**Incompatibility activity and expression of ribonucleotide reductase in
*Neurospora crassa***

A Thesis

Presented to

The Faculty of Graduate Studies

Of

Carleton University

by

Leila Haidari

In partial fulfilment of requirements

for the degree of

Master of Science

August, 2002

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Incompatibility activity and expression of ribonucleotide reductase in *Neurospora crassa*

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Abstract

Incompatibility activity and expression of ribonucleotide reductase in *Neurospora crassa*

In filamentous fungi a heterokaryon is formed when genetically distinct nuclei occur in the same cell. In *Neurospora crassa* 10 different heterokaryon incompatibility loci and the mating-type locus regulate heterokaryon formation. A difference at any one of these incompatibility loci leads to reduced growth and/or cell death of the heterokaryon. Two closely linked genes, *un-24*⁺ and *het-6*, at the *het-6* locus appear to have heterokaryon incompatibility activity in transformation-based assays. The heterokaryon incompatibility function of *un-24* is surprising since the gene encodes the large subunit of a type I ribonucleotide reductase (RNR), an enzyme found across kingdoms that converts ribonucleotides to the deoxy-ribonucleotides and is essential for the *de novo* synthesis of DNA.

To test the hypothesis that a second open reading frame overlapping with *un-24* is responsible for incompatibility activity in this region, two constructs, ORF1-stop and ORF3-stop were made using site-directed mutagenesis. The activities of these constructs were tested in a functional transformation assay, confirming that *un-24* has heterokaryon incompatibility activity. Therefore, *un-24* has dual functions in *N. crassa*; heterokaryon incompatibility and ribonucleotide reductase activity. This is the first demonstration of a nonself recognition function for the large subunit of RNR.

The specificity region of the *un-24* polypeptide was hypothesized to be within a unique 30 amino acids insert near the C-terminus. To test this, the entire *un-24*^{PA} sequence was determined and then two recombinant forms of *un-24*, OR/PA and PA/OR, were constructed. These chimeras were tested for incompatibility function and *un-24* temperature-sensitivity complementation by DNA transformation assays. The results verified that the carboxyl terminus of *un-24*^{OR} encodes heterokaryon incompatibility specificity.

It was hypothesised that the mechanism of *un-24* incompatibility was through protein aggregation. This hypothesis was tested through Western blots using antibody to the large subunit RNR from mouse. The results, which need to be verified with a more

specific antibody, suggest that UN-24 protein accumulates and may form insoluble aggregates in self-incompatible *un-24^{PA/OR}* partial diploids. Construction of a 6x His tagged vector containing *un-24^{OR}* was initiated that could be used to express, purify and subsequently raise antibodies to, UN-24 proteins.

Acknowledgements

I would like to express my sincere gratitude and appreciation and to allow recognition of some of many individual who have contributed to the completion of this work.

In particular, I would like to thank my supervisor, Dr. Myron L. Smith, who has been an inexhaustible source of patience, guidance and inspiration. His enthusiastic commitment to the research, and his regular words of encouragement helped me persist through the course of this work specially the writing process. I would also like to thank my research advisors, Dr. Linda Bonen and Dr. John Vierula for their numerous helpful suggestions regarding the subject of this thesis.

I am also grateful to Dr. Natalie Chaly for providing me the fibroblast 3T3 mouse protein and for allowing me to use her laboratory. Many thanks to Dr. Hirohisa Masuda from Cellular and Molecular Biology Laboratory, Wako, Japan, who kindly prepared and donated the anti-RNR-N antibody for Western analysis of *un-24* in *un-24* PA/OR self-incompatible strains.

I am deeply appreciative of my dear friend Cristina Micali, who provided the PA- λ B *cla/pst* construct for PA/OR chimera. I am also grateful to Cristina for her friendship, helpful suggestions, and constant encouragement and for bailing me out of trouble on numerous occasions, “Spanish Club”.

I would also like to thank Jen Crosthwait for helping me with the *un-24*^{PA} sequencing. Thanks to Suzann Patterson and Miguel Providenti for technical advice. Many thanks to my friend Moshi Kotierk, who generously gave of his time to help me and Natash Schokman for all her help.

I am grateful to my friends Muhammad, Osama and Omar Mussa for the much needed help they provided me.

I owe a debt of gratitude to my parents for having raised me to value education and for their support throughout my life.

A heartfelt thank you goes to my best friend, Rostam Mehdi-pour, for giving me a better perspective on life, for having the courage to marry me and the love to make it work. Without the love, encouragement, support and motivation of my husband, Rostam, I never would have been able to complete this thesis. Thanks for everything.

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List of Abbreviations

ATP	adenosine triphosphate
cDNA	complementary DNA
dH ₂ O	distilled water
DNA	Deoxyribonucleic acid
dATP	Deoxy-adenosine triphosphate
dADP	Deoxy-adenine diphosphate
dCDP	Deoxy-cytosine diphosphate
dGDP	Deoxy-guanine diphosphates
dNDP	Deoxy-nucleotide diphosphate
dNTP	Deoxy-nucleotide triphosphate
dTDP	Deoxy-thymine diphosphate
<i>het</i>	heterokaryon incompatibility gene
hyg ^R	hygromycin B resistance
kbp	Kilobase pair
LB	Luria-Bertani medium
<i>mat</i>	mating-type gene
<i>N. crassa</i>	<i>Neurospora crassa</i>
NDGA	Nordi hydroguaiavetic acid
OR	Oak-Ridge
ORFs	Open Reading Frames
PA	Panama
PCR	Polymerase Chain Reaction
RIP	Repeat Induced Point mutation
RNA	Ribonucleic acid
RNR	ribonucleotide reductase
rpm	rotation per minute
<i>vic</i>	vegetative incompatibility

Nomenclature of *un-24*:

Genotype designations

<i>un-24</i> ^{OR}	= wild-type "Oak-ridge" form of <i>un-24</i>
<i>un-24</i> ^{PA}	= wild-type "Panama" form of <i>un-24</i>
<i>un-24</i>	= temperature sensitive form of <i>un-24</i> derived from OR type
<i>un-24</i> ⁺	= either OR or PA wild-type form of <i>un-24</i> gene

Protein designations

UN-24 ^{OR}	= product of <i>un-24</i> ^{OR} allele
UN-24 ^{PA}	= product of <i>un-24</i> ^{PA} allele

Introduction

Nonsself recognition is ubiquitous amongst multicellular, eukaryotic organisms. It is essential for sexual reproduction, defence against pathogen invasion, and maintenance of integrity and individuality. It has long been recognized that nonsself recognition between different fungal individuals of the same species is under genetic control and occurs during both the sexual phase and the vegetative phase of the life cycle (reviews in: Glass and Kulda, 1992; Leslie, 1993; Esser and Blauch, 1994; Poggeler and Kuck, 2000). During the sexual phase, successful mating interactions require that two fungal individuals differ in mating type (Glass and Staben, 1990). The mating type is defined by one or more genetic loci, EACH containing one or more alleles (Glass and Kulda, 1992). For example, the ascomycete fungus *Neurospora crassa*, possesses two idiomorphs *mat a* and *mat A* at the mating-type locus (Glass et al., 1988; Shiu and Glass, 1999). Idiomorphs are genes with dissimilar DNA sequences present at the same genetic locus. During the sexual phase the vegetative incompatibility system is suppressed so that prior to meiosis there is a brief phase of heterokaryosis, i.e. where nuclei of different genetic origin occur in the same cytoplasm (Raju, 1980; Perkins, 1988).

Nonsself recognition during the vegetative phase of the life cycle is controlled by an incompatibility system that is distinct from that controlling mating type. The genes responsible are known as the heterokaryon incompatibility (*het*) genes in some species of fungi and vegetative incompatibility (*vic*) genes in others (Glass and Kulda, 1992; Leslie, 1993). *het* and *vic* genes are thought to be homologous, or at least analogous. Fusion of hyphae that are different at any *het* or *vic* loci during vegetative growth results in growth inhibition and/or death of the fusion cells (Beadle and Coonradt, 1944; Garnjobst 1953;

Jacobson et al., 1998). Thus, only individuals that are identical at all *het* or *vic* loci can fuse and undergo heterokaryosis. The genetic mechanisms of vegetative incompatibility is an area of active research (reviews in: Saupe 2000; Glass et al. 2000). Vegetative incompatibility genes have been classified into two types: allelic and non-allelic. In allelic incompatibility, alternative alleles at the same *het* (or *vic*) locus will interact in a negative manner to produce the incompatibility reaction. Non-allelic incompatibility involves the interaction between specific alleles at different *het* or *vic* loci (Loubradou and Turcq, 2000; Saupe, 2000).

N. crassa has one of the best characterized vegetative incompatibility systems. In *N. crassa*, heterokaryon incompatibility is controlled by at least eleven loci (Perkins, 1988); ten *het* loci and a single mating-type (*mat*) locus. All *N. crassa* *het* loci appear to be allelic incompatibility factors (Saupe, 2000). A difference at any one, or multiples, of these loci triggers an incompatibility reaction that results in compartmentalization and subsequent cell death (Jacobson et al. 1998).

Of the eleven *het* loci involved in heterokaryon incompatibility in *N. crassa*, allelic differences at *het-6* result in one of the most severe incompatibility reactions (Smith *et al.* 2000 b). *het-6* was discovered by Mylyk (1975) through an analysis of partial diploids in which a portion of the left arm of linkage group II (LGII L) was duplicated (Figure 1). The partial diploids were obtained by crossing a translocation strain, *T(II→III)AR18*, with a wild type strain. Strains with this duplication [*Dp(II→III)AR18*] can have a slow growth phenotype and produce a characteristic brown pigment in the medium. This phenomenon, termed “Dark Agar”, is an indication of a self-incompatibility reaction due to heteroallelism at an incompatibility gene in the translocated segment (Perkins, 1988).

Figure1. Chromosomal map of *N.crassa* linkage group II (LGII), left arm, in OR conformation showing the location of incompatibility genes *het-6*, *un-24* and *het-C*, and the breakpoint of the *T(AR18)* translocation fragment (based on: Smith et al., 1996 and Perkins et al., 1982).

<i>ro-7:</i>	ropy-7
<i>cys-3:</i>	cysteine-3
<i>hsps-1:</i>	heat shock protein-1
<i>un-24:</i>	unknown-24, temperature sensitive
<i>het-6:</i>	heterokaryon incompatibility-6
<i>cot-5:</i>	colonial temperature sensitive-5
<i>het-C:</i>	heterokaryon incompatibility-C
<i>pyr-4:</i>	pyrimidine-4
<i>ro-3:</i>	ropy-3
<i>thr-2:</i>	threonine-2

Two genes, *un-24* and *het-6* in the *T(II→III)AR18* translocated region, were identified that have incompatibility function in DNA transformation-based assays (Smith et al., 2000 b). There are two possible alleles of both *un-24* and *het-6*, designated Oak-Ridge (OR) and Panama (PA). Although both genes have heterokaryon incompatibility activities, they appear to be inherited as a block; only *un-24^{PA} het-6^{PA}* and *un-24^{OR} het-6^{OR}* combinations are detected in nature (Mir-Rashed et al., 2000). In the OR configuration, *un-24⁺* and *het-6* are separated by about 14 kbp. In the PA configuration, about 5 kbp separate the two genes.

The *un-24⁺* gene encodes the large subunit of a type I ribonucleotide reductase (RNR). RNR is an essential enzyme that reduces ribonucleotides (NDPs) to the deoxyribonucleotides (dNDPs) necessary for DNA synthesis and thus replication (Elledge et al., 1993). The type I RNR holoenzyme consists of two large subunits (R1) and two small subunits (R2) (Figure 2). The large subunit contains a catalytic site for substrate binding and two allosteric effector binding sites. The substrate binding site of R1 includes a cysteine triad that is involved in catalysis (Jordan and Reichard, 1998). The balance between the four deoxy-ribonucleoside diphosphates (dADP, dTDP, dGDP and dCDP), which are subsequently phosphorylated to the respective dNTP's prior to use in DNA synthesis, is regulated and maintained by the allosteric specificity site of R1 (Eklund et al., 1997; Eliasson et al., 1999). The overall enzymatic activity of ribonucleotide reductase is regulated by binding of either ATP (activating) or dATP (inhibitory) to the allosteric activity site located in the N-terminal region of the large subunit. R2 contains a dinuclear ferric center and a stable free tyrosyl radical (Figure 2)

Figure 2. Schematic representation of *E. coli* type I ribonucleotide reductase (Modified from Reichard, 1993).

necessary for the enzymatic activity (Nordlund and Eklund 1993). A long-range electron transfer is proposed to take place from the tyrosine radical in R2 to the catalytic site of the large R1 subunit during the ribonucleotide reduction process (Nordlund and Eklund, 1993; Eklund et al., 1997).

