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Trans-species expression of a nonself recognition domain.

By

Robert Phillip Smith

**A dissertation
submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Philosophical Doctorate in Biology**

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Elimu haina mwisho
(Education has no ending)

ABSTRACT

This thesis examines the effects of expressing of the *un-24*^{PA} nonself recognition domain from *Neurospora crassa* in *Saccharomyces cerevisiae*. During vegetative growth of *N. crassa*, cell fusion between conspecifics may result in the formation of heterokaryons. Proliferation of heterokaryons in *N. crassa* is governed by 13 genes including *un-24*, which has a dual function as it also encodes the large subunit of ribonucleotide reductase. *un-24* has two alleles, Oakridge (OR) and Panama (PA). Cells that contain both OR and PA alleles are non-viable or grow slowly with an aberrant morphology but can revert to a wildtype morphology after several days of growth in a phenomenon called escape. A literature review of background information is provided in Chapter 1. Chapter 2 documents how low-level expression of the PA incompatibility domain results in several heterokaryon incompatibility-like cytological characteristics in yeast. As in *N. crassa*, this incompatibility-like phenotype is associated with a protein complex comprised of the PA incompatibility domain and yeast Rnr1p. In Chapter 3, we examine why this incompatibility-like phenotype is not observed when the PA incompatibility domain is overexpressed in yeast. When overexpressed, the majority of the PA incompatibility domain complexes with two Hsp70 proteins. We show that three genes with Hsp70-like homology are significantly upregulated upon escape in *N. crassa*. Since Hsp70s function in eliminating protein aggregates in yeast, our results indicate that Hsp70-like proteins in *N. crassa* may eliminate aberrant UN-24 complexes and facilitate escape. Redox-reactive cysteines in the PA incompatibility domain were previously shown to potentiate OR-PA UN-24 complex formation in *N. crassa*. In Chapter 4, cysteine mutant constructs were expressed in yeast and incompatibility-like cytologies

were assayed. The observations with the yeast system are consistent with what is seen in *N. crassa*, namely that cysteine C907 is required for *un-24*-associated incompatibility. Furthermore, we expressed selected cysteine and proline substitution mutants of the PA incompatibility domain in *Escherichia coli* and observed that some of these changes altered the mobility of the protein in SDS-PAGE. These assays provided evidence that specific amino acids have a key role in both function and structure of the PA incompatibility domain.

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Abbreviations

aa	amino acids
amp	ampicillin
°C	degrees Celsius
C-terminus	carboxy terminus
cDNA	complimentary DNA
cm	centimeter
C _t	cycle at threshold
CW	calcofluor white
dH ₂ O	distilled water
ddH ₂ O	double distilled water
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
NDP	nucleotide diphosphate
dNDP	deoxynucleotide diphosphate
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
fg	femtogram
G418	geneticin
G418 ^R	geneticin resistant
<i>het</i>	heterokaryon incompatibility locus
HIS	histidine
HMG	high-mobility group
HU	hydroxyurea
h	hour
hygB	hygromycin B
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodaltons
lac	lactose
lac ^I	lactose inhibitor
lac ^o	lactose operon
LB	Luria-Bertani media
MADS	<i>MCMI</i> -agamous-deficiens-serum
MAT	mating type locus
MAP	mitogen activated protein
MCS	multiple cloning site
MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
mm	millimeters
mg	milligrams
MW	molecular weight
N-terminus	amino terminus

NAT	nourseothricin
Nat ^R	nourseothricin resistant
ng	nanogram
OD ₆₀₀	optical density at 600nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCD	programmed cell death
PCET	proton coupled electron transfer
PCR	polymerase chain reaction
PIPES	piperrazine-N,N'-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
PRE	pheromone response element
qPCR	quantitative PCR
RIP	repeat-induced point mutation
RNA	ribonucleic acid
RPM	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
SI	Self Incompatibility Complex
TBST	tris buffered saline with Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
T _m	melting temperature
U	unit(s)
μg	microgram
μL	microlitre
μM	micromolar
USA	ureidosuccinic acid
vATPase	vacuolar ATPase
<i>vic</i>	vegetative incompatibility loci
VMM	Vogel's minimal medium
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
YPD	yeast peptone dextrose
YPRaf/Gal	yeast peptone supplemented with 1.5% raffinose and 0.5% galactose

General Genetic Designations

N. crassa Designations

<i>un-24</i> ^{OR}	wildtype “Oakridge” form of <i>un-24</i>
<i>un-24</i> ^{PA}	wildtype “Panama” form of <i>un-24</i>
<i>un-24</i>	either OR or PA wild-type of <i>un-24</i>
PA	<i>un-24</i> Panama allele
OR	<i>un-24</i> Oak-ridge allele
<i>un-24</i> ^{ts}	<i>un-24</i> ^{OR} temperature sensitive mutant
xxx-#	In general, gene abbreviations are lower case and are italicized. A gene number, if present, is hyphenated.

Yeast Designations

<i>RNR1</i>	gene, large subunit ribonucleotide reductase, class I from yeast
<i>GAL1</i>	yeast locus YBR020W that is under the control of a galactose inducible promoter
<i>XXX#</i>	In general, gene abbreviations are upper-case and are italicized. A gene number, if present, is not hyphenated.

Protein Designations

General Designations

RNR	ribonucleotide reductase holoenzyme
R1	large subunit of ribonucleotide reductase
R2	small subunit of ribonucleotide reductase

N. crassa Designations

UN-24 ^{OR}	<i>un-24</i> OR protein
UN-24 ^{PA}	<i>un-24</i> PA protein
UN-24	either OR or PA wild-type from of UN-24
XX-Y ^Z	This designates the protein product of a gene in <i>N. crassa</i> . XX-Y represents the gene abbreviation of the locus while Z represents the allelic form of the protein.

Yeast Designations

Rnr1p	RNR1 protein
Leu1p	isopropylmalate isomerase protein
Xxxp	This designates the protein form of a gene in yeast. 'p' designates protein while 'Xxx' designates the gene abbreviation.

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

Nonself Recognition

Nonself recognition is ubiquitous in biological systems and the mechanisms by which nonself recognition is mediated are diverse at both the inter-species and intra-species levels. Arguably, the simplest forms of nonself recognition systems are found in bacteria. Here, one form of nonself recognition is mediated by restriction endonucleases, enzymes that recognize and cleave specific, mostly palindromic DNA sequences. These sequences are often not found in the host's genome or, if present, are not recognized by endogenous restriction enzymes due to chemical modification of the DNA sequence (e.g. DNA methylation). A strong evolutionary pressure to avoid palindromic sequences in the genome has been observed in several bacterial species (Rocha *et al.*, 2009). This primitive form of 'immunity' has been postulated to protect bacteria from bacteriophage infection (Rambach and Tiollais, 1974). For example, the endonuclease *PvuII* from *Proteus vulgaris* cleaves the non-methylated DNA sequence CAGCTG. Since bacterial genomic and endogenous non-genomic DNA elements are methylated, a bacteriophage that contains the DNA sequence CAGCTG in a non-methylated state will not be able to infect *P. vulgaris*. The endonuclease *PvuII* will recognize viral DNA containing this sequence as nonself, and cleave it, thus restricting bacteriophage infection (Rice and Blumenthal, 2000). One of the more complex nonself recognition systems is modulated by the Major Histocompatibility Complex (MHC) that is found in vertebrates and comprises a series of glycoproteins that present both self (derived from endogenous cells) and nonself (derived from non-endogenous elements) peptides to T-lymphocytes. If a T-lymphocyte recognizes an MHC-presented peptide as nonself, it stimulates an immune

response that involves a series of specialized cells. These cells target and eliminate the non-endogenous element that contains the nonself peptide (Yeager *et al.*, 1999).

Sexual Nonself Recognition

Nonself recognition occurs during both the sexual and vegetative/somatic stages of life. During the sexual stage, nonself recognition promotes fusion between individuals that are genetically different (e.g. male and female). Therefore sexual nonself recognition is thought to promote outbreeding that may increase genetic variability in offspring (reviewed in Casselton, 2002). The Self Incompatibility (SI) system in plants provides a well-characterized example of a sexual nonself recognition system. SI prevents self-fertilization in plants and is controlled by a single, multi-allelic locus called the S-locus (reviewed in Matton *et al.*, 1994). The S-locus is actually a multi-gene unit and the set of alleles at these genes within the S-locus is referred to collectively as a haplotype (Takayama and Isogai, 2005). During fertilization, the sexual cycle is arrested when both pollen (male) and pistil (female) express the same haplotype. The mechanisms by which different families of plants use the S-locus to control the sexual cycle are diverse. In the S-haplotype-rich Brassicaceae, the S-locus haplotype from the pollen is presented to SRK receptors found on the pistil membrane (Ockendon, 2000). If the SRK receptors detect a pollen haplotype that is similar to the pistil haplotype, a signal cascade is initiated which inhibits metabolic activation and hydration of the pollen grain (Kachroo *et al.*, 2002). In the Rosaceae, Solanaceae and Scrophulariaceae families, SI is mediated by an S-RNase. When pistil cells of these families recognize pollen with a similar haplotype, an S-RNase is produced in the elongating pollen tube which destroys RNA and halts pollen tube elongation thus preventing fertilization (Takayama and Isogai, 2005). In the

Papaveraceae family, if pollen presents a haplotype similar to that of the pistil, Ca^{2+} is released into the pollen tube. This triggers a signal cascade which results in pollen tube destruction (Franklin-Tong *et al.*, 1993).

Sexual Incompatibility in Fungi

In fungi, sexual nonself recognition involves mating-type loci and only individuals of different mating types can undergo sexual fusion (reviewed in Casselton, 2002). The unicellular ascomycete yeast *Saccharomyces cerevisiae* contains arguably the best characterized fungal sexual nonself recognition system (see reviews in Johnson 1995; Herskowitz, 1998; Casselton, 2002). *S. cerevisiae* has two mating types which are designated MAT α and MATa. The MAT α and MATa loci encode transcription factors that activate genes required for mating-type specific pheromone and pheromone-specific receptor production. The MAT α locus contains two actively transcribed genes, $\alpha 1$ and $\alpha 2$, whereas the MATa locus contains only one actively transcribed gene, $a 1$. Transcription of MAT α -specific genes occur when the constitutively translated *MCM1* protein (Mcm1p), a general transcription factor belonging to the *MCM1*-agamous-deficiens-serum (MADS) response factor family, binds to the protein product of the $\alpha 1$ locus (Bender and Sprague Jr., 1987; reviewed in Dolan *et al.* 1991). Mcm1p also forms a complex with the $\alpha 2$ protein, which then interacts with the pheromone response element (PRE) to ensure that $a 1$, the MATa-specific gene, is not transcribed (Keheler *et al.*, 1989). In MATa cells, Mcm1p binds upstream of the $a 1$ locus and activates transcription of MATa-specific genes (Ammerer, 1990). In both MAT α and MATa haploid cells, Mcm1p also interacts with Ste1p to drive expression of genes required for mating and fusion (Dolan *et al.*, 1989). Mating-type specific peptide pheromones allow cells from opposite mating-types

to detect and orient towards each other using chemotropism which produces elongated cells called 'schmoos' (Leberer *et al.*, 1997). MAT α and MATa cells produced peptide pheromones that are referred to as the α - and a-factor, respectively (Julius *et al.*, 1983). Mating-type specific G-protein coupled cell surface receptors detect the presence of the opposite pheromone in the medium. Binding of the α - or a-factor to a pheromone receptor located on a cell of the opposite mating-type results in a mitogen activated protein (MAP) kinase signal cascade. This causes G₁ arrest and activates Ste12p which augments transcription of mating-type specific genes thus increasing pheromone production (reviewed in Sprague and Thorner, 1992). Physical contact between MAT α and MATa cells results in cell fusion, plasmogamy and eventually karyogamy (reviewed in Rose, 1991). Once MAT α and MATa cells have fused and undergone plasmogamy, the α 2 and a1 protein products interact and suppress transcription of mating-type specific genes. The α 2/a1 protein complex also inhibits transcription of *RME1*, a gene that inhibits meiosis, thus allowing meiosis to proceed. When confronted with suboptimal growing conditions, the diploid cell will undergo meiosis and sporulate into four haploid progeny.

The *S. cerevisiae* mating system has provided a useful template for understanding mating processes in other fungi. Similar to yeast, filamentous ascomycetes such as *Neurospora crassa* contain two mating-type loci, designated *mat-A* and *mat-a*, which also encode transcription factors that control expression of mating-type specific peptide pheromones and pheromone specific G-protein coupled receptors (reviewed in Staben, 1996). Although the *N. crassa* sexual nonself recognition system shares many similarities with that of yeast, the *mat* locus in *Neurospora* is unique among fungi in that it also has vegetative nonself recognition function (reviewed in Debets and Griffiths, 1998). The

mat-a locus is comprised of one gene, *mata-1*, while the *mat-A* locus comprises of three genes, *matA-1*, *matA-2* and *matA-3*. Both *matA-1* and *mata-1* belong to the high-mobility group (HMG) domain family of transcription factors and, similar to yeast, control transcription of mating-type-specific genes that encode receptors and pheromones. Fusion of cells with opposite mating types involves a specialized sexual structure (female) called the ascogonium. The trichogyne, a specialized receptive hyphae associated with the developing ascogonium, grows towards cells of the opposite mating type to initiate plasmogamy. Following plasmogamy, nuclear fusion does not occur immediately as sexually compatible nuclei are replicated and maintained in dikaryotic cells (Phillee and Staben, 1994). However, once the fruiting body has fully developed, karyogamy occurs and is quickly followed by meiosis and sporulation (Coppin *et al.*, 1997).

Vegetative/Somatic Incompatibility

In contrast to sexual incompatibility systems, vegetative or somatic incompatibility involves the breakdown of *dissimilar* cell fusions and occurs during the mitotic growth phase of the life cycle. Vegetative nonself recognition promotes fusion between individuals that are genetically similar or identical. Vegetative cell fusion is often observed in fungi during times of high nutrient content and when growth is rapid. These fusion events likely facilitate genomic stability and resource sharing between different regions of the same individual (Micali and Smith, 2005). Several vegetative incompatibility systems have been observed across taxa especially in colonial organisms. In the ascidian invertebrate *Botryllus schlosseri*, chimeras resulting from the fusion of genetically distinct colonies are terminated shortly after fusion (Rinkevich and Weissman, 1987). This type of somatic recognition is also observed in the hydroids

(Shenk and Buss, 1991). For example, the colonial cnidarian *Hydractinia echinata* fuses with colonies in close proximity in order to share a common gastrointestinal tract. The outcome of a cell fusion event in *H. echinata* is governed by polymorphic nonself recognition loci. Fusions between *H. echinata* cells that are similar at nonself recognition loci are viable while those that are different at these loci are terminated (Grossberg 1988; Buss, 1990). Under nutritional stress, the cellular slime mold (related to animals and fungi) *Dictyostelium mucoroides* will co-aggregate to form a multi-cellular structure called a grex (Clarke and Steck, 1979). The grex will then differentiate into germline and somatic cells to function in dispersal and structure, respectively. Amoebae that are genetically identical are able to co-aggregate and form a grex. In contrast, amoebae that are genetically different are excluded from the grex (Buss 1982).

The examples described above represent a small subset of the diversity of sexual and vegetative nonself recognition systems that have been characterized (reviewed in Crampton and Hurst, 1994). However, the central focus of this literature review and thesis is nonself recognition as it relates to the yeast *Saccharomyces cerevisiae* and the filamentous fungus *N. crassa*.

Vegetative Incompatibility in Fungi

Vegetative incompatibility has been characterized in several species of filamentous fungi including *N. crassa*, *Podospora anserina*, *Aspergillus niger* and *Cryphonectria parasitica* (reviews in: Saupe, 2000; Micali and Smith, 2006). At least two types of vegetative incompatibility exist in filamentous fungi; mycelial incompatibility and heterokaryon incompatibility. In mycelial incompatibility, a barrage (also known as a

demarcation or zone line) occurs at or near the contact point between two fungal individuals. This aversive interaction has been observed in both basidiomycetes (reviewed in Worrall, 1977) and ascomycetes (Bernet, 1965; Anagnostakis, 1977; Brasier, 1984). Various types of barrage exist and are described based on their physical characteristics which include a region of dead or dying cells, a raised mycelium, brown pigment disposition and/or two rows of perithecia that flank the fusion area (Leslie, 1993; Micali and Smith, 2005).

Heterokaryon incompatibility involves a genetic system that prevents the proliferation of heterokaryons, cells that contain multiple nuclear types (Figure 1.1). Heterokaryons that form between individuals that are identical at all nonself recognition loci are viable and can persist and propagate as heterokaryons (reviews in: Saupe, 2000; Micali and Smith, 2005). However, heterokaryons that carry different alleles at any one of several nonself recognition loci are non-viable and undergo a type of programmed cell death (PCD).

Although once thought to be manifestations of the same phenomenon, mycelial incompatibility and heterokaryon incompatibility have been shown to involve independent genetic determinants in some cases in *N. crassa*, *C. parasitica* and *Sclerotinia sclerotiorum* (Ford, 1995; Micali and Smith, 2003; Smith *et al.*, 2006). For example, in *N. crassa*, barrages were observed to occur between strains that were identical at all heterokaryon incompatibility loci, including the *mat* locus. Conversely, a subset of pairs of strains that differed at nonself recognition loci did not form barrages (Micali and Smith, 2003). Although mycelial incompatibility remains an important

Figure 1.1: Schematic representation of vegetative cell fusion with or without subsequent heterokaryon incompatibility (modified from Saupe *et al.*, 2000a and Glass and Kaneko, 2003). When strains are identical at all nonself recognition loci, the resulting heterokaryon is viable. However, a difference at one or more nonself recognition loci results in PCD (hatch marks). White circles represent nuclei of individual A and black circles represent nuclei of individual B.

[Fig. 1.1]

component of vegetative incompatibility, it has been proposed that the mechanisms by which the two processes are mediated may differ in some cases. For example, it has been proposed that mycelia incompatibility may include extracellular or cell surface factors that lead to the formation of barrages, whereas heterokaryon incompatibility operates only intracellularly (Smith *et al.*, 2006).

Heterokaryon Incompatibility Loci and Incompatibility Reactions

The genetic basis of mycelial incompatibility (that operates independent of heterokaryon incompatibility) is unknown. However, several genes involved in heterokaryon incompatibility have been characterized at the molecular level. In the filamentous fungi *N. crassa* and *P. anserina* vegetative nonself recognition is mediated by heterokaryon (*het*) incompatibility loci. *N. crassa* also has two non-*het* loci, *mat* and *un-24*, that mediate nonself recognition. In general, distinct incompatibility loci appear to be dissimilar (reviewed in Saupe, 2000) although several *het* loci produce proteins which contain a HET domain, a fungal specific domain associated with nonself recognition. The HET domain is characterized by three conserved blocks of 15 to 30 aa and has been observed in three nonself recognition loci in *N. crassa* (*het-c*, *pin-c* and *tol*) and in two nonself recognition loci in *P. anserina* (*het-d*, *het-e*) (Smith *et al.*, 1996; Glass and Dementhon, 2006; Kaneko *et al.*, 2006; Paoletti *et al.*, 2007; Paoletti and Clave, 2007). The HET domain has been postulated to trigger PCD during heterokaryon incompatibility (Paoletti and Clave, 2007). For example, point mutations in the HET domain of *pin-c* result in the loss of *het-c*-associated incompatibility (Kaneko *et al.*, 2006) while deletion of the *vib-1* locus results in low-level expression of the HET containing genes *het-c*, *pin-c* and *tol* (Xiang and Glass, 2002, 2004; Glass and Dementhon, 2006). In addition to the

HET domain, some *het* loci contain an NTP binding domain commonly referred to as a NACHT domain (Paoletti *et al.*, 2007). Although this domain has yet to be directly implicated in causing PCD as a result of heterokaryon incompatibility, the NACHT domain has been observed in pathogen recognition in mammalian cells (Kufer *et al.*, 2005) and in PCD in animals and plants (Leipe *et al.*, 2004).

Genes involved in heterokaryon incompatibility have been molecularly characterized from only two fungi to date, *P. anserina* and *N. crassa* (Table 1.1). *P. anserina* contains nine *het* loci, including *het-s*, *-c*, *-r*, *-d*, *-e* and *-v* that have been characterized at the molecular level (Coustou *et al.*, 1999; Saupe *et al.*, 2000a; Saupe *et al.*, 2000b; Glass and Kaneko, 2003). In *N. crassa*, proliferation of heterokaryotic cells is governed by at least nine *het* loci, designated as *het-c*, *-d*, *-e*, *-i*, *-6*, *-7*, *-8*, *-9*, *-10* and five additional loci with heterokaryon incompatibility function, *pin-c*, *un-24*, *tol*, *mat* and *vib-1*. In *N. crassa*, genes involved in heterokaryon incompatibility that have been characterized at the molecular level include *het-c*, *het-6*, *un-24*, *tol*, *mat*, *vib-1* and *pin-c*.

In both *N. crassa* and *P. anserina*, destruction of the heterokaryon is mediated by PCD. Filamentous fungi contain several genes that are homologous to loci involved in PCD in *S. cerevisiae* (Pal *et al.*, 2007). Filamentous fungi also have homologs of genes known to be involved in PCD in metazoans. In addition, heterokaryon incompatibility leads to several hallmark cytological features associated with apoptosis in metazoans, such as DNA degradation, the presence of phosphatidylserine on the outer membrane and the production of reactive oxygen species (Glass and Dementhon, 2006). Formation of heterokaryons between incompatible cells of *P. anserina* results in a series of cytological changes, including isolation of the heterokaryon via septal plugging and vacuolar

Table 1.1: Genes involved in heterokaryon incompatibility that have been molecularly characterized from *N. crassa* and *P. anserina*.

Modifiers (^) and suppressors (*) for each locus are also listed.

Species and Incompatibility Gene	<i>het</i> Protein Domains	Suppressor (*)/Modifier (^) Gene	Suppressor/Modifier Protein Domains	Reference
<i>N. crassa</i>				
<i>mat-A1</i>	α domain	<i>tol</i> *	leucine rich repeats	Glass <i>et al.</i> , 1990; Shiu and Glass, 1999
<i>mat-a1</i>	HMG box	<i>tol</i> *		Staben and Yanofsky, 1990
<i>het-c</i>	glycine-rich repeats, signal peptide	<i>vib-1</i> ^		Saupe <i>et al.</i> , 1996
<i>het-e</i>	HET domain, similar to PIN-C, HET-6	<i>vib-1</i> ^	acid phosphatase	Xiang and Glass, 2004
<i>un-24</i>	ribonucleotide reductase large subunit	-		Smith <i>et al.</i> , 2000, Begin and Smith, unpublished
<i>pin-c</i>	HET domain, rich in tryptophan	-		Kaneko <i>et al.</i> , 2006

[Table 1.1 continued]

<i>P. anserina</i>				
<i>het-s</i>	prion protein			Turcq et al., 1990
<i>het-c</i>	glycolipid transfer	$\hat{mod-A}$	SH3-binding domain,	Saupe et al., 1994; Barreau et al., 1998,
	protein		proline rich	Paoletti and Clave, 2007
		$\hat{mod-B}$	proline rich	Paoletti and Clave, 2007
		$\hat{mod-C}$		
		$\hat{mod-D}$	α subunit G protein	Barreau et al., 1998
<i>het-e</i>	NACHT domain	$\hat{mod-A}, \hat{mod-B},$		Loubradou et al., 1999; Paoletti et al., 2007
	WD40 repeats	$\hat{mod-C}, \hat{mod-D}$		
<i>het-d</i>	NACHT domain	$\hat{mod-A}, \hat{mod-B},$		Espagne et al., 2002; Paoletti et al., 2007
	WD40 repeats	$\hat{mod-C}, \hat{mod-D}$		
<i>het-r</i>	NACHT domain	$\hat{mod-A}, \hat{mod-B},$		Chevanne et al., 2009
	WD40 repeats	$\hat{mod-D},$		
		$\hat{mod-E}$	Hsp90 heat shock	Loubradou et al., 1997
			protein	
<i>het-v</i>	Unknown	$\hat{mod-A}, \hat{mod-B},$		
		$\hat{mod-D}, \hat{mod-E}$		

enlargement (Pinan-Lucarre *et al.*, 2007). Increased vacuolarization has been implicated in mediating PCD (Pinan-Lucarre *et al.*, 2005, 2007). It has been suggested that the increased vacuolarization of the incompatible heterokaryon ultimately leads to vacuolar rupturing, lytic enzyme release and acidification of the cytoplasm which ultimately results in PCD (Pinan-Lucarre *et al.*, 2007). When heterokaryons form between incompatible *N. crassa* cells, the heterokaryotic cell is isolated via septal plugging and increased vacuolarization of the cytoplasm is evident. In the later stages of PCD, degradation of organelles and plasma membrane shrinkage are observed until a shrunken, unorganized cytoplasmic mass remains at the center of the former cell (Jacobson *et al.*, 1998). The following section will describe what is known about *het* gene determinants from *P. anserina* and *N. crassa*.

Allelic and Non-Allelic Incompatibility Systems

Two general types of heterokaryon incompatibility systems exist; allelic and non-allelic. In allelic systems, proteins from alternate alleles of the same gene interact to mediate nonself recognition (reviewed in Saupe, 2000; Smith *et al.*, 2000b). For example the *het-s* locus of *P. anserina* contains two alleles, *het-s* and *het-S*, that when found together in the same cell cause incompatibility. The protein product of the *het-s* allele exists in two forms; [Het-s], a protein that spontaneously converts into a prion which acts antagonistically with HET-S, and [Het-s*], a non-prion form that does not interact negatively with HET-S. A prion is a protein that undergoes a change in shape (either spontaneously or through mutation) which then allows it to interact with non-prion forms of the same protein converting them into prions themselves (Prusiner, 1998). In addition to causing vegetative incompatibility, [Het-s] causes death of spores that contain the *het-*

S allele during the sexual cycle (Dalstra *et al.*, 2005). The mating-type (*mat*) locus also has an allelic vegetative nonself recognition function in *N. crassa*. Interaction of the gene products, MAT-A1 and MAT-a1, leads to vegetative nonself recognition and result in PCD. Mutation of protein binding domains in either MAT-A1 or MAT-a1 abolishes vegetative nonself recognition function but does not affect sexual nonself recognition (Griffiths and Delange, 1978; Philley and Staben, 1994). *mat*-associated vegetative incompatibility is conditional upon the presence of TOL, a protein encoded by the *tol* locus. It has been suggested that a toxic complex formed between TOL and MAT-A1 and MATa1 leads to PCD. Indeed, mutations to the leucine rich region of TOL result in the loss of *mat*-associated vegetative incompatibility (Shiu and Glass, 1999). Since *tol* is only expressed during vegetative growth, its absence during the sexual cycle allows co-existence of nuclei expressing alternate *mat* alleles during the dikaryotic phase just prior to karyogamy.

In non-allelic systems, proteins from different *het* genes interact to mediate nonself recognition (reviewed in Saupe, 2000; Smith *et al.*, 2000b). For example, in the *het-c* system from *N. crassa*, two linked genes, *het-c* and *pin-c*, modulate incompatibility. Proper expression of *het-c* and *pin-c*, as well as their ability to interact and cause PCD, is dependent upon the expression of another locus that is involved in nonself recognition, *vib-1* (Dementhon *et al.*, 2006). Fusion between cells with genotypes *het-c1/pin-c1* and *het-c2/pin-c2* result in both homodimeric and heterodimeric incompatible protein interactions, which occur between HET-C1-HET-C2 (allelic), HET-C1-PIN-C2 (non-allelic) and HET-C2-PIN-C1 (non-allelic). It is proposed that formation of these incompatible protein complexes results in PCD (Kaneko *et al.*, 2006). The *het-r/het-v*

system from *P. anserina* provides another example of a non-allelic vegetative incompatibility system (Bourges *et al.*, 1998). Two alleles exist at each of these unlinked loci; *het-R* and *het-r* at the *het-r* locus and *het-V* and *het-V1* at the *het-v* locus. The *het-r/het-v* system is temperature sensitive; if grown at $< 26^{\circ}\text{C}$, strains that are *het-R/het-V* rapidly undergo PCD after germination. Conversely, if grown at $> 26^{\circ}\text{C}$, *het-R/het-V* strains are viable (Labarere, 1973).

Evolution of Nonspecific Recognition in Fungi

Two opposing theories have been proposed to explain the evolution and biological significance of vegetative incompatibility in filamentous fungi; the accident theory and the allorecognition theory (Saupe, 2000). The accident theory holds that nonspecific recognition in fungi does not have any biological significance and arose as an evolutionary ‘accident’ (Saupe, 2000). According to the accident theory, a series of neutral polymorphisms exist within a fungal population and some heteroallelic combinations of these polymorphisms are deleterious to the cell. In contrast, the allorecognition theory holds that fungal nonspecific recognition systems arose to reduce cytoplasmic mixing between individuals and thus to limit transmission of infectious elements such as viruses, plasmids and transposons. Indeed, vegetative transmission of mitochondrial plasmids, which contain sequences with homology to transposons, and viruses has been observed in *Neurospora* (Collins and Saville, 1990; Biella *et al.*, 2002). However, not all supposed nonspecific recognition loci have the ability to limit virus transfer. For example, the vegetative incompatibility locus *vic-4* of chestnut blight fungus *Cryphonectria parasitica* does not limit viral transfer (Biella *et al.*, 2002). Nonspecific recognition systems that aid in allorecognition should have at least three shared

characteristics. First, each locus should have multiple alleles which contain numerous non-synonymous substitutions in regions associated with nonself recognition. This implies that nonself recognition domains are under diversifying selection and that a high amount of diversity is preferred at these loci (Wu *et al.* 1998). In support of this, when compared to other regions of the protein, the frequency of non-synonymous substitutions is significantly higher in the C-terminus of UN-24 where the incompatibility domain is located (Micali and Smith, 2006). Second, incompatible alleles of nonself recognition genes should occur as trans-species polymorphisms and, as a third characteristic, be maintained under balancing selection. Trans-species polymorphisms are observed when an allelic form in one species is more closely related to an allele in a different species than to the alternate allele within the same species (Muirhead *et al.* 2002). For example, *Sordaria* and *Neurospora* diverged over 36 million years ago. However, alleles at the *het-c* locus retain ancestral polymorphisms despite multiple intervening speciation events (Wu *et al.*, 1998). During speciation events, polymorphisms at neutral alleles are expected to increase with time since speciation. However, balancing selection maintains ancestral alleles within a population for periods of time far exceeding those expected for neutral polymorphisms (Wu *et al.* 1998). In addition, balancing selection results in a specific allele frequency within a population. For example, the two *un-24* alleles are observed to occur in nearly equal frequencies in *N. crassa* populations suggesting balancing selection is occurring at this nonself recognition locus (Mir-Rashed *et al.*, 2000; Micali and Smith, 2006). The balance is maintained in cases where, if one allele drifts towards extinction, strains with the alternate allele have a selective advantage (e.g.

in not acquiring infectious agents) and thus contribute more progeny to the next generation.

Several studies have highlighted the importance of vegetative incompatibility in limiting the transfer of deleterious genetic elements or to prevent resource plundering by aggressive genotypes. For example, *Ophiostoma novo-ulmi*, the fungus responsible for Dutch elm disease, invaded Europe as several clonal populations with limited *vic* diversity. This lack of diversity in *vic* loci is considered to be an important factor in the subsequent spread of deleterious viruses within populations resulting in a 90% infection rate (Paoletti *et al.*, 2006). However, due to inter-species gene transfer during rare hybridization events occurring between *O. novo-ulmi* and a closely related species, *Ophiostoma ulmi*, additional *vic* loci were acquired by *O. novo-ulmi* and this apparently decreased the rate of virus proliferation (Brasier, 1988; Paoletti *et al.*, 2006). A similar phenomenon has been observed in *C. parasitica*. Differences at some *vic* loci (e.g. *vic*-2, *vic*-6 and *vic*-7) significantly limit viral transfer from infected to non-infected individuals (Cortesi *et al.*, 2001). In addition, *vic*-associated PCD rates are closely correlated with impedance of virus transfer (Biella *et al.*, 2002). As a final example in support the allorecognition theory, the vegetative nonself recognition function of *mat* in *N. crassa* impedes the transfer of plasmids during crosses. The *mat* locus functions as a vegetative nonself recognition locus only when *tol* is properly expressed. Strains that do not have a functional TOL do not have *mat*-associated vegetative nonself recognition. When crosses were performed between parents that had *mat*-associated vegetative nonself recognition (e.g. express a functional TOL) the frequency of paternal transmission of a circular plasmid to the resulting ascospores was low with only 5% of ascospores inheriting the

paternal plasmid. However, crosses performed between parents where neither parent had *mat*-associated vegetative nonself recognition (e.g. do not have a functional TOL) nearly 40% of the resulting ascospores received the paternal plasmid (Debets and Griffiths, 1998). These studies provide support for the allorecognition theory of vegetative nonself recognition evolution.

Studying Heterokaryon Incompatibility

To study heterokaryon incompatibility, two general methods are employed. In the first method, two strains containing different auxotrophic or antibiotic resistance markers are co-inoculated onto a minimal medium or a medium containing both antibiotics so that neither strain can grow on its own (Pittinger and Brawner, 1961). Growth observed on this plate will be due to the formation of a viable heterokaryon. If the two strains differ at one or more *het* loci, the resulting heterokaryon will grow slowly with an aberrant morphology or will not grow at all.

A second method utilized to study heterokaryon incompatibility is through partial diploids (Perkins, 1975). Partial diploids are synthesized so that the nucleus contains two copies of the incompatibility gene being studied. This can be done by crosses involving segmental duplications or through a transformation assay (Mylyk, 1975; Perkins, 1975; Smith *et al.*, 2000b). For the latter, a fungal strain containing an endogenous copy of the *het* locus being studied is transformed with DNA encoding the same (control) or the alternate allele of the *het* gene. For selection purposes, both the control and the alternate *het* allele are accompanied by a selectable marker such as an antibiotic resistance gene. When this is done with *N. crassa*, the transformed DNA randomly integrates into the

genome (Grant *et al.*, 1984). Transformants are haploid with the exception of the *het* locus being studied which occurs as two copies in the transformed nuclei, an endogenous and an ectopic (transformed) copy. Transformants with complimentary *het* loci have a wildtype morphology and wildtype growth rate. In contrast, transformants that express antagonistic *het* alleles are either non-viable, which is manifested by a significant reduction in transformation efficiency, or have a self-incompatible morphology. Self-incompatible colonies are morphologically distinguished by several characteristics including an aberrant colony form consisting of sparsely distributed, ‘spidery’ hyphae, the inability to conidiate or to produce aerial hyphae, and a slower than wildtype growth rate (Siahbazi, 2008; Wellman *et al.*, 2009). In addition, in the case of *un-24* in *N. crassa*, self-incompatible colonies yield less RNA per gram of mycelium than wildtype/self-compatible colonies, contain ~7 nuclei in each cell compared to wildtype/self-compatible cells that have ~30 nuclei per cell and have on average smaller cell volumes (~300 μm^3) compared to wildtype/self-compatible strains (~2000 μm^3 , Lafontaine, unpublished; Siahbazi, 2008). Electron micrographs of *un-24* self-incompatible colonies have revealed that branching hyphae are reduced in length and emerge from the main hyphae at ~90° angles. In contrast, wildtype and self-compatible transformants display significantly longer branching hyphae that emerge from the main hyphae at ~45° angles (Siahbazi, 2008). Figure 1.2 demonstrates some of these cytological differences between partial diploids that are self-incompatible (containing alternate *un-24* alleles) and self-compatible (containing two copies of the same *un-24* allele), the latter of which is indistinguishable from wildtype strains.

Figure 1.2: Cytological comparisons of self-incompatible (containing alternate *un-24* alleles) and compatible (containing two copies of the *un-24*^{OR} allele) *N. crassa* partial diploids. Scanning electron micrographs show that self-incompatible colonies have a sparse hyphal network, very narrow hyphae and an acute branch angles when compared to compatible colonies. Fluorescent micrographs following 4',6-diamidino-2-phenylindole (DAPI, stains nuclei) and calcofluor white (stains cell walls) staining reveal that cells from self-incompatible colonies have fewer nuclei per cell and smaller cell volumes compared to cells from compatible colonies. Micrographs from Siahbazi (2008).

Scanning Electron
Micrographs

Fluorescent Light
Micrographs

Self-Incompatible

Self-Compatible

[fig. 1,2]

An interesting characteristic of self-incompatible colonies is their ability to undergo escape. Escape is defined as a sudden shift from a self-incompatible morphology and growth rate to a wildtype morphology and growth rate (Smith *et al.*, 1996). The length of time that self-incompatible colonies require to undergo escape varies based on the nonself recognition locus. For example in *N. crassa*, escape from *un-24*-associated incompatibility requires 4-7 days while the majority of *het-c* self-incompatibles require 20 days to escape (Smith *et al.*, 1996; Siahbazi, 2008). Once colonies have undergone escape, reversion to a self-incompatible morphology has yet to be observed. One mechanism of escape is gene deletion. It has been observed that deletion of one of the alternate alleles in the *het-5*, *het-6* and *het-c* incompatibility systems in *N. crassa* results in escape (Jacobson, 1992; Smith *et al.* 1996; Xiang *et al.*, 2002) while independent or pairwise deletion of specific modifier loci (*mod-A*, *mod-B*, *mod-C* and *mod-D*) in *P. anserina* results in escape from *het-c/het-e*, *het-c/het-d*, and *het-r/het-v* non-allelic incompatibility (Belcourt and Bernet, 1969; Barreau *et al.*, 1998; Loubradou *et al.*, 1999; Xiang and Glass, 2002). A more thorough review of escape in both *N. crassa* and *P. anserina* is presented in Chapter 3.

