Developing a catalytically functional ribonucleotide reductase for
Neurospora crassa that lacks heterokaryon incompatibility activity

by

Ryan Reshke

A thesis submitted to the Faculty of Graduate and Postdoctoral
Affairs in partial fulfillment of the requirements
for the degree of

Master of Science

in

Biology

Carleton University
Ottawa, Ontario

© 2013
Ryan Reshke

Abstract

We are exploring the structure-function relationship of proteins that govern nonself recognition in the filamentous fungus *Neurospora crassa*. Heterokaryon incompatibility (HI) is a form of nonself recognition in fungi that prevents the proliferation of heterokaryons – cells that contain genetically dissimilar nuclei. In *N. crassa* HI is governed by *het* loci, one of which, *het-6*, comprises two tightly linked incompatibility genes called *un-24* and *het-6* that function together as a supergene complex. Each gene exists as one of two allelic variants, either Oak Ridge (OR) or Panama (PA), and only two possible haplotypes occur in nature: *un-24*^{OR}-*het-6*^{OR} or *un-24*^{PA}-*het-6*^{PA}. The *het-6* gene encodes a HET-domain protein with no known function outside of incompatibility whereas *un-24* encodes incompatibility function and the large subunit of ribonucleotide reductase (RNR). RNR is an enzyme that is essential for the conversion of ribonucleotides to deoxyribonucleotides, and thus is crucial for DNA synthesis and repair. The OR and PA allelic forms of UN-24 are nearly identical but differ significantly in the final ~60 residues of their C-termini, suggesting that incompatibility activity and specificity are encoded within this variable region. In this study we identify a single glutamic acid residue within the C-terminus of UN-24^{OR} that governs incompatibility specificity. In addition, by changing this glutamic acid to leucine we produced an enzymatically functional RNR in *N. crassa* that lacks incompatibility activity (UN-24⁰). Prior to this, any perturbation of *un-24* resulted in a loss of catalytic activity and cell death, creating a barrier to the study of possible *het-6* allelic interactions. Using this *un-24*⁰ strain, we provide insight into the role that HET-6 proteins play in nonself recognition.

Acknowledgements

First I would like to thank Dr. Myron Smith for welcoming me into his lab three years ago, for keeping me on to complete a Master's degree, and for continuing to provide endless guidance through it all. I would also like to thank the members of my advisory committee, Dr. John Vierula and Dr. Keith Seifert, for imparting some of their wisdom on me and helping me stay on track over the last two years. Next, I'd like to thank Denis Lafontaine for showing me the ropes in the lab and the rest of the Smith lab for all of their help and wonderful company. None of this would have been possible without my friends and family, so I must thank my parents for their support and encouragement. Lastly I would like to thank my girlfriend, Allison, for the hours she has spent involuntarily learning about *Neurospora* and for her endless support throughout this experience.

Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Tables	v
List of Figures	vi
List of Abbreviations	vii
Nutritional supplement abbreviations.....	vii
Introduction	1
Diversity of Nonself Recognition Systems	1
Nonself recognition in filamentous ascomycete fungi	4
Genetic basis of nonself recognition in fungi.....	8
Heterokaryon Incompatibility at the <i>het-6</i> locus in <i>Neurospora crassa</i>	11
Ribonucleotide Reductase	15
RNR associated incompatibility in <i>N. crassa</i>	19
The purpose of this study	20
Materials and Methods.....	22
Strains and culture conditions	22
DNA isolation, PCR amplification and TA cloning	22
Transformation of <i>N. crassa</i>	23
Development of <i>un-24</i> mutants.....	24
Crosses of <i>N. crassa</i>	29

Heterokaryon Incompatibility Tests.....	29
Results.....	33
Transformation of <i>Cryphonectria parasitica rnr1</i> into <i>N. crassa</i>	33
Production of mutant <i>un-24^{OR}</i> constructs	38
Determination of mutant construct incompatibility activity.....	42
<i>un-24⁰</i> , a catalytically active allele without incompatibility activity.....	44
Crosses and heterokaryon incompatibility tests	51
Structure/function analysis.....	53
Discussion.....	59
Appendix I	75
Alignment of <i>Neurospora crassa un-24^{OR}</i> , <i>un-24^{PA}</i> , and <i>Cryphonectria parasitica rnr1</i>	75
Literature Cited	79

List of Tables

Table 1. Strains used in this study.....	30
Table 2. Primers used in this study.	31
Table 3. Cloning vectors used in this study.....	32
Table 4. The ratio of wildtype-like colonies over total transformants formed by the transformation of the mutant <i>un-24^{OR}</i> constructs into C9-2 and C2(2)-1.	43
Table 5. Ratio of wildtype-like colonies over total transformants following C9-2 and C2(2)-1 transformation with the <i>un-24^{OR(E>L)}</i> mutant construct.	47

List of Figures

Figure 1. Venn diagram showing the relationship among nonself recognition processes in filamentous ascomycetes.....	7
Figure 2. . Organization and known interactions of incompatibility genes at the <i>het-6</i> locus	112
Figure 3. Representations of the protein products of <i>un-24</i> ^{OR} and <i>un-24</i> ^{PA}	14
Figure 4. The reaction mechanism of substrate reduction in the active site of RNR	18
Figure 5. Schematic representations of methods used to build mutant constructs of <i>un-24</i> ^{OR}	25
Figure 6. Characteristics of self-incompatible <i>un-24</i> ^{OR} <i>het-6</i> ^{OR} :: <i>un-24</i> ^{PA} transformants.	35
Figure 7. Graph depicting the indistinguishable growth rates of DLL7-23:: <i>rnr1</i> and DLL7-23:: <i>un-24</i> ^{PA} transformants following subculture from transformation plates onto VMM containing tryptophan.....	36
Figure 8. A clustalW alignment of the C-terminal residues of <i>N. crassa</i> UN-24 ^{OR} and UN-24 ^{PA} and <i>C. parasitica</i> RNR1 showing each of their incompatibility activities in <i>N. crass</i>	39
Figure 9. The four mutant <i>un-24</i> ^{OR} constructs created in this study	40
Figure 10. Obtaining the required fragments for production of <i>un-24</i> ^{OR(A)}	41
Figure 11. The incompatibility activity of four mutant <i>un-24</i> ^{OR} constructs created in this study	45
Figure 12. ClustalW alignment of the C-terminal residues of <i>N. crassa</i> UN-24 ^{OR} , UN-24 ^{PA} and UN-24 ^{OR(B)} with the large subunit of RNR from six other species	46
Figure 13. Diagnostic PCR of <i>un-24</i> ^{ts} gene replacement	49
Figure 14. Schematic representation of the recombination event required to select a full <i>het-6</i> -null strain (<i>un-24</i> ^{OR(E>L)} Δ <i>het-6</i> :: <i>hph</i>) from the cross RR1-3 X DLL8-16.....	52
Figure 15. Heterokaryon incompatibility tests verify that RR2-3 has no incompatibility activity associated with the <i>het-6</i> locus	54
Figure 16. Summary of the incompatibility activity UN-24 ^{OR} , UN-24 ^{PA} , UN-24 ^{OR(B)} and UN-24 ⁰	55
Figure 17. Structure-function analysis of UN-24 C-terminus.	57

Figure 18. A schematic representation of the <i>het-6</i> locus showing the test for allelic incompatibility interaction between <i>het-6</i> ^{OR} and <i>het-6</i> ^{PA}	63
Figure 19. The three cysteines conserved in UN-24 and most other RNR large subunits.....	66
Figure 20. A model of allelic RNR-associated incompatibility in <i>N. crassa</i>	68
Figure 21. Possible models of non-allelic incompatibility observed between <i>un-24</i> ^{PA} and <i>het-6</i> ^{OR}	72

List of Abbreviations

<i>het</i>	Heterokaryon incompatibility gene
HI	Heterokaryon incompatibility
HNWD	A group of incompatibility proteins sharing three conserved domains
<i>hph</i>	Hygromycin phosphotransferase
LG	Linkage group
<i>mat</i>	Mating type
MHC	Major histocompatibility complex
PCD	Programmed cell death
PCR	Polymerase chain reaction
RNR	Ribonucleotide reductase
<i>rnr1</i>	Encodes the large subunit of RNR in <i>C. parasitica</i>
ROS	Reactive oxygen species
SCM	Synthetic crossing medium
SI	Self-incompatible
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling
<i>un-24</i>	Encodes the large subunit of RNR in <i>N. crassa</i>
<i>vic</i>	Vegetative incompatibility gene
VMM	Vogel's minimal medium

Nutritional supplement abbreviations

Glu (E)	Glutamic acid
inl	Inositol
Leu (L)	Leucine
Lys (K)	Lysine
Thr (T)	Threonine
Trp (W)	Tryptophan
ura	Uracil
Val (V)	Valine

Introduction

Diversity of Nonself Recognition Systems

This thesis explores a gene involved in nonself recognition in the filamentous ascomycete fungus, *Neurospora crassa*. The results presented add to our understanding of a fundamental biological process: the ability to discriminate self from nonself. This process appears to have evolved independently in diverse organisms, and fulfills many functions, including the reduction of transmission of infectious agents, restriction of fusions between genetically dissimilar organisms, interaction of sexually reproductive partners and identification of other members of a common genetic background. The following sections briefly summarize some of the diversity of nonself recognition systems, drawing examples from bacteria, cellular slime moulds, colonial ascidians, and higher eukaryotes. Following this, I will provide a brief review of fungal systems, which arguably provide the most in depth picture of the molecular bases of nonself recognition, as they are largely characterized at both the genetic and biochemical level.

Bacteria employ a restriction/modification system that creates a barrier against unregulated uptake of foreign DNA during transformation, transduction and conjugation. This system utilizes endonucleases that cleave foreign DNA based on sequence and patterns of methylation and is essential for maintaining the genetic identity of different species (Jeltsch, 2003). Nonself recognition is also conveniently observed in the bacterium *Proteus mirabilis* that is able to move across solid surfaces through a coordinated translocation of cells called swarming. Boundaries form between colonies of different strains, but not between colonies of the same strain (Gibbs *et al*, 2008). This nonself recognition system was mapped to a six-gene locus termed *ids* (identification of self) and a second, unlinked, five-gene locus termed *idr* (identity recognition) that encode specific molecular identifiers that

are all exported from the cell by the same putative type VI secretion system encoded at the 17-gene cluster *tss* (Gibbs *et al*, 2008; Wenren *et al*, 2013).

Another example of a well-studied nonself recognition system is found in the Dictyostelids. The Dictyostelids are a group of cellular slime moulds, or social amoebae that undergo a unique developmental process that requires multiple forms of nonself recognition. These single celled organisms feed by phagocytosis, primarily engulfing bacteria and other microbes in the soil during their growth phase. When starvation conditions arise, the amoebae begin to aggregate, forming a mound of cells. At this point, cells begin to differentiate into either stalk cells or spore forming cells. Next, the mound of cells forms a slug that is able to migrate over the soil to a location where it forms a fruiting body comprised of a stalk bearing a ball of spores at its top (Shaulsky and Kessin, 2007). It is hypothesized that cell surface molecules are present on all types of slime moulds that inhibit individuals from ingesting other members of the same strain. In the case of *Dictyostelium caveatum*, however, cannibalistic strains have arisen through mutation that do not recognize these molecules and will eat neighbouring amoebae to avoid entering the starvation-induced aggregation phase (Waddell and Duffy, 1986). During sexual development, cells of opposite mating type fuse to form a giant cell, or zygote. It has been shown that bisexual strains of *Dictyostelium discoideum* are self-incompatible in cell fusion, but can fuse with a heterothallic strain. This process is hypothesized to also be governed by cell surface molecules and receptors (Higuchi *et al*, 1995). These giant zygote cells also abandon nonself recognition systems that prohibit them from ingesting members of the same strain, and they are able to phagocytise all nearby amoebae (Shaulsky and Kessin, 2007). Self-recognition in these amoebae is mediated by a pair of polymorphic alleles, *tgrB1* and *tgrC1*, that encode transmembrane proteins. A matching pair of *tgrB1*-*tgrC1* alleles is necessary and sufficient to facilitate self-recognition. The encoded proteins mediate recognition by direct interactions between extracellular protein domains of TgrB1 and TgrC1 on the surface of adjacent cells (Hirose *et al*, 2011).

Colonial ascidians also have a well-studied nonself recognition system that allows them to form chimeras in nature. Colonial ascidians are sessile, sac-like marine invertebrates that disperse as individual cells (zooids) that subsequently aggregate to form large colonies. These organisms form natural chimeras in the wild with other members of the same species. These fusions can only occur if both individuals share at least one codominantly expressed allele at the polymorphic fusibility/histocompatibility (Fu/HC) locus (Rinkevich and Weissman, 1992). Contacts between two colonies that have different alleles at this locus result in necrosis at the contact area (Watanabe and Taneda, 1982). Following fusion events between incompatible colonies, blastozooids of one of the partners can become resorbed, or the two pieces can separate. These processes of resorption and separation are also mediated by the Fu/MC locus and provide evidence for a second level of nonself recognition that acts after fusion (Saito *et al*, 1994). Microarray analysis of these incompatible fusions has shown that the partner that displays the morphological markers of rejection (the 'rejected' partner) also shows transcriptional inhibition of protein biosynthesis, cell structure and stress response genes, while the 'rejecting' partner does not. These results suggest that the 'rejected' colony activates a self-destructive mechanism to separate from the incompatible fusion (Oren *et al*, 2010).

In higher eukaryotes, nonself recognition is essential for detecting invading pathogens, ensuring species-specificity during fertilization and modulating cell and tissue fusion (Khalturin and Bosch, 2006). Multiple forms of recognition systems have evolved separately in plants to prevent self-fertilization. One such system is found in the flowering plant family, Brassicaceae, which have a set of closely linked genes at the 'S' locus. These genes have multiple alleles and can be expressed in the stigma and the pollen. When the expressed S specificities match, pollen tube growth is halted thus preventing self-fertilization (Sato *et al*, 2002). Additionally, plants protect themselves from pathogens through the use of receptor-like kinases as pattern recognition receptors that are able to detect specific molecular patterns on the surfaces of invading pathogens following pathogen invasion (Sanabria *et al*, 2010). Similarly, vertebrates

utilize a form of nonself recognition to identify microbes as well as cells that do not express sufficient levels of major histocompatibility complex (MHC) class I molecules, called missing or altered self (Reviews by: Ljunggren and Karre, 1990; Raulet, 2006). The MHC is a large vertebrate gene family that produces highly polymorphic MHC proteins (Kumanovics *et al*, 2003). Pathogen-associated molecular patterns are recognized and bound by these antigen-presenting molecules that ultimately results in MHC:peptide presentation to activate immune cells (Medzhitov and Janeway, 2002). It has been suggested that the vertebrate MHC has evolved directly from the more primitive Fu/HC locus found in the colonial ascidians (Saito *et al*, 1994).

Nonself recognition in filamentous ascomycete fungi

Among the fungi, nonself recognition in filamentous ascomycetes is controlled separately during the sexual and vegetative portions of the life cycle. During the sexual phase of the life cycle, when sexually compatible strains come into contact, cell fusion is followed by nuclear fusion to produce diploid nuclei. These diploids then undergo meiosis leading to the production of ascospores that may germinate to produce an interconnected network of hyphae called a mycelium. All known heterothallic ascomycetes have two mating types that determine mating compatibility (Kronstad and Staben, 1997). In *N. crassa*, for example, nonself recognition is controlled during the sexual cycle by the mating type (*mat*) locus that is comprised of two idiomorphs, *A* and *a*, located on the left arm of linkage group (LG) I (Griffiths and DeLange, 1977). Idiomorphs are genes that occupy the same genetic location (locus) in different strains, but are of non-homologous DNA sequence. The different mating-type genes are also embedded in chromosomal regions of high sequence dissimilarity that results in a lack of meiotic recombination within the locus (Metzenberg and Glass, 1990). Two *N. crassa* individuals are only capable of mating if they are of opposite mating type (*A* vs *a*). Pheromone production is transcriptionally controlled by the mating-type locus, and each mating type produces pheromones that are recognized by

strains of the opposite mating type. These pheromones are small, diffusible, hydrophobic factors produced by hyphae, macroconidia and microconidia (Staben, 1996). Similarly, in *Podospora anserina* mating type is controlled by the idiomorphs *mat+* and *mat-*, which work in a fashion similar to those found in *N. crassa*.

The need for nonself recognition is also apparent in filamentous fungi during vegetative growth. In this phase of the life cycle, filamentous fungi grow as multinucleated hyphal filaments by tip extension and branching and undergo frequent hyphal fusions, creating a mycelial network (Glass and Kaneko, 2003). Vegetative incompatibility between conspecific mycelia can be manifested in at least two ways: heterokaryon incompatibility and mycelial incompatibility (Smith and Lafontaine, 2012). Heterokaryon incompatibility can occur after fusion events between different individuals, leading to the formation of cells that contain genetically dissimilar nuclei in a common cytoplasm, called heterokaryons (Reviewed by: Saupe, 2000; Glass and Kaneko, 2003). Normal growth by heterokaryons requires that the different nuclei carry compatible alleles at all heterokaryon incompatibility (*het*) loci. Two nuclei that differ at any one of several *het* loci in the genome will result in aberrant growth or no growth by the heterokaryon and such strains are termed heterokaryon incompatible (HI). Heterokaryon incompatibility tests are used to identify vegetative incompatibility. These tests consist of forcing two strains, containing complementary auxotrophic markers, into forming a heterokaryon by co-innoculating them on a medium lacking the supplements required for the growth of either strain independently. Co-innoculated strains that are identical at all *het* loci will form a heterokaryon that resembles a wildtype strain for growth rate and morphology. Strains that differ at one or more *het* loci will exhibit growth that is partially or completely inhibited (Smith *et al*, 1996).

Mycelial incompatibility is most commonly studied by examining for barrage formation or by microscopic examination of fusion cells in the zone where two different strain make contact. A good

example of barrage formation can be observed in the chestnut blight fungus *Cryphonectria parasitica*, where mycelial interactions between strains differing at any vegetative incompatibility (*vic*) locus (akin to *het* loci in other species) display zone lines between strains. These zone lines stain green with the pH indicator bromocresol green, as a result of cell death and lysis after hyphal fusion (Smith *et al*, 2006). Under the microscope, heterokaryon incompatibility can be visualized with vital stains such as Evans Blue, that cannot be pumped out of the dying cells, which then appear red using fluorescent microscopy (Belov and Smith, unpublished). Additionally, following fusion of incompatible strains, there is a progression of stages that can be observed by light microscopy that is characteristic of programmed cell death (PCD), starting with cytoplasmic granulation, then vacuolization and finally cytoplasmic shrinkage and plasmolysis (Biella *et al*, 2002).

In summary, nonself recognition processes in filamentous ascomycetes are best represented by a partially overlapping set of interaction types (Figure 1). Heterokaryon incompatibility and mycelial incompatibility may be distinct processes or manifestations of what is apparently the same process. Mycelial incompatibility can be induced by HI (such is the case for five of six *vic* loci in *C. parasitica*), or can be apparently completely independent of HI, as in *N. crassa* where no correlation is observed between the two (Micali and Smith, 2003). In addition, while sexual and vegetative nonself recognition systems generally function independently of each other, there can be overlap between them in certain species. In the case of *N. crassa*, for example, the mating-type locus is implicated in nonself recognition during both the vegetative and sexual phases of the life cycle such that individuals must be of opposite mating types to reproduce sexually, and must be of the same mating type to form a viable vegetative heterokaryon (Perkins, 1988). In *P. anserina*, on the other hand, mating-type genes do not function as *het* genes and nuclei of opposite mating type can co-occur in heterokaryons (Glass and Kulda, 1992).

Figure 1. Venn diagram showing the relationship among nonself recognition processes in filamentous ascomycetes. Figure adapted from Smith and Lafontaine (2012).