Although type I RNRs are conserved in evolutionary diverse life forms, UN-24 in *N. crassa* is the first described example of a large subunit of RNR that has nonself recognition function (Smith et al., 2000b). In *N. crassa*, transformation of *un-24^{PA}* spheroplasts with DNA carrying the wild-type OR allele, *un-24^{OR}*, results in cell death, whereas the transformation of an *un-24^{OR}* strain with the *un-24^{PA}* allele results in a decrease in transformant numbers and a large proportion of the resulting transformants having a aberrant colony morphology (Smith et al., 2001a; C. Micali, unpublished). Because of the central role that RNR plays in all organisms and its apparently unique nonself recognition function in *N. crassa*, a more in depth examination of the phenomenon is warranted. In the following section I provide a brief description of what was known about *un-24* at the outset of my thesis project.

A temperature-sensitive mutation of *un-24^{OR}* was generated by Repeat Induced Point mutation (RIP) (Smith et al. 2001b). RIP is a process that occurs during the premeiotic phase of the sexual cycle, just before fusion of the parental nuclei (Selker, 1990), in which some nucleotides within the duplicated sequences are methylated or undergo transition mutations (GC→AT). *un-24* was isolated from a cross between a wild-type *N. crassa* strain and a *Dp(II→III) AR18* (Smith et al. 2001a, Figure 1). A comparison of the *un-24* and *un-24⁺* DNA sequences revealed three transition mutations near the N-terminus of *un-24* (Hubbard, 1997). Strains that carry *un-24* are characterized by near

wild-type growth rates at temperatures below 30 °C but do not grow at temperatures above 35 °C (Smith et al., 2001a).

The temperature sensitivity of *un-24* strains can be complemented by transformation with DNA carrying the OR, but not the PA form of the gene. The region of DNA that complements *un-24* actually contains three Open Reading Frames (*un-24*, ORF2 and ORF3; Figure 3). The striking identity between *un-24* and type I ribonucleotide reductase large subunits from other organisms (Smith et al., 2001b), and the low identity values of the other two ORFs to sequence database entries suggest that a single gene occurs in this region and has both the catalytic and incompatibility functions. Neither ORF2 nor ORF3 have good consensus transcription or translation start sites (Smith et al., 2000 b). Additional support for the dual function of *un-24* comes from transcript analysis in the region. An mRNA was detected that corresponded to the predicted transcript from *un-24* but no mRNAs corresponding to ORF2 or ORF3 transcripts were detected (Smith et al., 2000 b). However, based on results from transformation assays and a comparison of the DNA sequence of the fragment that complemented *un-24* to those that had incompatibility function, Smith et al. (2000 b) observed that *un-24* complementation and incompatibility activity are not coextensive. As depicted in Figure 3, complementation of the *un-24* temperature sensitive phenotype requires that the transformed DNA extend over the entire length of ORF1 (i.e. *un-24*; constructs A and B, Figure 3). Deletion of either end of ORF1 (constructs C and E, Figure 3) results in the loss of *un-24* complementation. However, incompatibility activity, but not *un-24* complementation, was observed with a DNA construct in which the 5' end of *un-24* was removed (construct

Figure 3. Map of region of p31Eco-16 with the *un-24^{OR}* gene showing selected restriction enzyme recognition sites and three open reading frames. Introns in ORF1 are indicated by parentheses. Selected cloned products shown at bottom were tested by DNA transformation assays for [a] the presence (+) or absence (-) of incompatibility activity and [b] ability to complement (+) or not complement (-) *un-24*, a temperature-sensitive mutation in the large subunit of ribonucleotide reductase (modified from Smith et al., 2000 b).

<i>uri-24</i> mutant complementation	Transformed DNA	Incompatibility activity	Construct
+		+	A
+		+	B
-		-	C
-		-	D
-		+	E
-		-	F

E, Figure 3). The lack of incompatibility activity in construct D, which contains all of ORF2, suggests that ORF2 does not have incompatibility function. These results could be explained by ORF3 encoding incompatibility activity and ORF1 (*un-24*) having catalytic (ribonucleotide reductase) activity.

Because ribonucleotide reductase is a well characterized and important enzyme in nucleic acid biochemistry, and because the putative nonself recognition function of RNR is apparently novel in *N. crassa*, it is important to examine the possibility that incompatibility function is associated not with *un-24*⁺ but with ORF3. Therefore, my first objective was to investigate whether or not the heterokaryon incompatibility function in this region is due to *un-24*⁺ activity. To do this, I first determined the entire *un-24*^{PA} sequence. Then, to test the hypothesis that *un-24* has heterokaryon incompatibility function, two constructs were made using site-directed mutagenesis. In one construct, “ORF1-stop”, a stop codon was introduced into the *un-24*^{OR} sequence, which caused a predicted change from glutamic acid to serine in ORF3. Given the hypothesis that heterokaryon incompatibility function is associated with *un-24*, we expect that the “ORF1-stop” mutation will result in a loss in incompatibility function and that it will not complement the temperature sensitive phenotype of *un-24* strains in transformation assays. An “ORF3-stop” was also constructed that resulted in a silent mutation in ORF1. This construct was also tested for incompatibility function and temperature sensitivity complementation in DNA transformation assays. I hypothesized that the ORF3-stop construct would have both incompatibility function and would complement temperature sensitivity when transformed into *un-24*^{PA} and *un-24* strains, respectively.

Based on restriction enzyme fragment pattern comparisons, the OR and PA forms of *un-24* are very similar aside from the segment corresponding to the C-terminus of the predicted protein (Figure 4). Furthermore, two forms of the gene were identified by sequencing in the 3' region and these correlate to OR and PA heterokaryon incompatibility function. Through sequencing the entire *un-24*^{PA} DNA, I verified that a small (predicted ~ 30 amino acid) segment near the C-terminus is the only markedly different region between the PA and OR forms.

A second objective of my thesis was to test the hypothesis that the specificity region of the *un-24*⁺ polypeptide lies within this variable domain near the C-terminus. For this, I constructed two recombinant forms of *un-24*⁺; the first construct was an "OR/PA chimera" in which the N-terminus was derived from the OR form and the C-terminus from the PA form of *un-24*⁺. Similarly, the second construct was a PA/OR chimera. These chimeras were evaluated for incompatibility function and *un-24* temperature sensitivity complementation by DNA transformation assays. Based on this hypothesis, I expected incompatibility function, but not temperature sensitive complementation, to be associated with the C-terminal region.

The third objective of my thesis was to initiate studies on how heteroallelism at *un-24*⁺ triggers incompatibility and leads to cell death. Ribonucleotide reductase, being a redox-reactive enzyme, might initiate a chain reaction of free radical generation in incompatible heterokaryons. This hypothesis was not supported by experiments that measured catalase activity in incompatible and compatible heterokaryons where no difference in catalase activity was observed (Tropiano and Smith, unpublished). Since increases in catalase activity is a good measure of oxidative stress, it was concluded from

Figure 4. Sequence analysis of the *un-24*⁺ C-terminus region reveals major differences between the two allelic forms, OR and PA, the top and the bottom predicted amino acid sequences, respectively. Different amino acid shown in red colour and insertion/deletion of about 18bp is indicated by [-] (based on Smith et al., 2000 b).

these experiments that *un-24*-mediated incompatibility is not associated with oxidative stress. Likewise, the addition of antioxidants ascorbic acid, vitamin E and NDGA (CalBiochem), which are all free radical scavengers, to the growth medium did not significantly increase growth rates of incompatible heterokaryons (Smith, unpublished). These experiments further suggested that heteroallelism at *un-24*⁺ does not cause incompatibility through a mechanism involving oxidative stress.

Another possibility, which has yet to be examined, is that heteroallelism at *un-24*⁺ results in a loss of RNR function. A final possibility, examined here, is that heteroallelism at *un-24*⁺ results in UN-24 protein aggregates, that, in turn, elicit a cell death response. A similar mechanism was proposed by Coustou et al. (1997) for *het-s* of *Podospora anserina* and by Iyer et al. (2001) for *het-c* of *Neurospora crassa*. Saupe and collaborators (2000) suggested that the protein encoded by the *het-s* gene behaves as a prion. Prions can convert the normal form of the protein to an altered conformation that is usually resistance to proteases and therefore cause a lethal disorder of some cellular function (Prusiner, 1994). To test the hypothesis that heteroallelism at *un-24* causes aggregation of UN-24 proteins, I performed Western analysis (using anti-mouse RNR-N antibody) on self-compatible and self-incompatible partial diploids. Qualitative and quantitative analyses of *un-24* proteins produced by compatible and incompatible strains were made. The results of these Western analyses were encouraging, but somewhat hampered by a lack of good specific *un-24* antibody. Therefore, the final objective of this thesis was to develop an expression construct for *un-24* with which polyclonal antibodies against *N. crassa* UN-24 could be generated.

Methods and Materials

Strains, plasmid vectors and culture conditions

N. crassa strains used in this study are listed in Table 1. Strains were grown and maintained on 1 x Vogel's medium (Vogel, 1964), with or without 1.5 % agar and 1 x growth supplements, where necessary (Davis and de Serres, 1970), at either room temperature or at 30 °C. Vectors used were pCB1004, the hyg^R vector (Carroll et al., 1994), pCRII (TA Cloning kit, Invitrogen, CA), pUC128 (Gibco / BRL), and pQE-30 (Qiagen, CA).

Preparation and use of plasmid vectors

DNA ligations were performed at 16 °C in a 10 µl reaction volume with T4 DNA ligase according to the specifications of the manufacturer (New England Biolabs, Mississauga, ON and Gibco BRL, Burlington, ON).

For plasmid DNA extraction, a single colony from the freshly prepared culture was inoculated into 6 ml LB broth supplemented with the appropriate antibiotic. Cultures were grown overnight (approximately 16 hours) at 37 °C with shaking at 250 rpm. The next day, 2-4 ml of the culture was centrifuged at 14,000 rpm for 2 minutes. Plasmid DNA was extracted from the cell pellet using the Wizard miniprep kit according to the manufacture's instructions (Amersham, Oakville ON).

Table 1: *N. crassa* strains used in this study.

Strain ^a	Origin
C2 (2)-1 <i>un-24</i> ^{PA} <i>het-6</i> ^{PA} <i>het-C</i> ^{OR} <i>thr-2 a</i>	Smith et al. (1996)
C9-2 <i>un-24</i> ^{OR} <i>het-6</i> ^{OR} <i>het-C</i> ^{PA} <i>thr-2 a</i>	C2 (2)-5 x RLM57-30
C8c-164 <i>un-24</i> <i>het-6</i> ^{OR} ; <i>trp-1</i> ; <i>inl a</i>	Smith et al. (2000)
C (01) 4-2 <i>un-24</i> <i>het-6</i> ^{OR} <i>het-C</i> ^{PA} <i>pyr-4</i> ; <i>inl a</i>	M.L.Smith (C9-2x C8c-164)

^aStrains are OR at all undesignated *het* loci.

Plasmids were transformed into *E. coli* DH5 α cells by electroporation. *E. coli* cells were prepared as described in Sambrook et al. (1989). Briefly, LB liquid medium (500 ml) was inoculated with 1/100 volume of fresh overnight *E. coli* DH5 α culture. The culture was grown at 37°C with shaking (200 rpm) for 3-4 hours, until the optical density of the culture was ~ 0.5-0.7 at a wavelength of 600 nm. The cells were then chilled on ice for 30 minutes and centrifuged at 8000 rpm, 15 minutes, 4 °C. The pellet was resuspended in 250 ml of ice-cold sterile 10% glycerol and the centrifugation step was repeated. The bacterial pellet was sequentially resuspended and washed with 125 ml and 5ml of 10% glycerol and was centrifuged for 15 minutes. The cells were finally resuspended into 750 μ l of 10% ice-cold glycerol and distributed as 40 μ l aliquotes into ice cold eppendorf tubes and stored at -80 °C.