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

The *het-6* locus of *N. crassa* was first identified through partial diploid analyses (Mylyk, 1975). The locus contains two tightly linked heterokaryon incompatibility genes, *het-6* and *un-24* (Figure 1.3A, Smith *et al.*, 2000b). Each of these genes has two alleles, designated Oakridge (OR) and Panama (PA). *un-24* lies ~5.2 kb and ~19 kb centromere distal to the *het-6* gene in PA and OR haplotypes, respectively (Micali and Smith, 2006). The *het-6* gene encodes a protein that has no known function outside of nonself

recognition (Smith *et al.*, 2000b). A conserved HET domain was characterized in the *het-6* protein that is now recognized as a motif common to about 30-60 genes in filamentous fungi, including incompatibility-associated proteins such as TOL and PIN-C of *N. crassa* and HET-E and HET-D of *P. anserina* (Smith *et al.*, 2000b). Not all HET containing proteins have been observed to have nonself recognition function. The OR and PA alleles of *het-6* share 78% identity and 68% similarity at the nucleotide and protein level, respectively, and sequence differences are spread throughout the entire gene (Micali and Smith, 2006). In contrast to *het-6* and all other characterized fungal incompatibility genes, *un-24* has a nonself recognition function and an essential role in cell function. *un-24* encodes the large subunit (R1) of a class I ribonucleotide reductase (RNR), the enzyme responsible for the conversion of nucleotides (NDPs) into deoxynucleotides (dNDPs). dNDPs are subsequently phosphorylated by endogenous kinases to become dNTPs which are used in DNA synthesis and repair. Relative to the *Escherichia coli* form of R1, UN-24 encoded by *N. crassa* has an insertion of ~160 aa within an otherwise highly conserved C-terminus domain while the N-terminus remains highly conserved between allelic forms. There are significant sequence differences in this C-terminus insertion that correlate to both nonself recognition activity and the specificity of the *un-24*^{OR} and *un-24*^{PA} allelic forms (Figure 1.3B). The two *un-24* alleles differ by ~30 aa in this 160 aa C-terminal region, including an insertion of six aa in the OR allele relative to the PA form (Smith *et al.*, 2000b). In contrast, there are only two aa differences in the N-terminus between the two allelic forms; serine and tyrosine in UN-24^{PA} replace phenylalanine and asparagine in UN-24^{OR} at amino acids positions 311 and 445, respectively (Smith *et al.*, 2000b).

Figure 1.3: The *het-6* locus of *N. crassa* encodes two genes, *het-6* and *un-24*, both of which have heterokaryon incompatibility activity. (A) Orientation of the *het-6* gene and *un-24* reveals a chromosomal inversion that is partly responsible for the complete linkage disequilibrium of these two genes. The right and left inversion breakpoints are designated *brk-1* and *brk-2*. In the *het-6*^{OR} locus, the *het-6*^{OR} gene and *un-24*^{OR} are separated by ~19 kb and are transcribed from alternate DNA strands as indicated. In contrast, the *het-6*^{PA} gene and *un-24*^{PA} are separated by ~5.2 kb and are transcribed from the same DNA strand. Genes and spacings are not drawn to scale. (B) The C-terminus of UN-24 (PA and OR) differs significantly from R1 subunits of other species, including closely related fungi such as *Magnaporthe grisea* (Mg). The identity plot was made by using a pairwise, sliding window analysis (60 aa in a window with 30 aa steps) between UN-24^{OR} and UN-24^{PA} (diamonds), UN-24^{PA} and R1 from *M. grisea* (triangles), and UN-24^{OR} and R1 from *M. grisea* (grey squares). Protein alignment of ~210 aa of the C-terminus of UN-24^{OR}, UN-24^{PA} and R1 from *M. grisea* is displayed at the bottom of the identity plot. Panel A is adapted from Micali and Smith (2006) and panel B is presented with permission from M.L. Smith.

A

B

[Fig. 1.3]

As a result of a paracentric inversion [*In(het-6)-I*] and associated sequence differences between the inversion breakpoints and these genes, *het-6* and *un-24* are in complete linkage disequilibrium. Only the allelic combinations *het-6*^{OR}/*un-24*^{OR} and *het-6*^{PA}/*un-24*^{PA} occur in nature and therefore these haplotypes represent distinct gene complexes. Regions between the inversion breakpoints and *het-6/un-24* of OR and PA haplotypes are highly dissimilar and thus expected to inhibit homologous recombination events that would result in *het-6*^{OR}/*un-24*^{PA} and *het-6*^{PA}/*un-24*^{OR} haplotypes. Interestingly, the right breakpoint (*brk-1*) of the *In(het-6)-I* inversion is associated with an element with inverted repeats that is represented several times in the *N. crassa* genome. Such characteristics are often signatures of transposable genetic elements. In the PA configuration, this putative transposon lies between the highly variable 3' end of *un-24* and *het-6*. These variable regions of *un-24* and *het-6* also exhibit a high frequency of non-synonymous substitutions between OR and PA alleles indicating that they are under diversifying selection (Micali and Smith, 2006).

The evolution of a 'super gene' at this locus could be a result of a strong selection pressure to maintain the *het-6*^{OR}/*un-24*^{OR} and *het-6*^{PA}/*un-24*^{PA} haplotypes as 'strong' incompatibility complexes. It also suggests that both non-allelic and allelic interactions among *het-6* and *un-24* gene products may be involved in the incompatibility mechanism. Introduction of the gene pairs *het-6*^{PA} and *un-24*^{PA} or *het-6*^{OR} and *un-24*^{OR} into an OR or PA strain, respectively, results in a significant reduction in transformation efficiency (Smith *et al.*, 2000; Micali and Smith, 2006). In contrast, transforming either *un-24*^{PA} or *het-6*^{PA} into an *un-24*^{OR} background strain results in 'weak' incompatibility. To demonstrate this, Micali and Smith (2006) transformed *het-6*^{PA} and *un-24*^{PA}, separately,

into OR strains. The resulting transformants were viable but exhibited a characteristic self-incompatible morphology and underwent escape within 4-7 days. Escape resulted in a loss of incompatibility activity by one or the other of the OR or PA alleles and resulted in the recovery of strains that were functionally *un-24^{PA}/het-6^{OR}* and *un-24^{OR}/het-6^{PA}*. Ascospores generated from separate crosses between functionally *un-24^{PA}/het-6^{OR}* escapes and wildtype *un-24^{OR}/het-6^{OR}* or wildtype *un-24^{PA}/het-6^{PA}* had a significantly lower germination frequency compared to progeny from crosses between escapes that were functionally *un-24^{OR}/het-6^{OR}* and wildtype (*un-24^{OR}/het-6^{OR}*). As such, the *un-24^{PA}/het-6^{OR}* combination has been proposed to confer a reduced fitness as it gives rise to significantly fewer viable progeny. In this same study, it was proposed that the presence of a strong 'super gene' complex would enhance the effectiveness of a protective function and reduce transmission of parasitic elements via heterokaryosis in the species.

Ribonucleotide reductase

Since *un-24* encodes a class I ribonucleotide reductase large subunit (R1) has opened up several interesting areas of research. Most notably, since the function of this enzyme is essential for DNA synthesis, it is possible that *un-24* incompatibility causes loss of enzyme function and results in a shortage of dNTPs and an interruption in DNA synthesis. Although it is currently unknown whether depletion of dNTP pools results in apoptosis in yeast and in *N. crassa*, depleted dNTP pools have been observed to cause apoptosis in mammalian cells (Roy *et al.*, 2004). Furthermore, since the *N. crassa* RNR holoenzyme is a tetramer, comprised of a large subunit dimer with a small subunit dimer, alternate allelic forms of UN-24 could interact and form a toxic complex. Three classes of RNR exist and are distinguishable based on functional differences, the cofactor used in

catalysis and their quaternary structure (reviewed in Reichard, 2002; Norlund and Reichard, 2006). All RNRs use free radical chemistry to complete catalysis. Class I RNRs are found in all eukaryotes and some prokaryotes and viruses. The holoenzyme functions mainly as a tetramer, comprised of two large subunits (R1) and two small subunits (R2). A diiron complex with R2 is used to generate the free radical. Class II RNRs are found primarily in prokaryotes and are expressed under both aerobic and anaerobic conditions. The holoenzyme comprises two R1 subunits and the enzyme uses cobalamin to generate the free radical used in catalysis. In contrast, class III RNRs, which are also found solely in prokaryotes, are expressed strictly under anaerobic conditions. The class III holoenzyme consists of two R1 and two R2 subunits while a 4Fe-4S cluster located in R2 is used to generate the free radical (Reichard, 2002).

This thesis is concerned with class I RNRs found in *N. crassa* and *S. cerevisiae*. Although the majority of structural and mechanistic work has been completed on the class I RNR from *E. coli*, the three dimensional structure and function of class I RNRs from *N. crassa* and *S. cerevisiae* are likely similar based on overall similarities in amino acid composition (72 % and 72.3% identity for OR and PA allelic forms, respectively) including highly conserved catalytically important residues (Xu *et al.*, 2006). A schematic overview of class I *E. coli* RNR structure and activity can be found in Figure 1.4. The *E. coli* R1 subunit contains the catalytic site for dNDP formation and two allosteric regulatory sites, one of which controls substrate specificity while the second controls enzyme activity (Mao *et al.*, 1992; Uhlin and Eklund, 1994). Substrate specificity is modulated by the presence of a particular nucleotide in the specificity site. Binding of each dNTP in the specificity site results in structural shifts within the

Figure 1.4: Schematic of class I RNR holoenzyme and reaction catalyzed in the catalytic site of R1. (A) The class I RNR holoenzyme consists of two R1 subunits and two R2 subunits. The allosteric activity (solid triangle) and specificity sites (solid circle) as well as the catalytic site (open circle) are located in the R1 subunit while the R2 subunit houses the diiron center that is used to generate the free radical. The catalytic site accepts NDPs and uses free radical chemistry to produce dNDPs. The allosteric activity site controls enzyme activity; binding of dATP inhibits enzyme activity while binding of ATP promotes enzyme activity. The allosteric specificity site controls substrate specificity of the catalytic site. (B) The overall enzymatic reaction of RNR. The 2' hydroxyl group of the ribose moiety is replaced with a hydrogen atom using free radical chemistry thus creating a dNDP. H_2O and a disulphide bond within the catalytic site of the large subunit are produced as a result of this reaction. C-terminal cysteines are involved in restoring sulfhydryl groups in the catalytic site after NDP reduction. The nitrogenous base of the nucleotide is represented by *. Adapted from Elledge *et al.* (1992) and structural model outlined from M. Hogbom, University of Stockholm.

A

B

[fig. 1.4]

enzyme's catalytic site. This allows a specific NDP located in the catalytic site to orient in close proximity to catalytically important residues and undergo catalysis (Xu *et al.*, 2006). Binding of ATP or dATP in the specificity site allows for the reduction of CDP and UDP within the catalytic site. Binding of dTTP and dGTP allow for reduction of GDP and ADP, respectively (Reichard, 2002). Catalytic activity is promoted by the presence of ATP in the R1 allosteric activity site, while inactivation is caused by the presence of dATP within this site (Reichard, 2002). Catalysis is initiated when the diiron center located in R2 generates a tyrosyl radical through a currently unknown mechanism. This radical undergoes a long-range transfer via amino acid R-groups to a properly located NDP substrate within the catalytic site of R1. In all, five cysteines are involved in class I RNR activity as demonstrated by the *E. coli* system. Prior to catalysis, C439 accepts the electron from the tyrosyl radical on R2, generating a thiyl group, which is essential in 2' hydrogen abstraction from the ribose sugar (Mao *et al.*, 1992). During 2' hydroxyl group removal from the ribose moiety, two cysteines located within the catalytic site (C225 and C462) are oxidized and form a disulphide bond. Once the dNDP is released, the flexible C-terminus of the adjacent R1 subunit within the holoenzyme enters the catalytic site to reduce the catalytic site disulphide bond. The disulphide bond is transferred from C225 and C462 (catalytic site residues) to residues C754 and C759, which are located in the C-terminus of the adjacent R1 subunit, by a mechanism that is currently unknown. The C-terminus then moves out of the catalytic site and the C754/C759 disulphide bond is subsequently reduced by either glutaredoxin or thioredoxin, thus reactivating the enzyme (Uhlund and Eklund, 1994; Mirand-Vizueté *et al.*, 1996).

Although the overall structure and mechanism of RNR is thought to be similar between *E. coli*, *S. cerevisiae* and *N. crassa*, there are several important differences among these systems. UN-24 in *N. crassa* and R1 from many other eukaryotes differ from R1 of *E. coli* in having an additional cysteine residue in the C-terminus. Based on mutational studies, this residue appears to be involved in UN-24 catalytic activity (Wellman *et al.*, 2009). Both UN-24^{OR} and UN-24^{PA} differ significantly between each other and other R1s in that they contain a highly variable C-terminus region. Despite these differences, the C-terminus of UN-24 is thought to retain its role in RNR catalytic activity (Wellman *et al.*, 2009). *S. cerevisiae* is unusual in that two forms of both the large and the small subunits are encoded in the genome. *RNR1* (R1 subunit) and *RNR2* and *RNR4* (R2 subunit) are involved in cell cycle related dNDP synthesis while *RNR3* (R1 subunit) is up-regulated in response to DNA damage (Elledge and Davis, 1990). In addition, *S. cerevisiae* contains Sm11p, a protein that inhibits RNR activity by blocking access to the R1 catalytic site. When dNDP synthesis is required, Sm11p is phosphorylated and subsequently removed from R1 allowing NDP access to the catalytic site (Chabes *et al.*, 1999; Zhao *et al.*, 2000).

Although their primary function is to create dNDP precursors for dNTP formation and hence DNA replication and repair, other roles of RNR have been reported. In *Xenopus* egg mitotic extracts, RNR has been observed to activate spindle pole bodies (Takada *et al.*, 2000). Sambade *et al.* (2005) observed that yeast strains that lack *RNR1* have a significant decrease in vacuolar ATPase (vATPase) activity indicating a potential role in modulating vacuolar acidification and cellular pH homeostasis. Interestingly, the class I R1 subunit of the β -herpesvirus subfamily is not involved in dNDP synthesis at

all. Instead, this R1 protein is able to block cellular signaling pathways that are involved in the immune response of host cells (Lembo and Brune, 2009). Finally, as mentioned previously, the nonself recognition function appears to be unique to the R1 subunit (UN-24) of *N. crassa*.

***un-24*-associated Incompatibility**

Although several heterokaryon incompatibility loci have been genetically characterized, few have been biochemically characterized. To study the *un-24* system from *N. crassa*, partial diploids were created by transforming *un-24*^{OR} into strains containing *un-24*^{PA} to obtain *un-24*^{PA/OR} partial diploids and by transforming *un-24*^{OR} strains with *un-24*^{PA} to obtain *un-24*^{OR/PA} transformants (Micali and Smith, 2006). In the partial diploid designation, the first superscripted allele represents the endogenous *un-24* allele while the second represents the ectopic *un-24* allele. Transformation of *un-24*^{OR} into *un-24*^{PA} resulted in a > 90% reduction in transformed colonies recovered compared to *un-24*^{OR} into *un-24*^{OR} transformations. This large reduction in transformation efficiency was subsequently called OR-like incompatibility. In contrast, transformation of *un-24*^{PA} into *un-24*^{OR} resulted in a 20 - 40% reduction in transformation efficiency compared to *un-24*^{PA} into *un-24*^{PA} transformations. The majority of these *un-24*^{OR/PA} transformants displayed a self-incompatible phenotype. On transformation plates, these colonies were small and had a sparse, irregularly shaped mycelium resembling, and consequently referred to as ‘spider’ or ‘star’ colonies. In contrast, *un-24*^{PA/PA} transformants were large, had a robust mycelium and conidiated. These self-compatible colonies resembled, and therefore, were referred to as ‘cloud’ colonies. When transferred to Vogel’s minimal medium (VMM) plates, these *un-24*^{OR/PA} colonies grew significantly

slower than *un-24*^{PA/PA} colonies, had an aberrant ‘spidery’ mycelial morphology, were aconidial and escaped within 4-7 days of growth. This was subsequently called PA-like incompatibility (Wellman *et al.*, 2009).

Incompatibility specificity was subsequently confirmed to be associated with the C-terminus of UN-24. Two chimeric constructs, OR/PA and PA/OR, were created such that each construct contained the N-terminus and C-terminus from alternate UN-24 alleles. The OR/PA construct contained the UN-24^{OR} N-terminus and ~500 aa of the C-terminus from UN-24^{PA} while the PA/OR construct contained the N-terminus from UN-24^{PA} and ~500 aa of the C-terminus from UN-24^{OR}. Transformation of the OR/PA chimera resulted in PA-like incompatibility while transformation of the PA/OR resulted in OR-like incompatibility. These results were consistent with the idea that the UN-24 incompatibility specificity domain was located in the highly variable C-terminus (Haidari, 2002).

An emerging theme in heterokaryon incompatibility is that PCD is elicited by the formation of a toxic protein complex formed from protein products of alternate *het* alleles. In the *het-s* system from *P. anserina*, when HET-s is in its prion form, [Het-s], it interacts with HET-S to form a toxic protein complex. It has been proposed that the presence of HET-S interrupts the formation of non-toxic fibrillar [Het-s] aggregates and results the formation of smaller and more toxic HET-S-[Het-s] oligomers (Coustou *et al.*, 1997; Balguerie *et al.*, 2004). The *het-c* system from *N. crassa* provides another putative example of a toxic protein complex formed between alternate *het* alleles. Alternate allelic forms of HET-C appear to form complexes and further interact with a non-allelic partner PIN-C. These HET-C heterodimers appear to localize to the cell membrane and cause

incompatibility by either disrupting membrane integrity or stimulating pathways that slow cell growth (Kaneko *et al.*, 2006). Recent evidence suggests that UN-24^{PA} and UN-24^{OR} form an incompatibility protein complex to trigger PCD (Figure 1.5). Using anti-mouse RNR1-(N) antibodies, it was shown that UN-24^{OR} and UN-24^{PA} form a non-reducible complex in *un-24^{OR/PA}* self-incompatibles. The intensity of this complex was positively correlated with the degree of self-incompatibility. *un-24^{OR/PA}* colonies that displayed the most severe growth inhibition had relatively more UN-24^{OR}-UN-24^{PA} complex than *un-24^{OR/PA}* colonies with less severe growth inhibition (Haidari, 2002; Wellman *et al.*, 2009). Upon escape, UN-24^{OR} and UN-24^{PA} were no longer observed to complex but rather appeared at the expected size (~100 kDa) of the monomeric UN-24. It was thus hypothesized that PCD results from the UN-24^{OR}-UN-24^{PA} toxic heterodimeric complex itself, or a subsequent perturbation in dNTP pools associated with a non-functional RNR holoenzyme (Wellman *et al.*, 2009).

Importance of Studying Heterokaryon Incompatibility

Investigating heterokaryon incompatibility in *Neurospora* and other filamentous fungi has led to several important discoveries and has basic evolutionary and life history implications (Micali and Smith, 2005). For example, the discovery of the *het-s* system in *P. anserina* has led to the extensive characterization of a novel prion and aggregation domain (Coustou *et al.*, 1997). Recently, [Het-s] has been shown to replicate in *S. cerevisiae* demonstrating that prions that do not contain Q/N rich regions can replicate in yeast (Taneja *et al.*, 2007). This has spurred research into finding prions endogenous to yeast that do not have Q/N rich regions. Increasing evidence suggests that death due to heterokaryon incompatibility is mediated by PCD (Glass and Dementhon, 2006).

Figure 1.5: Immunoblot of proteins extracted from $un-24^{OR/PA}$ self-incompatible strains probed with anti-mouse RNR1(N) provides evidence for formation of a non-reducible UN-24^{OR}-UN-24^{PA} protein complex. Protein extracts from mouse (lane 1), $un-24^{OR/PA}$ self-incompatible colonies (lanes 2-5), a post-escape $un-24^{OR/PA}$ self-incompatible colony (lane 6) and untransformed $un-24^{OR}$ (lane 7) and $un-24^{PA}$ strains (lane 8). The $un-24^{OR/PA}$ self-incompatible samples are arranged from most inhibited (lane 2) to least inhibited (lane 5) as indicated by the increasing height of the triangle. The white arrowhead indicates the UN-24^{OR}-UN-24^{PA} complex in $un-24^{OR/PA}$ self-incompatible colonies. The black arrowhead indicates the predicted size of the UN-24 monomer. The mouse R1 protein occurs at the expected size of 93 kDa. Protein size standards in kDa are indicated at left of figure. Adapted from Wellman *et al.* (2009).

[fig. 1.5]

Characterizing PCD in incompatible heterokaryons may lead to the discovery of novel apoptotic pathways in fungi which may be pertinent to anti-cancer studies in humans. Studying the mechanisms of heterokaryon incompatibility also has applications in environmental plant pathogen control. The classic example of this involves the filamentous fungus *C. parasitica*, the causal agent of chestnut blight. Introduction of this pathogen to North America decimated the American chestnut tree in the early 1900s. Biological control of chestnut blight via the introduction of *C. parasitica*-specific double stranded RNA viruses that inhibit chestnut infection was successful in Europe and in Michigan but not in the eastern United States and Canada (Nuss, 1992; reviewed in Milgroom and Cortesi, 2004). Infection of *C. parasitica* with these RNA viruses significantly inhibits the ability of the fungus to cause chestnut blight (Cummings-Carlson, 1998; Dawe and Nuss, 2001). Although interactions between trophic levels (virus, tree and fungus) as well as environmental factors modulate successful viral infection, the best correlated factor in determining the spread of hypovirulence success is the absence of allelic variants at the nonself recognition-mediating *vic* loci (Milgroom and Cortesi, 2004). Heteroallelism at *vic* loci prevents heterokaryon formation with the exception of the *vic-4* locus (Smith *et al.*, 2006). Since hypovirus transmission is through vegetative cell fusion, populations of *C. parasitica* with fewer vegetative compatibility groups have a greater probability of virus infection. In contrast, in areas where *vic* diversity is high, such as the eastern United States and Canada, spread of the virus within the population is low (Cortesi *et al.*, 2001; Milgroom and Cortesi 1999). Reduction in virus transmission by single and multiple *vic* differences has been demonstrated in laboratory studies (Cortesi *et al.*, 2001) and is correlated to the rate of *vic*-associated PCD

(Biella *et al.*, 2002). An understanding of incompatibility systems may therefore facilitate biological control strategies of pathogenic fungi and other pathogens. Finally, study of nonself recognition in fungi may lead to the development of chemotherapeutics that target specific organisms. The emergence of antifungal resistance in fungi that cause human mycoses and devastate food crops highlights the importance of developing novel strategies to control fungal growth (Alexander and Perfect, 1997; Jessulat *et al.*, 2006).

Yeast as a Model Organism

Both *N. crassa* and *S. cerevisiae* have been used as model organisms for decades due to ease of manipulation, rapid doubling rate, similarity to pathogenic fungi and their close relatedness to the animal kingdom (Taylor *et al.*, 1993). *S. cerevisiae* has been used as a model organism in several areas including studies on apoptosis, metal homeostasis, and population genetics (Madeo *et al.*, 2002; Zeyl, 2000; Sychrova, 2004). The first eukaryotic genome to be fully sequenced was that of *S. cerevisiae* (Goffeau *et al.*, 1997) while the complete genomic sequence of *N. crassa* was released in 2003 (Galagan *et al.*, 2003). Comparison of the genomic sequences between these two organisms revealed that *S. cerevisiae* and *N. crassa* share a high degree of similarity. 87% of ORFs in found in *N. crassa* have a homolog in *S. cerevisiae*. In addition, 17% of these shared ORFs are fungal specific (Mannhaupt *et al.*, 2003). Although the *N. crassa* genome has been fully sequenced and overexpression/gene deletion libraries are emerging, *S. cerevisiae* remains a better characterized organism. With the emergence of databases such as the Saccharomyces Genome Database (Cherry *et al.*, 1998), a library that contains knockouts of non-essential genes (Giaever *et al.*, 2002) and other well-developed resources, the

ability for researchers to express and characterize novel non-endogenous proteins in *S. cerevisiae* has increased drastically.

Objectives of this Thesis

The primary objective of this thesis was to study the *N. crassa un-24* incompatibility system using the model organism *S. cerevisiae*. Yeast was chosen due to its lack of endogenous vegetative incompatibility and it was reasoned that the *un-24* system could therefore be studied in yeast without interference from other vegetative incompatibility factors. In Chapter 2, *un-24* genes were cloned behind the *GALI* promoter in *S. cerevisiae*. We determined that the *un-24*^{PA} incompatibility domain is able to cause an incompatibility-like phenotype in yeast and explored the mechanisms by which incompatibility was caused. In Chapter 3, again using the yeast system, we determined a possible mechanism of escape from *un-24*-associated incompatibility. Our yeast studies led us to hypothesize that escape from *un-24*-associated incompatibility involved Hsp70-like protein interactions in *N. crassa*. Finally, in Chapter 4, we initiate a physical study of the C-terminus of UN-24^{PA} with an aim to understand how this domain interacts with the large subunit of RNR in yeast and UN-24^{OR} in *N. crassa*. Constructs containing site directed mutations were expressed in *S. cerevisiae* and examined for incompatibility activity. To compliment these results, we expressed mutant forms of the UN-24^{PA} incompatibility domain in *E. coli* and examined the effects of replacing key cysteine and proline residues on gel mobility of expressed proteins in SDS-PAGE.

CHAPTER 2: *un-24*-ASSOCIATED INCOMPATIBILITY IN YEAST

INTRODUCTION

Nonspecific recognition systems are ubiquitous across diverse organisms as exemplified by restriction enzymes in prokaryotes and the Major Histocompatibility Complex (MHC) in vertebrates. In filamentous fungi, nonspecific recognition occurs in both the sexual and vegetative phases. During vegetative growth, cell fusion between conspecific fungi may result in the formation of heterokaryons, cells that contain two or more genetically distinct nuclei (reviewed in Saupe, 2000; Smith *et al.*, 2000; Micali and Smith, 2003). Proliferation of such heterokaryons in *N. crassa* is governed by at least nine heterokaryon incompatibility (*het*) loci. The *het-6* locus is comprised of two genes with incompatibility activity, the *het-6* gene and *un-24*. *un-24* is unique in that it also encodes the large subunit (R1) of a class I ribonucleotide reductase (RNR). No other fungal incompatibility gene characterized to date has essential catalytic activity, and there have been no reports of nonspecific recognition function by RNR genes in organisms aside from *Neurospora* (Saupe, 2000; Micali and Smith, 2006).

The incompatible Oakridge (OR) and Panama (PA) allelic forms of UN-24 in *N. crassa* differ significantly from each other, and from other R1s, by their C-terminus region (Micali and Smith, 2006). When UN-24^{OR} and UN-24^{PA} occur together in the same cell during the asexual growth phase, an incompatibility reaction ensues as a result of the apparent formation of a non-reducible UN-24^{OR}-UN-24^{PA} complex (reviewed in Chapter 1). Since the C-terminus of R1 is involved in restoring redox-potential to the R1 catalytic site, these allelic differences suggested a possible mode of activity (Wellman *et al.*, 2009).

Several lines of evidence suggest that the C-termini of UN-24^{OR} and UN-24^{PA} mediate incompatibility. Haidari (2002) transformed chimeric PA/OR and OR/PA constructs, in which the N-terminal and C-terminal regions of alternate allelic forms were swapped, into *N. crassa* (see Chapter 1). Both PA-like incompatibility (transformants with a self-incompatible phenotype), and OR-like incompatibility (> 90% loss in transformation efficiency), was correlated with the identity of the C-terminus. This first suggested that the C-terminus of UN-24 was responsible for both specifying and mediating incompatibility. Wellman *et al.* (2009) further explored this hypothesis. In their study, they assessed both the incompatibility activity and the catalytic activity of UN-24 using partial diploids. Catalytic activity was assessed using a temperature sensitive form of *un-24*^{OR}. This mutant, designated *un-24*^{ts}, was created using repeat-induced point mutation (RIP; Smith *et al.*, 2000a). *un-24*^{ts} colonies have a wildtype morphology and growth rate when grown at $\leq 34^{\circ}\text{C}$ but do not grow at temperatures $> 34^{\circ}\text{C}$, except on high osmotic medium (Smith *et al.*, 2000a). When *un-24*^{OR} was transformed into *un-24*^{ts}, the resulting transformants were rescued from temperature sensitivity and were able to grow at temperatures $> 34^{\circ}\text{C}$. When ~ 384 aa was removed from the N-terminus of UN-24^{OR}, the construct was unable to complement the temperature sensitivity of *un-24*^{ts} strains suggesting that this area was required for RNR catalytic activity. However, this construct did cause OR-like incompatibility when transformed into *un-24*^{PA}. This result provided further evidence that the OR specificity domain was not located in N-terminus of UN-24^{OR} and that catalytic and incompatibility functions by UN-24 are somewhat independent.

To further rule out that the N-terminal region alone could act as a determinant for *un-24* incompatibility specificity, Wellman *et al.* (2009) created constructs in which N-terminal amino acids found in one allele of UN-24 were substituted to the corresponding residue of the alternate allelic form. In the N-terminal region, only two amino acids differentiate UN-24^{OR} and UN-24^{PA} allelic forms; serine and tyrosine in UN-24^{PA} replace phenylalanine and asparagine in UN-24^{OR} at amino acids positions 311 and 445, respectively. Substitution of both of these residues separately resulted in constructs that retained catalytic activity as determined by complementation with *un-24^{ts}*. Incompatibility specificity of these substitution constructs was correlated with the identity of the C-terminus and not the changes induced in the N-terminal substitutions. It was concluded that the N-terminus alone did not specify incompatibility activity.

To explore the role of the C-terminus in mediating *un-24*-associated incompatibility, constructs consisting of the hygromycin B resistance gene fused in-frame at the 5' end of varying portions of the *un-24^{OR}* or *un-24^{PA}* 3' end were transformed into *N. crassa* to create partial diploids (Wellman *et al.*, 2009). Hygromycin B was chosen as the resistance marker since it is highly selectable in *N. crassa* and in other higher eukaryotes and it is not cross-reactive with numerous other antibiotics (Blochliger and Diggelmann, 1984; Staben *et al.*, 1989). Transformation of hygunPA, a construct that encoded 135 aa of C-terminus from UN-24^{PA} (residues 788-923) into *N. crassa* strain C9-2 (*un-24^{OR}*) led to a PA-like incompatibility reaction. Incompatibility was also evident when 73 aa were removed from the N-terminal end of hygunPA demonstrating that 62 aa of the C-terminus (residues 861-923) conferred incompatibility activity. In contrast, the construct hygunOR, which incorporated a region of 141 aa including the UN-24^{OR} C-

terminus (residues 788-929) did not cause incompatibility when transformed into C2(2)-1 (*un-24^{PA}*). The construct *hygunOR(L)*, which consisted of UN-24^{OR} residues 355-929, thus containing N-terminal residues important in RNR catalytic activity, caused incompatibility when transformed into C2(2)-1. Removal of more than two amino acids from the C-terminus region of *hygunOR(L)* resulted in a loss of incompatibility activity. Thus, OR-like incompatibility required residues located in the C-terminus and the region that encoded the catalytic site of UN-24^{OR}. This suggested that residues located in the N-terminal region of UN-24^{OR} that incorporate the OR catalytic site are required for incompatibility but that the C-terminal region specifies OR-like incompatibility. Only the C-terminus of UN-24^{PA} is required for PA-like incompatibility activity and specificity.

Overall, then, incompatibility activity was evident with both OR and PA constructs that did not contain the N-terminal region and thus did not have RNR activity. However, constructs that lacked incompatibility activity but retained RNR activity have never been observed (Smith *et al.*, 2000b; M.L. Smith, pers. comm.). This suggested that residues essential for RNR activity were likely involved in mediating incompatibility (Wellman *et al.*, 2009). Through mutational analysis (reviewed in Chapter 4), Wellman *et al.* (2009) suggested that an aberrant disulphide bond between catalytic site cysteine residues in UN-24^{OR} and C-terminal cysteine residues in UN-24^{PA} potentiated the UN-24^{OR}-UN-24^{PA} complex. Interestingly, this complex was not broken down under strong reducing conditions (DTT, boiling) which suggested a non-reducible linkage was holding the complex together (see Chapter 1; Haidari, 2002). Since disulphide bonds are reduced in the presence of DTT, this intermolecular bond appears to only potentiate, and not be the major linkage between UN-24^{OR} and UN-24^{PA} in the complex.

The well-defined incompatibility domain of UN-24 offered a unique opportunity to investigate trans-species activity of an incompatibility system. To our knowledge, a vegetative incompatibility system does not occur in yeast. In addition, *in silico* experiments suggest that yeast lack genes homologous to several, well conserved *het* loci in fungi (Pal *et al.*, 2007). Yeast and filamentous fungi diverged roughly 1.1 billion years ago (Cai *et al.*, 2006) and, as a result of a unicellular lifestyle, yeast may not have evolved vegetative nonself recognition loci or may have lost the attribute. Therefore, by expressing *N. crassa* incompatibility factors in yeast it was hoped that the *un-24* system could be studied without interference from other vegetative nonself recognition factors endogenous to *N. crassa* and other filamentous fungi. In addition, we wanted to examine if the UN-24^{OR}-UN-24^{PA} complex alone could cause *het*-associated incompatibility in yeast or if additional protein partners or metabolic pathways specific to *N. crassa* are required to cause incompatibility and initiate PCD.

The original objective of this chapter was to examine the consequences of co-expression of *un-24*^{OR} and *un-24*^{PA} constructs in diploid yeast. While examining the effects of expressing *un-24* constructs in yeast, it was evident that the PA incompatibility domain itself caused several cytological changes in yeast that were reminiscent of *het*-associated incompatibility in filamentous fungi. In this chapter, we characterized this incompatibility phenotype in yeast strains and investigate whether or not this domain interacts with the large subunit of ribonucleotide reductase (Rnr1p) endogenous to yeast. Examining trans-species activity of the PA incompatibility domain in yeast provided us with novel insights into incompatibility function in *N. crassa*.

METHODS AND MATERIALS

Microbiology and Molecular Genetic Techniques.

S. cerevisiae strains listed in Table 2.1 were cultured by standard methods (Ausubel *et al.*, 1997) with either YPD [1% Bacto Yeast Extract (BD, New Jersey, USA), 2% Bacto Peptone (BD), 2% Glucose (BD) with or without 2% Agar (BD)] or YPRaf/Gal [1.5% Raffinose (Mississauga, Sigma-Aldrich, ON) and 0.5% Galactose (Sigma-Aldrich) in place of 2% glucose]. Antibiotic selective plating was performed with 100 µg/mL hygromycin B (hygB, Roche, Basel, Switzerland), 100 µg/mL nourseothricin (NAT, Werner Bioagents, Jena, Germany) and/or 200 µg/mL geneticin (G418, Bioshop, Oakville, ON). In all cases, when grown in YPD or YPRaf/Gal overnight, subsequent manipulation was performed in the same medium (unless otherwise indicated). To achieve log-phase growth, all overnight cultures were diluted one in five in fresh medium and incubated with shaking for a minimum of 2 h. *N. crassa* strain C9-2 (*un-24*^{OR} *het-6*^{OR} *thr-2 a*) was grown on medium with 1 X Vogel's salts and 250 mg/L threonine at 30°C with or without 1.5% agar (VMM, Vogel, 1964). Selection for hygB-resistant strains was performed on VMM supplemented with 200 µg/mL hygB.

All PCR reactions were completed in a 20 µL reaction volume using 1 X Reaction Buffer (Invitrogen, Burlington, ON), 1.5 mM MgCl₂ (Invitrogen), 0.2 mM of each dNTP (Bioshop), 1 unit (U) of Invitrogen Taq polymerase (10,000 U/mL), 0.5 mM forward primer, 0.5 mM of reverse primer and 100 pg – 1 µg of template. The reaction was made up to 20 µL using ddH₂O. PCR was performed in a Biometra T-Gradient Thermoblock (Biometra, Goettingen, Germany). PCR product sizes were verified by gel

Table 2.1: List of *S. cerevisiae* strains used in this chapter.

Strain	Genotype	Reference
Y2454	<i>MATα mfa1Δ::MFA1pr-HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0</i>	Tong <i>et al.</i> , 2001
Y3068	<i>MATα can1Δ::MFA1pr-HIS3 ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0</i>	Tong <i>et al.</i> , 2001
<i>yma11::G418^R</i>	<i>MATα/a his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0 YPL234CA::G418^R</i>	Giaever <i>et al.</i> , 1992
<i>cwp2::G418^R</i>	<i>MATα/a his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0 YKL096W-AΔ::G418^R</i>	Giaever <i>et al.</i> , 1992
pGAL-RNR1	<i>MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 pGAL-RNR1</i>	Sopko <i>et al.</i> , 2006
pGAL-RNR3	<i>MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 pGAL-RNR3</i>	Sopko <i>et al.</i> , 2006

electrophoresis in 0.8% agarose (Bioshop). All primers were manufactured by Sigma Aldrich. All constructs were sequenced (Stemcore, Ottawa, ON) and determined to be error free.

Restriction digests were performed as recommended by the enzyme manufacturer. For double digestions, the reaction buffer that allowed both enzymes to function at the highest efficiency was selected (New England Biolabs, Mississauga, ON). In all cases, each reaction was supplemented with 10 µg of BSA (New England Biolabs) and each reaction was performed in a volume of 20 µL.