Vegetative incompatibility in filamentous ascomycetes results in a form of PCD and interestingly demonstrates some similarities to apoptosis in other eukaryotes. Approximately 50 putative downstream components of the mammalian apoptotic pathway have been identified in the filamentous fungus *Aspergillus fumigatus*, including the apoptosis inducing factor AMID, inhibitor of apoptosis, BIR1, and the serine protease HtrA2 that cleaves inhibitors of apoptosis (Fedorova *et al*, 2005). Each of these proteins is more similar to their human counterparts than to yeast homologues. Additionally, HI-induced cell death commonly displays organelle degradation and plasmolysis (Jacobson *et al*, 1998) as well as nuclear DNA degradation identified by terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) (Marek *et al*, 2003). While these similarities to apoptosis in higher eukaryotes are striking, it has also been determined that reactive oxygen species (ROS) and calcium signalling pathways are involved in HI while certain homologues to apoptosis genes in higher eukaryotes like caspases and apoptosis-inducing factor are not (Hutchison *et al*, 2009). There is significant gap in our understanding of how vegetative incompatibility, initiated by *het* or *vic* gene interactions, triggers the PCD pathway.

Genetic basis of nonself recognition in fungi

The genetic basis of vegetative incompatibility systems are best understood in *C. parasitica*, *P. anserina*, and *N. crassa*. All three species are members of Sordariaceae but have distinct life histories: *C. parasitica* is a plant pathogen that caused the demise of the American chestnut tree in the early 1900s, *P. anserina* is a coprophilous fungus that lives on the feces of herbivorous animals and *N. crassa* is commonly found growing on dead plant matter after fires in tropical and subtropical regions. In the following sections a brief overview is provided of the genetic basis of vegetative incompatibility in each of these species.

Vegetative incompatibility in *C. parasitica* is governed by six unlinked *vic* loci, *vic1*, 2, 3, 4, 6, and 7, each with two allelic forms (Cortesi and Milgroom, 1998). Four of these loci, *vic2*, 4, 6 and 7, have been partially characterized at the molecular level (Choi *et al*, 2012). Allelic incompatibility reactions may occur at each of these loci, such that barrage formation occurs when strains are paired with partners containing the opposite allelic type at an incompatibility gene. In some cases, nonallelic interactions (i.e. interactions between specific alleles of different genes) are involved in incompatibility. For example, the *vic6* locus is comprised of two polymorphic, tightly linked genes, *vic6* and *pix6*, that are in linkage disequilibrium such that natural populations contain one of two possible haplotypes: *vic6-1/pix6-1* or *vic6-2/pix6-2*. Nonallelic interactions at this locus, *vic6-1/pix6-2* and *vic6-2/pix6-1*, cause strong incompatibility reactions. Allelic interactions between *vic6-1/vic6-2* and *pix6-1/pix6-2* were not detected at this locus (Choi *et al*, 2012). Cortesi *et al.* (2001) showed that transmission of viruses between individuals is controlled primarily by these *vic* genes. They showed that heteroallelism at some *vic* genes would reduce virus transmission, but that each *vic* gene differed in this effect. For example, strains that were heteroallelic at *vic2* displayed virus transmission in only 21% of pairings, while transmission was not restricted at all in pairings that were heteroallelic at *vic4*. Additionally pairings that were heteroallelic at multiple *vic* genes were seen to have additive effects on reduction of virus transmission. Biella *et al.* (2002) concluded that *vic* genes trigger a common PCD pathway and that asymmetric PCD exists in pairings that were heteroallelic at *vic1*, *vic2*, and *vic7*. In cases of asymmetric PCD, they observed reduced virus transmission into strains that die more quickly upon fusion. This type of asymmetry has also been observed in *P. anserina* and *N. crassa*.

Nine *het* loci have been identified in *P. anserina*, five of which are molecularly characterized: *het-s*, *het-r*, *het-c*, *het-d* and *het-e*. Amongst the characterized loci, there are five allelic and three non-allelic interactions governing heterokaryon incompatibility (reviewed by: Hutchison and Glass, 2012; Saupe, 2000). The *het-s* locus consists of two incompatible alleles: *het-S* and *het-s* that interact in an

allelic manner. Barrage formation is observed at the contact point between strains of opposite allelic variation at this locus. As little as a single amino acid difference can determine incompatibility between *het-s* alleles (Deleu *et al*, 1993). A second example of allelic incompatibility exists between *het-V* and *het-V1*. Additionally, three non-allelic incompatibility systems have been characterized in *P. anserina*, consisting of interactions between *het-c* and *het-d*, *het-c* and *het-e* and *het-r* and *het-v*. Interactions between strains bearing incompatible forms of these alleles also lead to incompatible fusions and barrage formation. Each of the proteins encoded by *het-d*, *het-e* and *het-r* encode proteins belonging to the HNWD family, a group of incompatibility proteins sharing three conserved domains (Chevanne *et al*, 2009). One of these conserved domains is the HET domain that has been identified in numerous other HI genes in fungi and some plants (Smith and Lafontaine, 2012). These HET domain genes have not been found to have any function outside of HI and frequently interact with linked or unlinked genes that have a second function outside of heterokaryon incompatibility. This is seen here, where *het-d* and *het-e* (encode HET domain proteins) both can interact with *het-c*, which encodes a glycolipid transfer protein, to induce an incompatibility reaction (Espagne *et al*, 2002).

In *N. crassa*, HI is controlled by 11 loci: 10 *het* loci and the mating-type (*mat*) locus, which is implicated in nonself recognition during both the vegetative and sexual phases of the life cycle (Perkins, 1988). Genes involved in heterokaryon incompatibility at three of these *het* loci have been cloned and are well characterized: *het-c* and *pin-c* (at the *het-c* locus), *het-6* and *un-24* (at the *het-6* locus), and *tol* and *mat* (unlinked genes involved in mating heterokaryon incompatibility). Interactions between *het-c* and *pin-c* function in a similar manner to *het-d*, *het-e*, and *het-c* in *P. anserina*. (Note that *het-c* in *P. anserina* is not homologous to *het-c* in *N. crassa*.) In *N. crassa* *het-c* encodes a glycine rich membrane protein while *pin-c* encodes a HET domain protein. The two genes are tightly linked, and exist in one of three haplotypes: *het-c1 pin-c1*, *het-c2 pin-c2*, and *het-c3 pin-c3*. Nonallelic interactions between different forms of *het-c* and *pin-c* give rise to heterokaryon incompatibility reactions, while allelic

interactions of incompatible alleles at *het-c* increase the severity of the response (Kaneko *et al*, 2006). During vegetative growth, the *mat* locus can also induce heterokaryon incompatibility; fusion of a *matA* and a *mata* strain, where at least one of the two strains carries a functional copy of *tol*, will result in an incompatibility reaction (Shiu and Glass, 1999). Finally, the *N. crassa* *het-6* locus is well characterized and is the primary focus of this study. The following sections will detail what is known about interactions among the genes at the *het-6* locus.

Heterokaryon Incompatibility at the *het-6* locus in *Neurospora crassa*

Heterokaryons or partial diploids having differences at the *het-6* locus have been found to exhibit one of the most severe HI reactions of all the HI loci in *N. crassa* (Smith *et al*, 2000). The *het-6* region maps to the left arm of linkage group II and comprises two genes tightly linked into a supergene complex: *un-24* and *het-6*, each having two allelic variants: Oak Ridge (OR) or Panama (PA) (Smith *et al*, 2000). The two genes in the supergene complex are under severe linkage disequilibrium conditions such that the complex occurs naturally in only two possible haplotype forms: *un-24*^{OR} *het-6*^{OR} or *un-24*^{PA} *het-6*^{PA} (Mir-Rashed *et al*, 2000). A paracentric inversion polymorphism has been identified within the *het-6* region and the lack of DNA sequence homology between these genes and the inversion breakpoints is likely responsible for the lack of recombination between the incompatibility genes (Figure 2A; Micali and Smith, 2006). There is noticeable asymmetry between incompatibility reactions associated with this locus (Lafontaine and Smith, 2012). For example, self-incompatible strains formed through the transformation of a *un-24*^{PA} allele into an *un-24*^{OR} *het-6*^{OR} strain give rise to incompatible colonies (designated *un-24*^{OR} *het-6*^{OR}::*un-24*^{PA}) that show growth rates that are 100 times less than wildtype rates and have a characteristic "spidery" morphology. On the other hand, transforming an *un-24*^{OR} allele into a PA-background strain, to obtain genotype *un-24*^{PA} *het-6*^{PA}::*un-24*^{OR}, results in complete growth

Figure 2. Organization and known interactions of incompatibility genes at the *het-6* locus. A)

Representation of the physical map of Oak Ridge (OR) and Panama (PA) haplotypes of the *un-24 het-6* supergene complex (adapted from Micali and Smith, 2006). The paracentric inversion polymorphism that is indicated by parentheses and dotted lines causes the *un-24*^{OR} gene to be in the reverse orientation relative to the *un-24*^{PA} gene. DNA sequence divergence around the inversion breakpoints prevents homologous recombination and leads to complete linkage disequilibrium of incompatibility genes at the locus. B) A schematic of the *het-6* locus outlining the known incompatibility interactions (red arrows), with the thicker arrow between *un-24*^{OR} and *het-6*^{PA} representing a stronger incompatibility reaction. Figure adapted from Lafontaine and Smith (2012).

inhibition and no transformed colonies are recovered (Micali and Smith, 2006). A schematic of the *het-6* locus displaying the known incompatibility interactions can be seen in Figure 2B.

Self-incompatible *un-24^{OR} het-6^{OR}:: un-24^{PA}* colonies can escape and revert back to wildtype growth patterns within 2 weeks. This 'escape' phenomenon is commonly observed in *P. anserina* (Chevanne *et al*, 2010) and *N. crassa* (Smith *et al*, 1996) and in most cases appears to be mediated by mutations in the *het* genes involved in the incompatibility reaction. At the *het-6* locus of *N. crassa*, deletion of the entire locus or point mutations in the *het-6* gene facilitate escape (Smith *et al*, 1996; Lafontaine and Smith, unpublished).

The OR and PA forms of the *het-6* gene share 78% DNA sequence identity and only 68% amino acid identity. The two forms do, however, each contain a HET domain, comprised of three conserved regions of 10, 27 and 21 amino acid segments, arranged in a specific order. The HET domain occurs, either partially or fully, in 57 *N. crassa* open reading frames and is also associated with heterokaryon incompatibility functions in *P. anserina* and *C. parasitica* (Smith and Lafontaine, 2012). The role of HET domain proteins in HI remains unknown, though it has been hypothesized that they act as signal transducers, relaying a death signal to a PCD pathway (Paoletti and Clavé, 2007).

Unlike *het-6*, *un-24^{OR}* and *un-24^{PA}* encode very similar protein sequences and have an overall DNA sequence identity of 95%. However the predicted proteins differ significantly in their C-terminus regions, sharing only 70% amino acid identity in the final ~60 residues. This suggests that incompatibility activity and specificity are encoded within this variable C-terminal region (Smith *et al*. 2013a). Figure 3A displays schematics and Figure 3B displays the alignment of the C-terminal regions of UN-24^{OR} and UN-24^{PA} proteins, highlighting the highly variable C-terminal domains. Both allelic (between different forms of *un-24*) and nonallelic (between *un-24^{OR}* and *het-6^{PA}* and between *un-24^{PA}* and *het-6^{OR}*) interactions give rise to incompatibility reactions in *N. crassa* (Lafontaine and Smith, 2012). In addition to its role in

A

B

UN-24 ^{OR}	APMPTASTSQILGNNECFEPYTSNIYQRRVLAGEFQVVNPWLLRDLVEMGLWSDAMKNRI
UN-24 ^{PA}	APMPTASTSQILGNNECFEPYTSNIYQRRVLAGEFQVVNPWLLRDLVEMGLWSDAMKNRI
UN-24 ^{OR}	IAEGGSIQNIQSIPNDIKALYKTVWEISQRTIVKMAADRGAFIDQSQSLNIHMREPTMGK
UN-24 ^{PA}	IAEGGSIQNIQSIPNDIKALYKTVWEISQRTIVKMAADRGAFIDQSQSLNIHMREPTMGK
UN-24 ^{OR}	ITSMHFAGWKMGLKTGMYYLRTQAAAQPIQFTVDQEALRATDDRVAPAHSGLKKRSPAG
UN-24 ^{PA}	ITSMHFAGWKMGLKTGMYYLRTQAAAQPIQFTVDQEALRATDDRVAPAHSGLKKRSPAG
UN-24 ^{OR}	TYTSIVLRENTSGPRPYAQTGVSGTSTPIGTRDVPTPASTPPPTTEVPEETLVQSDNRPRPL
UN-24 ^{PA}	TYTSIVLRENTSGPRPYAQTGVSGTSTPIGTRDVPTPASTPPPAEDSETLVQSDNRPRPL
UN-24 ^{OR}	VSPAKSAGFKADLPEPESPKALATDPIVKTEDIGSPLLERKEGQNEDEVDEDSQERDENIY
UN-24 ^{PA}	VSPAKNAGFKADLPEPESPKALATDPIVKTEAISRELEMKEGOSEDKDEESKEREEDIY
UN-24 ^{OR}	SNAPLSEQQVARCAWNPGADPSSCEMCSG
UN-24 ^{PA}	S-----DEVVSCPYPVPGADPSSCVMCSG

Figure 3. Representations of the protein products of *un-24^{OR}* and *un-24^{PA}*. A) UN-24^{OR} and UN-24^{PA} share highly conserved N-terminal regions (white areas) but considerable differences in the variable C-terminal domain (grey and black areas). The scale represents the number of amino acids in each protein. B) Alignment of UN-24^{OR} and UN-24^{PA} from amino acid 601 to the C-terminus, illustrating the lack of homology in the C-terminal region. Black shading represents identical amino acids, grey and white areas represent amino acids with similar and dissimilar R-groups, respectively.

HI, *un-24* encodes the large subunit of the enzyme ribonucleotide reductase (RNR). Therefore, much like the previously mentioned cases, *het-6/un-24* represents another instance of a non-allelic interaction involving a HET domain protein (HET-6) and a second protein with an additional function unrelated to HI (UN-24).

Ribonucleotide Reductase

RNR is an evolutionarily conserved, essential enzyme that catalyzes the rate limiting step in the production of deoxyribonucleotides, the reduction of the 2'-hydroxyl of ribonucleotides, and thus is crucial for DNA synthesis and repair (Uhlen and Eklund, 1994). The proper function and maintenance of RNR is required for a constant ratio of DNA precursors to cell mass throughout growth (Herrick and Sclavi, 2007). The enzyme is allosterically regulated by nucleotides at two distinct binding sites. The first site (the activity site) binds either ATP, which stimulates catalytic activity, or dATP, which inhibits activity (Elledge *et al*, 1992). The second site (the specificity site) binds either ATP, dATP, dGTP, or dTTP. Binding of ATP or dATP at this site promotes the reduction of CDP or UDP. Binding of dGTP promotes reduction of ADP and binding of dTTP promotes the reduction of GDP (Reichard, 2001). Overall the activity site is responsible for ensuring that a sufficient amount of dNTPs are present for DNA synthesis, while the specificity site ensures that the proportions of these nucleotides remains balanced (Reviewed by: Nordlund and Reichard, 2006). In addition to allosteric regulation, RNR has also been shown to be transcriptionally regulated during the cell cycle and under DNA damage conditions (Elledge *et al*, 1992).

Three different classes of RNR were all originally discovered in bacteria in the 1960's (Reichard *et al*, 1961). All three types of RNRs contain cysteine residues required to form thiyl radicals for ribonucleotide reduction and are allosterically regulated in a nearly identical manner (Jordan and Reichard, 1998). The main differences between the three classes (I, II and III) are related to the

generation of the radical used in reduction and the requirement of oxygen in this process. Class I RNRs generate a tyrosyl radical through interaction with a diferric centre in an oxygen dependent reaction, and thus function only under aerobic conditions. Class II enzymes also generate a tyrosyl radical, but in this case it arises from adenosylcobalamin in a reaction that does not require oxygen, but is not hindered by it. These enzymes function in aerobic or anaerobic conditions. Finally, class III RNRs generate a glycy radical with the aid of an iron-sulfur cluster and S-adenosylmethionine. This radical is destroyed by oxygen, causing the enzyme to only function under anaerobic conditions (Reichard, 2001). Type I RNRs are found in all eukaryotic species, including *N. crassa*, as well as some bacteria and viruses and will be the focus of the current study, referred to herein simply as RNR.

The type I RNR enzyme functions as a tetramer with an $\alpha_2\beta_2$ structure; consisting of a large subunit homodimer (called R1), and a small subunit homodimer (called R2) (Reichard *et al*, 1993). The large subunit contains the allosteric binding sites and the catalytic, or active, site while the small subunit contains an organic tyrosyl radical in close proximity to a dinuclear iron centre required for radical generation (Uhlen and Eklund, 1994). Furthermore, the large subunit is composed of three domains: a ~220 residue N-terminal domain, a ~480 residue 10-stranded α/β barrel domain and a ~70 residue flexible C-terminal domain that cannot be resolved in the crystal structure (Uhlen and Eklund, 1994).

The redox reactions of RNR are mediated by five cysteine residues that are conserved across all taxa having type I RNRs, corresponding to C225, C439, C462, C754 and C759 in *E. coli*. Substrate binding is initiated upon reduction of the redox-active cysteines in the active site (C462 and C225). C462 becomes buried and C225 moves into its place, making room for substrate binding (Eriksson *et al*, 1997). The substrate forms hydrogen bonds to the main chain nitrogen residues at the amino end of the $\alpha 24$ helix within the active site (Eriksson *et al*, 1997). Ribonucleoside diphosphates are the only substrates

able to bind the active site of RNR as a result of a highly narrow phosphate binding site and specific α -phosphate interactions (Eriksson *et al*, 1997).

The first step of the substrate reduction reaction involves the generation of a thiyl radical on the redox-active cysteine C439 in the active site. In *E. coli*, the radical is initially formed on Y122 of the R2 small subunit and is transferred through a long-range electron transfer to C439 of the R1 protein. This series of electron transfers is essential since the tyrosyl radical is buried deep within the R2 subunit (Jordan and Reichard, 1998). The R2 diiron site is coordinated by carboxylates and displays large structural flexibility in reactions with molecular oxygen, thus allowing for the generation of a tyrosyl radical (Nordlund and Reichard, 2006). The subsequent electron transfer involves the residues D237, W48 and Y356 of R2 and the R1 residues Y731 and Y730 (Jordan and Reichard, 1998). The following steps of the substrate reaction are all carried out within the active site, and are outlined in Figure 4A, while Figure 4B outlines the chemical equation associated with this mechanism. The 3'-hydrogen from the substrate ribose ring is removed by the C439 radical. Next, the 2'-OH-group is protonated by C225, leaving as a water molecule, thus converting the substrate into a 2-ketyl radical. Redox-active cysteines C225 and C462 then transfer a hydrogen atom to the substrate and are in turn oxidized to a disulfide anion radical. The radical electron is then transferred to the 2'-position of the substrate, which removes a hydrogen from C439, completing the production of the deoxyribonucleotide product, and reforming the thiyl radical on C439 (Kolberg *et al*, 2004; Eklund *et al*, 2001). The thiyl radical is then transferred back to the small subunit R2 where it was first generated.

During the substrate reaction, the redox-active cysteine pair (C225 and C462) is converted from an active, free dithiol reduced form to an inactive, disulfide-bonded oxidized form. This pair must be reduced to return the enzyme to the active state. Thioredoxin and glutaredoxin are both physiological reductants for R1 but are incapable of entering the active site to reduce the disulfide bond directly

A)

B)

Figure 4. A) Schematic of the reaction mechanism of substrate reduction in the active site of RNR. Note that certain active site residues that have putative roles in picking up protons were omitted in this figure for simplicity. Adapted from Mao *et al.* (1992). B) Balanced chemical equation of the substrate reduction mechanism shown in A.

