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Nonself recognition in *Neurospora crassa* and *Cryphonectria parasitica*

A thesis
presented to
the Faculty of Graduate Studies
of
Carleton University

by
Carmen Christine Gibbs

In partial fulfillment of the requirements
for the degree of
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ABSTRACT

Intraspecific nonself recognition is a widespread phenomenon that occurs during both the sexual and asexual life cycle phases of fungi. During vegetative growth nonself recognition is a result of a genetic-mediated process called vegetative incompatibility. Vegetative incompatibility is recognized macroscopically as a demarcation zone (barrage) where genetically different individuals meet on the same substratum. Vegetative incompatibility is also represented by heterokaryon incompatibility, a system that restricts the ability of genetically different nuclei to coexist in the same cytoplasm. This thesis focuses on *un-24* in *Neurospora crassa* and *vic4* in *Cryphonectria parasitica*. The *un-24* gene of *N. crassa* has heterokaryon incompatibility function and encodes the large subunit of a type I ribonucleotide reductase (RNR), an evolutionarily conserved enzyme that is essential for *de novo* DNA synthesis. I examined *un-24*-derived transcript levels in strains before and after escape from self-incompatibility due to heteroallelic duplications of *un-24*. In addition to documented mechanisms including deletions within *het* genes and spontaneous suppressor mutations, my results suggest that escape from incompatibility may involve a change in the balance of transcript levels. Surprisingly, transcript levels of both *un-24*^{PA} and *un-24*^{OR} increased, but to different extents, in stable self-incompatible strains grown on a low nitrogen medium that promotes the sexual cycle. Therefore, localized inactivation of *un-24*-mediated incompatibility in the perithecius may be through differential transcript regulation of the two allelic forms. The *vic4* gene of *C. parasitica* has barrage function but does not restrict heterokaryon formation. Heterokaryons with *vic4* differences were found to have growth rates,

morphology and protein profiles similar to heterokaryons with no *vic* differences. My results suggest that the compatibility of *vic4* heterokaryons may function through skewed nuclear proportions of the two parental nuclear types.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
<i>a</i>	mating type a
<i>A</i>	mating type A
AFLP	amplified fragment length polymorphism
AMV	Avian Myeloblastosis Virus
APS	ammonium persulphate
ATP	adenosine triphosphate
ben	benomyl
ben ^R	benomyl resistant
bp	base pairs
<i>bml</i> ^R	benomyl resistant beta-tubulin
cm	centimeters
CW	Calcofluor White
C _T	critical threshold
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DMPC	dimethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide triphosphate
<i>Dp</i>	partial diploid
EB	Evans blue
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay
FDA	fluorescein diacetate
FDSB	first-dimension sample buffer
g	grams
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
<i>het</i>	heterokaryon incompatibility locus
<i>hph</i>	hygromycin B phosphotransferase
HRP	horse radish peroxidase
hyg	hygromycin
hyg ^R	hygromycin resistant
IDV	integrated density value
IEF	isoelectric focusing
IgG	immunoglobulin G
kb	kilo base pairs
kDa	kilodaltons
L	litres
M	moles per litre
<i>mat</i>	mating-type locus
MOPS	4-morpholinepropanesulfonic acid
nm	nanometers
OR	Oak Ridge
PA	Panama

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PDAg	green potato dextrose agar
PEG	polyethylene glycol
pI	isoelectric point
PMSF	phenyl methyl sulfonyl fluoride
R1	ribonucleotide reductase large subunit
R2	ribonucleotide reductase small subunit
RFLP	restriction fragment length polymorphism
RIP	repeat induced point mutations
RNA	ribonucleic acid
RNR	ribonucleotide reductase
RPM	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
<i>sbr</i>	small brown
SCM	synthetic crossing medium
SIA	self-incompatible A
SIB	self-incompatible B
TBST	tris-buffered saline with Tween [®] 20
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
<i>thr-2</i>	threonine-2
<i>un-24</i>	unknown temperature sensitive mutation-24
UN-24	<i>un-24</i> protein product
UV	ultraviolet
V	volts
vc	vegetative compatibility
<i>vic</i>	vegetative incompatibility locus
VIC4	<i>vic4</i> protein product
VMM	Vogel's minimal medium
x g	centrifugal force

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- Appendix A *un-24* transcript levels in *N. crassa* homokaryons grown on low and high nitrogen media.
- Appendix B Polyclonal anti-UN-24 antibody development.

GENERAL INTRODUCTION

Self/nonself recognition

Self/nonself recognition is a universal phenomenon found across kingdoms (Micali & Smith, 2003). For example, self-fertilization of monecious plants is prevented through self-recognition (Nasrallah, 2002). Sessile, clonal invertebrates within Cnidaria, Porifera and Bryozoa, mediate fusion and aggressive behaviour through nonself recognition mechanisms (Grosberg, 1988). In humans, the major histocompatibility complex (MHC) recognizes nonself particles and presents them to the T-cell receptors of T lymphocytes for destruction (Nei *et al.*, 1997). In fungi too, nonself recognition occurs throughout the vegetative and sexual life cycle phases. Nonself recognition during the sexual phase is mediated by the mating-type locus. Mating compatibility is limited to strains that carry alternate alleles of mating-type (*mat*) loci (reviewed by Coppin *et al.*, 1997; Kronstad & Staben, 1997; Shiu & Glass, 2000).

Vegetative incompatibility in fungi

Filamentous fungi grow as vegetatively branching tubular filaments (hyphae) that grow together and often fuse to form a mycelial network. Incomplete septa permit the flow of cytoplasm and nuclei throughout the mycelium (Springer, 1993). Hyphal anastomosis (fusion) increases cytoplasmic flow and connectivity within the fungal individual (Rayner, 1996). Hyphal anastomosis can also occur between genetically different individuals to form heterokaryotic hyphae containing nuclei of both individuals (Bégueret *et al.*, 1994; Debets *et al.*, 1994).

During vegetative growth, nonself recognition is a result of a genetic-mediated process called vegetative incompatibility (Bégueret *et al.*, 1994). Vegetative

incompatibility is mediated by *het* (*heterokaryon incompatibility*) or *vic* (*vegetative incompatibility*) genes (Jacobson *et al.*, 1998) and is a widespread phenomenon that has been found in every fungal species tested (Glass & Kuldau, 1992). Vegetative incompatibility was first recognized as a macroscopic demarcation line, termed barrage, that occurs at the zone of contact between two genetically different strains meeting on the same substratum. Barrage has been used to differentiate vegetative compatibility groups within populations (Smith *et al.*, 1994; Cubeta *et al.*, 1997; Milgroom & Cortesi, 1999; Dettman & van der Kamp, 2001a, b) and identify *vic* loci (Cortesi & Milgroom, 1998). Microscopically, barrage is caused by fusion and death of incompatible hyphae (Xiang & Glass, 2002).

Heterokaryon incompatibility, the second manifestation of vegetative incompatibility, limits heterokaryosis such that nuclei must have identical alleles at all *het* or *vic* loci (Glass & Kuldau, 1992). When individuals with genetic differences at one or more *het* loci fuse, the heterokaryotic cells are destroyed through septal plugging and vacuolization or have severely inhibited growth rates, depending on which *het* genes are heteroallelic (Garnjobst & Wilson, 1956; Perkins, 1988; Bégueret *et al.*, 1994; Jacobson *et al.*, 1998).

Genetics of vegetative incompatibility

The number of *vic* or *het* loci varies among species. Of the best characterized species, there have been 17 *het* loci identified in *Podospora anserina*, at least 12 *het* loci in *Neurospora crassa* (Perkins, 1988; Glass & Kuldau, 1992; Saupe, 2000; Smith *et al.*, 2000b), 8 *het* loci in *Aspergillus nidulans* (Anwar *et al.*, 1993) and 6 *vic* loci in

Cryphonectria parasitica (Cortesi & Milgroom, 1998). Most often incompatibility operates through an allelic system where incompatibility is due to differences at a given *het* or *vic* locus. Certain allelic combinations at two separate loci (non-allelic system) may also cause heterokaryon incompatibility (Glass & Kulda, 1992).

Three *het* loci in *P. anserina* and 4 in *N. crassa* have been cloned and characterized. The putative functions of proteins encoded by these loci vary. Four of the cloned loci (*het-c* and *het-6* of *N. crassa* and *het-s* and *het-e* of *P. anserina*) have no known functions other than to mediate heterokaryon incompatibility (Xiang & Glass, 2002) although the *het-s* protein product behaves as a prion (Coustou *et al.*, 1997). *N. crassa* mating-type genes *mat A-1* and *mat a-1* function in mating incompatibility as well as vegetative incompatibility and encode putative transcription factors that are thought to regulate transcription of genes involved in mating and fruit-body formation (Glass *et al.*, 1990; Staben & Yanofsky, 1990). *het-c* of *P. anserina* encodes a putative glycolipid transfer protein required for ascospore production (Saupe *et al.*, 1994). In addition to having nonself recognition activity, the protein encoded by *un-24* of *N. crassa* functions as the large subunit of ribonucleotide reductase (Smith *et al.*, 2000a), an enzyme required in synthesis of *de novo* DNA precursors (Jordan & Reichard, 1998).

The widespread occurrence of vegetative incompatibility in fungi, and analogous systems across kingdoms, suggests that it is biologically advantageous (Debets & Griffiths, 1998). Vegetative incompatibility may have evolved as a defense mechanism (Biella *et al.*, 2002) protecting individuals against the transfer of infectious genetic elements (Caten, 1972) such as double stranded RNA viruses (Coenen *et al.*, 1997; Cortesi *et al.*, 2001) and senescence plasmids (Debets & Griffiths, 1998). The transfer of

cytoplasmic elements was shown to occur at a reduced frequency between incompatible individuals (Coenen *et al.*, 1997; Cortesi *et al.*, 2001). Also, heterokaryon incompatibility may protect against the exploitation of nuclei that are not advantageous in homokaryotes but have a fitness advantage over other nuclei in the heterokaryotic state (Hartl *et al.*, 1975; Debets & Griffiths, 1998). An alternate view, the null hypothesis, is that vegetative incompatibility systems occurred by chance through nonselective polymorphisms whose products are detrimental in the heterokaryotic state (Saupe, 2000).

The research presented here investigates the mechanisms of vegetative incompatibility mediated by *un-24* of *N. crassa* and *vic4* of *C. parasitica*. Transcription of *un-24* throughout the *N. crassa* life cycle and within heteroallelic self-incompatible strains, and strains that have escaped from incompatibility was studied to examine the role of this gene during sexual reproduction and incompatibility (Chapter 1). In Chapter 2, I explore whether or not *vic4* in *C. parasitica* has heterokaryon incompatibility function.

Chapter 1: *un-24* mediated heterokaryon incompatibility in *Neurospora crassa*.

INTRODUCTION

Neurospora crassa life cycle

N. crassa is a filamentous ascomycete. Cells are arranged in tubular filaments, called hyphae, that collectively comprise the mycelium. Each cell contains multiple nuclei and is separated by an incomplete septum. Each septum has a central pore that allows for movement of materials, including nuclei and other organelles, from cell to cell along the length of the hypha (Davis, 2000). In nutrient limiting conditions, aerial hyphae develop multinucleate asexual macroconidia (Springer, 1993; Figure 1.1). Uninuclear microconidia are also produced from hyphae on short microconidiophores (Lowry *et al.*, 1967). Micro- and macroconidia can detach and serve as asexual propagules (Davis, 2000). The sexual cycle is initiated in reduced nitrogen and/or carbon conditions (Westergaard & Mitchell, 1947). At the outset of the sexual cycle the female parent forms a multicellular bundle of hyphae (protoperithecium). A filamentous gametic trichogyne extends from the protoperithecium and fuses with a male conidium or hypha of the opposite mating type. The male nucleus travels through the trichogyne into the protoperithecium, which then develops into a perithecium, a black ascospore-bearing structure that can be seen with the naked eye. Within the perithecium, karyogamy is followed by meiosis and two mitotic divisions. The haploid nuclei develop into ascospores contained within elongate sacs (asci). The ascospores are forcibly ejected from the ascus and germinate on suitable medium with or without chemical or heat induction (Davis, 2000).

Figure 1.1. Generalized life cycle of *Neurospora crassa* (adapted from Davis, 2000).

During the sexual cycle of *N. crassa*, nonself recognition is mediated by the mating-type locus, *mat*, with two alleles, *A* and *a* (Glass *et al.*, 1990). Individuals must have alternate mating types to complete sexual reproduction. *mat* also mediates heterokaryon incompatibility during vegetative growth (Glass & Kulda, 1992). Interestingly, genetic differences at *het* loci do not restrict the sexual reproductive phase, fertility, or subsequent meiotic events between vegetatively incompatible strains. Therefore, heterokaryon incompatibility function must be inactivated during the sexual cycle. Inactivation is thought to occur through the downregulation of *het* genes (Perkins, 1988).

Heterokaryon incompatibility loci

Heterokaryon incompatibility in *N. crassa* is mediated by at least 12 *het* loci, including the mating-type locus. Each *het* locus has two or three alleles and genetic differences at any one *het* locus restricts growth of the heterokaryon or induces death of the heterokaryotic fusion cell (Xiang & Glass, 2004). Death of the fusion cell occurs through the formation of septal plugs and vacuolization of the protoplasm within the fusion cells (Garnjobst & Wilson, 1956; Perkins, 1988). As a result, the migration of incompatible nuclei through septal pores into adjacent cells is impeded (Perkins, 1988).

Heterokaryon incompatibility is assayed by growing strains with complementary auxotrophic or antibiotic genetic markers on minimal or selective antibiotic medium, respectively, that will not allow the independent growth of either strain alone. Compatibility is displayed as near wild-type growth rates, whereas incompatibility is displayed as no growth or slow growth and phenotypic abnormalities (Perkins, 1988).

The function of *N. crassa mat*, *het-c*, *het-d*, *het-e* and *het-i* in heterokaryon incompatibility were first discovered using heterokaryon incompatibility testing (Beadle & Coonradt, 1944; Garnjobst, 1955; Wilson & Garnjobst, 1966).

Heterokaryon incompatibility has also been studied using partial diploid strains, obtained by crossing translocation strains or by DNA transformation, that contain alternate alleles of one or more *het* locus in the same nucleus. Translocation partial-diploid analysis was used in the place of conventional fusion methods because near isogenic strains with single *het* locus differences were often not available (Perkins, 1975). In partial-diploid analysis one strain with a reciprocal translocation bearing a *het* gene is crossed with a wild-type configuration strain of unknown *het* genotype to generate a non-tandem *het* gene duplication (Perkins, 1975). Progeny that are homozygous for the translocated *het* locus have wild type growth and morphology, similar to compatible heterokaryons (Mylyk, 1975). Conversely, progeny heterozygous for the *het* locus of interest are assessed as incompatible by characteristics of heterokaryon incompatibility such as limited growth, brown pigment production on diagnostic medium, or abnormal growth (Mylyk, 1975; Perkins, 1975). In *N. crassa*, *het-5*, *het-6*, *het-7*, *het-8*, *het-9* and *het-10* were identified using partial diploid analyses (Mylyk, 1975).

As clones of *het* genes become available, heterozygous duplications are being constructed through transformation. Transformation in filamentous fungi generally involves ectopic integration into the genome (Coustou *et al.*, 1999). Introduction of an allele with alternate specificity via transformation results in heterokaryon incompatibility phenotypes. Transformants containing alternate alleles of the *N. crassa het-c* locus display the same phenotypic slow growth as *het-c* heteroallelic translocation partial

diploids and heterokaryons (Saupe *et al.*, 1996b). Similarly, self-incompatible *un-24* heteroallelic transformants show reduced growth and a spidery morphology (Micali, 2003). In the case of the *N. crassa mat*, transformants were nonviable, presumably due to the co-occurrence of alternate *het* alleles (Glass *et al.*, 1990).

un-24-mediated incompatibility

Mylyk (1975) localized the *het-6* locus to a region on linkage group II by genetic analysis of partial diploids. He found that the partial diploids, heterozygous for the *het-6* locus duplication (*Dp(AR18)*), were initially aconidiate, slow growing and restricted to within and on the surface of the growth medium. Partial diploids, homozygous for the duplication, displayed near wild-type growth and conidiation.

Within the *het-6* region, Smith *et al.* (2000b) identified two tightly linked genes, *un-24* and *het-6*, that both mediate heterokaryon incompatibility. Each locus has two allelic variants, Oak Ridge (OR) and Panama (PA). *un-24* and *het-6* are always inherited as a block (recombination between them has not been observed) and therefore each individual has either both OR (*un-24*^{OR} *het-6*^{OR}) or PA (*un-24*^{PA} *het-6*^{PA}) alleles for each gene (Mir-Rashed *et al.*, 2000).

Most *het* genes have polymorphic alternate alleles (Glass *et al.*, 2000) and the *un-24* locus is no exception. The *un-24*^{OR} allele differs from *un-24*^{PA} within the 1.5 kb 3' coding region; 33 amino acids differ between the two alleles in addition to a 18 bp insertion/deletion (Smith *et al.*, 2000b). Smith and coworkers (2000a) created a mutation of *un-24*^{OR} (denoted *un-24*^{OR-}) through repeat induced point mutations (RIP). The *un-24*^{OR-} allele carries three GC→AT transition mutations at positions 247, 613, and 618 of

un-24^{OR}. One of these mutations (cys26→ tyr) is predicted to influence the helical structure of the allosteric activity site of ribonucleotide reductase. Strains carrying this mutation are not able to grow at temperatures above 34°C but grow at near wild-type rates below 30°C. Interestingly, *un-24*^{OR-} strains are able to grow in temperatures above 34°C in conditions of high osmolarity (Smith, 2000a).

un-24 codes for the large subunit of ribonucleotide reductase

The *un-24* gene of *N. crassa* codes for the large subunit (R1; see below) of ribonucleotide reductase (RNR; Smith *et al.*, 2000a), which is also known as ribonucleoside diphosphate reductase (Elledge *et al.*, 1992). RNR catalyses the first step in the synthesis of DNA (the conversion of NDPs to dNDPs (Elledge *et al.*, 1992)) and maintains appropriate levels and ratios of the four dNTPs for DNA replication (Thelander & Reichard, 1979).

The type I RNR of *N. crassa* has a $\alpha_2\beta_2$ structure. The two homodimers are denoted R1 and R2 (Uhlen & Eklund, 1994). Monomers of the large subunit, R1, contain two allosteric binding sites for dNTPs. One site maintains balanced nucleotide pools and controls substrate specificity. The second allosteric site monitors ATP/dATP levels and modulates overall enzyme activity (Thelander *et al.*, 1980). The small subunit, R2, contains iron and tyrosyl free radical residuals involved in catalysis (Petersson *et al.*, 1980; Mann *et al.*, 1991). The R1 subunit of *N. crassa* (UN-24) has a similar amino acid sequence to those of other organisms, except for a lengthy unique insertion near the C-terminus which is postulated to contain the heterokaryon incompatibility specific domain (Smith *et al.*, 2000a).

Regulation of RNR

Expression of the RNR enzyme is highly regulated (Elledge *et al.*, 1993) with both the large and small subunits of RNR regulated during the cell cycle in *S. cerevisiae*. A greater than 10-fold fluctuation in *RNR1* mRNA and a 2-fold fluctuation in *RNR2* mRNA occurs throughout the cell cycle. Transcript levels are highest at the S phase (Elledge & Davis, 1990). A corresponding fluctuation in R1 and R2 protein has not been assayed. During the cell cycle of mammals the R2 protein fluctuates and the R1 protein remains constant but the mRNA of both are cell cycle regulated (Björklund *et al.*, 1990).