DNA cloning was performed with plasmids TOPO-TA pCR2.1 (Invitrogen) and pCB1004 (Carroll *et al.*, 1994), which contains the hygromycin phosphotransferase selectable marker conferring hygromycin resistance. Ligation of PCR products into TOPO-TA pCR2.1 was performed as per the manufacturer's specifications (Invitrogen). Plasmids were purified using a Wizard Mini Prep Kit (Promega, Madison, WI) as recommended by the manufacturer. Quantification of DNA and RNA was performed using a NanoDrop spectrophotometer (Model ND-1000, Nanodrop, Washington DC, USA).

Creation of Transgenic Yeast Strains

un-24 fusion constructs used are represented in Figure 2.1 and were prepared as described in Wellman *et al.* (2009). PA and OR *un-24* constructs were inserted into the *GAL1* (YBR020W) locus of strains Y2454 and Y3068, respectively, by homologous recombination. Likewise, hyg control constructs were inserted into the *GAL1* locus. Primers were designed with flank areas homologous to YBR020W (Table 2.2) and the

Table 2.2: Oligonucleotide primers used to create *un-24* constructs capable of homologous recombination at the *GAL1* locus in yeast.

The underline sequence indicates area identical to the *GAL1* locus

Primer	Sequence (5'-3')	~T _m (°C)
YEASTHYGFWD	<u>TTGTTAATATACCTCTATACTTTAACGTC</u> AAGGAGAGAAAACTATAATGACTAAAAATGAAA AAGCCTGAAC TCACC	62 ¹
YEASTUN24REV	<u>TCATATAGACAGCTGCCCAATGCTGGTTAGAGACGATGATAGCATT</u> TTTCTAGCTGTGCGG GCTTAACCGCTGCA	74 ¹
YEASTHYGR1	<u>TCATATAGACAGCTGCCCAATGGTTAGAGACGATGATAGATT</u> TTTCTAGCTCTATTCCTTTG CCCTCGGAC	65 ¹
HYGFLAGREV	<u>TCATATAGACAGCTGCCCAATGCTGGTTAGAGACGATGATAGCATT</u> TTTCTAGCTTCCCCTTG TCATCGTCGTCCTTGTAATC	72 ¹
GALFWD	<u>TTAACGTC</u> AAGGAGAAAAAACTATA	59 ¹
GALREV	<u>TGCTGGTTTAGAGACGATGATAGCA</u>	68 ¹
HYGSEQFWD	TGGCTGTGTAGAAAGTACTGCGCCGAT	70

¹ estimated based on sequence homologous to *un-24* construct

construct of interest. Addition of *GALI* homologous ends to hyg_{un}PA, hygFLAG_{un}PA, hyg_{un}OR and hyg_{un}OR(L) was completed using primers YEASTFRW and YEASTREV with 35 cycles of the following program: 30 s at 95°C (denaturation), 30 s at 56°C (annealing) and 2 min 30 s at 72°C (extension). For constructs hyg and hygFLAG, the forward primer, YEASTFRW, was used in conjunction with either YEASTHYGR1 or HYGFLAGREV reverse primers, respectively, in the following PCR program: 35 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing) 1 min 30 s at 72°C (extension). We used the standard FLAG epitope sequence in this study (DYKDDDDK).

Yeast strains were transformed using lithium acetate as described in Geitz and Woods (2002) and selected on YPRaf/Gal plates containing hygB. To verify homologous recombination, genomic DNA was obtained from transformants by the bead beater method as described in Adams *et al.* (1997). Primers GALFRW and GALREV were complimentary to the area flanking the *GALI* locus and therefore produced an amplicon of the putative replaced region. These primers were used in the following PCR program that was cycled 35 times: 30 s at 95°C (denaturation), 30 s at 48°C (annealing), 2 min 30 s for 72°C (extension). The resulting amplicon was digested with either *PvuI* or *HindIII*. Successful digestion with *PvuI*, but not with *HindIII*, indicated a successful replacement of *GALI* with the construct of interest. The PCR amplicons were subsequently sequenced using the primer HYGSEQFWD and verified.

Yeast Standard Curves

Yeast strains were grown in liquid YPD or YPRaf/Gal to log phase. At various OD₆₀₀ values, duplicate aliquots of the cell suspension were removed, diluted 10⁻⁵ and plated on either YPD or YPRaf/Gal agar plates. After two days incubation at 30°C,

colonies on these agar plates were counted and the cell density (cells/mL) of the original culture at each OD₆₀₀ measurement was calculated. Standard curves of cell density versus OD₆₀₀ were thus produced. The equations of these standard curves (APPENDIX 1) were used to calculate yeast based on OD₆₀₀ of liquid cultures throughout this thesis.

Quantitative Mating Assay

Cells of opposite mating-type were grown overnight in either YPD or YPRaf/Gal liquid medium. Cell density was determined using OD₆₀₀ measurements and 10⁶ cells of both mating-types were collected on a 0.45 µm nitrocellulose filter (Type HA, Millipore, Bedford, MA) by vacuum filtration. The respective filters were incubated cell side up at 30°C for 5 h on either YPD or YPRaf/Gal agar plates and then the cells were resuspended in liquid medium (YPD or YPRaf/Gal) contained in a Petri dish by gentle shaking. The liquid cultures were then transferred into sterile tubes and subjected to sonication for 5 s using a Branson 1210 bath sonicator (Branson Ultrasonic Corporation, Danbury, CT). For diploid selection, mated cultures were centrifuged at 2,000 x g for 2 min, the pellet was resuspended in 100 µL of fresh medium and was plated on YPD or YPRaf/Gal agar plates containing NAT and G418 to select for diploids. For haploid selection, cultures were diluted 10⁻⁴ prior to centrifugation and plated on YPD or YPRaf/Gal agar plates which contained either NAT or G418. Filters containing only MAT α or MATa cells, separately, were prepared and plated as described above to control for sexual activity prior to the 5 h incubation. All plates were incubated for 5 days at 30°C to allow diploids to develop. The number of diploids (NAT^R/G418^R) and haploid MAT α (NAT^R) and MATa (G418^R) cells were counted and mating efficiency was determined using the following equation:

$$\text{Mating efficiency} = \frac{\text{\# diploids (per mL)}}{\text{\# of MAT}\alpha \text{ and MATa cell (per mL)}}$$

Differences in mating efficiency among different mating combinations were tested for significance.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) values for hygB and hydroxyurea (HU, Lot # 1932H, Bioshop) were determined with yeast using standard methods as described in Rex *et al.* (2001). The MIC was recorded as the lowest concentration of inhibitor at which no growth was visible after two days of growth at 30°C. Yeast strains were grown overnight and cell density was determined as described in ‘Quantitative Mating Assay.’ Each culture was diluted to the equivalent of 1 cell/μL in fresh medium and 100 μL of culture was added to each well in a 96-well microtitre plate. The highest concentration of hygB and HU in YPD was set to 200 μg/mL and 300 μM, respectively. In YPRaf/Gal, the highest concentration was 3000 μg/mL and 300 μM for hygB and HU, respectively. For both compounds, dilution series of 1/2 and 1/3 were performed across the plate. MIC values for calcofluor white (CW, Lot # M2R, Cyanamid of Canada, ON) were determined using a drop plate method as described in Chen *et al.* (2003). A 10-fold dilution series of cells was performed on YPD and YPRaf/Gal plates containing CW at concentrations of 0, 25, 50, 75, 100, 200, 300 and 400 μg/mL. Plates were incubated at 30°C for 48 h and growth was monitored. Five independent replica trials were performed in each medium and for each strain. Differences in MIC values for hygB and HU among different yeast strains were tested for significance.

Growth Rate

To measure growth rate, strains were grown overnight in YPD or YPRaf/Gal liquid medium. The cells were diluted in fresh medium to a concentration of 1,000 cells/ μ L and 100 μ L of each strain was placed in a 96-well plate and shaken at 30°C at 150 RPM. OD₆₀₀ was measured at ~1 h intervals without resuspending the cells using a Molecular Devices SpectraMax 340 96-well plate reader (Molecular Devices, Sunnyvale, CA). The mean cell density values, calculated from standard curves of each strain, were plotted as a function of time based on 3 independent trials. Differences in growth rate for yeast strains were tested for significance.

Settling Rate

Mid log-phase cells were cultured and one mL of culture was placed in a 4.5 mL polystyrene cuvet (Fischer). The cuvet was inverted to resuspend the culture and placed in a Biochrom Novapac Plus spectrophotometer (Amersham, Uppsala, Sweden). OD₆₀₀ values were measured ~15 min intervals without resuspending the cells or removing the cuvet from the spectrophotometer. The reduction in OD₆₀₀ values relative to the starting OD₆₀₀ of each culture was plotted as a function of time based on the mean and standard deviation (SD) of 3 independent trials. Differences in settling rate for yeast strains were tested for significance.

Phase Contrast Microscopy

To examine yeast strain cytology, mid-log phase cells were mounted in 5% Ficoll solution (Sigma-Aldrich) on glass slides and observed at 4000 X using phase-contrast on an Axiovision II microscope (Zeiss, Feldbach, Switzerland). Granulated and non-

granulated cells were tallied based on a minimum of 100 cells for each of five independent trials using a double-blind design. Differences in the frequency of granulated cells were tested for significance.

Yeast cell volume was estimated by measuring the length and width of cells at 4000 X using similar methods described in Martegani *et al.* (1990). Cell volume was determined by calculating the volume of an ellipsoid as follows:

$$\text{Volume} = 4/3\pi(a/2 \times b/2 \times b/2)$$

Where a and b equal length and width/depth, respectively. Five independent trials were performed with at least 50 cells being counted in each trial. Differences in the range of cell volumes between yeast strains were tested for significance.

To determine the number of cells per colony, colonies with diameters of 1.0 mm were selected, resuspended in 1 X PBS (pH 7.4), diluted and counted using a hemocytometer (C.A. Hausser and Sons, Philadelphia, PA). Five colonies per strain were selected. Differences in the number of cells per colony between yeast strains were tested for significance.

Evan's Blue Staining

Cell death was measured using the vital stain Evan's Blue in lag, mid-log and stationary phase cells. For lag-phase and log-phase cultures, overnight cultures were diluted in fresh medium and examined using OD₆₀₀ to determine growth phase based on the growth curve generated in 'Growth Rate'. For stationary phase, strains were cultured on YPD or YPRaf/Gal plates for 72 h and suspended in 1 X PBS (pH 7.4). For cell

staining, cultures were washed three times, resuspended in 1 X PBS, mixed with an equal volume of 1% w/v Evan's Blue (Fisher Scientific, Pittsburgh, PA), held for 5 min at room temperature and examined at 4000 X using bright-field microscopy. A minimum of 100 cells were monitored for each trial and three independent trials were performed using a double-blind design. Cells that were stained blue throughout the cytoplasm were scored as dead. Differences in the frequency of cells that stained blue throughout cytoplasm between yeast strains were tested for significance.

Neutral Red Staining

Mid-log phase cells were centrifuged at 800 x g, washed three times in 1X PBS (pH 7.4), and resuspended in 1 X PBS containing 0.025% Neutral Red (Lot # 619389, BDH, New York, NY). The cell suspension was kept at 4°C for 15 min and examined at 4000 X by bright-field microscopy. The frequency of cells that were completely red (as opposed to those with a bright red central vacuole) was determined. A minimum of 100 cells were counted for each of five independent trials using a double-blind design. Differences in the frequency of cells that stained red throughout the cytoplasm between yeast strains were tested for significance.

Generation of Self-Incompatible *N. crassa* Strains

Spheroplasts of *N. crassa* strain C9-2 were produced using Novozyme 234 (Lot # 4859, Interspex Products, Forster City, CA) and transformed using similar methods as detailed in Micali and Smith (2006). Constructs that had *un-24*^{PA} incompatibility activity were identified when 50 - 90% of the resulting transformants had a self-incompatible 'spidery' morphology on the transformation plate. Upon transfer to VMM, the self-

incompatible colonies had a slower than wildtype growth rate, few aerial hyphae and produced few or no conidia. Self-incompatible colonies were purged of non-transformed nuclei through three colony-margin subcultures. Self-incompatible colonies were maintained in a pre-escape state by subculture at < 4 day intervals. Growth rate and colony morphology were monitored to ensure maintenance of the self-incompatible phenotype. Some self-incompatible colonies were allowed to escape, as evident from a sudden increase in colony growth rate and conversion to a wildtype morphology. Post-escape colonies were maintained by routine subculture as with wildtype strains.

Cloning of UN-24^{OR} N-terminal Construct

To verify that antibodies specific to yeast Rnr1p were able to detect UN-24, we cloned 600 aa of the N-terminus of UN-24^{OR} into the protein expression plasmid pQE30-UA (Qiagen, Valencia, CA). Oligonucleotide primers used to create the UN-24^{OR} N-terminal construct are listed in Table 2.3. *un-24^{OR}* cDNA cloned into the plasmid pBluescript (Smith *et al.*, 2000b) was used as the PCR template. PCR using primers pqe30UAF and un-24RTrunc was performed: 30 cycles of 30 s at 95°C (denaturation), 30 s at 65°C (annealing) and 1 min 30 s at 72°C (extension). The resulting PCR product was cloned into pQE30-UA and was transformed into TOP10F' One Shot chemi-competent cells (Invitrogen) as recommended by the manufacturers. Putative transformants were selected from LB agar plates containing 100 µg/mL of ampicillin (amp) after overnight incubation at 37°C. Several colonies were isolated and grown overnight in 3 mL of liquid LB supplemented with 50 µg/mL amp. Plasmids were then isolated and digested with *Bam*HI to verify insert presence and orientation. Sequencing was performed using the primer pQE30F and was verified.

Table 2.3: Oligonucleotide primers used to create the N-terminal construct of UN-24^{OR} expressed in *E. coli*.

Primer	Sequence (5'-3')	~T _m (°C)
pQE30F	CGGATAACAATTTACACAG	58
pqe30UAF	ATGTATGTCAAGAAGCGCGA	63
un-24RTrunc	GAGCCTTCGTAGGTGGCGTA	66

Protein Extractions

E. coli strains were grown overnight at 37°C in 5 mL of liquid LB containing 50 µg/mL amp. One mL of each strain was then transferred into 50 mL of LB liquid medium (with 50 µg/mL amp) supplemented with either 2% glucose or 100 mM IPTG and shaken for 12 h at 30°C and 100 RPM. The cells were then pelleted at 4,500 x g for 20 min at 4°C, resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and were lysed using a Vibra Cell Sonicator (Sonics and Materials, Connecticut) at 50% duty cycle and an output of 4. Four rounds of sonication were applied to the cells for 30 s time intervals followed by 2 min on ice. Cell debris was removed by centrifugation at 16,000 x g for 20 min at 4°C. Batch purification of 6xHIS-tagged proteins was performed using Ni-NTA agarose beads as recommended by the manufacturer (Qiagen). Proteins were quantified by Bradford assay (Bradford, 1976) using a standard curve of bovine serum albumin (Sigma) and loaded directly on SDS-PAGE.

To extract proteins from *S. cerevisiae*, mid-log phase cells were harvested from YPD by centrifugation at 800 x g for 5 min, washed once with ice-cold ddH₂O, and resuspended in 2 volumes of extraction buffer [20 mM Tris HCl (pH 7.9), 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, 0.3 M ammonium sulphate, 10 mM DTT, 1 mM PMSF and 1 Complete Mini-Protean tablet (Roche)]. If proteins were extracted under non-reducing conditions, DTT was omitted from the extraction buffer. Strains containing the yeast *RNR1* or *RNR3* genes driven by a *GAL1* promoter were grown in YP supplemented with 2% galactose. Chilled glass beads (0.5 mm diameter, BioSpec, Bartlesville, OK) were added at a ½ volume of the cell pellet and the mixture was vortexed for 30 s at maximum

speed and then placed on ice for 2 min. Vortexing and ice-chilling of the cell-glass bead mixture was repeated four times. The supernatant (cytosolic fraction) was collected, transferred to a new tube and placed on ice. The glass beads and cell debris were washed with 1 volume of extraction buffer and combined with the cytosolic fraction. Cell debris from the cytosolic fraction was removed by centrifugation at 16,000 x g for 60 min at 4°C. Proteins were quantified, combined with 2X Laemmli and used directly in SDS-PAGE.

To extract proteins from *N. crassa*, mycelia were grown on cellophane overlayed on agar medium. To obtain enough material for a protein extraction, pre-escape transformants were allowed to grow to a diameter of 2 cm at which time they were harvested, sectioned into smaller fragments, and subcultured on cellophane overlaying VMM plates containing hygB. Mycelia were harvested, frozen in liquid N₂ and ground with a mortar and pestle. The ground material was resuspended in extraction buffer [20 mM Tris HCl (pH 7.9), 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, 0.3 M ammonium sulphate, 10 mM DTT, 1 mM PMSF and one Complete Mini-Protean tablet (Roche)] and lysed using a modified bead beater method (Adams *et al.*, 1997). Cell debris was removed by centrifugation at 16,000 x g for 1 h at 4°C. Proteins were quantified and used directly in SDS-PAGE.

Size Exclusion Chromatography

Crude cell lysates were combined with 100 µL of 2 mg/mL dextran blue (Pharmacia Corporation, North Peapack, NJ) and 100 µL of 2 mg/mL NiCl (BDH, Poole, England) and subjected to size exclusion chromatography at 4°C (30 cm column length,

10 mL bed volume) using Sephacryl 300 HR (Sigma Aldrich) pre-equilibrated in 0.1 M NaCl. Proteins were eluted at a flow rate of 200 μ L/min and collected in 1 mL fractions. Fractions were collected once the dextran blue had begun to elute from the column and stopped once the NiCl had eluted from the column. The eluted proteins were concentrated by adding trichloroacetic acid to a final concentration of 13% and incubated overnight at 4°C. The proteins were centrifuged at 16,000 x g for 15 min, the supernatant was removed and the pellet was dried in a Savant speed vac concentrator (Model SV100H, Hicksville, NY). The proteins were resuspended in extraction buffer, quantified and used directly in SDS-PAGE. All protein extracts were quantified using a Bradford assay.

SDS-PAGE

SDS-PAGE gels were prepared using a Mini Protean II (Biorad, Hercules, CA) dual slab gel (1.0 mm thick) as described by the manufacturer. Proteins were subjected to SDS-PAGE (4% stacking gel, 10% resolving gel) in a Tris-glycine running buffer (25 mM Tris, 200 mM glycine and 1% SDS). The stacking gel contained 4 mL of ddH₂O, 66 μ L of 10% SDS, 1.6 mL of 1.0 M Tris HCl (pH 6.8), 880 μ L of acrylamide/bis 37.5:1 solution (Biorad), 66 μ L of 10% ammonium persulphate (Sigma) and 6.6 μ L of TEMED (Sigma). The resolving gel contained 4.8 mL of ddH₂O, 150 μ L of 10% SDS, 3.8 mL of 1.5 M Tris HCl (pH 8.8), 6.3 mL of acrylamide/bis 37.5:1 solution (Biorad), 150 μ L of 10% ammonium persulphate (Sigma) and 10 μ L of TEMED (Sigma). Thirty μ g of crude protein was combined with 2 X Laemmli buffer [125mM Tris HCl (pH 6.8), 20% glycerol, 4% SDS, 0.004% bromophenol blue, 15.4 μ g/mL DTT, 0.7M β -mercaptoethanol] and boiled for 10 min. If proteins were extracted under non-reducing conditions, both DTT and β -mercaptoethanol were omitted from 2 X Laemmli loading

buffer. For molecular weight standards, 5 μ L of a pre-stained Kaleidoscope protein ladder (Biorad) was used. Electrophoresis was performed at 75 volts until the proteins had reached the resolving gel whereupon which the voltage was increased to 150 volts. The voltage was applied to all gels until the bromophenol blue band had exited the gel.

Coomassie Brilliant Blue Staining

To ensure consistent protein loading for immunoblotting experiments, all SDS-PAGE gels were prepared in duplicate and one of the duplicate gels was stained with Coomassie Brilliant Blue. The gel was fixed overnight in 25% methanol and 5% acetic acid. Following fixation, the gel was stained for 2 h in 0.25% Coomassie Brilliant Blue (Sigma) in 50% methanol and 7.5% acetic acid. The gel was then destained in 25% methanol and 5% acetic acid until the protein bands were clearly visible. Gels were stored at 4°C in ddH₂O. In each figure, protein bands that were representative of total protein loading in each lane are shown.

Size Exclusion Chromatography Standards

One hundred μ L of HiMark pre-stained HMW Protein Standard (Invitrogen) was subjected to size exclusion chromatography as previously described. The elution fractions were then assessed for presence of each molecular weight marker by SDS-PAGE and Coomassie Brilliant Blue staining. Two independent replicas were performed to determine the protein sizes eluted in each fraction.

Immunoblotting

To detect the 6xHIS epitope, proteins were transferred overnight at 30 volts at 4°C in transfer buffer [48 mM Tris HCl (pH 8.3), 39 mM glycine, 20% methanol] to a pre-wetted nitrocellulose membrane (Biorad). The nitrocellulose was blocked for 2 h in blocking solution {5% skim milk in TBST [20 mM Tris HCl (pH 7.6), 500 mM NaCl and 0.1% Tween 20 (Sigma)]}. Western blots were incubated for 1 h with a primary mouse anti-6xHIS antibody in blocking solution (1:4000 dilution, Sigma), followed by two, 30 min washes in TBST and a 1 h incubation with a secondary rabbit, anti-mouse antibody in blocking solution (1:8000 dilution, Promega). Three 15 min washes in TBST were performed and proteins were visualized using a Chemiluminescent Peroxidase Substrate-3 kit (Sigma-Aldrich), as recommended by the manufacturer, and exposed to autoradiographic film (Kodak BioMax Light Film, Sigma Aldrich). Films were developed for 2 min in GBX developer (Sigma Aldrich) and fixed for 5 min in GBX fixer (Sigma Aldrich).

To detect Rnr1p and UN-24^{OR}, proteins were transferred to nitrocellulose as described above. The nitrocellulose membranes were incubated for 2 h in blocking solution followed by 1 h with rabbit, anti-Rnr1p primary antibody in blocking solution (1:7500, J. Stubbe, MIT, Cambridge, MA). The nitrocellulose was washed twice for 30 min in TBST, incubated for 1 h with donkey, anti-rabbit secondary antibody in blocking solution (1:15000, Promega, Madison, WI) and washed three additional times for 30 min in TBST. Proteins were visualized as described above.

To detect constructs containing the FLAG epitope, proteins were transferred to nitrocellulose as described above. The nitrocellulose membranes were incubated for 2 h in blocking solution followed by 1 h with anti-FLAG antibody diluted in blocking solution (1:1000, Invitrogen). The nitrocellulose was washed once for 10 min in TBST and proteins were visualized as described above.

For all immunoblotting, the molecular weight of signals was determined by using a Kaleidoscope molecular weight marker (Biorad) in conjunction with the sizing tool in AlphaEase v4.0 (Alpha Innotech Corporation, San Leandro, CA).

Affinity Purification of Interaction Partners

Proteins containing the FLAG epitope, as well as their interaction partners, were purified using anti-FLAG M2-agarose from mouse (Sigma-Aldrich). To remove excess glycerol from the bead suspension, the agarose beads from 200 μ L of M2-agarose suspension were collected at 800 x g and washed three times in Buffer 0.5 {50% 2 X Buffer [40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 20% glycerol, 0.2% Tween 20], 25% 2 M KCl, 0.1% PMSF, 0.07% β -mercaptoethanol, and one Mini-Protean tablet (Roche)}. Five mg of crude extract protein was incubated with $\sim$ 100 μ L of agarose beads for 2 h at 4°C with gentle shaking. Unbound proteins were removed by collecting the beads by centrifugation at 800 x g and washing the beads three times with Buffer 0.5. Proteins bound to the M2-agarose were eluted by gently shaking the beads with 30 μ L of Tri-FLAG Peptide (10 μ g/mL, Sigma Aldrich) for 1 h at 4°C. All 30 μ L of eluent was used in SDS-PAGE.

Mass Spectrometry

Mass spectrometry was performed at the Ottawa Institute of Systems Biology (Ottawa, ON). Protein bands were axenically excised from Coomassie stained SDS-PAGE gels and digested in the gel using porcine trypsin (Promega). Mass spectrometry was performed using an electrospray ion-trap in a Mass Spectrometer (LTQ, Thermofinnigan, San Jose, CA). Protein identity was determined using Mascot (Matrix Science Inc., Boston, MA).

Data Analysis

For all statistical tests, $P < 0.05$ was considered to be significant. Microsoft Excel (version 2007, Microsoft, Redmond, WA) and JMP (version 7, SAS, Cary, NC) were used for data analysis and statistical tests. To determine significant differences between the mean of two data series, we utilized a two-tailed paired t-test. Percentages and frequencies were arcsine transformed in order to fit the data to a normal distribution. To determine significant differences between the ranges of two different data sets, we used the non-parametric Wilcoxon/Kruskal Wallis test.

RESULTS

The formation of yeast diploids is slightly inhibited by co-expression of alternate alleles of un-24

The original objective of this study was to examine whether co-expression of *un-24^{OR}* and *un-24^{PA}* constructs (Figure 2.1) in a conjugate yeast would lead to an incompatibility reaction that could be measured through a reduction in mating efficiency. For this we engineered MAT α and MATa yeast strains to express PA and OR constructs, respectively. These constructs were placed under the control of a *GALI* promoter. When cultured on glucose-containing medium such as YPD, the *GALI* promoter yields low-level expression of downstream elements (Zachariae *et al.*, 1992). When cultured on medium containing raffinose and galactose, such as YPRaf/Gal, expression of elements under *GALI* control is increased more than 1000 fold (Johnston *et al.*, 1994). It was observed that co-expression of PA and OR constructs in diploid yeast led to a 19% reduction in mating efficiency compared to when MAT α and MATa control strains were crossed on YPD ($P = 0.06$, non-significant reduction). However, during these experiments it was observed that yeast expressing the hyg⁺PA construct yielded significantly fewer colonies when streaked out on YPD plates (Figure 2.2A) compared to when the same MAT α strain expressing the hyg control construct was streaked out. The construct hyg⁺PA was composed of the hygromycin phosphotransferase gene fused in-frame with 136 aa of the incompatibility domain of UN-24^{PA}. The control strain used for comparative purposes was the same MAT α strain but had the hygromycin phosphotransferase gene inserted in the *GALI* locus instead of the hyg⁺PA construct. When grown on YPRaf/Gal medium, single colonies from both the control strain and the

Figure 2.1: *un-24* fusion constructs used in Chapter 2. Segments of the *un-24*^{OR} or *un-24*^{PA} 3' end were fused in-frame with the hygromycin phosphotransferase gene (white oval) as described in Wellman *et al.* (2009). For immunoblotting, a FLAG epitope (diamond) was inserted between the hygromycin phosphotransferase gene and the UN-24^{PA} C-terminus of hygunPA and after the hygromycin phosphotransferase gene in the hyg control construct. The construct or gene name is indicated to the left of the figure. Key cysteine residues are designated 'C'. The hygromycin phosphotransferase and the FLAG tag are not drawn to scale relative to the amino acid scale bar at the bottom of the figure which refers to the UN-24 residues only.

Construct

[Fig. 2.1]

Figure 2.2: Expression of hygunPA at low-levels results in significantly fewer and larger cells per colony when grown on YPD. (A) Colony streaks of hyg- and hygunPA-expressing yeast strains. A hygunPA-expressing colony grown on YPD contains fewer cells per colony relative to a colony of the control strain. (B) The number of cells per 1 mm diameter colony was determined by cell counts with a hemocytometer (white bar = hyg, black bar = hygunPA). After growth on YPD, there were significantly fewer cells in hygunPA-expressing colonies in comparison to colonies of the same size from the control strain when grown on YPD (5 trials, $P = 0.003$, two-tailed t-test) (C) Cell volume of hygunPA-expressing and control cells grown on YPD and YPRaf/Gal. Cell volume was determined by measuring width and length of more than 100 cells from light micrographs taken from five independently grown cultures. These measurements were converted to volume using the equation of an ellipsoid and grouped into $100 \mu\text{m}^3$ bin sizes. Cells expressing hygunPA (black bars) had significantly larger cell volumes compared to the control strain (white bars, 5 trials; $P < 0.001$, Wilcoxon/Kruskal Wallis test). When grown in YPRaf/Gal medium, the cell volumes of hygunPA-expressing cells were reduced overall compared to when growth was on YPD, yet remained significantly larger when compared to cell volumes of the control strain grown on YPRaf/Gal (5 trials, $P < 0.001$, Wilcoxon/Kruskal Wallis test).

[Fig. 2.2]

hygunPA-expressing strain yielded nearly identical colony numbers when streaked onto YPRaf/Gal (not shown). Yeast colonies harboring the OR constructs hygunOR or hygunOR(L) did not exhibit low cell numbers when streaked onto YPD or YPRaf/Gal plates. These initial observations suggested that the hygunPA construct, that causes incompatibility in *N. crassa*, also elicited an incompatibility reaction when expressed at low-levels in yeast. This was explored further as described below.

To determine if these lower cell counts in hygunPA-expressing yeast colonies were due to a reduced rate of cell division or an increased rate of cell death, yeast cells from 1 mm diameter colonies were suspended in 1 X PBS, diluted and counted using a hemocytometer (Figure 2.2B). hygunPA-expressing cells had significantly fewer cells per colony (2.02×10^7 cells/colony $\pm 1.25 \times 10^6$) compared to cells from the control strain (4.43×10^7 cells/colony $\pm 8.18 \times 10^6$; $P = 0.003$). To examine if an increased rate of cell death was associated with low-level expression of hygunPA, we used Evan's Blue, which is not pumped out of, and thus accumulates, within dead cells (Autio and Mattila-Sandholm, 1992). A minimum of 100 cells grown in YPD were counted per strain for each of five trials and the frequency of cells that stained with Evan's Blue was calculated. There was no significant difference in Evan's Blue staining of cells expressing hygunPA and the control strain in lag phase ($P = 0.27$), log phase ($P = 0.67$) or stationary phase ($P = 0.26$, Figure 2.3). This indicated that the earlier observation of relatively fewer cells in single hygunPA-expressing colonies was not due to an increase in the frequency of cell death.

Figure 2.3: The vital stain Evan's blue shows that low-level expression of hygunPA does not lead to increased frequencies of dead cells in lag, log and stationary phase. Cells from the control strain (white bars) or the hygunPA-expressing strain (black bars) were grown in YPD to lag, log or stationary phase, washed in PBS, stained with Evan's Blue and examined using light microscopy. Mean frequencies and SD from five independent trials (each with $n > 100$) are shown. The frequencies of cells that stained dark blue (dead) in hygunPA-expressing and control strains were determined to be not significantly different ($P \geq 0.26$, two-tailed t-test).

[Fig. 2.3]

Low-level expression of hygunPA affects progression through the cell cycle in yeast

hygunPA-expressing colonies grown on YPD but not YPRaf/Gal contained relatively fewer cells than equivalent sized colonies from the control strain. This suggested that low-level expression of hygunPA may interfere with cell division perhaps by decreasing RNR catalytic activity and thus slowing progression through the cell cycle. RNR activity increases during transition into S-phase to create sufficient dNDP precursors for DNA replication (Zhao and Rothstein, 2002). Disrupting the cell cycle at the G₁:S transition and arresting or prolonging the S-phase in yeast leads to an increase in cell size (Reed, 1980; Cross, 1990; Muller, 1991; Futcher, 1996). We hypothesized that if expression of hygunPA interferes with RNR catalytic activity, it would cause disruption of the S-phase and result in an increase in cell volume. Cell volumes of at least 100 randomly selected cells in each of five trials from the hygunPA-expressing cells and cells from the control strain were calculated from light micrographs. Figure 2.2C depicts the cell volume measurements that have been grouped into 100 μm^3 bin sizes. When grown in YPD, cells expressing hygunPA were distributed into significantly larger size classes compared to the control strain ($P < 0.001$) and *vma11::G418^R* ($P < 0.001$) cells (not shown). Cell volumes of the control strain and *vma11::G418^R* strains did not differ ($P = 0.4152$, not shown). Cells expressing hygunPA were of a smaller size, overall, when they were grown in YPRaf/Gal medium, rather than YPD medium, but still tended to be significantly larger than cells from the control strain ($P < 0.001$) grown in YPRaf/Gal.

To confirm our assessment that cells expressing hygunPA at low-levels are larger overall than cells from the control strain, we examined the rate of cell settling in a standard cuvet (Figure 2.4). According to Stoke's Law, settling velocity is greater for

spherical objects with larger radii (Stokes, 1849). Yeast strains were grown until mid-log phase and placed in a cuvet. The cells were resuspended by inverting the cuvet and the cuvet was directly placed in a spectrophotometer. Cell settling was measured by taking OD₆₀₀ readings at 15 min intervals. We then plotted the reduction of OD₆₀₀ values for each culture relative to its respective starting OD₆₀₀ value. A faster settling rate would result in a more rapid decrease in OD₆₀₀ values over time. When grown in YPD, cells expressing hyg_{un}PA had a significantly greater settling rate ($-2.21 \times 10^{-3} \text{ OD}_{600}/\text{min} \pm 1.63 \times 10^{-4}$) compared to the cells from the control strain ($-1.41 \times 10^{-3} \text{ OD}_{600}/\text{min} \pm 3.52 \times 10^{-4}$, $P = 0.018$). This increased settling rate confirmed our previous results indicating that, overall, cells expressing hyg_{un}PA at low-levels have on average larger cell volumes. The increased volume of hyg_{un}PA-expressing cells contrasted with the cell volumes of *N. crassa un-24^{OR/PA}* self-incompatibles, which have on average smaller cell volumes than wildtype and self-compatible *N. crassa* cells.

We next tested whether expression of hyg_{un}PA in yeast caused a reduction in yeast growth rate (Figure 2.5). Cells grown overnight were resuspended in fresh medium, grown until mid-log phase and diluted to 1 cell/ μL . One-hundred μL aliquots of this cell culture were placed into the wells of a 96-well microtitre plate and shaken at 30°C. The OD₆₀₀ was measured at various times and cell number per mL was calculated using the strain specific standard curves located in APPENDIX 1. We identified the log-phase growth area of the curve by selecting the two points that were linear and farthest from each other that gave the highest R^2 value for both strains. In YPD, hyg_{un}PA-expressing cells had a significantly slower log phase growth rate ($2.41 \times 10^6 \text{ cells/mL/h} \pm 6.5 \times 10^4$) compared to cells from the control strain ($4.06 \times 10^6 \text{ cells/mL/h} \pm 1.0 \times 10^5$, $P > 0.001$).

Figure 2.4: Low-level expression of hygunPA results in an increased cell settling rate.

Mean settling rate was determined by resuspending mid-log phase cell cultures in a cuvet (time = 0) and measuring the decrease of OD₆₀₀ values over time. Cells grown in YPD that express hygunPA (squares) had a faster settling rate (-2.21×10^{-3} OD₆₀₀/min $\pm$ 1.63×10^{-4}) compared to cells from the control strain (diamonds, -1.41×10^{-3} OD₆₀₀/min $\pm$ 3.52×10^{-4} , P = 0.018, two-tailed t-test). Mean OD₆₀₀ values with SD are plotted relative to the initial value at 0 min from three independent trials.

[fig. 2.4]

Interestingly, the OD₆₀₀ values for each strain increase at the same rate (Figure 2.5 inset). It is only when these OD₆₀₀ values are converted to number of cells using strain-specific standard curves (APPENDIX 1) that a significance difference in growth rate is evident. This suggests that, overall, the same amount of total growth is occurring in the hygunPA-expressing and control strains but that cell division rates are reduced for the hygunPA-expressing strain. In YPRaf/Gal, there was no difference in log-phase growth rate between these two strains (not shown). Therefore, similar to *N. crassa un-24^{OR/PA}* self-incompatible transformants, yeast cells expressing hygunPA at low-levels have a slower growth rate than wildtype yeast.

These experiments suggested that the hygunPA protein (hygunPAp) interferes with cell division when hygunPA is expressed at low-levels (glucose) but not at high-levels (raffinose/galactose). The large cell sizes and reduced growth rates were also consistent with the hypothesis that hygunPAp interacts with Rnr1p in yeast to impede progression through the cell cycle.

Low-level expression of hygunPA in yeast causes incompatibility-like cytology.

Low-level expression of hygunPA also resulted in yeast with an altered morphology that was readily apparent when the cells were viewed with phase contrast microscopy (Figure 2.6A). On YPD, significantly more of hygunPA-expressing cells ($20.5\% \pm 3.8$) displayed dark, cytoplasmic granules as compared to only $8.0\% \pm 3.4$ of control cells ($P = 0.007$). When grown in YPRaf/Gal, the proportion of hygunPA-expressing cells with a granulated morphology was reduced ($12.3\% \pm 2.4$) and was not significantly greater than control cells ($2.3\% \pm 1.7$, $P = 0.054$, not shown). Similar cytoplasmic granulation is observed after hyphal fusion between *het*-incompatible strains

Figure 2.5: Mean cell numbers ($\pm$ SD, $n = 3$) over time of yeast strains grown in YPD. hygunPA-expressing cells have a slower log-phase growth rate (2.41×10^6 cells/mL/h $\pm$ 6.5×10^4) compared to the control strain (4.06×10^6 cells/mL/h $\pm$ 1.0×10^5 , $P < 0.001$, two-tailed t-test). Straight lines indicate duration of log-phase growth used in regression analysis. Inset: Plot of mean OD₆₀₀ over time of hygunPA-expressing (squares) and control cells (diamonds) grown in YPD. The mean OD₆₀₀ values depicted in this inset are those from which cell numbers used in the growth curve were derived.