For electroporation, 40 μ l of competent cell suspension was thawed on ice prior to addition of 1-2 μ l of plasmid DNA. The mixture was transferred to an ice-cold 0.2 cm electroporation cuvette (Invitrogen). The cuvette was then placed in the electroporation chamber and subjected to 2.50 k voltage. SOC medium (1 ml) was immediately added to the cuvette and the cells were incubated at 37 °C at 200 rpm for 1-2 hours. The SOC medium consisted of 2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 20 mM glucose. 150 μ l of the culture were spread onto an LB agar plate containing the appropriate antibiotic. 40 μ l of 40 mg/ml X-Gal was mixed with 40 μ l of dH₂O and spread onto each plate 30 minutes prior to the plating of the cells for blue/white selection, where required.

DNA hybridization

DNAs were labelled by the random primer method (Sambrook et al., 1989) using Klenow and [α - 32 P] dCTP (Amersham, Oakville, ON). Prehybridization and hybridization conditions (60 °C) and washes were performed according to the membrane manufacturer's recommended protocols (Amersham). Hybridization signals were visualized following exposure to Kodak Bio-Max-1 film at -80 °C for 2 or more hours.

For screening bacterial colonies for plasmids, approximately 100 putative DH5 α transformants were replica plated onto LB containing the appropriate antibiotic and subsequent colony lifts were performed with Hybond-N hybridization membranes (Amersham) as per the supplier's protocol. The replica plate was chilled to 4 °C for 1-2 hours prior to lifting. The dry membrane disc was placed directly onto the agar plate for 1-2 minutes. The disc was asymmetrically marked with a needle to later aid with the orientation of the autoradiogram. The membrane was removed from the plate and placed on 2 layers of filter paper (Whatman 3MM no. 1) soaked with denaturation solution (1.5 M NaCl, 0.5M NaOH) for 15 minutes (colony side up). The membrane was blotted briefly on Whatman 3MM paper. The above steps were repeated with neutralization solution (1M Tris HCl; pH7.4, 1.5 M NaCl) and 2x SSC. The membrane was allowed to air dry for 30 minutes, prior to fixing by UV-light for 30 minutes prior to use in DNA hybridization.

Polymerase Chain Reaction (PCR) amplification and DNA sequencing

Oligonucleotide primers for PCR amplifications were synthesized either with an Applied Biosystems 390 PCR-Mate DNA synthesizer (Carleton University, Biology Department) based on the DNA sequence of Oak-Ridge allele at *un-24*, or synthesized at Life Technology (University of Ottawa, Ottawa ON). Primer sequences used in this study are given in Table 2. PCR reactions were performed using *Taq* DNA polymerase according to the manufacturer's recommendations (Gibco BRL, Burlington, ON) in a Barnstead/ Thermolyn Thermocycler (Ducuque, IA) programmed for 30 cycles of: 15 seconds at 95 °C (denaturation), 30 seconds at 54-56 °C (annealing) depending on the primer set and 2 minutes /1kbp DNA at 72 °C (extension). Amplification products were visualized by agarose gel electrophoresis with or without digestion with an appropriate restriction enzyme. Where required, PCR products were cloned into PCRII and sequenced with universal primers at Canadian Molecular Biology Research Services Inc. (Ottawa, ON).

Spheroplasts and DNA transformation of *N. crassa*

Spheroplasts of *N. crassa* strains were prepared with Novozym 234 (lot 4859, Interspex Products, Foster City, Calif.) by methods similar to that of Schweizer et al (1981), as modified by Royer and Yamashiro (1992). DNA transformation of *N. crassa* was carried out by a modified method of Akins and Lambowitz (1985). Strains C8c-164, C9-2 and C2 (2)-1 (Table 1) were used as recipients for transformation assays.

Table 2: List of primers used for PCR amplification and sequencing in this study.

Primer	Sequence (5'→3')	~Tm* (°C)
6JP3	GGTGACACGGCGCTGTG	58
6JP6	GTGCGGGCTTAACCGCTG	60
6JP7	CGAGGGTCTGAAGCCG	64
6JP9	TTGCCCATGGTGGGTTCG	58
6JP10	GGACGAGTTCGACTCGGC	60
6JP15	GTTGCTCAGAAAGTGGAG	60
6JP30	GTAGACCAGGAGGCTCTCAG	64
6JP33	CCCACACATCAGACATTATG	58
ORF1-stop	TGGATCCCCGATCTTTGAATGAAG	60
ORF3-stop	CTCTGGATCCCCGATCTATTCA	66
6x His/for	GGAAGATCTTATGTCAAGAAGCGCGATGG	60**
6xHis/rev	GCATTGGTTCTGCAGTGCGGGCTTAACCGCTG	60**
Puc/R	CCAGCTGGCGAAAGGGGGATG	56

* An approximate melting temperature

** Annealing temperature was calculated based on 20 nucleotides at 3' end.

Approximately 1-2 μ g of plasmid DNA was mixed with 100 μ l of spheroplast suspension ($\sim 8.0 \times 10^7$ spheroplast/ml) for each transformation. Spheroplasts transformed with the hygromycin resistance vector pCB1004 (Carroll et al., 1994) were selected on plates with a final volume of 235 units/ ml of hygromycin B (Smith et al., 2000). Transformants of the *un-24* strain were selected at the restrictive temperature of 37 °C.

An Oak Ridge (OR) cDNA clone was obtained from the Fungal Genetics Stock Center (FGSC, University of Kansas Medical Center, Kansas City, KS). The identity of the cDNA clone was confirmed by sequencing. To further verify the *un-24^{OR}* cDNA, it was tested for incompatibility activity by co-transformation with pCB1004 (Carroll et al., 1994) as the hygromycin resistance vector into OR and PA spheroplasts.

DNA sequencing of the *un-24^{PA}* allele

In order to sequence *un-24^{PA}*, several subclones were constructed using a series of oligonucleotide primers derived from the *un-24^{OR}* sequence (Figure 5). *un-24^{PA}* was first cloned into pCB1004 vector (in the same orientation as the *un-24^{OR}*), for use as a DNA template for PCR amplification. The PCR products were cloned into the pCRII vector and transformed into DH5 α . Transformants were selected on plates containing either kanamycin or ampicillin. Putative clones were first verified by *Eco*RI digestion and then sequenced with both universal forward and reverse T7 primers.

Figure 5. Selected restriction sites in *un-24^{PA}* allele, showing the location of the primers. Arrow at top indicates *un-24^{PA}* coding region. Arrows at the bottom indicate location, extent and direction of DNA sequencing.

Construction of stop codons in ORFs 1 and 3 of *un-24^{OR}*

Stop codons were introduced in ORFs 1 and 3 of *un-24^{OR}* by site-directed mutagenesis. Oligonucleotide primers were synthesized near the *Bam*HI site of *un-24^{OR}* (Figure 5). For introducing a stop codon in ORF1 that resulted in a conservative mutation in ORF3, a primer carrying two mismatches to the template sequence, was designed as shown below. The bold, italic nucleotides ***GA*** represent the mismatches, altering ORF1 codon from TTC (phenylalanine) to TGA (stop), and ORF3 codon from GAA (glutamic acid) to TCA (serine).

un-24^{OR} wild-type 5' –TGGATCCCCGATCTTTTCATGAAG – 3'

ORF1-stop 5' –TGGATCCCCGATCTTTGA ATGAAG –3'
*Bam*HI

For introducing a stop codon in ORF3 that resulted in a silent mutation in ORF1, the ORF3-stop primer was designed as shown below, that has only one mismatched nucleotide with the template sequence. The bold, italic nucleotide *A* represents the mismatch, altering ORF3 codon from AAG (lysine) to TGA (stop), and ORF1 codon from CTT to CTA both of which encode leucine.

un-24^{OR} wild-type 5' – CTCTGGATCCCCGATCTTTCA – 3'
ORF3-stop 5' – CTCTGGATCCCCGATCTATTCA – 3'
 *Bam*HI

The wild-type template DNA, *un-24^{OR}* was PCR amplified using one or the other mutagenesis primer and the 6JP6 primer (Table 2). The PCR product was ligated into

PCRII vector, transformed into DH5 α cells and transformants were selected on plates containing ampicillin. The putative clones and the *un-24*^{OR} in pCB1004 vector were double digested with *Bam*HI and *Not*I prior to electrophoresis on an agarose gel and the bands corresponding to the 3' end of *un-24*^{OR} gene (carrying the mismatch nucleotides) and the 5' end were recovered from the gel via band purification and ligated together. Clones of each construct (ORF1-stop and ORF3-stop) were sequenced through the *Bam*HI site to confirm that the ligation was in frame and that the mismatches were incorporated. These constructs were further evaluated for incompatibility function and temperature sensitivity complementation in DNA transformation assays.

Construction of *un-24* chimeras

un-24 chimeras were constructed using restriction enzyme-derived fragments of both OR and PA alleles. For the OR/PA chimera, both *un-24*^{OR} and *un-24*^{PA} fragments were obtained by PCR amplification with the primer pair 6JP3/6JP6. The 6JP3/6JP6 fragment containing *un-24*^{OR} was cloned in pCB1004 (hyg^R) and was used for the 5' end of OR/PA chimera. *un-24*^{PA} was also cloned into pCB1004 in the same orientation as the *un-24*^{OR} clone, and was used for the 3' end of OR/PA chimera. In the first step of constructing the chimeric gene, both the OR/pCB1004 and PA/pCB1004 clones were digested with *Bam*HI (Figure 6). The products of the *Bam*HI digests of both clones were two fragments of approximately 6.5 kbp and 2 kbp. These fragments were separated by electrophoresis on an 0.7% agarose gel and purified (Sambrook et al., 1989). The large fragment (6.5 kbp), carrying the 5' end of OR and the vector, was ligated to the small (2 kbp) 3' end

Figure 6. Schematic showing the construction of chimeric alleles of *un-24*.

An additional step was used in constructing the PA/OR chimera. The 3' end of OR was first cloned into the *EcoRI/BamHI* sites of pCB1004 vector. The 5' end of PA was excised from pCB1004 vector by a *BamHI/NotI* digest and then inserted into the same sites of the 3'OR clone.

fragments of PA in order to make OR/PA chimera (Figure 6). The ligation mix was purified using Gene Clean (Qiagen, CA) prior to transformation into *E. coli* DH5 α cells. The chimeric clones were confirmed by a *Bam*HI restriction digest. Furthermore, clones putatively containing the chimeric constructs were screened by PCR with 6JP6/P10 and 6JP10/P15 to confirm the orientation of the insert in the vector. Primer 6JP15 is specific to the 3' end of *un-24*^{OR}, thus only the constructs carrying the 3' end of OR such as PA/OR and *un-24*^{OR} (OR/OR) which was used as positive control, were expected to show PCR products with 6JP10/P15. However, PCR products from both OR/PA and PA/OR chimeras were expected to amplify with 6JP6/6JP10 primers. The chimeric PCR products were then cloned into the cloning site of PCRII and digested with *Eco*RI to confirm the clone. Finally, the insert was sequenced with universal primers to verify the *Bam*HI junction and the orientation of the chimeric genes.

For the PA/OR chimera, attempts to insert the 3' end of OR into the 5' end of PA to generate the PA/OR chimera using a similar procedure to that described above were not successful. We then altered our strategy to construct the PA/OR chimera in the following way. First, the 3' end of OR was cloned into pCB1004. Both *un-24*^{OR} and pCB1004 vector were double digested with *Eco*RI and *Bam*HI restriction enzymes. Two fragments were obtained from this digestion; one of ~1.5 kbp, carrying the 3' end of OR and one of ~ 6.5 kbp, carrying the 5' of OR and the vectors (Figure 6). The small fragment (3'OR) was cloned into the *Eco*RI / *Bam*HI sites of pCB1004 vector. To determine whether the 3' OR was correctly oriented within pCB1004 vector, asymmetric restriction digest analysis was performed using *Hind*III, an endonuclease which cuts within the pCB1004 vector as well as the 3' end of OR. The PA- λ B *clalpst* construct was prepared and kindly

provided by Cristina Micali for use as the *un-24^{PA}* sequence for PA/OR chimera. The 5' end of PA was excised from the pCB1004 vector using a double *Bam*HI/ *Not*I digest and inserted into *Bam*HI/*Not*I sites of the 3'OR clone. The chimeric clone was identified by DNA hybridization of colony lifts. Clones putatively containing PA/OR constructs were screened by PCR with 6JP6/P10, to verify the orientation of the insert in the pCB1004 vector and further verified by sequencing.