There have been few studies of RNR activity during the reproductive cycle, except for a few marine invertebrates (Noronha *et al.*, 1972; Standart *et al.*, 1985; Standart *et al.*, 1986). Standart and coworkers (1986) found that during the sexual stage of the surf clam, *Spisula solidissima*, mature oocytes (female gametes) contain the maximum level of R1 protein. In this species, the mRNA for the small subunit of RNR is stored until after fertilization, when it is translated and joins with R1 to form an active enzyme. After fertilization, the activity of R1 decreases to trace levels. The stores of R1 protein and R2 mRNA allow for rapid dNTP synthesis necessary for the early embryonic cell divisions (Standart *et al.*, 1986).

Regulation of RNR during the *N. crassa* life cycle has recently been explored. Gibbs and Smith (unpublished; Appendix A) found that the concentration of *un-24^{PA}* mRNA increased 2-fold when strains were grown on a medium that promotes the sexual cycle. The results suggested that the levels of *un-24^{PA}* (but not *un-24^{OR}*) transcripts increase at the onset of the sexual cycle to increase the pool of dNTPs in preparation for meiotic division and subsequent mitotic events during hyphal extension. This is

surprising since one would expect that *un-24* incompatibility function would be downregulated in sexual structures to prevent a heterokaryon incompatibility reaction, however *un-24* transcript levels have not been tested within protoperithecia or perithecia.

Escape from incompatibility

Heterokaryons (DeLange, 1975; Pittenger, 1964), translocation partial diploids (Newmeyer & Taylor, 1967; Perkins, 1972, 1975; Smith *et al.*, 1996) and incompatible transformants (Coustou *et al.*, 1999; Xiang & Glass, 2002; Xiang *et al.*, 2002; Xiang & Glass, 2004) escape from *het*-mediated growth inhibition. Mechanisms of escape are diverse, and often more than one is employed (Xiang & Glass, 2002; Micali, 2003; Xiang & Glass, 2004). Escape has been linked to nuclear segregation (Micali, 2003; Xiang & Glass, 2004), deletion or mutation of one *het* allele, and mutations at unlinked suppressor loci (Newmeyer, 1970; DeLange & Griffiths, 1975; Vellani *et al.*, 1994; Smith *et al.*, 1996; Xiang & Glass, 2002; Xiang & Glass, 2004).

Inactivation of either the endogenous or ectopic duplicated allele during escape occurs through mutational events (Nga & Roper, 1968; DeLange & Griffiths, 1975; Griffiths, 1982; Smith *et al.*, 1996; Coustou *et al.*, 1999). Molecular characterization of *het* alleles after escape shows that the mutational events range from large deletions (Smith *et al.*, 1996) to single amino acid replacements (Coustou *et al.*, 1999). The ectopic (Smith *et al.*, 1996) or endogenous allele (Coustou *et al.*, 1999) may be preferentially inactivated.

Escape also occurs through mutations in suppressor loci (Smith *et al.*, 1996; Newmeyer, 1970; DeLange & Griffiths, 1975; Vellani *et al.*, 1994; Xiang & Glass, 2002;

Xiang & Glass, 2004; Pittenger, 1964). Suppressors of incompatibility have been identified in escape strains that are either incompatible (recessive suppressor) or compatible (dominant suppressor) with tester strains carrying either allele (Smith *et al.*, 1996). Two suppressors have been identified in *N. crassa*. The *tol* mutation is a recessive suppressor of mating-type incompatibility (Newmeyer, 1970). In bisexual heterokaryons ($A + a$) where *tol* is present in one nuclear type but not both, escape may occur through deletion or mutation of the wild-type *tol*⁺ copy resulting in two recessive suppressor mutations (DeLange & Griffiths, 1975). *vib-1* is a dominant loss of function mutation that suppresses *het-c*-mediated incompatibility (Xiang & Glass, 2002). The mutational events in *vib-1*⁺, a gene required for *het-c*-mediated incompatibility, have been shown through molecular characterization to result from complex chromosome rearrangements including deletions that are sometimes coupled with chromosome rearrangements such as inversions and translocations. The deletions occur at double stranded breakpoints and are repaired through non-homologous end joining (Xiang & Glass, 2004).

Escape from un-24-mediated incompatibility

Escape from incompatibility in translocation partial diploids containing heteroallelic duplications of both *un-24* and *het-6* (*Dp(AR18)*) occurs infrequently (~24%) and slowly (after ~20 days). Escape most often occurs through a 70 kb to 270 kb (the entire translocated region) deletion of the ectopic *un-24*^{OR} *het-6*^{OR} region (Smith *et al.*, 1996).

Escape has also been characterized in self-incompatible partial diploids heteroallelic for *un-24* but homoallelic for *het-6*. The partial diploids were constructed through transformation of *un-24*^{PA} into an *un-24*^{OR} strain. Escape strains either returned to the pre-transformation *un-24*^{OR} phenotype and/or acquired a novel *un-24*^{PA} phenotype. Escape occurred through putative mechanisms including nuclear segregation, inactivation of either the ectopic or endogenous allele, and mutations in suppressor loci (Micali, 2003).

Objectives

The objectives of this study were to (1) quantify transcript levels in stable incompatible strains grown in media that promote either vegetative or sexual growth cycles, (2) verify the presence or absence of *un-24*^{OR} and *un-24*^{PA} alleles in escape strains as previously detected by RFLP analysis, (3) semi-quantify transcript levels in escapes transcribing both *un-24*^{OR} and *un-24*^{PA} copies, and (4) test the hypothesis that *col-PA* escapes are functionally *un-24*^{PA} *het-6*^{OR}.

METHODS AND MATERIALS

The general approach of this research was to use qualitative and semi-quantitative RT-PCR (reverse transcription PCR) to estimate the presence and number of *un-24*^{OR} and *un-24*^{PA} transcripts present in (1) C9-2 *un-24*^{OR/PA} stable self-incompatible strains, SIA and SIB, grown in low and high nitrogen media, (2) C9-2 *un-24*^{OR/OR} partial diploid self-compatible strains, (3) C9-2 *un-24*^{OR/PA} partial diploid self-incompatible strains before

escape, (4) C9-2 *un-24*^{OR/PA} escape strains, and (5) col(4)-2 *un-24*^{OR/PA} escape strains.

In addition, an attempt to develop a polyclonal antibody against UN-24 was unsuccessful but documented in Appendix B.

N. crassa strains, characteristics and growth conditions

Strains used in the study of *un-24* transcription are given in Table 1.1. Strains were maintained on Vogel's minimal medium (VMM; 1X Vogel's salts (3 g Na₃citrate·5H₂O, 2 g NH₄NO₃, 5 g KH₂PO₄, 200 mg MgSO₄·7H₂O, 100 mg CaCl₂·2H₂O, 250 mg citric acid·H₂O, 250 mg ZnSO₄·7H₂O, 50 mg Fe(NH₄)₂(SO₄)₂·6H₂O, 12.5 mg CuSO₄·5H₂O, 2.5 mg MnSO₄·H₂O, 2.5 mg H₃BO₃, 2.5 mg Na₂MoO₄·2H₂O, 5 pg biotin per litre; (Vogel, 1964)), 1.5% (w/v) sucrose, 1.5% (w/v) agar and nutritional additives (250 µg/mL threonine for *thr-2* mutants and 250 µg/mL uridine and 50 µg/mL inositol for *pyr-4 inl* mutants (Davis & De Serres, 1970))). col-PA1 and col-PA4 were maintained at 37°C; the remaining strains were grown at 30°C.

un-24^{OR/PA} escape strains e17, e18, e22, e26, e28, e36, e38, e40, e42, and e48 were characterized by Micali (2003). Escape strains of either *un-24*^{OR}, *un-24*^{PA}, or both *un-24*^{OR} and *un-24*^{PA} phenotypes (see Table 1.1 for designations) were assayed through heterokaryon incompatibility testing with tester strain C8c-32 (*un-24*^{OR} *het-6*^{OR}) and functionally *un-24*^{PA} *het-6*^{OR} strains col-PA1 and col-PA4 (described below). Escape strains having one or both *un-24* alleles were identified using *Bam*HI RFLP (Restriction Fragment Length Polymorphism) analysis and PCR RFLP analysis (Table 1.2). SIA (Micali, 2003) and SIB (from the current study; derivation given below) are self-incompatible strains that did not escape from *un-24*^{OR/PA} incompatibility.

Table 1.1. *N. crassa* strains used in this study. All strains have the Oak Ridge allele at *het* loci not mentioned below.

Strain	Genotype ^a	<i>un-24</i> phenotype ^b	Origin
C9-2	<i>un-24</i> ^{OR} <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR}	Smith <i>et al.</i> , 2000b
C2(2)-1	<i>un-24</i> ^{PA} <i>het-6</i> ^{PA} <i>het-c</i> ^{OR} <i>thr-2 a</i>	<i>un-24</i> ^{PA}	Smith <i>et al.</i> , 1996
col(4)-2	<i>un-24</i> ^{OR-} <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>pyr-4; inl a</i>	<i>un-24</i> ^{OR}	ML Smith
SIA	<i>un-24</i> ^{OR/PA} <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>pyr-4; inl a</i>	<i>un-24</i> ^{OR} and <i>un-24</i> ^{PA}	Micali, 2003
SIB	<i>un-24</i> ^{OR/PA} <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>pyr-4; inl a</i>	nt ^c	This study
col-PA1	<i>un-24</i> ^{OR-/PA} (esc ^a) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>pyr-4; inl a</i>	<i>un-24</i> ^{PA}	Micali, 2003
col-PA4	<i>un-24</i> ^{OR-/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>pyr-4; inl a</i>	<i>un-24</i> ^{PA}	Micali, 2003
e13	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR}	Micali, 2003
e26	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR}	Micali, 2003
e28	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR}	Micali, 2003
e12	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR}	Micali, 2003
e38	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR}	Micali, 2003
e17	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{PA}	Micali, 2003
e18	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{PA}	Micali, 2003
e36	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{PA}	Micali, 2003
e40	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{PA}	Micali, 2003
e42	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{PA}	Micali, 2003
e22	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR} and <i>un-24</i> ^{PA}	Micali, 2003
e48	<i>un-24</i> ^{OR/PA} (esc) <i>het-6</i> ^{OR} <i>het-c</i> ^{PA} <i>thr-2 a</i>	<i>un-24</i> ^{OR} and <i>un-24</i> ^{PA}	Micali, 2003

^aesc indicates that transformants had escaped from self-incompatibility

^b*un-24* incompatibility phenotype determined through heterokaryon incompatibility testing with tester strains C9-2 or C8c-32 (*un-24*^{OR} *het-6*^{OR}) and functionally *un-24*^{PA} *het-6*^{OR} strains col-PA1 and col-PA4.

^cnot tested

Table 1.2. Characteristics of C9-2 *un-24*^{OR/PA} escape strains based on heterokaryon incompatibility testing, RFLP analysis and allele-specific PCR and RT-PCR assays. Heterokaryon compatibility and incompatibility with C8c-32 (*un-24*^{OR}) and co1-PA strains (functionally *un-24*^{PA} *het-6*^{OR}) is indicated by + and -, respectively. C9-2-like and C9-2-unlike indicates that strains displayed the same RFLP pattern as C9-2 or C9-2 plus additional bands, respectively. The presence or absence of *un-24*^{OR} and/or *un-24*^{PA} copies in escape strains as assayed by PCR RFLP analysis and allele specific PCR is indicated by + and -, respectively. (+) indicates a faint PCR product. The presence or absence of *un-24*^{OR} and/or *un-24*^{PA} transcripts in escape strains as assayed by allele specific RT-PCR is indicated by + and -, respectively. The time after escape at which the assay was performed is given in parentheses. Escape strains are arranged by similarity.

Escape strain	C8c-32 (<i>un-24^{OR}</i>) compatible (day 9) ^a	<i>Bam</i> HI RFLP analysis (days 27-52) ^{a,b}	PCR RFLP analysis (days 41-60) ^{a,c}		col-PA (<i>un-24^{PA}</i>) compatible (1 year) ^a	Allele specific PCR assay (~2 years) ^d		Allele specific RT-PCR assay (~2 years) ^e	
			<i>un-24^{OR}</i>	<i>un-24^{PA}</i>		<i>un-24^{OR}</i>	<i>un-24^{PA}</i>	<i>un-24^{OR}</i>	<i>un-24^{PA}</i>
e13	+	C9-2 like	+	-	-	-	-	+	-
e26	+	C9-2 like	+	-	-	+	-	+	-
e28	+	C9-2 like	+	-	-	+	-	+	-
e12	+	C9-2 unlike	+	+	-	+	-	+	-
e38	+	C9-2 unlike	+	+	-	+	-	+	-
e22	-	C9-2 unlike	+	+	-	+	+	+	+
e48	-	C9-2 unlike	+	+	-	+	(+)	+	+
e17	-	C9-2 unlike	+	+	+	(+)	-	+	+
e18	-	C9-2 unlike	+	+	+	+	(+)	+	+
e36	-	C9-2 unlike	+	+	+	+	(+)	+	+
e40	-	C9-2 unlike	+	+	+	-	-	+	+
e42	-	C9-2 unlike	+	+	+	+	+	+	+

^adata provided by Micali (2003).

^bDNA digested with *Bam*HI and probed with *un-24^{OR}* DNA. Strains with restriction fragments in addition to C9-2 pattern are "C9-2 unlike"

^c6J-P11/6J-P6 primer (Smith *et al.*, 2000b) PCR products digested with *Pvu*I.

^dFigure 1.11

^eFigure 1.12

Strains col-PA1 and col-PA4 are escape strains derived from self-incompatible transformants of col(4)-2 provided by OC Micali that are functionally *un-24^{PA} het-6^{OR}*. The strains were created by transforming *un-24^{PA}* into col(4)-2, which carries a temperature sensitive allele of *un-24^{OR}*, denoted *un-24^{OR-}*. The self-incompatible transformants (denoted *un-24^{OR-/PA}*) were allowed to escape from incompatibility at 37°C. Growth at 37°C selects for escapes that express the ectopic *un-24^{PA}* copy but not the temperature sensitive *un-24^{OR-}* copy (Micali, 2003).

For the purpose of DNA and RNA isolation, the above strains were grown for 2-6 days on liquid 1X Vogel's salts, 1.5% (w/v) sucrose with the same temperatures (37°C for col(4)-2 escapes and 30°C for all others) and supplements as described above.

Obtaining pre-escape self-incompatible strains

Fungal transformation was used to create self-incompatible and -compatible partial diploids with an endogenous *un-24^{OR}* copy and an ectopic *un-24^{PA}* or *un-24^{OR}* copy, respectively. The reciprocal transformation (*un-24^{OR}* transformed into C2(2)-1), was not performed because Smith *et al.* (2000b) showed that these transformants exhibit complete heterokaryon incompatibility (i.e. no growth).

Escherichia coli DH5α containing the plasmid pCB1004 (Carroll *et al.*, 1994) carrying either *un-24^{OR}* (bound by primer sequences 6J-P3 and 6J-P6) or *un-24^{PA}* (bound by restriction sites *Cla*I and *Pst*I), were grown overnight in liquid LB medium containing 15 µg/mL chloramphenicol. pCB1004 contains hygromycin and chloramphenicol resistance genes which are selectable markers for *N. crassa* and *E. coli*, respectively.

From each *E. coli* strain, plasmid DNA was extracted using the Wizard™ kit (Promega, Madison, Wisconsin). This procedure involves the alkaline lysis of bacterial cells, digestion of RNA, removal of genomic DNA through renaturation and differential centrifugation, adhesion of plasmid DNA to Wizard™ resin and elution of plasmid DNA.

N. crassa spheroplasts of strain C9-2 were created and provided by OC Micali using methods similar to those described by Royer and Yamashiro (1992). Using the *un-24^{OR}* and *un-24^{PA}* pCB1004 DNA isolated from *E. coli* cells, two transformations were performed. Strain C9-2 *N. crassa* spheroplasts (*un-24^{OR}*) were transformed separately with *un-24^{PA}* and *un-24^{OR}* to acquire *un-24^{OR/PA}* and *un-24^{OR/OR}* partial diploids, respectively. Approximately 1 µg of DNA was added to 100 µl of spheroplasts ($\sim 10^8$) and incubated on ice for 30 minutes. Each sample was gently mixed in 1 mL of 40% (v/v) polyethylene glycol (PEG)/10 mM 4-morpholinepropanesulfonic acid (MOPS)/50 mM CaCl₂ and incubated at room temperature for 20 minutes. Spheroplasts plus DNA was mixed with 6 mL of top agar (1X Vogel's salts, 182.2 g/L sorbitol, 20 g/L L-sorbose, 0.5 g/L fructose, 0.5 g/L D-glucose, 0.2 g/L myo-inositol, 1.25% (w/v) agar) and poured evenly over plates containing 25 mL of bottom agar (1X Vogel's salts, 20 g/L L-sorbose, 0.5 g/L fructose, 0.5 g/L D-glucose, 0.2 g/L myo-inositol, 3.95 µL/mL hygromycin (Roche Molecular Biochemicals, Mannheim, Germany), 1.5% (w/v) agar, 250 µg/mL threonine). Sorbose contained in the bottom agar induces colonial growth of transformants (Micali, 2003), and sorbitol in the top agar maintains a high osmotic pressure and therefore prevents osmotic shock and breakage of the spheroplasts (O.C. Micali, personal communication).

N. crassa spheroplasts usually contain more than one nucleus, resulting in a mixed population of transformed and untransformed nuclei within the transformed cell. The *un-24^{OR}* or *un-24^{PA}* gene contained in the vector is randomly integrated into the genomic DNA, however not every nucleus contained within each spheroplast cell will accept foreign DNA. To reduce the co-occurrence of transformed and non-transformed nuclei within each *N. crassa* cell, a series of subcultures was performed. After four days of growth on transformation medium, transformed colonies were transferred onto hygromycin-free solid VMM containing threonine (one transformant per petri dish) and grown at 30°C. After one day's growth, a 1 mm block of medium was taken from the edge of each colony and subcultured onto VMM with threonine. Each transformant was subcultured twice more. *un-24^{OR/PA}* transformants that escaped from incompatibility were discarded.

Transformants were grown in 25 mL of liquid Vogel's medium plus threonine at 30°C in the dark. *un-24^{OR/OR}* transformants were grown for 2-4 days and each *un-24^{OR/PA}* transformant was grown for 2-8 days in three separate flasks. Only slow growing (i.e. presumably unescaped) *un-24^{OR/PA}* mycelia were used in nucleic acid extraction. One *un-24^{OR/PA}* self-incompatible strain (SIB) remained slow growing.

Stable incompatible strains grown on low and high nitrogen media

Self-incompatible, hygromycin resistant strains SIA and SIB (Table 1.1) were used to study the transcription of *un-24^{OR}* and *un-24^{PA}* during vegetative and sexual life cycle phases. To induce the asexual and sexual life cycle phases of *N. crassa*, stable incompatible strains were grown for five days in liquid VMM and Synthetic Crossing

Medium (SCM; Westergaard & Mitchell, 1947; Vogel, 1964), respectively, at room temperature (20°C - 25°C) and with ambient light. SCM is a low nitrogen medium with 1 g/L KNO₃ (compared to Vogel's with 2 g/L NH₄NO₃) that promotes sexual reproduction (Westergaard & Mitchell, 1947). Threonine concentration in SCM was reduced to 62.5 µg/mL.