[Fig. 2.5]

of *N. crassa* and other filamentous fungi (Jacobson *et al.*, 1998; Biella *et al.*, 2002).

As *het*-associated incompatibility reactions progress in filamentous fungi, cytoplasmic vacuolization and ruptured vacuoles are often observed and it has been proposed that the resulting cytoplasmic acidification and/or lytic enzyme release leads to PCD in filamentous fungi (Jacobson *et al.*, 1998; Pinan-Luccare *et al.*, 2007). To examine cell vacuoles in yeast expressing hygunPA, we used neutral red, a stain that accumulates in cellular compartments with low pH such as vacuoles (Cartledge *et al.*, 1977). This dye clearly differentiated control cells grown on YPD from those expressing hygunPA (Figure 2.6B). The majority of control cells had a large, red-staining central vacuole with only $6.9\% \pm 4.8$ of the cells staining throughout the cytoplasm. A significantly larger proportion of hygunPA-expressing cells stained red throughout the cytoplasm ($17.4\% \pm 2.8$, $P = 0.015$). This hygunPA staining pattern was indistinguishable from that of *vma11::G418^R*, a mutant strain that lacks subunit c' of the vacuolar ATPase (vATPase), and thus cannot effectively sequester H^+ in the vacuole (Kane, 2006). Therefore, neutral red staining indicated that, like the vATPase mutant strain, vacuolar function is compromised in hygunPA-expressing yeast. In contrast, when grown in YPRaf/Gal, *vma11::G418^R*, and cells hygunPA-expressing and control strains all had ~9% of cells with neutral red staining cytoplasms. Strains expressing OR constructs hygunOR and hygunOR(L) (Figure 2.1) did not display the above *het*-associated phenotypes apparent with the hygunPA-expressing strain. Therefore, when under low-level expression in yeast, hygunPA contributed to at least three aberrant cytologies, recognizable as large cell sizes, increased frequencies of cells with

Figure 2.6: Low-level expression of hygunPA leads to an aberrant cytology that is similar to that seen during heterokaryon incompatibility reactions in filamentous fungi. (A) Phase contrast microscopy revealed that significantly more hygunPA-expressing cells had a granulated cytoplasm compared to control cells ($n > 100$, $P < 0.007$, two-tailed t-test). (B) A higher frequency of cells from the hygunPA-expressing strain and the *vmal1::G418^R* knockout strain stained with neutral red throughout the cytoplasm compared to control cells ($n > 100$, $P = 0.015$, two-tailed t-test). This indicates that, like *vmal1::G418^R*, cells expressing hygunPA are defective in maintaining vacuolar acidification. Mean frequencies and SD in five independent trials are shown. Significant differences from the hyg-expressing control strain are indicated by ‘*’.

[Fig. 2.6]

cytoplasmic granulation and with decreased cytoplasmic pH. The latter two traits are reminiscent of incompatibility reactions in filamentous fungi.

Low-level expression of hyg_{un}PA causes enhanced sensitivity to chemical inhibitors

Increased sensitivities to hygB (Figure 2.7A), hydroxyurea (HU) (Figure 2.7B) and calcofluor white (CW, Figure 2.7C) were observed when yeast expressed hyg_{un}PA at low-levels compared to control strains. Overexpression of hyg_{un}PA on YPRaf/Gal medium remediated these increased sensitivities. In YPD, cells expressing hyg_{un}PA had a significantly lower hygB MIC value ($15.4 \mu\text{g/mL} \pm 0.0$) than control cells ($25.0 \mu\text{g/mL} \pm 0.0$, $P = 0.02$). In YPRaf/Gal, hygB MIC values for both cell types were $3000.0 \mu\text{g/mL} \pm 0.0$ and did not differ ($P = 1.0$). These hygB MICs observed in both YPD and YPRaf/Gal were significantly greater than those of Y2452 [$6.4 \mu\text{g/mL} \pm 0.0$ in both media ($P < 0.001$)], the untransformed progenitor strain (not resistant to hygB) from which both the hyg_{un}PA-expressing and the control strain were derived. Note that these hygB MIC results confirmed that our constructs inserted downstream of the *GAL1* promoter are expressed at low-levels on YPD and are properly up-regulated on YPRaf/Gal medium. hygB interferes with ribosomal translocation and thus causes mistranslation (Gritz and Davies, 1983). Although it has been previously unreported that self-incompatible *N. crassa* strains are sensitive to aminoglycosides, a synergistic effect created by the hyg_{un}PA protein (hyg_{un}PAp) and a general growth inhibitor such as hygB may cause a non-specific reduction in viability such as was seen when hyg_{un}PA was expressed at low-levels in these experiments.

Hydroxyurea (HU) specifically inhibits both prokaryotic and eukaryotic RNRs by abstracting the tyrosine radical located in the R2 subunit (Akerblom *et al.*, 1983). We hypothesized that if hyg_{un}PA complexes with endogenous Rnr1p in yeast, as is evident in *N. crassa*, fewer catalytically active RNRs would exist and therefore less HU would be required to inhibit the cell cycle. When grown in YPD, cells expressing hyg_{un}PA had a significantly lower HU MIC ($198.6 \text{ mM} \pm 18.11$) compared to the control strain ($300.0 \text{ mM} \pm 0.0$, $P < 0.001$). Surprisingly, both of these strains had identical MICs ($300.0 \text{ mM} \pm 0.0$) when grown in YPRaf/Gal. Therefore, similar to hygB MICs, enhanced HU sensitivity by cells expressing hyg_{un}PA was ameliorated by up-regulation of the hyg_{un}PA construct.

Similar inhibitory trends were observed with calcofluor white (CW). CW binds to chitin to prevent co-crystallization of β -1,4 glucan chains into microfibrils and thus weakens the cell wall (Roncero *et al.*, 1988). On YPD drop-out plates (described in Chen *et al.*, 2003) cells expressing hyg_{un}PA were more sensitive to CW compared to the control strain and to *cwp2::G418^R*, a mutant lacking a cell wall mannoprotein with a previously described sensitivity to CW (van der Vaart *et al.*, 1995). The hyg_{un}PA strain had a similar sensitivity to CW as *vmal1::G418^R*, which is defective in vacuolar ATPase activity. When grown on YPRaf/Gal, *vmal1::G418^R*, hyg_{un}PA-expressing and control strains all displayed equivalent sensitivities to CW while cells from *cwp::G418^R* were more sensitive (data not shown). Yeast strains harboring OR constructs hyg_{un}OR and hyg_{un}OR(L) (see Figure 2.1) did not display an increased sensitivity to the inhibitors mentioned above on either YPRaf/Gal or YPD. Therefore, low-level, but not high-level expression of hyg_{un}PA leads to increased sensitivity to hygB, HU and CW.

Figure 2.7: Increased sensitivity by hyg_{un}PA-expressing cells grown in YPD to selected inhibitors. Mutant *vma11::G418^R* lacks a vacuolar ATPase subunit and therefore cannot acidify vacuoles (Kane, 2006). Mutant *cwp2::G418^R* is defective in cell wall components and thus has a pre-described sensitivity to CW (van der Vaart *et al.*, 1995). When grown in YPD, hyg_{un}PA was significantly more sensitive to hygB (A, $P = 0.02$, two-tailed t-test), HU (B, $P < 0.001$, two-tailed t-test) and CW (C) compared to the control strain. hygB and HU MICs were determined by challenging yeast strains that were growing in liquid medium in a 96-well plate with increasing concentrations of inhibitor. In A and B, the values presented are mean MICs ($\pm$ SD) based on 5 independent trials. The MIC was recorded as the first concentration at which no growth was observed. Statistically significant differences (*) in comparison to the hyg-expressing control strain were determined by a two-tailed t-test. CW sensitivities were evaluated using a drop-plate method where serial dilutions of cell cultures were inoculated onto YPD or YPRaf/Gal plates containing the indicated concentrations of CW. In C, images are representative of 5 independent drop-plate assays.

[Fig. 2.6]

Anti-Rnr1p antibodies raised against yeast Rnr1p are cross-reactive to both N. crassa and yeast RNR1 proteins

Due to a limited supply of the anti-mouse RNR-(N) antibody that was used to detect the UN-24 complex in *N. crassa* (Figure 1.5; Wellman *et al.*, 2009), we obtained antibodies that detected yeast Rnr1p (anti-Rnr1p, courtesy of Joanne Stubbe, MIT). Reactivity of these antibodies to *N. crassa* UN-24^{OR} was determined by fusing a 6xHIS epitope to the N-terminal 600 aa of the UN-24^{OR} (encompassing residues 1-600). The construct was cloned into the protein expression vector pQE30-UA and transformed into *E. coli* strain TOP10F'. Constructs cloned into the multiple cloning site of this vector are placed under the control of a phage T5 promoter and two, tandem *lac* operator (*lac*^o) sequences that allow repression of the T5 promoter. When grown in the presence of glucose, the *lac*^I repressor (encoded by *lacI*^q on an F' episome harbored by TOP10F') binds to the *lac*^o and represses construct expression. In the absence of glucose, and in the presence of a lactose analog, in this case IPTG, the *lac*^I repressor is removed from the *lac*^o and the T5 promoter is able to transcribe the construct. TOP10F' cells containing either the pQE30-UA vector (control), or pQE30-UA containing the UN-24^{OR}(1-600) construct were grown in LB containing either 2% glucose or 100 mM IPTG (no glucose) and crude proteins were extracted under native conditions. Crude proteins were subjected to affinity purification using Ni-NTA agarose beads, subjected to SDS-PAGE under reducing conditions and immunoblotted with both anti-6xHIS and anti-Rnr1p antibodies. The reactivity of anti-Rnr1p antibodies to the truncated UN-24^{OR} protein was confirmed by comparing the molecular weight and intensity of the bands produced using both anti-Rnr1p and anti-6xHIS antibodies (Figure 2.8A). Both antibodies detected a

signal slightly below the 75 kDa marker as expected for the ~70 kDa truncated UN-24^{OR}(1-600) protein. When cells expressing the truncated UN-24^{OR}(1-600) construct were grown in the presence of glucose, significantly less of the truncated UN-24^{OR}(1-600) protein was detected on immunoblots with both antibodies. Immunoblotting with anti-Rnr1p or anti-6xHIS antibodies did not produce signals in proteins extracted from cells containing the pQE30-UA vector (control), whether cells had been grown in glucose or with IPTG (no glucose). These immunoblots demonstrated that the anti-Rnr1p antibodies that were raised against yeast Rnr1p detect UN-24. This was expected given their high levels of sequence similarity in the N-terminal region (Smith *et al.*, 2000a).

Yeast is unusual in that two forms (paralogs) of each RNR subunit exist. Rnr1p (large subunit) and Rnr2p (small subunit) and Rnr4p (small subunit) are involved in cell cycle related dNDP synthesis while Rnr3p (large subunit) is up-regulated in response to DNA damage (Elledge and Davis, 1990). Both Rnr1p and Rnr3p have similar molecular weights of 99.5 and 97.5 kDa, respectively, and thus would migrate to nearly the same area on SDS-PAGE. To confirm that anti-Rnr1p antibodies were specific to Rnr1p and not other RNR subunits found in yeast, specifically Rnr3p, we extracted proteins under non-reducing conditions from two yeast strains, *pGAL-RNR1* and *pGAL-RNR3*, that when grown in a galactose containing medium, overexpressed yeast *RNR1* and *RNR3*, respectively. We found that wildtype levels of Rnr1p were detected in proteins extracted from *pGAL-RNR3* and highly intense Rnr1p bands were observed in proteins extracted from the *RNR1*-overexpressing strain, *pGAL-RNR1* (Figure 2.8B). This demonstrated that the anti-Rnr1p antibody was specific to Rnr1p and not to Rnr3p. Camier *et al.* (2007) reported that the oxidation state of yeast Rnr1p can be determined by

SDS-PAGE. They report that under non-reducing conditions, yeast Rnr1p was resolved as two bands; a lower mobility oxidized form, presumably with a disulphide bond between C218 and C443, and a higher mobility, reduced form. In proteins extracted under non-reducing conditions from p*GAL-RNR1*, the anti-Rnr1p antibody detected two intense bands slightly above the 93 kDa marker that corresponded to the ~100 kDa oxidized and reduced forms of Rnr1p (Figure 2.8B). When proteins were extracted under reducing conditions, only the lower (reduced) band of Rnr1p was evident as previously reported by Camier *et al.* (2007).

hygunPA-expressing strains are deficient in oxidized Rnr1p and contain a non-reducible hygunPAp-Rnr1p complex when grown in YPD but not in YPRaf/Gal

Immunoblotting methods were used to test whether hygunPAp was binding to, and thus interfering with, the yeast Rnr1p. Using a similar protocol to that used in Camier *et al.* (2007), we extracted proteins under non-reducing conditions (without β -mercaptoethanol or DTT) from hygunPA-expressing and hyg-expressing control cells grown in YPD. In proteins extracted from control cells, we found that Rnr1p was resolved into reduced Rnr1p and oxidized Rnr1p at a ratio of ~2:1. In comparison, Rnr1p from cells expressing hygunPA had a reduced to oxidized ratio of ~10:1 (Figure 2.9). In addition, proteins from cells expressing hygunPA showed an intense band slightly above the 131 kDa marker that putatively represented a complex consisting of hygunPAp (53 kDa) and Rnr1p (100 kDa). The total expected molecular weight of such a hygunPAp-Rnr1p complex is ~153 kDa. When proteins were extracted under reducing conditions (with β -mercaptoethanol and DTT), the band corresponding to the oxidized form of Rnr1p was absent in both strains while both strains exhibited the reduced form of Rnr1p.

Figure 2.8: The anti-Rnr1p antibody detects UN-24 and the yeast endogenous Rnr1p but not yeast Rnr3p. (A) The 6xHIS-tagged UN-24 construct consisting of 600 aa from the N-terminus of UN-24^{OR} expressed in *E. coli* was detected by both anti-Rnr1p and anti-6xHIS antibodies. When grown in the presence of IPTG (induced), both anti-6xHIS and anti-Rnr1p antibodies detected abundant signal of the ~70 kDa UN-24^{OR}(1-600) protein. When grown in the presence of glucose (non-induced), these signals were reduced in intensity demonstrating repression of the T5 promoter. Proteins extracted from *E. coli* containing the pQE30-UA vector (no insert control) when grown in both inducing (IPTG) and non-inducing (glucose) conditions failed to produce this ~70 kDa protein when probed with either antibodies. These immunoblots demonstrated that UN-24 is detected with the anti-Rnr1p antibody. (B) Anti-Rnr1p antibodies bind to yeast Rnr1p but not to yeast Rnr3p. Proteins were extracted under non-reducing conditions from yeast strains pGAL-RNR1 and pGAL-RNR3, that when grown in YPRaf/Gal, overexpress Rnr1p and Rnr3p, respectively. When crude protein extracts from these strains were immunoblotted with the anti-Rnr1p antibody, two protein bands corresponding to the reduced (100 kDa, black arrowhead) and the oxidized (equivalent to 110 kDa, white arrowhead) forms of yeast Rnr1p were detected. The Rnr1p signals detected in proteins extracted from pGAL-RNR1 were ~100 X stronger than those detected in proteins extracted from pGAL-RNR3. The significantly lower intensities of these signals from proteins extracted from pGAL-RNR3 indicated that the anti-Rnr1p antibody did not have cross-reactivity with Rnr3p. The same 6xHIS and Rnr1p signals were detected in three independent protein samples. Equivalent protein loading was confirmed using Coomassie staining as shown across the bottom.

A

B

[fig. 2.8]

Interestingly, the ~153 kDa band in the hyg_{un}PA-expressing strain, that putatively represented the hyg_{un}PAp-Rnr1p complex, remained prominent under these strong reducing conditions, again indicating that this putative hyg_{un}PAp-Rnr1p complex is not resolved into monomeric forms under reducing conditions. Proteins extracted under non-reducing conditions from hyg_{un}PA-expressing and control cells that were grown in YPRaf/Gal medium had no observable differences in the total amount of Rnr1p, nor in their ratios of reduced to oxidized Rnr1p (~2:1), and the ~153 kDa band from both was absent (not shown). Likewise, when extracted under reducing conditions, proteins from hyg_{un}PA-expressing and control cells that were grown in YPRaf/Gal medium had no observable differences in the total amount of Rnr1p, contained only the reduced form of Rnr1p and lacked the ~153 kDa band (not shown). This was consistent with our view that the putative 153 kDa hyg_{un}PAp-Rnr1p complex was formed only with low-level expression of hyg_{un}PA construct and that it is correlated to the aberrant phenotype described earlier in this chapter.

Co-fractionation and co-localization of hygFLAG_{un}PAp and the R1 protein in both N. crassa and yeast.

A complex consisting of UN-24^{OR} and UN-24^{PA} is thought to form in self-incompatible *un-24^{OR/PA}* *N. crassa* colonies and to cause PCD. It was hypothesized that hyg_{un}PAp was also able to complex with UN-24^{OR} thus resulting in PCD in *N. crassa*. We utilized size exclusion chromatography and immunoblotting to investigate this idea further. We hypothesized that expressing the hyg_{un}PA protein in *N. crassa* would result in a complex between HYGUNPA and UN-24^{OR} that should elute in high molecular

Figure 2.9: Immunoblot of crude protein extracts from cells grown in YPD showing putative hygunPAP-Rnr1p ~153 kDa complex (*), and both the oxidized (white arrowhead) and reduced (black arrowhead) forms of Rnr1p monomer. Under non-reducing conditions, proteins extracted from cells expressing hygunPA contained relatively less oxidized Rnr1p with respect to the reduced form of Rnr1p, when compared to proteins extracted from the control strain. A ~153 kDa signal specific to the hygunPA-expressing strain was detected under these conditions. A marked reduction in the oxidized form of Rnr1p is evident when proteins were extracted under reducing conditions; however the ~153 kDa band persists. This 153 kDa signal putatively represented a non-reducible complex of hygunPAP (53 kDa) and yeast Rnr1p (100 kDa). Molecular weight markers are presented at the left of the figure. Similar Rnr1p signals were detected in five independent protein samples. Even protein loading across all lanes was confirmed by Coomassie staining of replicate SDS-PAGE gels as shown at the bottom.

NON REDUCING REDUCING

[fig. 2.9]

weight fractions during size exclusion chromatography. To detect the hygunPA protein on Western blots, we developed an internally FLAG-tagged construct, hygFLAGunPA, and a control construct, hygFLAG, which expressed the hygromycin phosphotransferase protein and the FLAG-tag components without the PA C-terminus (Figure 2.1). Both constructs were transformed into *N. crassa* C9-2 (*un-24^{OR}*). Transformation of hygFLAGunPA resulted in hygromycin B-resistant *N. crassa* transformants that had a *un-24^{OR/PA}* self-incompatible phenotype. Therefore, the internally fused FLAG epitope did not diminish the incompatibility activity of the incompatibility domain. Transformation of the hygFLAG construct into *N. crassa* resulted in hygB resistant transformants with wildtype morphology which indicated that this construct does not have incompatibility activity. Crude proteins extracted under native conditions from *N. crassa* transformed separately with hygFLAGunPA and hygFLAG were subjected to size exclusion chromatography and SDS-PAGE. Each fraction was immunoblotted with anti-Rnr1p and anti-FLAG antibodies (Figure 2.10). In these westerns, UN-24^{OR} from the hygFLAGunPA-expressing strain was observed to elute in higher molecular weight fractions in comparison to hygFLAG-expressing control strain. A signal slightly above the 40 kDa marker, which putatively represented the expected 39 kDa hygFLAG protein, was detected in extracts from *N. crassa* transformed with the hygFLAG construct. The 54 kDa signal corresponding to HYGFLAGUNPA was not observed in proteins extracted from *N. crassa* transformed with hygFLAGunPA. Rather, FLAG signals were detected in fractions 1 – 3 as two bands with molecular weights at and slightly above the 127 kDa marker. The size of the larger of the two signals was consistent with the expected size of

Figure 2.10: Size exclusion chromatography and immunoblotting show that UN-24^{OR} and HYGFLAGUNPA co-fractionate and co-localize in *N. crassa*. Native proteins extracted from C9-2 transformed with either hygFLAGunPA or hyg-FLAG constructs were fractionated by size exclusion chromatography, subjected to reducing conditions and immunoblotted with anti-Rnr1p and anti-FLAG antibodies. In proteins extracted from the hygFLAG-expressing control strain, anti-Rnr1p antibodies revealed that UN-24^{OR} eluted primarily in fraction 3 and was resolved slightly below the 127 kDa marker on immunoblots. This is close to the expected ~110 kDa size of the monomeric form of the UN-24 protein. In contrast, UN-24^{OR} from the hygFLAGunPA-expressing strain was distributed primarily in fractions 1-3 encompassing proteins between ~110 – 250 kDa. On the immunoblots, the anti-Rnr1p signal extended well above the ~127 kDa size standard in proteins from all three fractions. Anti-FLAG antibodies detected the 39 kDa HYGFLAG predominantly in fraction 5 (~30 – 100 kDa size range) in proteins extracted from the control strain. In proteins extracted from the hygFLAGunPA expressing strain, the HYGFLAGUNPA was not evident in its 54 kDa monomeric form but was observed in fractions 1 -3 and evidently co-localized with UN-24^{OR} at ~160 kDa. These immunoblots demonstrate that HYGFLAGUNPA associates with UN-24^{OR}. This trend was observed in three independent fractionation experiments. Size exclusion chromatography fraction number and protein weights that eluted in each fraction are indicated across the top and bottom, respectively. Molecular weight markers in kDa are indicated at the right of the figure. Even protein loading across all lanes was confirmed by Coomassie staining of SDS-PAGE replicate gels. FLAG signals and UN-24^{OR} signals were detected in three independent protein samples.

[fig. 2.10]

the putative HYGFLAGUNPA-UN-24^{OR} complex (~160 kDa) whereas the second ~127 kDa signal was unexpected and may represent a partially degraded form of the complex. The absence of the 54 kDa HYGFLAGUNPA monomer suggested that all of the expressed HYGFLAGUNPA was bound to UN-24^{OR}. Similar to earlier western analyses of *un-24*^{OR/PA} self-incompatible colonies of *N. crassa* (Wellman *et al.*, 2009), treating the proteins with reducing conditions (DTT and β -mercaptoethanol) and boiling prior to SDS-PAGE failed to separate the HYGFLAGUNPA-UN-24^{OR} complex into its components. These results suggested that HYGFLAGUNPA and UN-24^{OR} form a non-reducible complex in self-incompatible colonies which, similar to the UN-24^{OR}-UN-24^{PA} complex, leads to incompatibility.

Given that the above protein complex is associated with incompatibility in *N. crassa*, we tested whether or not a similar complex forms in yeast expressing hygunPA. The aberrant neutral red staining pattern when hygunPA is expressed at low-levels, as described earlier, also occurs in hygFLAGunPA-expressing strains. This shows that the hygFLAGunPA construct retains incompatibility-like function in yeast. We subjected crude proteins extracted under native conditions from control and hygFLAGunPA-expressing yeast grown in YPD to size exclusion chromatography (Figure 2.11). Prior to SDS-PAGE, the protein extracts were reduced (β -mercaptoethanol and DTT) and subjected to boiling. Reduced, but not oxidized, Rnr1p was detected using anti-Rnr1p antibodies in fractions 2, 3 and 4 in crude protein extracts from both strains. The putative ~154 kDa hygFLAGunPAp-Rnr1p complex was detected by anti-Rnr1p antibodies slightly above the 131 kDa marker in fraction 3 in proteins extracted from yeast expressing hygFLAGunPA. This signal was not detected in protein extracts from the

Figure 2.11: Size exclusion chromatography and immunoblotting indicate that the ~154 kDa band found in yeast cells expressing hyg_{un}PA is composed of hygFLAG_{un}Pap and Rnr1p. We performed size exclusion chromatography on native form proteins extracted from cells separately expressing hygFLAG and hygFLAG_{un}PA that were grown in YPD. Proteins eluted in each fraction were subjected to reducing conditions so that reduced Rnr1p (black arrowhead), but not oxidized, was detected in proteins extracted from both strains and eluted in fractions 2 (molecular weight range = 71-460 kDa, not shown), 3 (71-460 kDa) and 4 (55-238 kDa). In addition, a signal (*), which represented the putative ~154 kDa hygFLAG_{un}Pap-Rnr1p complex, was detected slightly above the 131 kDa marker in fraction 3 using both anti-FLAG and anti-Rnr1p antibodies. This signal was not detected with anti-FLAG antibodies in proteins extracted from the hygFLAG-expressing control strain. Detection of a ~154 kDa signal in size-fractionated proteins extracted from hygFLAG_{un}PA cells with both anti-FLAG and anti-Rnr1p antibodies confirmed that, similar to *N. crassa*, hygFLAG_{un}Pap and Rnr1p form a non-reducible complex. FLAG and Rnr1p signals were detected in two independent protein samples. The molecular weight range of each fraction was determined using a HiMark Pre-Stained HMV Protein Standard. Equal protein loading across all lanes was confirmed using Coomassie staining of replicate SDS-PAGE gels as shown at the bottom.

[Fig. 2.11]

control strain. Likewise, anti-FLAG antibodies bound to a protein in fraction 3 from hygFLAGunPA-expressing cells that was slightly above the 131 kDa marker. This signal was not detected in proteins extracted from the hygFLAG-expressing control cells. Therefore, similar to *N. crassa*, hygunPAp appears to associate with Rnr1p as a non-reducible complex in yeast.

hygunPA interacts with Leu1p, a protein involved in leucine biosynthesis

The previous results are consistent with the hypothesis that, in yeast, hygunPAp interacts with Rnr1p to illicit a reaction reminiscent of *het*-associated incompatibility. Whether other proteins were involved in mediating incompatibility remained unclear. To examine if hygunPAp was interacting with proteins other than Rnr1p we affinity purified native form interaction partners of cells expressing hygFLAGunPAp grown in YPD. Affinity purification was performed with anti-FLAG agarose beads. Purified proteins were subjected to SDS-PAGE and stained with Coomassie brilliant blue. One distinct band at ~90 kDa was observed, excised from the gel, digested with trypsin and subsequently subjected to mass spectrometry. Through this process the 90 kDa band was identified as isopropylmalate isomerase (Leu1p) in two separate affinity purifications. High Mascot scores of 341 and 391 were obtained in each separate purification which indicated a high probability that this protein was identified correctly as Leu1p. Leu1p is a protein with a molecular weight of 87 kDa that contains an iron-sulphur group, has homology to aconitase and catalyzes the second step in the leucine biosynthetic pathway (Gross *et al.*, 1962; Kohlhaw, 1988). Peptides detected by the mass spectrometer that correspond to Leu1p are shown in Table 2.4. The Leu1p interaction partner was not observed in affinity purified proteins from control cells or when proteins were extracted

from strains grown in YPRaf/Gal. Since this protein was not detected on immunoblots with anti-FLAG antibodies, Leu1p and hygFLAGunPAp interact via a reducible or non-covalent bond. We had expected, but did not observe, immunoprecipitation of the hygFLAGunPAp-Rnr1p complex. Attempts at purifying the hygFLAGunPAp-Rnr1p complex using rabbit anti-Rnr1p antibodies and anti-rabbit antibodies covalently linked to agarose beads also failed. Since hygFLAGunPA is expressed at low-levels, one would expect the hygFLAGunPAp-Rnr1p complex to be present in equally low-levels in the cell. Therefore, although the hygFLAGunPAp-Rnr1p complex may be successfully purified using the above purification method, silver staining (not shown) and Coomassie staining may not be able to detect the immunoprecipitated complex.

Table 2.4: Leu1p, a protein involved in leucine biosynthesis, co-immunoprecipitates with hygFLAGunPAp. Native form proteins extracted from yeast cells expressing hygFLAGunPA that were grown in YPD were subjected to affinity purification using anti-FLAG agarose beads. When purified proteins were subjected to SDS-PAGE and Coomassie staining, one protein band of ~90 kDa was observed. This protein band was identified through mass spectrometry as Leu1p, a protein containing aconitase homology that is involved in leucine biosynthesis. When crude proteins extracted from the control strain were affinity purified, SDS-PAGE and Coomassie staining failed to detect any co-immunoprecipitated proteins. Mascot scores indicate a high probability that Leu1p is the true identity of the protein that co-immunoprecipitated with hygFLAGunPAp. Therefore, it appears that Leu1p and hygFLAGunPAp interact in yeast when hygFLAGunPA is expressed at low-levels. Detection of hygFLAGunPA interaction partners was performed twice in two independent trials.

[table 2.4]

Purification Experiment	Protein Candidate	Theoretical Mass (kDa)	Mascot Score	Peptide Sequences
1	Isopropylmalate Isomerase (Leulp)	86.078	341	- LDQQIHK
				- LAMVVPGSLVK + oxid(M)
				- ALAYMGLEPNTPLK + oxid(M)
				- AGMIKPDETTFYTK + oxid(M)
				- LDQQIHKLIPIANK
				- HLVEVTSPQAFEGLENAG R
2	Isopropylmalate Isomerase (Leulp)	86.078	391	- ILSDSDGNVLVDHFEIEPFR
				- GLFYEW R
				- YSFLEGGSK
				- LDQQIHK
				- ANVDTDAIIPK
				- LAMVVPGSLVK + oxid(M)
				- ALAYMGLEPNTPLK + oxid(M)
				- DQETDFVLNVEPWR
				- SIAPSYGDIFYNNSFK
				- ILSDSDGNVLVDHFEIEPFR

DISCUSSION

Identification of a distinct incompatibility domain in the C-terminus of the *N. crassa* large subunit of ribonucleotide reductase protein (UN-24) offered a unique opportunity to investigate trans-species activity of an incompatibility system. To our knowledge, yeast lacks an endogenous vegetative nonself recognition system. Therefore, by utilizing yeast, the *un-24* system could be studied without interference from other vegetative nonself recognition systems that are found in *N. crassa*. As yeast has been extensively characterized, we postulated that we could gain further insight into the mechanism of *un-24* incompatibility.

There have been few studies that examine whether or not incompatibility factors can function interspecifically. Interestingly the *het-c*^{OR} allele does not confer incompatibility when expressed in species other than *N. crassa*. *P. anserina* contains a *het-c* homolog, *hch*, which has no apparent endogenous vegetative incompatibility activity. Expression of *het-c*^{PA}, but not *het-c*^{OR}, from *N. crassa* in *P. anserina* elicits a response reminiscent of a *het*-associated incompatibility reaction (Saupe *et al.*, 2000b). *Fusarium proliferatum* also contains a *het-c* homolog with no apparent endogenous vegetative incompatibility function. Expression of *het-c*^{OR}-like construct in this species does not result in an incompatibility response (Kerenyi *et al.*, 2006). Expression of *het-c*^{PA}, but not *het-c*^{OR}, from *N. crassa* in *Aspergillus niger* results in a reaction comparable to a *het*-associated incompatibility reaction (van Diepeningen *et al.*, 2009). One could therefore infer based on this trans-species activity, that *het-c*^{PA} is the derived form and *het-c*^{OR} is the ancestral form of *het-c*. Similarly, the ability of the hygunPA protein to elicit incompatibility in yeast suggests that UN-24^{PA} is a derived form of UN-24 and that

it has gained a secondary function of nonself recognition. We found that low-level, but not high-level, expression of *hygunPA* resulted in cytological characteristics, including cytoplasmic granulation and a decrease in cytoplasmic pH, that were reminiscent of *het*-associated incompatibility in filamentous fungi. Low-level expression of *hygunPA* also resulted in an increase in cell volume, a decrease in log-phase cell division and an increased sensitivity to chemical inhibitors (*hygB*, HU and CW) relative to control cells. Immunoblotting revealed that *hygunPA*p and yeast Rnr1p form a non-reducible complex that we postulate to be responsible for these aberrant cytologies. Interestingly, expression of constructs that contained the OR incompatibility domain [*hygunOR*, *hygunOR(L)*] did not illicit an incompatibility response in yeast. In pairwise comparisons, UN-24^{OR} shares 72%, and UN-24^{PA} shares 72.2% overall similarity to yeast Rnr1p. However these proteins are very dissimilar in the C-terminus region wherein lies the UN-24 incompatibility domain. We hypothesize that yeast Rnr1p is perceived by the PA incompatibility domain to be an ‘OR-type’ R1.

Increased frequencies of cells with cytoplasmic granulation are observed in *N. crassa* cells undergoing *het*-associated incompatibility (Jacobson *et al.*, 1998; Glass and Kaneko, 2003). Similarly, we found that low-level expression of *hygunPA* in yeast led to a higher frequency of cytoplasmic granulation relative to the control strain. In *N. crassa*, the factors that influence the degree of granulation and the make-up of the granules themselves remain unknown (Jacobson *et al.*, 1998). Another aberrant cytology observed when *hygunPA* was expressed at low-levels is an increased frequency of cells with intense cytoplasmic staining by neutral red. While this staining pattern provides evidence for a decrease in cytoplasmic pH, it is unclear if this decrease in pH is due to the

hygunPAp-Rnr1p complex or an interaction between hygunPAp and some other protein such as a vATPase subunit. In support of the later, the neutral red staining pattern associated with low-level expression of the PA incompatibility domain was observed to be nearly identical to that of a *vma11* deletion mutant. Also, deletion of *vma-1*, a gene coding for subunit A of the vATPase in *N. crassa*, terminates vATPase activity and results in strains that, similar to *un-24*^{OR/PA} self-incompatible transformants, grow slowly and are unable to conidiate (Bowman *et al.*, 2000). These phenotypic similarities could reflect a direct interaction of incompatibility factors and vacuolar components. On the other hand, yeast that lack *RNR1* have a significant decrease in vATPase activity which would result in a loss of vacuolar function and a decrease in cytoplasmic pH (Sambade *et al.*, 2005). Our observations suggest that the PA incompatibility domain non-reducibly links to, and inactivates, Rnr1p. This inactivation of Rnr1p would likely partially mimic a deletion of *RNR1*, and this would promote cytoplasmic acidification. Given its central role in cell division, inhibiting Rnr1p may have pleiotrophic effects on the cell that include interruption of vacuolar acidification. Additional evidence for a pleiotrophic effect was that, when expressed at low-levels, hygunPA caused increased sensitivity to the inhibitors hygB, HU and CW. Since each of these inhibitors specifically targets a different cellular component (translation, reduction of ribonucleotides and the cell wall), our hypothesis that interrupting Rnr1p activity has multiple effects on the cell is supported. Indeed several phenotypes associated with either a lack of, or reduced, activity of Rnr1p have been described. These phenotypes include an increased sensitivity to HU, cyclosporin A, and metal ions, a decreased competitive fitness in glucose medium, and decreased telomere length (Wolstencroft *et al.*, 2006; Sambade *et al.*, 2005;

Yadav *et al.*, 2007; Giaever *et al.*, 2002; Askree *et al.*, 2004). Furthermore, deletion of *RNR1* or interference with RNR catalytic activity has been observed to cause slow growth in yeast cells (Zhou and Elledge, 1992; Hughes *et al.*, 2000; Florio *et al.*, 2007). The slow growth rate of hygunPA-expressing yeast cells mirror these previous findings and suggests that, similar to the situation in self-incompatibles in *N. crassa*, hygunPA interferes with RNR catalytic activity resulting in a slower than wildtype growth rate. With respect to growth rate effects, it should be noted that low-level expression of hygunPA resulted in reduced cell division rates. This reduction was offset by larger cell sizes such that changes in OD₆₀₀ over time were not appreciably different between hygunPA-expressing and control strains.

Results from immunoblotting were consistent with the idea that hygunPA-associated phenotypes in yeast are mediated by a non-reducible hygunPAp-Rnr1p complex. When size-fractionated proteins that were extracted from hygFLAGunPA-expressing cells grown in YPD were immunoblotted with anti-Rnr1p and anti-FLAG antibodies separately, both Rnr1p and FLAG signals were detected at ~154 kDa, the expected size of a hygFLAGunPA-Rnr1p complex. Treatment of these proteins with reducing agents (DTT and β -mercaptoethanol) and boiling failed to separate this complex. Therefore, similar to *N. crassa*, the hygunPA-Rnr1p complex appears to be due to a non-reducible interaction. The reducing agents DTT and β -mercaptoethanol are used in protein extraction buffers to reduce disulphide bonds. Since our protein extraction buffer contained both reagents, a complex held together by a disulphide bond would be broken down into its components. It has been hypothesized that in *N. crassa* this complex is potentiated by a disulphide bond between cysteines located in the UN-24^{OR} catalytic

site and the UN-24^{PA} C-terminus (Wellman *et al.*, 2009). The non-reducible nature of the UN-24^{OR}-UN-24^{PA} and HYGUNPA-UN-24^{OR} complexes in *N. crassa* and the hygunPAp-Rnr1p complex in yeast suggest that these complexes may be initiated by disulphide bonding but are ultimately held together by another non-reducible intermolecular linkage. For example, aberrant localization of the radical in the catalytic pocket of Rnr1p could cause the linkage of two tyrosine residues (Salazar *et al.*, 2009) or two lysine residues (Stadtman and Levine, 2003).