Strains and growth conditions for *un-24* protein isolation

un-24 protein was extract from C9-2 (Table 1), C9-2 transformed with *un-24^{OR}* and C9-2 transformed with *un-24^{PA}* (self-incompatible). To avoid extracting proteins from *un-24^{PA}* transformed C9-2 colonies that have escaped, through deletion of one of the *un-24* copies (Smith et al., 1996), several self-incompatible colonies were selected and placed to petri plates containing 1x Vogels and threonine (250 mg/L). Self-incompatible colonies were grown for 2-3 day at 30 °C, until they were about 3mm in diameter. Each of these colonies was cut up into several small blocks and each block was transferred to a 250 ml flask containing 50 ml liquid medium (1x Vogels and threonine). Colonies of C9-2 transformed with *un-24^{OR}* were transferred directly to a 250 ml flask containing 50 ml liquid medium. For the untransformed OR, conidia were used to inoculate the liquid medium. Mycelium was harvested during active growth, prior to conidiation. For this, the untransformed C9-2 and the *un-24^{OR}*-transformed C9-2 strains were grown at 30 °C for 4 days and the *un-24^{PA}*-transformed C9-2 strains were grown at 30 °C for 6-7 days. The liquid medium was decanted and mycelium was washed 2-3 times in minimal medium. The mycelium was subsequently placed in a Waring Commercial Blender and

homogenized. The sample was then centrifuged at 4°C for 10 minutes at 8000 rpm in a Sorval RC-5B refrigerated centrifuge using an SS-34 rotor. The supernatant was decanted and the pellet resuspended in minimal medium and centrifuged as above. The washing step was repeated twice. The pellet was resuspended in 1-2 ml of minimal medium. Total protein of each sample was extracted by sonication using a Kontes micro ultrasonic cell disruptor. Sonication was performed on ice by applying 120-watt short pulses of four 20 seconds intervals separated by 40 seconds recovery intervals. Cell debris was removed by centrifugation at 4 °C for 5 minutes at 12,000 rpm in an Eppendorf centrifuge model 5403. Proteins were quantified by the Bradford protein assay, using a Pharmacia Biotech Ultraspec 3000 spectrophotometer. Protein samples were stored at -20 °C until they were required.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

N. crassa proteins were separated on a Biorad Mini-Protean II dual slab cell (0.75 mm thick) as outlined in the supplier's instructions. Protein gels were prepared to provide a 4% stacking gel and 10% resolving gel. The resolving gel was made by adding 3.9 ml of dH₂O, 500 µl of 1.5% ammonium persulphate (APS), 100 µl of 10% SDS, 1.25 ml of resolving gel buffer (3M Tris-HCl, pH 8.8) and 4.1 ml of acrylamide-bisacrylamide (30:0.8). Acrylamide-bisacrylamide was prepared by adding 30g of acrylamide and 0.8 g of bisacrylamide into 100 ml of dH₂O that was filtered through Whatman no.1 filter paper and degassed under vacuum for 20 minutes. TEMED (15 µl) was added to the resolving gel solution prior to pipetting the solution into the gel

apparatus, to activate polymerization. The resolving gel was then overlaid with dH₂O and allowed to polymerize for 45 minutes.

The stacking gel contained 5.6 ml of dH₂O, 500 µl of 1.5% APS, 100 µl of 10% SDS, 2.5 ml of stacking gel buffer (0.5M Tris-HCl, pH 6.8), 1.25 ml of acrylamide-bisacrylamide (30:0.8) and 15 µl of TEMED. Before adding the stacking gel solution, water was decanted from polymerized resolving gel and a comb with 10 wells (0.75 mm thickness) was inserted and more stacking gel was added. Once polymerization of the stacking gel was completed (30 minutes), gels were loaded into gel chamber with approximately 350 ml of reservoir buffer (3g Tris, 14.4 g glycine, and 1g SDS were dissolved and brought to 1 litre with dH₂O). Approximately 30 µg (25 µl) of each protein sample was mixed with 10 µl of SDS loading buffer (0.154 g of DTT in 10 ml of 1x SDS) and the mixture was boiled for 5 minutes prior to loading onto the gel. For molecular weight standard, 10 µl (0.1 mg /ml) of a broad range, prestained protein marker (New England Biolabs) was used. Electrophoresis of protein gels was at 80V until the bromophenol blue reached the resolving layer, then the voltage was increased to 100V for 90 minutes. One gel was stained with Coomassie Brilliant Blue staining solution (1.25g Coomassie Blue R-250 was added to 225 ml methanol, 50 ml acetic acid and 225 ml dH₂O), for 30 minutes. Background staining was removed by destaining with 125 ml isopropanol, 100 ml acetic acid and 775 ml dH₂O, with gentle rocking overnight. The next day, the destained gel was sandwiched between 2 sheets of cellophane and dried overnight. The second gel was used for Western blot analysis.

Western analysis of *un-24* in *un-24*^{PA/OR} self-incompatible strains

Following protein electrophoresis (~ 90 minutes), the gel was removed from the electrophoresis chamber and the stacking gel was removed from the resolving gel. The gel was then transferred to a pre-wetted (in transfer buffer) nitrocellulose membrane (Millipore) and sandwiched between 3 pre-wetted layers of Whatman no.1 filter paper and two foam pads for support. Proteins were transferred using a Biorad Transblot electrophoretic transfer cell, in blot transfer buffer (3g Tris, 14.4 g glycine, 200ml methanol and up to 1 litre of dH₂O) at 30V overnight. The nitrocellulose membrane was then washed 3x with dH₂O and then stained using Ponceau red solution (0.2% Ponceau S in 0.3% TCA) for 10 minutes to determine the efficiency of transfer. The membrane was washed with dH₂O (3x) before it was destained with 1x PBS (10 mM sodium phosphate, pH7.4; 140 mM NaCl), it was then covered with cellophane and stored at 4 °C until immunoblot detection.

Immunoblot detection

Immunoblot detection of *N. crassa* proteins was carried out using the IEC chemiluminescent detection protocol. The destained blot (with 1x PBS) was rinsed with TBS-T (20mM Tris.Cl, pH 7.5, 500 mM NaCl; 0.05% (v/v) Tween 20) three times for 10 minutes. The blot was then blocked in 5% nonfat milk in TBS-T for 60 minutes at room temperature with gentle shaking. Primary antibody, anti-mouse-RNR-N (Takada et al., 2000) ,and secondary antibody, goat anti-rabbit IgG/horseradish peroxidase, were both diluted in 1% nonfat milk in TBS-T. The affinity-purified anti-RNR-N (Table 3) was a donation kindly prepared by Dr. Hirohisa Masuda from Cellular and Molecular Biology

Table 3. Comparison of amino acid sequence used to generate the polyclonal anti-mouse- RNR-N with amino acids of the UN-24^{OR}.

Species	Amino acid No.	Peptide name	Sequences
Mouse	5-17	RNR-N*	KRDGRQERV MFDK
<i>N. crassa</i>	5-17	UN-24 ^{OR}	KRDGRQERVQFDK

* The RNR-N antibody was isolated from rabbits immunized against a synthetic peptide composed of 13 residues shown above. It is corresponding to the N-terminus of mouse large subunit (R1) of RNR (Takada et al., 2000).

Laboratory, Wako, Japan. The membrane was incubated with a 1:1000 dilution of anti-mouse-RNR-N for 2.5 hours at room temperature on a rocking platform. Once the incubation with primary antibody was finished, the immunoblot was washed six times for 5 minutes in TBS-T. The membrane was then incubated in a 1: 5000 dilution of goat anti-rabbit IgG/ horseradish peroxidase for 1 hour at room temperature with gentle shaking. The washing step (six times for 5 minutes with TBS-T) was repeated, prior to chemiluminescent detection. 500 µl of luminol reagent (Biolynex) solution was mixed with 500 µl of oxidizing solution (1:1). The mixture was added to the membrane for 5 minutes. The membrane was blotted to remove excess solution and wrapped in cellophane and exposed to autoradiography film.

Construction of His-tag expression vector of *un-24^{OR}*

A number of attempts were made to clone the *un-24^{OR}* cDNA into pQE-30 vector, which contains 6xHis tag at the N-terminus. However, most of these efforts were unsuccessful. In order to both amplify and introduce suitable restriction site into the *un-24^{OR}* cDNA, the “His/forward” primer was designed (Table 2) based on the *un-24^{OR}* sequence (Smith *et al.*, 2000 a). The “His/forward” primer was designed to carry a *Bgl*II restriction site in place of the ATG start codon at the 5’end of the gene in the same reading frame as the 6xHis affinity tag.

5’- GGA AGA TCT TAT GTC AAG AAG CGC GAT GG-3’
*Bgl*II ^ *un-24* insert start

*Bgl*II has compatible cohesive ends with the *Bam*HI present in the multiple cloning site of the pQE-30 expression vector (Figure 7). The 6JP6 primer (Table 2) was used as a reverse primer. The amplified *un-24^{OR}* cDNA was first cloned into pCRII (TA Cloning kit, Invitrogen), for blue/white colony selection. Putative clones were digested with *Bgl*II to confirm the presence of the site in the insert. They were also digested with *Apa*I and *Hind*III, to confirm the orientation of the insert in the vector. The insert was liberated from the vector by an *Eco*RI digest. From the above digestions, only the *Eco*RI and the *Hind*III digestions gave the expected fragments, therefore, a different reverse primer was used to amplify the *un-24^{OR}* cDNA.

In the second attempt, the universal reverse primer, “Puc R” (Table 2) and the forward primer, “His/forward” were used to amplify OR cDNA gene and a similar procedure was carried out as explained above to clone the OR cDNA into pCRII vector. However, again, clones with the expected restriction sites were not detected. Thus, a new primer, “His/reverse” (Table 2) for the 3’end of OR cDNA gene was designed. In addition to the *Pst*I recognition site, which is present in the cloning site of pQE-30 vector, this primer carries the entire 6JP6 primer at the 3’end of *un-24*.

5’-GCA TTG GTT CTG CAG TGC GGG CTT AAC CGC TG-3’

*Pst*I ^ 6JP6 primer at the 3’end of *un-24*

Upon amplification of the *un-24^{OR}* cDNA with His/forward and His/reverse primers, the PCR product was digested with *Bgl*II and *Pst*I and ligated into pQE-30 vector. Prior to the ligation, the vector was digested first with *Bam*HI, gene cleaned and then it was cut with *Pst*I and gel-purified. The ligation product was then transformed into DH5α cells

Figure 7. Steps used in cloning *un-24^{OR}* cDNA in pQE-30 vector.

(Figure 7). Transformants were selected on plates containing ampicillin. Alkaline lysis miniprep was performed on the 20 colonies that resulted from the transformation. All the 20 colonies were double digested with *Bgl*II / *Pst*I to release the insert DNA but none of the colonies contained the correct insert.

Therefore, the amplified OR cDNA gene was first cloned into pCRII vector. The ligation product was then transformed into DH5 α cells and transformants were plated onto LB plates containing AP. Wizard-minipreps were performed on white colonies and plasmid DNAs were digested with both *Bgl*II/*Pst*I and *Bam*HI prior to electrophoresis on a 0.7% agarose gel. The above digestions confirmed the presence of the OR cDNA in pCR II. Two of the positive clones were cut with *Eco*RI to liberate the insert DNA completely from the pCRII vector and the band corresponding to the OR cDNA was recovered from the gel via band purification and double digested with *Bgl*II / *Pst*I. The double-digested DNA was gene cleaned prior to ligation into the pQE-30 vector. The vector was first digested with *Bam*HI, gene cleaned and then it was cut with *Pst*I and gel-purified prior to ligation. The ligation product was then transformed into DH5 α cells. Transformants were selected on plates containing ampicillin. All the colonies from this transformation were transformed to a membrane and probed with α -³²P labelled *un-24^{OR}* 6JP3/P6 DNA. Wizard-minipreps were performed on two positive clones obtained from this probe. Both colonies were digested with *Hind*III to further verify the presence of the OR cDNA in the pQE-30 vector. One of the positive clones, pUNEXP was further verified by sequencing to ensure that the insert DNA was in frame.