RNA and DNA extraction and quantification

All metal and glass instruments for RNA extraction were made free of RNase by baking overnight at 200°C and all solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) or dimethyl pyrocarbonate (DMPC) and autoclaved. Plugged RNase free pipette tips were used to dispense solutions.

Mycelia were vacuum-filtered through 3 mm paper, washed with sterile 0.9% (w/v) NaCl to remove growth medium, and dried briefly under vacuum. Mycelia used in RNA extractions were weighed, flash-frozen in liquid nitrogen, and stored at -76°C until processed.

Frozen tissue (approximately 100 mg) was ground in liquid nitrogen using mortar and pestle and RNA was extracted using the TRIzol reagent (Invitrogen, Burlington, ON). One mL of TRIzol was added to the ground tissue and homogenized thoroughly using mortar and pestle. All subsequent steps of the extraction were performed on ice. The homogenate was transferred to a 1.5 mL microfuge tube and mixed with 200 µl of chloroform by vortexing for 15 seconds. To separate protein, DNA and RNA, samples were centrifuged at 12000 x g for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred to a new tube and the interphase and lower aqueous

phase were retained for DNA extraction (see below). To further remove contaminating DNA and protein, the RNA samples were centrifuged at 12000 x g for 1 minute at room temperature and the upper aqueous phase was again transferred to a new tube. To precipitate the RNA, 0.5 mL of isopropanol was added, the tubes were inverted twice and incubated at room temperature for 10 minutes. To pellet the RNA, samples were centrifuged at 12000 x g for 10 minutes at 4°C. The supernatant was decanted and 1 mL of 70% ethanol was added to the RNA pellet. The tubes were inverted twice and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was decanted and the pellet was allowed to dry approximately 20 minutes at room temperature. The RNA was dissolved in 50 µL of DEPC treated water and stored at -76°C.

To quantify RNA samples, total RNA from each strain was subjected to electrophoresis on a denaturing 1.3% (w/v) agarose gel (8.9% (v/v) formaldehyde) in 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate trihydrate and 1 mM EDTA, pH7). To prepare the samples, 6 µL of RNA loading buffer (1X MOPS buffer, 51.3% (v/v) formamide, 6.6% (v/v) formaldehyde, 5.1% (v/v) glycerol, 25.6 mM EDTA, pH 8.0, 0.051% (w/v) ethidium bromide and 0.25% (w/v) bromophenol blue) was added to 2 µL of total RNA. Samples were heated at 65°C for 20 minutes, placed on ice for 5 minutes, and loaded into wells. The RNA samples were electrophoresed for approximately 3 hours (or until the bromophenol blue migrated half the length of the gel) at 55 V. The gel was photographed over UV light. 3 µg of a 0.24 – 9.5 Kb RNA ladder (Invitrogen, Burlington, ON) was used to quantify the amount of RNA in each sample. Densitometry was performed using the autogrid function of AlphaEaseFC (Alpha Innotech Corp., San

Leandro, CA), which averages the pixel intensities along the width of a gel lane and then sums the average pixel intensities along the length of the lane for each band.

The lower aqueous phase and interphase of the TRIzol extractions described above were retained to extract DNA. The remaining upper aqueous phase was removed from the samples. The DNA was precipitated by adding 0.3 mL of ethanol, mixing by inversion, and centrifuging at 2000 x g for 5 minutes at 4°C. The phenol ethanol supernatant was removed and the DNA pellets were washed three times in a solution containing 0.1 M sodium citrate in 10% (v/v) ethanol. During each wash, 1 mL of the solution was incubated with the pellet at room temperature for 30 minutes with periodic mixing, samples were centrifuged at 2000 x g for 5 minutes at 4°C, and the supernatant was decanted. The DNA pellet was suspended in 1.5 mL of 75% (v/v) ethanol, incubated for 20 minutes at room temperature and centrifuged at 2000 x g for 5 minutes at 4°C. The supernatant was removed and the pellets were air dried at room temperature for 20 minutes. The DNA was dissolved in 300 µL of 8 mM NaOH, incubated at 4°C overnight and neutralized with 86 µL of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). To remove insoluble materials the samples were centrifuged at 2000 x g for 5 minutes at 4°C and the supernatant was retained.

Qualitative PCR

Qualitative PCR was used to detect *un-24*^{OR} and/or *un-24*^{PA} DNA sequences in self-incompatible and -compatible partial diploids, escape strains and stable self-incompatible strains grown in low and high nitrogen media. Allele specific primers were designed to anneal to the 3' polymorphic region of *un-24* DNA. The *un-24*^{OR} specific

reverse primer UN24OR-1 (5'-CTTGTTGCTCAGAAAGTGGAGCATT-3') and the *un-24^{PA}* specific reverse primer UN24PA-1 (5'-TGAGACTACCTCATCAGAGTAGATG-3') when used with the forward primer 6JP-35 (5'-CAGCCCATCCAGTTCACTGTAGA-3') produce 490 bp (*un-24^{OR}*) and 480 bp (*un-24^{PA}*) PCR products (Figure 1.2). The *un-24^{OR}* specific primer was designed to amplify both wild type *un-24^{OR}* and mutant *un-24^{OR}* DNA. Each DNA sample was amplified separately using the *un-24^{PA}* and *un-24^{OR}* specific primers. Each 25 µL PCR reaction contained 1 µL of DNA, 1X PCR buffer, 1 µM 6JP-35, 1 µM UN24OR-1 or UN24PA-1, 1.5 mM MgCl₂, 200 µM dNTPs, 1 u/µL *taq* DNA polymerase and the reaction conditions were 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 66°C for thirty seconds, 72°C for 1 minute; 72°C for 10 minutes, 4°C soak, and was performed in a Biometra® (Göettingen, Germany) TGradient thermocycler.

Semi-quantitative and qualitative RT-PCR

Qualitative RT-PCR was used to detect the presence or absence and levels of *un-24^{OR}* and/or *un-24^{PA}* transcripts from self-incompatible and -compatible partial diploids, escape strains and stable self-incompatible strains grown in low and high nitrogen media.

Reverse primers used in RT-PCR were designed to be both cDNA and allele specific (Figure 1.2). For allele specificity, reverse primers were designed near the polymorphic 3' end of the *un-24* coding region. The *un-24^{OR}* specific reverse primer 6JP-OR2 (5'-CGGGATTCCAGGCGCAAGCAG-3') and the *un-24^{PA}* specific primer 6JP-PA2 (5'-CGGGAACGTAGGGGCATGAGA-3') when used with the forward primer 6JP-35 (5'-CAGCCCATCCAGTTCACTGTAGA-3') gave PCR products of 514

Figure 1.2. 662 bp alignment of the of *un-24*^{OR} and *un-24*^{PA} 3' coding region. The allele specific primer sequences used in RT-PCR (6JP-OR2 and 6JP-PA2) and PCR (UN24OR-1 and UN24PA-1) and the universal forward primer 6JP-35 are indicated with arrows. 2334 bp of the upstream *un-24* coding region is not shown.

un - 24 PA GGGTCTCAAGACGGGCATGTACTACCTCCGTACCCAGGCTGCTGCTCAGCCCCATCCAGTTCACCTGTAGACCAGGAGGCTCTCAGGGCCACGGATGACCGAG
un24OR GGGTCTCAAGACCGGCATGTACTACCTCCGTACGCAGGCTGCTGCTCAGCCCCATCCAGTTCACCTGTAGACCAGGAGGCTCTCAGGGCCACGGATGACCGAG

un - 24 PA TGGCCCCAGCCCACTCGGGTCTTAAGAAAGCGTCCCCCTCCCGCTGGCACTTATACGCTATCGTGTGAGGGAGAACACTTTCGGGGCCCCCGCCCGTATGCC
un24OR TGGCCCCAGCCCACTCGGGTCTTAAGAAAGCGTCCCCCTCCCGCTGGCACTTATACGCTATCGTGTGAGGGAGAACACTTTCGGGGCCCCCGCCCGTATGCC

un - 24 PA CAGACTGGTGTAGTGGCACTAGCACACCCATCGGCACCTAGGGATGTTCCACACCCGCCAGCACACCTCCTCTGCTGAAGATTCTGAGACTCTTGATACA
un24OR CAGACTGGTGTAGTGGCACCTAGCACACCCATCGGCACCCAGGGATGTTCCACACCCGCCAGCACACCTCCTCCCACTGAGGTTCTGAAAACCTTTGTACA

un - 24 PA GAGTGACAAAGAGGCCCGGCCACCTGGTCTCGCCCGCCAGAAATGCCGGCTTCAAGCCGATCTGCCCGAGCCTGAGAGCCCAAGGCTCTGGCCACTGACCG
un24OR GAGTGACAAAGAGGCCCGGTCCTCACTTGTCTCGCCCGCCAGAGTGCCGGTTTCAAGCCGATTTGCCCGAGCCTGAGAGCCCAAGGCTCTGGCCACTGACCG

un - 24 PA CAATTGTCAAGACCGAGGCCATTAGTCTCCGGGAGCTGGAGATGAAGGAGGGCCAGAGTGAAGCAAGGACGAGGAGAGCAAGGACAGACAGCGCGCA
un24OR CGATTGTTAAGACCGAGGACATTGGTCCACCGTCTGGAGAGAAAGGAGGGCCAGAAATGAGGACGTGGACGGAAGATAGCCAGGACGAGGACGAGAA

bp (*un-24^{OR}*) and 496 bp (*un-24^{PA}*). To prevent the primers from binding to *un-24* DNA, the allele specific reverse primers spanned a 63 bp intron of the *un-24* locus (Figure 1.2). Note that the *un-24^{OR}* specific reverse primer was designed to amplify both *un-24^{OR}* and *un-24^{OR-}* (the temperature sensitive mutant). To ensure that RNA only was amplified using RT-PCR, RNA from each strain was amplified without reverse transcriptase and omitting the reverse transcription incubation step.

Both end point RT-PCR and semi-quantitative RT-PCR were performed using the Access RT-PCR System (Promega, Madison, WI). Each RT-PCR reaction contained 1X AMV/*tfl* reaction buffer, 0.2 mM each dNTP, 1 μ M 6JP-35, 1 μ M 6JP-PA2 or 6JP-OR2, 1 mM MgSO₄, 0.1 u/ μ L AMV reverse transcriptase, and 0.1 u/ μ L *tfl* polymerase. End point PCR and semi-quantitative RT-PCR reactions contained 2.5 ng/ μ L and 1 ng/ μ L of total RNA, respectively. Conditions for RT-PCR were as follows: 30°C soak; 48°C for 45 minutes; 94°C for 2 minutes; 40 cycles of 94°C for 30 seconds, 62°C for 1 minute, 68°C for 1 minute; 68°C for 7 minutes; 4°C soak performed in a Biometra® (Göettingen, Germany) TGradient thermocycler. To detect the presence or absence of *un-24^{OR}* and *un-24^{PA}* transcripts, all RNA samples were amplified separately using the primer pairs 6JP-OR2/6JP-35 and 6JP-PA2/6JP-35, respectively.

To compare the amount of RNA in each sample, semi-quantitative RT-PCR was performed with primers specific to the *sbr* gene of *N. crassa*. *sbr* is a low abundance, constitutively expressed gene (PJ Vierula, pers. comm.). The forward primer *sbr-yy11* (5' -GATGGAGGACCAAGATGCCTCTGC- 3') and the reverse primer *sbr-yy9a* (5' -TGGGGGTTGCGCGTTTCGTGAA- 3') gave an estimated product of 712 bp. The reverse primer *sbr-yy9* spanned a 64 bp intron of the *sbr* gene.

The *un-24*^{OR}, *un-24*^{PA} and *sbr* transcript levels were quantified using RT-PCR in (1) three control strains (2) three self-incompatible and three self-compatible C9-2 partial diploids, (3) two *col(4)-2* escape strains, (4) three C9-2 escape strains where both alleles of *un-24* were previously detected, and (5) two stable self-incompatible strains grown in low nitrogen (at 25°C) and high nitrogen (at 25°C and 30°C) media. Semi-quantitative RT-PCR was performed with three samples at a time. First, a grand master mix of water, AMV reaction buffer, dNTPs, MgSO₄, AMV RT and *tfl* polymerase was made. Second, the grand master mix was separated into three and to each an RNA sample was added. Third, each RNA master mix was separated into three and one of the primer sets 6JP-OR2 and 6JP-35, 6JP-PA2 and 6JP-35, and *sbr-yy9a* and *sbr-yy11* was added to each. The result was nine 100 µL PCR reactions: three RNA samples each with three primer sets. Each 100 µL volume was separated into 10 * 10 µL aliquots. After every fourth temperature cycle, one 10 µL aliquot for each of the nine reactions was removed from the thermocycler and placed at 4°C.

To establish a relationship between the amount of PCR product and transcript copy number, semi-quantitative RT-PCR was performed on a 1.2 kb kanamycin resistance gene mRNA ($5 * 10^4$ copies/µL) provided with the Access RT-PCR System. Amplification of this mRNA using the control RNA upstream primer 5'-GCCATTCTCACCGGATTCAAGTCGTC- 3' and control RNA downstream primer 5'-AGCCGCCGTCCCGTCAAGTCAG- 3' produces a 323 bp product. Semi-quantitative RT-PCR was performed over a range of five fold dilutions ranging from 2 to 200000 copies/µL.

Five μL of each RT-PCR aliquot was electrophoresed on a 1% acrylamide gel in 1X TAE (242 g/L Tris base, 5.71% (v/v) glacial acetic acid, 50 mM EDTA pH 8), stained in 0.25 mg/L ethidium bromide for 30 minutes and destained in water for 30 minutes. The molecular weight was determined using a 100 bp DNA ladder (Invitrogen, Burlington, ON).

Densitometry was performed using AlphaEaseFC (Alpha Innotech Corp., San Leandro, CA). In general, the intensity of PCR products increased with the PCR cycle. The pixel intensity of a rectangular area surrounding each band, and including some background, was quantified by summing the intensity of each pixel in the area. The pixel intensity of each band was quantified starting at the 40 cycle reaction and in decreasing order until there was no longer a change in intensity. The intensity of the last two aliquots with unchanging intensities was assigned as the background intensity for that reaction. The sum of the pixel intensity in the background band was subtracted from the intensity for each aliquot of the reaction to give integrated density values (IDVs).

In general, the IDVs in each reaction increased linearly with cycle number and then reached a plateau. The x intercept of a line of best fit drawn through the linear portion of the curve was taken as the critical threshold (C_T) given in cycle number.

The number of *un-24^{OR}*, *un-24^{PA}* and *sbr* transcripts in each reaction was extrapolated from a standard curve of control RNA C_T related to copy number. The copy number in each reaction was normalized using the *sbr* copy number to account for variations in RNA concentration.

RESULTS

Primer specificity

Reverse primers UN24OR-1 and UN24PA-1 were tested for selective amplification of *un-24^{OR}* and *un-24^{PA}* DNA, respectively. When DNA was amplified with primers UN24OR-1 and 6JP-35, a ~500 bp product was observed for strains C9-2 (*un-24^{OR}*) and col(4)-2 (*un-24^{OR}*) but not C2(2)-1 (*un-24^{PA}*). Conversely, DNA amplified with primers UN24PA-1 and 6JP-35 yielded products for C2(2)-1 (*un-24^{PA}*) but not C9-2 (*un-24^{OR}*) nor col(4)-2 (*un-24^{OR}*; Figure 1.3).

The RT-PCR reverse primers 6JP-OR2 and 6JP-PA2 with forward primer 6JP-35 did not amplify a product without the reverse transcriptase and the reverse transcription step (Figure 1.4) and were allele specific (Figure 1.5). Using 6JP-OR2, a ~500 bp product was amplified by RT-PCR from C9-2 (*un-24^{OR}*) and col(4)-2 (*un-24^{OR}*) but not C2(2)-1 (*un-24^{PA}*) RNA, respectively. Conversely, using 6JP-PA2, a ~500 bp product was amplified from C2(2)-1 (*un-24^{PA}*) but not C9-2 (*un-24^{OR}*) nor col(4)-2 (*un-24^{OR}*) RNA (Figure 1.5). Some non-specific product was evident in the 700 bp – 400 bp size range following semi-quantitative RT-PCR (C9-2 *un-24^{PA}* = 0.03 copies/*sbr* copy, C2(2)-1 *un-24^{OR}* < 0.01 copies/*sbr* copy, col(4)-2 *un-24^{PA}* = 0.01 copies/*sbr* copy).

un-24^{OR} and un-24^{PA} DNA and transcripts in self-incompatible strains grown in low and high nitrogen media

Transcript levels of *un-24^{OR}* and *un-24^{PA}* were assayed in stable self-incompatible strains SIA and SIB grown separately in low (SCM) and high nitrogen (VMM) media at

Figure 1.3. Selective amplification of *un-24*^{OR} (both C9-2 and col(4)-2) and *un-24*^{PA} (C2(2)-1) DNA with reverse primers UN24OR-1 and UN24PA-1, respectively, and forward primer 6JP-35.

Figure 1.4. Specificity of *un-24* allele-specific primers 6JP-OR2 and 6JP-PA2 to RNA. *un-24*^{OR} cDNA (positive control), and RNA from C9-2, C2(2)-1 and co1(4)-2 were amplified using the same conditions as with RT-PCR (see text for conditions) without AMV reverse transcriptase nor the reverse transcription incubation step. Amplification was performed with both primer pairs 6JP-OR2/6JP-35 and 6JP-PA2/6JP-35 as indicated. A cDNA of *un-24*^{PA} is not available.

Figure 1.5. Selective RT-PCR amplification of *un-24*^{OR} (both wild type C9-2 and *un-24* mutant col1(4)-2) and *un-24*^{PA} (C2(2)-1) transcripts with reverse primers 6JP-OR2 and 6JP-PA2, respectively, and forward primer 6JP-35.

room temperature. Both $un-24^{OR}$ and $un-24^{PA}$ alleles were detected in DNA samples of SIA (the VMM DNA extraction most likely failed) and SIB (Figure 1.6).

Results of qualitative RT-PCR indicate that $un-24^{OR}$ transcripts are absent when SIA was grown on SCM (Figure 1.7). Conversely, the results of semi-quantitative RT-PCR indicate that the transcript levels of $un-24^{OR}$ was 5 times greater than $un-24^{PA}$ when SIA was grown on SCM (Figure 1.8). Previous qualitative RT-PCR reactions were able to detect $un-24^{OR}$ transcripts in SIA grown on SCM (not shown) and therefore the qualitative RT-PCR reaction shown here may have failed. $un-24^{PA}$ transcript levels increased 3 times when SIA was grown on SCM however $un-24^{PA}$ transcripts were an average 8 times less abundant than $un-24^{OR}$.

$un-24^{PA}$ transcripts were absent or present at very low levels in SIB grown in both VMM and SCM. The $un-24^{PA}$ RT-PCR product was very weak using qualitative RT-PCR (VMM and SCM; Figure 1.7). Using semi-quantitative RT-PCR, $un-24^{PA}$ transcripts were undetected in SIB grown in VMM and less than 1 copy per *sbr* copy with SCM (Figure 1.8). In SIB, the $un-24^{OR}$ transcript level was greater than $un-24^{PA}$ (25 times greater on SCM), and $un-24^{OR}$ increased 12 times when SIB was grown on SCM.