(Holmgren and Sengupta, 2010). To overcome this, a conserved cysteine pair on the C-terminal end of R1, C754 and C759 in *E. coli*, enters the catalytic site of the neighboring large subunit monomer and acts *in trans* to reduce the active R1 cysteine pair by disulfide bond transfer (Zhang *et al*, 2007). This C-terminal cysteine pair exits the catalytic pocket and is in turn reduced by either thioredoxin or glutaredoxin.

All of the structural and mechanistic information discussed in the previous paragraphs refers to the *E. coli* RNR. The structure of the yeast ortholog, Rnr1p, has a very similar 3-dimensional structure to the *E. coli* protein (Xu *et al*, 2006). In yeast, the conserved, redox-active cysteines are C218, C428, C443, C883, C886. The C-terminal cysteines of the yeast RNR are found in the characteristic CX₂C motif of eukaryotic R1s (Zhang *et al*, 2007). Rnr1p also contains an insertion of 128 amino acids located inside the C-terminal domain, which is the most variable domain between different RNRs.

RNR associated incompatibility in *N. crassa*

The *N. crassa* RNR protein, UN-24, contains an N-terminal domain that is highly conserved when compared to all other type I RNRs, including the yeast forms. It follows that UN-24 will share structural and functional features with Rnr1p in yeast and RNR1 in *E. coli*. Similar to yeast Rnr1p, UN-24 contains a relatively large insertion of 160 amino acids in the variable C-terminal region, as compared to the *E. coli* R1 protein. In most eukaryotic species, the C-terminal region usually contains three terminal cysteines. The C-terminal region is not resolved by crystallography and is thought to be of irregular structure. The two forms of UN-24 (OR and PA) are also identical in the N-terminal regions, while in the C-terminal regions, 30 of 106 amino acids differ (Lafontaine and Smith, 2012). This C-terminus of UN-24 is the highly variable arm that is responsible for the reduction of the active site of the neighbouring monomer and is encoded by the 3' end of the *un-24* gene. UN-24-associated incompatibility is correlated with the

formation of a high molecular weight protein complex likely composed of one UN-24^{OR} and one UN-24^{PA} in self-incompatible *un-24^{OR} het-6^{OR}::un-24^{PA}* strains (Smith *et al*, 2013a). It has been shown that after escape from incompatibility in these strains, the complex resolves into UN-24 protein that corresponds to the monomeric size (Smith *et al*, 2013a). Mutational analyses demonstrated that all three C-terminal cysteines are required for incompatibility activity for the UN-24^{OR} form, while two of the three cysteines in the UN-24^{PA} form are redundant (Smith *et al*, 2013a). In another study, it was shown that only 135 residues from the C-terminal tail of UN-24^{PA} are required to induce incompatibility activity and that this truncated UN-24^{PA} can also induce incompatibility when expressed in *Saccharomyces cerevisiae* (Smith *et al*, 2013b). Taking all of this information together suggested that an aberrant disulfide bond is formed between the UN-24^{PA} C-terminus and the UN-24^{OR} catalytic site, producing an aberrant heterodimeric complex that causes an incompatibility reaction following the reduction of the active site by the C-terminal cysteines (Smith *et al*, 2013a). The role that HET-6 plays in this model is unknown, but it has been suggested that it either acts in transducing a signal to a cell death pathway when an aberrant UN-24 protein complex has formed, or that it functions by physically stabilizing a toxic UN-24^{OR}-UN-24^{PA} protein complex. Interestingly, nonallelic interactions between *un-24^{PA}* and *het-6^{OR}* give rise to an incompatibility reaction suggesting that HET-6^{OR} interacts specifically with the C-terminus of UN-24^{PA}.

The purpose of this study

Nonspecific recognition is a ubiquitous, fundamental process in living organisms. A full understanding of these types of systems has the potential to unravel numerous questions related to pathogen response and immunity and the evolution of multicellular organisms. In the case of *N. crassa*, however, our understanding of *het-6*-associated nonspecific recognition is incomplete since any study of allelic interactions between *het-6^{OR}* and *het-6^{PA}* has been hampered by the fact that any modifications that result in a loss of *un-24* associated incompatibility also cause loss of catalytic function of this

essential gene, and thus cell death. The *N. crassa* UN-24 protein is the only known RNR that is involved in nonself recognition, suggesting that this additional function has been recruited to the essential enzyme secondarily. This makes the *het-6* locus, and the UN-24 protein in particular, highly unique. Evolutionarily speaking, what is the benefit of having an essential enzyme that is also involved in nonself recognition, and how exactly does this system work?

Additionally, vegetative incompatibility has only been identified in filamentous fungi - it does not occur in the yeast, *S. cerevisiae*, for example. However, Smith *et al.* (2013b) showed that transforming *S. cerevisiae* with the incompatibility-inducing C-terminal region of *un-24*^{PA} from *N. crassa* elicits a HI response, as evidenced by cytoplasmic granulation, perturbation of vacuole integrity and growth inhibition, all of which are characteristics observed in incompatibility in filamentous fungi. This makes UN-24 an interesting candidate for the production of small peptide antibiotics. This work in yeasts begs the question: In what other types of cells could *un-24* induce a nonself recognition cell death response? In this regard it is possible that *un-24* could be used as an antifungal or other type of antibiotic, or as a tumor suppressor. But precluding these types of promising applications, we do not have a complete understanding of the functioning of the *het-6* locus and the structure-function relationship of UN-24. In this study, we explore this structure-function relationship. We use site directed mutagenesis techniques to identify the residues governing UN-24-associated incompatibility. Additionally we produce a catalytically functional *un-24* gene that lacks incompatibility activity. This *un-24*⁰ allele will facilitate the study of allelic interactions of *het-6* and provide insights into the role that HET-6 proteins play in heterokaryon incompatibility.

Materials and Methods

Strains and culture conditions

All *Neurospora crassa* strains used in this study were cultured on Vogel's minimal medium (VMM) containing 1.5% sucrose, 1.5% agar and supplements as required (Davis and De Serres, 1970), unless otherwise indicated. The *Cryphonectria parasitica* strain (EP155) and *Saccharomyces cerevisiae* strain (FY834) used were grown in liquid potato dextrose broth (PDB) and yeast potato dextrose (YPD), respectively. Details of all strains used are included in Table 1. All fungal cultures were grown at 30°C without shaking unless otherwise indicated.

DNA isolation, PCR amplification and TA cloning

Genomic DNA from *C. parasitica* and *N. crassa* was isolated using the method described by Oakley *et al.* (1987). Strains were grown in liquid medium, vacuum filtered and dried under vacuum in a desiccator. The dried mycelia were then pulverized and resuspended in extraction buffer before chloroform extraction and ethanol precipitation of DNA. Concentrations of genomic and plasmid DNA preparations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Routine PCR amplification was carried out using Taq DNA polymerase (Bioshop, Burlington, Ontario) in a Biometra T-gradient thermocycler (Biometra, Germany). Initial denaturation was for 5 minutes at 95°C, followed by 30 cycles of a 1 minute denaturation at 95°C, 30 second annealing at 62°C and an extension at 72°C for 1 minute per kilobase. The final extension was for 10 minutes at 72°C. Amplification for the purpose of cloning and transformation was conducted under the same conditions, using iProof™ High-Fidelity DNA polymerase (BioRad, Mississauga, Ontario). Oligonucleotide primers used in this study were synthesized by Sigma-Aldrich (Oakville, Ontario) or IDT (Toronto, Ontario), and are included in Table 2. Following iProof™ amplification, PCR products

underwent an extension at 72°C for 1 hour using Taq DNA polymerase to generate products containing 3'-A overhangs for TA cloning. All amplicons intended for transformation into *N. crassa* were inserted into the pCR2.1TOPO cloning vector and were transformed into chemically competent Top10 *E. coli* cells according to the TOPO cloning reaction and transformation protocol (Invitrogen, Burlington, Ontario) and were selected on LB medium with 50 µg/mL ampicillin and 50 µg/mL kanamycin. Table 3 includes details of the vectors used in this study. Successfully transformed colonies were grown overnight in 5 mL of selective LB broth in a 37°C shaking incubator and plasmids were extracted using the Promega Wizard miniprep kit (Fischer Scientific, Nepean, Ontario). All constructs were verified by DNA sequencing at Genome Quebec Innovation Centre (Montreal, PQ). Sequence alignments were performed using ClustalW (European Bioinformatics Institute). PCR products intended for direct transformation into *N. crassa* were purified using the FroggaBio Gel/PCR DNA extraction Kit (FroggaBio Inc, Toronto, Ontario).

Transformation of *N. crassa*

Transformations of *N. crassa* were conducted using methods similar to those used previously (Royer and Yamashiro, 1992). One µg of plasmid DNA was mixed with 100 µL of spheroplast suspension ($\sim 8.0 \times 10^7$ spheroplasts/mL) on ice for 30 minutes prior to the addition of 1 mL sterile 40% PEG/10 mM MOPS/50 mM CaCl₂ and an additional incubation of 20 minutes at room temperature. Transformed spheroplasts were mixed with Vogel's agar containing 18% sorbitol and were plated on regeneration Vogel's agar containing supplements and 1X FIGS (fructose, inositol, glucose and sucrose). Transformed *N. crassa* colonies were selected in one of two ways: either at 30°C on regeneration agar containing hygromycin B (200 µg /100 mL; Roche, Laval, Quebec), or at 39 °C where selection was based on complementation of the temperature sensitive allele, *un-24^{ts}*.

Development of *un-24* mutants

Three different strategies were used to create mutants in the C-terminal region of *un-24*^{OR}. The mutations were designed to convert the C-terminal form of UN-24^{OR} into the UN-24^{PA} form in a stepwise manner. Schematics of the protocols used to produce each mutant construct are included in Figure 5. The first construct (*un-24*^{OR(A)}) consisted of a 6 amino acid (NAPLSE) deletion at position 901-907 of UN-24^{OR}. This deletion was introduced using recombination-mediated PCR-directed plasmid construction in yeast, as described by Oldenburg *et al.* (1997) (Figure 5A). A pair of complementary internal primers (unOR6aaDELfw and unOR6aaDELrv) were designed with ~20 bases of homology and 18 base deletion. External primers were designed ~800 bp upstream (pRS426unORHybFW) and ~2 Kbp downstream (pRS426unORHybRV) of the *un-24*^{OR} coding region. These external primers had 3' ends that contained ~20 bases of homology with the selectable (URA3) yeast plasmid pRS426 (Table 3), and that overlapped the restriction sites for XhoI and EcoRI. These primers were used in a PCR reaction to produce a 4kb fragment "1" and a 2kb fragment "2" that overlapped at the desired deletion (Figure 5A). The pRS426 plasmid was linearized by a double restriction digestion with XhoI and EcoRI (fragment "3", Figure 5A) and co-transformed with the fragments 1 and 2 into the yeast strain FY834 using the method described by Gietz and Woods (2006). In the FY834 strain, the three DNA fragments were recombined via homologous recombination into the circular plasmid, which was subsequently selected on synthetic complete medium lacking uracil (SC-ura) but containing all other necessary amino acids for growth of this yeast strain (Winston *et al.*, 1995). Thirty colonies were chosen from the transformation plate and were grown overnight in liquid SC-ura at 30°C. Fifteen cultures were pooled into two separate tubes and plasmids were isolated. The mutant construct was then amplified using primers sm5F840fw and pRS426unORHybRV (Figure 5A) and was cloned into pCR2.1TOPO as described above. Following TOPO cloning, the insert was transferred into the vector pCB1004 (Table 3), which contains hygromycin B resistance as a selectable marker for transformations into *N. crassa*. For this, constructs were excised

Figure 5. Schematic representations of methods used to build mutant constructs of *un-24*^{OR}. A) The yeast transformation method used to produce *un-24*^{OR(A)}. Numbers 1, 2 and 3 represent the three fragments of DNA that were recombined together in the yeast strain FY834. B) The direct PCR method used to produce *un-24*^{OR(B)}. C) The modified QuikChange™ Site-Directed Mutagenesis method used to produce *un-24*^{OR(C)}. Red sections in each panel represent the mutated region and are not represented to scale. Primer sequences are provided in Table 2.

from the pCB2.1-TOPO vector using XbaI and SacI (New England Biolabs, Pickering, Ontario), and were ligated using T4 ligase (New England Biolabs, Pickering, Ontario) into the pCB1004 vector, which had been cut with the same restriction enzymes. Two μL of the ligation reaction was then transformed into chemically competent DH5 α cells (Invitrogen, Burlington, Ontario). Transformants were replica transferred onto chloramphenicol and ampicillin plates to ensure that the gene, and not the linearized pCR2.1TOPO plasmid, had been successfully ligated into pCB1004. If the insert had been properly ligated into the pCB1004 vector, it would be resistant to chloramphenicol (resistance is encoded by pCB1004). Had the pCR2.1TOPO plasmid been ligated into pCB1004, the resulting unwanted construct would be resistant to both chloramphenicol and ampicillin (resistance is encoded by pCR2.1TOPO).

The next mutation (*un-24*^{OR(B)}) consisted of replacing the glutamic acid (codon GAG) at position 925 with a valine residue (codon GTG). A reverse primer (unORe-vRV) that ended at the stop codon was designed to contain the desired mutation, and used to amplify a 4 kbp fragment with the forward primer (sm5F840fw) situated 800 bp upstream of the *un-24*^{OR} start codon (Figure 5B). The construct was then cloned into pCR2.1TOPO, and inserted into pCB1004 as described above.

A replacement of an aspartic acid at position 895 with a glutamic acid (codon GAC > GAG) and an asparagine at position 897 with an aspartic acid (codon AAT > GAT) represented the third mutation (*un-24*^(C)). These two changes were introduced using a modified version of the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA; Figure 5C). A set of overlapping complementary primers (unORde-ndfw and unORde-ndrv) were designed with the two desired mutations situated near the centre. A standard 20 μL PCR reaction using iProof™ polymerase was used to amplify a 7.9 kbp fragment that included the entire pCB1004 plasmid and inserted gene with duplications of the mutated region at the termini. For this amplification, PCR extension time was increased to 8 min to allow for amplification of the entire gene and plasmid. Template DNA was removed by digestion with DpnI (New

England Biolabs, Pickering, Ontario) before 10 µL of the digested reaction was transformed into competent Top10 cells that were plated on selective LB plates. Homologous recombination in *E. coli* at the ends of the PCR product resulted in the mutated construct becoming re-circularized as part of the pCB1004 plasmid.

In addition to the mentioned single mutations, combinations were also constructed, including *un-24*^{OR(AB)} and *un-24*^{OR(ABC)}. One final mutation consisted of replacing the valine at position 925 (codon GTG) with a leucine residue (codon TTG) in the *un-24*^{OR(B)} construct, following the same protocol that was used to produce *un-24*^{OR(B)}. The leucine substitution was chosen for this final mutant to make it more similar to the RNRs of many other species that are not reported to have heterokaryon incompatibility activity. These other species have leucine at the same location as the glutamic acid in UN-24^{OR} and valine in UN-24^{PA} (refer to Figure13).

Determination of mutant construct incompatibility activity

To evaluate incompatibility activity, pCB1004 constructs were transformed into the OR-background *N. crassa* strain, C9-2, and the strain C(2)2-1 (*un-24*^{PA} *het-6*^{PA}). Transformants were counted, and the morphology and growth rates of the transformed colonies was analysed and compared to self-incompatible and compatible control transformations. Compatible transformants are identified as large, round or "cloud-like" colonies that grow at rates of approximately 1 mm/hour when transferred to VMM and incubated at 30 °C. In contrast, self-incompatible transformants (*un-24*^{OR} *het-6*^{OR}::*un-24*^{PA}) form small, "star-like" colonies that grow at rates of 1 – 2 mm/day on VMM at 30 °C. As mentioned, *un-24*^{PA} *het-6*^{PA}::*un-24*^{OR} self-incompatibles are not recovered from transformations. Radial growth rates were determined daily after subculture onto VMM containing hygromycin at 30 °C and compared to growth rates of typical C9-2 and C(2)2-1 strains transformed with either *un-24*^{OR} or *un-24*^{PA}.

Crosses of *N. crassa*

Crosses of *N. crassa* were conducted on plates containing Synthetic Crossing Medium (SCM) and supplements as required (Davis and De Serres, 1970). Single drops of conidial suspensions were inoculated at opposite sides of the plate and colonies were allowed to grow together at room temperature under natural light. Compatible mating interactions produced perithecia after approximately two weeks and ascospores were produced after an additional two weeks. Ascospores were collected, plated on selective VMM plates and were heat treated at 60°C for 55 minutes. Plates were then incubated overnight to allow for the identification of germinating spores, which were transferred to individual tubes containing selective medium.

Heterokaryon Incompatibility Tests

Following the determination of the incompatibility activity of the mutant constructs produced in this study, multiple new strains of *N. crassa* were generated by gene replacements with these mutant genes. Heterokaryon incompatibility tests were used as described in Smith *et al.* (1996) to deduce the incompatibility activities of these strains. For this, two strains containing complementary auxotrophic markers are each inoculated separately on either side of a plate, and are co-inoculated at the centre of the plate, on a medium lacking the supplements required for the growth of either strain alone. Co-inoculated strains that are identical at all *het* loci will form a heterokaryon that grows at wildtype rates. Strains that differ at one or more *het* loci will exhibit growth that is partially or completely inhibited. This test was also used to verify the mating type of new, unmated strains, since in *N. crassa*, the *mat* locus acts as an incompatibility factor during vegetative growth. Once mating type was known, all further heterokaryon incompatibility tests were conducted with strains of the same mating type; *mat-a* in this study.

Table 1. Strains used in this study.

Strain	Genotype ^a	Origin
<i>N. crassa</i> strains used for transformations and crosses		
C9-2	<i>un-24^{OR} het-6^{OR} thr-2 a</i>	M.L. Smith
C2(2)-1 ^b	<i>un-24^{PA} het-6^{PA} thr-2 a</i>	Smith <i>et al.</i> (1996)
DLL7-23	<i>un-24^{ts}, trp-1 a</i>	D.L. Lafontaine
DLL1-26	<i>un-24^{ts}, Δmus-52::bar; inl a</i>	Lafontaine and Smith (2012)
DLL15-6	<i>un-24^{PA} Δhet-6::hph, Δmus-52::bar; inl a</i>	Lafontaine and Smith (2012)
DLL30-16 ^b	<i>un-24^{ts} Δhet-6::hph; thr-2, pan-2 A</i>	D.L. Lafontaine
DLL8-16	<i>un-24^{ts} Δhet-6::hph, Δmus-52::bar; inl A</i>	Lafontaine and Smith (2012)
6(3)	<i>arg-1 ad3B, un-24^{PA} het-6^{PA}; inl A</i>	M.L. Smith
<i>N. crassa</i> strains used for heterokaryon incompatibility testing		
A1	<i>ad3A nic-2 (cyh-1) a</i>	M.L. Smith
DLL3-15	<i>Δhet-6::hph trp-1 a</i>	Lafontaine and Smith (2012)
DLL30-10	<i>un-24^{ts} Δhet-6::hph; pan-2 a</i>	Lafontaine and Smith (2012)
<i>N. crassa un-24⁰ strains</i>		
RR1-3	<i>un-24⁰ het-6^{OR}; Δmus-52::bar; inl a</i>	DLL1-26:: <i>un-24⁰</i>
RR2-3	<i>un-24⁰ Δhet-6::hph; Δmus-52::bar; inl a</i>	RR1-3 x DLL8-16
<i>C. parasitica</i> strain		
EP155	<i>matA</i>	ATCC38755 ^b
<i>S. cerevisiae</i> strain		
FY834	<i>Mat-α his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63</i>	Winston <i>et al.</i> (1995)

^a All strains have Oak Ridge (OR) alleles at all het loci unless specified. *un-24^{ts}* designates the temperature sensitive allele that has OR incompatibility activity. (cyh-1) indicates untested possibility that the strain carries cycloheximide resistance.

