATAGAHTGAYCGATYATGAGGTA
GGCAGT

APPENDIX 2

Summary of spheroplast transformation assays with p λ B and p λ B subclones. DNAs corresponding to each construct were introduced into C9-2 (OR) and C2(2)-1 (PA) spheroplasts as described in the Materials and Methods section and transformants were allowed to regenerate at 30°C for 3 to 5 days. The number of regenerating colonies and their phenotype (cloud or star) were recorded. Average and standard deviation (SD) values were calculated for each construct.

C9-2	1	2	3	4	5	6	7	8	9	10	11	12	Average cloud	SD cloud	Average star	SD star
Trial #																
pCB1004	131	167	65	21	90	185	46	155	73	204	204	204	128.55	67.04	-	-
pCOR-1	-	219	185	49	-	-	-	-	-	-	-	-	151.00	89.96	-	-
p λ B	23	54	0	11	-	-	-	-	-	-	-	-	21.67	23.31	-	-
p λ BC/P	-	-	13(7)	25(81)	-	13	-	-	-	-	-	-	46.33	51.79	29.33	44.88
p λ BC/N	-	-	24	35	-	-	11	-	-	-	-	-	23.33	12.01	-	-
p λ BB/H	-	-	-	32	-	-	-	43(23)	14(7)	-	-	-	39.67	23.46	10.00	11.79
p λ BB/B	-	-	-	16	-	-	-	21(11)	1(1)	-	-	-	16.33	14.50	4.00	6.08
p λ BX/C	-	-	-	-	19(87)	90(142)	11(87)	-	-	-	-	-	145.33	75.16	105.33	31.75
p λ BN/P	-	-	-	-	-	187	-	109	15	-	-	-	103.67	86.12	-	-
p λ BB/A	-	-	-	-	-	-	5	-	-	18	7	11	10.25	5.74	-	-
6V-PA3/6V-PA4	140	-	-	-	-	-	-	-	-	-	-	-	140	-	-	-

APPENDIX 3

Growth characteristics of C9-2 *un-24*^{PA} transformants before and after escape from *un-24* associated self-incompatibility. Growth rate was measured on Vogel's solid (1.5% agar) medium at 30°C. Hygromycin B resistance was scored as ability (+) or inability (-) to grow on Vogel's solid (1.5% agar) medium containing 12.6 µg/ml hygromycin B (Roche) after 24 hours of incubation at 30°C. Strains that were able to grow at a low rate on hygromycin B medium were scored as +/-.

Isolate	Growth rate before escape (mm/day)	Growth rate after escape (mm/day)	Time of escape (days)	Growth on hygromycin B
14	1.2	40	3	-
16	1.3	25	3	-
23	1.5	30	4	-
31	1.5	30	4	-
44	2.5	40	4	-
27	2.8	44	5	-
29	3	36	5	-
20	2.4	70	6	-
39	2.5	30	7	-
17	3	48	7	-
25	2.3	65	7	-
26	2.6	38	7	-
47	2.8	30	7	-
22	2.5	70	8	-
33	2.8	68	8	-
24	3.1	65	8	-
34	1.3	42	8	-
41	2.75	55	8	-
48	2.3	4.5	9	-
11	4.4	48	10	-

[Appendix 3 continued]

9	7	30	3	+
21	1.6	48	5	+
37	2.8	50	7	+
10	2.5	45	7	+
45	1.5	53	8	+
42	2.5	50	8	+
40	2	50	5	+/-
18	1.5	58	8	+/-
36	4.4	55	8	+/-