Transcript levels of $un-24^{OR}$ and $un-24^{PA}$ in self-compatible and -incompatible partial diploids

C9-2 spheroplasts transformed with $un-24^{PA}$ ($un-24^{OR/PA}$ partial diploids) developed into "star-shaped" colonies after growth at 30°C for 4 days. When subcultured, these colonies grew very slowly, with a "spidery" morphology similar to self-incompatibility due to differences at *het-c* or mating type (Beadle & Coonradt, 1944;

Figure 1.6. The presence or absence of *un-24^{OR}* (UN24OR-1) and *un-24^{PA}* (UN24PA-1) DNA sequence as assayed by PCR in *un-24^{OR/PA}* stable incompatible strains SIA and SIB grown in low (SCM) and high (VMM) nitrogen media at room temperature. The DNA extraction from SIA VMM most likely failed.

Figure 1.7. The presence or absence of *un-24^{OR}* (6JP-OR2) and *un-24^{PA}* (6JP-PA2) transcripts as assayed by RT-PCR in *un-24^{OR/PA}* stable incompatible strains SIA and SIB grown in low (SCM) and high (VMM) nitrogen media at room temperature.

Figure 1.8. Transcript levels of *un-24^{OR}* and *un-24^{PA}* as assayed by semi-quantitative RT-PCR in *un-24^{OR/PA}* stable incompatible strains SIA and SIB grown in low (SCM) and high (VMM) nitrogen media at room temperature. *un-24^{OR}* and *un-24^{PA}* transcripts detected using qualitative RT-PCR are indicated with stars.

Saupe *et al.*, 1996b). Self-incompatible partial diploids returned to pre-transformation growth rates (escaping from incompatibility) between 4 and 11 days after transfer from transformation medium to hygromycin-free VMM. Only pre-escape self-incompatible transformants were included in the experiment and four of the 12 partial diploids were discarded because escape occurred before harvesting of mycelium. One partial diploid (denoted self-incompatible B (SIB)) did not escape from incompatibility and was able to grow on VMM with hygromycin (similar to SIA from Micali (2003)). C9-2 spheroplasts transformed with *un-24^{OR}* (*un-24^{OR/OR}* partial diploids) displayed near wild type growth rates.

Using allele specific RT-PCR primers, the presence or absence of *un-24^{OR}* and *un-24^{PA}* transcripts was assayed in both self-incompatible and -compatible partial diploids. As predicted, *un-24^{OR}* transcripts were detected in both self-incompatible and -compatible partial diploids. All transformants carry the endogenous *un-24^{OR}* copy and self-compatible partial diploids (*un-24^{OR/OR}*) carry an additional *un-24^{OR}* copy. Also as predicted, *un-24^{PA}* transcripts were detected in self-incompatible partial diploids (*un-24^{OR/PA}*) carrying an ectopic *un-24^{PA}* copy (Figure 1.9).

The *un-24^{PA}* and *un-24^{OR}* transcript levels of 3 self-compatible and 3 self-incompatible partial diploids were estimated using semi-quantitative RT-PCR (Figure 1.10). The median *un-24^{OR}* transcript level in self-compatible *un-24^{OR/OR}* strains (3.29 copies/*sbr* copy, n=3) was 7 times greater than that of strain C9-2 (0.46 copies/*sbr* copy, n=1). Some non-specific product amplification of RNA from self-compatible strains was observed using *un-24^{PA}* transcript specific primers (between 0.09 and 1.0 copies/*sbr* copy).

Figure 1.9. The presence or absence of *un-24*^{OR} (6JP-OR2) and *un-24*^{PA} (6JP-PA2) transcripts as assayed by RT-PCR in (1) control strains C9-2 and C2(2)-1, (2) *un-24*^{OR/OR} partial diploid self-compatible strains OR 2.1, OR 2.2, OR 2.3 and OR 2.4 and (3) pre-escaped *un-24*^{OR/PA} partial diploid self-incompatible strains PA 2.1, PA 2.2, PA 2.5, PA 2.8, PA 2.9 and PA 2.10. The *un-24* genotypes *un-24*^{OR} (OR), *un-24*^{PA} (PA), *un-24*^{OR/OR} (OR/OR) and *un-24*^{OR/PA} (OR/PA) are given above the strain designations.

Figure 1.10. Transcript levels of *un-24^{OR}*, *un-24^{OR-}* and *un-24^{PA}* as assayed by semi-quantitative RT-PCR in (1) strains C9-2, C2(2)-1 and co1(4)-2, (2) co1(4)-2 *un-24^{OR/PA}* escape strains co1-PA1 and co1-PA4, (3) *un-24^{OR/OR}* self-compatible strains OR 2.1, OR 2.2, OR 2.3, (4) *un-24^{OR/PA}* self-incompatible strains PA 2.1, PA 2.2, PA2.5 (5) C9-2 *un-24^{OR/PA}* escape strains e18, e22, e42, and (6) *un-24^{OR/PA}* stable self-incompatible strains SIA and SIB. *un-24^{OR}*, *un-24^{OR-}* and *un-24^{PA}* transcripts detected using qualitative RT-PCR are indicated with stars.

Self-incompatible partial diploids contained either about equal $un-24^{PA}$ and $un-24^{OR}$ transcript levels (PA 2.1) or greater $un-24^{OR}$ transcript levels (mean 4 times greater, $n=2$; PA 2.2, 2.5). The mean self-incompatible partial diploid $un-24^{OR}$ transcript level (5.65 copies/*sbr* copy, $n=3$) was 12 times greater than that of C9-2 (0.46 copies/*sbr* copy, $n=1$); the mean $un-24^{PA}$ transcript level (4.47 copies/*sbr* copy, $n=3$) was 3 times greater than that of C2(2)-1 (1.44 copies/*sbr* copy, $n=1$; Figure 1.10).

$un-24^{OR}$ and $un-24^{PA}$ DNA and transcripts in C9-2 escape strains

Using allele specific PCR and RT-PCR, the presence or absence of $un-24^{OR}$ and $un-24^{PA}$ DNA and transcripts was assayed in C9-2 self-incompatible $un-24^{OR/PA}$ partial diploids that had escaped from incompatibility (Table 1.2, Figures 1.10, 1.11, 1.12). The $un-24$ phenotype and presence or absence of $un-24^{OR}$ and $un-24^{PA}$ copies assayed with RFLP analysis is based on the results of Micali (2003).

Escape strains e13, e26, and e28 are phenotypically $un-24^{OR}$ (compatible with $un-24^{OR}$ strain C8c-32 and incompatible with col-PA strains) and carry only $un-24^{OR}$ DNA based on RFLP analysis. Using the allele specific PCR assay, $un-24^{OR}$ but not $un-24^{PA}$ copies were detected in e26 and e28. Neither $un-24^{OR}$ nor $un-24^{PA}$ was detected in e13 most likely due to insufficient DNA quality or quantity (Figure 1.11, Table 1.2). Using the allele specific RT-PCR assay, $un-24^{OR}$ but not $un-24^{PA}$ transcripts were detected in e13, e26 and e28 (Figure 1.12, Table 1.2). Transcript levels in these three strains were not quantified using semi-quantitative RT-PCR.

Escape strains e12 and e38 are phenotypically $un-24^{OR}$ and carried $un-24^{OR}$ and $un-24^{PA}$ DNA based on RFLP analysis at 41-60 days after escape. Using allele specific

Figure 1.11. The presence or absence of *un-24^{OR}*, *un-24^{OR-}* (UN24OR-1) and *un-24^{PA}* (UN24PA-1) DNA as assayed by PCR in (1) strains C9-2, C2(2)-1 and col(4)-2, (2) col(4)-2 *un-24^{OR-/PA}* escape strains col-PA1 and col-PA4, (3) C9-2 *un-24^{OR/PA}* escape strains e12, e13, e17, e18, e22, e26, e28, e36, e38, e40, e42 and e48 and (4) *un-24^{OR/PA}* stable incompatible strains SIA and SIB. Note where DNA extractions failed (marked with arrows).

Figure 1.12. The presence or absence of *un-24^{OR}*, *un-24^{OR-}* (6JP-OR2) and *un-24^{PA}* (6JP-PA2) transcripts as assayed by RT-PCR in (1) haplotype strains C9-2, C2(2)-1 and col(4)-2, (2) col(4)-2 *un-24^{OR-PA}* escape strains col-PA1 and col-PA4, (3) C9-2 *un-24^{OR/PA}* escape strains e12, e13, e17, e18, e22, e26, e28, e36, e38, e40, e42 and e48 and (4) *un-24^{OR/PA}* stable incompatible strains SIA and SIB.

PCR and RT-PCR assays, *un-24^{OR}* but not *un-24^{PA}* DNA nor transcripts were detected in both escape strains (Table 1.2, Figures 1.11, 1.12). Transcript levels in these two strains were not quantified using semi-quantitative RT-PCR.

Escape strains e22, e48, e17, e18, e36, e40 and e42 carry *un-24^{OR}* and *un-24^{PA}* DNA based on RFLP analysis. e22 and e48 are incompatible with both *un-24^{OR}* strain C8c-32 and *un-24^{PA}* col-PA strains. e17, e18, e36, e40 and e42 are phenotypically *un-24^{PA}* (incompatible with *un-24^{OR}* strain C8c-32 and compatible with col-PA strains; Micali, 2003). The allele specific PCR assay detected DNA copies of either *un-24^{OR}* but not *un-24^{PA}* (e17; probably a very low DNA template concentration), *un-24^{OR}* and *un-24^{PA}* (e18, e22, e36, e42, e48) or neither *un-24^{OR}* nor *un-24^{PA}* (e40; Table 1.2, Figure 1.11); however, both *un-24^{OR}* and *un-24^{PA}* transcripts were detected in all using the RT-PCR allele specific assay (Table 1.2, Figure 1.12). The *un-24^{OR}* and *un-24^{PA}* transcript levels in strains e18, e22, and e42 were measured using semi-quantitative RT-PCR. *un-24^{PA}* transcript levels were about the same as (e42), or significantly in excess of (e18 and e22; 11 times greater) *un-24^{OR}* transcript levels. The mean *un-24^{PA}* transcript level of these three escape strains (12.22 copies/*sbr* copy, n=3) was 3 times greater than that of self-incompatible partial diploids (4.47 copies/*sbr* copy, n=3) and 9 times greater than C2(2)-1 (1.44 copies/*sbr* copy, n=1; Figure 1.10). The *un-24^{OR}* transcript level in escapes (mean = 1.30 copies/*sbr* copy, n=3) was similar to that of C9-2 (0.46 copies/*sbr* copy, n=1) but less than self-incompatible partial diploids (mean = 5.65 copies/*sbr* copy, n=3; Figure 1.10).

un-24^{OR-} and un-24^{PA} DNA and transcripts in col(4)-2 escape strains

The col(4)-2 *un-24^{OR-/PA}* escape strains, col-PA1 and col-PA4 (Micali, 2003), were assayed for the presence or absence of *un-24^{OR-}* and *un-24^{PA}* DNA and transcripts. *un-24^{OR-}* DNA was detected in both strains but *un-24^{PA}* DNA was detected only in col-PA4 (Figure 1.11). Both *un-24^{OR-}* and *un-24^{PA}* transcripts were detected in col-PA1 and col-PA4 (Figure 1.12) and further analyzed using semi-quantitative RT-PCR. The mean transcript level of *un-24^{PA}* (3.25 copies/*sbr* copy, n=2) was 5 times greater than *un-24^{OR-}* (0.61 copies/*sbr* copy, n=2) in col-PA strains. The mean transcript level of *un-24^{OR-}* in col-PA strains was 3 times less than that of col(4)-2 (2.04 copies/*sbr* copy, n=1); the transcript level of *un-24^{PA}* in col-PA strains was 2 times greater than that of C2(2)-1 (1.44 copies/*sbr* copy, n=1; Figure 1.10).

DISCUSSION

The expression of *het* genes is thought to be downregulated during the sexual cycle to prevent an incompatibility reaction between anastomosing hyphae (Perkins, 1988). A systemic downregulation of *un-24*, however, presents a paradox. The protein encoded by *un-24*, RNR, is essential for DNA synthesis and repair (Elledge *et al.*, 1993). Downregulation of *un-24* would, therefore, starve the hyphae of DNA precursors necessary for meiotic division and subsequent mitotic events during the sexual cycle. I found that *un-24* was transcriptionally regulated in response to low nitrogen conditions (a signal to enter into the sexual cycle), instigating further studies on the role of *un-24* transcription regulation in both heterokaryon incompatibility and escape from

incompatibility. The current study suggests that transcription regulation, in addition to other mechanisms such as nuclear sorting and deletion, may play a role in escape.

Transcription of un-24 throughout the N. crassa life cycle

There are two opposing hypotheses of *un-24* transcription during the sexual and vegetative life-cycle phases. The first hypothesis is that transcription of heterokaryon incompatibility genes (including *un-24*) decreases during sexual reproduction to prevent a heterokaryon incompatibility reaction between genetically different individuals (Perkins, 1988). The second hypothesis is that transcription of *un-24* should increase during sexual reproduction to provide the necessary dNTPs for the generation of ascospores.

In the current study, stable incompatible strains that carry a resident *un-24*^{OR} and an ectopic *un-24*^{PA} were used as a model of what would happen when two genetically different nuclei share a common cytoplasm. At mating initiation, nuclei of the *A* and *a* mating-types do not fuse until ~10 mitotic divisions occur. The diploid stage is immediately followed by meiosis (Davis, 2000 and references within). Therefore, during mating, alternate alleles are essentially expressed from different nuclei sharing a common cytoplasm. Transformed strains differ because alternate alleles are expressed from the same nucleus.

Using semi-quantitative RT-PCR as a measure of transcript abundance, *un-24*^{OR} transcript levels showed approximate 5 and 12 fold increases when SIA and SIB, respectively, were grown on medium promoting the sexual cycle. A ~3 fold increase in *un-24*^{PA} transcript levels were observed in SIA grown on medium promoting the sexual cycle. In a previous study, Northern analyses of *un-24* transcript levels in *un-24*^{OR-}, *un-*

24^{OR} and $un-24^{PA}$ strains suggested that $un-24^{PA}$ has the capacity to be regulated during the sexual cycle. Transcripts of $un-24^{PA}$ were approximately 2 times more abundant when strains were grown on SCM and $un-24^{OR}$ transcripts remained fairly constant (Appendix A). Contrary to expectations, results of the current study indicate that when both alleles are co-expressed in the same nucleus, $un-24^{OR}$ transcript levels increase more than $un-24^{PA}$ on medium promoting the sexual cycle.

Some precautions should be considered, however, when interpreting these data. First, the ectopic $un-24^{PA}$ allele in stable transformants is inserted randomly into the *N. crassa* genome and may no longer be associated with important *cis*-acting regulatory elements such as enhancers and DNA binding sites for tissue-specific transcription factors. Similarly, the ectopic $un-24^{PA}$ allele is no longer associated with $het-6^{PA}$. Second, a different measure of transcript levels was employed in each experiment and the results of Northern analysis were not replicated using semi-quantitative RT-PCR. Nevertheless, both Northern and RT-PCR results indicate that both $un-24^{OR}$ and $un-24^{PA}$ alleles are differentially regulated during the sexual cycle. A lack of incompatibility during mating may be prevented through local downregulation of one or both $un-24$ alleles in the sexual structures.

Further studies of un-24 transcription during the N. crassa life cycle

The transcript levels of $un-24$ alleles should be estimated in sexual structures as well as vegetative and pre-meiotic cultures (grown on SCM). Perithecia formed during mating can be harvested and the transcript levels estimated using quantitative RT-PCR. Similar experiments using Northern analysis have explored transcript regulation of *N.*

crassa mat A-1 (Saupe *et al.*, 1996a) and *tol* (required for mating type incompatibility; Shiu & Glass, 1999) throughout the *N. crassa* life cycle. These experiments showed that *tol* but not *mat A-1* transcription is downregulated in sexual structures.

Untranslated regions of un-24

The differential *un-24*^{OR} and *un-24*^{PA} transcript levels suggest that the regulatory mechanisms between alleles are different. When 1037 bp of the *un-24*^{OR} and *un-24*^{PA} 5' untranslated regions are aligned, there are several non-identical regions that may include allele specific *cis*-acting elements (Figure 1.13). The identification of important *un-24* transcriptional signal regions is difficult for *N. crassa* due to a general lack of promoter consensus sequences (Davis, 2000). Transcriptional start sites are often found within or within one or two bp of the consensus sequence TCATCANC (Bruchez *et al.*, 1993) however this sequence does not exist within ~1000 bp upstream of the *un-24* start codon (Figure 1.13). Two upstream regions have been implicated in the regulation of the *N. crassa* RNA polymerase II, responsible for mRNA synthesis. CAAAT boxes occurring in approximately one-eighth of *N. crassa* promoters are 76 to 88 bp upstream of the transcriptional start (Bruchez *et al.*, 1993) and similar in sequence and position to enhancers and promoters of mammalian gene transcription (Davis, 2000). Two CAAAT boxes occur upstream of the *un-24* start codon. One is present 666 bp upstream of the putative transcription start sites (Smith *et al.*, 2000a) for both alleles and the second is present in a non-identical region 624 bp upstream of the *un-24*^{PA} putative transcription start site and absent for *un-24*^{OR}. The later CAAAT box may play a role in differentially regulating *un-24*^{OR} and *un-24*^{PA}. In *N. crassa* TATATAA boxes occur 34 to 44 pb from

Figure 1.13. Sequence alignment of the region upstream of the *un-24*^{OR} (top) and *un-24*^{PA} (bottom) start codons. CAAAT sequences are enclosed in boxes. The putative transcription start site is taken from Smith *et al.* (2000a). Sequence information for *un-24*^{OR} is from the Information Centre for Protein Sequences, *N. crassa* genome project (<http://www.mips.biochem.mpg.de/desc/neurospora/>). Sequence information for *un-24*^{PA} is from ML Smith (unpublished)

1 TGGGACCTGACAGGGGCCATGCTTGGCTCGCTGCAGCTCATCCGCTGAGTC
 TTTGGMCTGACASGGGGCCATGCTTGGCTCGCTGCAGCTCATCCKCTKASTC
 * * ***** ** * **

 52 GCTGCAGGGCGCCCATCCATGATTACTTAAGCACCGTGTTTTTGGGCGGAT
 GCTGCAGGGCGCCCATCCATGATTACTTAAGCACCGTGTTTTTGGGCGGAT

 103 TCAAGGTGCCGATGGCTGGAGTTACGGATCCGCGCGGGTCTGGGATTTTGC
 TCAAGGTGCCGATGGCTGGAGTTACGGATCCGCGCGGGTCTGGGATTTTGC

 154 TAGTGTGAGACTGTCAGGCAGCAGGGCGCGATGTAAACAAATGGGTGACAC
 TAGTGTGAGACTGTCAGGCAGCAGGGCGCGATGTAAACAAATGGGTGACAC

 205 GCGCTGTGAATATCTGTGAATTTTGTCTCAACTTGAAAAGGCCAAAGGCA
 GCGCTGTGAATATCTGTGAATTTTGTCTCAACTTGAAAAGGCCAAAGGCA
 ***** ** *****

 256 AAGAGTAATGTTTACGGTAATTCACAATTCGCATTGCTTGCGATGATGTA
 AAGAGTAATGTTTACGGTAATTCACAATTCGCATTGCTTGCGATGATGTA

 307 ATTTACAGACGCGTCGTTCTGGACGTGTAAAGTGGTGCTAGCGCTGTTAAA
 ATTTACAGACGCGTCGTTCTGGACGTGTAAAGTGGTGCTAGCGCTGTTAAA

 358 AGGTGGGGTGGCAGCTGCAGTGTTTTGTTTAAACATCGGGCCAGGGGAGGGG
 AGGTGGGGTGGCAGCTGCAGTGTTTTGTTTAAACATCGGGCCAGGGGAGGGG

 409 GGGACGAGGAGACTTTTGGTGGAGCCCCCTCCCTCCAGCCCCGGGCTACAA
 GGGACGAGGAGACTTTTGGTGGAGCCCCCTCCCTCCAGCCCCAGGCTACAA

 460 CGCGCTCCAAAAGAACAACAACTGTAGCAGCCCGCAGTGAGACGCGTCCTT
 CGCGCTCCAAAAGAACAACAACTGTAGCAGCCCGCAGTGAGACGCGTCCTT

 511 GTGCTCCCAGTACTCCAAAATGCACACATACTGCTACTGCAGTGACACTG
 GTGCTCCCAGTACTCCAAAATGCACACATACTGC-AGTGCA-CACCTACTG
 ***** * **** * ****