^b ATCC = American Type Culture Collection, Manassas, VA

Table 2. Primers used in this study.

Primer Name	Sequence (5' → 3')
<i>rnr1</i> Isolation and Sequencing Primers	
RNR1fw	GACAGGCTGACTTGAACAACAGCG
RNR1rv	CCGTTCCGCCATTTGAAGAGCAC
CpRNR1-1fw	AGTTTGACAAGATCACGGGCA
CpRNR1-2fw	AAGAGCAACCAGAAGAACCTG
CpRNR1-3rv	TGGTGACTTCATGCAGCAGCT
CpRNR1-4fw	CCCCTGACTCCTGTCGTTGACA
CpRNR1-5rv	CTCTTCGGTAGGCTTCTCGCCA
CpRNR1-6fw	TACATGACCGTCACCCACCCA
CpRNR1-7rv	TCCTGGGAGATCATAGGCGAC
<i>un-24</i> Isolation and Sequencing Primers^a	
sm5F840fw	ACGGCGCTGTGAATATCTGTGA
5F50tsfw	GTTCTTGTTCCCTCTTCTCACC
3F1092fw	AAGAACCACGGTAAGGAAGAGG
3F1182rv	GTTCTGCTCAACACGCTTCA
5F1408fw	TACCATCCGCAGCTCCAA
5F2410rv	ACGACGACTTGAGTGACCTC
un-24fw	GTACAGAGTGACAACAGGC
3F3969rv	AAGAAGCCCTATCCATGCTGCTGA
3F5130rv	TTCCGAGGGTAGCTGTCTGTCT
<i>het-6</i> Isolation and Sequencing Primers^a	
het-6OR1fw	GTTGTCCGATTCTGAGAAGGC
het-6OR2fw	TCTTCCTTCATACTCTTCGCCC
het-6OR3rv	TGTGGCTATCGCCGTAGGAA
het-6OR4fw	GCGACTGTGGACTATAGAGGAA
het-6OR5rv	TAGGTGTGTCAGGGGTCTC
het-6OR6rv	TCACGATGGAGTGTCCAAGG
Primers for Mutant Construct Formation, Diagnostics and Transformation	
unORe-vRV	TTAACCGCTGCACATCACGCAGGAGGAGGGGTCGGCACCG
un-24ORV-Lrv	TTAACCGCTGCACATCAAGCAGGAGGAGGGGTCGGCACCG

unOR6aaDELfw	ACGAGAATATCTACTCTCAACAAGTTGCTCGTTGTAAGTGACAAG
unOR6aaDELrv	AACGAGCAACTTGTTGAGAGTAGATATTCTCGTCCCTCTC
6aaDELdiagRV	CTCAGAAAGTGGAGCATTAGAG
unORde-ndFW	CCAGGAGAGGGAGGAGGATATCTACTC
unORde-ndRV	GAGTAGATATCCTCCTCCCTCTCCTGG
un-24PAv-eRV	TTAACCGCTGCACATCTCGCAGGAGGAGGGATCGGCGCCG
un-24PAv-Lrv	TTAACCGCTGCACATAAGGCAGGAGGAGGGATCGGCGCCG
un-24OR-L	TTAACCGCTGCACATCAA
un-24OR-E	TTAACCGCTGCACATCTC
un-24ORV-L3Frv1	ACACTGCACATTTTTGACCATAAGCTT
un-24ORV-L3'flankRV1	ACACTGCACATTTTTGACCATAAGCTTCATATACCCACCG GACAGACAAGGGTGCGGGCTTAACCGCTGCACATCAAGC
pRS426unORHybFW ^a	ATAGGGCGAATTGGGTACCGGGCCCCCGGCGCTGTGAATA
pRS426unORHybRV ^a	ACTAGTGGATCCCCGGGCTGCAGGAATTCCGAGGGTAGCTG

^a - Primers designed by D.L. Lafontaine. All other primers designed by R. Reshke.

Table 3. Cloning vectors used in this study.

Vector Name	Size (bp)	Features
pCR2.1TOPO	3931	Bacterial expression vector with ampicillin and kanamycin resistance genes
pRS426	5726	Yeast episomal vector with URA3 marker and the ampicillin resistance gene
pCB1004	4800	Phagemid vector derived from pBluescript II with chloramphenicol and hygromycin resistance genes

Results

Transformation of *Cryphonectria parasitica* *rnr1* into *N. crassa*

The *rnr1* gene from *Cryphonectria parasitica* encodes the large subunit of RNR, and is the ortholog of *un-24* in *N. crassa*. However, unlike *un-24*, *rnr1* is not known to be involved in nonself recognition. Based on this, *rnr1* was identified as a candidate to replace *un-24* in *Neurospora crassa*. The expectation was that replacing *un-24* with *rnr1* would fulfill the catalytic RNR requirement, but would result in a loss of nonself recognition function. Sequences of *N. crassa* *un-24*^{OR} and *un-24*^{PA} were obtained from the NCBI genbank (AF171697.1 and DQ525966.2, respectively) and the sequence of *C. parasitica* *rnr1* was obtained from the JGI genome portal (ID: 351299). The three sequences were then aligned using ClustalW and *rnr1* was found to share 80% sequence identity with both *un-24* genes, with the major region of variation being found in the final ~500 bases of the C-terminal end. Full alignments are included in Appendix one.

DNA was then isolated from the *C. parasitica* strain EP155 and the *rnr1* gene was PCR amplified using the RNR1fw and the RNR1rv primers and cloned into the pCR2.1TOPO vector. Next, the DNA sequence of the entire gene was determined using the CpRNR1-1 to -7 primers (Table 2) to ensure there were no undesired mutations present in the cloned copy. One µg of the *rnr1* DNA was transformed into the *N. crassa* strain DLL7-23 (*un-24*^{ts}, *trp-1 a*). DLL7-23 contains a temperature sensitive form of *un-24*^{OR}, *un-24*^{ts}, characterized by three transition mutations in the N-terminal region, one of which results in a non-conservative amino acid substitution within a four-helix bundle required for allosteric control of RNR activity (Smith *et al.* 2000a). The temperature-sensitive mutant allele is non-functional at temperatures above 34°C, and consequently strains that carry this allele are unable to grow at these restrictive temperatures. Taking this into account, the formation of DLL7-23 colonies at restrictive

temperatures following ectopic introduction of the *rnr1* gene would suggest that *rnr1* is able to complement the temperature sensitive *un-24^{ts}* mutation, and to function as a catalytic replacement for *un-24* in *N. crassa*. *un-24^{OR}* (which should form wildtype, non-incompatible colonies when transformed into DLL7-23) and H₂O were used as positive and negative controls, respectively. About 20 colonies of DLL7-23::*rnr1* were observed to grow at 39°C from this transformation. These were subcultured onto VMM plates with and without tryptophan (trp+/trp- respectively), a supplement required for growth of DLL7-23, to ensure that colony formation was not a result of contamination by another strain. Slow growth that ceased after ~2 days was observed on the trp- plates, signifying that the transformants did require tryptophan in the medium for growth and were likely therefore not contaminants.

Approximately 50% of the colonies on the original transformation plate had the small, star-like colony morphology that is characteristic of self-incompatible *un-24^{OR} het-6^{OR}::un-24^{PA}* SI transformants (Figure 6). When these star-like colonies were subcultured onto Vogel's trp+ medium and allowed to grow for ~1 week at 39°C they showed characteristic PA incompatibility-like phenotypes. These strains grew with a slow, spidery morphology for ~5 days before escaping to a wildtype growth rate and morphology, covering the plate within the next 2 days (Figure 7A). These growth patterns were indistinguishable from the typical growth patterns of *un-24^{OR} het-6^{OR}::un-24^{PA}* self-incompatible strains (Figure 7B).

This surprising observation of PA-like incompatibility function by *rnr1* was verified using heterokaryon incompatibility tests as follows. DLL7-23::*rnr1* transformants that escaped at 39°C (reverting to wildtype growth rate and morphology) were found to form incompatible heterokaryons when co-inoculated with the *un-24^{OR}* strain, A1, similar to heterokaryons formed between A1 and an *un-24^{PA}* strain. On the other hand, these DLL7-23::*rnr1* strains formed compatible heterokaryons with the *un-24^{PA} Δhet-6* strain, DLL15-6.

Figure 6. Characteristics of self-incompatible *un-24^{OR} het-6^{OR}::un-24^{PA}* transformants. Transformation of the Oak Ridge background *N. crassa* strain, C9-2, with A) *un-24^{OR}*, B) *un-24^{PA}*, and C) H₂O. Four or five poorly defined colonies can be observed on the negative control plate (C), upon subculture, these were found to not grow on hygromycin B plates and deemed to be non-transformed colonies. Approximately 50 colonies can be observed on the other two plates. The C9-2::*un-24^{OR}* transformants are large and round, indicative of compatible colonies; an enlarged view is included in panel D. The C9-2::*un-24^{PA}* plate contains many transformants that appear small and star-like in shape. When subcultured, these star-like colonies grow slowly and with a spidery morphology on VMM medium containing hygromycin B. These slow growing colonies escape to wildtype morphology and growth rate within 2 weeks. These are characteristics of *un-24^{OR} het-6^{OR}::un-24^{PA}* self incompatibles. An enlarged view of a *un-24^{OR} het-6^{OR}::un-24^{PA}* self incompatible colony is included in panel E. DLL7-23::*rnr1* transformants were indistinguishable from these C9-2::*un-24^{PA}* transformants suggesting that *rnr1* from *C. parasitica* carries PA-like incompatibility function.

Figure 7. Graph depicting the indistinguishable growth rates of DLL7-23::*rnr1* and DLL7-23::*un-24*^{PA} transformants following subculture from transformation plates onto VMM containing tryptophan. A). The drastic increase in growth rate at approximately 4 days corresponds to escape, where colony growth rate and morphology revert to that of wildtype. B) Growth pattern on VMM of an Oak Ridge strain (C9-2) transformed with *un-24*^{PA} showing the typical slow, spidery growth for the first 4-7 days (1) followed by an escape event to wildtype growth characteristics. Two separate escape events can be observed that produce distinct areas of wildtype growth (white arrows). Black lines correspond to traces of the growth front at ~24 hour intervals.

A

B

Production of mutant *un-24*^{OR} constructs

The previous results suggest that *C. parasitica rnr1* carries PA-like incompatibility activity when transformed into *N. crassa*. Taking this into account, we sought to identify amino acid sequence similarities between RNR1 and UN-24^{PA} that differed from UN-24^{OR} in the C-terminal domains, the putative location of heterokaryon incompatibility activity and specificity. Using a ClustalW alignment, three separate sections of sequence were identified as sites that could potentially be responsible for governing incompatibility (Figure 8). 'A' is a section of six amino acids (NAPLSE) only observed in the OR form. 'B' consists of a glutamic acid in the OR form, as opposed to a valine residue in the PA and RNR1 forms. 'C' comprises three continuous amino acids; aspartic acid-glutamic acid-asparagine in the OR form as opposed to glutamic acid-glycine-aspartic acid in the PA form and glutamic acid-methionine-aspartic acid in RNR1. While there were other residues that differed across these C-terminal regions, these three sites were investigated due to the fact that they consisted of identical, or nearly identical, residues in UN-24^{PA} and RNR1 that were distinct from the UN-24^{OR} form. In addition, these polymorphic sites were of interest due to their proximity to the three C-terminal cysteine residues known to be involved in both catalysis and incompatibility associated with UN-24 (Smith *et al*, 2013a).

A total of four mutant *un-24*^{OR} constructs were produced based on the above analysis, each consisting of one or more of the A, B and C mutations (Figure 9). The first construct (*un-24*^{OR(A)}) was produced using a yeast recombination system. Two sets of primers, outlined in Figure 3, were used to produce fragments of ~2kb and ~4kb flanking the desired deletion using PCR and verified with gel electrophoresis (Figure 10). Additionally the selectable (URA3) plasmid, pRS426, was cut with restriction enzymes XhoI and EcoRI and was also verified via gel electrophoresis. All three fragments were then transformed into the yeast strain FY834 on SC-ura medium to take advantage of homologous recombination to combine the fragments providing the ability for growth on the selective (URA-)

Figure 8. A clustalW alignment of the C-terminal residues of *N. crassa* UN-24^{OR} (NcUN-24OR) and UN-24^{PA} (NcUN-24PA) and *C. parasitica* RNR1 (CpRNR1) showing each of their incompatibility activities in *N. crassa* in red boxes at right. 'A' (an insertion of the six amino acids NAPLSE), 'B' (E>V) and 'C' (D>E and N>D) represent sites of sequence similarity between UN-24^{PA} and RNR1 that differ in UN-24^{OR} that could be responsible for governing incompatibility specificity. Black shading represents identical amino acids, grey and white areas represent amino acids with similar and dissimilar R-groups, respectively.

Figure 9. The four mutant *un-24*^{OR} constructs created in this study. The arrows represent the final ~700 bp of each construct and indicate the positions and relative sizes of the introduced mutations. *un-24*^{OR} was altered to consist of sequence that would encode the desired 'A', 'B' and/or 'C' amino acid sequences from UN-24^{PA} and RNR1 as seen in Figure 8.

Figure 10. Obtaining the required fragments for production of *un-24*^{OR(A)}. Ethidium bromide stained gel visualized under UV light following electrophoresis at 120V for 30 minutes in 1% agarose. Lanes contain a 1kb DNA ladder from NEB (lane 1), ~2kb bands representing the C-terminal and ~2kb downstream portion of the *un-24*^{OR(A)} construct (lanes 2 and 3), ~4kb bands representing the N-terminal and ~800bp upstream portion of the *un-24*^{OR(A)} construct (lanes 5 and 6). Primers used to produce these fragments are included in Table 2 and a schematic outlining the yeast homologous recombination method to obtain the *un-24*^{OR(A)} construct is included in Figure 3.

medium. Hundreds of yeast colonies formed on the SC-ura plate, thirty of which were selected for plasmid isolation, which were verified for the presence of *un-24*^{OR(A)} using PCR. *un-24*^{OR(B)}, *un-24*^{OR(AB)}, and *un-24*^{OR(ABC)} all had mutations introduced via PCR, as outlined in the materials and methods section. The mutations were verified by DNA sequencing. Following the successful introduction of mutations, each construct was inserted into the pCR2.1TOPO cloning vector and was transformed into the temperature sensitive *N. crassa* strain DLL7-23 to ensure that they complemented the *un-24*^{ts} mutation (i.e. that they were enzymatically functional).

Determination of mutant construct incompatibility activity

After verification that each of the four constructs did retain enzymatic function and that each was capable of producing colonies at 39°C following ectopic transformation, each constructs was separately inserted into the hygromycin resistance vector, pCB1004, for additional assays. In each case, both the construct in the pCR2.1TOPO vector and the pCB1004 vector were cut using XbaI and SacI restriction enzymes, combined together in a ligation reaction, and transformed into DH5α cells. Transformant colonies were isolated and tested for chloramphenicol- and ampicillin-resistance to indicate that the desired constructs were cloned into pCB1004. If the ligation reaction produced a new construct consisting of two plasmids together, clones would be expected to grow in the presence of both ampicillin and chloramphenicol since pCB1004 and pCR2.1TOPO confer chloramphenicol and ampicillin resistance, respectively. However, all clones grew in the presence of chloramphenicol and not ampicillin, suggesting that the desired ligation had occurred.

Once they had been inserted into the hygromycin resistance vector, each construct was transformed into both an OR (C9-2) and a PA [C2(2)-1] *N. crassa* strain to perform a preliminary test for incompatibility activity. Activity was evaluated by comparing the proportion of large, wildtype-like

colonies formed in each transformation (Table 4), and by comparing the growth rates of subcultured transformants to rates observed in wildtype OR and PA transformants.

Table 4. The ratio of wildtype-like colonies over total transformants formed by the transformation of the mutant $un-24^{OR}$ constructs into C9-2 and C2(2)-1.

Construct		# of wildtype-like transformants total # of transformants	
		C9-2	C2(2)-1
1)	$un-24^{OR(A)}$	12/18	0/1
2)	$un-24^{OR(AB)}$	6/26	22/42
3)	$un-24^{OR(B)}$	7/40	23/52
4)	$un-24^{OR(ABC)}$	4/37	10/60

From Table 4, it can be observed that $un-24^{OR(A)}$ produced only one non-wildtype colony when transformed into the PA strain C2(2)-1, suggesting that it remains functionally OR in terms of incompatibility activity. $un-24^{OR(AB)}$, $un-24^{OR(B)}$, and $un-24^{OR(ABC)}$ (constructs 2, 3 and 4, respectively) all produced more colonies and multiple wildtype-like colonies when transformed into C2(2)-1. Additionally, the C2(2)-1 transformants containing constructs 2, 3 and 4, in each case, grew in a manner that closely resembled the wildtype C2(2)-1:: $un-24^{PA}$ transformants. As seen in Figure 1A, there is a noticeable asymmetry to the different incompatibility reactions such that when $un-24^{OR}$ is transformed into a PA background strain, the reaction is severely incompatible and no transformed colonies are formed (as seen in the $un-24^{OR(A)}$ case). This is defined as 'strong' incompatibility. In contrast, $un-24^{PA}$ transformed into an OR-background strain results in slow growing, 'star' colonies and is termed 'weak' incompatibility. Here, we see the formation of colonies when mutant $un-24^{OR(AB)}$, $un-24^{OR(B)}$, and $un-24^{OR(ABC)}$ genes are transformed into the PA strain, suggesting that they have switched from an OR to a PA specificity. However based on these analyses of growth rates and morphology, it does not appear as though these constructs are fully PA. While each of these modified OR constructs gained the ability to form colonies in the PA-background strain, the transformed colony growth rates were, on average, only

46% of wildtype growth rates and, in each case, when grown on VMM the transformed colonies produced a sparse mycelium in comparison to wildtype colonies. This suggests that some incompatibility with PA still existed. Figure 11 presents a summary of the activities of the four mutant *un-24*^{OR} constructs.

***un-24*⁰, a catalytically active allele without incompatibility activity**

The previous findings suggest that the 'B' site is the critical site in determining incompatibility specificity, since all three constructs *un-24*^{OR(AB)}, *un-24*^{OR(B)}, and *un-24*^{OR(ABC)} switched, to a degree, from an OR- to PA-activity form, and the E>V mutation at the 'B' site was the only one found in each of the three constructs.

Following this observation, an alignment was made using RNR1 protein sequences from a larger number of species including the additional fungi *Saccharomyces cerevisiae*, *Aspergillus fumigatus* and *Laccaria bicolor*, in addition to the plant, *Arabidopsis thaliana*, and the mammals *Mus musculus* and *Homo sapiens*, to examine what residues occur at the 'B' site in a broader context (Figure 12). All of the other species in Figure 12, excluding *S. cerevisiae*, possess a leucine residue at the 'B' site, in place of the *N. crassa* glutamic acid (OR) or valine (PA) and do not possess any known RNR-associated nonself recognition function. Like *N. crassa* UN-24^{OR}, Rnr1p of *S. cerevisiae* has a glutamic acid at this position and is already known to exhibit incompatibility-like phenotypes when transformed with *un-24*^{PA} (Smith *et al*, 2013b). This suggested that an E>L mutation at position 'B' in *un-24*^{OR} might exhibit a null (neither OR nor PA) incompatibility phenotype.