We were unable to affinity purify the putative hygFLAGunPAp-Rnr1p complex using anti-FLAG antibodies cross-linked to agarose beads. However, through this process, Leu1p, was immunoprecipitated in two independent purifications. Immunoprecipitation of Leu1p was unexpected as this protein shares no common domains or functions with R1 and has not been observed to interact with RNR subunits or non-specifically with the FLAG epitope (Cherry *et al.*, 1997; Ho *et al.*, 2002). Leu1p is a protein involved in leucine biosynthesis that has homology to aconitase and contains a 4Fe-4S cluster (Kohlhaw, 1988). A negative interaction between hygunPAp and Leu1p would likely result in leucine auxotrophy. However, since the hygunPA-expressing yeast strain is auxotrophic for leucine (*leu2Δ*), expression of hygunPA in a non-auxotrophic yeast strain would be required to examine this hypothesis. Interestingly, the *het-6* gene, one of two genes at the *het-6* locus which also has incompatibility function and is inextricably linked to *un-24* in *N. crassa*, contains a domain with 51% similarity to bacterial aconitases (Micali and Smith, 2006). Recent experiments have implicated non-allelic interactions between alternate alleles of *het-6* and *un-24* (Lafontaine, unpublished). It is possible that hygunPAp interacts with the aconitase-like domains of Leu1p in yeast

and of HET-6 in *N. crassa*. If so, this would provide further evidence that non-allelic interactions occur between alternate allelic forms of HET-6 and UN-24 in *N. crassa*.

The increase in cell volume range was less prominent when cells expressing hygunPA were grown in YPRaf/Gal than when they were grown in YPD. It is possible that the observation of this phenotype in YPRaf/Gal was due to a carry-over effect from previous growth in YPD. With each division, a budding cell increases in cell size relative to its daughter cells (reviewed in Laun *et al.*, 2006). hygunPA-expressing cells transferred from YPD medium, some of which have a larger cell volume compared to wildtype cells, into YPRaf/Gal would retain their cell volume. When these enlarged cells divide, they would produce relatively smaller cells that, because they do not experience the increase in cell volume as a result of hygunPA-associated incompatibility, would resemble daughter cells produced by budding of wildtype yeast. After several rounds of doubling in YPRaf/Gal, cells that had experienced hygunPA-associated incompatibility (e.g. those that had been transferred from YPD) would begin to die and daughter cells that had arisen from these cells in YPRaf/Gal (e.g. that had not experience hygunPA-associated incompatibility) would make up the majority of the population. This would lead to an overall decrease in the range of cell volume.

Although low-level expression of hygunPA in yeast resulted in several cytologies that were similar to cells undergoing *het*-associated incompatibility in filamentous fungi, two differences were noted. Firstly, in *N. crassa*, the majority of cells within a self-incompatible colony display *het*-associated cytologies (Siahbazi, 2008). In contrast, only ~20% of yeast cells expressing hygunPA at low-levels had highly granulated cytoplasm and ~20% of cells expressing hygunPA had cytoplasm that stained with neutral red. This

suggested that only a subset of yeast cells experience the *hygunPA*-associated incompatibility at any given time. This may be explained by considering that yeast is unusual in having paralogs of R1, Rnr1p and Rnr3p. Rnr1p is produced prior to entering S-phase and is the main R1 subunit used for dNDP synthesis (Elledge *et al.*, 1992). When Rnr1p subunits are not required for dNDP synthesis, Sml1p, a protein found only in yeast, binds to Rnr1p and prevents access to the catalytic site (Chabes *et al.*, 1999; Zhao *et al.*, 2000). Once Rnr1p is required for dNDP synthesis, Sml1p is removed allowing NDPS access to the catalytic site (Zhang *et al.*, 2007). Rnr3p is produced primarily during DNA damage and has limited RNR activity under optimal growth conditions. However, a cross-talking event with Rnr1p substantially increases the activity of Rnr3p in the reduction of ribonucleotides (Domkin *et al.*, 2002). It has been suggested that when Rnr1p is limiting, Rnr1p and Rnr3p form a heterodimer and thereby increase dNDP production (Domkin *et al.*, 2002). It is plausible that during S-phase when dNDP synthesis is required, Sml1p is removed from the Rnr1p catalytic site. *hygunPAp* gains access to the catalytic site and interacts with Rnr1p reducing RNR activity and causing *hygunPA*-associated incompatibility. The reduction of RNR activity in the cell causes a cross-talking event to occur between Rnr1p and Rnr3p and thus activates Rnr3p to participate in dNDP synthesis. Since Rnr3p ‘rescues’ dNDP synthesis, a subset of cells are able to proceed through the cell cycle and do not exhibit *hygunPA*-associated incompatibility. If this hypothesis were correct, it would suggest that a lack of dNTPs, and not the presence of a toxic *hygunPAp*-Rnr1p complex, causes incompatibility.

A second difference between this yeast system and *het*-associated incompatibility in filamentous fungi is that some yeast cells expressing *hygunPA* at low-levels had an

larger cell volume when compared to the control strain. This contrasts with *N. crassa un-24^{OR/PA}* self-incompatibles, which have on average smaller cell volumes ($\sim 300 \mu\text{m}^3$) compared to wildtype strains ($\sim 2000 \mu\text{m}^3$, Figure 1.2; Siahbazi, 2008). In yeast, we surmise that hygunPAp forms a non-reducible complex with Rnr1p, compromising dNTP production and thus causing an extended G₁ or S-phase. Extending G₁, S-phase or the G₁-S interface is known to cause an increase in cell volume (Reed, 1980; Cross, 1990; Muller, 1991; Futcher, 1996). It has been previously shown that disruption of RNR proteins in mammalian, *Arabidopsis* and yeast cells leads to cell cycle arrest (Wang and Liu, 2006). Furthermore, reduction-of-function mutations in yeast Rnr1p lead to an increased duration of S-phase (Camier *et al.*, 2007). Interestingly, when a single colony of cells deficient in *RNR1* is streaked out on agar plates, fewer colonies are observed relative to strains that have a functional *RNR1* (Zhang *et al.*, 2007). This phenotype is identical to when a single colony of the hygunPA-expressing yeast strain is streaked out on YPD plates and suggests that, similar to hygunPA-expressing cells, yeast deficient in *RNR1* have fewer cells per colony. The smaller cell observed in *N. crassa un-24^{OR/PA}* self-incompatibles may be due to a non-allelic interaction between *un-24^{PA}* and *het-6^{OR}* which may lead or contribute to the smaller cell volume observed. Non-allelic interactions have been described for *het* loci in *P. anserina* and *N. crassa* (reviewed in Chapter 1). In addition, as mentioned earlier, *N. crassa* and other filamentous fungi have a well developed vegetative nonself recognition system not found in yeast, although yeast does retain a sexual nonself recognition system. It is possible that other components of this system are involved in downstream processes to limit cell size during *un-24*-associated incompatibility.

In conclusion, low-level, trans-species expression of hygunPA in yeast causes several abnormal phenotypes and cytologies that are reminiscent of *het*-associated incompatibility reactions in *N. crassa*. In both yeast and in *N. crassa*, the incompatibility-like phenotypes are correlated with the presence of a non-reducible protein complex that putatively comprises the C-terminal incompatibility domain of UN-24^{PA} and the large subunit of ribonucleotide reductase, either Rnr1p or UN-24^{OR}, respectively. This research demonstrates that yeast can be used to characterize nonself recognition factors from filamentous fungi.

**CHAPTER 3: ESCAPE FROM *un-24*-ASSOCIATED INCOMPATIBILITY IN
YEAST**

INTRODUCTION

Self-incompatible *un-24*^{OR/PA} colonies of *N. crassa* are characterized by a sparse, aconidial (do not make mitotic spores) morphology and have a significantly slower growth rate when compared to wild-type strains (Wellman *et al.*, 2009). However, within about one week of continuous growth at room temperature, *un-24*^{OR/PA} self-incompatible colonies will spontaneously escape to a wildtype morphology and growth rate. This escape phenomenon was first identified by Newmeyer (1970) who observed a sudden suppression of incompatibility in *N. crassa* strains that were vegetatively incompatible due to differences at the mating-type locus. In 1975, DeLange and Griffiths similarly studied escape in *N. crassa* mating-type incompatible heterokaryons. In these studies, escape was associated with deletion of one or the other of the duplicated mating-type loci. It was found that mutation of *tol*, a gene required for proper incompatibility function by the mating-type locus in *N. crassa*, may also result in escape (DeLange and Griffiths, 1975; Shiu and Glass, 1999). *tol* mutants have since been observed to retain incompatibility activity for all other *het* loci (Leslie and Yamashiro, 1997). The evolutionary significance of escape has yet to be examined. Vegetative cell fusion between incompatible strains may result in the transfer of genetic material (Collins and Saville, 1990). Therefore escape may allow incompatible heterokaryons to utilize mitotically transferred genetic elements in the environment to determine if they confer a selective advantage to the colony.

Escape from incompatibility is not limited to *N. crassa*. In *P. anserina*, heterokaryon incompatibility can be remediated by the deletion of any of three modifier loci, *mod-A*, *mod-B* or *mod-D*, that are involved in *het-c/het-e*, *het-c/het-d*, and *het-r/het-v*

non-allelic incompatibility systems (Belcourt and Bernet, 1969; Barreau *et al.*, 1998; Loubradou *et al.*, 1999; Xiang and Glass, 2002). Deletion of *mod-C* has also been described to cause escape from the *het-r/het-v* system but has no effect on other non-allelic incompatibility systems (Labarère and Bernet 1977).

Characteristics of DNA deletions that occur during escape in *N. crassa* were further investigated at the molecular level Smith *et al.* (1996). It was observed that large deletions of either the ectopic copy (*un-24^{OR} het-6^{OR}*) or the endogenous copy (*un-24^{PA} het-6^{PA}*) resulted in escape. Similarly, escape from *het-c* incompatibility is accompanied by either deletion of one of the duplicated *het-c* alleles or by mutations in the *vib-1* (Xiang and Glass, 2002; Xiang *et al.*, 2002). *vib-1* is a putative transcription regulator that localizes to the nucleus during heterokaryon incompatibility and is required for proper expression of HET domain genes including *het-6*, *tol* and *het-c* (Xiang and Glass, 2004; Dementhon *et al.*, 2006). In addition, *vib-1* may be required for proper expression of signaling proteins involved in PCD caused by heterokaryon incompatibility (Dementhon *et al.*, 2006). The identity of downstream signaling proteins and effector proteins involved in PCD is currently unknown in *N. crassa*.

From the above studies, it is evident that escape processes have several features in common. Escape occurs spontaneously and usually after several weeks of growth in about 25% - 85% of self-incompatible colonies. In addition, escape is characterized by mutations that either inactivates one or the other of the duplicated incompatibility genes, or that give rise to a suppressor of incompatibility. The mechanism of escape from *un-24*-associated incompatibility appears to be distinct from these previously reported systems. For example, in previously described systems, escape usually occurs after 20 days of

growth and then only 25% of self-incompatibles escape (Smith *et al.*, 1996). In contrast, *un-24*^{OR/PA} partial diploids escape within 4-7 days of growth. In addition, virtually all *un-24*^{OR/PA} partial diploids undergo escape (Siahbazi, 2008). Furthermore, unlike previously described systems in which escape times are random, subcultured colonies obtained from the same *un-24*^{OR/PA} transformant escape within a narrow window of times that are characteristic for a given transformant (Siahbazi, 2008). This indicates that escape time is likely influenced by the area in which the transgene integrates into the genome. Since transgene integration is random (Grant *et al.*, 1984), areas where the transgene integrates may be more or less accessible to endogenous transcriptional machinery. Furthermore, there appears to be no correlation between pre-escape growth rates and escape time (Siahbazi, 2008). For example, slower growing pre-escape strains do not necessarily escape earlier or later than faster growing pre-escape strains. In 2003, Micali and Smith demonstrated that, unlike the previously characterized mechanisms, escape in *un-24*^{OR/PA} self-incompatible transformants was not mediated by deletion of one copy of *un-24*. Both PCR and Southern blot analysis confirmed the presence of both *un-24*^{PA} (ectopic) and *un-24*^{OR} (endogenous) in these escaped partial diploids (Micali and Smith, 2003). Together, these results indicate that escape from *un-24*-associated incompatibility is through an undescribed process.

Several unpublished studies in our laboratory have excluded specific possible mechanisms for escape from *un-24*-associated incompatibility in *N. crassa*. In 2005, Saeed and Smith (unpublished) used a methylation deficient strain, *dim-2*, to generate *un-24*^{OR/PA} self-incompatible strains. There was no difference in the timing or the frequency of escape for *dim-2* self-incompatible colonies compared to *dim-2*⁺ self-incompatible

colonies. This provided strong evidence that escape did not involve methylation-associated gene silencing of *un-24* alleles. In a study done in 2006, Zymantas and Smith (unpublished) inferred that RNA silencing (Romano and Macino, 1992) was also not involved in escape. In their study, they created self-incompatible *un-24*^{OR/PA} transformants using a quelling deficient strain, *qde2*⁻. This strain lacks RNA silencing mechanisms and thus allows overexpression of duplicated transgenes (Cogoni and Macino, 1997). There was no difference in timing or frequency of escape for *qde2*⁻ self-incompatible *un-24*^{OR/PA} colonies compared to *qde2*⁺ self-incompatible colonies indicating that RNA silencing of *un-24* alleles was not involved in escape. In support of the idea that transcript levels of one or both copies of *un-24* are not altered upon escape, Siahbazi (2008) utilized quantitative PCR to show that transcript levels of both the transgene and the endogenous *un-24* gene were equivalent in pre- and post-escape partial diploids. Overall, it appeared that changes in transcript levels of both the endogenous and ectopic copy of *un-24* were not involved in escape.

One genetic mechanism that may be involved in the *un-24* escape process that has yet to be formally studied is mutation. For example, repeat induced point mutation (RIP) is a mechanism by which *N. crassa* can inactivate a duplicated gene. This pre-meiotic process involves detection and inactivation of duplicated genes through numerous G to C and A to T point mutations (Cambareri *et al.*, 1991). However RIP only occurs during the sexual cycle and is therefore not likely causing mutations in *un-24* transgenes during vegetative growth. To our knowledge, *Neurospora* lacks a system similar to RIP that operates during vegetative growth. In fact, several lines of evidence suggest that escape from *un-24*-associated incompatibility by a mutation mechanism is unlikely. The

predictability in timing of *un-24* escape mentioned above is not a characteristic of mutation, which usually follows a stochastic pattern of occurrence. More direct evidence that mutation does not account for *un-24* escape is based on observations of escape by *un-24*^{OR} strains transformed with *hygunPA*, a construct in which the hygromycin resistance gene is fused to the UN-24^{PA} incompatibility domain. Mutation of either *hygunPA* or UN-24^{OR} in a self-incompatible *un-24::hygunPA* transformant would result in either a loss of hygromycin resistance or in essential UN-24 catalytic activity, respectively, and thereby strains containing such DNA mutations would not be viable on VMM containing hygB. Therefore, the genetic mechanisms of methylation, RNA interference and mutation do not appear to be involved in *un-24*-associated escape and this led us to explore mechanisms that may be acting at the protein level.

A protein-based mechanism of escape is plausible given our observations that co-expression of *un-24*^{OR} and *un-24*^{PA} leads to the formation of protein complexes. There are several structural similarities between UN-24 and fungal prion proteins such as HET-s from *P. anserina* and Sup35p and Ure2p from yeast. The aforementioned prions contain a structural domain connected to a flexible domain, which is required for prion formation and is often observed in the center of amyloid aggregates (Balguerie *et al.*, 2004; Chiti and Dobson, 2006). The C-terminus of UN-24 is thought to be highly flexible whereas the N-terminus is highly structured. Wellman *et al.* (2009) showed that the flexible C-terminal domain of both UN-24^{OR} and UN-24^{PA} is required for incompatibility while the N-terminal structured domain is required for incompatibility activity of UN-24^{OR}, but not UN-24^{PA}. These shared characteristics suggested that UN-24 may have prion-like properties since these structural features are thought to lead to aggregation formation by

prions. An aggregate is an association of two or more misfolded proteins. Small aggregates are often soluble, however larger aggregates, which can arise from association of smaller aggregates or by the addition of multiple misfolded proteins to an existing smaller aggregate, are insoluble under physiological conditions. The term amyloid applies to protein aggregates that are composed of amyloid fibers, which are thermodynamically stable and highly insoluble proteins organized in repeating β -sheets (Ross and Poirier, 2005).

An emerging theme in prion research is the role that heat shock proteins play in identifying and eliminating protein aggregates and this may be relevant to the *un-24*-associated escape phenomenon. The most widely studied examples of this occur in the yeast prion systems. Three prions are endogenous to yeast; $[PSI^+]$, $[URE3]$ and $[RNQ^+]$ or $[PIN^+]$. The $[PSI^+]$ prion is encoded by the *SUP35* gene. Under non-prion conditions, $[psi^-]$, *SUP35* encodes a translation termination factor. However, when Sup35p takes on a prion form, a nonsense suppressor phenotype is conferred upon the cell (Cox, 1965; Cox, 1971; Wickner, 1994). $[URE3]$ is encoded by the *URE2* locus. When the $[URE3]$ prion is present cells can uptake ureidosuccinic acid (USA). USA is generally excluded from cells when Ure2p is not in its prion state $[ure-0]$ (Lacroute, 1971; Aigle and Lacroute, 1975; Wickner, 1994). Finally, the $[RNQ^+]$ or $[PIN^+]$ prion is encoded by the *RNQ1* locus. Presence of $[RNQ^+]$ increases the probability that Sup35p and Ure2p will undergo conversion into their respective prion forms (Derkatch *et al.*, 1997). It has been suggested that the presence of $[RNQ^+]$ amyloid fibers act as a template upon which other prions can assemble (Derkatch *et al.*, 2001).

Heat shock proteins have been observed to modulate the above yeast prion functions. Hsp70p and Hsp40p bind to Sup35p and disassemble oligomers thus sequestering them from $[PSI^+]$ prion formation. In addition, Hsp70p and Hsp40p recognize and disassemble mature $[PSI^+]$ prion proteins (Shorter and Lindquist, 2008). In contrast, physiological levels of Hsp104p stimulates the formation of $[PSI^+]$ prions whereas overexpression or deletion of Hsp104p alleviates the $[PSI^+]$ phenotype (Chernoff *et al.*, 1995). It is hypothesized that at physiological levels, Hsp104p causes an increased amount of Sup35p oligomers which results in increased prion nucleation (Shorter and Lindquist, 2004, 2006). When expressed at high levels, Hsp104p utilizes ATP hydrolysis to disaggregate $[PSI^+]$ into two forms; an SDS soluble form and a non-SDS soluble form which has a diminished ability to nucleate prions (Shorter and Lindquist, 2004, 2006). In the $[URE3]$ system, overexpression of Hsp40p stimulates Hsp70p to dismantle $[URE3]$ aggregates (Sharma *et al.*, 2009). Interestingly, yeast heat shock proteins have been observed to disassemble non-endogenous aggregating proteins that naturally aggregate in their endogenous host. For example, when the amyloid forming Huntington protein from humans is expressed in yeast, overexpression of yeast Hsp104p enables the identification and disassembly of the Huntington aggregate (Cashikar *et al.*, 2005). Furthermore, when yeast Hsp104p and the Huntington gene are co-expressed in *Caenorhabditis elegans*, the Huntington protein is disaggregated by yeast Hsp104p (Satyal *et al.*, 2000).

As indicated in Chapter 2, overexpression of hygPA in yeast led to a response reminiscent of escape from heterokaryon incompatibility in *N. crassa*. In this chapter, we explore the mechanism by which hygPA-associated incompatibility is remediated in yeast. We focused on identifying proteins that may interact with the hygPA protein to

alleviate hygPA-associated incompatibility in yeast. We then examined whether or not proteins similar to those identified in yeast are involved in mediating escape from *un-24*-associated incompatibility in *N. crassa*.

METHODS AND MATERIALS

Microbiology and Molecular Genetic Techniques.

Yeast strains were cultured as described in Chapter 2. Quantification of DNA and RNA was performed as described in Chapter 2. To obtain *SSA1* knockout strains carrying the hygFLAGunPA or hygFLAG constructs, strains carrying these respective constructs in the *GAL1* locus were mated with *ssa1::G418^R* (*MATa his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 YAL005C::G418^R* from Giaever *et al.*, 2002). Haploids were obtained following these crosses through random sporulation as described in Ausubel *et al.* (1997) and were liberated from asci using 10 μL β-glucuronidase (Type H-3 from *Helix pomatia*, 99000 U/mL, Sigma Aldrich) with 100 μL of cell suspension at room temperature for 1 h. Double mutants (those that contained the hygFLAG or hygFLAGunPA constructs and *ssa1::G418^R*) were selected on medium containing both G418 and hygB.

Protein Methods

To induce high-level expression of the *GAL1* locus, yeast strains were transferred from YPD to YPRaf/Gal liquid medium. Proteins were extracted from yeast as described in Chapter 2. Size exclusion chromatography was performed on crude protein extracts from yeast as described in Chapter 2. SDS-PAGE, protein quantification and Coomassie staining and transfer of proteins to nitrocellulose were all performed as described in Chapter 2. For immunoblotting, the nitrocellulose was incubated for 2 h in blocking solution followed by 1 h with anti-FLAG antibody diluted in blocking solution (1:1000, Invitrogen). The nitrocellulose was washed three times for 30 min in TBST and proteins were visualized as described in Chapter 2. Affinity purification of the FLAG-tagged

proteins was performed as described in Chapter 2. Protein bands were isolated from SDS-PAGE gels following Coomassie staining and identified using mass spectrometry as described in Chapter 2.

Yeast 'Escape' Assay

Yeast cells expressing hygFLAGunPA and the hygFLAG-expressing control strain were grown overnight in 5 mL YPD liquid medium. The following day, both yeast strains were resuspended separately in 50 mL fresh YPD liquid medium and allowed to achieve mid-log growth. Cultures were then centrifuged at 800 x g for 2 min to pellet the cells, washed three times in 1 X PBS (pH 7.4) and resuspended in 50 mL of pre-warmed YPRaf/Gal liquid medium. After two h of shaking at 30°C, two 1 mL aliquots of each cell culture in YPRaf/Gal were removed and immediately processed as described below. These aliquots were considered to be time zero in our assay. Cells from each culture were then pelleted at 800 x g for two min, resuspended in 50 mL of pre-warmed (30°C) YPD liquid medium and incubated at 150 RPM, 30°C. At 15 min intervals (15, 30, 45 and 60 min) two, 1 mL aliquots of each yeast culture were removed. Cells from one aliquot were stained with neutral red and the percentage of cells that stained red throughout the cytoplasm was counted (as describe in Chapter 2). Proteins were extracted from the second 1 mL aliquot for immunoblotting with anti-FLAG antibodies in order to detect FLAG-tagged proteins (described in Chapter 2). For immunoblotting, 10 µg of total protein from each sample was loaded on SDS-PAGE. Equal protein loading was determined by Coomassie staining of replicate gels (described in Chapter 2). As control lines, the same procedure as described above was done except that, instead of transferring cells into YPD after two h in YPRaf/Gal, the cells were transferred into pre-warmed

(30°C) YPRaf/Gal liquid medium. Aliquots were taken at 15 min intervals and neutral red staining, protein extraction and immunoblotting were performed as described above.

Protein band intensity was determined using the densitometry tool in AlphaEase v4.0. For densitometry measurements, the integrated density value (IDV) was calculated relative to a protein band at ~50 kDa on a Coomassie stained replicate gel. Therefore, any differences in protein loading would be represented in the IDV value.

RNA Isolation from Self-Incompatible *N. crassa* Strains

N. crassa strain C9-2 (*un-24*^{OR}) was transformed with hygPA and self-incompatible colonies that had a ‘spidery’ morphology were selected and cultured as described in Chapter 2. Lysing enzymes from *Trichoderma harzianum* (Sigma Aldrich) were used in place of Novozyme 234 to spheroplast cells. For isolating cDNA from pre-escape colonies, small (~0.5 mm) samples of self-incompatible transformants were subcultured from the original transformation plate onto VMM containing threonine and hygB. These primary cultures were incubated for 3 days and then ~1.0 mm³ inocula of a colony were transferred onto the surface of sterile cellophane disks overlayed on VMM with threonine and hygB. Subcultures of individual transformants were allowed to grow for 3 days at 30°C, removed from the cellophane and ~75 mg of mycelium was from each transformant was pooled for RNA extractions. Harvested mycelia were homogenized in a MM301 homogenizer (Retsch , Haan, Germany) for 5 min at a frequency of 1/30 s. RNA was extracted using an RNeasy Plant Minikit (Qiagen) using the optional in-column DNase treatment (Qiagen). For post-escape cDNA samples, each of several self-incompatible transformants was allowed to grow at room temperature and observed daily for fast-growing, wildtype escape sectors. Escaped hyphae were transferred to cellophane

on VMM with threonine and hygB and ~75 mg of mycelium was used to obtain post-escape cDNA as described below.

cDNA was created by resuspending a total of 100 ng of total RNA in 11 μ L of ddH₂O. One μ L of a 50 mM oligo d(18T) primer (Sigma-Aldrich) was added, the mixture was heated to 70°C for 5 min and cooled on ice for 10 min. Five μ L of a 4 mM dNTP mixture (Invitrogen) along with 2 μ L of 10 X M-MuLV Reverse Transcriptase Reaction Buffer (New England Biolabs) and 1 μ L of RNase inhibitor (New England Biolabs) was added to the mixture. The mixture was incubated at 37°C for 5 min, 1 μ L of M-MuLV Reverse Transcriptase (200,000 U/mL, New England Biolabs) was added and cDNA synthesis was performed at 42°C for 1 h. The reaction was terminated by incubating the mixture at 70°C for 10 min and stored at -20°C.

Quantitative PCR of *N. crassa* Loci with Hsp70-like Characteristics

Quantitative PCR (qPCR) was done in a Rotor Gene RG-3000 (Corbett Research, Sydney, Australia) using IQ SYBR Green Supermix as recommended by the manufacturer (Biorad). PCRs were performed using the primer pairs in Table 3.1. Each PCR product was generated using 40 cycles of the following PCR program: 30 s at 95°C (denaturation), 30 s of annealing at temperatures indicated in Table 3.1 and 30 s at 72°C (extension). To verify that equal amounts of cDNA were added to each reaction mix, we performed qPCR using primers Sbr-yy9 and sbrL-02RC that were specific to the SBR locus (Campsall, 1998).

All gene-specific PCR products were confirmed to be of the correct size using gel electrophoresis. The copy number (ng/ μ L) of each Hsp70 cDNA was estimated by setting

the cycle at threshold (C_t) to 0.1 and using the equation of a relative qPCR standard curve. A relative standard curve was prepared by adding known concentrations of the plasmid pUC19 into each *N. crassa* cDNA mixture and doing PCR amplification with M13 forward and reverse primers to obtain the C_t value (C_t threshold set at 0.1) for each concentration. The C_t value was plotted against the negative logarithm of the DNA concentration to obtain a standard curve as described in Providenti *et al.* (2006). The copy number was subsequently converted to fg of transcript per ng of RNA. To control for DNA contamination, qPCR was performed with RNA samples that had not undergone reverse transcriptase treatment. cDNA from pre- and post-escape cells of four independent transformants were used. Amplicons from each independent transformant were detected once. Differences in transcript amounts from Hsp-70 like loci between pre- and post-escape strains were tested for significance.

Cloning of Genes that have Hsp70-like Characteristics

The inducible quinic acid promoter region (*qa-2P*) and two of the three loci that had Hsp70-like characteristics (NCU02075 and NCU01499) that were shown to be significantly upregulated in post-escape self-incompatible *un-24^{OR}::hygunPA N. crassa* colonies were amplified with primers found in Table 3.2. The proofreading polymerase iProof was used to amplify each gene according to manufacturer's specifications (BioRad). *qa-2P* was amplified using the following PCR program: 30 cycles of 30 s at 95°C (denaturation), 30 s at 58°C (annealing) and 1 min at 72°C (extension). ~150 bp upstream and coding sequences of NCU02075 and NCU01499 were amplified using the following PCR program: 30 cycles of 30 s at 95°C (denaturation), 30 s at 55°C

Table 3.1: Primers and annealing temperatures used to examine pre- and post-escape transcript levels for ten *N. crassa* genes with similarity to Hsp70.

Locus	Primer	Sequence (5'-3')	~Tm (°C)	Annealing Temperature (°C)	Amplicon Size (bp)
NCU09602	9602 for	TTCCGTGAGGACCGCT	63	60	164
	9602 rev	CCAATGAGACGCTTGGC	63		
NCU02075	2075 for	GGTCCTCACCAAGCCC	62	63	146
	2075 rev	CGGTGAAGACACCACCC	63		
NCU03982	3982 for	GCTTGAGGAGAATGCCG	62	58	145
	3982 rev	AAGCTCGTCATGGCCAG	63		
NCU05269	5269 for	GCGGTGTCGATGTCATC	61	62	170
	5269 rev	GTCGTCGAGAGTGCGG	62		
NCU00692	0692 for	AACGAGGATGGCGACC	62	60	153
	0692 rev	GACGGCCGCAAAGTC	62		
NCU08693	8693 for	GGCTTACCAGGGCTCTG	61	60	161
	8693 rev	GGCGGAGTTGGTGGTA	61		
NCU01499	1499 for	GCATATTGCCAAGGGC	60	60	148
	1499 rev	GTCGCGCGTGTACGA	62		
NCU00726	0726 for	CTCGGCCCCAACAA	60	-	197
	0726 rev	GAATAGCACCGCTGCCT	62		
NCU00573	0573 for	GGCAACAGCCGTAGC	59	-	163
	0573 rev	GGACGGTGGGGATCT	59		
NCU02455	2455 for	CCAATGGCCAGCAATG	62	-	172
	2455 rev	GGCTTGGGGACGC	60		
Sbr	Sbr-yy9	GGTTGCGCGTTTCGTCAA	68	58	175
	sbrL-02RC	CTCTGTTCGCTCACATTGCT	63		

- primer pair did not yield a qPCR product at several annealing temperatures

Table 3.2: Primers used to amplify the quinic acid inducible promoter region (*qa-2P*) and genes with Hsp70-like characteristics that were shown to be upregulated in post-escape samples of *N. crassa*.

Primer	Sequence (5'-3')	~T _m (°C)
Qa2pf	TAGGATCCAATCGACGGACCGGC	61
Qa2pr	TAACTAGTTGCCGAGGAGGTTGAG	66
2075F	TAGCGGCCGCTTAGCGGGAAGACATGGCCT	83
2075R	TAACTAGTACGTAGCCCACTCTTTCAGC	66
1499F	TAACTAGTAAGTTGATAGCGGAGGTTC	63
1499R	TAGCGGCCGCCTAGTAAAACTCGGCATCAAC	79

(annealing) and 2 min 30 s at 72°C (extension). Since iProof Taq does not incorporate dATP as a 3' overhang to the amplicon, all iProof PCR products were gel purified as described in Vogelstein and Gillespie (1979) and incubated at 72°C for 30 min with Taq polymerase to facilitate cloning into TOPO-TA vectors (Bioshop). Gel purified amplicons of *qa-2P*, NCU01499 and NCU02075 were cloned into TOPO-TA pCR2.1 (Invitrogen), creating pqa-2P, pNCU01499 and pNCU02075, respectively. To create qa-2P-NCU01499 and qa-2P-NCU02075 fusion constructs, pqa-2P and either pNCU01499 or pNCU02075 were digested with *SpeI* followed by heat inactivation at 80°C for 20 min. NCU01499 or NCU02075 were ligated separately to *qa-2P*, following manufacturer's specifications (Invitrogen). The resulting ligated constructs were subsequently amplified with iProof Taq using the primer pair Qa2pf and either 2075R or 1499R, for NCU02075 and NCU01499, respectively, with the following PCR program: 30 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing) and 3 min 30 s at 72°C (extension). dATP was incorporated as a 3' overhang on the amplicon as described above. The constructs were then cloned into TOPO-TA pCR2.1 as QP1499 (*qa-2* promoter region upstream of NCU01499) and QP2075 (*qa-2* promoter region upstream of NCU02075). These constructs were sequenced using the primers listed in Table 3.3. The constructs QP1499 and QP2075 were subsequently ligated separately into pRS41N, a vector that confers NAT resistance to *N. crassa* (Smith and Smith, 2006). To insert QP1499 and QP2075 into pRS41N, both pRS41N (no insert) and pCR2.1 containing either QP1499 or QP2075 were digested with *Bam*HI and *Not*I for 90 min and gel purified as described above. QP1499 and QP2075 were ligated separately into pRS41N, following manufacturer's specifications (Invitrogen) creating two vectors, p(NAT)QP1499 and p(NAT)QP2075.

Table 3.3: Primers used to sequence amplicons of the quinic acid inducible promoter region (*qa-2P*), the Hsp70-like genes NCU01499 and NCU02075, and the constructs QP1499 and QP2075.

Primer	Sequence (5'-3')	~T _m (°C)
1499 Fi	GCTTCCAGTTCCGACC	59
1499 Fii	TCACTAGGGCCGATC	55
1499 Ri	GCGCGTGTAGTGGAAG	59
1499 Rii	GGACGAGGTCACAGACC	59
2075 Fi	TTGTCGGCAACAGTGG	60
2075 Fii	TGATACGGAGGACGTTG	53
2075 Ri	ACTTCGACACCAACCTTC	58
2075 Rii	GTTTACGATGGTGCC	53
M13 Forward	CGCCAGGGT TTTCCCAGTCACGAC	76
M13 Reverse	TCACACAGGAAACAGCTATGAC	62

Transformation of *N. crassa*

To obtain self-incompatible colonies containing quinic acid inducible Hsp70 paralogs, spheroplasts of *N. crassa* strain *qde-2* (*un-24*^{OR} *het-6*^{OR} *qde2⁻* *a*, Cogoni and Macino, 1997) were produced as described in Chapter 2. Transformation was performed as described in Chapter 2 using 2 µg of p(NAT)QP1499, p(NAT)QP2075 and pRS41N (control). In addition, we co-transformed 0.1 µg of *pqa-2P* and 0.9 µg of pRS41N as a second control. Transformation plates containing 200 µg/mL NAT were used to select for transformants. NAT resistant transformants were subcultured and genomic DNA was extracted as follows. Transformants were transferred to 5 mL of liquid VMM and shaken at 30°C for 5 days. The mycelium were harvested and placed in a 1.5 mL Eppendorf tube along with a cap full of 0.5 mm glass beads and 1 mL of TENS solution [100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 100 mM EDTA (pH 8.0), 2% SDS]. The mixture was vortexed for 10 min and subsequently centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed, combined with 1/6 volume of 3 M NaOAc and was kept on ice for 30 min. The mixture was centrifuged at 12,000 x g for 10 min, the supernatant was removed and was mixed with an equal volume of isopropanol. After incubating this mixture overnight at -20°C, it was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 50 µL of ddH₂O. Presence of either QP1499, QP2075 and *qa-2P* in the *N. crassa* genome was verified by PCR using the M13 forward and reverse primers in the following PCR program: 35 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing) and 3 min 30 s at 72°C (extension). The resulting amplicons were gel purified as described above and presence of each Hsp70-

like gene and *qa-2P* was verified using the primers Qa2pf and either Qa2pr, 1499R or 2075F for *qa-2P*, QP1499 and QP2075, respectively (Table 3.2).

RESULTS

hygunPAp interacts with Hsp70 paralogs when overexpressed in yeast

As described in Chapter 2, yeast that expressed hygunPA at low-levels (when grown on YPD) exhibited cytological abnormalities reminiscent of *het*-associated incompatibility in *N. crassa*. However, these traits were ameliorated when hygunPA was overexpressed in yeast (grown on YPRaf/Gal). To examine protein interactions that may be remediating hygunPA incompatibility during overexpression, we extracted and immunoblotted proteins under reducing conditions from yeast strains expressing either hygFLAGunPA or the hygFLAG (control) that had been grown in YPRaf/Gal (Figure 3.1). Using anti-FLAG antibodies, a single band was detected slightly above the 38 kDa marker in proteins extracted from control cells expressing hygFLAG. This corresponded to the expected size (41 kDa) of the hygFLAG protein (hygFLAGp) monomer. Protein extracts from cells expressing hygFLAGunPA yielded two bands when probed with anti-FLAG antibodies; a faint band located above the 38 kDa marker and a second intense band slightly below the 93 kDa marker. The signal that was above the 38 kDa marker corresponded to the expected size of hygFLAGunPA protein (54 kDa, hygFLAGunPAp) monomer. The second signal, which was determined to be ~85 kDa, was of unknown identity. Both protein bands from the hygFLAGunPA-expressing strain were subsequently separately purified using M2 anti-FLAG agarose beads. Mass spectrometry analysis identified fragments of the hygromycin phosphotransferase protein in the 54 kDa band and therefore, as expected, this band corresponded to hygFLAGunPAp. The 85 kDa signal was identified by mass spectrometry as Ssa1p, a member of the Hsp70 family of proteins in yeast. The peptides obtained by mass spectrometry, their respective protein

Figure 3.1: When overexpressed in yeast, hygFLAGunPAp binds to Ssa1p, a member of the Hsp70 family. Proteins were extracted under reducing conditions (DTT, β -mercaptoethanol) from cells grown in YPRaf/Gal that expressed either the hyg, hygFLAG, hyggunPA or hygFLAGunPA constructs. These proteins were subsequently immunoblotted with anti-FLAG antibodies. FLAG signals were not detected in proteins extracted from the hyggunPA-expressing and the hyg-expressing control strains. This demonstrated that signals detected on these immunoblots were specific to FLAG-tagged proteins. An intense ~ 41 kDa signal (c), that represented the hygFLAGp monomer, was detected in proteins extracted from the hygFLAG-strain. A weak ~ 54 kDa signal (b) and an intense ~ 85 kDa signal (a) were detected in proteins extracted from hygFLAGunPA-expressing yeast. The ~ 54 kDa signal was identified by mass spectrometry as the hygFLAGunPAp monomer. The ~ 85 kDa signal, which was identified by mass spectrometry as Ssa1p, was on average 10 X more intense than the ~ 54 kDa signal. Since the ~ 85 kDa signal was larger than the expected size for Ssa1p (~ 70 kDa) and because it was detected by anti-FLAG antibodies, we infer that this signal represents a complex between hygFLAGunPA and Ssa1p. Similar anti-FLAG signals were detected in five independent immunoblots. Equal protein loading was verified by Coomassie staining of replicate gels as shown across the bottom of the figure.