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562      CCGTGGGTGACGCGCTTTTGACGGCCGACCCATAGAGGTCCCGCATTGGCA
      CCGTGGGTGACGCGCTTTTGACGGCCGACCCATAGAGGTCCCGCATTGGCA
      *****

613      CTGGCAGTTTCCAGTCCAAGACGAGCGCTAGAAAAGAGTGGACGCGCCAAT
      CTGGCAGTTTCCAGTCCAAGACGAGCGCTAGAAAAGAGTGGACGCGCCAAT
      *****

664      CTCGAAAAACCATCCTTCTCGCTGCCTTCTCCTTCGATCCGTCTCTTCGTT
      CTCGAAAAACCATCCTTCTCGCTGCCTTCTCCTTC- - -CATCTCTTCTCT
      *****          *          *

715      CTCTCCCTTCCCCTTCTCTCCCGTCTCTTCCGTGACTGTGACTACTTCAAG
      CCCTTCCA-CTTCTCTCCTCCCGTCTCTTCCGTGACTGTGACTACTTCAAG
      *** * * * * *****

766      TTACTGTCCTTCCCTCTCCTCCTTCTCCTCCATCCTATACCCAATTTCCCC
      TTACTGTCCTTCCCTCTCCTCCTTCTCCTCCATCCTATACCCAATTTCCCC
      *****

                                     Putative transcription start site ┐→
817      CAAGACAACCTTCGAGATTGCTTGGGCCTATCTTGGTGTAGTCGTCTTCCT
      CAAGACAACCTTCGAGATTGCTTGGGCCTATCTTGGTGTAGTCGTCTTCCT
      *****

868      GCTCGTGCCATTTGTCAGCGTAGTCGAATCCTGTCTACGGCCCAGCAGCAG
      GCTCGTGCCATTTGTCAGCGTAGTCGAATCCTGTCTACGGCCCAGCAGCAG
      *****

919      CAGCAGCACTTCAAGTTTTCGTGTTCTTCTGTTTTGTCCCCTCCATTTTGG
      CAGCAGCACTTCAAGTTCAAGTGTTCTTCTGTTTTGTCCCCTCCATTTTGG
      *****          *****

970      TTGTCAATAACTGTACTT- - -GTTCTTGTTCCCTCTTCTTCACCTTCTCC
      TTGTCAATAACTGTACTTCCTTGTTCTTGTTCCCTCTTCTTCACCTTCTCC
      *****          *****

1021     CCCACACATCAGACATTATG
      CCCACACATCAGACATTATG
      *****

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Translation start site

the transcriptional start in approximately one in ten upstream regions (Bruchez *et al.*, 1993). This sequence has not been identified upstream of *un-24*.

Escape from un-24-mediated incompatibility

When *N. crassa* strains carrying the *un-24*^{OR} allele are transformed with *un-24*^{PA}, the alternate *un-24* allele, transformants show slower growth than the wild type and exhibit a spidery morphology. The inhibited phenotype is generally unstable, where transformants escape from incompatibility and growth returns to wild-type rates (Micali, 2003).

The mechanism of escape may be the result of nuclear segregation (Xiang & Glass, 2004), inactivation of either the endogenous or ectopic duplicated allele through mutational events (Griffiths, 1982; Smith *et al.*, 1996; Coustou *et al.*, 1999), or mutations in suppressor loci (DeLange & Griffiths, 1975; Vellani *et al.*, 1994; Xiang & Glass, 2002; Xiang & Glass, 2004; Pittenger, 1964). Other possible mechanisms such as transcript regulation have not been previously reported in *N. crassa* or other fungal species. This study used allele selective PCR and RT-PCR to investigate mechanisms of escape from *un-24*-mediated incompatibility.

Escape through loss of transformed nucleus

Escape strains displaying the *un-24*^{OR} phenotype and *un-24*^{OR} RFLP pattern (Micali, 2003) did not carry an ectopic *un-24*^{PA} copy nor transcripts as assayed using allele specific PCR and RT-PCR, respectively. The loss of *un-24*^{PA} function and DNA may have occurred through complete deletion of the ectopic copy. Translocation partial

diploids containing heteroallelic duplications of both *un-24* and *het-6* (*Dp(AR18)*), most often escape through deletions of the ectopic translocated *un-24*^{OR} *het-6*^{OR} segment and retain the resident *un-24*^{PA} *het-6*^{PA} loci (Smith *et al.*, 1996). A more simple explanation is the loss of the transformed nucleus (Micali, 2003; Xiang & Glass, 2004). Fungal protoplasts used in transformation may contain multiple nuclei, not all of which accept foreign DNA. As the transformed protoplast grows into a mycelium, transformed and untransformed nuclei will replicate and segregate into sectors of the mycelium. Escape may occur when an untransformed fast growing sector overtakes the transformed inhibited sector.

Escape through deletion and nuclear sorting may be distinguished using Southern blotting with the vector pCB1004 DNA sequence as a probe. If pCB1004 DNA is detected, then escaped nuclei were transformed and subsequently lost the ectopic *un-24*^{PA} copy through deletion. The size of RFLP fragments would help to determine the extent of the deletion. If pCB1004 DNA is not detected then escape may have occurred through deletion of vector and *un-24*^{PA} DNA or through sectoring of the transformed nuclei.

Escape through a temporary mechanism leading to deletion

Three escape strains displaying the *un-24*^{OR} phenotype were previously found through RFLP analysis to contain both *un-24*^{OR} and *un-24*^{PA} alleles (Micali, 2003). Unexpectedly, the *un-24*^{PA} allele was not amplified using allele specific primers. This may indicate that a post-escape deletion occurred in the ectopic allele. The extent of the deletion is unknown but would include the ~500 bp of the 3' region meant to amplify in PCR and RT-PCR. Escape may occur through initial events such as methylation or

mutation that both destroy the function of the ectopic copy through either transcription inhibition, stability of transcripts or function of the protein product, and also mark the allele for further deletion as suggested by Micali (2003). An alternative to deletion is that mutations may have occurred in the *un-24^{PA}* primer binding-sites, preventing primer annealing during PCR.

Escape through transcript regulation

Escape strains that carried both *un-24^{PA}* and *un-24^{OR}* alleles based on RFLP analyses were incompatible with an *un-24^{OR}* tester strain (C8c-32 *un-24^{OR} het-6^{OR}*) indicating that the *un-24^{PA}* ectopic copy remained functional through the escape process. Some of these escape strains (e22 and e48) were also incompatible with *col-1-PA* strains (*un-24^{PA} het-6^{OR}*), indicating that the endogenous *un-24^{OR}* copy is functional (Micali, 2003). I verified that five of seven escapes had both PA and OR-specific *un-24* DNAs. Of the remaining two, one had only *un-24^{OR}* DNA, suggesting deletion or mutation within *un-24^{PA}*, and the other had neither DNA copy, most likely due to poor DNA quality or quantity. Regardless of whether one or both DNA copies were detected, transcripts of both *un-24^{PA}* and *un-24^{OR}* were detected in all seven escapes tested.

Through semi-quantitative RT-PCR the transcript level of *un-24^{PA}* was found to be similar to (e42) or greater than (e18 and e22) that of *un-24^{OR}* in all three of the above escape strains that were tested. This contrasts with incompatible strains before escape, where *un-24^{OR}* transcripts were proportionately equal to or greater than *un-24^{PA}*. The switch from slow to fast growth during escape leading to a functional *un-24^{PA}* appears to be accompanied, or caused by, a switch from relatively high *un-24^{OR}* to relatively high

un-24^{PA} transcript levels. Interestingly, previous experiments have shown that the two *un-24* alleles are asymmetrical in strength of incompatibility. Incompatible reactions from *un-24*^{PA} transformed into the *un-24*^{OR} background are weaker than are those of *un-24*^{OR} transformed into the *un-24*^{PA} background (Micali, 2003). Therefore, the increase in proportion of *un-24*^{PA} over *un-24*^{OR} transcripts may alone facilitate escape.

Alternatively, the endogenous *un-24*^{OR} allele may carry point mutations or chromosome rearrangements that inhibit translation and/or protein function. Crosses could be performed between escapes and tester strains to test if *un-24*^{OR} function is retained in these escape strains.

Another possibility is that spontaneous suppressor mutation(s) may allow strains transcribing both *un-24* alleles to escape from incompatibility. Wild-type suppressors may interact as co-factors with the products of alternate *het* alleles in incompatibility reactions and when mutated cannot interact to cause incompatibility (Vellani *et al.*, 1994). An alternate function of suppressors of incompatibility is to act as transcription factors involved in the regulation of cellular processes influencing vegetative incompatibility. *vib-1*⁺, required for *het-c*-mediated incompatibility, is implicated in the regulation of *N. crassa* conidiation and nonrepressible acid phosphatase (Xiang & Glass, 2002). Similarly, a suppressor of *un-24*-mediated incompatibility may act as an *un-24* transcription factor and regulate the transcriptional switch from relatively high *un-24*^{OR} transcripts before escape to relatively high *un-24*^{PA} transcripts after escape.

Methylation is known to play a role in controlling gene expression (Eden and Cedar, 1994) and may also regulate *un-24* transcript levels. Similar to other fungi, the DNA of *N. crassa* is not abundantly methylated (Russell *et al.*, 1985) but spontaneous

methylation often occurs in transformed DNA sequences within vegetatively growing strains (Bull & Wootton, 1984). Methylation has been shown in *N. crassa* to inhibit elongation of transcripts but not to interfere with the promoter region (Rountree & Selker, 1997). Micali (2003) found some evidence of *un-24* methylation in strains escaping from incompatibility. Transcription of duplicated *un-24* loci in transformation partial diploids could be selectively suppressed through methylation of nucleotides.

Can escape be used to create novel $un-24^{PA}$ $het-6^{OR}$ genotype?

The *un-24^{PA} het-6^{OR}* genotype has not been discovered in nature (Mir-Rashed *et al.*, 2000). *un-24* and *het-6* are tightly linked and an inversion between the loci is thought to prevent recombination (OC Micali and ML Smith, unpublished). To create a functional *un-24^{PA} het-6^{OR}* strain, col(4)-2 carrying a temperature sensitive copy of *un-24* was transformed with wild-type *un-24^{PA}* and allowed to escape from incompatibility at a restrictive temperature, selectively silencing the endogenous *un-24^{OR-}* copy. The resulting col-PA1 and col-PA4 strains were known to contain a functional *un-24^{PA}* copy because (1) they grew at a restrictive temperature and (2) they exhibit heterokaryon incompatibility with an *un-24^{OR}* tester strain (Micali, 2003). Using allele specific PCR and RT-PCR both *un-24^{OR-}* and *un-24^{PA}* DNA and transcripts were detected. When quantified, however, *un-24^{PA}* transcripts were approximately 5 times greater than *un-24^{OR-}* transcripts. The presence of *un-24^{OR-}* transcripts suggests that col-PA strains have not silenced *un-24^{OR-}*; however, the *un-24^{OR-}* transcripts may not be translated into functional UN-24 proteins. Current efforts are focused on recovering functional copies

of *un-24^{OR}* and *un-24^{PA}* in the progeny of crosses between col-PA strains and *un-24^{OR}* and *un-24^{PA}* wild-type tester strains.

Stable self-incompatible strains

Two self-incompatible transformants (SIA and SIB) carrying a randomly inserted ectopic copy of *un-24^{PA}* did not escape from incompatibility. The mechanism allowing these strains to stably carry both copies is unknown. One possibility explaining the stability of SIA, which transcribed both *un-24^{OR}* and *un-24^{PA}*, is that insertion of the ectopic copy disrupted a gene essential for the escape process. For example, escape may require a gene such as *dim-2*, a DNA methyltransferase gene that is responsible for all known methylation of cytosine in *N. crassa* (Kouzminova & Selker, 2001). Insertion into a gene involved in escape would result in a stable self-incompatible strain. *un-24^{PA}* was not transcribed in SIB indicating that escape did occur but that the ectopic allele may have disrupted a gene responsible for growth of *N. crassa*.

Limitations of semi-quantitative RT-PCR

The technique of semi-quantitative RT-PCR includes a number of sources of error including (1) variation in RNA concentration between samples, (2) slight variations in the concentration of RNA and reagents between aliquots of each RT-PCR reaction, (3) pipetting error when PCR products are loaded onto an agarose gel, (4) measurement of PCR product using densitometry, and (5) defining the data points included in the linear portion of the amplification curve used to calculate the C_T value. The experiments presented here should be replicated using real time RT-PCR (qRT-PCR). qRT-PCR is a

technique where the amplification product is measured at each PCR cycle. The double stranded DNA produced during PCR binds to SYBR green, a fluorescent compound that can be measured (Walker, 2002). The C_T value obtained through qRT-PCR is more precise because the amplification product concentration is measured at each PCR cycle. Also, the error associated with aliquoting each sample into several smaller reactions (such as gel loading and reaction concentration variations) is removed. qRT-PCR requires fewer reactions which reduces the cost of PCR reagents and preparation time (however the cost of a real-time PCR machine is significant) and allows for increased replication.

Summary

The results of this study suggest that *un-24*, a heterokaryon incompatibility gene, is regulated during the *N. crassa* life cycle. Both *un-24^{OR}* and *un-24^{PA}* transcript levels increased in stable self-incompatible strains grown on a low nitrogen medium that promotes sexual reproduction. The role of *un-24* transcription in heterokaryon incompatibility and escape from incompatibility was also investigated. Escape strains phenotypically *un-24^{OR}* and carrying only *un-24^{OR}* DNA, transcribed only *un-24^{OR}* indicating that escape occurred by complete loss of the *un-24^{PA}* DNA, or more likely, through loss of the transformed nucleus. *un-24^{OR}* escape strains previously found to contain both *un-24^{OR}* and *un-24^{PA}* DNA, but that in this study were found to carry and transcribe *un-24^{OR}* only, can be explained through temporary inactivation of *un-24^{PA}* followed by deletion or chromosome rearrangements. Finally, escape strains transcribing both *un-24^{OR}* and *un-24^{PA}* had a disproportionately large number of *un-24^{PA}* transcripts,

suggesting that escape may occur through changes in the ratio between both transcript levels.

Chapter 2: *vic4*-mediated vegetative incompatibility in *Cryphonectria parasitica*.

INTRODUCTION

In fungi, nonself recognition during vegetative growth is known as vegetative or somatic incompatibility. Vegetative incompatibility is manifested as two phenomena: mycelial and heterokaryon incompatibility. Mycelial incompatibility results in a macroscopic antagonistic reaction between two mycelia that grow together (Worrall, 1997), whereas heterokaryon incompatibility limits the ability of individuals to form viable heterokaryons, cells that contain different nuclear types (Glass & Kulda, 1992). Both methods have been used to study vegetative incompatibility in fungi.

Barrage

Mycelial incompatibility is assayed by a macroscopic aversive reaction (called barrage) at the zone of contact between strains. Barrage has been described in several basidiomycete (Worrall, 1997) and ascomycete species (Micali and Smith, 2003 and references within). Microscopically, barrage is thought to be caused by fusion and death of incompatible hyphae (Esser & Meinhardt, 1984; Xiang & Glass, 2002) although Micali and Smith (2003) suggest that barrage could also result from chemical signalling between distant hyphae, interaction of surface molecules during cell-cell contact, or mechanisms of heterokaryon incompatibility after hyphal fusion.

The phenotype of barrage varies interspecifically and sometimes intraspecifically (Esser & Meinhardt, 1984). In *Neurospora crassa* (Micali & Smith, 2003), *Cryphonectria parasitica* (Anagnostakis, 1977) and *Sclerotinia sclerotiorum* (Kohn *et al.*, 1990), a zone of low hyphal density between adjacent colonies may become visible

shortly after mycelial contact. This clear zone phenotype appears to be due to the degradation of fusion cells after hyphal anastomosis (Anagnostakis, 1977; Kohn *et al.*, 1990). Second, dark lines due to pigment deposition have been observed between adjacent mycelia of *C. parasitica* (Newhouse & Macdonald, 1991), *N. crassa* (Micali & Smith, 2003) and numerous basidiomycetes (Worrall, 1997). Dark lines may be observed on agar media or natural substrates as dark interaction lines, e.g. spalted wood (Esser & Meinhardt, 1984). A third phenotype known as “bourelet” (Rizet, 1952) is observed in *N. crassa* (Micali & Smith, 2003), *S. sclerotiorum* (Kohn *et al.*, 1990) and *P. anserina* (Rizet, 1952), where hyphal tips at the zone of interaction branch profusely, producing a raised hyphal aggregate.

Heterokaryon incompatibility

Heterokaryon incompatibility (reviewed in Chapter 1) is tested through complementation of genetic markers. Strains are inoculated on a medium that will not support growth of either strain individually but will support growth of heterokaryons containing nuclei from both strains (Micali & Smith, 2003). Compatible heterokaryons grow at near wild-type rates and with a similar morphology to the wild type and incompatible heterokaryons display either no growth or slow growth and aberrant morphology (Perkins, 1988).

Genetic basis of heterokaryon incompatibility and barrage

It is often assumed that the genetic and biochemical processes that mediate heterokaryon incompatibility also control mycelial incompatibility (Micali & Smith,

2003). Both phenomena are clearly “heterogenetic incompatibility systems”, i.e. brought about by genetic differences (Esser & Meinhardt, 1984). For all barrage types, genetic differences are necessary since barrages will not occur in confrontations of the same strain (Micali & Smith, 2003). Likewise, heterokaryon incompatibility occurs when one or more *het* loci have alternate allelic specificities (Perkins, 1988). Indeed, there is a correlation between barrage and heterokaryon incompatibility in *P. anserina* where genetic differences at *het* loci are associated with barrage (Rizet, 1952). However, recent studies have shown that mycelial and heterokaryon incompatibility do not always occur between the same individuals. In *S. sclerotiorum*, members of the same mycelial compatibility group (assayed using barrage) may be members of different vegetative compatibility groups (assayed using heterokaryon incompatibility) and *vice versa* (Ford *et al.*, 1995). In *N. crassa*, the barrage phenotypes of dark line, raised hyphal aggregate (bourelet) and clear zones, are under complex genetic controls that are not well understood but appear to be independent of the heterokaryon incompatibility system (Micali & Smith, 2003). The results of these studies with *S. sclerotiorum* and *N. crassa* suggest that mycelial and heterokaryon incompatibility may measure different genetic properties in some species (Ford *et al.*, 1995; Micali & Smith, 2003).