Results

Sequence of *un-24^{PA}* allele

The incompatibility reaction caused by heteroallelism at *het-6* results in severe growth inhibition in partial diploids and heterokaryons (Smith et al., 2000 a). The *het-6* locus was initially identified by Mylyk (1975), through an analysis of partial diploids (Figure 1). But it was just recently shown by Smith et al. (2000 b) that two distinct but tightly linked genes occur in this region that both have incompatibility activity. These heterokaryon incompatibility genes are designated as *un-24⁺* and *het-6* and they both have at least two allelic forms, designated OR and PA. The OR and PA alleles of *het-6* have been cloned and sequenced entirely (Smith et al., 2000 b). The 6JP3/6JP6 PCR fragment containing the entire *un-24^{OR}* was sequenced and 3' end of *un-24^{PA}* was sequenced prior to my thesis research.

To compare both the sequence and the function of *un-24^{PA}* to the alternative OR allele, the entire *un-24^{PA}* gene was sequenced using the scheme presented in Figure 5. This DNA sequence is presented in Figure 8. The predicted polypeptides encoded by *un-24^{OR}* and *un-24^{PA}* are 929 and 923 residues in length, respectively (Figure 9). As predicted based on restriction analysis (Smith et al., 2000 b), the two alleles of *un-24* are very similar at the 5' end of the gene, however, they have low sequence identity at both DNA and protein levels at the 3' end (C-terminus). Overall, the OR and PA forms of *un-24* gene have 78 % sequence identity at the DNA level and 96% at the protein level but are different at 30 of the 113 amino acids at the C-terminus (Figure 4). The isoelectric points for the *un-24^{OR}* and *un-24^{PA}* at this variable region were calculated to be 4.33 and 4.36

respectively. Notably, an insertion/deletion of 18bp also distinguishes the PA and OR forms of *un-24* in the 3' region (Smith et al., 2000 b). Similar to the *un-24^{OR}* sequence (Smith et al., 2000 a), two additional putative ORFs overlap with *un-24^{PA}*.

Figure 8. Nucleotide and deduced amino acid sequence of the *un-24^{PA}* gene of *N. crassa*.

Nucleotide numbers are given above and amino acids are shown below the DNA sequence.

5' ATG TAT GTC AAG AAG CGC GAT GGC CGC GAG GAG CGC GTC CAG TTC GAC AAG ATC ACA GCT GGT TCG AGG CTC TGT TAT
 M Y V K K R D G R Q E R V Q F D K I T A R V S R L C Y 81
 90 GGC CTC GAC ATG AAC CAT GTT GAT CCT GTA GCC GAT GGC GGC GTC AAG GTC TCT TCT GGC GTC TAC TAC GGT GGC GTC ACC ACA GCA
 G L D M N H V D P V A I T Q CAA AAG GTC ATC ATC V I S G V Y G G V T T A 162
 171 CAA CTA GAC GAT CTG GCT GCC GAG ACT GCC GAT GGC GGC GTC ATG ACC GTC ACC CAC CCC GAT TAT GGC ATC CTC GCC GGC CGT ATC
 Q L D D L A A E T A A Y M T V T H P D Y A I L A A R I 243
 252 GCC GTG TCC AAC CTC CAC AAG CAG ACC AAG AAG CAA TGG TCG CTC GTC ATC AGC GAG CTG TAC AAC TAT GTC AAC CCC AGG
 A V S N L H K Q T K K Q W S L V I S E L Y N Y V N P R 324
 333 ACC GGA AAG CAC TCC CCC ATG ATC GGC AAG GAC GTA TAC GAG TGT GTC ATG CGC CAC AAG GAC GAG TTC GAC TCT GCA ATT
 T G K H S P M I A K D V Y E C V M R H K D E F D S A I 405
 414 GTC TAC GAC CGT GAC TTC AAC TAC CAA TAC TTC GGC TTC AAG ACC CTC GAA CGA TCA TAC CTT CTT AAG CTC GAT GGC CAG
 V Y D R D F N Y Q Y F G G F K T L E R S Y L L K L D G Q 486
 495 ATT GCT GAG CGT CCC CAG CAC ATG ATC ATG CGT GGT GTC GTC GGT ATC TGG GGA GAC GAC GGT GAA CGG GTC ATT GAG ACT
 I A E R P Q H M I M R V A V G I W G D D V E R V I E T 567
 576 TAC AAC CTC ATG TCC CTC AAG ACC TTC ACC CAC GAT TCT TCT CCC ACC CTC TTC AAC GCC GGT ACT CCC CAG CCC CAG CTC TCT
 Y N L M S L K T F T H A S P T L F N A G T P Q P Q L S 648
 657 TCG TGC TTC CTG GTC GAC ATG AAG GAA GAC AGC ATC GAG GGC ATC TAC GAT ACC GAT GGC GGC TCG ACC ATC TCT AAG
 S C F L V D M K E D S I E G I Y D T L K T C A M I S K 729
 738 ATG GCT GGC GGT ATC GGT CTC AAC ATC CAC CGC GAT ATT CGT GGC ACT GGC TCC TAC ATC GCT GGT ACC AAC GGC ACA TCC AAC
 M A G G I G L N I H R I R A T G S Y I A G T N G T S N 810

1602 1611 1620 1629 1638 1647 1656 1665 1674
 ATT TTC GAG ACC ATC TAC CAC GCC GGT CTT ACT GCC TCT TGC CAG CTC GGC CAG GGC CCG TAC GCC ACC TAC GAA
 I F E T I Y H A A L T A S C Q L A K E Q G P Y A T Y E

1683 1692 1701 1710 1719 1728 1737 1746 1755
 GGC TCG CCC GTC TCC CAG GGA ATC CTC CAA TAC GAC ATG TGG AAC GTC ACC CCT ACC AAC CTC TGG GAC TGG ACT GCT CTC
 G S P V S Q G I L Q Y D M W N V T P T N L W D W T A L

1764 1773 1782 1791 1800 1809 1818 1827 1836
 AAG GCG GAC ATC AAG AAG TAC GGT GTT CGC AAC AGT CTG CTG CTT GCT CCT ATG CCC ACT GCT TCC ACT TCT CAG ATT CTT
 K A D I K K Y G V G R N S L L L A P M P T A S T S Q I L

1845 1854 1863 1872 1881 1890 1899 1908 1917
 GGC AAC AAC GAG TGC TTC GAG CCT TAC ACC TCT AAC ATC TAC CAA CGA CGT GTG CTC GGC GGC GAG TTC CAG GTC GTC AAC
 G N N E C F E P Y T S N I Y Q R R V L A G E F Q V N

1926 1935 1944 1953 1962 1971 1980 1989 1998
 CCC TGG CTT CTC AGG GAT CTT GTG GAG ATG GGC CTT TGG TCT GAT GCC ATG AAG AAC CGC ATC ATT GCT GAG GGC GGT TCT
 P W L L R D L V E M G L W S D A M K N R I A E G G S

2007 2016 2025 2034 2043 2052 2061 2070 2079
 ATC CAG AAC ATC CAG AGC ATT CCC AAT GAC ATC AAG GCC CTC TAC TCC CAG TCC CTC AAG ACC GTC TGG GAA ATT TCC CAG CGT ACC ATT GTC
 I Q N I I Q S I P N D I K A L Y K T V W E I S Q R T I V

2088 2097 2106 2115 2124 2133 2142 2151 2160
 AAG ATG GCT GCC GAC CGT GGT GCC TGC GGT ATT GAT CAG TCC CAG TCC CAG TCC CTC AAT ATT CAC ATG CGC GAA CCC ACC ATG GGT AAG
 K M A A D R G A F I D Q S Q S L N I H M R E P T M G K

2169 2178 2187 2196 2205 2214 2223 2232 2241
 ATT ACC AGC ATG CAT TTC GCC GGC TGG AAG ATG GGT CTC AAG ACG GGC ATG TAC TAC CTC CGT ACC CAG GCT GCT GCT CAG
 I T S M H F A G W K M G L K T G M Y Y L R T Q A A Q

2250 2259 2268 2277 2286 2295 2304 2313 2322
 CCC ATC CAG TTC ACT GTA GAC CAG GAG GCT CTC AGG GCC ACG GAT GAC CGA GTG GCC CCA GCC CAC TCG GGT CTT AAG AAG
 P I Q F T V D Q E A L R A T D D R V A P A H S G L K K

2331 2340 2349 2358 2367 2376 2385 2394 2403
 CGC TCC CCT CCC GCT GGC ACT TAT ACG TCT ATC GTG TTG AGG GAG AAC ACT TCG GGC CCC CGC CCG TAT GCC CAG ACT GGT
 R S P P A G T Y T S I V L R E N T S G P R P Y A Q T G

2412 GTT AGT GGC ACT AGC ACA CCC ATC GGC ACT AGG GAT GTT CCC ACA CCC GCC AGC ACA CCT CCT CCT GCT GAA GAT TCT GAG 2484
 V S G T S T P I G T R D V P T P T P P P A E D S E
 2493 ACT CTT GTA CAG AGT GAC AAC AGG CCC CGC CCA CTG GTC TCG CCC GCC AAG AAT GCC GGC TTC AAG GCC GAT CTG CCC GAG 2565
 T L V Q S D N R P R P L V S P A K N A G F K A D L P E
 2574 CCT GAG AGC CCC AAG GCT CTG GCC ACT GAC CCA ATT GTC AAG ACC GAG GCC ATT AGC TCC CGG GAG CTG GAG ATG AAG GAG 2646
 P E S P K A L A T A T D P I V K T E A I S S R E L E M K E
 2655 GGC CAG AGT GAG GAC AAG GAC GAG GAG AGC AGC AAG GAG AGA GAG GGG GAC ATC TAC TCT GAT GAG GTA GTC TCA TGC CCC TAC 2727
 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
 2736 GTT CCC GGC GCC GAT CCC TCC TCC TGC GTT ATG TGC AGC GGT TAA 3' 2772
 V P G A D P S S C S V M C S G *

Figure 9. Predicted amino acid sequence of *un-24^{OR}* aligned with *un-24^{PA}*. Amino acid positions are given above and below OR and PA sequences, respectively. Conserved amino acids predicted at allosteric site (3, 5, 6, 13, 17, 18, 53, and 88) and the active site (218, 427, 429, 431, 444, 738 and 739) are indicated in blue colour. Amino acid differences are shown in red colour and an insertion/deletion is represented by *.