The *un-24*^{OR(E>L)} construct was produced using the same method as *un-24*^{OR(B)}, incorporating the base substitution in a unique reverse primer (un-24ORV-Lrv) to engineer the appropriate amino acid change. The construct was then transformed into DLL7-23 to ensure that it encoded an enzyme with

Figure 11. The incompatibility activity of four mutant *un-24^{OR}* constructs created in this study. The arrows on the left represent the final ~700 bp of each construct and indicate the positions and relative sizes of the introduced mutations. *un-24^{OR}* was altered to consist of sequence that would encode the desired 'A', 'B' and/or 'C' amino acid sequences from UN-24^{PA} and RNR1 as seen in Figure 7.

catalytic activity intact, and subsequently inserted into pCB1004, similarly to previous constructs. The incompatibility activity of *un-24*^{OR(E>L)} was determined using the same set of criteria used to determine that *un-24*^{OR(B)} had gained PA-like activity (Table 5). These criteria for identifying PA activity were the ability to form colonies in the PA strain, C2(2)-1, and growth rates approaching those observed in wildtype C2(2)-1 transformants.

Table 5. Ratio of wildtype-like colonies over total transformants following C9-2 and C2(2)-1 transformation with the *un-24*^{OR(E>L)} mutant construct.

Construct	# of wildtype-like transformants total # of transformants	
	C9-2	C2(2)-1
5) <i>un-24</i> ^{OR(E>L)}	7/35	9/40

As seen in Table 5, similar numbers of transformants were produced when *un-24*^{OR(E>L)} was transformed into either C9-2 or C2(2)-1. Additionally, when transformants were transferred to VMM, both C9-2:: *un-24*^{OR(E>L)} and C2(2)-1:: *un-24*^{OR(E>L)} transformants had growth rates and morphologies virtually identical to those of wildtype colonies. These results indicate that *un-24*^{OR(E>L)} does not have incompatibility activity; it is a null allele for incompatibility activity. To further verify this, *un-24*^{OR(E>L)} was transformed into DLL1-26 (*un-24*^{ts}, Δ *mus-52::bar*; *inl a*). The Δ *mus-52* mutation results in a loss of non-homologous end joining (NHEJ), thus facilitating gene replacement by homologous recombination. This allows for the replacement of the *un-24*^{ts} present in DLL1-26 with the new *un-24*^{OR(E>L)} via homologous recombination. To accomplish this, DNA sequence homologous to the *un-24*^{OR} downstream of the C-terminal had to be added to the 3' end of the *un-24*^{OR(E>L)} construct to allow for appropriate strand invasion during homologous recombination. About 60 bases of homologous DNA sequence was added on downstream of *un-24*^{OR(E>L)} using the *un-24*ORV-L3 flankRV1 primer (Table 2).

The resulting PCR product was cleaned using the Gel/PCR cleanup kit from FroggaBio, yielding ~30 ng/μL of DNA. *N. crassa* spheroplasts are typically transformed with ~1 μg of DNA to produce a large number of transformant colonies. In this case, spheroplasts were transformed with 750 ng (25 μL) of DNA that was mixed with 25 μL of 2.5 M sorbitol to maintain an appropriate osmotic pressure and avoid bursting of the spheroplasts. Two transformations were conducted in this manner and plates were incubated at the restrictive temperature of 39°C for selection. The two transformations yielded two and three colonies, respectively, from which DNA was isolated. A set of diagnostic PCR primers was then used to ensure that an entire replacement of the endogenous *un-24*^{ts} had been achieved, as opposed to a partial N-terminal replacement (replacing the temperature sensitive mutation and allowing for growth at restrictive temperatures), or an ectopic insertion of the *un-24*^{OR(E>L)} gene.

The two diagnostic primers (un-24OR-L and un-24OR-E) are identical aside from the two 3' nucleotides, which sit directly within the 'B' site that was mutated in the E>L construct. The un-24OR-E primer is designed to anneal with regular *un-24*^{OR} DNA while un-24OR-L should preferentially anneal with *un-24*^{OR(E>L)} DNA. PCR was done on DNA from each of the 5 transformants using one of the reverse primers paired with the un-24fw primer to produce an expected fragment of ~500 bp. If a complete replacement of *un-24*^{ts} had been accomplished, the PCR using the un-24OR-L primer would be the only one to produce a band. If a partial replacement was carried out (corresponding to the N-terminal region of *un-24*^{ts}), then only the un-24OR-E primer pair would be expected to produce a band. If the transformation resulted in an ectopic insertion of *un-24*^{OR(E>L)} then both sets of primers would be expected to produce bands. As controls, *un-24*^{OR} and *un-24*^{OR(E>L)} plasmid constructs were also amplified using the same sets of primers to ensure that the diagnostic primers were amplifying the expected templates. A gel showing the diagnostic bands from three of the five transformants is shown in Figure 13.

Figure 13. Diagnostic PCR of *un-24^{ts}* gene replacement. Ethidium bromide stained gel visualized under UV light following electrophoresis at 120V for 30 minutes in 1% agarose. Lane 1 represents a 1kb ladder that did not separate fully. All bands in lanes 2-11 represent amplifications using the un-24fw primer in combination with either un-24OR-E or un-24OR-L to produce fragments of ~500bp. Lanes 2 and 3 show amplification of *un-24^{OR}* with un-24OR-E and un-24OR-L reverse primers, respectively. Lane two is brighter than three showing that the un-24OR-E reverse primer preferentially binds when the sequence at the 'B' site is unaltered from the OR form. Lanes 4 and 5 show amplification of *un-24^{OR(E>L)}* with un-24OR-E and un-24OR-L reverse primers, respectively. Lane five is brighter than four, showing that the un-24OR-L primer binds preferentially when the sequence at the 'B' site has been altered from 'GA' to 'TT' to produce the E>L mutation. Lanes 6 and 7 show amplification of RR1-3 with un-24OR-E and un-24OR-L reverse primers respectively. Lane seven is brighter than six suggesting that RR1-3 contains a single, fully replaced, copy of *un-24^{OR(E>L)}*. Lanes 8 and 10 show amplification of RR1-4 and RR1-5 DNA, respectively, with un-24OR-E while lanes 9 and 11 show amplifications of the same DNA with the un-24OR-L primer. These bands indicate that the 'B' sites of the RR1-4 and RR1-5 DNA have not been altered from the OR form, suggesting that a partial replacement of the 5' end of the gene has occurred in these transformants to allow for growth of the strain at restrictive temperatures.

	Controls				Clones					
Template DNA	<u><i>un-24</i>^{OR}</u>		<u><i>un-24</i>^{OR[E>L]}</u>		<u>RR1-3</u>		<u>RR1-4</u>		<u>RR1-5</u>	
Reverse primer (<i>un-24</i> OR-E/L)	E	L	E	L	E	L	E	L	E	L

E>L

OR

OR

Figure 13 shows that a brighter band (more PCR product) is produced using the un-24OR-E primer with *un-24*^{OR} as the template, while a brighter band is observed from the reaction containing the un-24OR-L primer and *un-24*^{OR(E>L)} template, implying that the two primers bind preferentially to appropriate templates. Also of note in Figure 13 is that RR1-3 (lanes 6 and 7) appears to be a full, endogenous replacement of the *un-24*^{ts} with *un-24*^{OR(E>L)}, evidenced by the fact that a band is visible in lane 7 (un-24OR-L) and not lane 6 (un-24OR-E). RR1-4 and RR1-5 as well as RR1-1 and RR1-2 (not shown in this report) show the opposite pattern, suggestive of a partial N-terminal replacement of the temperature sensitive mutation, but not of the C-terminal domain. A PCR amplification of *un-24* from RR1-3 was later sent to be sequenced to verify that it did contain the GA>TT mutation indicative of the E>L switch.

Crosses and heterokaryon incompatibility tests

The next objective was to produce a strain that is fully null at the *het-6* locus. For this I selected *un-24*^{or(E>V)} Δ *het-6::hph*; Δ *mus-52::bar*; *inl* progeny from the cross: RR1-3 (*un-24*^{or(E>L)} *het-6*^{OR}; Δ *mus-52::bar*; *inl a*) X DLL8-16 (*un-24*^{ts} Δ *het-6::hph*, Δ *mus-52::bar*; *inl A*). About three weeks following set up of the cross, spores were harvested and were spread on a plate containing VMM *inl*⁺ medium with hygromycin, and were treated at 55°C for 50 minutes to initiate germination. Double selection on hygromycin and at the restrictive temperature allowed for only those progeny having undergone the appropriate recombination event to grow. This recombination event is expected to occur at a frequency of ~1.5% (M.L. Smith, personal communication), and is illustrated in Figure 14.

About 16 hours after heat treatment to initiate germination of ascospores, germlings were aseptically excised and placed into individual tubes containing VMM *inl*⁺ with hygromycin B and incubated at 39°C. Progeny selected in this way were subjected to heterokaryon incompatibility tests

Figure 14. Schematic representation of the recombination event required to select a full *het-6*-null strain ($un-24^{OR(E>L)} \Delta het-6::hph$) from the cross RR1-3 ($un-24^0 het-6^{OR}$; $\Delta mus-52::bar$; *inl a*) X DLL8-16 ($un-24^{ts} \Delta het-6::hph$, $\Delta mus-52::bar$; *inl A*). The frequency of the crossover event was anticipated to be ~1.5% and was actually observed in 1 – 3% of the progeny.

with A1 (*mat a*) and DLL30-16 (*mat A*) to identify mating type. Since mating type acts as an incompatibility gene during vegetative growth, compatible growth should only be observed between strains of identical mating type. The strain RR2-3 was identified as *mat a* (*un-24*^{OR(E>L)} Δ *het-6::hph*; Δ *mus-52::bar*; *inl a*) using this test.

Strain RR2-3 was then used in a second series of heterokaryon incompatibility tests to confirm that the *un-24*^{OR(E>L)} gene that appeared to be null following the transformation assays was indeed null. Heterokaryon incompatibility tests were done with strains that included an OR strain (A1), a PA strain [C2(2)-1], and a *un-24*^{OR} Δ *het-6* strain (DLL3-15). If null, RR2-3 was expected to form a compatible heterokaryon with each of these three strains; that is, the resultant heterokaryons should grow at wildtype rates and display wildtype morphology. Figure 15 displays the results of these heterokaryon tests. RR2-3 produced heterokaryons that grew at wildtype rates with all three of the tested strains, including an OR strain and a PA strain. This shows that RR2-3 is fully null at the *het-6* locus, and thus I conclude that the *un-24*^{OR(E>L)} construct produced in this study encodes a catalytically active RNR that has no incompatibility activity. From this point forward, *un-24*^{OR(E>L)} will be referred to as *un-24*⁰. Figure 16 presents a summary of the amino acid differences between UN-24^{OR}, UN-24^{PA}, UN-24^{OR(B)} and UN-24⁰ and their respective incompatibility activity activities in *N crassa*.

Structure/function analysis

Once substitutions at a single amino acid were identified to produce the differences between *un-24*^{OR}, *un-24*^{PA} and *un-24*⁰, a series of structural analyses was performed using online prediction tools to attempt to elucidate the structure-function relationship of the C-terminal arm of UN-24. The following caveats apply to using polypeptide structure prediction tools. Each prediction tool uses a distinct algorithm for prediction, leading to potentially non-identical structures being predicted with

Figure 15. Heterokaryon incompatibility tests verify that RR2-3 has no incompatibility activity associated with the *het-6* locus. Heterokaryon incompatibility was assayed by co-inoculation of RR2-3 and OR strain A1 (A), PA strain C2(2)-1 (B) and *un-24^{OR}Δhet-6* strain DLL3-15 (C). Growth rates at 30°C and colony morphologies in each case were similar to wildtype indicating that RR2-3 is compatible with both OR and PA background strains, and is thus completely null at the *het-6* locus.

Figure 16. Summary of the incompatibility activity UN-24^{OR}, UN-24^{PA}, UN-24^{OR(B)} and UN-24⁰. The black lines within grey rectangles represent the positions and relative sizes of the amino acid differences relative to the OR form for the C-terminal ~175 amino acid residues. The C-terminal ends of UN-24^{OR} and UN-24^{PA} differ at many positions. *un-24^{OR}* was altered to produce *un-24^{OR(B)}* and *un-24⁰* (which encode UN-24^{OR(B)} and UN-24⁰ respectively) by altering DNA to consist of sequence that would encode the desired forms E>V (to correspond to UN-24^{PA}, Figure 7) or E>L (to correspond with the large subunits of RNR from many other species, Figure 12). The UN-24⁰ protein was found to have no incompatibility activity and will be referred to as a null mutation.

different tools. Also, the majority of tools employ a BLAST search to examine other proteins with similar sequence motifs to the protein of interest (query), frequently consulting a database of proteins for which structures have been solved by crystallography. To date, elucidation of the structure of the RNR C-terminal domain by crystallography has not been possible due to its mobile nature; assigning secondary and tertiary to such protein domains based on structure predictions based on crystallography may not be accurate.

These tools can, however, be useful in preliminary and comparative structural studies, such as for this study. The final 63 residues (the highly variable C-terminal region shown to be capable of inducing incompatibility activity) of four forms of UN-24 were run through a series of web-based structure prediction tools. The four proteins examined were UN-24^{OR}, UN-24^{OR(B)}, UN-24⁰, and UN-24^{PA}. The former three proteins are identical, aside from the single amino acid substitution at the 'B' site, while the UN-24^{PA} is more variable throughout the 63 amino acids, though the final 13 residues are also identical with the exception of the 'B' site. When analyzed with the structure prediction tool Phyre² (Kelley and Sternberg, 2009), it can be observed that UN-24^{PA} is strikingly dissimilar to the three forms of UN-24^{OR}, which all form α -helices around the 'B' site (Figure 17 A1-D1). Interestingly, the Phyre² analysis also suggests that the number of amino acids involved in these C-terminal helices decreases from UN-24^{OR} (7 residues) to UN-24⁰ (5 residues) to UN-24^{OR(B)} (4 residues), suggesting that UN-24^{OR(B)} resembles UN-24^{PA} more closely than the other two proteins in the C-terminal region.

Phyre² (Protein Homology/analogY Recognition Engine V 2.0) produces predicted structures based on a generalized alignment of sequences with a profile hidden Markov model (HMM). It is used with BLAST, PSI-BLAST, HMMER, PROF_SIM and COMPASS to perform a comparison with a database of 3691 protein domains of known structure. It makes predictions of secondary structure using a two-stage

Figure 17. Structure-function analysis of UN-24 C-terminus. Phyre² polypeptide structure predictions of the 63 C-terminal amino acids of UN-24^{OR} (A1), UN-24⁰ (B1), UN-24^{OR(B)} (C1), and UN-24^{PA} (D1) and PEP-FOLD polypeptide predictions of the final 36 residues of the same four polypeptides (A2, B2, C2 and D2, respectively). The colours are indicative of sequence where blue is the N-terminus and red is the C-terminus of the 63 amino acid domain. α -helices are present in the C-terminal ends of UN-24^{OR}, UN-24⁰ and UN-24^{OR(B)} and are not observed in the prediction of UN-24^{PA} using either tool. It should be noted that the larger helices observed in the PEP-FOLD prediction are also predicted using Phyre² secondary structure prediction, but are not shown in the 3D structure provided.

neural network based on a position specific scoring matrix from PSI-BLAST and employs a system that provides a probable structure determination based on similarities in related proteins.

A second structure predictor, PEP-FOLD (Maupetit *et al*, 2009), was also used to generate 3D structures of the four proteins, and similar structures were obtained to those determined with Phyre² (Figure 17 A2-D2). Unlike UN-24^{OR}, UN-24^{PA} again did not form a helix in the C-terminus. Similar to the Phyre² analysis, UN-24⁰ had a reduced terminal α -helix. Unlike the BLAST approach of Phyre², PEP-FOLD predicts peptide structures based on amino acid sequence. It employs a Hidden Markov Model Structural alphabet (SA-HMM) approach that uses a limited number of recurrent structural elements of proteins (letters), which are governed by logic rules to form the words of protein structures. These letters are then assembled by an enhanced 'greedy' algorithm, which attempts to make a logically optimal choice at each step of assembly to approximate a global optimum in a reasonable time.

In addition to the 3D structures predicted for these four proteins by the two mentioned tools, secondary structure elements were predicted using an array of other tools including FOLDpro (Cheng and Baldi, 2006), PSSpred (Zhang, 2012), CONCORD (Wei *et al*, 2011) and Jpred3 (Cole *et al*, 2008). The common theme that was observed was the striking difference between the PA form and the other three forms in every case.

The presence of disulfide-forming cysteine residues was also predicted for each peptide using Dipro (Donald Bren School of Information and Computer Sciences, University of California, Irvine). Dipro functions in a two-step process: first it uses a support vector machinery to identify disulfide bonds in the sequence, and secondly it uses a 2D recurrent neural network and a regression algorithm to predict the number and pattern of the bonds. Dipro analysis predicted that UN-24^{PA} will not contain any disulfide bonded cysteine residues, while UN-24^{OR}, UN-24^{OR(b)} and UN-24⁰ all contain disulfide-forming cysteines. Furthermore, it identifies the cysteines at positions 913 and 924 as the residues most likely to form

disulfide bonds, excluding the C-terminal-most cysteine at position 927 from the bonds. The significance of these results will be examined in the following discussion.

Discussion

In this study we identified a single glutamic acid in the C-terminal region of the Oak Ridge form of the large subunit of the *Neurospora crassa* RNR, UN-24, that governs incompatibility specificity and activity. Upon switching the glutamic acid at position 925 in UN-24^{OR} to a valine (the homologous residue in UN-24^{PA}), the construct becomes PA-like in incompatibility specificity. When the same glutamic acid is substituted with a leucine residue (the residue found at the same location in RNRL of numerous other species) the construct loses incompatibility activity altogether in *N. crassa*.

These insights were gained following the transformation of the *rnr1* gene from *Cryphonectria parasitica* (*Cprnr1*), which also encodes the large subunit of RNR, into *N. crassa*. When transformed into *N. crassa*, the *Cprnr1* gene functioned in a manner almost identical to *un-24*^{PA}. This observation was initially unexpected considering that there is no RNR-associated incompatibility found in *C. parasitica*, and in fact, there are no known cases of RNR-associated incompatibility in any species other than *N. crassa*. It was for this reason that an RNR from *C. parasitica* was originally selected in an attempt to produce a catalytically functional RNR in *N. crassa* with no incompatibility activity.

Although the initial finding that *Cprnr1* contained PA-like activity in *N. crassa* was surprising, identifying a glutamic acid at the 'B' site, amino acid position 925, as at least partially responsible for incompatibility supported the idea. UN-24^{OR} contains the glutamic acid at the 'B' site, while UN-24^{PA} and CpRNR1 (the protein product of *Cprnr1*) both contain valine, and function in a PA-like manner. Additional support comes from a study by Smith *et al.* (2013b) where the incompatibility-inducing C-terminal domain of *un-24*^{PA} (termed *hygunPA*) was transformed into *Saccharomyces cerevisiae*. Much

like *C. parasitica*, *S. cerevisiae* does not possess RNR-associated incompatibility activity, yet when it was transformed with *hygunPA*, it exhibited a phenotype highly reminiscent of the incompatibility reaction seen in *N. crassa*, evidenced by cytoplasmic granulation, perturbation of vacuole integrity and growth inhibition. This suggests that Rnr1p in *S. cerevisiae* exhibits OR-like RNR-associated incompatibility activity, despite the fact that it does not apparently undergo this type of incompatibility reaction naturally. As seen in Figure 12, *S. cerevisiae* possesses a glutamic acid residue at the RNRL 'B' site that is akin to UN-24^{OR} from *N. crassa*, supporting the idea that this site is responsible for incompatibility specificity.