[Fig. 3.1]

candidates as well as Mascot scores are listed in Table 3.4. We inferred that the 85 kDa band was a hygFLAGunPAp-Ssa1p complex since it was larger than the expected size of Ssa1 (69.7 kDa) and was detected by anti-FLAG antibodies only from the hygFLAGunPA-expressing strain. Peptides corresponding to the UN-24^{PA} C-terminus portion of hygFLAGunPAp were not detected. This putative hygunPAp-Ssa1p complex was not dissociated under reducing conditions of 9 M urea and β -mercaptoethanol (not shown). We also infer that the majority of hygunPAp in these YPRaf/Gal-grown cells was associated with Ssa1p. The 85 kDa signal was, on average, about ten times more intense than the 54 kDa hygunPAp signal in each of five independent immunoblots. This banding pattern and ratio were also observed when proteins from yeast expressing hygFLAGunPA grown in YPRaf/Gal were extracted under non-reducing conditions (boiling, no DTT, no β -mercaptoethanol). Interestingly, the expected size of a complex between Ssa1p and hygFLAGunPAp is ~124 kDa whereas the band we observe was at ~85 kDa. This indicates that Ssa1p may be bound to a degraded form of hygFLAGunPA. Ssa1p participates in a multitude of cellular functions, is moderately expressed during normal growth and is highly induced during stress (Werner-Washburne *et al.*, 1987). In addition, as mentioned previously, Hsp70 paralogs have been observed to disassemble protein aggregates and modulate prion formation in yeast. We hypothesize that when the PA domain is expressed at high levels, this hygFLAGunPAp-Ssa1p complex impedes the formation of the hygFLAGunPAp-Rnr1p complex and/or leads to its degradation.

A different chaperone protein sequesters hygunPAp in a yeast strain that is deleted for *SSA1*. We determined this by first crossing cells containing hygFLAGunPA with *ssa1::G418^R*, an *SSA1* knockout strain, to obtain cells that expressed hygFLAGunPAp but

Table 3.4: Mascot results of anti-FLAG purified protein bands from hygFLAGunPA-expressing yeast grown in YPRaf/Gal. The ~54 kDa and ~85 kDa protein bands generated peptide sequences that corresponded to hygromycin phosphotransferase protein and Ssa1p, respectively. The theoretical molecular weight (MW) of the protein candidate identified by mass spectrometry and the transgenic protein or complex expected in yeast is given.

Protein Candidate	MW on SDS-PAGE (kDa)	Theoretical MW (kDa) of Protein Candidate	Mascot Score	Peptide Sequences
Hygromycin phosphotransferase protein	54	38.32*	111	- AFSFDVGGR
				- FDSVSDLMQLSEGESR
				- SAAVWTDGCVLADSGNR + Carb (C)
Heat Shock Protein 70 (Ssa1p)	85	69.615	325	- DAGTIAGLNVLR
				- FEELCADLFR + Carb (C)
				- SQVDEIVLVGGSTR
				- TTPSFVAFTDTER
				- ARFEELCADLFR
				- NFTPEQISSMVLGK

* this is the theoretical MW of the hygromycin phosphotransferase protein without in-frame addition of the FLAG epitope and the *un-24^{PA}* incompatibility domain. The estimated MW of the hygFLAGunPAp monomer is 54 kDa.

lack Ssa1p. Similar to our previous observations, when grown in YPD, ~20% of *ssalp::G418^R* cells that express hygFLAGunPA have a highly granulated cytoplasm and ~20% of cells stained red throughout the cytoplasm with neutral red (results not shown). These incompatibility-like traits did not occur when these cells were grown in YPRaf/Gal. To examine if hygFLAGunPAp could complex with other Hsp70 proteins, *ssalp::G418^R* cells that expressed hygFLAGunPA were grown in YPRaf/Gal and proteins were extracted under reducing conditions and immunoblotted with anti-FLAG antibodies (Figure 3.2). Similar to our previous observations, proteins from *ssalp::G418^R* expressing hygFLAGunPA yielded two signals on these immunoblots, a very faint ~54 kDa signal and an intense ~85 kDa signal. By mass spectrometry, the ~85 kDa band was identified as another Hsp70 family member, Ssb2p, and/or a mitochondrial precursor Hsp60p. Peptides detected with this mass spectrometry analysis, their corresponding protein candidate and Mascot scores are listed in Table 3.5. Similarly, because the ~85 kDa protein was larger than the expected sizes of either Ssb2p (67 kDa) or Hsp60p (61kDa) and since it was detected by anti-FLAG antibodies, this signal appears to represent a complex between the C-terminus of UN-24^{PA} and one of the heat shock proteins. As was observed previously, peptides corresponding to the UN-24^{PA} C-terminus portion of hygFLAGunPAp were not detected. The majority of hygunPAp in *ssalp::G418^R* cells expressing hygFLAGunPA was associated with Ssb2p and/or Hsp60p. The presence of this complex suggests that when absent, the function of Ssa1p is replaced by Ssb2p and/or Hsp60p. Hsp60p is a mitochondrial chaperone protein that is involved in ATP-dependent folding of polypeptides, in preventing aggregation of proteins and has been observed to interact with mtDNA and has thus been implicated in

Figure 3.2: When overexpressed in *ssal::G418^R* cells, hygFLAGunPAp interacts with Ssb2p and/or Hsp60p. Proteins from hygFLAG- and hygFLAGunPA-expressing cells as well proteins from *ssal::G418^R* cells expressing hygFLAG and hygFLAGunPA were extracted under reducing conditions (DTT and β -mercaptoethanol) and immunoblotted with anti-FLAG antibodies. Anti-FLAG antibody bound to a ~41 kDa protein (c) in *ssal::G418^R* cells expressing hygFLAG. The ~41 kDa signal was the expected size of hygFLAGp monomer (c). Two protein bands at ~54 kDa and ~85 kDa were detected in proteins extracted from *ssal::G418^R* that expressed hygFLAGunPA. We infer that the ~54 kDa band represents the hygFLAGunPAp product (b). Similar to the hygFLAGunPAp-Ssa1p complex observed in hygFLAGunPA expressing cells (Figure 3.1), nearly all of the anti-FLAG antibody bound to the ~85 kDa protein (a), which was identified by mass spectrometry as Ssb2p and/or Hsp60p. We infer that nearly all of the hygFLAGunPA protein is linked in a non-reducible complex to Ssb2p and/or Hsp60p (a). Anti-FLAG antibodies detected similar signals to the one shown in two independent immunoblots. Equal protein loading was confirmed using Coomassie stained replicate gels (not shown).

ssa1::G418^R

hygFLAGunPA

hygFLAG

hygFLAGunPA

hygFLAG

a
b
c

191 kDa —
93 kDa —
38 kDa —

[fig. 3.2]

Table 3.5: Mascot results of anti-FLAG purified protein from *ssal::G418^R* cells that expressed hygFLAGGunPA. The ~ 85 kDa protein band yielded peptides that corresponded to the mitochondrial chaperone Hsp60 and to the cytosolic Hsp70 homolog, Ssb2p. The theoretical molecular weight (MW) of the protein candidate as identified by mass spectrometry as well as the theoretical mass involving the protein candidate and hygFLAGGunPAp is indicated.

Protein Candidate	MW on SDS-PAGE (kDa)	Theoretical MW of Protein Candidate (kDa)	Mascot Score	Peptide Sequences
Heat shock protein Hsp60 precursor, mitochondrial	85	60,999	401	- VGGASEVEVGEK
				- NVLIEQPFGPPK
				- GSIDITTTNSYEK
				- VLDEVVVDNFDQK
				- GVETLAEAVAATLGPK
				- TLEDELEVTEGMR
				- NEAAGDGTTSATVLGR
				- ISSIQDILPALEISNQRS
				- TGLDISDDAR
				- STLEPVEQVLK
Heat Shock Protein 70 (Ssb2p)	85	66,537	333	- FEDLNAALFK
				- VTPSFVAFTPQER
				- TFSPQEISAMVLTK
				- AVITVPAYFNDAQR
				- IINEPTAAAIAYGLGAGK
				- VIDVDGNPVEVQYLEETK
				- LESYVASIEQTVTDPVLS SK

mitochondrial DNA transmission (Martin *et al.*, 1992; Dubaquié *et al.*, 1997; Kaufman *et al.*, 2003). Ssb2p is an Hsp70 paralog that is involved in the folding of newly-synthesized polypeptides (Pfund *et al.*, 1998). It is unclear if just one or both of these heat shock proteins is involved in complexing with hygFLAGunPA. Nonetheless, the presence of these complexes appears to be associated with the alleviation of aberrant hygFLAGunPA-associated phenotypes.

Treatment of the hygFLAGunPAp-Ssa1p complex with reducing agents and boiling liberates some, but not all, hygFLAGunPAp from the complex

To explore the nature of the linkage between the UN-24^{PA} C-terminus and Ssa1p we subjected proteins extracted under native conditions from hygFLAG- and hygFLAGunPA-expressing yeast grown in YPRaf/Gal to size exclusion chromatography. Following size exclusion chromatography, fractionated proteins were treated with reducing conditions (DTT, β -mercaptoethanol and boiling) and immunoblotted with anti-FLAG antibodies. We hypothesized that if all of the hygFLAGunPAp in the cell was associated with Ssa1p, then we would observe co-elution of hygFLAGunPAp monomer and the hygFLAGunPAp-Ssa1p complex. Alternatively, if detectable amounts of hygFLAGunPAp were not associating with Ssa1p then we would be able to detect hygFLAGunPAp monomer in fractions that did not contain the hygFLAGunPAp-Ssa1p complex. In immunoblots, both the 85 kDa hygFLAGunPAp-Ssa1p complex and the 54 kDa hygFLAGunPAp eluted in fractions 3, 4 and 5 (Figure 3.3). Interestingly, the hygFLAGunPAp monomer was not present in fractions 6-8 (lanes 7-8 not shown) even though our size exclusion chromatograph eluted the monomeric form of hygFLAGp from hygFLAG-expressing cells in fractions 4-8. This indicated that all detectable hygunPAp

Figure 3.3: Virtually all of overexpressed hygFLAGunPAp interacts with Ssa1p. Proteins extracted under native conditions from hygFLAGunPA- and hygFLAG-expressing cells grown in YPRaf/Gal were subjected to size exclusion chromatography and immunoblotted with anti-FLAG antibodies. The ~54 kDa and ~85 kDa signals were detected in fractions 3-5 but not detected in fractions 6-8. Apparently, the 54 kDa protein signal represents the hygFLAGunPAp (b) monomer and the 85 kDa signal represents the hygFLAGunPAp-Ssa1p complex (a). The intensity of both signals were similar to previous results in that the hygFLAGunPA-Ssa1p complex signal was ~10 X brighter than the hygFLAGunPAp monomer signal. In contrast, the hygFLAGp monomer was detected in fractions 4-8. Since the hygFLAGunPAp monomer (c) was detected in the same fractions as the hygFLAGunPApSsa1p complex, but not in fractions 6-8 where proteins with a molecular weight of 54 kDa are expected to elute, we infer that, when overexpressed, virtually all of hygFLAGunPAp associates with Ssa1p. Similar anti-FLAG signals were detected in two independent immunoblots. Equal protein loading was verified by Coomassie staining of a replicate gel as shown across the bottom of the figure.

hygFLAGunPA hygFLAG

monomer in yeast associates with Ssa1p. Reducing conditions (DTT and β -mercaptoethanol) and boiling failed to liberate the remainder of the hygFLAGunPAp monomer from the complex and indicated that ~90% of the hygFLAGunPAp monomer was covalently complexed to Ssa1p. Only ~10% of the monomer was liberated from the complex under these strong reducing conditions.

The disappearance of the aberrant incompatibility-like cytology is closely correlated to the formation of the hygFLAGunPAp-Ssa1p complex

We examined the relationship between neutral red staining and the hygFLAGunPAp-Ssa1p complex to further test whether the presence of this complex was correlated with the absence of *het*-associated incompatibility. Cells expressing hygFLAG or hygFLAGunPA constructs were grown in YPD to mid-log phase, resuspended in YPRaf/Gal for 2 h and subsequently transferred back into YPD. Upon transferring to YPD, and at 15 min intervals thereafter, two, 1 mL aliquots of cells were removed. Cells from one aliquot were stained with neutral red while proteins were extracted under reducing conditions from the second aliquot (Figure 3.4). In this figure, aberrant neutral red staining was scored as present (+) when $\geq 15\%$ of cells had entire cytoplasm that stained bright red. It can be seen that neutral red staining of control hygFLAG-expressing cells was not pronounced at any time. At 0 min (in YPRaf/Gal just prior to transfer into YPD) and after 15 min in YPD medium, neutral red staining was not pronounced in cells expressing hygFLAGunPA. When sampled at and after 30 min after transfer to YPD, pronounced neutral red staining of hygFLAGunPA-expressing cells was observed. To monitor the presence or the absence of the hygFLAGunPAp-Ssa1p complex, we extracted and immunoblotted proteins with anti-FLAG antibodies from both hygFLAG-

and hygFLAGunPA-expressing cells. To estimate their intensity, we calculated the integrated density value (IDV) of the ~41 kDa, ~54 kDa and ~85 kDa signals. Note that IDV is not measured in values. As is clearly seen in Figure 3.4, the hygFLAGp monomer detected in proteins extracted from the hygFLAG-expressing strain was present at all sampling points and decreased in intensity over the first 45 min of incubation in YPD as expected for a gene driven by the *GALI* promoter. When proteins were sampled from hygFLAGunPA-expressing cells at time 0 (in YPRaf/Gal just prior to transfer into YPD) the intensity and ratio of both the hygFLAGunPAp monomer and the hygFLAGunPAp-Ssa1p complex was similar to previously obtained results. Upon transfer into YPD medium, the intensity of the hygFLAGunPAp-Ssa1p complex was reduced at a rate of ~25% at each 15 min. The hygFLAGunPAp monomer also decreased in intensity with time but at a lower rate. At 30 min, when the IDV of the hygFLAGunPAp-Ssa1p complex was reduced about 75000, a pronounced neutral red staining pattern was observed in hygFLAGunPA-expressing cells. From this point on, as the signal from the hygFLAGunPAp-Ssa1p complex decreased, a pronounced neutral red staining pattern was apparent in hygFLAGunPA-expressing cells. This correlation suggested that formation of the hygFLAGunPAp-Ssa1p complex was remediating hygunPA-associated incompatibility.

Three genes with Hsp70-like characteristics are upregulated in post-escape in N. crassa un-24^{OR/PA} self-incompatibles

The above observations suggested that formation of the hygFLAGunPAp-Ssa1p complex remediates hygunPA-associated incompatibility in yeast. The incompatibility remediating nature of Ssa1p and Ssb2p/Hsp60p in yeast led us to examine the expression

Figure 3.4: Correlation between disappearance of the hygFLAGunPAp-Ssa1p complex and onset of hygunPA-associated incompatibility in yeast. hygFLAG- and hygFLAGunPA-expressing yeast were grown overnight in YPD, transferred to YPRaf/Gal for 2 h and subsequently resuspended in YPD. At 15 min intervals after transfer to YPD, hygunPA-associated incompatibility was monitored with neutral red staining and protein extraction/immunoblotting with anti-FLAG antibodies was used to examine the abundance of the hygFLAGunPAp monomer and hygFLAGunPAp-Ssa1p complex. We calculated the IDV of each protein band to determine its intensity as shown at the bottom of figure. As expected, the anti-FLAG antibody bound to a single ~41 kDa protein in the hygFLAG expressing control strain. This protein (c) is the expected size of hygFLAGp and was observed to gradually decrease in abundance with time after transfer to YPD (diamonds). A pronounced neutral red staining pattern was not observed at any time point with these hygFLAG-expressing cells. Anti-FLAG antibodies detected two bands at ~54 kDa and ~85 kDa in proteins extracted from the hygFLAGunPA-expressing strain. From previous results, these signals represented the hygFLAGunPAp monomer (b) and the hygFLAGunPAp-Ssa1p complex (a), respectively. Upon transfer to YPD, the hygFLAGunPAp-Ssa1p complex (square) and the hygFLAGunPAp monomer (circle) were observed to decrease by ~25% at each 15 min time point sampled. Pronounced neutral red staining patterns was observed with hygFLAGunPA-expressing cells starting at 30 min in YPD. Similar anti-FLAG signals and neutral red staining were observed in two independent trials. Note that the IDV values are from one experiment but are representative of both trials. Equal protein loading was confirmed using a replicate SDS-PAGE gel stained with Coomassie blue as shown.

[Fig. 3.4]

of genes in *N. crassa* that have Hsp70-like characteristics during escape from hygunPA-associated incompatibility. We utilized the *Neurospora crassa* Database at the Broad Institute to identify loci that had Hsp70-like characteristics which include Hsp70 domains (an N-terminal ATPase binding domain and a C-terminal peptide binding domain) and/or high sequence similarity to known Hsp70 proteins. *N. crassa* contains twelve loci that have Hsp70-like characteristics and one locus homologous to *HSP60* from yeast. Two of these loci (NCU09485 and NCU00714) with Hsp70-like characteristics were annotated while this thesis was being written and therefore were not included in our analysis. We utilized qPCR to examine the expression level of the remaining Hsp70-like genes in four *un-24^{OR}::hygunPA* self-incompatible transformants at pre- and post-escape stages. Three loci (NCU00573, NCU02455 and NCU00726) did not produce amplicons at several temperatures used in our study. Therefore, we used qPCR to examine transcript levels for the seven remaining *N. crassa* loci with Hsp70-like characteristics. For these assays, we used the same quantity of RNA in each cDNA/qPCR based on Nanodrop analysis. We also employed the small brown locus (SBR) as a standard across qPCR reactions. The SBR locus encodes a putative regulatory element that may be involved in tubulin distribution (Campsall, 1998). We reasoned that expression of this locus would not be affected by incompatibility and indeed, qPCR data with primers specific to the SBR locus demonstrated that there was no significant difference between pre- and post-escape SBR cDNA concentrations ($P = 0.98$, Figure 3.6). To determine the amount of transcript found in total cDNA, we determined the C_i of each primer pair and cDNA sample. We then used a standard curve (Figure 3.5) to estimate the amount of transcript (in fg) for each gene with Hsp70-like characteristics. The standard curve was produced by seeding

Figure 3.5: qPCR standard curve relating qPCR cycle at threshold (C_t) and ng of DNA. Known concentrations of pUC19 DNA were seeded into cDNA extracts from pre- and post-escape self-incompatible colonies. We then used M13 forward and reverse primers to amplify 103 bp of the multiple cloning site of pUC19. We determined the C_t value (C_t threshold = 0.1) for each pUC19 template concentration. Since the standard curves produced from pUC19 DNA seeded into both pre- (diamonds) and post-escape (squares) cDNA were nearly identical, we combined the data sets for a single regression line that was subsequently used to estimate concentrations of transcripts from genes with Hsp70-like characteristics in pre- and post-escape samples (Figure 3.6).

[Fig. 3.5]

Figure 3.6: Cycle at threshold values (C_t) of genes with Hsp70-like characteristics from pre- and post-escape self-incompatible transformants as determined using qPCR. Each RNA extract was quantified and adjusted so that all samples had nearly identical amounts of RNA added to the cDNA/qPCR reactions. We performed qPCR using primers specific to seven loci with Hsp70-like characteristics in *N. crassa*. The C_t values from qPCR of cDNA from four independent *un-24*^{OR/PA} pre- (grey bars) and post-escape (black bars) self-incompatibles are shown. The respective agarose gel showing the size and intensity of the final qPCR amplicon is shown below the C_t value histogram. Control primers to the SBR locus verified no significant differences in the amount of cDNA from the pre- and post-escape samples. DNA size standards (bp) are located to the left of each agarose gel series. The self-incompatible colony number is indicated at the bottom of the figure.

1 2 3 4

1 2 3 4

[Fig. 3.6]

known amounts of pUC19 plasmid DNA into both pre- and post-escape cDNA mixtures. We then amplified a 103 bp region of the multiple cloning site of pUC19 using M13 forward and reverse primers. The two curves were, overall, very similar and the two data sets were therefore combined to calculate a single regression line ($y = -3.85x + 16.85$). qPCR of the seven Hsp70-like genes revealed that three loci, NCU02075, NCU01499 and NCU08693 were consistently more abundant in post-escape cells (Figure 3.6).

Amplicons from NCU01499 and NCU02075 were not detected in any pre-escape self-incompatibles while NCU08693 cDNA was detected in pre-escape samples but at consistently lower levels than in post-escape samples. These qPCR results are summarized in Figure 3.7 where the mean amount ($\pm$ SD) of transcript (fg) per ng of total RNA for each of the seven Hsp70-like genes in pre- and post-escape RNA samples is shown. All other Hsp70-like loci showed no significant difference in expression between pre- and post-escape samples (Figure 3.6 and 3.7). qPCRs performed on all RNA samples prior to reverse transcription did not yield detectable amplicons indicating that all samples were relatively free of contaminating DNA. Based on sequence and structural similarity, locus NCU02075 is most similar to the yeast heat shock protein Ssb1p. Functions of this Ssb1p homolog have yet to be specified in *N. crassa* although it has been implicated in conidiation (Kasuga *et al.*, 2005). Locus NCU01499 corresponds to a conserved hypothetical protein with motifs similar to Hsp70 proteins. Locus NCU08693 corresponds to an Hsp70 mitochondrial precursor that is upregulated in response to changes in pH (Leal *et al.*, 2009). To examine the relatedness between NCU02075, NCU08693 and NCU01499 and Ssa1p and Ssb2 from yeast, we used ClustalW (Larkin *et al.*, 2007) to align the predicted protein sequences for the 12 Hsp70-like proteins with

Ssa1p, Ssb2p and Hsp60p. We then created a neighbor joining tree using NJPlot (Perrière and Gouy, 1996) using the method described in Saitou and Nei (1987). The neighbor joining tree (APPENDIX 2) suggests that Ssa1p and Ssb2p are most closely related to NCU09602 and NCU02075, respectively. This latter relationship is most interesting considering that we implicate Ssb2p and NCU02075 in escape from *un-24*-associated incompatibility in yeast and in *N. crassa*, respectively.

Figure 3.7: Mean ($\pm$ SD) amount of transcript (fg) per ng RNA from seven loci with Hsp70-like characteristics and *sbr* in pre- (grey bars) and post- (black bars) escape *N. crassa* self-incompatible *un-24^{OR}::hygunPA* colonies. We utilized qPCR to obtain a cycle at threshold value ($C_t = 0.1$) for each Hsp70-like locus in pre- and post-escape samples. Each threshold value was then converted to copy number of transcript using a standard curve of shown in Figure 3.5. Transcripts from NCU02075, NCU08693 and NCU01499 were detected in significantly higher concentrations in post-escape colonies compared to pre-escape colonies (*). Control primers to the SBR locus verified that there were no significant differences in the amount of cDNA from the pre-and post-escape samples ($n = 4$, $P = 0.98$, two-tailed t-test).

Amount of Transcript
(fg of transcript /ng of RNA)

[fig. 3.7]

DISCUSSION

We had originally expected that overexpression of the hygunPA construct would lead to increased hygunPA-associated incompatibility in yeast. In contrast, overexpression of hygunPA resulted in a decrease in incompatibility; symptoms that were concurrent with the formation of a covalent complex with Hsp70 proteins Ssa1p and, in the *ssa1::G418^R* strain, with Ssb2p and/or Hsp60p. Hsp70s are involved in a multitude of housekeeping functions including intracellular translocation of proteins and protein folding (reviewed in Rudiger *et al.*, 1997). Yeast has 12 Hsp70 paralogs. Among these are four Ssa paralogs (Ssa1, Ssa2, Ssa3, Ssa4), which are involved in protein folding, translocation and prevention of protein aggregation, and two Ssb paralogs (Ssb1 and Ssb2), which interact and help fold polypeptides as they emerge from the ribosome (Albanese *et al.*, 2006). Yeast contains one Hsp60 protein that is involved in mitochondrial DNA transmission, preventing protein aggregation and in polypeptide folding. This protein is primarily located in the mitochondria, however, it has been observed to interact with cytosolic proteins including other heat shock proteins such as Hsp90p and Hsp10p (Rospert *et al.*, 1993; Millson *et al.*, 2005). Given the predescribed nature of Hsp70 and their interactions with yeast prions, our observation that Hsp70 paralogs mediate an escape-like process in yeast is noteworthy.

Both Ssa and Ssb proteins have been observed to directly interact with the aggregating proteins Sup35p (encodes the $[PSI^+]$ prion) and Ure2p (encodes the $[URE3]$ prion, Allen *et al.*, 2005; Bagrianstev *et al.*, 2008). Basal level expression of both *SSA1* and *SSB1* cures weak $[PSI^+]$ variants, but has no effect on strong variants (Kushnirov *et al.*, 2000; Kryndushkin *et al.*, 2002). When overexpressed in *HSP104* knockout strains,

Ssa1p eliminates protein aggregates and antagonizes prion formation (Allen *et al.*, 2005). However, overexpression of *SSA1* reduces prion curing when *HSP104* is expressed at basal levels (Allen *et al.*, 2005). It has been proposed that during $[PSI^+]$ aggregate degradation, Ssa1p maintains active Sup35p oligomer ends thus allowing $[PSI^+]$ prions to form. Therefore, when *SSA1* is overexpressed, more active oligomer ends are available for Hsp104p to convert into the prion form (Shorter and Lindquist, 2008). Conversely, in *HSP104* knockout strains, Hsp104p is not available to create new prion seeds and Ssa1p alleviates the $[PSI^+]$ phenotype (Shorter and Lindquist, 2008). In contrast to Ssa1p, both basal and enhanced expression of Ssb1p and Ssb2p antagonize $[PSI^+]$ prion formation (Chernoff *et al.*, 1999; Allen *et al.*, 2005; Shorter and Lindquist, 2008). As for the $[URE3]$ prion, Hsp104p propagates $[URE3]$ prions whereas Ssa1p antagonizes their aggregation $[URE3]$ (Moriyama *et al.*, 2000; Schwimmer and Masison 2002, Savistchenko *et al.*, 2008). In addition to modulating prion aggregation, Hsp70 proteins also sequester proteins of a non-aggregating nature including proteins involved in DNA replication and apoptosis (Hoffman *et al.*, 1992; Ruchalski *et al.*, 2006). However, given the proposed mechanism of how *un-24* incompatibility results from UN-24^{OR}-UN-24^{PA} protein complexes in *N. crassa*, and that similar hygunPAp-Rnr1p complexes appear to form in yeast, it is not surprising that Ssa and Ssb proteins might interact with or target such complexes and be involved in reducing the incompatibility symptoms associated with overexpression of the UN-24^{PA} incompatibility domain.

The appearance of the hygFLAGunPAp-Ssa1p complex correlated to ‘escape’ in yeast. Similarly, the disappearance of the hygFLAGunPAp-Ssa1p complex resulted in a return of hygunPA-associated incompatibility in yeast. There are two plausible

explanations for how the Hsp70 paralogs inhibit the incompatibility-like effect of hyg_{un}PA in yeast. The first explanation is that when hyg_{un}PA is overexpressed, Hsp70 proteins such as Ssa1p and Ssb2p recognizes hyg_{un}PAP as a protein that is causing, or has the potential to cause, aggregation and thus sequester it. Other implications of this type of function are observed in *Drosophila melanogaster* where Hsp70 proteins sequester endogenous proteins that have poly-glutamine repeats to inhibit their aggregation (Bettencourt *et al.*, 2007). It has also been suggested that Ssa1p, in conjunction with Hsp40p, sequesters Sup35p to prevent $[PSI^+]$ formation in yeast (Krzewska and Melki, 2006). Additional roles of members of the Hsp70 family aside from aiding protein folding, stress tolerance and possibly conidiation have yet to be confirmed in *N. crassa* (Kasuga *et al.*, 2005).

Most Hsp70s, including the *E. coli* Hsp70 homolog DnaK, consist of two domains, an N-terminal ATPase domain and a C-terminal substrate recognition domain (Zhu *et al.*, 1996). The DnaK binding site recognizes peptide sequences consisting of 4-5 hydrophobic residues flanked by basic amino acids. Interestingly, examination of the sequence of the UN-24^{PA} incompatibility domain reveals that it does not contain such a putative Hsp70 binding site. Similarly, a putative Hsp70 binding motif has yet to be identified in Sup35p/ $[PSI^+]$ (Bagrianstev *et al.*, 2008). As observed in our size exclusion chromatography experiments, strong reducing conditions failed to liberate ~90% of hygFLAG_{un}PAP from Ssa1p suggesting that a non-reducible interaction links the hygFLAG_{un}PAP-Ssa1p complex. Since hydrophobic bonds normally drive interactions between Ssa1p and their substrates, including $[PSI^+]$ (Bagrianstev *et al.*, 2008), it appears

that the non-reducible interaction between Ssa1p and hygunPAp is unique. It is unclear what amino acids are mediating this non-reducible bond between Ssa1p and hygunPAp.

A second explanation for how Hsp70 proteins inhibit hygunPA activity in yeast is that overexpression of hygunPA causes activation of an undetermined degradation pathway which results in elimination of hygunPAp-Rnr1p complexes. Under this hypothesis, Hsp70 paralogs would perform or assist in degradation of the hygunPAp-Rnr1p complex. Ssa1p fragments have been detected in partially degraded aggregates that contain $[PSI^+]$, although they are not covalently associated (Bagrianstev *et al.*, 2008). Both Hsp104p and Hsp70 have been observed to target misfolded proteins to the proteasome (Ohba *et al.*, 1994; Lee *et al.*, 1996). Mutation of Ub6p, a protein that removes poly-ubiquitin from ubiquitinated proteins prior to degradation by the 20S proteasome, results in increased $[PSI^+]$ propagation (Chernova *et al.*, 2003). Removal of both Ub6p and Ubc4p, an ubiquitin conjugating enzyme, results in increased $[PSI^+]$ formation (Allen *et al.*, 2007). It should be noted that *N. crassa* contains an ubiquitination system that is similar to the ubiquitination system found in yeast. Therefore proteasomal degradation of UN-24 complexes is possible in *N. crassa* (He *et al.*, 2005). However, the absence of a laddering pattern in immunoblots of Sup35p (and hygunPAp in this study) indicates that these proteins are not poly-ubiquitinated and thus suggests that these complexes are degraded through alternative pathways (Peng *et al.*, 2003; Allen *et al.*, 2007). Hsp70 paralogs have also been observed to facilitate lysosomal/vacuolar degradation of protein aggregates such as Huntington aggregates via autophagy (Ravikumar *et al.*, 2002; Dice, 2007). Autophagic degradation (reviewed in Abeliovich and Klionsky, 2001) involves sequestering cytoplasmic elements in double-membrane

bound vesicles called autophagosomes. Autophagosomes are trafficked to the vacuole where they fuse and release their contents into the vacuolar lumen. Vacuolar hydrolases then degrade the contents previously contained in the autophagosome. Direct evidence that yeast prions are degraded through autophagy is lacking. Interestingly, it has been observed in *P. anserina* that knockouts of key genes involved in autophagy increases the rate and/or frequency of PCD associated with heterokaryon incompatibility. The inference is that autophagy may protect *het*-incompatible cells in *P. anserina* by eliminating toxic complexes formed by *het* products (Pinan-Lucarre *et al.*, 2006). Therefore it is conceivable that autophagy pathways are somehow involved in escape in filamentous fungi.

Our qPCR results showed that at least three loci with Hsp70-like characteristics in *N. crassa*, specifically NCU02075, NCU08693 and NCU01499, are upregulated upon escape from *un-24*-associated incompatibility. We did not examine the transcript levels of the *HSP60* homolog in *N. crassa*. Admittedly, upregulation of a locus does not necessarily indicate that it is involved in escape. For example, prior to escape, *un-24*^{OR/PA} self-incompatibles do not conidiate. However, once these self-incompatible colonies undergo escape, they regain the ability to conidiate. Since NCU02075 has been observed to be involved in conidiation, it is plausible that transcript from this locus is observed to be in higher amounts as post-escape colonies initiate conidiation. It is also conceivable that regulation of escape does not occur at the transcript level but at the post-transcriptional level. As such, Hsp70s may become post-transcriptionally activated and target deleterious UN-24 complexes. In yeast, however, the presence of [*PSI*⁺] aggregates has been shown to promote an increase in Hsp70 transcription (Jung *et al.*, 2000). We are

now testing whether or not upregulation of construct QP1499 (NCU01499) and construct QP2075 (NCU02075) will facilitate escape. Such a result would suggest that, similar to yeast, either one or both of these Hsp70-like proteins alleviates *un-24*^{OR/PA} associated incompatibility likely by forming a hygunPAp-Hsp70p complex.

In conclusion, overexpression of hygunPA in yeast reduces phenotypic abnormalities that are characteristics of low-level expression of the UN-24^{PA} incompatibility domain. This remediation in yeast resembles escape in *N. crassa*. Overexpressed hygunPAp was observed to complex with yeast proteins in the Hsp70 family, Ssa1p and Ssb2p, and possibly Hsp60p. Although formation of these complexes was negatively correlated with incompatibility, it was unclear if their overexpression causes Hsp70-assisted degradation of the hygunPAp-Rnr1p complex or if Hsp70 was sequestering hygunPAp prior to its interaction with Rnr1p. In *N. crassa*, the homolog of Ssb1 (NCU02075), another putative Hsp70-like protein (NCU01499) and a protein that resembles a mitochondrial chaperone (NCU08693) were significantly upregulated in post-escape colonies. Future overexpression of these Hsp70-like genes in *N. crassa* will allow us to test if they are involved in mediating escape from *un-24*-associated incompatibility in *N. crassa*. If Hsp70-like proteins are mediating escape, this would be the first example of a non-genetic mechanism for escape from *het*-associated incompatibility.

**CHAPTER 4: THE ROLE OF SELECTED CYSTEINE AND PROLINE
RESIDUES IN DETERMINING FUNCTION AND STRUCTURE OF THE *un-24*
INCOMPATIBILITY DOMAIN**

INTRODUCTION

un-24–associated incompatibility in *N. crassa* is hypothesized to occur when UN-24^{OR} and UN-24^{PA} form a non-reducible protein complex. The 136 aa segment of the UN-24^{PA} C-terminus (hygunPA) was observed to cause incompatibility when transformed into strains carrying *un-24*^{OR} and to cause aberrant symptoms when expressed in yeast. Low-level expression of this construct in yeast led to yeast with large cell volumes, reduced cell division rates, a decrease in cytoplasmic pH as evident by neutral red staining, a granulated cytoplasm as evident by phase contrast microscopy and a sensitivity to chemical inhibitors (hygB, HU and CW) as evident by lower MIC values (Chapter 2). Size exclusion chromatography and immunoblotting showed that hygFLAGunPAp and Rnr1p form a complex when hygFLAGunPA was expressed at low-levels (Chapter 2). This chapter investigates how alterations to key aa residues in the C-terminus of UN-24^{PA} influence the function and structure of this incompatibility domain from *N. crassa*.

The tertiary structure of the well studied *E. coli* R1 subunit was shown to be similar to that of yeast (Xu *et al.*, 2006). Given that the predicted protein sequence of UN-24 is 85% similar to Rnr1p in *S. cerevisiae* it is hypothesized that the UN-24 also has conserved class I RNR structural features. RNR exists as a heterotetramer consisting of two large subunits (R1) and two small subunits (R2). R1 contains the catalytic site, which performs reduction of ribonucleotides, a specificity site, which dictates the ribonucleotide to be reduced, and an allosteric site, which controls enzyme activity. R2 contains a diiron center which generates a stable tyrosine radical essential for catalysis (Eriksson *et al.*, 1997). For a review of RNR structure and function consult Chapter 1 or Fontecave

(1994). Based on studies done in the *E. coli* system and recent evidence in yeast, the reaction mechanism of RNR involves five conserved cysteines and a radical intermediate (Figure 4.1; Aberg *et al.*, 1989; Mao *et al.*, 1992; Camier *et al.*, 2007). Catalysis is initiated when the tyrosil radical on R2 is transferred to R1 via a long range proton coupled electron transfer (PCET) to generate a thiyl radical within the catalytic site (C439 in *E. coli*) (Uhlen and Eklund, 1994; Stubbe *et al.*, 2003). This thiyl moiety is essential for 2' hydrogen abstraction from the ribose sugar (Mao *et al.*, 1992). The free radical is transferred to the 3' carbon of the ribose sugar. Concurrently, the 2' hydroxyl group is stabilized by two cysteine residues, C225 and C462, located in the R1 catalytic site (Mao *et al.*, 1992). The free radical is then transferred to the 2' carbon of the ribose moiety causing the 2' hydroxyl group to be removed thus creating the dNDP. As a result, a disulphide bond is formed between C225 and C462 and a single water molecule is produced as a byproduct of the reaction (Mao *et al.*, 1992). Once the dNDP is released from the catalytic site, the flexible C-terminus of the adjacent R1 monomer enters the catalytic site to transfer this disulphide bond between C225 and C462 to two R1 C-terminal cysteines, C754 and C759 (Mao *et al.*, 1992; Uhlin and Eklund, 1994). The C-terminus moves out of the catalytic site where the C754/C759 disulphide bond is subsequently reduced by either glutathione reductase or thioredoxin thus reactivating the enzyme (Uhlen and Eklund, 1994).