Cryphonectria parasitica

The ascomycete fungus *C. parasitica* causes the chestnut blight that decimated populations of the American chestnut throughout North America beginning in 1904 (Merkel, 1906; Guerin *et al.*, 2001). The pathogen continues to girdle and kill chestnut tree sprouts before they can reach sexual maturity (Anagnostakis, 1987). A variant of *C.*

parasitica with significantly reduced virulence (hypovirulence) that competes with virulent strains in cankers (Dawe & Nuss, 2001 and references within) was first observed in Italy (Biraghi, 1953), instigating biocontrol efforts of chestnut blight in both Europe and North America. *C. parasitica* strains displaying virulence attenuation were infected with a double-stranded RNA virus (hypovirus; reviewed by Dawe and Nuss (2001)). However, the horizontal transmission of this virus through hyphal fusion was found to be largely inhibited by mycelial incompatibility (Cortesi *et al.*, 2001). Efforts to use the double-stranded RNA virus as a biological control agent of chestnut blight have focused attention on vegetative incompatibility in *C. parasitica* and its role in preventing virus transmission (Nuss, 1992).

Mycelial incompatibility in *C. parasitica* is mediated by six unlinked vegetative incompatibility (*vic*) loci, *vic1*, *vic2*, *vic3*, *vic4*, *vic6*, and *vic7*, each with two alleles (Cortesi & Milgroom, 1998). Strains differing at any one *vic* locus produce a barrage between confronting colonies (Cortesi & Milgroom, 1998). Barrage between mycelial incompatible strains is associated with the cell death of fusion cells through granulation, vacuolization, and subsequent cytoplasmic condensation (Biella *et al.*, 2002).

There is a good correlation between mycelial incompatibility and heterokaryon incompatibility in *C. parasitica*. Heterokaryons with a difference at *vic1*, *vic2*, *vic3*, *vic6* or *vic7* do not grow and are therefore incompatible. Heterokaryons with differences at *vic4*, however, grow as well as heterokaryons with no *vic* differences (ML Smith and MG Milgroom, unpublished results). The focus of my research was to further characterize heterokaryons with *vic4* differences and investigate mechanisms that may permit *vic4* heterokaryosis. First, to further test the hypothesis that differences at *vic4* do not result in

a heterokaryon incompatibility phenotype, the cell death frequencies of *vic*-homoallelic (no *vic* differences) and *vic4*-heteroallelic (*vic4* difference) heterokaryons were compared. Second, the protein profiles were compared to determine if protein aggregation, a putative mechanism of heterokaryon incompatibility (Haidari, 2002; Sarkar *et al.*, 2002; Glass & Kaneko, 2003), occurs in *vic4*-heteroallelic heterokaryons. Last, the proportions of parental nuclei in *vic4* heterokaryons were compared to investigate if heterokaryon incompatibility may be prevented through skewed nuclear proportions.

METHODS AND MATERIALS

C. parasitica strains and growth conditions

Strains used in this study are presented in Table 2.1. *C. parasitica* strains EP155B, P4-4B and P5-1B were transformed to benomyl resistance by electroporating spheroplasts with either one of two plasmids, pSV50 (Vollmer & Yanofsky, 1986) or pMYX2 (Campbell *et al.*, 1994) carrying identical *bml^R* genes, and selecting stable benomyl resistant colonies (ML Smith, unpublished). Benomyl resistant strains were maintained on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD) supplemented with 0.3 µg/mL benomyl (PDA ben; Bonide Products, Inc., Yorkville, NY) and hygromycin resistant strain 22.1H carrying the *E. coli* hygromycin B phosphotransferase gene *hph* was maintained on PDA supplemented with 30 µg/mL hygromycin (PDA hyg; Roche Molecular Biochemicals, Mannheim, Germany).

Table 2.1. Isolates of *C. parasitica* used in the study of *vic4*.

Isolate	<i>vic</i> genotype ^a	vc type ^a	antibiotic resistance	origin
22.1H	2211-22	EU5	hygromycin	A. Churchill – hygromycin resistant strain of EP155-2
EP155B	2211-22	EU5	benomyl	M.L. Smith – benomyl resistant strain of EP155 (A. Churchill - ATCC#38755)
P4-4B	2212-22	EU1	benomyl	M.L. Smith - benomyl resistant strain of P4-4 (Cortesi & Milgroom, 1998)
P5-1B	2212-22	EU1	benomyl	M.L. Smith - benomyl resistant strain of P5-1 (Cortesi & Milgroom, 1998)
P11-23	1211-22	EU31	none	Cortesi & Milgroom, 1998

^aVegetative incompatibility genotype and vegetative compatibility group (EU type) is based on Cortesi & Milgroom, 1998.

The concentration of hygromycin or benomyl that restricted growth of *C. parasitica* strains 22.1H (*hph*), P5-1B (*bml^R*), P4-4B (*bml^R*) and EP155B (*bml^R*) in PDB was measured to be able to selectively grow heterokaryons carrying *hph* and *bml^R* in potato dextrose broth (PDB; Becton, Dickinson and Company, Sparks, MD). Each strain was inoculated into 25 mL of PDB containing 15 µg/mL, 20 µg/mL, 25 µg/mL or 30 µg/mL hygromycin or 0.1 µg/mL, 0.2 µg/mL, 0.3 µg/mL or 0.4 µg/mL benomyl. After seven days the percent area of the flask bottom covered by fungal colonies was recorded.

Mycelial compatibility testing

Mycelial compatibility testing between *vic4*-compatible and *vic4*-incompatible strains was performed using PDAG medium (Powell, 1995; Cortesi & Milgroom, 1998). PDAG contained 39 g PDA, 7 g malt extract, 2 g yeast extract, 0.8 g tannic acid, 0.1 g methionine, 2 mg biotin, 2 mg thiamine (added after autoclaving), 20 g agar, and 50 mg bromocresol green per litre. Bromocresol green is a pH indicator that is yellow at pH below 3.8 and blue-green at pH above 5.4. Cubes were cut from the actively growing margin of <7-day-old cultures grown on PDA ben or PDA hyg and placed ~5mm apart on PDAG. There were five pairings per PDAG plate: two vegetatively compatible control pairings (EP155B/EP155B; EP155B/22.1H), one *vic1* vegetatively incompatible control pairing (EP155B/P11-23) and two experimental pairings (EP155B/P4-4B; EP155B/P5-1B). Plates were incubated at 25°C for 5 days in the dark. Colony pairs separated by a dark line were scored as mycelial incompatible; pairings that produced no dark line were scored as compatible (Powell, 1995).

Creating vic-homoallelic and vic4-heteroallelic heterokaryons

C. parasitica strains EP155B, P4-4B and P5-1B (*bml^R*) were separately confronted with 22.1H (*hph*). Sterile cellophane (UCB Films, Atlanta, GA) was laid over PDA in petri dishes. The cellophane is permeable to nutrients and moisture necessary to fungal growth. Four confrontations per petri dish were performed by inoculating two strains (22.1H and either EP155B, P4-4B or P5-1B) 1-3 mm apart on the cellophane. As a control, each strain was inoculated separately without confrontation to another strain (Figure 2.1).

The strains were grown for 2 to 3 days, so that the mycelia of confronting colonies were in contact for ~1 day. Using aseptic technique, the cellophane with growing colonies was transferred to PDA containing 0.3 µg/mL benomyl and 30 µg/mL hygromycin (PDA ben hyg; benomyl and hygromycin were added to 50°C molten agar). Immediately after transfer to selective medium, the colony margins were marked on the petri dish bottom and the plates were incubated at room temperature. After 2 to 4 days the plates were examined for mycelial growth extending from the region of contact between the two parental strains. Hyphae growing from the contact zone were assumed to represent heterokaryotic sectors.

Differential vital staining of live and dead cells

Parental strains 22.1H, EP155B, P4-4B, and P5-1B, one of each heterokaryon EP155B+22.1H, P4-4B+22.1H and P5-1B+22.1H, and two double resistant conidial-derived isolates of P5-1B+22.1H #1 were inoculated onto ~1 cm² cubes of PDA plus selective antibiotics with a coverslip placed on each cube. After incubation for 1-2 days

Figure 2.1. Method of forcing heterokaryons between 22.1H and EP155B, P4-4B or P5-1B. The symbol “x” represents the point of inoculation of isolate 22.1H and “o” represents the point of inoculation of EP155B, P4-4B or P5-1B. Widely spaced inoculations in the centre of the petri dish serve as negative controls.

x = hygromycin
resistant 22.1H

o = benomyl resistant strain
(EP155B, P4-4B or P5-1B)

at 30°C the mycelium adhering to the cover slips was removed gently from the cube and stained separately with fluorescein diacetate (FDA; Polysciences Inc., Warrington, PA) and Evans blue (EB; [2,4-(NaOSO₂)₂C₁₀H₃-1-NH₂-8-OH-7-N:N-4-C₆H₃-3-CH₃]₂; Fisher, Ottawa, ON). FDA diffuses through the membranes of living cells. Once inside the cell, FDA is converted by nonspecific esterases into fluorescent products that are retained by cells with intact membranes (Molecular Probes, 2004). FDA was used to count live cells. EB is pumped out of cells with intact membranes and was used to identify dead or dying cells (Jacobson *et al.*, 1998). All mycelia were stained with the cell wall stain Calcofluor White (CW; Cyanamid of Canada Ltd., Montréal, QC) to count total cells.

Mycelia were grown onto coverslips, incubated for five minutes in 2 mg/mL FDA dissolved in acetone or 1% (w/v) EB dissolved in water and emersed in 40 mL dH₂O for five minutes. Following EB or FDA staining mycelia were then incubated in 1 mg/mL CW for five minutes and emersed in 40 mL dH₂O for five minutes.

Samples were examined using a Zeiss Axioplan 2 imaging microscope with a Zeiss AxioCam HRm camera. For total cell counts, samples were viewed under UV light (365 nm) to visualize the cell walls stained with CW. Both live (FDA stained) and dead (EB stained) cells were counted. Live cells stained with FDA appear bright green when viewed under blue light (450 to 490 nm). Brightfield microscopy was used to visualize dark blue dead or dying cells stained with EB.

Proteomic evaluation of vic4-heteroallelic and vic-homoallelic C. parasitica heterokaryons

The proteomes of heterokaryons created from parents with identical (*vic*-homoallelic) and different (*vic4*-heteroallelic) *vic4* specificities were compared. The objective was to identify differences in these proteomes that may be associated with *vic4* incompatibility. Total cellular protein was separated in two-dimensions by charge and molecular weight. Both autoradiography of ³⁵S labeled protein and silver staining of unlabeled protein was used to visualize proteins on denaturing gels.

Protein extraction from unlabeled heterokaryons

Heterokaryons of 22.1H and either EP155B, P4-4B or P5-1B were made as previously described. Two to four day old heterokaryotic sectors were excised from cellophane using a sterile loop and grown for 12-15 days in PDB containing 0.2 µg/mL benomyl and 15 µg/mL hygromycin (PDB ben hyg). Total protein was isolated from P4-4B+22.1H, P5-1B+22.1H and EP155B+22.1H. The mycelia in PDA ben hyg were filtered under vacuum with Whatman[®] filter paper contained in a Buchner funnel and washed with 0.9% (w/v) NaCl. Mycelia were crushed at room temperature in first-dimension sample buffer (FDSB; 9.5 M urea, 2.0% (w/v) Triton[®] X-100, 5% (v/v) β-mercaptoethanol, 1.6% Bio-Lyte[®] 5/7 ampholyte (Bio-Rad Laboratories, Hercules, CA), 0.4% Bio-Lyte[®] 3/10 ampholyte (Bio-Rad Laboratories, Hercules, CA)) using a mortar and pestle for 3-4 minutes and the homogenate was centrifuged for 10 minutes at 3500 RPM. The supernatant was transferred to a new tube and stored at -20°C. 50 µg of

unlabeled cellular protein was electrophoresed in two-dimensions and visualized using silver staining (methods to follow).

³⁵S labeling and extraction of cellular protein

To increase the sensitivity of protein detection in two-dimensional gels, cellular protein was labeled with ³⁵S. Heterokaryons of 22.1H and either EP155B, P4-4B or P5-1B were made as previously described. For seven days each heterokaryon was grown at room temperature in natural light conditions in 25 mL of PDB ben hyg and each of the parental strains in 25 mL PDB containing 0.3 µg/mL benomyl (PDB ben; strains EP155B, P4-4B and P5-1B) or 30 µg/mL hygromycin (PDB hyg; strain 22.1H). The colonies were transferred separately to 25 mL of defined medium (0.48 mg H₃BO₃, 1.12 mg MnCl₂·4H₂O, 3.2 mg ZnCl₂, 0.32 mg Na₂MoO₄·2H₂O, 0.8 mg FeCl₃·6H₂O, 3.2 mg CuSO₄·5H₂O, 24 g NH₄NO₃, 16 g KH₂PO₄, 4 g Na₂SO₄, 8 g KCl, 2 g MgSO₄·7H₂O, 1 g CaCl₂ per litre; Puhalla & Anagnostakis, 1971) maintaining the same antibiotic conditions as with PDB. Each culture was amended with 10 µCi/mL of Promix (Amersham Pharmacia Biotech, Piscataway, NJ), which is composed of approximately 30% ³⁵S methionine and 70% ³⁵S cysteine. After 4 hours growth at 25°C in the dark, total proteins were isolated by filtering the colonies under vacuum, crushing in FDSB with mortar and pestle, centrifuging at maximum speed for two minutes, and collecting the supernatant. Proteins were stored at -20°C. To estimate the relative amount of ³⁵S methionine and/or ³⁵S cysteine incorporated into the cellular protein, 200 µg of protein from each sample was subjected to electrophoresis on an 8% acrylamide gel, dried under vacuum with a Bio-Rad (Mississauga, ON) model 583 gel dryer at 60°C for 30 minutes,

and exposed to Kodak Bio-Max MR film. The ^{35}S labeled protein samples were electrophoresed in two dimensions. The amount of protein used for each sample depended on the relative incorporation of ^{35}S methionine and/or ^{35}S cysteine into the cellular protein; 20 μg of ^{35}S labeled 22.1H, EP155B+22.1H, P5-1B+22.1H, 40 μg of P5-1B, P4-4B+22.1H and 80 μg of EP155B, P4-4B protein was electrophoresed. Protein was visualized using autoradiography (method to follow).

Two-dimensional gel electrophoresis

Protein samples were electrophoresed in two dimensions using the Mini-PROTEAN[®] 2-D Cell (Bio-Rad). Total proteins were first separated based on their isoelectric point (pI) using isoelectric focusing (IEF; pH range 3-10). During IEF, proteins at the alkaline end of an immobilized pH gradient gel are subjected to a strong electric field. The proteins migrate towards the positive pole but stop at the point where they are neutralized by the gel and lose their net charge.

The first dimension gel monomer solution consisted of 9.2 M Urea, 4% (w/v) acrylamide, 20% (v/v) Triton[®] X-100, 1.6% (v/v) Bio-Lyte[®] 5/7 ampholyte (Bio-Rad Laboratories, Hercules, CA), 0.4% (v/v) Bio-Lyte[®] 3/10 ampholyte (Bio-Rad Laboratories, Hercules, CA), 0.01% (w/v) ammonium persulfate (APS), and 0.1% (v/v) TEMED. All ingredients except APS and TEMED were combined and degassed for 15 minutes. APS and TEMED were added immediately before casting the first dimension tube gels.

To cast the tube gels, one end of a casting tube was sealed with Parafilm[®] (Pechiney Plastic Packaging Menasha, WI). The casting tube was filled with capillary

gel tubes and the first dimension gel monomer solution was added using a pipette. The tip of the pipette was inserted below the top of the capillary tubes so that air bubbles were not introduced. The first dimension gel was polymerized with the casting tube in a vertical position. After polymerization the capillary tubes were removed from the casting tube and wiped of any excess gel adhering to the outside of the capillary tubes. The tube gels were inspected for air bubbles, and attached to sample reservoirs using tubing connectors.

To run the first dimension, tube gels were inserted into the tube adaptor using the sample reservoirs. Any unused positions in the tube adaptor were blocked with stoppers to prevent the upper buffer from leaking into the lower chamber. Lower chamber buffer (degassed 10 mM H_3PO_4) was forced into the bottom of the tube gels to expelled air bubbles. The tube adaptor with gel tubes was placed into the lower buffer chamber filled with lower chamber buffer. The upper chamber was filled with upper chamber buffer (degassed 100 mM NaOH) until the sample reservoirs were submerged. The air trapped in the sample reservoir was expelled by forcing upper chamber buffer through a long narrow pipette tip into the top of the gel tube and sample reservoir. Protein samples were loaded into the gel tubes and overlaid with 30-35 μl of sample overlay buffer (9 M urea, 0.8% (v/v) Bio-Lyte[®] 5/7 ampholyte (Bio-Rad Laboratories, Hercules, CA), 0.2% (v/v) Bio-Lyte[®] 3/10 ampholyte (Bio-Rad Laboratories, Hercules, CA), 0.0025% (w/v) Bromophenol Blue). The gels were run at 500 V for 10 minutes and then 750 V for 3.5 hours. The gels were removed from the capillary tubes using a tube gel ejector that forces air into one end of the capillary tube gently expelling the gel from the tube. The gels were laid on Parafilm[®] and stored at -20°C .

The first dimension gels containing total protein extract were individually laid along the top of a 1 mm thick 8% acrylamide gel (0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 26.7% (v/v) acrylamide/bisacrylamide (30%T, 2.67%C), 0.05% (w/v) APS, 0.0005% (v/v) TEMED) with a 3% acrylamide stacking gel (0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 10% (v/v) acrylamide/bisacrylamide (30%T, 2.67%C), 0.05% (w/v) APS, 0.001% (v/v) TEMED) lacking wells. The proteins were electrophoresed for one hour at 120 volts.

Silver staining

Silver staining was used to visualize the protein in two-dimensional gels not labeled with ^{35}S . The proteins were fixed in a solution of 50% (v/v) ethanol, 5% (v/v) acetic acid for 30 minutes on a rocking platform. The gels were washed once in 50% (v/v) ethanol for 10 minutes, and twice in water for 10 minutes. The protein was made sensitive to the silver nitrate by placing the gels in freshly prepared 0.02% (w/v) sodium thiosulphate for three minutes. Excess sensitizing solution was removed by washing the gels twice for five minutes in water. To stain the proteins, gels were placed in 0.1% (w/v) silver nitrate and incubated without shaking for 30 minutes at 4°C. The gels were rinsed briefly in water and twice in a small amount of freshly prepared developer (0.04% (v/v) formaldehyde in 2% (w/v) sodium carbonate). The gels were placed in developer until brown spots appeared, rinsed again in water and placed in 5% (v/v) acetic acid to halt the action of the developer. Gels were stored in 1% (v/v) acetic acid before photographing.

Autoradiography

Two-dimensional gels containing ^{35}S labeled protein were dried onto Whatman[®] filter paper in a model 583 gel dryer (Bio-Rad) at 60°C under vacuum for 30 minutes, and exposed to Kodak Bio-Max MR film for 102 days.

Nuclear proportions

The proportions of nuclei from each parental strain in *vic4*-heteroallelic (P5-1B+22.1H and P4-4B+22.1H) and *vic*-homoallelic (EP155B+ 22.1H) heterokaryons were estimated by testing cultures derived from conidia for benomyl and hygromycin resistance (Figure 2.2). Some assumptions of using conidial phenotypes to estimate nuclear proportions in heterokaryons should be addressed. The first assumption is that each conidium contains a single nucleus. In *C. parasitica*, 99.8% of conidia are uninucleate (McGuire *et al.*, 2004). The second assumption is that nuclei are randomly incorporated into developing conidia (Prout *et al.*, 1953). Third, that the two spore types survive and/or germinate at similar rates on nonselective medium (Beadle & Coonradt, 1944; Prout *et al.*, 1953).