With *N. crassa* being the only known species to demonstrate RNR-associated incompatibility activity, we can pose an interesting evolutionary question: why would UN-24, an enzyme as fundamentally essential for DNA replication, evolve in *N. crassa* to have nonself recognition function? It seems probable that it signifies a requirement for a highly capable and severe form of nonself recognition. A form of nonself recognition involving inactivation of an essential enzyme like RNR is highly efficient. Hall *et al.* (2010) demonstrated that in the case of another *N. crassa* incompatibility locus, *het-c* (containing genes *het-c* and *pin-c*), haplotypes evolved as a result of a genomic rearrangement event that facilitated the insertion of *pin-c* near the *het-c* locus in an ancestor of *Sordaria* and *Neurospora*. These haplotypes subsequently diverged through mutation and limited recombination. This nonself recognition ability was determined to be a gain-of-function consequence of the genome rearrangement and divergence and not a disruption of proper function of the two involved genes. They suggest that the HET domain found in *pin-c* acts as a death effector and that following the rearrangement that formed *het-c pin-c* haplotypes, it enabled *pin-c* to act as a death effector upon nonself recognition, likely mediated by interactions between HET-C and PIN-C. We observe a similar phenomenon at the *het-6* locus, where *het-6* may have inserted next to *un-24*, and recruited UN-24 to have nonself recognition function. This again would represent a gain of function for the *un-24* gene. These findings indicate that

the strain RR2-3, developed in this study using *un-24*⁰, which is fully null at the *het-6* locus is not only severely 'immuno-compromised' but is likely much more similar to a *Neurospora* ancestor that did not possess RNR-associated incompatibility. This mutant could provide novel insights into the evolution of nonself recognition in *N. crassa*.

While it is plausible that those genes that encode HET-domain proteins have recruited other nearby genes into nonself recognition on an evolutionary scale, it is unknown whether sequence proximity remains imperative for the function of all of these nonself recognition systems. Proximity is at least not essential for the *tol* gene, (HET-domain protein, LGIV), which interacts with *mat* (LGI) in *N. crassa* to mediate heterokaryon incompatibility during vegetative growth. Experiments are currently ongoing in our lab to determine if sequence proximity between *het-6* and *un-24* is essential for nonself recognition function by moving *het-6* from its natural location (LGII) into the *csr-1* locus (LGI). *csr-1* encodes a cyclosporin A-binding protein, which when disrupted leads to a cyclosporin resistant phenotype, and provides an ideal location for single-copy gene insertion (Bardiya and Shiu, 2007). The experimental process consists of cloning *het-6* into a *csr-1* knock-in vector [developed by Bardiya and Shiu (2007)], which will be transformed into a *het-6* knockout strain. This will allow for the generation of an *N. crassa* strain containing a single copy of *het-6*, which will be found in the *csr-1* locus on LGI, that can be used in heterokaryon incompatibility assays to assess its nonself recognition activity.

In this study, a null form of UN-24 was created by switching a glutamic acid residue at position 925 of UN-24^{OR} with a leucine residue. Leucine was selected as a result of its presence at the same location in the RNR large subunits of numerous other species. It should be noted, however, that methionine and threonine are also commonly found at that location in other species and could also potentially be used to produce a null UN-24. The *un-24*⁰ construct produced in this study provides an extremely useful tool in examining heterokaryon incompatibility at the *het-6* locus. The first use of this

construct will be in the investigation of possible allelic interactions between the two variants of *het-6* (Figure 18). To date, any perturbation of *un-24* has resulted in a loss of catalytic RNR activity, and subsequent cell death. This has made it impossible to investigate whether or not *het-6* is involved in allelic interactions. By using *un-24*⁰ we will be able to investigate phenotypes produced through possible *het-6* associated incompatibility without interference from *un-24*-associated incompatibility. This will allow for a much clearer picture of the mechanisms governing incompatibility at this puzzling locus by clarifying the role that HET-6 proteins play in incompatibility. These studies are currently ongoing.

While we identified a single glutamic acid in the C-terminal region of UN-24^{OR}, that governs incompatibility specificity in *N. crassa*, it should be noted that a complete switch in specificity from OR to PA was not obtained, suggesting that there must be some additional minor difference(s) involved in PA-specificity. However, with the major residues governing RNR-associated incompatibility specificity identified, and the production of an RNR with no incompatibility activity, we seek to investigate the structure/function relationship of UN-24 in inducing incompatibility in *N. crassa*. This is a novel role for incompatibility, wherein it is used as a tool to understand the structure of a protein domain that cannot be crystallized, and to use that information to develop a model of structure-function relationship of a protein-protein interaction.

In terms of initial structural investigation, the ability of a single amino acid switch that is sufficient to bring about a change in incompatibility activity is worth discussion. While this might seem surprising, a similar effect was observed in the *het-s/het-S* system of *Podospora anserina* by Deleu *et al.* (1993). These two alleles differ by only 14 amino acids and eight of them were deemed to be potentially important in determining specificity based on sequence comparisons with another related species. Using site directed mutagenesis to alter amino acids and then examining the resulting phenotypes, they

Figure 18. A schematic representation of the *het-6* locus showing the test for allelic incompatibility interaction between *het-6*^{OR} and *het-6*^{PA} (red arrow) that can be investigated by transforming *het-6*^{PA} into an OR strain containing *un-24*⁰ (RR1-3).

identified one proline (P) > histidine (H) substitution that is sufficient to modify the activity from *het-S* specificity to *het-s*. Interestingly, they also determined that the inverse substitution, from H > P, did not result in a switch in specificity from *het-s* to *het-S*. Rather, two amino acid substitutions were required; aspartic acid and proline were substituted with alanine and histidine respectively to induce this *het-s* to *het-S* switch. The single amino acid substitution observed in this system is comparable to the *un-24^{OR}* to *un-24^{PA}* switch we observe. However, we have not yet introduced the full inverse mutation into *un-24^{PA}* to determine if the number of amino acids required is conserved between the two alleles. These experiments are also currently ongoing.

Following the identification of the glutamic acid/valine/leucine residues that alter the incompatibility activity and specificity of UN-24, structural predictions of polypeptides containing each of these residues were done using online web tools (Figure 17). The insights gained in this process will be applied to the current model of RNR-associated incompatibility in *N. crassa* in the following section.

With the reduction of the substrate in the catalytic pocket of one large subunits of the functional RNR tetramer, the redox-active cysteine pair within the active site is converted from the active dithiol form to an inactive disulfide-bonded form. Once this disulfide bond is formed in both RNRL subunits, the enzyme is inactive and the disulphide bond in the catalytic site must be reduced to return the enzyme to an active state. To accomplish this, the C-terminal arm of the neighbouring monomer enters the active site and conserved C-terminal cysteine residues reduce the active site disulfide bond. The current model of RNR-associated incompatibility by Smith *et al.* (2013a) hypothesizes that when RNR is comprised of an UN-24^{OR}/UN-24^{PA} heterodimer, cysteine residues in the C-terminal arm of the PA monomer form an aberrant disulfide bond when it reduces the disulfide bond of the OR catalytic pocket, forming an aberrant heterodimeric complex. A numbering system for the three C-terminal cysteine

residues is provided in Figure 19. The following list includes the information that was already taken into consideration in forming the current model, and the new insights provided in this study.

1. The C-terminal domain of one UN-24 subunit acts *in trans* to reduce the active site of the neighbouring monomer (Zhang *et al*, 2007; and also inferred in Smith *et al*, 2013a using the UN-24 system).
2. UN-24 forms a high molecular weight protein in incompatible *un-24^{OR} het-6^{OR}::un-24^{PA}* strain that is non-reducible until escape, whereby it resolves into monomeric sized proteins (Smith *et al*, 2013a).
3. The PA C-terminus incompatibility domain localizes to the pellet fraction in self-incompatible colonies following treatment with reductants and centrifugation, consistent with findings regarding large protein complexes, and to the lysate fraction following escape (Smith *et al*, 2013a).
4. UN-24^{OR} incompatibility activity is eliminated upon modification of any of the three cysteine residues in the C-terminal domain. UN-24^{PA}-associated incompatibility, on the other hand, is maintained when either of C2 or C3 is altered, suggesting that they operate in a redundant manner. In both the OR and PA forms, C1 is essential for incompatibility activity (Smith *et al*, 2013a).
5. Incompatibility specificity can be partially inversed by switching the UN-24^{OR} glutamic acid at position 925 with the UN-24^{PA}-specific valine. Activity can be eliminated by replacing the glutamic acid with a leucine residue at this same position.
6. Polypeptide structural predictions indicate that the C-terminal end of UN-24^{PA} does not form an α -helix, while the different forms of UN-24^{OR} do. Additionally, structural predictions suggest that the helix present in UN-24^{E>V} more closely resembles UN-24^{PA} than either the OR or null forms.

$\underline{\text{C}}$ A W N P G A D P S S $\underline{\text{C}}$ E M $\underline{\text{C}}$ S G
 1 2 * 3

Figure 19. The 17 C-terminal-most amino acid residues of UN-24^{OR} highlighting the three cysteines conserved in UN-24^{PA} and most other RNR large subunits. From N-terminus to C-terminus, the three cysteines are designated C1, C2 and C3, respectively. Also shown is the glutamic acid residue that was substituted with a leucine to produce UN-24⁰ (*).

7. A separate prediction suggests that UN-24^{PA} will not contain disulfide-bonding cysteine residues in the C-terminal domain, while UN-24^{OR}, UN-24^{OR(B)} and UN-24⁰ all would.
8. Prediction software also indicates that the most likely disulfide bond formed in the C-termini of various forms of UN-24 would be between C1 and C2.
9. Hydrophobic residues like valine and leucine generally are not found in close sequence proximity to cysteines that are active in disulfide bridge formation, while polar and charged residues like glutamic acid do tend to be found in proximity to those cysteines and favour disulfide bridge formation (Petersen *et al*, 1999).

The above points allow for a more in depth picture of UN-24-associated incompatibility and allow us to make more inferences concerning the model. As a caveat, it should be noted again that there are uncertainties to structural predictions based on available software based on mathematical algorithms and sequence comparisons. Furthermore, there remains a substantial amount of information to be uncovered concerning this *het-6* locus, and its functioning. With these caveats in mind, the structural analyses presented herein present novel pieces to this puzzle and allow for inferences to be made and the model to be updated.

Nevertheless, we present a model of allelic UN-24 incompatibility (Figure 20) in the current study similar to that suggested in Smith *et al.* (2013a), whereby the C-terminal arm of the PA monomer becomes trapped in the OR active site following entry to reduce the disulfide bond formed during the substrate reaction. The N-terminal domains of UN-24^{OR} and UN-24^{PA} are identical, and there is no incompatibility observed in cases where the UN-24 complex is homodimeric. This suggests that a structural rearrangement must occur in the active site before either arm could become trapped in the active site. In our model, the UN-24^{OR} arm enters the active site of the UN-24^{PA} monomer and reduces the disulfide bond, bringing about a structural rearrangement within its active site pocket. Subsequently,

Figure 20. A model of allelic RNR-associated incompatibility in *N. crassa*. Each panel displays the functional holoenzyme consisting of two small subunits (RNRS) and two large subunits (UN-24). Note that the subunits are not drawn to scale. A) displays a compatible interaction between two subunits of a homodimer (UN-24^{PA}, in this case). A1) Following the substrate reaction, a redox-active cysteine pair within the active site of both monomers is in an inactive disulfide bonded form, while a cysteine pair on the C-terminal arm of either monomer is in the free dithiol form. A2 & A3) To return the enzyme to the active form, the C-terminal arms enter the active sites of their neighbouring monomer and reduce the disulfide bonds, becoming disulfide bonded themselves. A4) The C-terminal arm exits the catalytic pocket and the disulfide bonded cysteine pair is reduced by either thioredoxin or glutaredoxin, returning the enzyme to the active form. B) represents the incompatible interaction between UN-24^{OR} (red) and UN-24^{PA} (blue). B1) Following the substrate reaction, a redox-active cysteine pair within the active site of both monomers is in an inactive disulfide bonded form, while a cysteine pair on the C-terminal arm of either monomer is in the free dithiol form. B2) To return the enzyme to the active form, the C-terminal arms enter the active sites of their neighbouring monomer to reduce the disulfide bond. The active site of the PA monomer is reduced by the OR first, as the cysteine pair on the OR arm more willingly forms a disulfide bond. This bond formation brings about a physical alteration in the OR active site. B3) Following the physical alteration of the OR active site, one of the cysteines on the flexible C-terminal arm of the PA monomer forms an aberrant disulfide bond with an active site cysteine. This aberrant bond causes the arm to become trapped in the active site, and a non-reducible, toxic aggregate is formed. It should be noted that in both cases, only the cysteine residues involved in disulfide bond formation are shown.

when the UN-24^{PA} arm enters the modified UN-24^{OR} active site, it becomes trapped. Based on the structural predictions of the two different proteins, the C-terminal end of UN-24^{PA}, where the redox active cysteines are found, is much more flexible as compared to the OR form, that contains an α -helix. This flexibility may allow one of the C-terminal cysteines to form an aberrant disulfide bond with one of the cysteines within the active site. In the PA form, only C1 and one of C2 or C3 is required for incompatibility activity to remain intact. In our model, the flexibility inherent in the C-terminus allows for either of the C-terminal cysteines to form this aberrant bond in the rearranged active site.

We have shown that replacing the glutamic acid at position 925 of UN-24^{OR} with the UN-24^{PA}-specific valine alters the incompatibility specificity, causing it to gain PA-like activity. Using secondary structure prediction tools we showed that this E>V mutation results in a significantly shorter stretch of α -helix at its C-terminus, giving it more flexibility than the unmutated OR form, and giving it an appearance closer to that of the PA form, supporting our model in which a lack of the rigid helix allows for aberrant bond formation. Interestingly, the *un-24*^{OR(E>V)} construct did not completely lose OR activity upon gaining PA activity, nor does the predicted structure completely lose its C-terminal helix. These two observations are potentially linked and warrant further investigation.

Prediction software and neighbouring amino acid residue analysis indicate that the C-terminal arm of UN-24^{PA} is less capable at forming disulfide bonds than its OR counterpart. We do know that disulfide bond formation must be possible, however, as it is required for proper catalytic activity by UN-24^{PA}. In our model, the UN-24^{PA} C-terminal arm does not form an aberrant bond until after the OR arm reduces the PA active site, as this brings about a structural change in the OR active site, facilitating the formation of an aberrant bond between an active site cysteine and a cysteine on the flexible PA C-terminal arm. This information suggests that even if the two arms enter the neighbouring active sites at the same time, the OR arm will reduce the PA cysteine first, facilitating the initiation of the aberrant

bond by the PA arm. This reduces the probability of the PA arm reducing the OR active site first, leading to the reactivation of a functioning, compatible RNR.

While a weak allelic incompatibility is observed in *un-24^{OR}::un-24^{PA}* strains when no functional *het-6* is present (as outlined in the model presented in Figure 20), the reaction is significantly more severe in the presence of *het-6* (Lafontaine and Smith, 2012). Additionally an intermediate nonallelic incompatibility reaction is observed between *un-24^{PA}* and *het-6^{OR}*. The role that HET-6 plays in this RNR-associated incompatibility remains unclear but it has been hypothesized that it may down regulate transcription of *un-24*, or function in a protein-protein interaction with UN-24, allowing it to transduce a death signal to a PCD pathway, or it may physically interact with UN-24 to form a toxic protein. We present possible models for *het-6*-induced incompatibility (Figure 21). *het-6* is able to induce an intermediate incompatibility in the presence of the other allelic form of *un-24* even when the UN-24 heterodimer is not present. In our model, HET-6 would bind to the C-terminal arm of UN-24 following disulfide bond formation within the active sites. HET-6 would then act in a signal transducer only if it interacted with the unique structure of the arm of UN-24 of the opposite allelic type. We also hypothesize that if HET-6 interacts with the incompatible form of UN-24 following the formation of an aberrant disulfide bond, it recognizes an altered structure on the C-terminal arm, and transmits a signal to a different pathway, resulting in the more severe incompatibility reaction seen in *un-24^{OR} het-6^{OR}* / *un-24^{PA} het-6^{PA}* incompatible strains.

The RR2-3 (*un-24⁰ Δhet-6*) strain produced in this study showed no incompatibility with an *un-24^{PA} het-6^{PA}* or an *un-24^{OR} het-6^{OR}* strain. This indicates that switching the OR glutamic acid to leucine produced a new construct lacking both allelic and non-allelic incompatibility activity. This single amino acid substitution in the incompatibility-active region of UN-24 inhibits any non-allelic incompatibility, which indicates that HET-6 can no longer bind to UN-24 to elicit the reaction. This suggests that the

Figure 21. Possible models of non-allelic incompatibility observed between *un-24^{PA}* and *het-6^{OR}*.

Interactions between only *un-24^{PA}* and *het-6^{OR}* induce an intermediate incompatibility reaction (stronger than the allelic reaction seen between *un-24^{OR}* and *un-24^{PA}*), while interactions between *un-24^{OR}* *het-6^{OR}* and *un-24^{PA}* *het-6^{PA}* bring about a strong incompatibility reaction. A) One model of non-allelic incompatibility consists of *het-6* down regulating the transcription of *un-24*. B) The second model involves protein-protein interactions between UN-24 (the subunits are not drawn to scale) and HET-6. On the left is the interaction between UN-24^{PA} and HET-6^{OR} that induces an intermediate incompatibility reaction. Here, HET-6^{OR} binds to the unique structure of the C-terminal arm of UN-24^{PA} following reduction of the active site cysteines and subsequently transduces a signal to a death pathway. On the right side, is the interaction between UN-24^{OR} HET-6^{OR} and UN-24^{PA} HET-6^{PA} that causes a strong incompatibility reaction. Here, again, HET-6 binds to the C-terminal arm of UN-24, and acts in a signal transduction pathway only if interacting with the unique structure of the arm of UN-24 of the opposite allelic type. Furthermore, we hypothesize that if HET-6 interacts with the opposite form of UN-24 following the formation of an aberrant disulfide bond, it induces a secondary mechanism, resulting in a more severe incompatibility.

A

B

protein-protein interaction role for HET-6 is more likely than the downregulation of transcription, and warrants further investigation.

The information uncovered in this study provides novel insights into the function of a highly complex heterokaryon incompatibility locus in *N. crassa*. Heterokaryon incompatibility in *N. crassa* shares many parallels with nonself recognition systems seen in other eukaryotes. As we move closer to a thorough understanding of incompatibility in *N. crassa* we will start to be able to make new inferences in higher eukaryotes, humans included. In addition to this, the UN-24 (RNR) protein examined herein represents a very interesting candidate for the production of small peptide antibiotics, since it has been shown to induce PCD in species that do not possess their own intrinsic RNR-associated incompatibility (Smith *et al*, 2013b). In this study we identified a single amino acid that governs specificity in RNR-associated incompatibility. This has the interesting potential to identify what strains or species could, theoretically, be targeted with UN-24 small peptide antibiotics, and could potentially be utilized in the adjustment of such drugs to differentiate between host and pathogen.

This study can also be viewed from a different angle, where we take advantage of nonself recognition properties to shed new light on a structure-function relationship that had previously been unclear. This represents the first time nonself recognition has been used in this capacity. Based on information obtained from nonself recognition characteristics, and using peptide prediction software, we produced a model of protein-protein interactions that cannot be visualized by crystallization. This technique could be utilized in future studies to understand a wide range of other nonself recognition-associated proteins even when crystal structures are not obtainable.

Appendix I

Alignment of *Neurospora crassa* *un-24^{OR}*, *un-24^{PA}*, and *Cryphonectria parasitica* *rnr1*.