$un-24^{or}$
1 10 20 30 40 50 60 70 80 90 100
MYVKKRDGRQERVQFDKITARVSRCLCYGLDMNHVDPVAITQKVISGVYGGVTTAQLDDLAETAAYMTVTHPDYAILAARIAVSNLHKQTKKQWSLVISE

 $un-24^{2A}$
1 10 20 30 40 50 60 70 80 90 100
MYVKKRDGRQERVQFDKITARVSRCLCYGLDMNHVDPVAITQKVISGVYGGVTTAQLDDLAETAAYMTVTHPDYAILAARIAVSNLHKQTKKQWSLVISE

101 110 120 130 140 150 160 170 180 190 200
LYNVNPRGTGKHSPMIAKDVYECVMRHKDEFDSAIVYDRDFNYQYFGFKTLERSYLLKLDGQIAERPQHMINRVAVGWGGDDVERVETYNLMSLKTFFTH

101 110 120 130 140 150 160 170 180 190 200
LYNVNPRGTGKHSPMIAKDVYECVMRHKDEFDSAIVYDRDFNYQYFGFKTLERSYLLKLDGQIAERPQHMINRVAVGWGGDDVERVETYNLMSLKTFFTH

201 210 220 230 240 250 260 270 280 290 300
ASPTLFNAGTPQPLSSCFLVDMKEDSIEGIYDTLKT CAMISKMAGGIGLINIHRIRATGSYIAGTNGTSNGIVPMLRVFNNTARYVDQGGNKRPGAFAIY

201 210 220 230 240 250 260 270 280 290 300
ASPTLFNAGTPQPLSSCFLVDMKEDSIEGIYDTLKT CAMISKMAGGIGLINIHRIRATGSYIAGTNGTSNGIVPMLRVFNNTARYVDQGGNKRPGAFAIY

301 310 320 330 340 350 360 370 380 390 400
LEPWHADVFEFLDLRKNHGKEEVRRDLFLALWIPDLFMKRVEQNGQWTLMCPHECPGLADVYGDEFEALYKEYEKEGKGRKTVKAQKLWYAILLEAQTTET

301 310 320 330 340 350 360 370 380 390 400
LEPWHADVFEFLDLRKNHGKEEVRRDLFLALWIPDLFMKRVEQNGQWTLMCPHECPGLADVYGDEFEALYKEYEKEGKGRKTVKAQKLWYAILLEAQTTET

401 410 420 430 440 450 460 470 480 490 500
GNPFFMLYKDACNRKSNQNLGTIRSSNLCETELIEYSAPDEVAVCNLASLALSAFIDYENASYDFKKLHEVTQVVVRNLNKIIDINHYPVKEAHNSNMRRH

401 410 420 430 440 450 460 470 480 490 500
GNPFFMLYKDACNRKSNQNLGTIRSSNLCETELIEYSAPDEVAVCYLASLALSAFIDYENASYDFKKLHEVTQVVVRNLNKIIDINHYPVKEAHNSNMRRH

501 510 520 530 540 550 560 570 580 590 600
 PIGVGQGLADAFALRMPFDSDAASKLNIQIFETIYHAALTASCOLAKEQGPYATYEGSPVSQGILOYDMWNVPTPTNLWDWTALKADIKKYGVNRNSLLL

 PIGVGQGLADAFALRMPFDSDAASKLNIQIFETIYHAALTASCOLAKEQGPYATYEGSPVSQGILOYDMWNVPTPTNLWDWTALKADIKKYGVNRNSLLL
 501 510 520 530 540 550 560 570 580 590 600

 601 610 620 630 640 650 660 670 680 690 700
 APMPASTSQILGNNECFEYTSNIYQRRVLAGEFQVVPNPWLLRDLVEMGLWSDAMKNRIIAEGGSIQNIQSIPNDIKALYKTVWEISQRTIVKMAADRG
 APMPASTSQILGNNECFEYTSNIYQRRVLAGEFQVVPNPWLLRDLVEMGLWSDAMKNRIIAEGGSIQNIQSIPNDIKALYKTVWEISQRTIVKMAADRG
 601 610 620 630 640 650 660 670 680 690 700

 701 710 720 730 740 750 760 770 780 790 800
 AFIDQSQSLNIHMREPTMGKITSMHFAGWKMGKLTGMYYLRTQAAAQPIQFTVDQEALRATDDRVAPAHSGLKKRSPPGAGTYTSIVLRENTSGPRPYAQT
 AFIDQSQSLNIHMREPTMGKITSMHFAGWKMGKLTGMYYLRTQAAAQPIQFTVDQEALRATDDRVAPAHSGLKKRSPPGAGTYTSIVLRENTSGPRPYAQT
 701 710 720 730 740 750 760 770 780 790 800

 801 810 820 830 840 850 860 870 880 890 900
 GVSGTSTPIGTRDVPPTASTPPPTTEVPETLVQSDNRPRPLVSPAKSAGFKADLPEPESPKALATDPIVKTEDIGSPLLERKEGQNEVDDEESQERDENIYS
 GVSGTSTPIGTRDVPPTASTPPPTTEVPETLVQSDNRPRPLVSPAKNAGFKADLPEPESPKALATDPIVKTEALSSRELENKEGQSEDKDEESKEREEDIYS
 801 810 820 830 840 850 860 870 880 890 900

 901 910 920 929
 NAPLSEQQVARCAMNPGADPSSCEMCSG -C terminus
 *****DEVSCFVVPGADPSSCVMCSG -C terminus
 901 910 923

Transformation results with ORF1-stop and ORF 3-stop constructs

DNA carrying *un-24*^{OR} was previously shown to trigger an incompatibility reaction when transformed into *un-24*^{PA} spheroplasts (Smith et al., 2000 a). However, two additional large open reading frames, ORF2 and ORF3, overlap with *un-24*⁺. ORF2 was shown to be an unlikely candidate, since a small subclone containing all of ORF2 did not have incompatibility function in DNA transformation assays. However, the hypothesis that ORF3 encodes incompatibility activity was not formally rejected. To examine which of *un-24* or ORF3 encodes incompatibility function, two constructs, ‘ORF1-stop’ and ‘ORF3-stop’, were made using site-directed mutagenesis and tested for heterokaryon incompatibility function. The ORF1-stop construct has mismatches at nucleotide positions 1256 and 1257 of *un-24* resulting in T→G and C→A transversions that cause a stop (UGA) in ORF1 (Figure 10). These sequence alterations are predicted to convert the glutamic acid codon (GAA) to serine (UCA) in ORF3 (Figure 10). The stop within ORF3 in the ORF3-stop construct is due to a T→A transversion mutation (at position 1254 of *un-24*^{OR}) that results in a silent mutation in *un-24* through changing CUU to CUA, both of which encode leucine (Figure 10).

Figure 10. Changes in proteins predicted for *un-24^{OR}* region due to ORF1-stop and ORF3-stop mutations.

ORF1-stop

Predicted changes in proteins

ORF3-stop

Predicted changes in proteins

As shown in Figure 11 and Table 4, the introduction of ORF1-stop DNA into spheroplasts of *un-24^{PA}* gave equivalent numbers of transformants as when the same construct was transformed into C9-2 (*un-24^{OR}* haplotype) spheroplasts. Apparently, the ORF1-stop construct does not display incompatibility activity. However, when the construct ORF3-stop was used, significantly fewer C2(2)-1 colonies regenerated compared to when C9-2 was used as the recipient of the transforming DNA (Figure 11, Table 4). Thus, the mutation in ORF3 does not abolish incompatibility activity and is not likely an incompatible gene. The representative transformation experiment is shown in Figure 12.

The mutation constructs, ORF1-stop and ORF3-stop were also tested for their ability to complement *un-24*, a temperature-sensitive mutation in the large subunit of RNR. As shown in Figure 13, C8c-164 (*un-24⁻*) transformed with the wild type *un-24^{OR}* allele (OR-P3/P6) grew well both at permissive temperature, 30 °C, and at restrictive temperature, 37 °C. Plates that were grown at 37 °C required 2-3 days longer to grow than those that kept at 30 °C. However, when ORF1-stop DNA was transformed into C8c-164 spheroplasts, few transformants were recovered at either 30 °C or 37 °C temperatures (Figure 13). On the other hand, about 174 and 143 transformants were observed when ORF3-stop DNA was transformed into C8c-164 spheroplasts at 30 °C and 37 °C, respectively (Figure 14, Table 5). As expected, the mutations in ORF1-stop resulted in a loss of *un-24* complementation function, whereas the mutation in ORF3-stop did not disrupt complementation of *un-24*. Thus, the complementation of *un-24* required ORF1 but not ORF3.

Figure 11. Numbers of transformed colonies with wild-type morphology recovered following DNA transformation. Histogram depicts the number of C9-2 (*un-24^{OR}*) and C2(2)-1 (*un-24^{PA}*) colonies that grow on medium containing hygromycin B after transformation with water (no DNA control), pCB1004 (hyg^R plasmid), OR-P3/P6 (hyg^R + *un-24^{OR}* wild type), PA-P3/P6 (hyg^R + *un-24^{PA}* wild type), PA-λBcl/pst (hyg^R + *un-24^{PA}* genomic fragment), OR/PA (hyg^R + chimeric *un-24^{OR}* (5' end)-*un-24^{PA}* (3' end)), PA/OR (hyg^R + chimeric *un-24^{PA}* (5' end)-*un-24^{OR}* (3' end)), ORF1-stop (hyg^R + ORF1-stop construct) and ORF3-stop (hyg^R + ORF3-stop construct). Values are means (n=5) with standard deviations. Significant difference (X^2 , $P \leq 0.05$) between C9-2 and C2(2)-1 transformant numbers for each construct are indicated by asterix.

Table 4. The average number and standard deviation of *N. crassa* colonies obtained after DNA transformation with plasmid constructs (n =5) into both Oak Ridge (C9-2) and Panama [C2(2)-1] spheroplasts.

Transformed strain														ttest OR vs PA	
Construct	C9-2 (OR)					Notes ¹		Mean	SD	C2(2)-1 (PA)					P value ²
H2O	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PCB1004	250	300	288	200	300	wt	268	43	319	155	271	301	200	wt	0.717
OR-P3/P6	100	120	98	131	120	wt	114	14	3	7	4	8	4	wt	0
PA-P3/P6	18	38	19	16	34	wt	25	10	91	68	86	52	43	wt	0.023
PA-P3/P6	14	19	12	17	18	star	16	3	- ³	-	-	-	-	-	
PA-λB cla/pst	45	23	29	35	27	wt	32	9	124	103	143	113	127	wt	0
PA-λB cla/pst	22	48	39	41	23	star	35	12	-	-	-	-	-	-	
OR/PA	61	29	51	39	43	wt	45	12	124	109	74	127	133	wt	0.005
OR/PA	33	19	39	23	29	star	29	8	-	-	-	-	-	-	
PA/OR	118	81	105	89	97	wt	98	14	4	2	3	3	2	wt	0
ORF1-stop	51	93	84	99	79	wt	81	19	79	52	64	92	98	wt	0.756
ORF3-stop	268	192	186	95	235	wt	195	65	13	14	9	11	8	wt	0.003

¹ In some transformation experiments, two types of colony morphologies are observed. Wild-type (wt) morphology transformants are cloud-like in shape, “star”-shaped colonies (star) are self-incompatible *un-24^{PA/OR}* partial diploids.

² P value based on t-test (n =5) comparing numbers of C9-2 and C2(2)-1 transformants.

³ - star-shaped colonies not observed.

Figure 12. Spheroplasts of C9-2 and C(2)2-1 were transformed with either *un-24^{OR}*, ORF1-stop, or ORF3-stop DNA (site-directed mutagenesis constructs) and plated onto medium containing hygromycin B.

Plasmid Construct	Spheroplasts	
	C9-2 (OR)	C(2)2-1 (PA)
<i>un-24^{OR}</i>
ORF1-stop
ORF3- stop

Figure 13. Spheroplasts of C8c-164 (*un-24* and has the *het-6^{OR}* incompatibility functions) were transformed with *un-24^{OR}*, ORF1-stop and ORF3-stop plasmid DNA, plated onto medium containing hygromycin B, and allowed to regenerate at 30 °C or 37 °C.

Plasmid Construct	C8c-164 Spheroplasts	
	30 °C	37 °C
<i>un-24^{OR}</i>
ORF1-stop
ORF3-stop

Figure 14. Total numbers of transformed colonies recovered following DNA transformation. Histogram depicts the number of C8c-164 (*un-24*) colonies that grow on medium with or without hygromycin B at 30 and 37 °C, respectively, after transformation with water (no DNA control), pCB1004 (*hyg*^R plasmid), OR-P3/P6 (*hyg*^R + *un-24*^{OR} wild type), PA-P3/P6 (*hyg*^R + *un-24*^{PA} wild type), OR/PA (*hyg*^R + chimeric *un-24*^{OR} (5'end)-*un-24*^{PA} (3'end)), PA/OR (*hyg*^R +chimeric *un-24*^{PA} (5'end)- *un-24*^{OR} (3'end)), ORF1-stop(*hyg*^R + ORF1-stop construct) and ORF3-stop (*hyg*^R + ORF3-stop construct). Values are means (n =3) with standard deviations. The * indicates significant difference (χ^2 , $P \leq 0.05$) in transformant numbers for each construct between the respective 30 °C or 37 °C control transformation with the OR-P3/P6 construct..

Table 5. The average number and standard deviation of *N. crassa* colonies obtained following DNA transformations of plasmid DNAs C8c-164 spheroplasts (n =3) at both 30 °C and 37 °C.