Four *vic*-homoallelic (EP155B+22.1H), and three each of *vic4*-heteroallelic (P5-1B+22.1H and P4-4B+22.1H) heterokaryons were grown on PDA ben hyg until the colonies developed conidia. Sterile water was added to each plate and the colony surface was abraded with a sterile bent glass rod. The spores in liquid were collected with a sterile pipette. Four ten-fold dilutions of the conidial suspensions were spread onto PDA with a sterile glass rod. The next day single germinated conidia (germlings) viewed with a dissecting microscope were carefully excised from the medium, transferred to PDA and

Figure 2.2. Testing the parental nuclear proportions of heterokaryons. One parental nuclear type is indicated by dark circles and the other as light circles. Conidia derived from heterokaryons were spread onto PDA. Single germinated conidia (germlings) viewed with a dissecting microscope were excised from the medium, transferred to PDA and grown at room temperature for two to three days. Each conidial-derived isolate was inoculated separately onto PDA hyg and PDA ben and after two days the presence or absence of growth was recorded for each.

grown at room temperature for two to three days. A culture derived from a single conidium will be referred to as a “conidial-derived isolate”. There were so few spores collected for heterokaryon P5-1B+22.1H #1a that the spores on the highest dilution plate were grown for three extra days until small colonies formed. These isolates are denoted as “colony-derived isolates”.

Primary conidial- or colony-derived isolates (Figure 2.3) were examined for resistance to each of benomyl and hygromycin to estimate the nuclear proportions of each heterokaryon. This was done by inoculating each 1° conidial-derived and colony-derived isolate separately onto PDA hyg and PDA ben and recording the presence or absence of growth for each after two days.

Conidial- and colony-derived isolates of heterokaryons that exhibited resistance to both hygromycin and benomyl that may have resulted from isolation of (1) heterokaryotic hyphal fragments rather than single conidia, (2) more than one conidium that subsequently fused to form a heterokaryon (3) rare heterokaryotic conidia, or (4) homokaryotic mitotic recombinants were investigated further. Each single spore isolate able to grow on both PDA ben and PDA hyg was inoculated onto PDA ben hyg. Those that did not grow were inoculated on both PDA and PDA ben hyg to ensure that inoculum was viable. To determine whether the double resistant 1° conidial/colony-derived isolates were homokaryotic or heterokaryotic, 2° conidial-derived isolates (Figure 2.3) were tested for resistance to benomyl and hygromycin.

Figure 2.3. 1° and 2° conidial- and colony-derived isolates. Isolates derived from single conidia or colonies of heterokaryons (1° conidial/colony-derived isolates) are characterized as being either, or both, benomyl (ben^R) or hygromycin (hyg^R) resistant. Isolates derived from 1° conidial/colony-derived isolates (2° conidial/colony-derived isolates) resistant to both benomyl and hygromycin were further characterized for benomyl and/or hygromycin resistance.

Nuclear sorting in heterokaryons grown in non-selective conditions

Five each of homokaryotic isolates, *vic*-homoallelic heterokaryons, *vic4*-heteroallelic heterokaryons and doubly resistant isolates were inoculated onto one end of 7.5 cm by 1 cm sterile pieces of cellophane placed on PDA in petri dishes (Figure 2.4). The isolates were incubated at room temperature (21°C - 22°C) and each day the colony margins were marked on the petri dish bottoms. After twelve days, the cellophane with growing mycelium was aseptically transferred to selective PDA ben hyg. The growth regions along the mycelial strips were monitored over a period of 8 days. The location that either the benomyl or hygromycin resistant nuclear type was lost in heterokaryons grown on non-selective medium was taken as the epicenter of the sector that grew furthest from the colony origin after transfer to PDA ben hyg (see example in Figure 2.4). The corresponding location on the PDA petri dish was used to determine the day at which double resistance was lost between 0 days (no growth) and 12 days (growth from the colony margin).

RESULTS

Growth of C. parasitica strains in antibiotic liquid medium

The restrictive concentration of benomyl and hygromycin in liquid potato-dextrose (PDB) medium was determined by growing strains 22.1H, EP155B, P4-4B and P5-1B in liquid medium amended with a range of concentrations of either benomyl or hygromycin (Figure 2.5). The growth of 22.1H (*hph*) increased from 2% to 30% colony area as the concentration of hygromycin diminished from 30 µg/mL to 15 µg/mL. 22.1H

Figure 2.4. Testing the loss of resistance to either benomyl or hygromycin in heterokaryons grown on non-selective medium. Each heterokaryon was inoculated onto a strip of cellophane overlaid on non-selective PDA. After 12-days growth the cellophane strips were transferred to PDA ben hyg where only mycelia resistant to both benomyl and hygromycin were able to grow. Examples of maintenance of resistance to both benomyl and hygromycin throughout the experiment (bottom left) and loss of resistance to either benomyl or hygromycin in a growing heterokaryon (bottom right; epicentre is marked) are given.

Mycelial strip
on cellophane
transferred to
PDA ben hyg

Incubate
(4-8 days)

Double resistance maintained in
growing colony

Double resistance lost in
growing colony

Figure 2.5. Growth of isolates 22.1H (circles), EP155B (triangles), P4-4B (squares) and P5-1B (diamonds) in hygromycin (A) and benomyl (B) amended PDB measured as the percent area of the flask bottom covered by fungal colonies after growth for seven days at room temperature.

did not grow at benomyl concentrations of greater than 0.2 $\mu\text{g/mL}$, however a 2% colony area was observed with a 0.1 $\mu\text{g/mL}$ benomyl concentration. The growth of EP155B (*bml^R*) was completely inhibited at ≥ 15 $\mu\text{g/mL}$ hygromycin and varied between 30% and 75% coverage throughout the benomyl concentration range (0.1-0.4 $\mu\text{g/mL}$). The growth of P4-4B (*bml^R*) was completely inhibited at ≥ 15 $\mu\text{g/mL}$ hygromycin and varied between 50% and 80% throughout the benomyl concentration range. The growth of P5-1B (*bml^R*) was completely inhibited by 15 $\mu\text{g/mL}$ of hygromycin but 0.5% colony cover was observed in ≥ 20 $\mu\text{g/mL}$ hygromycin. P5-1B growth varied between 50% and 70% coverage throughout the benomyl concentration range. Therefore, 0.2 $\mu\text{g/mL}$ benomyl and 15 $\mu\text{g/mL}$ hygromycin was used in liquid medium to select for heterokaryons resistant to both benomyl and hygromycin.

Heterokaryon incompatibility vs. mycelial incompatibility associated with vic4 differences

Double selection on PDA ben hyg permitted the growth of heterokaryotic hyphae (with resistance to both benomyl and hygromycin) and restricted the growth of monokaryotic hyphae (with resistance to either benomyl or hygromycin but not to both). Both *vic4*-heteroallelic and *vic4*-homoallelic heterokaryons grew from the regions of contact between colonies, producing an outgrowth of mycelium (Figure 2.6).

The *vic4* genotypes of the strains used to create heterokaryons (as determined by Cortesi and Milgroom (1998)) were verified with barrage (mycelial compatibility testing). Mycelial compatibility testing of *vic*-homoallelic and *vic4*-heteroallelic strains was repeated four times using PDAG medium. After 5 days growth on PDAG, a dark line

Figure 2.6. Heterokaryotic mycelium formed from two *C. parasitica* parental isolates 22.1H and P4-4B grown on PDA without antibiotics and then transferred to PDA amended with benomyl and hygromycin.

was observed between positive control strains EP155B/P11-23 (*vic1*-heteroallelic) and experimental strains EP155B/P4-4B and EP155B/P5-1B (*vic4*-heteroallelic). No dark line was observed between the *vic*-homoallelic controls EP155B/EP155B and EP155B/22.1H (Figure 2.7).

Percent dead or dying cells

The percentage of dead or dying cells was estimated in homokaryons, *vic4*-heteroallelic and *vic*-homoallelic heterokaryons, and two double resistant isolates of P5-1B+22.1H #1b using EB and FDA (FDA given as 100 - percentage of living cells; data provided by A. Moss; Figure 2.8). The two methods did not give significantly different results ($p = 0.08$) and the percentage of dead cells was significantly different between strains ($p < 0.0001$). The percent of dead cells among heterokaryons was greater than that of homokaryons. The *vic*-homoallelic heterokaryon EP155B+22.1H displayed approximately twice the percentage of dead cells as the *vic4*-heteroallelic heterokaryons P4-4B+22.1H and P5-1B+22.1H. The cell death frequencies of two double resistant isolates of P5-1B+22.1H #1b were similar to the *vic4*-heteroallelic heterokaryons (Figure 2.8).

Proteomic evaluation of vic4-heteroallelic and vic-homoallelic C. parasitica heterokaryons

The proteomes of *vic4*-heteroallelic and *vic4*-homoallelic heterokaryons were compared to identify candidate VIC4 or VIC4-associated proteins. Total *C. parasitica* protein was separated using two-dimensional gel electrophoresis and detected using ^{35}S

Figure 2.7. Mycelial compatibility test of *C. parasitica* strains. EP155B/EP155B and EP155B/22.1H are homoallelic at all *vic* loci and served as compatible controls. EP155B and P11-23 are *vic1*-heteroallelic and served as an incompatible control. EP155B/P4-4B and EP155B/P5-1B are *vic4*-heteroallelic. A dark coloured line between two colonies indicates an incompatibility reaction.

Figure 2.8. Percentage of dead cells in (1) parental strains 22.1H, EP155B, P4-4B, P5-1B, (2) *vic*-homoallelic EP155B+22.1H, (3) *vic4*-heteroallelic P4-4B+22.1H, P5-1B+22.1H and (4) two double resistant conidial-derived isolates of P5-1B+22.1H measured using Evans blue (EB) and fluorescein diacetate (FDA; given as 100 – percent FDA stained cells). The error bars indicate standard deviation (n=3).

labeling or silver staining. Using both methods of detection, we did not observe a distinct protein in the proteome of *vic4*-heteroallelic heterokaryons that was absent in *vic4*-homoallelic heterokaryons (Figure 2.9, 2.10). Conversely, when ^{35}S protein labeling was used to detect proteins, one protein spot with a mid-range pI and at a relatively low molecular weight (indicated with an arrow in Figure 2.10) was more prominent in EP155B+22.1H *vic*-homoallelic heterokaryon and haplotype proteomes than in *vic4*-heteroallelic heterokaryons.

Nuclear proportions

The proportion of nuclei contributed by each parental strain to EP155B+22.1H, P4-4B+22.1H and P5-1B+22.1H heterokaryons was estimated by testing the antibiotic resistance of conidia originating on the heterokaryons (see Table 2.2 for sample sizes). For each conidium there were three possible outcomes: resistance to benomyl only (denoted benomyl resistant), resistance to hygromycin only (denoted hygromycin resistant), and resistance to both benomyl and hygromycin (denoted doubly resistant; Figure 2.3). ML Smith provided data for three P5-1B+22.1H and five EP155B+22.1H heterokaryons.

The proportion of hygromycin resistant conidia for each heterokaryon was calculated as a percent of the total conidia tested for that heterokaryon minus conidia doubly resistant. The proportion of conidia doubly resistant was calculated as a percent of the total conidia for that heterokaryon (Table 2.2). The proportion of hygromycin resistant conidia derived from *vic4*-heteroallelic heterokaryons (P4-4B+22.1H and P5-1B+22.1H) had a bimodal distribution meaning that these heterokaryons contained

Figure 2.9. Two-dimensional gel electrophoresis of EP155B+22.1H, P4-4B+22.1H and P5-1B+22.1H unlabelled total proteins. The proteins were separated horizontally by isoelectric focusing (pH range of 3 on right to 10 on left) and vertically by molecular weight and visualized using silver staining.

EP155B+22.1H

P4-4B+22.1H

P5-1B+22.1H

Figure 2.10. Two-dimensional gel electrophoresis of EP155B, P4-4B, P5-1B, EP155B+22.1H, P4-4B+22.1H and P5-1B+22.1H ³⁵S labelled proteins. The proteins were separated horizontally by isoelectric focusing (pH range of 3 on right to 10 on left) and vertically by molecular weight and exposed to x-ray film. A protein with decreased intensity in P4-4B+22.1H and P5-1B+22.1H heterokaryons is indicated by an arrow.

EP155B

P4-4B

P5-1B

EP155B+22.1H

P4-4B+22.1H

P5-1B+22.1H

Table 2.2. Proportion of single spore isolates of *vic*-homoallelic and *vic4*-heteroallelic heterokaryons either hygromycin or benomyl resistant or resistant to both hygromycin and benomyl.

Vegetative compatibility ^a	Heterokaryon ^b	Proportion hyg resistant ^d	n ^c	Proportion ben hyg resistant ^f	n ^g
≠	P4.4B+22.1H#1	0	26	0	26
	P4.4B+22.1H#2	0.87	23	0	23
	P4.4B+22.1H#3	0	66	0	66
	P5.1B+22.1H ^c	0.82	38	0	38
	P5.1B+22.1H ^c	0.88	50	0	50
	P5.1B+22.1H ^c	1	50	0	50
	P5.1B+22.1H#1a	0.20	15	0.06	16
	P5.1B+22.1H#1b		0	1	28
	P5.1B+22.1H#2	1	41	0.07	44
	P5.1B+22.1H#3	0.29	14	0	14
=	EP155B+22.1H ^c	1	44	0	44
	EP155B+22.1H ^c	0.87	46	0	46
	EP155B+22.1H ^c	0.76	45	0	45
	EP155B+22.1H ^c	0.67	49	0	49
	EP155B+22.1H ^c	0.04	70	0	70
	EP155B+22.1H#1	0.56	36	0	36
	EP155B+22.1H#2	0	13	0	13
	EP155B+22.1H#3	0.24	25	0	25
	EP155B+22.1H#5	0.31	32	0	32

^a Vegetative compatibility of parental strains based on *vic4* genotype. Different or identical alleles at *vic4* between parental isolates are indicated by ≠ and =, respectively.

^b Heterokaryon designations are indicated by the two parental isolates followed by the heterokaryon number.

^c Data provided by ML Smith.

^d Hygromycin resistant isolates as a proportion of the total isolates minus isolates resistant to both benomyl and hygromycin.

^e Total isolates minus isolates resistant to both benomyl and hygromycin.

^f Isolates resistant to both benomyl and hygromycin as a percentage of the total isolates.

^g Total isolates

almost all benomyl resistant nuclei or almost all hygromycin resistant nuclei.

Conversely, the proportion of each nuclear type in EP155B+22.1H heterokaryons was more evenly distributed (Figure 2.11).

The nuclear proportion of single spore isolates resistant to both benomyl and hygromycin was estimated to ensure that resistance was not due to the isolation of a hyphal fragment or multiple conidia. Six P5-1B+22.1H 1° single spore isolates resistant to both antibiotics (one from P5-1B+22.1H #1a, two from P5-1B+22.1H #1b and three from P5-1B+22.1H #2) also had conidia resistant to both antibiotics (15, 52, 52, 15, 16 and 20 spores tested, respectively). Conversely, single spore isolates of EP155B+22.1H and P4-4B+22.1H heterokaryons able to grow on PDA ben and PDA hyg were either unable to grow on PDA ben hyg or had conidia resistant to either benomyl or hygromycin. Three 1° conidial-derived isolates of EP155B+22.1H (one from EP155B+22.1H #1 and two from EP155B+22.1H #3) were recovered on PDA ben hyg and found to have conidia resistant to either, but not both, benomyl or hygromycin (5, 9 and 5 conidia tested, respectively). One 1° conidial-derived isolate of P4-4B+22.1H #2 was recovered on PDA ben hyg and found to have conidia resistant to either, but not both, benomyl or hygromycin (13 conidia tested). As a result, EP155B+22.1H and P4-4B+22.1H 1° single spore isolates having resistance to both benomyl and hygromycin were assumed to be hyphal fragments or multiple-spore isolates and excluded from further analyses.

After excluding doubly resistant 1° colony/conidial isolates that did not themselves produce doubly resistant 2° colony/conidial isolates, no EP155B+22.1H (n=9) nor P4-4B+22.1H (n=3) heterokaryons bore conidia resistant to both benomyl and

Figure 2.11. Distribution of the proportion of hygromycin resistant single spore isolates (hygromycin resistant / total – doubly resistant conidia) of EP155B+22.1H, P4-4B+22.1H and P5-1B+22.1H heterokaryons.

hygromycin. Conversely, three of the seven P5-1B+22.1H heterokaryons had doubly resistant conidia: one had all doubly resistant conidia and two had less than 10% doubly resistant conidia (Table 2.2).

Nuclear sorting in heterokaryons grown under non-selective conditions

As expected, homokaryons 22.1H, EP155B, P4-4B and P5-1B grown on PDA were not resistant to both benomyl and hygromycin. Growth of these strains was not observed after transfer to PDA ben hyg (n=5; Figure 2.12). Doubly resistant conidial-derived isolates of P5-1B+22.1H heterokaryons (1a-15, 1b-35, 2-6) grown on non-selective PDA for 12 days retained resistance to both benomyl and hygromycin (growth after transfer to PDA ben hyg; n=5; Figure 2.12, 2.13).

In general, *vic4*-heteroallelic heterokaryons retained both nuclear types for longer than did *vic*-homoallelic heterokaryons. Four of nine EP155B+22.1H, two of seven P4-4B+22.1H and two of eleven P5-1B+22.1H heterokaryons did not grow after transfer to PDA ben hyg. Of those that did grow, resistance to both benomyl and hygromycin was generally retained between 3 and 4 days in *vic*-homoallelic EP155B+22.1H. Two *vic4*-heteroallelic P4-4B+22.1H heterokaryons maintained resistance for the entire 12 days, and the other three maintained heterokaryons for ≤ 7 days. The time at which *vic4*-heteroallelic P5-1B+22.1H heterokaryons lost double resistance was clustered into two distinct groups; two heterokaryons maintained resistance the entire 12 days and the other seven for ≤ 4 days (Figure 2.13).

Figure 2.12. Growth of homokaryons 22.1H, EP155B, P4-4B, P5-1B, and double resistant P5-1B+22.1H single spore isolates 1a-15, 1b-35 and 2-6 on PDA ben hyg 7 days after transfer from non-selective PDA.

22.1H

EP155B

P4-4B

P5-1B

P5-1B+22.1H #1a-15

P5-1B+22.1H #1b-35

P5-1B+22.1H #2-6

Figure 2.13. Day that (1) *vic*-homoallelic heterokaryons (EP155B+22.1H), (2) *vic4*-heteroallelic heterokaryons (P4-4B+22.1H, P5-1B+22.1H) and (3) double resistant 1° conidial-derived P5-1B+22.1H isolates grown on nonselective PDA lost the ability to grow on selective PDA ben hyg.

DISCUSSION

Mycelial incompatibility is correlated with heterokaryon incompatibility function in *C. parasitica* at all *vic* loci except for *vic4* (ML Smith and MG Milgroom, unpublished results). As shown in this study, the *vic4* locus mediates mycelial incompatibility but permits heterokaryosis. In this study *vic4*-heteroallelic and *vic*-homoallelic heterokaryons were compared for frequency of cell death, protein profiles and nuclear segregation and sorting.

Cell death

vic4-heteroallelic heterokaryons had a greater frequency of cell death than homokaryons indicating that differences at *vic4* may elicit a weak incompatibility reaction that does not significantly reduce growth rates (ML Smith and MG Milgroom, unpublished). Unexpectedly, *vic*-homoallelic heterokaryons had a greater percentage (~50%) of dead or dying cells compared to *vic4*-heteroallelic heterokaryons (~20%). The two homokaryotic strains, EP155B and 22.1H, forming *vic*-homoallelic heterokaryons are nearly isogenic with differences putatively only at antibiotic resistance loci (*N. crassa* benomyl-resistance gene *bml^R* and the *Escherichia coli* hygromycin B phosphotransferase gene *hph*, respectively). Increased cell death due to the co-occurrence of *bml^R* and *hph* is unlikely since both *vic4*-heteroallelic and *vic*-homokaryotic heterokaryons carry both genes.