	Start	Introns
Ncun-24OR	CACATCAGACATTATGTATGTCAAGAAGCGCGGTATGTTACGACTCTCCCTTCCTACCC	
Ncun-24PA	-ACATCAGACATTATGTATGTCAAGAAGCGCGGTATGTTACGACTCTCCCTTCCTAC--	
Cprnr1	-----ATGTTTGTCAAGAAAGCGAGGTATGT-----CTCTCATGGAATATAT	
Ncun-24OR	CAATACCATCATCACGCCCA--ATGCTAACTCTCTCCTCATAGATGGCCGCCAGGAGCG	
Ncun-24PA	TAATACCATCATCACGCCCA--ATGCTAACTCTCTCCTCATAGATGGCCGCCAGGAGCG	
Cprnr1	CAACATCAGTATT-CAAACAGGCCAACTAA-TCTATTCTTACAGACGGACGCCAGGAGCG	
Ncun-24OR	CGTCCAGTTCGACAAGATCACAGCTCGTGTTCGAGGCTCTGTTATGGCCTCGACATGAA	
Ncun-24PA	CGTCCAGTTCGACAAGATCACAGCTCGTGTTCGAGGCTCTGTTATGGCCTCGACATGAA	
Cprnr1	CGTCCAGTTCGACAAGATCACAGCTCGTGTTCGAGGCTCTGTTATGGCCTCGACATGAA	
Ncun-24OR	CCATGTTGATCCTGTAGCCATTACACAAAAGGT CATCTCTGGCGTCTACGGTGGCGT CAC	
Ncun-24PA	CCATGTTGATCCTGTAGCCATTACACAAAAGGT CATCTCTGGCGTCTACGGTGGCGT CAC	
Cprnr1	GCATGTTGATCCTGTAGCCATTACACAAAAGGT CATCTCTGGCGTCTACGGTGGCGT CAC	
Ncun-24OR	CACAGCACAACCTAGACGATCTGGTAAGCACC-TTCTCCCCA--GTCCCTCTCCT-----	
Ncun-24PA	CACAGCACAACCTAGACGATCTGGTAAGCACC-TTCTCCCCA--GTCCCTCTCCT-----	
Cprnr1	AACAGTCCAGCTCGACGATCTGGTCCGTACCATTCATCCCATAGTTCTTTTCCCCTTCTCT	
Ncun-24OR	-----TGTACCGATTAT-----AAATCACGAGCTAAC-ACATAATCTTTCCT--AGGCTG	
Ncun-24PA	---CTTGTACCGATTAT-----AAATCACGAGCTAAC-ACATAATCTTTCCT--AGGCTG	
Cprnr1	GGCCTTCCACCGCACATCTGGGGCCCAAATGCTGACTATGTTGTCTTACCTTCCAGGCTG	
Ncun-24OR	CCGAGACTGCCGCCTACATGACCGTCACCCACCCCGACTATGCCATCCTCGCCGCCCGTA	
Ncun-24PA	CCGAGACTGCCGCCTACATGACCGTCACCCACCCCGATTTATGCCATCCTCGCCGCCCGTA	
Cprnr1	CCGAGACTGCCGCCTACATGACCGTCACCCACCCAGACTATGCCCTCCTCGCCGCCCGTA	
Ncun-24OR	TCGCCGTGTCCAACCTCCACAAGCAGACCAAGAAGCAATGGTCGCTCGTTATCAGCGAGC	
Ncun-24PA	TCGCCGTGTCCAACCTCCACAAGCAGACCAAGAAGCAATGGTCGCTCGTCATCAGCGAGC	
Cprnr1	TCGCCTGTGTCCAACCTCCACAAGCAGACCAAGAAGCAATGGTCATCGGTCTGAGCGATC	
Ncun-24OR	TGTACAACCTATGTCAACCCCAGGACCGGAAAGCACTCCCCCATGATCGCCAAGGACGTAT	
Ncun-24PA	TGTACAACCTATGTCAACCCCAGGACCGGAAAGCACTCCCCCATGATCGCCAAGGACGTAT	
Cprnr1	TGTACCACTATGTCAACCCAAAGGAACAATCGCCCGTCTGCCTATGATCTCCAGGAGACCT	
Ncun-24OR	ACGAGTGTGT CATGCGCCACAAGGACGAGTTCGACTCGGCAATTGTCTACGACCGTGACT	
Ncun-24PA	ACGAGTGTGT CATGCGCCACAAGGACGAGTTCGACTCTGCAATTGTCTACGACCGTGACT	
Cprnr1	ACGACTGTGTGATGCGTCACAAGGAGGAGTTTGACTCCGCCATCGTCTATGACCGAGACT	
Ncun-24OR	TCAACTACCAATACTTCGGCTTCAAGACCCTCGAACGATCATACCTTCTTAAGCTCGATG	
Ncun-24PA	TCAACTACCAATACTTCGGCTTCAAGACCCTCGAACGATCATACCTTCTTAAGCTCGATG	
Cprnr1	ACCACTACCAATACTTCGGCTTCAAGACCCTGGAGCGTTCTGTATCTTCTTAAGCTCAACG	

Ncun-24OR GCCAGATTGCTGAGCGTCCCCAGCACATGATCATGCGTGTTGCTGTCCGGTATCTGGGGAG
 Ncun-24PA GCCAGATTGCTGAGCGTCCCCAGCACATGATCATGCGTGTTGCTGTCCGGTATCTGGGGAG
 Cprnr1 GCAAAGTTGTGAGCGGCCACAGCACATGCTGATGCGTGTCGCGTGGCATCTGGGGAG

Ncun-24OR ACGACGTTGAGCGGGTCATTGAGACTTACAACCTCATGTCCCTCAAGACCTTCACCCACG
 Ncun-24PA ACGACGTTGAACGGGTCATTGAGACTTACAACCTCATGTCCCTCAAGACCTTCACCCACG
 Cprnr1 ACAACATCGAGAGGGTCTGAGACGTACAACCTACCTCTCCAAACAGTTTTTCACAACAG

Ncun-24OR CTTCTCCACCCCTCTTCAACGCCGGTACTCCCCAGCCCCAGCTCTCTTCGTGCTTCCTGG
 Ncun-24PA CTTCTCCACCCCTCTTCAACGCCGGTACTCCCCAGCCCCAGCTCTCTTCGTGCTTCCTGG
 Cprnr1 CCTCGCCGACTCTGTTCAATGCCGGCACGCCCTCAGGCAACAGCTGTGTCGTGCTTCCTTG

Ncun-24OR TCGACATGAAGGAAGACAGTATCGAGGGCATCTACGATACCCTCAAGACCTGCGCCATGA
 Ncun-24PA TCGACATGAAGGAAGACAGCATCGAGGGCATCTACGATACCCTCAAGACCTGCGCCATGA
 Cprnr1 TAGACATGAAGGAGGACAGCATCGAGGGTATTTACGATACCCTCAAGACCTGCGCCATGA

Ncun-24OR TCTCTAAGATGGCTGGCGGTATCGGTCTCAACATCCACCGCATTTCGTGCCACTGGCTCCT
 Ncun-24PA TCTCTAAGATGGCTGGCGGTATCGGTCTCAACATCCACCGCATTTCGTGCCACTGGCTCCT
 Cprnr1 TTTCCAAGATGGCCGGAGGCATTGGTCTCAATGTCCACCGCATCCGTGCCACTGGCTCTT

Ncun-24OR ACATCGCTGGTACCAACGGCACATCCAACGGTATCGTTCCCATGCTCCGTGTCTTCAACA
 Ncun-24PA ACATCGCTGGTACCAACGGCACATCCAACGGTATCGTTCCCATGCTCCGTGTCTTCAACA
 Cprnr1 ATATTGCCGGCACTAACGGCACGTCCAATGGCATCATCCCCATGCTGCGTGTCTTCAACA

Ncun-24OR ACACCGCCCGCTACGTTGACCAGGGTGGCAACAAGCGTCCTGGTGCTTTGCCCATCTACC
 Ncun-24PA ACACCGCCCGCTACGTTGACCAGGGTGGCAACAAGCGTCCTGGTGCTTTGCCCATCTACC
 Cprnr1 ACACTGCTTCGCTATGTGACCAGGGTGGCAACAAGCGTCCTGGCGCCTTTGCCCATCTACC

Ncun-24OR TCGAGCCTTGGCACGCCGATGTCTTCGAGTTCCTTGACCTCCGCAAGAACCACGGTAAGG
 Ncun-24PA TCGAGCCTTGGCACGCCGATGTCTTCGAGTCCCTTGACCTCCGCAAGAACCACGGTAAGG
 Cprnr1 TGGAGCCATGGCACGCTGATGTCTTTGAGTTCCTTGACCTGCGCAAGAACCACGGAAGG

Ncun-24OR AAGAGGTGCGCGCTCGCGATCTCTTCCTTGCCCTCTGGATCCCCGATCTTTTCATGAAGC
 Ncun-24PA AAGAGGTGCGCGCTCGCGATCTCTTCCTTGCCCTCTGGATCCCCGATCTTTTCATGAAGC
 Cprnr1 AAGAGGTTCGTGCCCGTGACCTCTTCCTGGCCCTCTGGATCCCCGATCTCTTCATGAAGC

Ncun-24OR GTGTTGAGCAGAACGGCCAGTGGACTCTCATGTGCCCTCACGAGTGCCCTGGCCTTGCCG
 Ncun-24PA GTGTTGAGCAGAACGGCCAGTGGACTCTCATGTGCCCCACGAGTGCCCTGGCCTTGCCG
 Cprnr1 GTGTGGAGAAGAACGGTGACTGGACCCTCATGTGCCCCAACGAGTGCCCTGGCCTGGCCG

Ncun-24OR ACGTTTACGGTGACGAGTTCGAAGCTCTGTACGAAAAGTACGAAAAGGAAGGCAAGGGTC
 Ncun-24PA ACGTTTACGGTGACGAGTTCGAAGCTCTGTACGAAAAGTACGAAAAGGAAGGCAAGGGTC
 Cprnr1 ACTGCTACGGTGAGGAGTTCGAGGCTTTGTACGAGAAATATGAGCGCGAGGGTCGCGGCC

Ncun-24OR GCAAGACCGTCAAGGCCCAGAAGCTTTGGTATGCCATCCTCGAGGCCCAGACTGAGACGG
 Ncun-24PA GCAAGACCGTCAAGGCCCAGAAGCTCTGGTATGCCATCCTCGAGGCCCAGACTGAGACGG
 Cprnr1 AGAAGACCATGAAGGCAAGAAGCTCTGGTATGCCATCCTCGAGGCCGACAGACCGAGACCG

Ncun-24OR GCAACCCCTTCATGCTCTACAAGGATGCCTGCAACCGCAAGAGTAACCAGAAGAACCTGG
 Ncun-24PA GCAACCCCTTCATGCTCTACAAGGATGCCTGCAACCGCAAGAGCAACCAGAAGAACCTGG
 Cprnr1 GCAACCCATTTCATGCTGTATTAAGGACCAAGCCGCAACCGCAAGAGCAACCAGAAGAACCTGG

Ncun-24OR GTACCATCCGACGCTCCAACCTGTGCACTGAGATTATCGAGTACTCTGCTCCGGATGAGG

Ncun-24PA GTACCATCCGCAGCTCCAACCTGTGCACTGAGATTATCGAGTACTCTGCTCCGGATGAGG
Cprnr1 GCACCATCCGCAGCTCCAACCTTTGCACTGAGATCATCGAGTACTCTGCCCTGATGAGG

Ncun-24OR TTGCCGTTTGCAACCTTGCTCTCTCGCCCTTTCCGCCTTTATCGACTACGAGAATGCCT
Ncun-24PA TTGCCGTTTGCTTACCTTGCTCTCTCGCCCTTTCCGCCTTTATCGACTACGAGAATGCCT
Cprnr1 TTGCTGTATGCAACCTGGCGTCTATCGCCCTGCCTCAGTTTCTGCTCGACTACGAGAATGACT

Ncun-24OR CTTACGACTTCAAGAAGCTGCACGAGGTCACTCAAGTCGTCTGCGCAACCTCAACAAGA
Ncun-24PA CTTATGACTTCAAGAAGCTGCACGAGGTCACTCAAGTCGTCTGCGCAACCTCAACAAGA
Cprnr1 GCTACAACCTTCAAGCTGCTGCATGAAGTCACCCAGGTGTGCTGTCTGCAATCTGAACAAGA

Ncun-24OR TCATTGACATCAACCACTACCCCGTTAAGGAGGCCACAACAGCAACATGCGTCACCGTC
Ncun-24PA TCATTGACATCAACCACTACCCCGTTAAGGAGGCCACAACAGCAACATGCGTCACCGTC
Cprnr1 TCATCGATTATCAACCACTATCCAGTTCCAGGAGGCTCGCAACAGCAACATGCGGCACCGGC

Ncun-24OR CCATTGGTGTGCGGTGTTCAAGGCTCTTGTGATGCTTTCTTGGCTCTCCGCATGCCCTTCG
Ncun-24PA CCATTGGTGTGCGGTGTTCAAGGCTCTTGTGATGCTTTCTTGGCTCTCCGCATGCCCTTCG
Cprnr1 CTATCGGCGTGGGTGTTCAAGGCTCTGGCTGATGCTTTCTTGGCTCTCCGCCTTCCCTTCG

Ncun-24OR ATTCTGACGCGGCTAGCAAGCTCAACATCCAGATTTTCGAGACCATCTACCACGCCGCTC
Ncun-24PA ATTCTGACGCGGCTAGCAAGCTCAACATCCAGATTTTCGAGACCATCTACCACGCCGCTC
Cprnr1 AGTCGCCCGAGGCTCGTCAGCTGAACAAGCAATCTTTGAGACCATCTACCACGCCGCGC

Ncun-24OR TTA CTGCCTCTTGCCAGCTCGCCAAGGAGCAGGGCCCGTACGCCACCTACGAAGGCTCAC
Ncun-24PA TTA CTGCCTCTTGCCAGCTCGCCAAGGAGCAGGGCCCGTACGCCACCTACGAAGGCTCGC
Cprnr1 TCACCGCATCGTGCAGCATTGCCAAGGTGGACGGTCCCTACTCATCGTACGAGGGATCTC

Ncun-24OR CCGTCTCCCAAGGAATCCTCCAATACGACATGTGG- - AACGTCACCCCTACCAACCTCT
Ncun-24PA CCGTCTCCCAGGGAATCCTCCAATACGACATGTGG- - AACGTCACCCCTACCAACCTCT
Cprnr1 CCGTCTCTCAGGGGATCCTCCAGTATGATATGTGGGACAAACGTCAAGCCCTCTGACCTCT

Ncun-24OR GGGACTGGACTGCTCTCAAGGCGGACATCAAGAAGTACGGTGTTCGCAACAGTCTGCTGC
Ncun-24PA GGGACTGGACTGCTCTCAAGGCGGACATCAAGAAGTACGGTGTTCGCAACAGTCTGCTGC
Cprnr1 GGGACTGGGACTCCCTGAAAGCCAAGATCAAGCAGCATGGTGTTCGCAACAGCTTGTGA

Ncun-24OR TTGCTCCTATGCCCACTGCTTCCAATTCTCAGATTCTTGGCAACAACGAGTGCTTCGAGC
Ncun-24PA TTGCTCCTATGCCCACTGCTTCCAATTCTCAGATTCTTGGCAACAACGAGTGCTTCGAGC
Cprnr1 TGGCGCCTATGCTTACTGCCTCGACTTCGCAGATCCTGGGCAACAACGAGTGCTTTGAGC

Ncun-24OR CTTACACCTCTAACATCTACCAACGACGTGTGCTCGCCGGCGAGTTCAGGTCGTCAACC
Ncun-24PA CTTACACCTCTAACATCTACCAACGACGTGTGCTCGCCGGCGAGTTCAGGTCGTCAACC
Cprnr1 CCTATACGTCCAACATCTACCAACGACGTGTGCTCGCTGGTGAGTTCAGGTCGTCAACC

Ncun-24OR CCTGGCTTCTCAGGGATCTTGTGGAGATGGGCCTTTGGTCTGATGCCATGAAGAACCGCA
Ncun-24PA CCTGGCTTCTCAGGGATCTTGTGGAGATGGGCCTTTGGTCTGATGCCATGAAGAACCGCA
Cprnr1 CGTGGCTGCTCAAGGACCTTGTGCAGTGTGGTCTGTGGTCCGAGGACCTGAAGAACCGCA

Ncun-24OR TCATTGCTGAGGGCGGTTCTATCCAGAACATCCAAAGCATTCCAATGACATCAAGGCTC
Ncun-24PA TCATTGCTGAGGGCGGTTCTATCCAGAACATCCAGAGCATTCCAATGACATCAAGGCTC
Cprnr1 TCATTGCCGAGGATGGTTCTATTCCAGAACATTCGCAGCATCCCGGATGATATCAAGGCTC

Ncun-24OR TCTATTAAGACCGTCTGGGAGATTTCTCAGCGTACCATTGTCAAGATGGCTGCCGATCGTG
Ncun-24PA TCTACAAGACCGTCTGGGAATTTCTCAGCGTACCATTGTCAAGATGGCTGCCGACCGTG