In 2009, Wellman *et al.* proposed a mechanistic model to explain how the R1 subunit from *N. crassa* (UN-24) also acts as a nonself recognition factor. PA-like incompatibility, which is manifested as very slow growth rate and an aberrant morphology, was evident in *N. crassa* when 136 aa from the UN-24^{PA} C-terminus

Figure 4.1: The reduction of ribonucleotides by class I ribonucleotide reductase.

Catalysis is initiated (1) when a proton from R2 is used to generate a thiyl radical in the R1 catalytic site. As a consequence of 2' OH abstraction from the ribose moiety, a disulphide bond between two cysteine residues is formed in the catalytic site of R1 (2). The flexible C-terminus of the adjacent R1 protein within the tetrameric holoenzyme enters the catalytic site and transfers the disulphide bond out of the catalytic site to cysteine residues located on the C-terminus of the adjacent R1 subunit in the holoenzyme (3). During this process, the proton is transferred out of the catalytic site of R1 and back to the tyrosine radical in R2 via proton coupled electron transport (PCET). Thioredoxin or glutathione reductase remove the disulphide bond from the C-terminus of R1 (4) thus reactivating the holoenzyme. Figure adapted from Jie *et al.* (2003).

(hygunPA) was transformed into C9-2 (*un-24^{OR}*). This construct contained three residues, C907, C918 and C921, (the later two of which are equivalent to C754 and C759 in *E. coli*) that are likely involved in RNR catalytic activity. OR-like incompatibility activity, which is manifested as a > 90% reduction in the number of transformants recovered, was evident when 574 aa of UN-24^{OR} were transformed into *N. crassa* strain C2(2)-1 (*un-24^{PA}*). This construct, called hygunOR(L), contained all five cysteine residues involved in RNR catalytic activity. Site directed mutagenesis revealed the importance of these catalytically active cysteine residues in mediating incompatibility (Figure 4.2). In the naming system presented in this figure, the residues in parentheses after the construct name represented the C-terminal cysteine residues and their substitution residues. For example, the construct hygunPA(CCG) has C921 substituted with a glycine residue while cysteines C907 and C918 remain unchanged. Substitution of any of the three C-terminal cysteine residues (C907, C918, C921) with glycines in the hygunOR(355-929) construct resulted in a loss of incompatibility activity when these constructs were transformed into C2(2)-1 (*un-24^{PA}*). In addition, substitution of C444 with glycine also led to a loss of incompatibility activity. These results showed that OR-like incompatibility required all three C-terminal cysteines as well as the catalytic site cysteine C444. In contrast, substitution of C-terminal cysteines C907 [hygunPA(GCC)] or both C918 and C921 [hygunPA(CGG)] resulted in a loss of incompatibility activity when hygunPA was transformed into C9-2 (*un-24^{OR}*). Interestingly, constructs hygunPA(CGC) and hygunPA(CCG) retained incompatibility activity, demonstrating a redundant role for C918 and C921 in mediating incompatibility (Wellman *et al.*, 2009). Overall, then, incompatibility of both UN-24^{OR} and UN-24^{PA} was associated with C-terminal cysteine residues that are

also required for restoring RNR catalytic potential. Therefore, it was proposed that an aberrant interaction during the restoration of the catalytic site potentiated a non-reducible linkage between the catalytic site of the OR component and the C-terminus of the PA component (Wellman *et al.*, 2009).

A remarkable feature of the C-terminus region of UN-24 is that it is rich in proline residues. The 102 aa incompatibility domain of the C-terminus of UN-24^{PA} has 12 proline residues. Although the last 108 aa region of UN-24^{OR} is not sufficient in itself to cause incompatibility activity, this area also contains a large number (14) of proline residues. This abundance of proline residues may allow for unique structural characteristics of the UN-24 C-terminus. Proline residues impart structural constraints which lead to kinks in the peptide backbone. In addition, proline is the only amino acid that is often observed in biologically relevant quantities in *cis* form (Grathwohl and Wuthrich, 1976; reviewed in Anderotti, 2003). In an unfolded peptide, there is an approximately equal frequency of proline residues in the *cis* and *trans* state however the *trans* form is more energetically favored (Eyles, 2001). In contrast, fully folded proteins contain ~5% of prolines in the *cis* form (Nelson *et al.*, 2006). Proline isomerization, which involves a one step transition through a planar state, is extremely slow and is often the rate limiting step in protein folding (Fischer, 2000; Wedemeyer, 2002). When a 6xHIS epitope was added to the N-terminus of the UN-24^{PA} C-terminus (108 aa) [construct called PA(CCC)] and was expressed in *E. coli*, three unique protein species that resolved at sizes equivalent to ~20 kDa, ~18 kDa and ~16 kDa were observed (Siahbazi, 2008). These sizes were larger than the expected 13 kDa for the PA(CCC) protein. It has been observed that peptides that contain many proline residues migrate

more slowly than expected (Mochida *et al.*, 2002). Therefore, the apparent increase in molecular weight of the PA(CCC) protein bands may be due to the multiple proline residues located in the PA(CCC) protein. One possibility is that the three forms result from differential post-translational modifications, for example phosphorylation of threonine, serine or tyrosine residues (Siahbazi, 2008). It was also observed that site directed mutagenesis of C907, C918 and C921 of the PA(CCC) protein alters the intensity and presence of the ~20 kDa, ~18 kDa and ~16 kDa bands (Siahbazi, 2008). However, an explanation for the existence of the multiple, low mobility (in SDS-PAGE) forms of the PA(CCC) protein has yet to be put forth. In this chapter we aim to determine why these three forms PA(CCC) exist with a specific focus on the role that C907, C918 and C921 as well as selected proline residues play in mediating the ~20 kDa, ~18 kDa and ~16 kDa forms of the PA(CCC) protein.

The objectives of this chapter were, therefore, two-fold. First, we wanted to see whether or not there were parallels in the gel-mobility characteristics and incompatibility function of the PA C-terminal protein. For this, we examined the roles that C907, C918 and C921 of hygunPA play in forming the hygunPAp-Rnr1p complex in yeast. Second, we wanted to verify and further examine what residues in the PA C-terminus protein are important in determining the unusual SDS-PAGE banding patterns of the PA C-terminus protein. We utilize this structure/function information to enhance the preliminary model put forth by Wellman *et al.* (2009) as to how UN-24^{OR}-UN-24^{PA} complexes are formed.

METHODS AND MATERIALS

Microbiology and Molecular Genetic Techniques.

N. crassa strains C2(2)-1 (*un-24^{PA} het-6^{PA} thr-2 a*) and C9-2 (*un-24^{OR} het-6^{OR} thr-2 a*) and yeast strains were cultured as described in Chapter 2. PCR and restriction digests were performed as described in Chapter 2. Ligation into TOPO TA-pCR2.1, plasmid isolation and DNA/RNA quantification were performed as described in Chapter 3. *un-24* derived constructs expressed in yeast are shown in Figure 4.2.

E. coli cultures were grown as described in Chapter 2. Constructs expressed in *E. coli* are shown in Figure 4.3 were derived from plasmid pQE31 (Qiagen), fused in-frame with an N-terminal 6xHIS epitope and under the regulation of the lac operator. These plasmids were originally in *E. coli* strain TOP10 as described in Siahbazi (2008). Plasmids were isolated as previously described (Chapter 2).

For preparing cDNA, *N. crassa* strain C2(2)-1 was inoculated into liquid VMM supplemented with threonine and shaken at 30°C (150 RPM) for 3 days. The mycelium was harvested and RNA was extracted as described in Chapter 3. cDNA was created by using 1 µg of total RNA as described in Chapter 3.

Site Directed Mutagenesis

UN-24^{PA} cysteine residues C907, C918 and C921 were replaced by glycines as described in Wellman *et al.* (2009) or by serines as described below using site directed mutagenesis. A reverse oligonucleotide primer was synthesized that, when used in PCR, replaced the original cysteine codon (TGC) with a serine codon (TCC) as exemplified in Figure 4.4. The sequences of oligonucleotide primers used to create *un-24^{PA}*(SSS) and

Figure 4.2: Constructs expressed in yeast in this chapter. For each construct, key cysteine residues are designated 'C'. C-terminus cysteines replaced with glycines (G) or serines (S) are indicated. FLAG-tagged constructs were created as described in Chapter 2. The hygromycin phosphotransferase protein is represented as a white oval. The ability of each construct to cause an incompatible phenotype when transformed into *N. crassa* strain C9-2 (*un-24^{OR}*) is indicated on the right of each construct as determined in Wellman *et al.* (2009). '+' indicates that the construct causes incompatibility when transformed into C9-2 while '-' indicates that the construct does not cause incompatibility when transformed into C9-2. 'NT' indicates that incompatibility function in *N. crassa* was not tested. The hygromycin phosphotransferase and the FLAG tag are not drawn to scale relative to the amino acid scale bar at the bottom of the figure which refers to the UN-24 residues only. Figure based on work by Wellman *et al.* (2009).

[fig. 4.2]

Figure 4.3: Constructs expressed in *E. coli*. For each construct, key cysteine residues are designated 'C' and C-terminus cysteines replaced with glycines (G) are indicated. 6xHIS-tagged constructs were created as described in Siahbazi (2008). The 6xHIS tag (oval) is not drawn to scale relative to the amino acid scale bar at the bottom of the figure.

Construct

[fig. 4.3]

the hygunPA(SSS) construct are given in Table 4.1. To create *un-24^{PA}*(SSS), PCR was performed using forward primer 6JP100 and reverse primer un-24PA R(SSS) using the following cycling program for 30 cycles: 30 s at 95°C (denaturation), 30 s at 62°C (annealing) and 3 min at 72°C (extension). The resulting product was cloned into TOPO-TA pCR2.1 and sequenced. To create the hygunPA(SSS) construct, hygunPA contained within pCR2.1 was isolated from glycerol stocks. PCR was performed using forward primer HPHF1.P1 and reverse primer un-24PA R(SSS) using 30 cycles of the following program: 30 s at 95°C (denaturation), 30 s at 60°C (annealing) and 1.5 min at 72°C (extension). The resulting products were cloned into TOPO-TA pCR2.1 and sequenced.

To substitute proline residues in the PA(CCC) construct, we introduced point mutations using PCR that altered selected proline residues to either valine or glycine (Figure 4.5). We chose to focus on mutating six specific proline residues; three residues located in the N-terminus of the PA(CCC) construct (P822, P837, P839) and three residues located in the C-terminus of the PA(CCC) construct (P908, P911, P915). In our naming scheme, the proline mutations are indicated in [] after PA(CCC). For example, a construct where P911 and P915 have been changed to valine is represented by the construct PA(CCC)[PPPPVV]. For each primer set, 30 cycles of the following PCR program were used: 30 s at 95°C (denaturation), 30 s at 55°C (annealing) and 30s at 72°C (extension). To create the constructs PA(CCC)[VVVVVV] and PA(CCC)[GGGGGG] we used primers PA(CCC) PtoV For with PA(CCC) PtoV Rev and PA(CCC) PtoG For and PA(CCC) PtoG Rev, respectively. To substitute P908, P911 and P915 with valine we utilized the forward primer PA(CCC) For in conjunction with reverse primers

Table 4.1: Oligonucleotide primers used for site directed mutagenesis of the UN-24^{PA} C-terminus.

Primer	Sequence (5'-3')	~T _m (°C)
6JP100	GTCGAATCCTGTCTACGGC	62
HPHF1.P1	GGAAGATCTGTAACTGATATTGAAGGAGC	67
un-24 ^{PA} R(SSS)	TTAACCGCTGGACATAACCGGAGGAGGGATCGGGCGCGGAAACGTAGGGGGATGAGACTAC	93
PA(CCC) PtoG For	GGCGCTGAAGATTCTGAGACTCTTGTACAGAGTGACAACAGGGGCCGGACTGGT	91
PA(CCC) PtoG Rev	ACCGCTGCACATAACGCAGGAGGAGCCATCGGGCGCGCCAAACGTAGCCGCA	98
PA(CCC) PtoV For	GTCGCTGAAGATTCTGAGACTCTTGTACAGAGTGACAACAGGGTCCGCGTCCTGGT	88
PA(CCC) PtoV Rev	ACCGCTGCACATAACGCAGGAGGAGACATCGGGCGCGCCGACAAACGTAGACGCA	94
PA(CCC) PtoV 1/2P Rev	ACCGCTGCACATAACGCAGGAGGAGACATCGGGCGCGGACAAC	91
PA(CCC) PtoV 3P Rev	ACCGCTGCACATAACGCAGGAGGAGGATCGGGCGCGGAAACGTAGACGCA	95
PA(CCC) For	ACCGCTGCACATAACGCAGG	70

Figure 4.4: Sequence of the C-terminus of UN-24^{PA} showing the codon conversions used to substitute serines in place of C907 (C₁), C918 (C₂) and C921 (C₃). The bold, italicized nucleotides represent the nucleotide substitution while the bold amino acids represent the resulting predicted change.

un-24^{PA} wildtype

GTA GTC TCA TGC CCC TAC GTT CCC GGC GCC GAT CCC TCC TCC
V V S C₁ P Y V P G A D P S S
TGC GTT ATG TGC AGC GGT TAA
C₂ V M C₃ S G Stop

un-24^{PA} (SSS) and *hygunPA*(SSS)

GTA GTC TCA TCC CCC TAC GTT CCC GGC GCC GAT CCC TCC TCC
V V S S P Y V P G A D P S S
TCC GTT ATG TCC AGC GGT TAA
S V M S S G Stop

Figure 4.5: Constructs expressed in *E. coli* that contain proline to glycine or proline to valine substitutions. For each construct, key cysteine residues are designated 'C' and key proline residues (P822, P837, P839, P908, P911 and P915) are indicated by 'P'. Site directed mutagenesis was performed using PCR. In each construct, proline residues that have been substituted by either a glycine or a valine are indicated by 'G' or 'V', respectively. The 6xHIS tag (oval) is not drawn to the scale relative to the amino acid scale bar at the bottom of the figure.

[Fig. 4.5]

PA(CCC) PtoV Rev, PA(CCC) PtoV 3P Rev or PA(CCC) PtoV 2P Rev for constructs PA(CCC)[PPPVVV], PA(CCC)[PPPVPP] and PA(CCC)[PPPPVV], respectively. The resulting products were cloned into pQE30-UA (Qiagen) as described in Chapter 1 and verified by DNA sequencing. The amino acid sequences of each proline to glycine or proline to valine PA(CCC) construct is located in APPENDIX 3.

Ligation into pCB1004

To clone *un-24^{PA}*(SSS) into pCB1004, both pCR2.1 containing *un-24^{PA}*(SSS) and pCB1004 were digested with *Hind*III and *Not*I. Prior to ligation, *un-24^{PA}*(SSS) and pCB1004 were gel purified as described in Chapter 3. The ligation reaction mixture was made following the manufacturer's recommendations (Invitrogen) using a 3 molar excess of *un-24^{PA}*(SSS) relative to pCB1004. Ligation was performed at 4°C for three days using T4 DNA ligase (Invitrogen). The resulting ligation was transformed into TOP10 One-Shot chemi-competent cells (Invitrogen) according to manufacturer's specifications. Transformants were selected at 37°C on LB (Sigma) plates containing 50 µL/mL chloramphenicol and 80 µg/mL of X-gal (Sigma). White colonies were selected, inoculated into LB liquid medium containing 25 µg/mL chloramphenicol and grown overnight at 37°C. Plasmids were isolated and screened for successful ligation by agarose gel analysis after digestion with *Hind*III and *Not*I.

Incompatibility Activity Test

Production of *N. crassa* strain C9-2 spheroplasts and subsequent transformation was performed as described in Chapter 2. Criteria for determination of incompatibility

were as outlined in Chapter 2. As controls, transformation of C9-2 with hyg⁺PA (positive) and with pCB1004 (negative) was performed.

Transformation of Yeast Strains with C to G Replacement Constructs

Yeast was transformed as described in Chapter 2. Primers used to create C to G replacement constructs are listed in Table 4.2. Addition of *GAL1* homologous ends to hyg⁺PA(CGC) and hyg⁺PA(GCC) was done using primers YEASTFRW and YEASTREV as described in Chapter 2. Addition of *GAL1* homologous ends to hyg⁺PA(CCG) was completed using YEASTHYGFWD primer and YEASTUN24REV(CCG). The PCR program used was 30 cycles of: 30 s at 95°C (denaturation), 30 s at 47°C (annealing) and 2 min 30 s at 72°C (extension). C to G replacement constructs containing the FLAG epitope were amplified as described above. PCR product transformation, colony screening and sequencing were performed as described in Chapter 2.

Preparation of *E. coli* Chemically Competent Cells

A stock culture of *E. coli* TOP10F' {F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ⁻ } was obtained and grown overnight at 37°C in 5 mL of LB. The culture was placed at 4°C for 8 hours, one mL was inoculated into 500 mL of LB and the flask was shaken overnight at 18°C and 200 RPM. The culture was chilled on ice for 30 min, centrifuged at 2,600 x g for 10 min at 4°C and resuspended in 40 mL of ice cold IT buffer [10 mM PIPES (pH 6.7), 15 mM CaCl₂, 250 mM KCl and 55 mM MnCl₂]. The suspension was kept on ice for 10 min, centrifuged at 2,500 x g for 10 min at 4°C and

Table 4.2: Oligonucleotide primers used to introduce ends homologous to *GAL1* in C to G replacement constructs. The underlined sequence represents the area of the primer that was identical to the *GAL1* locus of yeast.

Primer	Sequence (5'-3')	$\sim T_m^i$ (°C)
YEASTHYGFWD	TTGTTAAATATACCTCTATACTTTAACGTCAAGGAGAGAAAACTATAATG <u>ACTAAAATGAAAAAGCCTGAACTCACC</u>	62
YEASTUN24REV	TCATATAGACAGCTGCCCAAATGCTGTGTTTAGAGACGATGATAGCATTI <u>TCTAGCTGTGCGGGCTTAACCGCTGCA</u>	75
YEASTUN24REV(CCG)	TCATATAGACAGCTGCCCAAATGCTGTGTTTAGAGACGATGATAGCATTI <u>TCTAGCTGTGCGGGCTTAACCGCTGCC</u>	74

ⁱ estimated based on sequence identical to the *un-24* construct (not underlined)

resuspended in 9.3 mL of ice cold IT buffer containing 0.7 mL of DMSO. Two hundred μ L aliquots of the suspension were distributed into ice cold 1.5 mL eppendorf tubes. The cells were then frozen in an ethanol/dry ice bath and stored at -80°C .

Transformation of *E. coli*

Chemically competent TOP10F' cells were thawed on ice. One μ g of plasmid was incubated with the cells for 30 min. The cells were heat shocked at 42°C for 30 s and cooled on ice for 5 min. Nine hundred μ L of SOC [0.5% yeast extract (BD), 2% Tryptone (Bioshop), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , 20 mM glucose (Bioshop)] pre-warmed to 37°C was added and the cells were incubated at 37°C , 200 RPM for 90 min. The culture was then plated on LB plates containing 100 $\mu\text{g/mL}$ amp and incubated overnight at 37°C .

Minimum Inhibitory Concentration and Neutral Red Staining

The minimum inhibitory concentration (MIC) of yeast stains harboring *un-24*^{PA} cysteine mutant constructs was determined for hygB and HU as described in Chapter 2. Neutral red staining was performed as described in Chapter 2.

Protein Methods

Protein extraction from yeast and *E. coli* was performed as described in Chapter 2. To extract proteins under native conditions, crude protein extracts were kept at 4°C at all times. Protein concentration was determined as described in Chapter 2. SDS-PAGE was performed on protein extracted from yeast as described in Chapter 2. To observe the affects of reducing agents on proteins extracted from *E. coli*, 5 μ g of total protein was

combined with 2 X Laemmli buffer (containing 15.4% DTT and 0.7 M β -mercaptoethanol) and boiled for 10 min prior to loading on to the gel. Under non-reducing conditions, both DTT and β -mercaptoethanol were omitted from the loading buffer. For PAGE under native conditions, 5 μ g of total protein was combined with 2 X Laemmli buffer (that did not contain SDS, DTT and β -mercaptoethanol and was not boiled) and was directly subjected to discontinuous PAGE. PAGE was performed as described in Chapter 2 however SDS was not included in the gel or the running buffer. The running buffer was adjusted to pH 8.0 prior to electrophoresis. Rnrp1 and the 6xHIS epitope were detected using anti-Rnrp1 and anti-6xHIS antibodies, respectively, as described in Chapter 2.

RESULTS

un-24^{PA}(SSS) does not cause self-incompatibility in N. crassa

Previous studies in *N. crassa* suggested that C907, C918, and C921 of UN-24^{PA} were involved in incompatibility function. This inference was based on mutational studies where each of the three cysteines was sequentially and pairwise replaced with glycine (Figure 4.2, Wellman *et al.*, 2009). Due to its small size and its biochemical characteristics that allow it to be found on both the inside and outside of proteins, glycine was originally chosen as the replacement amino acid. However, we wanted to use cysteine to serine replacements as these would likely maintain a protein's three dimensional characteristics since serine is structurally and biochemically more similar to cysteine than is glycine. To confirm that the original choice of glycine did not in itself compromise incompatibility activity, we replaced C907, C918 and C921 of UN-24^{PA} and hyg_{un}PA_{ap} with serines creating *un-24^{PA}(SSS)* and the *hyg_{un}PA(SSS)* construct, respectively. Transformation of C9-2 (*un-24^{OR}*) with either of *un-24^{PA}(SSS)* or *hyg_{un}PA(SSS)* did not produce colonies with the self-incompatible morphology. Furthermore, when the resulting transformants were transferred to fresh VMM, their growth rate and morphology were indistinguishable from C9-2 transformed with pCB1004 (negative control). In comparison, transformation of hyg_{un}PA into C9-2 (positive control) resulted in transformants having a self-incompatible phenotype (e.g. slow growth, aconidial). Since *un-24^{PA}(SSS)* and *hyg_{un}PA(SSS)* transformant colonies did not display PA-like incompatibility, that these cysteine residues are essential for incompatibility function was confirmed. It was not confirmed if *un-24^{PA}(SSS)* could complement *un-24^{ts}*. However, given the essentially nature of the cysteine residues

mutated, one would expect that this protein would not be able to rescue *un-24^{ts}* at the non-permissive temperature.

Incompatibility-like cytology is modulated by C-terminal cysteine residues

As described in Chapter 2, low-level expression of hygunPA in yeast resulted in three distinct aberrant cytologies; an increased frequency of cells with cytoplasmic granulation, an increased frequency of cells with a low cytoplasmic pH as evident by neutral red staining and cells with a large cell volume. The first two of these aberrant yeast cytologies were reminiscent of *het*-associated incompatibility in *N. crassa*. To examine if C907, C918 and C921 mediated incompatibility in yeast as in *N. crassa*, we transformed yeast with the cysteine to glycine replacement constructs hygunPA(GCC), hygunPA(CGC) and hygunPA(CCG). We then compared yeast strains expressing these constructs at low-levels to hygunPA- (positive control) and hyg- (negative control) expressing yeast to determine if expression of these constructs does, or does not, cause *het*-associated neutral red staining in yeast (Figure 4.6). When grown in YPD, $20.5\% \pm 3.8$ of hygunPA-expressing (positive control) and $8.0\% \pm 3.4$ hyg-expressing (negative control) yeast cells have cytoplasms that stain bright red with neutral red. In YPD, the frequency of cells with cytoplasms that stained with neutral red was $2.0\% \pm 2.0$ and $4.6\% \pm 4.6\%$ cells expressing hygunPA(GCC) and hygunPA(CCG), respectively. Therefore, neutral red staining characteristics of strains expressing these two mutant constructs does not significantly differ from cells of the hyg-expressing (negative) control strain ($P \geq 0.25$). An intermediate frequency of yeast cells that had cytoplasms that stained with neutral red was observed in the hygunPA(CGC)-expressing strain ($12.7\% \pm 2.0$). This

Figure 4.6: Neutral red staining of yeast cells grown in YPD that express wildtype and selected cysteine mutants of hygunPA. The mean frequency from five independent trials ($\pm$ SD) of cells that had neutral red staining cytoplasms is based on light microscopy observations of at least 100 cells for each strain. hygunPA(GCC)- and hygunPA(CCG)-expressing cells had frequencies of red staining cells that were indistinguishable from the hyg-expressing (negative) control strain ($P = 0.25$ and $P = 0.75$, respectively, two-tailed t-test). In contrast, an intermediate frequency of cells expressing hygunPA(CGC) had cytoplasms that stained with neutral red that was not significantly different from that of either hygunPA-expressing (positive) or hyg-expressing (negative) control cells ($P = 0.46$ and $P = 0.09$, respectively, two-tailed t-test). Significant differences in the frequency of red staining cells in comparison to the hyg-expressing (negative) control strain are indicated by '+' while '+/-' indicates that the frequency of cells with red staining cytoplasms does not differ from either the hygunPA-(positive) or the hyg-expressing (negative) the control strain.

[Fig. 4.6]

frequency that did not differ from either the hygunPA-expressing (positive control) or hyg-expressing (negative) control yeast strains ($P = 0.46$ and 0.09 , respectively). When grown in YPRaf/Gal, yeast strains expressing wildtype and cysteine mutant constructs showed no significant difference in the frequency of cells with neutral red staining cytoplasm when compared to the hyg-expressing (negative) control strain (not shown). Therefore, independently mutating C907 and C921 resulted in a loss of hygunPA-associated neutral red staining while mutation of C918 resulted in an intermediate level of this staining cytology. This suggests that C907 and C921 are the primary residues responsible for incompatibility-like function in yeast and that C918 plays a redundant or enhancing role in mediating incompatibility.

Increased sensitivity to chemical inhibitors is modulated by C-terminal cysteine residues

Low-level expression of hygunPA in yeast was also associated with increased sensitivity to hygB and HU while overexpression of hygunPA remediated these sensitivities (Chapter 2). To examine if expressing mutant forms of hygunPA in which C907, C918 and C921 had been separately replaced with glycines resulted in a loss of chemical inhibitor sensitivity, we challenged yeast cells expressing hygunPA(GCC), hygunPA(CGC) and hygunPA(CCG) with hygB and HU, separately, in an MIC assay (Figure 4.7). When grown in YPD, cells separately expressing the cysteine mutant constructs had significantly higher hygB MIC values ($25.0 \mu\text{g/mL} \pm 0.0$ for all three strains) compared to hygunPA-expressing (positive control) cells ($15.4 \mu\text{g/mL} \pm 4.86$, $P = 0.017$) but did not differ from the hyg-expressing (negative) control strain ($25.0 \mu\text{g/mL} \pm 0.0$, $P = 1.0$). When grown in YPRaf/Gal, the MIC values increased for the hyg-expressing (negative) control strain and strains separately expressing hygunPA (positive

control), hygunPA(CGC) and hygunPA(CCG) to 3000.0 ± 0.0 $\mu\text{g/mL}$ as expected for constructs controlled by the *GALI* promoter. Interestingly, when grown in YPRaf/Gal hygunPA(GCC)-expressing cells had an MIC of $1500.0 \mu\text{g/mL} \pm 0.0$, which was significantly lower than the other strains tested ($P < 0.001$). It may be argued that this increased sensitivity to hygB is due to a mutation in the *GALI* promoter leading to a decreased rate of hygunPA(GCC) construct expression. However, similar hygB MIC values of $1500.0 \mu\text{g/mL} \pm 0.0$ were observed in three independently derived hygunPA(GCC)- and hygFLAGunPA(GCC)-expressing clones. It is most likely, therefore, that overexpression of the hygunPA(GCC) construct in yeast causes an increased sensitivity to hygB.

Somewhat different trends were observed when these same strains were challenged with HU (Figure 4.8). When grown in YPD, unlike the MIC value for hygunPA-expressing (positive control) strain ($198.6 \text{ mM} \pm 18.11$), cells expressing any of the cysteine mutant constructs had MIC values ($300.0 \text{ mM} \pm 0.0$) that did not differ from the hyg-expressing (negative) control strain ($284.0 \text{ mM} \pm 32.0$, $P = 0.37$). This indicated that replacement of any one of C907, C918 or C921 with glycine resulted in a loss of enhanced sensitivity to HU. When grown in YPRaf/Gal, the MICs for yeast expressing the cysteine mutant constructs ($\text{MIC} = 300.0 \pm 0.0 \text{ mM}$) did not differ from MIC values for hygunPA-expressing (positive control) and the hyg-expressing (negative) control strains ($300.0 \text{ mM} \pm 0.0$, $P = 1.0$). So, unlike the situation with hygromycin resistance, increased expressing of the hygunPA(GCC) construct does not yield enhanced sensitivity to HU.

Figure 4.7: Hygromycin B (hygB) MIC values for yeast cells that express either wildtype or a selected cysteine mutant of hygunPA. When grown in YPD (top graph, low-level expression of construct), hygB MIC values for yeast separately expressing the cysteine mutant constructs were identical to the MIC of the hyg-expressing (negative) control strain ($n = 3$, $P = 1.0$, two-tailed t-test). On YPRaf/Gal, cells expressing hygunPA(CGC) and hygunPA(CCG) had MIC values identical to the hygunPA- (positive control) and hyg- expressing (negative) control strains ($n=3$, $P < 0.001$, two-tailed t-test). However, high-level expression of hygunPA(GCC) resulted in a significantly lower hygB MIC when compared to cells expressing cysteine mutant constructs and the positive and negative control strains ($n = 3$, $P < 0.001$, two-tailed t-test). MICs were recorded as the first concentration at which no growth was observed. Mean MIC values and SD in three independent trials are shown. A significant difference in MIC value from the hyg-expressing (negative) control strain is indicated by *.

Figure 4

Figure 4.8: Hydroxyurea (HU) MIC values for yeast cells expressing wildtype and selected cysteine mutants of hyg_{un}PA. When grown in YPD, HU MICs for yeast cells separately expressing the cysteine mutant constructs were identical to the MIC value of the hyg-expressing (negative) control strain ($n = 3$, $P = 1.0$, two-tailed test). When grown in YPRaf/Gal, yeast cells separately expressing any one of the three mutant constructs resulted in MIC values identical to hyg_{un}PA- (positive control) expressing and the hyg-expressing (negative) control strains ($n = 3$, $P = 1.0$, two-tailed t-test). The MIC was recorded as the first concentration at which no growth was observed. Mean MIC values and SD in three independent trials are shown. A significant difference in MIC value from the hyg-expressing (negative) control strain is indicated by *.

[fig. 4.8]

Overall, then, strains having low-level expression of constructs where any one of C907, C918 and C921 was replaced with glycine were of equivalent sensitivity to chemical inhibitors as was the hyg-expressing negative control strain. This demonstrated that all three cysteine residues were required for an increase in sensitivity to these chemical inhibitors.

Mutation of C907 abolishes the formation of the hygunPAp-Rnr1p complex

Immunoblotting methods were used to examine the effect that separately replacing C907, C918 and C921 with glycine had on the formation of the hygunPAp-Rnr1p complex. In Chapter 2, we observed that when proteins were extracted under non-reducing conditions from the hyg-expressing (negative) control strain, immunoblotting with the anti-Rnr1p antibody yielded two bands at ~100 kDa and ~110 kDa that represented the reduced and oxidized forms of Rnr1p, respectively. The reduced and oxidized forms of Rnr1p were observed to occur at a ratio of ~2:1. In contrast, when proteins were extracted under non-reducing conditions from hygunPA- (positive control) expressing strain, the ratio of Rnr1p in reduced and oxidized forms was ~10:1. In addition, a non-reducible ~153 kDa complex, apparently comprising of hygunPAp and Rnr1p, was observed in these (positive control) proteins. To examine the effects of separately replacing C907, C918 and C921 with glycine on the formation of the hygunPAp-Rnr1p complex, we extracted proteins under both non-reducing (no β -mercaptoethanol or DTT) and reducing (β -mercaptoethanol and DTT) conditions from cells separately expressing hygunPA(GCC), hygunPA(CGC) and hygunPA(CCG) that were grown in YPD. Under non-reducing conditions (Figure 4.10), proteins extracted from hygunPA(GCC)-expressing cells had amounts of Rnr1p in reduced and oxidized

forms at a ratio of ~1:2 whereas hyg_{un}PA(CGC)- and hyg_{un}PA(CCG)-expressing cells had similar or equal amounts of Rnr1p in the reduced and oxidized forms. These three cysteine mutants all differed from the hyg_{un}PA- (positive control) expressing strain that had no or little of Rnr1p in the oxidized form. Under both non-reducing and reducing (not shown) conditions, protein extracts from yeast separately expressing the hyg_{un}PA(CGC) and hyg_{un}PA(CCG) constructs had a unique signal detected slightly above the 150 kDa at a size expected for the hyg_{un}PAp-Rnr1p complex. This signal is of the size expected for a complex between hyg_{un}PA(CGC)p or hyg_{un}PA(CCG)p and Rnr1p, respectively. It is evident from Figure 4.9 that this complex is most pronounced in the hyg_{un}PA-expressing (positive control) strain. This ~153 kDa signal was not observed in proteins extracted under both non-reducing and reducing conditions from cells expressing hyg_{un}PA(GCC), suggesting that hyg_{un}PA(GCC)p and Rnr1p do not form a complex in this strain. Therefore, mutation of C907 fails to form the hyg_{un}PAp-Rnr1p complex, whereas this complex is still visible when either C918 or C921 are substituted with glycine. These results were similar to results from site-directed mutagenesis studies performed previously in *N. crassa* (Figure 4.2). When *N. crassa* C9-2 (*un-24*^{OR}) was transformed with the hyg_{un}PA(GCC) construct, the resulting transformants did not display PA-like incompatibility. However, transformation of C9-2 (*un-24*^{OR}) with hyg_{un}PA constructs hyg_{un}PA(CGC) or hyg_{un}PA(CCG) resulted in transformant colonies with PA-like self-incompatibility (Wellman *et al.*, 2009).

Analysis of hygFLAG_{un}PAp-Ssa1p complex in selected cysteine mutants

It was previously observed that overexpressed hygFLAG_{un}PA protein formed a complex with Ssa1p. Formation of this complex was clearly negatively associated with,

Figure 4.9: Cysteine-to-glycine replacements of C907, C918 or C921 led to varying levels of the detectable hygunPAp-Rnr1p complex. We extracted proteins under non-reducing and reducing (not shown) conditions from hygunPA-, hygunPA(GCC)-, hygunPA(CGC)- and hygunPA(CCG)-expressing strains grown in YPD and immunoblotted with anti-Rnr1p antibodies. Anti-Rnr1p antibodies detected a ratio of reduced (black arrowhead) to oxidized (white arrowhead) forms of Rnr1p at ~1:2 in hygunPA(GCC)-expressing cells. Nearly equal amounts of oxidized and reduced forms of Rnr1p were detected in proteins extracted from hygunPA(CGC)- and hygunPA(CCG)-expressing cells. Interestingly, the ~153 kDa signal (*), that putatively represented a complex between hygunPAp and Rnr1p, was not pronounced in proteins extracted from hygunPA(GCC)-expressing cells while this signal was detected in proteins extracted from hygunPA-, hygunPA(CGC)- and hygunPA(CCG)-expressing cells. Therefore it appears that C907 is required for formation of the hygunPAp-Rnr1p complex in yeast.

Approximately even protein loading across all lanes is indicated by the intensity of non-specific signals produced by the anti-Rnr1p antibody at the bottom of the figure.

Molecular weight markers are presented at the left of the gel.

hyguntPA(CCG)

hyguntPA(CCG)

hyguntPA(GCG)

hyguntPA

200 kDa —
150 kDa —

100 kDa —

Loading Control

[Fig. 4, 9]

and may remediate, the aberrant incompatibility-like phenotypes associated with low-level expression of the C-terminal domain of UN-24^{PA} in yeast (Chapter 3). We examined the incidence and abundance of this complex in yeast that separately overexpressed the wildtype or the FLAG-tagged cysteine mutant hyg_{un}PA constructs. This was done by extracting proteins under reducing conditions from each of these strains when grown in YPRaf/Gal (Figure 4.10). Immunoblotting with anti-FLAG antibodies revealed a signal from all strains that was slightly below the 93 kDa marker, corresponding to the hyg_{un}PAp-Ssa1p complex. A protein slightly larger than 50 kDa, the expected size of the hygFLAG_{un}PAp monomer, was also detected in all strains except the hygFLAG_{un}PA(GCC)-expressing strain. Interestingly, both bands were significantly less intense in the strain expressing the hygFLAG_{un}PA(GCC) mutant construct than in all other strains. This difference in signal intensity was observed in three independent extractions and was not due to a difference in protein loading as Coomassie staining of a duplicate SDS-PAGE gel revealed approximately equal protein loading of all samples in all replicates. The weak signal intensities observed in proteins extracted from hygFLAG_{un}PA(GCC)-expressing cells indicate that hygFLAG_{un}PA(GCC)p might lack stability or may be sequestered, or degraded by Ssa1p at a faster rate compared to other hygFLAG_{un}PA proteins. It was noteworthy that, of all the mutants constructs examined here, the hygFLAG_{un}PA(GCC) mutant had the fewest indicators of incompatibility-like activities. Table 4.3 summarizes and compares how these glycine replacements of C907, C918 and C921 effect hyg_{un}PA-associated phenotypes in *N. crassa* and yeast.