Transformed strain
C8c-164

Construct	30°C			Notes ¹	ttest vs control			37°C	Notes	ttest vs control		
					Mean	SD	P value			Mean	SD	P value
H ₂ O	0	0	0		0	0	0.017	0	0	0	0	0.015
PCB1004	180	191	172		181	10	0.211	3	2	2	0.6	0.016
OR-P3/P6	175	280	249		235	54		151	232	184	189	40.7
PA-P3/P6	12	18	6	star	12	6	0.018	8	10	9	9	1.0
OR/PA	8	9	4	star	7	3	0.018	5	7	4	5	1.5
PA/OR	163	196	220		193	29	0.195	96	174	261	177	82.5
ORF1-stop	18	29	12		20	9	0.018	10	16	26	17	8.1
ORF3-stop	176	196	151		174	23	0.190	129	168	132	143	21.7
												0.066

¹"Star"-like colonies represent self-incompatible *un-24^{PA/OR}* transformants. Where not noted, transformants had the cloud-like, wild-type colony morphology.

Complementation and specificity of *un-24* chimeras, OR/PA and PA/OR

A PA-specific incompatibility reaction in this study was defined as a marked reduction in the number of transformant *un-24*^{OR} colonies when compared to those of the *un-24*^{PA} genotype. An OR-specific incompatibility reaction was defined as a marked reduction in the number of transformant *un-24*^{PA} spheroplast recovered following transformation in comparison to where an *un-24*OR strain is the recipient. For each transformation experiment the pCB1004 (hyg^R) vector and water were used as positive and negative controls, respectively. The pCB1004 vector was used as an indicator of the hygromycin resistance gene expression in the transformed spheroplasts and to compare spheroplast competency. Water was used to measure the number of spheroplasts able to grow on hygromycin medium without the hyg^R vector DNA. As shown in Figures 11 and 14, little or no background growth was detected when water was used to transformed C9-2, C2(2)-1 and C8c-164 spheroplasts.

To test the hypothesis that the *het*-specificity region of *un-24* lies in the variable C-terminus domain, I constructed chimeric recombinant forms of the gene and determined their *het* phenotype and whether they complemented temperature sensitivity of *un-24* strains by DNA transformation. The *un-24*^{OR} and the *un-24*^{PA} were cloned separately into the pCB1004 (hyg^R) vector and used for construction of chimeras. The OR/PA chimera was constructed using restriction enzyme derived fragments of both alleles, by replacing the 3' end of *un-24*^{OR} with that from *un-24*^{PA}. I observed that this recombinant gene did not complement the temperature sensitivity of *un-24* (Figure 15). This construct produced a significantly greater number of wild-type morphology transformants in *un-24*^{PA} than in C9-2 *un-24*^{OR} (Figure 16, Table 4).

Figure 15. Spheroplasts of C8c-164(*un-24* and has the *het-6*^{OR} incompatibility function) were transformed with *un-24*^{OR}, *un-24*^{PA}, OR/PA and PA/OR plasmid DNA, plated onto medium containing hygromycin B, and allowed to regenerate at 30 °C or 37 °C.

Plasmid Construct	C8c-164 Spheroplasts	
	30 °C	37 °C
<i>un-24^{OR}</i>
<i>un-24^{PA}</i>
OR/PA
PA/OR

By replacing the 3' end of *un-24^{PA}* with that from *un-24^{OR}* the PA/OR chimera was produced. I observed that this recombinant gene complemented the temperature sensitivity of *un-24* (Figure 15) and had OR-like specificity based on transformation assays (Figure 16).

When *un-24^{OR}* allele was transformed into C2(2)-1 *het-6^{PA}* haplotype strain, there was a dramatic drop in the number of transformant colonies (Figures 11 and 16) indicating that the introduction of *un-24^{OR}* into a PA background results in strong incompatibility reaction. The incompatibility of the *un-24^{PA}* allele in the transformation assays was less pronounced than the reciprocal transformation of the *un-24^{OR}* allele (Figure 16). In addition, as noted previously (C. Micali, unpublished), incompatibility via transforming *un-24^{PA}* into *un-24^{OR}* spheroplasts results in a significant number of “star-shape” transformants. Upon transfer, these colonies grow slowly and with a “spidery” phenotype similar to *het-C* and *mat* incompatibility reactions. The PA-λ B Cla/Pst construct gave consistently stronger PA-specific incompatibility reactions (Table 4). This construct extends about 1 kb upstream of *un-24* start codon and approximately 2.25 kb downstream from the stop codon of *un-24* and was subcloned directly from genomic DNA. The PA-P3/P6 construct is smaller, extending about 840 bp upstream from *un-24* and has the P6 primers annealing site on the translation stop site.

Figure 16. Physical appearance of C9-2 and C2(2)-1 transformants containing *un-24^{OR}*, *un-24^{PA}*, OR/PA and PA/OR constructs. The vector pCB1004 (hyg^R) was used to examine the relative transformation competence of *N. crassa* spheroplasts.

Plasmid Construct	Spheroplasts	
	C9-2 (OR)	C2(2)-1 (PA)
PCB1004
<i>un-24</i> ^{OR}
<i>un-24</i> ^{PA}
OR/PA
PA/OR

Western analysis of *un-24* in *un-24* PA/OR self-incompatible strains

Iyer et al, (2001) proposed that heteroallelism at *het-c* in *N. crassa* may cause cell death through HET-C protein aggregation. The possibility that *un-24* incompatibility is also due to UN-24 protein aggregation was examined in *un-24^{OR/PA}* self-incompatible partial diploid strains. We hypothesized that heteroallelism at *un-24⁺* may result in a loss of RNR function through aggregation, or that protein aggregation may elicit a death response. To determine whether heteroallelism at *un-24* causes aggregation of UN-24 proteins, Western blots were performed using anti-mouse RNR-N antibody on self-compatible and self-incompatible partial diploids. The anti- mouse RNR-N that we used is a polyclonal antibody that binds to an epitope of 13 amino acids in the N-terminus region (Table 3, Takada et al., 2000). Overall, this region is very similar in UN-24 and RNR-N, with just one amino acid difference.

In Figure 17, a representative Coomassie blue-stained gel and Western blot is shown. In total, five sets of gels were analysed. Figure 17A shows a Coomassie blue stained gel of proteins from C9-2 (*un-24^{OR}*), untransformed, and transformed with *un-24^{OR}* and *un-24^{PA}*. Prestained Protein Marker (NEB) that covers a broad range (6-175 kDa) is shown in lane 1. Lane 2 represents fibroblast 3T3 mouse protein kindly provided by Dr. Chaly (Carleton University), used as a positive control for RNR detection. Lane 3 contains protein from untransformed C9-2. Lanes 4-9 represent C9-2 colonies that were transformed with *un-24^{PA}*. These colonies were self-incompatible star colonies, and arranged from slowest growing (lane 4) to fastest growing (lane 9). Lane 10 contains protein from a C9-2 strain that was transformed with *un-24^{OR}*.

Figure 17. (A) Acrylamide gel of Coomassie blue-stained mouse and *N. crassa* proteins. Lanes are; 1) NEB Prestained Protein Marker, 2) Mouse Protein, 3) OR untransformed, 4-9) *un-24^{PA}* transformed into C9-2, 10) *un-24^{OR}* transformed into C9-2. The self-incompatible star colonies represented in lane 4-9 are arranged from slowest growing (lane 4) to fastest growing (lane 9).

(B) and (C) Immunostain of Western blots using anti-mouse-RNR-N and goat anti-rabbit IgG/horseradish peroxidase as primary and secondary antibodies, respectively. Lanes 1-10 are the same as in the Coomassie-stained protein gel in panel A.

The Western blot was immunostained with anti-mouse-RNR-N and then with the secondary antibody, goat anti-rabbit IgG/horseradish peroxidase. Immunodetection as shown in Figures 17B and 17C revealed a well-defined band of about 90 kDa in lane 2 corresponding to the predicted molecular weight of the mouse RNR large subunit monomer. Protein from untransformed C9-2 in lane 3 shows a shaded area starting above the mouse band and extending well below the mouse band to about the 48 kDa region. The shaded area is evident as multiple bands in Figures 17C and in other Western blots (not shown). A dark band is evident ~175 kDa in lanes 4, 5, 6 and 7. These lanes contain protein from the four slowest growing self-incompatible star colonies. The intensity of this high molecular weight band is greatest in lane 4, representing protein from the slowest growing colony. That these high-intensity bands are ~ 175 kDa, much larger than the expected 103 kDa UN-24 protein, may suggest that covalently linked aggregates are present in the slowest growing self-incompatible colonies. No bands were detected in lane 8, from the second fastest growing self-incompatible colony. A very intense, broad band, appearing as a doublet band in these and other Western blots, is present at ~103 kDa in lane 9, from the fastest growing self-incompatible colony. Lane 10 contains protein from a C9-2 that was transformed with *un-24^{OR}* and shows a shaded area similar to lane 3, ranging from about 103-48 kDa. The immuno-stained Western blot presented in Figure 17 B and C are similar to the other three Western blots analysed although in several of the blots non-specific binding was evident in the *N. crassa* lanes. However, in all cases the immunodetection of the Western blots suggested that the amount of UN-24 protein in self-incompatible strains (star colonies) is greater than in the untransformed

C9-2 and in *un-24^{OR}*-transformed C9-2 colonies, which provides preliminary support for the hypothesis that incompatibility is associated with UN-24 protein aggregation.

Discussion

Heterokaryosis is an important part of many fungal life cycles where it may function to mask deleterious alleles through complementation and increase an individual's ability to deal with different substrates and environmental conditions while at the same time providing an opportunity for parasexual recombination (Leslie, 1993; Begueret et al., 1994). However, cell fusion between different individuals and heterokaryon formation may result in the transmission of deleterious genetic elements, such as viruses, plasmids and organelles. The heterokaryon incompatibility systems in filamentous fungi may have evolved as a protective mechanism against such disease transmission (Caten, 1972).

In the ascomycete *N. crassa*, ten different heterokaryon incompatibility loci (*het* genes) and a single mating-type locus (*mat*) govern incompatibility function. Smith et al. (2000 b) characterized two closely linked genes, *het-6* and *un-24*, at the *het-6* locus that both display heterokaryon incompatibility based on transformation assays. Both genes occur as two possible allelic variants, Oak Ridge (OR) and Panama (PA). *un-24*⁺ is located 14 kbp and 5 kbp away from *het-6* in the OR and PA configurations, respectively. However, only two combinations of alleles at these genes have been detected in *N. crassa*, *un-24*^{OR}-*het-6*^{OR} and *un-24*^{PA}-*het-6*^{PA}.

Incompatibility was evident when *het-6*^{OR} was transformed into strains containing the *het-6*^{PA} but not when *het-6*^{PA} was transformed into *het-6*^{OR} recipient strains (Smith et al. 2000 b). Likewise incompatibility was not apparent in transformations of *un-24*^{PA} into *un-24*^{OR}, as it was in reciprocal transformations. Thus it was hypothesized that the incompatibility function at the *un-24* and *het-6* genes may be mediated in a nonallelic manner (Smith et al., 2000 b). Further to suggest a non-allelic association of these

incompatibility genes, preliminary studies of *het-6* indicated the presence of three conserved regions between HET-6 and two other heterokaryon incompatibility-associated proteins (Smith et al. 2000 b), HET-E of *Podospira anserina*, which is involved in incompatibility of the nonallelic type (Saupe et al., 1995), and TOL of *N. crassa*, which plays a major role in determining the heterokaryon incompatibility function of mating type (Shiu and Glass, 1999). This similarity between *het-6* and other non-allelic incompatibility genes, the lack of *un-24^{PA}-het-6^{OR}* and *un-24^{OR}-het-6^{PA}* variants, and the asymmetric incompatibility function in transformations suggested that genes in the *het-6* region are inherited as a unit and may function as a gene complex.