Cprnr1 TCTACAAGACCGTATGGGAGATCTCAGAGGACCATCGTTCAGATGGCCGCCGACCGTG

Ncun-24OR GTGCCTTCATTGATCAGTCCCAGTCCCTCAACATTACATGCGCGAACCCACCATGGGCA

Ncun-24PA GTGCCTTCATTGATCAGTCCCAGTCCCTCAACATTACATGCGCGAACCCACCATGGGTAA

Cprnr1 GTGCCTTCATCGATCAGTCTCAGTCCCTGAACATCCACATGCGTGAACCTTCTATGGGCA

Ncun-24OR AGATTACCAGCATGCATTTGCGCGGCTGGAAGATGGGTCTCAAGACCGGCATGTACTACC

Ncun-24PA AGATTACCAGCATGCATTTGCGCGGCTGGAAGATGGGTCTCAAGACCGGCATGTACTACC

Cprnr1 AGATCACGAGCATGCATTTACCGGGTGGAAGCTGGGTCTCAAGACTGGCATGTACTACC

Ncun-24OR TCCGTACGCAGGCTGCTGCTCAGCCC--ATCCAGTTCACTGTAGACCAGGAGGCTCTCAG

Ncun-24PA TCCGTACCCAGGCTGCTGCTCAGCCC--ATCCAGTTCACTGTAGACCAGGAGGCTCTCAG

Cprnr1 TCCGCACCCAGGCTGCGGC--AGCCCGATTCAGTTCACCGTCGACCAGCAGGCCCTCAA

Ncun-24OR GGCCACGGATGACCGAGTGGCCCCAGCCACTCGGGTCTTAAGAAGCGCTCCCCTCCCGC

Ncun-24PA GGCCACGGATGACCGAGTGGCCCCAGCCACTCGGGTCTTAAGAAGCGCTCCCCTCCCGC

Cprnr1 GATCCAGGA--GCAGGCTGGCGTGAAGC--ACAGCGGCCTGGGCAACGTCGCGCTCCGGC

Ncun-24OR TGGCACTTATACGTCTATCGTGTTGAGGGAGAACAACCTTCGGGCCCCCGCCCGTATGCCCA

Ncun-24PA TGGCACTTATACGTCTATCGTGTTGAGGGAGAACAACCTTCGGGCCCCCGCCCGTATGCCCA

Cprnr1 TGGCTACTACACTTCCACCTCTCT-----CGTCCGCCCCATGCACGTG-ACCAA

Ncun-24OR GACTGGTGTAGTGG--CACTAGCACACCCATCGGCACCAGGGATGTTCCCACACCCGC

Ncun-24PA GACTGGTGTAGTGG--CACTAGCACACCCATCGGCACCTAGGGATGTTCCCACACCCGC

Cprnr1 GGATCTTCTGTGAGAACACCAACATTACCATGAGCAACAATGGAATCCCTACCCCTTC

Ncun-24OR CAGCACACC--TCCTCCCACTGAGGTTCTTGAACTCTTGT-----ACAGAGTGACAA

Ncun-24PA CAGCACACC--TCCTCCTGCTGAAGATTCTGAGACTCTTGT-----ACAGAGTGACAA

Cprnr1 CGTTACGCCAAAGTCTC--GTCGCCACCCTG--ACTCTGTGCGTTGACAAGGAGGCA

Ncun-24OR CAGGCCCGGTCCACTTGTCTGCCCCGCCAA-----GAGTGCCGGTTTCAAGGCCGATT

Ncun-24PA CAGGCCCGGCTCCACTGGTCTGCCCCGCCAA-----GAATGCCGGCTTCAAGGCCGATCT

Cprnr1 CTGCACGCATC--ATGGCCTCTCCCTCCAAACCCAGGAATGTGCCATTCAAGGCCGATCA

Ncun-24OR GCCCGAGCCTGAGAGCCCCAAGGCTCTGGCCACTGACCCGATTGTTAAGA-----CCGA

Ncun-24PA GCCCGAGCCTGAGAGCCCCAAGGCTCTGGCCACTGACCCAATTGTCAAGA-----CCGA

Cprnr1 GGCCGAAGGCAGAGAGCCCCAAGGCCTGCTGTGTGAGCCCACTGGCGAGAAGCCTACCGA

Ncun-24OR GGA--CATTGGCTCACCGCTGCTG-----GAGAGAAAG--GAGGGCCAGAATGAGGACG

Ncun-24PA GGC--CATTAGCTCCCGGGAGCTG-----GAGATGAAG--GAGGGCCAGAGTGAGGACA

Cprnr1 AGAGGCACCTT-CCCGAGGCTGCTGTCAACGGCAACAAGCCTGATGGCCAGACCAGGAGACA

Ncun-24OR TGGACGAAGATAGCCAGGAGAGGGACGAGAATATCTACTCTAATGCTCCACTTTCTGAGC

Ncun-24PA AGGACGAGGAGAGCAAGGAGAGAGAGGGGGACATCTACTCTGATG-----

Cprnr1 AGGATGGAGATAGCAAGGAGCGGGAGATGGACATCTACTCTGAGGCT-----

Ncun-24OR AACAAAGTTGCTGCTGTAAAGTGACAAGACTTAGCCCTACAGACCTTGAGACACACACT

Ncun-24PA ---AGGTAGTCTCATGTAAAGCGACAACACT-ATCCTCTATGAAACCTTGAGACACACGCT

Cprnr1 -----GTCCTTGCTGTAAAGTGCCGTGCGCGACCTCTCTCGAATAAACTGACAGAGGCT

Ncun-24OR AACCACATCAA--ATAA-CAGGCGCCTGGAATCCCGGTGCCGACCCCTCCTCCTGCGA

Ncun-24PA AACCACATCAA--ACAAAAGGGCCCTACGTTCCCGGCGCCGATCCCTCCTCCTGCGT

Cprnr1 AACATCATCGTCATCACAGGCAG-CATTGAGAATCCCGAGGCA-----TGCGT

Stop

```

Ncun-24OR  GATGTGCAGCGGTTAAGCCCCGAC- CCTTGTCT- - - GTCCGGTGGGTATATGAAGCTT
Ncun-24PA  TATGTGCAGCGGTTAAGCACGCAG- TCTTGCCT- - - GCCTGGTATGGTGCATGATGCTT
Cprnr1     TATGTGCAGCGGTTAGATCAACATACCTCGACTAGAA GTCCGAGTGAAGTATGAAG- - -

Ncun-24OR  ATGGTCAAAA- ATGTGCAGTGTT- ATGACAAAGGTACGGTGTGATTTGTT CAGA- - - - -
Ncun-24PA  AAGGTTAAAAGATGTGCAGTGTT- ATGATGTGGTACGGTGTGATATGTT CAGGAATAATG
Cprnr1     GTGACTACGA- - TCCTTGATGTTGATGTTG- GCAACGG- - - - - AAAA- - - - -

```

Literature Cited

- Bardiya, N., Shiu, P. K. T., 2007. Cyclosporin A-resistance based gene placement system for *Neurospora crassa*. *Fungal Genet. Biol.* **44**: 307-314
- Bertrand, H., 2000. Role of mitochondrial DNA in the senescence and hypovirulence of fungi and potential for plant disease control. *Annu. Rev. Phytopathol.* **38**: 397-422
- Biella, S., Smith, M. L., Aist, J. R., Cortesi, P., Milgroom, M. G., 2002. Programmed cell death correlates with virus transmission in a filamentous fungus. *Proc. R. Soc. Lond. B* **269**: 2269-2276
- Cheng, J., Baldi, P., 2006. A machine learning information retrieval approach to protein fold recognition. *Bioinformatics* **22**: 1456-1463
- Chevanne, D., Bastiaans, E., Debets, A., Saupe, S. J., Clavé, C., Paoletti, M., 2009. Identification of the *het-r* vegetative incompatibility gene of *Podospora anserina* as a member of the fast evolving *HNWD* gene family. *Curr. Genet.* **55**: 93-102
- Chevanne, D., Saupe, S. J., Clavé, C., Paoletti, M., 2010. WD-repeat instability and diversification of the *Podospora anserina* *hnwd* non-self recognition gene family. *BMC Evolutionary Biology* **10**:134
- Choi, G. H., Dawe, A. L., Churbanov, A., Smith, M. L., Milgroom, M. G., Nuss, D. L., 2012. Molecular characterization of vegetative incompatibility genes that restrict hypovirus transmission in the chestnut blight fungus *Cryphonectria parasitica*. *Genetics* **190**: 113-127
- Cole, C., Barber, J. D., Barton, G. J., 2008. The Jpred3 secondary structure prediction server. *Nucl. Acids Res.* **36**: 197-201
- Cortesi, P., McCulloch, C. E., Song, H., Lin, H., Milgroom, M. G., 2001. Genetic control of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* **159**: 107-118
- Cortesi, P., Milgroom, M. G., 1998. Genetics of vegetative incompatibility in *Cryphonectria parasitica*. *Appl. Environ. Microbiol.* **64**: 2988-2994
- Davis, R. H., de Serres, F. J., 1970. Genetic and microbiological research techniques for *Neurospora crassa*. *Meth. Enzymol.* **17**: 79-143

- Debets, A. J. M., Griffiths, A. J. F., 1998. Polymorphism of *het*-genes prevents resource plundering in *Neurospora crassa*. *Mycol. Res.* **102**: 1343-1349
- Deleu, C., Clavé, C., Bégueret, J., 1993. A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*. *Genetics* **135**: 45-52
- Eklund, H., Uhlin, U., Farnegardh, M., Logan, D. T., Nordlund, P., 2001. Structure and function of the radical enzyme ribonucleotide reductase. *Prog. Biophys. Mol. Biol.* **77**: 177-268
- Elledge, S. J., Zhou, Z., Allen, J. B., 1992. Ribonucleotide reductase: regulation, regulation, regulation. *Trends Biochem. Sci.* **17**: 119-123
- Eriksson, M., Uhlin, U., Ramaswamy, S., Ekberg, M., Regnstrom, K., Sjoberg, B. M., Eklund, H., 1997. Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure* **5**: 1077-1092
- Espagne, E., Balhadère, P., Penin, M., Barreau, C., Turcq, B., 2002. HET-E and HET-D belong to a new subfamily of WD40 proteins involved in vegetative incompatibility specificity in the fungus *Podospora anserina*. *Genetics* **161**: 71-81
- Fedorova, N. D., Badger, J. H., Robson, G. D., Wortman, J. R., Nierman, W. C., 2005. Comparative analysis of programmed cell death pathways in filamentous fungi. *BMC Genomics* **6**:177
- Gibbs, K. A., Urbanowski, M. L., Greenberg, E. P., 2008. Genetic determinants of self identity and social recognition in bacteria. *Science* **321**: 256-259
- Gietz, R. D., Woods, R. A., 2006. Yeast transformation by the LiAc/SS carrier DNA/PEG method. *Methods Mol. Biol.* **313**: 107-120
- Glass, N. L., Dementhon, K., 2006. Non-self recognition and programmed cell death in filamentous fungi. *Curr. Op. Microbiol.* **9**: 553-558
- Glass, N. L., Kaneko, I., 2003. Fatal attraction: nonself recognition and heterokaryon incompatibility in filamentous fungi. *Eukaryotic Cell* **2**: 1-8
- Glass, N. L., Kulda, G. A., 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. *Annu. Rev. Phytopathol.* **30**: 201-224
- Griffiths, A. J. F., DeLange, A. M., 1977. Mutations of the *a* mating-type gene in *Neurospora crassa*. *Genetics* **88**: 239-254
- Hall, C., Welch, J., Kowbel, D. J., Glass, N. L., 2010. Evolution and diversity of a fungal self/nonself recognition locus. *PLoS ONE* **5**:11
- Herrick, J., Sclavi, B., 2007. Ribonucleotide reductase and the regulation of DNA replication: an old story and an ancient heritage. *Mol. Microbiol.* **63**: 22-34

- Higuchi, I., Kanemura, Y., Shimizu, H., Urushihara, H., 1995. Self- and non-self-recognition in bisexual mating of *Dictyostelium discoideum*. *Develop. Growth Differ.* **37**: 311-317
- Hirose, S., Benabentos, R., Ho, H., Kuspa, A., Shaulsky, G., 2011. Self-recognition in social amoebae is mediated by allelic pairs of tiger genes. *Science* **333**: 467-470
- Holmgren, A., Sengupta, R., 2010. The use of thiols by ribonucleotide reductase. *Free Rad. Biol. Med.* **49**: 1617-1628
- Hutchison, E. A., Glass, N. L., 2012. Programmed cell death and heterokaryon incompatibility in filamentous fungi. In: Biocommunication of Fungi. G. Witzany, Ed. Springer Netherlands.
- Hutchison, E., Brown, S., Tian, C., Glass, N. L., 2009. Transcriptional profiling and functional analysis of heterokaryon incompatibility in *Neurospora crassa* reveals that reactive oxygen species, but not metacaspases, are associated with programmed cell death. *Microbiology* **155**: 3957-3970
- Jacobson, D. J., Beurkens, K., Klomparens, K. L., 1998. Microscopic and ultrastructural examination of vegetative incompatibility in partial diploids heterozygous at *het* loci in *Neurospora crassa*. *Fungal Genet. Biol.* **23**: 45-56
- Jeltsch, A., 2003. Maintenance of species identity and controlling speciation of bacteria: a new function for restriction/modification systems? *Gene* **317**: 13-16
- Jordan, A., Reichard, P., 1998. Ribonucleotide reductases. *Annu. Rev. Biochem.* **67**: 71-98
- Kaneko, I., Dementhon, K., Xiang, Q., Glass, N. L., 2006. Nonallelic interactions between *het-c* and a polymorphic locus, *pin-c*, are essential for nonself recognition and programmed cell death in *Neurospora crassa*. *Genetics* **172**: 1545-1555
- Kelley, L. A., Sternberg, M. J. E., 2009. Protein structure prediction on the web: a case study using the Phyre server. *Nature Protocols* **4**: 363-371
- Khalturin, K., Bosch, T. C. G., 2006. Self/nonself discrimination at the basis of chordate evolution: limits on molecular conservation. *Curr. Opin. Immunol.* **18**: 1-6
- Kolberg, M., Strand, K. R., Graff, P., Andersson, K. K., 2004. Structure, function and mechanism of ribonucleotide reductases. *Biochim. et biophys. Acta.* **1699**: 1-34
- Kronstad, J. W., Staben, C., 1997. Mating type in filamentous fungi. *Annu. Rev. Genet.* **31**: 245-276
- Kumanovics, A., Takada, T., Lindahl, K. F., 2003. Genomic organization of the mammalian MHC. *Annu. Rev. Immunol.* **21**: 629-657
- Lafontaine, D. L., Smith, M. L., 2012. Diverse interactions mediate asymmetric incompatibility by the *het-6* supergene complex in *Neurospora crassa*. *Fungal Genet. Biol.* **49**: 65-73
- Ljunggren, H. G., Karre, K., 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology Today* **11**: 237-244

- Mao, S. S., Holler, T. P., Yu, X., Bollinger Jr, J. M., Booker, S., Johnston, M. I., Stubbe, J., 1992. A model for the role of multiple cysteine residues involved in ribonucleotide reduction: amazing and still confusing. *Biochemistry* **31**: 9733-9743
- Marek, S. M., Wu, J., Glass, N. L., Glichrist, D. G., Bostock, R. M., 2003. Nuclear DNA degradation during heterokaryon incompatibility in *Neurospora crassa*. *Fungal Genet. Biol.* **40**: 126-137
- Maupetit J., Derreumaux, P., Tuffery, P., 2010. A fast and accurate method for large-scale de novo peptide structure prediction. *J. Comput. Chem.* **31**: 726-738
- Medzhitov, R., Janeway Jr., C. A., 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* **296**: 298-300
- Metzenberg, R. L., Glass, N. L., 1990. Mating type and mating strategies in *Neurospora*. *BioEssays* **12**: 52-59
- Micali, C. O., Smith, M. L., 2003. On the independence of barrage formation and heterokaryon incompatibility in *Neurospora crassa*. *Fungal Gen. Biol.* **38**: 209-219
- Micali, C. O., Smith, M. L., 2005. Biological concepts of vegetative self and nonself recognition in fungi. In: Evolutionary Genetics of Fungi. J. Xu, Ed. Horizon Scientific Press, UK.
- Micali, C. O., Smith, M. L., 2006. A nonself recognition complex in *Neurospora crassa*. *Genetics* **173**: 1991-2004
- Mir-Rashed, N., Jacobson, D. J., Dehghany, M. R., Micali, O. C., Smith, M. L., 2000. Molecular and functional analyses of incompatibility genes at *het-6* in a population of *Neurospora crassa*. *Fungal Genet. Biol.* **30**: 197-205
- Nordlund, P., Reichard, P., 2006. Ribonucleotide reductases. *Annu. Rev. Biochem.* **75**: 681-706
- Oakley, C. E., Weil, C. F., Kretz, P. L., Oakley, B. R., 1987. Cloning of the *riboB* locus of *Aspergillus nidulans*. *Gene* **53**: 293-298
- Oren, M., Paz, G., Douek, J., Rosner, A., Fishelson, Z., Goulet, T. L., Henchel, K., Rinkevich, B., 2010. 'Rejected' vs. 'rejecting' transcriptomes in allogeneic challenged colonial urchinates. *Mol. Immun.* **47**: 2083-2093
- Paoletti, M., Clavé, C., 2007. The fungus-specific HET domain mediates programmed cell death in *Podospora anserina*. *Eukaryotic Cell* **6**: 2001-2008
- Perkins, D. D., 1988. Main features of vegetative incompatibility in *Neurospora*. *Fungal Genet. Newsl.* **35**: 44-46
- Petersen, M. T. N., Jonson, R. H., Petersen, S. B., 1999. Amino acid neighbours and detailed conformation analysis of cysteines in proteins. *Prot. Eng.* **12**: 535-548

- Raulet, D. H., 2006. Missing self recognition and self tolerance of natural killer (NK) cells. *Seminars in Immunology* **18**: 145-150
- Reichard, P., 1961. Formation of deoxycytidine phosphates from cytidine phosphates in extracts from *Escherichia coli*. *J. Biol. Chem.* **236**: 1150-1157
- Reichard, P., 1993. From RNA to DNA, why so many ribonucleotide reductases? *Science* **260**: 1773-1777
- Reichard, P., 2001. Ribonucleotide reductases: the evolution of allosteric regulation. *Arch. Biochem. Biophys.* **397**: 149-155
- Rinkevich, B., Weissman, I. L., 1992. Allogenic resorption in colonial protochordates: consequences of nonself recognition. *Dev. comp. immunology* **16**: 275-286
- Royer, J. C., Yamashiro, C. T., 1992. Generation of transformable spheroplasts from mycelia, macroconidia, microconidia and germinating ascospores of *Neurospora crassa*. *Fungal Genet. Newsl.* **39**: 76-79
- Saito, Y., Hirose, E., Wantanabe, H., 1994. Allorecognition in compound ascidians. *Int. J. Biol.* **38**: 237-247
- Sanabria, N. M., Huang, J., Dubery, I. A., 2010. Self/nonself perception in plants in innate immunity and defense. *Self/Nonself* **1**: 40-54
- Sato, K., Nishio, T., Kimura, R., Kusaba, M., Suzuki, T., Hatakeyama, K., Ochikendon, D. J., Satta, Y., 2002. Coevolution of the S-locus genes *SRK*, *SLG* and *SP11/SCR* in *Brassica oleracea* and *B. rapa*. *Genetics* **162**: 931-940
- Saupe, S. J., 2000. Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. *Microbiol. Mol. Biol. Rev.* **64**: 489-502
- Shaulsky, G., Kessin, R. H., 2007. The cold war of the social amoebae. *Current Biology* **17**: 684-692
- Shiu, P. K. T., Glass, N. L., 1999. Molecular characterization of *tol*, a mediator of mating-type-associated vegetative incompatibility in *Neurospora crassa*. *Genetics* **151**: 545-555
- Smith, M. L., Gibbs, C. C., Milgroom, M. G., 2006. Heterokaryon incompatibility function of barrage-associated vegetative incompatibility genes (*vic*) in *Cryphonectria parasitica*. *Mycologia* **98**: 43-50
- Smith, M. L., Hubbard, S. P., Jacobson, D. J., Micali, O. C., Glass, N. L., 2000b. An osmotic-remedial, temperature-sensitive mutation in the allosteric activity site of ribonucleotide reductase in *Neurospora crassa*. *Mol. Gen. Genet.* **262**: 1022-1035
- Smith, M. L., Lafontaine, D. L., 2012. The fungal sense of nonself. In: *Neurospora Genomics and Molecular Biology*. Eds. Kevin McCluskey and D. Kasbekar, Horizon Scientific Press, Norfolk UK.

- Smith, M. L., Micali, O. C., Hubbard, S. P., Mir-Rashed, N., Jacobson, D. J., Glass, N. L., 2000b. Vegetative incompatibility in the *het-6* region of *Neurospora crassa* is mediated by two linked genes. *Genetics* **155**: 1095-1104
- Smith, M. L., Yang, C. J., Metzenberg, R. L., Glass, N. L., 1996. Escape from *het-6* incompatibility in *Neurospora crassa* partial diploids involves preferential deletion within the ectopic segment. *Genetics* **144**: 523-531
- Smith, R. P., Wellman, K., Haidari, L., Masuda, H., Smith, M. L., 2013. Nonspecific recognition through intermolecular disulfide bond formation of ribonucleotide reductase in *Neurospora*. *Genetics* **193**: 1175-1183
- Smith, R. P., Wellman, K., Smith, M. L., 2013b. Trans-species activity of a nonspecific recognition domain. *BMC Microbiology* **13**:63
- Staben, C., 1996. The mating-type locus of *Neurospora crassa*. *J. Genet.* **75**: 341-350
- Uhlir, U., Eklund, H., 1994. Structure of ribonucleotide reductase protein R1. *Nature* **370**: 533-539
- Waddell, D. R., Duffy, K. T. I., 1986. Breakdown of self/nonspecific recognition in cannibalistic strains of the predatory slime mold, *Dictyostelium caveatum*. *The Journal of Cell Biology* **102**: 298-305
- Watanabe, H., Taneda, Y., 1982. Self or non-self recognition in compound ascidians. *Amer. Zool.* **22**: 775-782
- Wei, Y., Thompson, J., Floudas, C. A., 2011. CONCORD: A consensus method for protein secondary structure prediction via mixed integer linear optimization, *Proc. R. Soc. A* Published online November 18, 2011.
- Wenren, L. M., Sullivan, N. L., Cardarelli, L., Septer, A. N., Gibbs, K. A., 2013. Two independent pathways for self-recognition in *Proteus mirabilis* are linked by type VI-dependent export. *mBio* **4**:4
- Winston, F., Dollard, C., Ricupero-Hovasse, S. L., 1995. Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11**: 53-55
- Xu, H., Faber, C., Uchiki, T., Fairman, J. W., Racca, J., Dealwis, C., 2006. Structures of eukaryotic ribonucleotide reductase I provide insights into dNTP regulation. *Proc. Natl. Acad. Sci. USA* **103**: 4022-4027
- Zhang, Y., 2012. <http://zhanglab.ccmb.med.umich.edu/PSSpred>
- Zhang, Z., Yang, K., Chen, C., Feser, J., Huang, M., 2007. Role of the C terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. *Proc. Natl. Acad. Sci. USA* **104**: 2217-2222