Figure 4.10: Immunoblot of crude protein extracts from cells overexpressing hygFLAGunPA constructs (on YPRaf/Gal) with selected cysteine residues replaced with glycine. Proteins were extracted under reducing conditions from cells separately expressing wildtype and cysteine mutant constructs and immunoblotted with anti-FLAG antibodies. Anti-FLAG antibodies revealed a protein (a) that was slightly below the 93 kDa marker in all samples that corresponded to the expected size of the hygFLAGunPAp-Ssa1p complex. In addition, all but the hygFLAGunPA(GCC) mutant had a signal (b) positioned slightly above the 50 kDa marker, corresponding to the expected size of the hygFLAGunPAp monomer. A third band, positioned between the putative complex and monomer form is of unknown identity but may represent a degraded product of the complex. The putative complex in the hygFLAGunPA(GCC)-expressing strain was ~20 X less intense than that of other strains examined. In addition, the hygFLAGunPA(GCC)p monomer was nearly undetectable. These same anti-FLAG signals were detected in three independent immunoblots. Approximately even protein loading across all lanes is indicated by Coomassie stained proteins at the bottom of the figure. Molecular weight markers are presented at the left of the figure.

[fig. 4.10]

Table 4.3: Summary table showing the effects of low-level expression of UN-24^{PA} C-terminal constructs where C907, C918 and C921 have been replaced separately with glycine. ‘+’ and ‘-’ indicate the presence and absence, respectively, of the cytology or protein complex. +/- indicates an intermediate level of the cytology or protein complex was observed. N/A indicates that the cytology was not assessed.

In <i>N. crassa</i>		In Yeast					
Construct	Incompatibility activity	Neutral Red Staining	Increased Sensitivity to Chemical Inhibitors when Grown on YPD			Protein Complex	
			hygB	HU	CW	hygunPAp:Rnr1p	hygunPAp:Ssa1p
hyg control	-	-	-	-	-	-	-
hygunPA	+	+	+	+	+	+	+
hygunPA(GCC)	-	-	-	-	N/A	-	+/-
hygunPA(CGC)	+	+/-	-	-	N/A	+	+
hygunPA(CCG)	+	-	-	-	N/A	+	+

Inducible expression of the UN-24^{PA} incompatibility domain in E. coli results in three distinct bands with higher than expected molecular weights

To further our understanding of how the C-terminus of UN-24^{PA} causes incompatibility, we added a 6xHIS tag to the N-terminus of the 108 aa of the C-terminus of UN-24^{PA} [construct subsequently called PA(CCC)] for expression in *E. coli*. In addition, we expressed several constructs where C907, C918 and C921 had been replaced with glycine, separately or in combination (Figure 4.3). Previous studies (Siahbazi, 2008) indicated that expression of this PA(CCC) construct in *E. coli* resulted in three protein bands that resolved at sizes of about 20 kDa, 18 kDa and 16 kDa. These sizes were larger than the predicted size of 13 kDa for the PA(CCC) protein. However, the previous study was not performed with an *E. coli* strain that allowed inducible expression of each construct. In addition, loading controls were not included and the presence of signals with weak intensities may therefore have been overlooked in under-loaded samples. To confirm the results obtained in the previous study, we isolated previously created and characterized PA(CCC)-derived constructs and transformed them into *E. coli* strain TOP10F'. This *E. coli* strain allows repression and inducible expression of genes cloned into the multiple cloning site of pQE30-UA. Genes cloned into the multiple cloning site of pQE30-UA are controlled by two tandemly placed lac operators (*lac*^o). In the presence of glucose, the lac repressor (*lac*^I) that is harbored by TOP10F' binds to *lac*^o and prevents transcription of downstream elements. In contrast, in the absence of glucose and in the presence of a lactose analog such as IPTG, *lac*^I is removed from *lac*^o and transcription of downstream elements is facilitated, in this case the PA(CCC)-derived constructs.

To confirm inducible expression of the PA(CCC)-derived constructs, proteins were extracted under reducing conditions from TOP10F' *E. coli* carrying the PA(CCC) construct that had been grown in LB medium containing 2% glucose (without IPTG) or in the presence of IPTG (without glucose) and immunoblotted with anti-6xHIS antibodies (Figure 4.11). Under non-inducing conditions (medium containing 2% glucose, no IPTG), anti-6xHIS signals were not detected on immunoblots. However, immunoblots of protein extracted from cells grown in inducing conditions (medium containing IPTG, no glucose) showed three bands with approximate molecular weights of 20 kDa, 18 kDa and 16 kDa. This indicated that the system allowed for repression and induction of the PA(CCC)-derived constructs. In these immunoblots, both the 20 kDa and 18 kDa bands were of equal intensity while the band at 16 kDa was ~10 X less intense than the 20 kDa and 18 kDa bands. These results were identical to previous results (Siahbazi, 2008). Proteins extracted from *E. coli* harboring the pQE30-UA vector without an insert (negative control) did not produce any signals. This indicated that the signals observed in proteins extracted from *E. coli* expressing PA(CCC) were specific to the 6xHIS tagged PA(CCC) protein.

We next extracted proteins under both non-reducing and reducing conditions from strains grown in the presence of IPTG (without glucose) that expressed UN-24^{PA} C-terminus constructs where C907, C918 and C921 had been replaced separately or in combination with glycine residues (Figure 4.12). Under reducing conditions, replacement of C907 with glycine, [constructs PA(GCC), PA(GGC), PA(GCG)], resulted in the absence of the ~16 kDa band. Replacement of either C918 [PA(CGC)] or C921[PA(CCG)] or both C918 and C921 concurrently [PA(CGG)] with glycine yielded

Figure 4.11: Expression of a 6xHIS-tagged construct of PA(CCC) in *E. coli* strain TOP10F' reveals three proteins at ~20 kDa, ~18 kDa and ~16 kDa on immunoblots probed with anti-6xHIS antibodies. We grew TOP10F' *E. coli* that separately harbored PA(CCC) and pQE30-UA with no insert (negative control) in LB medium containing either glucose without IPTG (non-inducing) or IPTG without glucose (inducing), extracted proteins under reducing conditions and immunoblotted with anti-6xHIS antibodies. Proteins detected by this antibody were seen only under inducing conditions with PA(CCC)-expressing cells. These PA(CCC)-expressing cells produced three signals at ~20 kDa (black arrowhead), ~18 kDa (white arrowhead) and ~16 kDa (open-ended arrowhead). Similar anti-6xHIS signals were detected in three independent immunoblots. Approximately even protein loading across all lanes is indicated by Coomassie stained proteins at the bottom of the figure. Molecular weight markers are presented at the right of the gel.

the same protein banding pattern as seen in PA(CCC). Replacement of all three cysteines [PA(GGG)] resulted in the loss of the ~16 kDa band and a significant decrease in the intensity of the ~18 kDa band. Proteins extracted under non-reducing conditions displayed similar banding patterns as described above. Therefore, similar to previous results (Siahbazi, 2008), replacement of selected cysteine residues in the PA(CCC) protein altered the intensity and the presence or absence of the ~18 kDa and ~16 kDa protein signals.

The PA(CCC) proteins resolve to one band in their native state

These observations of greater than expected molecular weight and multiple forms led us to examine the PA(CCC) protein extracted under native conditions. It had been previously suggested that phosphorylation, which would contribute additional negative charges to the PA(CCC) protein, may explain the multiple protein bands and apparent increase in molecular weight of the PA(CCC) protein following SDS-PAGE (Siahbazi, 2008). Therefore by examining the PA(CCC) protein using native PAGE we could determine if multiple forms of the PA(CCC) protein existed under physiological conditions and if these multiple forms differed in their charge. We extracted native form proteins from *E. coli* separately expressing the wildtype and glycine replacement constructs of PA(CCC). These extracted proteins were subjected to native PAGE at pH 8.3 and immunoblotted with anti-6xHIS antibody (Figure 4.13). This pH level was chosen as the pK_a value of the sulfhydryl R group of cysteine is 8.4 (Voet *et al.*, 1999). At pH 8.3, the sulfhydryl group will be protonated and not confer a charge to the protein. Since native PAGE separates proteins based on size and charge, we wanted to ensure that

Figure 4.12: Cysteine replacement constructs of PA(CCC) have an altered protein banding pattern in SDS-PAGE. The presence and intensity of the ~20 kDa (black arrowhead), ~18 kDa (white arrowhead) and ~16 kDa (open-ended arrowhead) bands were monitored by examining proteins expressed in *E. coli* and extracted under reducing and non-reducing conditions. Under reducing conditions, replacement of C907 [PA(GCC), PA(GGC) and PA(GCG)] with glycine resulted in the absence of the ~16 kDa protein band. Replacement of all three cysteines [PA(GGG)] with glycines resulted in the absence of the ~16 kDa band and a significant reduction in intensity of the ~18 kDa band. Replacement of the terminal two cysteines (C918 and C921), separately or together resulted in protein banding patterns that were similar to those of the PA(CCC) wildtype from. When proteins were extracted under non-reducing conditions, the banding patterns remained the same as described above. Comparable anti-6xHIS signals were detected in all three independent immunoblots performed. Approximately even protein loading across all lanes is indicated by Coomassie stained proteins at the bottom of the figure. Molecular weight markers are presented at the right of the gel.

[fig. 4.2]

deprotonation of C907, C918 and/or C921 was not contributing to differences in banding patterns of PA(CCC) wildtype and glycine replacement proteins. If we had chosen a pH greater than 8.4, the sulfhydryl groups of the three cysteine residues in PA(CCC) would become deprotonated and confer a 3^- charge to the protein. Similarly, deprotonation of the sulfhydryl groups in the PA(GCC) and PA(GGC) proteins would confer charges of 2^- and 1^- , respectively. By running our native PAGE at pH 8.3, we avoided these charge differences between PA(CCC) wildtype and replacement proteins so that any differences in banding pattern would be a result of difference in charge not directly related to replacement of selected cysteines residues with glycine. Under native conditions, proteins extracted from cells expressing PA(CCC) showed one intense signal. This signal putatively represented the PA(CCC) protein. Proteins extracted from *E. coli* separately expressing each of the seven cysteine replacement constructs yielded a single intense signal at the same area as the signal produced from proteins extracted from PA(CCC)-expressing cells. The presence of this intense signal for wildtype and cysteine mutant PA(CCC) constructs suggest that, under physiological conditions, PA(CCC) proteins do not differ in charge. Therefore, post-translational modifications, such as phosphorylation, do not likely contribute to the multiple PA(CCC) protein species.

Mutation of selected proline residues results in fewer protein bands and increased mobility of PA(CCC) and related proteins based on SDS-PAGE

We replaced selected proline residues with either valine or glycine to examine their role in the unusual SDS-PAGE mobility characteristics of the PA(CCC) protein. Since the C-terminus of UN-24^{PA} is hypothesized to have a labile structure, we chose two replacement residues with different chemical properties. We chose glycine as a

Figure 4.13: Native PAGE immunoblot of proteins extracted from *E. coli* separately expressing wildtype and cysteine replacement PA(CCC) constructs. One predominant signal occurred at a position just above the 37 kDa marker for all strains following native PAGE. Note that because of the native PAGE conditions used, these MW markers (shown at right) can not be used to accurately estimate protein sizes. Native PAGE experiments indicated that under physiological conditions, there were no differences in gel mobility of wildtype and cysteine replacement PA(CCC) proteins. Comparable anti-6xHIS signals were detected in two independent immunoblots performed. Approximately even protein loading across all lanes is indicated by Coomassie stained proteins at the bottom of the figure.

[fig. 4, 13]

replacement residue due to its small size and its biochemical characteristics that allow it to be found on the outside or inside of a protein. Valine was chosen because it has a similar molecular weight to proline and is hydrophobic. We expressed PA(CCC)-derived constructs containing proline substitutions in *E. coli*, extracted protein under reducing conditions and immunoblotted using anti-6xHIS antibodies (Figure 4.14). In each case, replacement of selected proline residues resulted in two signals that were separated by ~0.5 kDa. When P822, P837, P839, P908, P911 and P915 were replaced with either valine {PA(CCC)[VVVVVV]} or glycine {PA(CCC)[GGGGGG]} (not shown), these two signals were detected at a molecular weight of ~17 kDa. This indicated that our selection of either valine or glycine as the replacement residue did not, in itself, alter the mobility of these proteins. Replacement of P822, P837 and P839 {PA(CCC)[VVVPPP]} or P908, P911 and P915 {PA(CCC)[PPPVVV]} with valine resulted in two signals that resolved to a molecular weight of ~17 kDa and ~18 kDa, respectively. When P911 and P915 were concurrently replaced with valine {PA(CCC)[PPPPVV]}, two signals at ~18 kDa were detected. Similarly, when P908 was replaced valine {PA(CCC)[PPPVPP]}, two signals at ~18 kDa were detected. Similar trends were observed when proteins were extracted under non-reducing conditions. Overall, the replacement of the selected proline residues resulted in an increased SDS-PAGE mobility of the protein and resolution of two electrophoretic forms that are separated by ~0.5 kDa. This suggests that these proline residues are responsible for both the apparent increase in molecular weight of the PA(CCC) protein and for the multiple banding forms of the PA(CCC) protein.

Figure 4.14: Replacement of selected proline residues resulted in an altered mobility of the PA(CCC) protein. We extracted proteins from *E. coli* that expressed PA(CCC) containing selected proline residues that were substituted for valine, extracted proteins under reducing and non-reducing conditions and immunoblotted with anti-6xHIS antibodies. As previously observed, when extracted under both non-reducing and reducing conditions, the non-mutated PA(CCC) protein resolved into three bands with molecular weights of ~20 kDa (black arrowhead), ~18 kDa (white arrowhead) and ~16 kDa (open-ended arrowhead). When extracted under reducing and non-reducing conditions, proteins from proline-replacement constructs were resolved into two major bands separated by ~0.5 kDa and with sizes ranging from ~17 kDa to ~18 kDa. Overall, these proline residues replacements resulted in an increased SDS-PAGE gel mobility of the PA(CCC) protein. Comparable anti-6xHIS signals were detected in three independent immunoblots. Approximately even protein loading across all lanes is indicated by Coomassie stained proteins at the bottom of the figure. Molecular weight markers are presented at right.

[fig. 4.14]

DISCUSSION

Previous studies performed in *N. crassa* suggested that catalytically important cysteine residues are required for the formation of the UN-24^{OR}-UN-24^{PA} complex. In UN-24^{PA}, three cysteine residues, C907, C918 and C921, were implicated in mediating incompatibility. It was proposed that these residues formed a disulphide bond with cysteine residues located in the catalytic site of UN-24^{OR} and potentiated the formation of a non-reducible UN-24^{OR}-UN-24^{PA} complex. In *N. crassa*, separate replacement of C907, C918 and C921 in the hygunPA protein with glycine altered hygunPA-associated incompatibility (Wellman *et al.*, 2009). It was observed that replacement of C907 or both C918 and C921 with glycine in the hygunPA protein abolished incompatibility activity. This suggested that C907 was required for PA-like incompatibility and that C918 and C921 have a redundant, but necessary role in incompatibility function. We hypothesized that these same cysteine residues would be important in hygunPA function in yeast. If correct, we could further enhance our understanding of how catalytically important cysteine residues allow hygunPAp to complex with Rnr1p in both yeast and in *N. crassa*.

We found replacement of C907 with glycine resulted in complete loss of hygunPA-associated incompatibility function in yeast. Separate replacements of C918 and C921 with glycine resulted in partial loss of hygunPA-associated incompatibility function. Overall, similar to the situation in *N. crassa*, these results suggested that C907 is required for hygunPA-associated incompatibility in yeast while C918 and C921 have an enhancing role in mediating incompatibility. Interestingly, replacement of C918 or C921 led to intermediate levels of incompatibility in yeast. Although proteins extracted from hygunPA(CGC)- and hygunPA(CCG)-expressing yeast cells revealed the presence

of the hygunPAp-Rnr1p complex, only the hygunPA(CGC) expressing strain had an increased frequency of neutral red staining cytoplasm relative to the hyg-expressing (negative) control strain. Furthermore, neither hygunPA(CGC)- nor hygunPA(CCG)-expressing yeast displayed an increased sensitivity to hygB and HU. These observations closely parallel those with *N. crassa* but the subtle phenotypic differences associated with expressing these constructs in yeast indicate that additional resolution of the protein interactions can be obtained from the yeast system.

Site directed mutagenesis has led to varying levels of incompatibility in other *het* systems as well as systems where the formation of an aberrant complex confers a deleterious phenotype. In the *het-s* system from *P. anserina*, removal of HET-s residues 83 to 120 resulted in an intermediate level of incompatibility as determined through barrage testing. However, this construct retains the ability to convert into the [Het-s] prion form and to confer *het*-associated incompatibility when present in cells containing alternate *het-s* allele, *het-S* (Coustou *et al.*, 1999). Similarly, point mutations in *SUP35* lead to varying levels of $[PSI^+]$ aggregate formation and thus alter the degree to which nonsense suppression, the phenotype conferred by $[PSI^+]$ aggregation, is observed in yeast cells (Derkatch *et al.*, 1999; Zhou *et al.*, 1999; Chien *et al.*, 2003; Kryndushkin *et al.*, 2003).

Disulphide bonding that leads to non-reducible protein complexes in the form of aggregates has been studied in the mammalian prion PrP^C and in the onset of Alzheimer's disease (Welker *et al.*, 2001; Cumming and Schubert, 2005). It has been suggested that reorganization of an intramolecular disulphide bond to an intermolecular disulphide bond incorporates PrP^C (non-prion form) into PrP^{Sc} (prion form) oligomers and facilitates PrP^C

to PrP^{Sc} conversion (Welker *et al.*, 2001). In the mammalian PrP^C protein, NMR studies have detected an intramolecular disulphide bond located between residues C179 and C214. In PrP^{Sc} oligomers, this disulphide bond is converted into an intermolecular bond between residues C179 and C214 between adjacent PrP proteins (Knaus *et al.*, 2001). In addition, the presence of thiol blocking agents, chemicals that prevent the formation of disulphide bonds, inhibit the conversion of PrP^C into PrP^{Sc} *in vitro* (Lucassen *et al.*, 2003). It has been suggested that this intermolecular disulphide bond leads to domain swapping, causing PrP^C to convert to PrP^{Sc} (Cho *et al.*, 2005). Domain swapping is a three dimensional rearrangement where domains from two proteins intertwine and form a stable complex. However, disulphide bonds are not present in the final aggregate (Welker *et al.*, 2003). Therefore, similar to the proposed model of how the UN-24^{OR}-UN-24^{PA} and hyg_{un}PAp-Rnr1p complexes form in *N. crassa* and in yeast, respectively, intermolecular disulphide bonding appears to initiate formation of a non-reducible complex.

It was previously observed that overexpression of the hygFLAG_{un}PA construct resulted in a complex that formed between hygFLAG_{un}PAp and Ssa1p (Chapter 3). When overexpressed, we observed that hygFLAG_{un}PA(CGC)p and hygFLAG_{un}PA(CCG)p formed a complex with Ssa1p. The intensity of both of these complexes was identical to the intensity of hygFLAG_{un}PAp-Ssa1p complex. Interestingly, overexpression of hygFLAG_{un}PA(GCC), which has no apparent hyg_{un}PA-associated incompatibility in yeast, nor in *N. crassa* strain C9-2, led to a weak hygFLAG_{un}PA(GCC)p-Ssa1p signal and to a nearly undetectable hygFLAG_{un}PA(GCC)p monomer signal. This suggests that Ssa1p is targeting a specific protein domain or structure found in the C-terminus of UN-24^{PA} and is not necessarily

responding to the ability of the hygunPA protein to complex with Rnr1p. This same type of interaction pattern has been observed in the $[PSI^+]$ prion. In the $[PSI^+]$ prion, Ssa1p interacts strongly with the prion form of Sup35p but interacts with non-prion forming Sup35p variants at significantly lower, yet detectable levels (Bagrianstev *et al.*, 2008). Therefore it is plausible that, similar to $[PSI^+]$, Ssa1p recognizes the potential ability of the hygFLAGunPA(GCC) protein to form complexes or aggregates and thus sequesters or targets it for degradation.

To enhance our understanding of the role that cysteines C907, C918 and C921 and selected proline residues play in hygunPA-associated incompatibility and in the structure of the UN-24^{PA} C-terminus, we expressed 108 aa of the UN-24^{PA} C-terminus in *E. coli*. We observed that replacing selected cysteine residues with glycine resulted in changes to the PA(CCC) protein banding pattern on SDS-PAGE gels. Significantly, under both reducing conditions and non-reducing conditions in all cases, replacement of C907 resulted in the absence of the ~16 kDa band. These same 907 substitutions are also of note in having a loss of incompatibility function in both yeast and *N. crassa* (Table 4.3). This may suggest that the ~16 kDa band represents a structurally ‘active’ form of the UN-24^{PA} incompatibility domain while the ~20 kDa and ~18 kDa proteins may represent folding intermediates that are inactive for incompatibility function. The only exception to this is the PA(CGG) protein; it does not have incompatibility activity when transformed into *N. crassa* strain C9-2, but produces a ~16 kDa band. This may indicate that, although this protein can structurally change into its active (~16 kDa) form, due to the concurrent mutations of C918 and C921 it cannot interact with the active site of UN-24^{OR} in *N. crassa*. Interestingly, expression of the PA C-terminus in *E. coli* did not

produce any immediately observable negative effects. This may indicate that the PA C-terminus is unable to interact with the *E. coli* R1.

The C-terminus of UN-24^{PA} contains a relatively high number (12) of proline residues. Similar to previous studies (Mochida *et al.*, 2002) we observed that these abundant proline residues contributed to a low mobility during SDS-PAGE. When we replaced selected proline residues with valine or glycine, we observed increased electrophoretic mobility of the PA(CCC)-derived proteins. This suggests that the unique structural constraints imparted by these proline residues are causing the PA(CCC) protein to migrate more slowly in the gel. Presumably these prolines cause kinks in the PA(CCC) peptide that do not allow it to run as a linear protein even when treated with SDS. In addition, mutation of selected proline residues resulted in an apparent reduction in the number of protein bands on SDS-PAGE gels. This could indicate that proline isomerization or other structural constraints associated with these proline residues leads to formation of the ~20 kDa, ~18 kDa and ~16 kDa forms of the PA(CCC) protein. Interestingly, proline residues have been observed to contribute to the formation of non-reducible complexes. Proline isomerization has been shown to initiate domain swapping and protein aggregation (Guo and Eisenberg, 2006). The amyloidogenic protein stefin B oligomerizes through domain swapping which is dependent upon *trans* to *cis* isomerization of highly a conserved proline residue (Jenko Kokalj *et al.*, 2007; Morgan *et al.*, 2008; Skerget *et al.*, 2009). In addition, a *cis* to *trans* proline isomerization initiates amyloid formation in the naturally amyloidogenic protein β_2 -microglobulin (Liang *et al.*, 2008; Eichner and Radford, 2009). When proline residues occur next to a cysteine residue involved in a disulphide bond, *cis/trans* isomerization of the proline residue

increases 12 fold (Shi *et al.*, 2004). The disulphide bond stabilizes *cis* proline such that it occurs more often in the polypeptide (Shi *et al.*, 2004, 2006). Although very hypothetical at this point, interplay between proline residues and C907, C918 and C921 of the UN-24^{PA} domain may be responsible for the formation of the UN-24^{OR}-UN-24^{PA} complex *N. crassa*. In *N. crassa*, the UN-24^{OR}-UN-24^{PA} complex is hypothesized to be potentiated by a disulphide bond between cysteine residues found in the UN-24^{PA} C-terminus and the UN-24^{OR} catalytic site. It is possible that after the disulphide bond is formed between C444 of UN-24^{OR} and one of the C-terminal cysteines in UN-24^{PA} selected proline residues located in the UN-24^{PA} C-terminus undergo isomerization. This change in structure may either cause a change in shape of the UN-24^{PA} C-terminus or may lead to domain swapping between the UN-24^{PA} C-terminus and the catalytic pocket of UN-24^{OR}. Notable in this respect C907 sits next to a proline residue (P908, see Figure 4.4). Evaluating incompatibility function of UN-24^{PA} constructs with proline substitutions in *N. crassa* may provide an initial test of this hypothesis of cysteine-proline interactions.

In conclusion, similar to the *N. crassa* system, C907 of UN-24^{PA} is required while C918 and C921 play enhancing roles for hygPA-associated incompatibility in yeast. This research initiates a bio-physical characterization of the UN-24^{PA} C-terminus and suggests that the non-reducible linkage formed between UN-24^{OR} and UN-24^{PA} in *N. crassa* could be initiated by disulphide bonding but may be non-reducibly complexed as a result of proline isomerization and/or domain swapping. Further studies that examine how the UN-24^{OR} and UN-24^{PA} subunits interact to form a non-reducible complex could lead to the first bio-physical characterization of the mechanisms by which two alternate

allelic protein forms of a heterokaryon incompatibility factor in *N. crassa* complex to initiate heterokaryon incompatibility and PCD.

SUMMARY AND CONCLUSIONS

Adversity is like a strong wind. It tears away from us all but the things that cannot be torn, so that we see ourselves as we really are.

- Arthur Golden

This thesis examined the expression of the incompatibility domain of UN-24^{PA} from *N. crassa* in yeast and in *E. coli*. We identified incompatibility-like affects of expressing this factor when expressed at low-levels in yeast and initiated a biophysical characterization of the incompatibility domain.

In Chapter 2, we observed that low-level expression of the UN-24^{PA} incompatibility domain in yeast resulted in several incompatibility-like cytologies, including an increased frequency of cells with a highly granulated cytoplasm, an increased frequency of cells that stained bright red with the stain neutral red and a slower than wildtype growth rate. These phenotypes correlated with the presence of a non-reducible hygunPAp-Rnr1p complex. Furthermore, we observed that hygunPA interacts reducibly with Leu1p, a protein that functions in leucine biosynthesis and has domains similar to aconitase. Overall, this study revealed that the UN-24^{PA} incompatibility domain of *N. crassa* functions in yeast. It appears that a similar incompatibility mechanism, a complex formed between Rnr1p or UN-24^{OR} and the UN-24^{PA} incompatibility domain, occurs in both yeast and in *N. crassa*. These results lend support to a mechanistic model for incompatibility activity by UN-24 put forth by Wellman *et al.* (2009). This study demonstrates that fungal nonself recognition systems can be expressed and characterized in yeast. As such, we can now utilize the yeast system to study nonself recognition systems from filamentous fungi that have yet to be characterized at the molecular level (e.g. *het-5* from *N. crassa*). Furthermore, due to the availability of overexpression and knock-out libraries, as well as wide scale genomic and proteomic resources in yeast, nonself recognition systems from filamentous fungi may be transferred to yeast to increase our understanding of how incompatibility from these systems is mediated. For

example, in the *het-c* system from *N. crassa*, it has been postulated that homodimeric and heterodimeric complexes formed between alternate allelic forms of HET-C and PIN-C (reviewed in Chapter 1) initiates PCD itself or activates downstream effectors resulting in PCD. Since yeast and filamentous fungi share a large number of genes involved in PCD (Pal *et al.*, 2007) successful expression and characterization of this system in yeast could enhance our understanding of how these complexes cause PCD. Furthermore, expression of these systems in yeast could identify new interaction partners, as was the case in our study where Leu1p was observed to interact with hygunPAp. Although we postulate that the reason for this interaction is that hygunPA interacts with the aconitase-like domain shared by both HET-6 and Leu1p, additional novel interactions partners, such as those involved in initiating the incompatibility-like cytologies, should be identified in the future. Although the current study initiates the study of *un-24* in yeast, abundant information can still be gained from further study of this system. For example, how does low-level expression of hygunPA cause acidification of the cytoplasm? Does hygunPAp interact with vATPase subunits leading to a loss of vATPase activity? Or does the hygunPAp-Rnr1p complex trigger acidification of the cytoplasm and if so how? Since vacuolar rupturing and acidification of the cytoplasm are hallmark cytological traits of *het*-associated incompatibility in filamentous fungi, identifying how nonself recognition protein complexes initiate this cytology in yeast would have broad applications in the study of heterokaryon incompatibility in general.

Interestingly, high-level expression of the PA incompatibility domain resulted in a loss of incompatibility activity in yeast. In Chapter 3, we observed that the vast majority of overexpressed hygunPAp non-reducibly complexes with the Hsp70 paralog Ssa1p and

in the absence of *SSA1*, *Ssb2p* and/or *Hsp60p*. This complex was observed to be negatively correlated with the onset of incompatibility-like cytologies in yeast and suggests that these complexes remediate the effects of the UN-24^{PA} domain. Using quantitative PCR, we observed that three genes (NCU01499, NCU02075 and NCU08693) with Hsp70-like characteristics are upregulated upon escape from hygPA-associated incompatibility in *N. crassa*. As such, we have potentially identified the first non-genetic mechanism of escape from incompatibility in filamentous fungi. The results presented in Chapter 3 are consistent with previous studies where heat shock proteins have been implicated in propagating and antagonizing protein complexes, prions and aggregates in other organisms, including in yeast. We have initiated a study of two of the three aforementioned Hsp70-like genes from *N. crassa* that are upregulated in post-escape colonies by separately cloning each gene and placing each one under the regulation of a quinic acid-inducible promoter. We hypothesize that upregulation of these genes in an *N. crassa un-24*^{OR/PA} self-incompatible colony will result in induction of escape. An alternative approach to studying the role of these proteins in causing escape would be to generate *un-24*^{OR/PA} self-incompatible colonies in strains where NCU01499, NCU02075 and NCU08693 have been separately knocked out. We would expect that self-incompatible transformants generated in these strains would not escape. However, studies involving the yeast [*PSI*⁺] prion have indicated that numerous heat shock proteins are involved in antagonizing [*PSI*⁺] propagation and that their roles can change depending on the presence or absence and quantity of other heat shock proteins in the cell (Shorter and Lindquist, 2008). Therefore, it is possible that we are underestimating the complexity of the roles that these Hsp70-like proteins may play in mediating escape from

un-24-associated incompatibility in *N. crassa* and that simple overexpression or deletion studies as described above will not generate the hypothesized results. Further study of this system would also enhance the understanding of how Hsp70 proteins complex to and sequester or degrade proteins that lack a traditional Hsp70 binding motif. Similar to the UN-24^{PA} C-terminus, the prion forming proteins Sup35p and Ure2p ([*PSI*⁺] and [*URE*], respectively) from yeast do not contain a putative Hsp70 binding motif (Bagrianstev *et al.*, 2008). Therefore, if we were able to identify the sequence of the UN-24^{PA} C-terminus that Hsp70 was recognizing and binding to, we could examine the sequences of Sup35p and Ure2p for similar motifs.

In Chapter 4, we initiate a biophysical characterization of the incompatibility domain of UN-24^{PA}. First, we expressed at low-levels hygunPA constructs with cysteine substitutions in yeast and examine the presence or absence of incompatibility-like cytologies. We observed that substitution of C907 most convincingly abolished the incompatibility activity of hygunPA in yeast. This paralleled previous studies in *N. crassa* where C907 of the hygunPA construct had been substituted for glycine did not carry PA-like incompatibility. Also in Chapter 4, we expressed the UN-24^{PA} domain in *E. coli* and examined the effect of substituting cysteine and selected proline residues on the mobility of the UN-24^{PA} domain in SDS-PAGE. We observed that substitution of C907 results in a loss of the ~16 kDa protein band while substitution of selected proline residues resulted in increased SDS-PAGE mobility, overall, of the PA incompatibility domain. Based on these results from both yeast and *E. coli*, we postulate that the non-reducible UN-24^{OR}-UN-24^{PA} complex observed in *un-24*^{OR/PA} self-incompatible colonies and the hygunPAp-Rnr1p observed in yeast is potentiated by a disulphide bond between

the C-terminus of the UN-24^{PA} incompatibility domain and catalytic site of UN-24^{OR} and of Rnr1p in *N. crassa* and yeast, respectively. We speculate that due to this disulphide bond, increased isomerization of one or more proline residues in the UN-24^{PA} C-terminus causes domain swapping to occur between the UN-24^{PA} incompatibility domain and the UN-24^{OR} (*N. crassa*) or Rnr1p (yeast) catalytic sites. Admittedly, this hypothesis would require numerous studies to be substantiated. Initially, PA constructs that contain selected proline substitutions could be transformed into C9-2 to examine if these constructs retain the ability to cause PA-like incompatibility. UV circular dichroism analysis of the UN-24^{PA} C-terminus may reveal details regarding the structure of this domain. In addition, rotating frame nuclear Overhauser effect spectroscopy could detect proline isomerization and its effect on the formation of the UN-24 complex (Shi *et al.*, 2006). Ultimately, purification of the UN-24^{OR}-UN-24^{PA} and/or the hygunPAP-Rnr1p complexes from *N. crassa* and yeast, respectively, would be desirable. Purification of this complex could allow structural studies, such as x-ray crystallography, to be performed that could demonstrate the role of domain swapping in non-reducibly linking UN-24^{OR} and Rnr1p and the UN-24^{PA} incompatibility domain in *N. crassa* and yeast, respectively. Purification of this complex *in vivo* has been elusive, likely because the complex appears to be insoluble. Overall, the formation of this UN-24^{OR}-UN-24^{PA} complex in *N. crassa* and the hygunPAP-Rnr1p complex in yeast is likely highly complex and the hypothesis put forth in this chapter likely explains some, but not all, of the intricate mechanisms involved in linking these protein complexes.

The work in this thesis has described how yeast can be used to gain insight into nonself recognition systems from filamentous fungi, has provided leads into what is

possibly the first non-genetic mechanism by which escape from incompatibility is mediated, and presents a new model for how UN-24^{OR} and the UN-24^{PA} incompatibility domain form a non-reducible complex in *N. crassa*. As I reflect on my time spent as a PhD student, I am grateful for having the opportunity to work on this incompatibility system. It has shown me how complex and simple both science and life can be.

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APPENDIX 1

Standard relating cells/mL and OD₆₀₀ for curves for hyg- (squares, solid line) and hygunPA- (diamonds, hatched line) expressing yeast strains grown in YPD. Strains were grown in YPD liquid medium, one mL aliquots were removed at indicated times and OD₆₀₀ was measured in a spectrophotometer. The aliquot was diluted and plated on YPD plates. The number of viable colonies on these plates was used to estimate the number of cells/mL of the original culture at each OD₆₀₀ value. The equation of both lines as well as their respective R² values are indicated.

APPENDIX 2

Unrooted neighbor joining tree of yeast Hsp70 paralogs and Hsp60 that were observed to interact with hygFLAGunPA [Ssa1p, Ssb2p and Hsp70] and the 12 Hsp70-like proteins in *N. crassa*. Ssa1p is most closely related to NCU09602 while Ssb2p is most closely related to NCU02075. ClustalW alignments (Larking *et al.*, 2007) were done of protein sequences and the neighbor joining tree was constructed using NJPlot (Perrière and Gouy, 1996) using the method described in Saitou and Nei (1987). ‘*’ indicates that the Hsp70-like protein in from *N. crassa* was upregulated upon escape from *un-24*-associated incompatibility.

[app. 2]

APPENDIX 3

The predicted full amino acid sequences of PA C-terminal proteins containing an N-terminal 6xHIS-tag and proline to glycine or valine replacement mutations. The mutated residues in each construct are indicated in bold. The underline residues represent residues contained within the construct that are found in the vector.

PA(CCC)[PPPPPP]

M R G S H H H H H T D P P A E D S E T L V Q S D N R P R P L
V S P A K N A G F K A G L P E P E S P K A L A T D P I V K T E
A I S S R E L E M K E G Q S E D K D E E S K E R E G D I Y S D E
V V S C P Y V P G A D P S S C V M C S G V D L Q P S L I S -

PA(CCC)[GGGGGG]

M R G S H H H H H G S H V I S S I A S G A E D S E T L V Q S
D N R G R G L V L V S P A K N A G F K A D L P E P E S P K A L
A T D P I V K T E A I S S R E L E M K E G Q S E D K D E E S K E
R E G D I Y S D E V V S C **G Y V G G A D G S S C V M C S G** E A
I E E I -

PA(CCC)[VVVVVVV]

M R G S H H H H H G S H V I S S I A S V A E D S E T L V Q S
 D N R V R V L V L V S P A K N A G F K A D L P E P E S P K A L
 A T D P I V K T E A I S S R E L E M K E G Q S E D K D E E S K E
 R E G D I Y S D E V V S C V Y V V G A D V S S C V M C S G E A I
E E I -

PA(CCC)[VVVPPP]

M R G S H H H H H G S H V I S S I A S V A E D S E T L V Q S
 D N R V R V L V L V S P A K N A G F K A D L P E P E S P K A L
 A T D P I V K T E A I S S R E L E M K E G Q S E D K D E E S K E
 R E G D I Y S D E V V S C P Y V P G A D P S S C V M C S G E A I
E E I -

PA(CCC)[PPPVVV]

M R G S H H H H H G S H V I S S I A S P A E D S E T L V Q S D
 N R P R P L V L V S P A K N A G F K A D L P E P E S P K A L A T
 D P I V K T E A I S S R E L E M K E G Q S E D K D E E S K E R E
 G D I Y S D E V V S C V Y V V G A D V S S C V M C S G E A I E
E I -

PA(CCC)[PPPVPP]

M R G S H H H H H G S H V I S S I A S P A E D S E T L V Q S D
 N R P R P L V L V S P A K N A G F K A D L P E P E S P K A L A T
 D P I V K T E A I S S R E L E M K E G Q S E D K D E E S K E R E
 G D I Y S D E V V S C V Y V P G A D P S S C V M C S G E A I E E
I -

PA(CCC)[PPPPVV]

M R G S H H H H H G S H V I S S I A S P A E D S E T L V Q S D
 N R P R P L V L V S P A K N A G F K A D L P E P E S P K A L A T
 D P I V K T E A I S S R E L E M K E G Q S E D K D E E S K E R E
 G D I Y S D E V V S C P Y V V G A D V S S C V M C S G E A I E E
I -