The molecular mechanism by which the incompatibility reaction of *het-6* and *un-24⁺* occurs is not known. This thesis deals with *un-24*, a single copy gene in *N. crassa* that encodes the large subunit of a type I ribonucleotide reductase (RNR) (Smith et al., 2000 a). Unlike any other known large subunit of RNR, *un-24* appears to have a non self recognition function. Previous studies showed that of three possible open reading frames (ORFs), which occur within the region of *un-24⁺*, transcripts were detected from only ORF1, corresponding to *un-24* (Smith et al., 2000 b). No transcripts were detected by RT-PCR from either ORF2 or ORF3. The large subunit of RNR encoded by *un-24^{OR}* is predicted to be 929 amino acids long (Smith et al., 2000 a). ORF2 is predicted to be ~300 amino acids long and has sequence similarity to mucin-like proteins. Mucin is a large, highly polymorphic glycoprotein that is the major macromolecular constituent of epithelial mucus secretions (Wu et al., 1994). The protein core of mucin consists of several repeated domains rich in serine, proline and threonine residues (Eckhardt et al., 1997). ORF3 is predicted to be about 400 amino acids long and has little similarity to

data base sequences. Neither ORF2 nor ORF3 have good consensus transcription or translation start sites. Based on these analyses, Smith et al. (2000 b) hypothesized that *un-24^{OR}* is the gene with incompatibility activity. The possibility that ORF2 functions in nonself recognition can be ruled out based on DNA transformation assays, however, this possibility has not been rejected for ORF3. Therefore, the first objective of this thesis was to further test the putative heterokaryon incompatibility function of *un-24*.

To determine whether ORF1 or ORF3 encode incompatibility function, 'ORF1-stop' and 'ORF3-stop' were constructed using site-directed mutagenesis. Incompatibility was assessed following transformation of these constructs into OR and PA background spheroplasts. As hypothesized, introducing a stop codon in ORF1, which caused a missense mutation in ORF3; completely abolished the incompatibility activity of *un-24^{OR}*. Alternatively, introducing a stop codon in ORF3, which resulted in a silent mutation in ORF1, did not affect the incompatibility activity of ORF3-stop construct. These results provide strong evidence that ORF1 is responsible for heterokaryon incompatibility function.

To determine if the mutation constructs, ORF1-stop and ORF3-stop, complemented temperature sensitivity in *un-24* strains, we introduced these constructs into C8c-164 spheroplasts. C8c-164 transformed with ORF3-stop DNA gave higher number of transformants at both permissive (30 °C) and restrictive (37 °C) temperatures than when ORF1-stop DNA was used for transformation. It is unexpected, however, that only few colonies were observed at permissive (30 °C) temperatures following transformation with ORF1-stop. Quelling (gene silencing) could be a possible explanation for this. Quelling is believed to be the result of a constitutive mechanism that recognizes gene duplications

and transiently methylates them (Cogoni et al., 2001). This appears to directly reduce the level of transcription. It seems that, the introduction of ORF1-stop DNA into C8c-164 spheroplasts potentially generates inactivated genes through the mechanism of quelling and as a result not many colonies were observed upon transformation. Transformation of the ORF1-stop construct into strain C8c-164 resulted in no *un-24* complementation at restrictive temperature, 37 °C. These results further confirm that the heterokaryon incompatibility and ribonucleotide reductase catalytic activity are both associated with *un-24*. This dual function of *un-24* in *N. crassa*, is unique for a type I ribonucleotide reductase.

The large subunit of RNR in *N. crassa* is markedly similar to protein sequences from other organisms except for an unique long insertion near the C-terminus (Smith et al., 2000 a). Analysis of over 200 wild type isolates of *N. crassa* (Mir-Rashed et al., 2000) differentiated two major forms of this inserted C-terminus region which correlate perfectly to OR and PA heterokaryon incompatibility function. It is within this region that heterokaryon incompatibility is thought to be specified.

In this study, the variable region between *un-24*⁺ alleles was verified by sequencing the entire *un-24*^{PA} coding region. *un-24*^{PA} putatively encodes a polypeptide of 923 amino acids which has 96% identity with the protein coded by the OR allele. A comparison of *un-24*^{OR} and *un-24*^{PA} sequences at the DNA level revealed that they have 78% overall sequence identity that translates into 30 amino acids differences. The variation between the two allelic forms is found almost entirely at the 3'end (C-terminus) of *un-24*⁺, between what would be amino acid positions 820-929 of the OR form. At the 5'end of

the gene (N-terminus), the OR and PA alleles are predicted to differ by 2 amino acids; serine and tyrosine in PA take the place of phenylalanine and asparagine in OR at positions 311 and 445, respectively. A comparison of *un-24^{OR}* and the large subunit of type I RNR of different organisms such as, human, mouse, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Vaccinia virus*, *Schizosaccharomyces pombe* and *Escherichia coli*, revealed that phenylalanine and asparagine are conserved at the same positions in all organisms except *E. coli* (Smith et al., 2000 a). However, it was found that the first 220 amino acids at the N-terminus in *E. coli* play an important role in the enzymatic activity of RNR. These residues form a 12-helix allosteric activity site in the large subunit of RNR (Eriksson et al., 1997). In addition, the non-conserved amino acids at positions 311 and 445 in *N. crassa* are not part of the conserved residues (Figure 9, amino acids shown in blue colour) that are predicted to be involved in RNR activity at the catalytic site or the allosteric activity site. Thus, in this context, it is unlikely that differences in amino acids at positions 311 and 445 in the N-terminus of UN-24^{PA} with respect to UN-24^{OR} are associated with allelic specificity.

In addition to the 30 amino acids that differ in the C-terminus regions between the PA and OR forms, the PA allele of *un-24⁺* gene lacks 18 bp present at the C-terminus of *un-24^{OR}*. These 18 nucleotides would encode six amino acids, asparagine, alanine, proline, leucine, serine and glutamic acid at positions 901-906, respectively (Figure 9). Clearly, the C-terminus is the most variable region found between OR and PA alleles of the gene and this suggests it may be responsible for allelic specificity.

For the second objective of this thesis, therefore, I tested the hypothesis that the specificity region of *un-24⁺* is within the unique ~30 amino acid insert near the

C-terminus. To do this, two recombinant forms of *un-24*⁺, OR/PA and PA/OR, were made and incompatibility function of *un-24*⁺ was tested using these chimeric forms through DNA transformation assays. First, the OR/PA chimera, in which the N-terminus was derived from OR and the C-terminus from PA, had PA-like specificity based on transformation assays and did not complement the temperature sensitive *un-24* mutant strain. Alternatively, the PA/OR chimera, with the PA N-terminus and the OR C-terminus, had OR-like specificity and complemented temperature sensitivity of *un-24* strains. Thus, heterokaryon incompatibility specificity is associated with the 3' end of *un-24* (the C-terminus). The lack of complementation by constructs carrying PA sequences at the 3' end can be explained by recognizing that *un-24* was derived from *un-24*^{OR} and, while *un-24* is defective for catalytic activity at high temperature, it is not defective for heterokaryon incompatibility function (Smith et al., 2000 b). Therefore, complementation of *un-24* requires the presence of wild-type sequence (either PA or OR) at the 5' end but it only occurs if the 3' end is derived from the OR allele.

These results clearly indicate that the heterokaryon incompatibility specificity region of *un-24*⁺ is associated with the 3' region of the gene. Further, identification of specificity region will lead to understanding of how *un-24* functions as an incompatible gene. For example, it is still unknown whether one or more of the amino acid differences, or the insertion/deletion that differentiates the PA and OR forms, is/are responsible for the incompatibility specificity. Replacement of histidine by proline at amino acid position 33 in HET-S protein in *Podospora anserina* gives rise to protein of the HET-s specificity (Deleu et al., 1993). It is also unknown whether the specificity region functions by differentially controlling transcription or translation of *un-24*^{OR} or

un-24^{PA}, or by mediating a protein-protein interaction in some way. The precise definition of specificity region and how it functions will, therefore, require additional work.

Interesting is the observation that *un-24^{OR}* and *un-24^{PA}* act differently in terms of incompatibility. The *un-24^{OR}* allele elicits a very strong incompatibility response when transformed into spheroplasts that carry the *un-24^{PA}* allele. The PA allele does not cause the same dramatic effect when transformed into OR-background cells. This “asymmetric” incompatibility could be due other factor(s) that are involved in *un-24⁺* incompatibility activity. Another possibility is that some region upstream or downstream of *un-24⁺* is necessary for PA activity but not for OR activity. Turcq et al. (1990) showed that the sequences up and down stream of the *het-S* and *het-s* alleles in *P. anserina* vary by a 2 kb deletion and the presence of a transposon long terminal repeat. Several other examples of asymmetry in incompatibility reactions, including *het-c* and *het-d* in *N. crassa* (Wilson et al., 1961), suggest that this interesting phenomenon is quite wide spread among incompatibility factors.

Preliminary studies of the *het-c* locus in *N. crassa* suggested that heterokaryon incompatibility is associated with a formation of a HET-C protein complex that, in turn, results in aggregation of the protein and leads to hyphal compartmentation and death (Iyer et al., 2001). A similar mechanism was proposed by Coustou et al. (1997) for the *het-s* of *Podospora anserina*. The third objective of this thesis was to test whether aggregation of UN-24 protein can be detected in partial diploid cells that are heterozygous at *un-24*.

To determine whether heteroallelism at *un-24* causes aggregation of UN-24 proteins, five Western blots were performed using anti-mouse RNR-N antibody on self-compatible and self-incompatible partial diploids. The following general points can be made based on these Western blots. First, more primary antibody binds to lanes with protein from incompatible *un-24*^{PA/OR} partial diploids than to the control lanes representing the untransformed OR strain and a compatible *un-24*^{PA/PA} partial diploid. Second, the signal in control lanes appear to represent fragments of UN-24 protein, ranging in size from 103 kDa (the expected size for *un-24*) to 48 kDa. Third, in the slow growing incompatible partial diploids a very abundant (~ 175 kDa) protein band is evident.

The 175 kDa protein in self-incompatible strains is of greater intensity than signals from the untransformed C9-2 and in *un-24*^{OR}-transformed C9-2 colonies. The range of sizes of immuno-stained products in untransformed C9-2 and *un-24*^{OR/OR} partial diploid colonies, compared to the discrete, more intense bands in the self-incompatible colonies, suggest that UN-24 degradation is interrupted in the self-incompatible colonies. It is possible to explain this high molecular weight band in a non-denaturing gel from the slow growing self-incompatible partial diploids as an insoluble aggregate. Another possibility for the high molecular weight protein in these lanes could be due to cysteine cross linkage between monomers of UN-24 protein. While the results of Western analyses were encouraging, the poor resolution of *N. crassa* UN-24 protein and relatively high back-ground signals in *N. crassa* lanes suggests that a more specific antibody is required. It is hoped that the development of an expression construct for *un-24* will enable the generation of polyclonal antibodies against *N. crassa* UN-24 in the future.

Conclusion

In spite of important progress achieved in the molecular characterization of *het* genes, the molecular mechanism by which *het* gene interactions lead to cell death is still poorly understood. Some incompatibility factors are thought to be cytoplasmic and proteinaceous (Wilson et al., 1961). Early studies on *N. crassa* *het-C* performed by Wilson et al. (1961) showed that microinjection of incompatible cytoplasmic extracts into the recipient cells triggered incompatibility. The cytoplasmic factors could be inactivated by heat (60 °C) or proteases. A direct protein-protein interaction between the incompatible products was thought to be involved in the incompatibility reaction; however, this is about all that is known about the incompatibility mechanism to date. Since a single gene mutation was shown to suppress the incompatibility reactions associated with multiple *het* genes in *N. crassa* (Arganza et al., 1994), it was hypothesised that a common incompatibility reaction pathway must exist. However, molecular characterization of *het* genes from *N. crassa* and *P. anserina* have revealed that a great diversity of genes can act as *het* genes (Saupe, 2000). It has been shown that some of the *het* genes have cellular functions in addition to their role in incompatibility. For example, enzymatic activity associated with the *un-24* gene in *N. crassa* and its involvement in redox chemistry makes it a suitable and interesting candidate for studying the precise cellular function of *het* genes.

The results gathered so far from transformation assays, site-directed mutagenesis, presence of variable region and chimeras all indicate that the large subunit of RNR in *N. crassa* has nonself recognition function in addition to catalytic activity. Even though type I RNRs are conserved across all Kingdoms, where they function in converting RNA

precursors to DNA precursors, this is the first example of a large subunit RNR gene having nonself recognition function. This function could be considered for development of new antiviral and antimicrobial drugs. Since RNR is an important enzyme in regulating cell division and cell proliferation, it is also of interest as a potential target for antitumour drugs.

In this study I explored the allelic differences between the two forms of *un-24*, verified the heterokaryon incompatibility function of *un-24* and identified the specificity region of the factor. In addition, I initiated studies that quantify the *un-24* protein product in compatible and incompatible heterokaryons that implicate protein aggregation as a general mechanism to heterokaryon incompatibility function.

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