Similarity of protein profiles

The protein profiles of *vic*-homoallelic and *vic4*-heteroallelic heterokaryons were compared to identify VIC4 or a VIC4-associated protein. The pattern of protein separation from two-dimensional gel electrophoresis was similar between both monokaryons and heterokaryons, and between *vic*-compatible and *vic4*-incompatible heterokaryons. The similarity of the protein profiles may have been due to the apparent lack of an incompatibility reaction in heterokaryons that carry both *vic4-1* and *vic4-2*. One protein detected using ³⁵S protein labelling, however, was less abundant in the *vic4*-heteroallelic heterokaryons. This protein may function in *vic4*-mediated heterokaryon incompatibility through protein aggregation, precipitated during protein extraction and was discarded. Mass spectrometry could be used to determine the amino acid sequence of this protein. The sequence would give information on the protein structure and function and could be used to clone the corresponding gene through reverse genetics.

Nuclear proportions in vic-homoallelic heterokaryons

vic-homoallelic *C. parasitica* heterokaryons had a wide range of nuclear proportions that tended to be evenly distributed between 0 and 1. This is similar to the results of Beadle and Coonradt (1944) and Pittenger and Atwood (1956) who found a wide range of *N. crassa* nuclear proportions in compatible heterokaryons (same at all *het* loci) with wild-type growth rates. Stable submaximal growth rates occurred only in compatible heterokaryons with extreme disproportion (0.919 - 0.994) in the initial nuclear proportions (Pittenger & Atwood, 1956).

The range of nuclear proportions in *vic*-homoallelic *C. parasitica* heterokaryons may correlate to the nuclear proportions at time of heterokaryon formation. In *N. crassa*, compatible heterokaryons exhibiting both maximal and submaximal growth have stable nuclear proportions over prolonged growth (input ratios approximately equal output ratios). The mechanism stabilizing nuclear proportions is nonadaptive, meaning that nuclear ratios in *N. crassa* remain constant even when a change may facilitate growth through complementation of auxotrophic mutations (Pittenger & Atwood, 1956). Monitoring the nuclear ratios in *vic*-homoallelic *C. parasitica* heterokaryons throughout growth would show if changes in nuclear proportions occur.

Skewed nuclear ratios may facilitate vic4-heteroallelic heterokaryon formation

vic4-heteroallelic heterokaryons had nuclear proportions that tended to be skewed towards one or the other nuclear type, which may explain the apparent lack of a *vic4*-heterokaryon incompatibility reaction. The population of minority nuclei may be small enough to avoid an incompatibility reaction but large enough to confer antibiotic resistance to the colony. In addition, the growing heterokaryon at the time of conidiation may be a mosaic of partly heterokaryotic and partly homokaryotic sections (Atwood & Mukai, 1955). Prout *et al.* (1953) found that hyphal tips from *N. crassa* heterokaryons are often homokaryotic and Atwood and Mukai (1955) found that in *N. crassa*, like nuclei tend to occur together in compatible heterokaryons. If *vic4*-heteroallelic heterokaryons were a mosaic of homokaryotic and heterokaryotic hyphal segments then relatively few cells would be truly heterokaryotic.

The disproportion of nuclear types comprising *vic4*-heteroallelic heterokaryons implies that (1) nuclear proportions become skewed throughout growth of the heterokaryon and/or (2) homokaryons contribute disproportionate number of nuclei during heterokaryosis to result in skewed nuclear proportions. Changes in nuclear proportion throughout growth was shown with studies of *N. crassa het-i* (Pittenger & Brawner, 1961), in which nuclei carrying *het-I* are thought to replicate at a greater relative rate and inhibit replication of *het-i* nuclei under certain conditions. Inherently different rates of *vic4-1* and *vic4-2* nuclear division, however, cannot explain skewed nuclear ratios in *vic4*-heteroallelic heterokaryons because one nuclear type was not consistently in the majority. Alternatively, the rate of *vic4-1* and *vic4-2* nuclear division may be dependent on nuclear proportion. For example, an initial nuclear proportion less than 70% of *het-i* nuclei in *N. crassa* heterokaryons leads to a drastic increase in *het-I* nuclei resulting in labile or no growth (Pittenger & Brawner, 1961). Sampling nuclear ratios at growth intervals of *vic4*-heteroallelic heterokaryons would be useful to study the dynamics in nuclear proportions during colony growth.

Nuclear sorting

Both *vic*-homoallelic and *vic4*-heteroallelic heterokaryons were grown on nonselective medium and a radial-oriented elongate mycelial sample was transferred to selective medium to test the maintenance of double resistance in growing heterokaryons. Heterokaryons grown on nonselective medium are not expected to be under a selective pressure to maintain heterokaryosis. Either nuclear type is adequate for survival and growth on nonselective medium. Loss of double resistance is presumably due to loss of

one nuclear type and the associated antibiotic resistance. All *vic*-homoallelic EP155B+22.1H heterokaryons and some of each P4-4B+22.1H and P5-1B+22.1H *vic4*-heteroallelic heterokaryons lost one nuclear type during growth on nonselective medium. Other *vic4*-heteroallelic heterokaryons, however, retained resistance to both antibiotics throughout the 12-day growth period. These heterokaryons may permanently retain both nuclear types or take longer than 12 days to lose one nuclear type. These results suggest that nuclei with differences at *vic4* are more stable as heterokaryons than nuclei without *vic* differences. Alternately, some *vic4*-heteroallelic heterokaryons may have undergone diploidization (see *The parasexual cycle* below) resulting in homokaryons resistant to both antibiotics. Testing the nuclear proportions of doubly resistant mycelia growing from the colony margin would differentiate between the above two possibilities. Heterokaryons would have conidia resistant to either antibiotic and recombinant homokaryons would have all conidia resistant to both antibiotics.

The parasexual cycle

Since the conidia of *C. parasitica* are nearly all uninucleate (McGuire *et al.*, 2004), conidial-derived cultures from heterokaryons are expected to be of one parental type and not both. If the parents carry different antibiotic resistance genes, then isolates derived from conidia would not be expected to grow on medium supplemented with both antibiotics. Unexpectedly, I found that some P5-1B+22.1H heterokaryons produced homokaryotic conidia resistance to both benomyl and hygromycin.

P5-1B+22.1H doubly resistant isolates may have arisen through diploidization with or without mitotic recombination characteristic of the parasexual cycle (Figure

Figure 2.14. The parasexual cycle. Two homokaryons fused to form a heterokaryon with genetically different nuclei. The nuclei fuse to form a heterozygous diploid which may experience crossing over during mitosis. Frequent errors in mitosis of diploid nuclei yield aneuploid daughter nuclei that lose chromosomes to restore either diploidy or haploidy.

Adapted from Charlie *et al.*, 2001.

2.14). The parasexual cycle has been suggested to occur in *C. parasitica* (Puhalla and Anagnostakis, 1971) but has not been characterized. To initiate the parasexual cycle, genetically different nuclei in a heterokaryon fuse to form a diploid. The diploid nucleus can divide mitotically, during which mitotic crossing over can occur. Errors are common during mitosis of diploids resulting in aneuploid daughter nuclei. $2n+x$ aneuploid nuclei will lose any one chromosome present in three copies to restore diploidy; $2n-x$ aneuploid nuclei will lose either of each homologous pair to become haploid. Haploidization may take several mitoses to complete. Mitotic recombination therefore occurs not only through mitotic crossing over but also through chromosome loss (Charlie *et al.*, 2001). If the P5-1B+22.1H isolates acquired resistance through the parasexual cycle, then the resistant nuclei may be haploid, aneuploid, or diploid. Aneuploid strains generally grow poorly (Charlie *et al.*, 2001) and therefore haploid and diploid nuclei would most likely be favoured. We are currently using AFLP (Amplified Fragment Length Polymorphisms) analysis to compare the genotypes of parental and doubly resistant isolates. This information will help to determine the ploidy level of these strains. The occurrence of double resistance only in conidial isolates of P5-1B+22.1H may be an artefact of a small sample size or perhaps due to unknown properties of P5-1B+22.1H heterokaryons whereby diploidization is favoured.

Summary

Significant heterokaryon incompatibility was found not to occur between *C. parasitica* strains that differed at *vic4*, a gene that mediates mycelial incompatibility. No protein involved in protein aggregation was identified through protein profiling and cell

death frequencies were greater than homokaryons but less than a heterokaryon with no *vic* differences. Heterokaryons with *vic4* differences had skewed nuclear ratios that may prevent a heterokaryon incompatibility reaction. Unexpectedly, heterokaryons with no *vic* differences appeared to be more incompatible than heterokaryons heteroallelic at *vic4*. Heterokaryons with no *vic* differences had greater cell death and quickly returned to homokaryosis on nonselective medium compared to heterokaryons with both *vic4-1* and *vic4-2* alleles. Other *vic* homoallelic heterokaryons with different genetic backgrounds need to be investigated to test if high cell death and rapid homokaryosis are restricted to EP155B+22.1H or occur among all *vic*-homoallelic heterokaryons.

CONCLUSION

This thesis investigated nonself recognition in two ascomycete fungi, *N. crassa* (Chapter 1) and *C. parasitica* (Chapter 2). In Chapter 1, I investigated transcription of *un-24*, an *N. crassa* heterokaryon incompatibility gene, throughout the *N. crassa* life cycle, during incompatibility and escape from incompatibility. In Chapter 2, I characterized *C. parasitica* heterokaryons differing at *vic4*, a gene that mediates mycelial incompatibility.

In *N. crassa*, *un-24* has both heterokaryon incompatibility and ribonucleotide reductase function (Smith *et al.*, 2000a). Using semi-quantitative RT-PCR, I found that transcript levels of both *un-24* alleles, *un-24*^{OR} and *un-24*^{PA}, increased when strains were cultured on synthetic crossing medium. If *un-24* is upregulated at the outset of the sexual cycle, how is incompatibility avoided during mating? Is *un-24* locally downregulated in reproductive structures to prevent an incompatibility reaction? Are dNTPs transported into protoperithecia from surrounding regions? These questions could be explored by comparing *un-24* transcript levels in perithecia, sexual structures visible to the naked eye, to transcript levels in pre-meiotic (grown in limiting nitrogen conditions) and vegetative cultures (where nitrogen is not limiting).

The ability of *un-24* alleles to be differentially regulated during the *N. crassa* life cycle suggests that transcript regulation may play a role in *un-24*-mediated heterokaryon incompatibility and escape from incompatibility. The results of this and previous research (Micali, 2003) suggest that unstable self-incompatible strains carrying a resident *un-24*^{OR} allele and an ectopic *un-24*^{PA} allele escape from incompatibility through a number of mechanisms including loss of the transformed nucleus, deletion of either the ectopic or endogenous copy, temporary inactivation of the ectopic allele, and

spontaneous suppressor mutation. The results of my research also implicate transcription regulation as a mechanism of escape. Escape strains transcribing both alleles underwent a transcriptional switch from relatively high *un-24^{OR}* transcripts before escape to relatively high *un-24^{PA}* after escape, suggesting that escape may occur through altering the balance in allelic transcript levels. Transcript analysis identifying key upstream DNA elements and their interaction with activators and/or repressors to regulate *un-24^{OR}* and *un-24^{PA}* transcription would be valuable to the understanding of how *un-24* alleles, and possibly the alleles of other *het* genes, are differentially regulated during incompatibility and mating.

Mycelial incompatibility (assayed by barrage) and heterokaryon incompatibility are two forms of vegetative incompatibility. The two forms are often assumed to be manifestations of the same phenomenon (Micali & Smith, 2003), however studies of *N. crassa* (Micali & Smith, 2003), *S. sclerotiorum* (Ford *et al.*, 1995) and this study on the *vic4* gene of *C. parasitica*, suggest that there are at least two pathways involved in nonself recognition in fungi. In *C. parasitica*, six genes are involved in mycelial incompatibility. All except one, *vic4*, also mediate heterokaryon incompatibility. Significant heterokaryon incompatibility, characterized by low growth rates (ML Smith and MG Milgroom, unpublished), protein profiles and cell death frequency, did not occur in heterokaryons with *vic4* differences. The absence of incompatibility characteristics of *vic4* heterokaryons may be due to skewed nuclear proportions of the two parental nuclear types. This is suggested by the observation that the proportions of parental nuclear types in *vic4* heterokaryons were biased toward one or the other, whereas heterokaryons with no *vic* differences had a wide range of nuclear proportions including balanced ratios. The

mechanisms responsible for the observed differences in nuclear proportions between heterokaryons with and without *vic4* differences are unknown. One possible explanation is that in *vic4* heterokaryons, each nuclear type inhibits or alters the nuclear division rate of the other. Alternatively, *vic4*-heteroallelic nuclear types may compete for resources required for nuclear division, thereby skewing nuclear proportions.

Finally, I observed that conidia of one *vic4* heterokaryon frequently carried antibiotic markers from both parents, suggesting that a parasexual mechanism occurs in *C. parasitica*. A more extensive genetic analysis involving multiple genetic markers is needed to test whether or not somatic recombination occurs in these strains.

Characterization of the parasexual cycle in *C. parasitica* may have significant implications for genetic studies. Crossing strains of *C. parasitica* is a difficult and laborious task and somatic recombination could be an important tool in mapping *C. parasitica* chromosomes.

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APPENDIX A: *un-24* transcript levels in *N. crassa* homokaryons grown on low and high
nitrogen medium

Figure A.1. Total RNA isolated from *N. crassa* strains col(4)-2 (lanes 1 and 2), C9-2 (lanes 3 and 4) and C2(2)-1 (lanes 5 and 6) grown on Vogel's medium (lanes 1, 3, 5) and SCM (lanes 2, 4, 6) separated on a 1.3% denaturing agarose gel. (A) Replicate 1 containing 2 µg of total RNA. (B) Replicate 2 containing 10 µg of total RNA.

Figure A.2. Autoradiographs of Northern blots testing the expression of *un-24* during the vegetative (lanes 1, 3, 5; VMM) and sexual (lanes 2, 4, 6; SCM) growth phases of *N. crassa* strains col(4)-2 (lanes 1 and 2; *un-24*^{OR-}), C9-2 (lanes 3 and 4; *un-24*^{OR}) and C2(2)-1 (lanes 5 and 6; *un-24*^{PA}). (A) Replicate 1 containing 2 µg of total RNA. (B) Replicate 2 containing 10 µg of total RNA.

Appendix B: Polyclonal anti-UN-24 antibody development

A rabbit polyclonal antibody was raised against the UN-24 peptide KRDGRQERVQFDKIT (BioCarta, San Diego, CA). The peptide sequence was derived from amino acids 5 to 19 in the N-terminus of UN-24. This sequence was chosen because a peptide sequence with one amino acid difference was used to make an anti-mouse-RNR-N antibody (Takada *et al.*, 2000) that hybridizes weakly to RNR R1 of *N. crassa* (Haidari, 2002). The sequence is also common between the UN-24 Panama and UN-24 Oak Ridge protein sequences (Figure B.1). The peptide was synthesized, conjugated to a keyhole limpet hemocyanin peptide via a N-terminal cysteine and injected into two rabbits.

Eight weeks after immunization the rabbit serum (provided by BioCarta) was tested for specificity to UN-24 using immunoblotting. To extract protein from *N. crassa*, strains C9-2 (Smith *et al.*, 2000a) and C2(2)-1 (Smith *et al.*, 1996) were grown in liquid Vogel's medium ((Vogel, 1964); 3 g Na₃Citrate·5H₂O, 2 g NH₄NO₃, 5 g KH₂PO₄, 200 mg MgSO₄·7H₂O, 100 mg CaCl₂·2H₂O, 250 mg L-threonine, 250 mg citric acid·H₂O, 250 mg ZnSO₄·7H₂O, 50 mg Fe(NH₄)₂(SO₄)₂·6H₂O, 12.5 mg CuSO₄·5H₂O, 2.5 mg MnSO₄·H₂O, 2.5 mg H₃BO₃, 2.5 mg Na₂MoO₄·2H₂O, 0.005 ng biotin, 15 g sucrose per litre), filtered under vacuum through Whatman[®] paper, washed with 0.9% (w/v) NaCl, and frozen in liquid nitrogen. The tissue was ground in liquid nitrogen and protein was extracted using the TRIzol reagent (Invitrogen, Burlington, ON). 1 mL of TRIzol was added to the ground tissue and homogenized thoroughly by vortexing. All subsequent steps of the extraction were performed on ice. The homogenate was transferred to an epitube, supplemented with 200 µl of chloroform, and vortexed 15 seconds. To separate protein, DNA, and RNA, samples were centrifuged at 12000 x g for 15 minutes at 4°C.

Figure B.1. Alignment of the UN-24 Oak Ridge (top) and UN-24 Panama proteins. The peptide sequence used to make a polyclonal anti-UN-24 antibody is given in italics.


```

1  MYVKKRDGRQERVQFDKITARVSRLCYGLDMNHVDPVAITQKVISGVYGGVTTAQLDDLA
1  MYVKKRDGRQERVQFDKITARVSRLCYGLDMNHVDPVAITQKVISGVYGGVTTAQLDDLA
*****

61 AETAAYMTVTHPDYAILAARIAVSNLHKQTKKQWSLVISELYNYVNPRTGKHSPMIKDV
61 AETAAYMTVTHPDYAILAARIAVSNLHKQTKKQWSLVISELYNYVNPRTGKHSPMIKDV
*****

121 YECVMRHKDEFDSAIVYDRDFNYQYFGFKTLERSYLLKLDGQIAERPQHMIMRVAVGIWG
121 YECVMRHKDEFDSAIVYDRDFNYQYFGFKTLERSYLLKLDGQIAERPQHMIMRVAVGIWG
*****

181 DDVERVIETYNLMSLKTFTTHASPTLFNAGTPQPQLSSCFLVDMKEDSIEGIYDTLKTCAM
181 DDVERVIETYNLMSLKTFTTHASPTLFNAGTPQPQLSSCFLVDMKEDSIEGIYDTLKTCAM
*****

241 ISKMAGGIGLNIHRIRATGSYIAGTNGTSNGIVPMLRVFNNTARYVDQGGNKRPGAFAIY
241 ISKMAGGIGLNIHRIRATGSYIAGTNGTSNGIVPMLRVFNNTARYVDQGGNKRPGAFAIY
*****

301 LEPWHADVFEFLDLRKNHGKEEVRARDLFLALWIPDLFMKRVEQNGQWTL MCPHECPGLA
301 LEPWHADVFEFLDLRKNHGKEEVRARDLFLALWIPDLFMKRVEQNGQWTL MCPHECPGLA
*****

361 DVYGDEFEALYEKYEKEGKGRKTVKAQKLWYAILEAQTTETGNPFMLYKDACNRKSNQKNL
361 DVYGDEFEALYEKYEKEGKGRKTVKAQKLWYAILEAQTTETGNPFMLYKDACNRKSNQKNL
*****

421 GTIRSSNLCTEII EYSAPDEVAVCNLASLALSAFIDYENASYDFKKLHEVTQVVRNLNK
421 GTIRSSNLCTEII EYSAPDEVAVCYLASLALSAFIDYENASYDFKKLHEVTQVVRNLNK
*****

481 IIDINHYPVKEAHNSNMRHRPIGVGVQGLADAF LALRMPFSDAASKLNIQIFETIYHAA
481 IIDINHYPVKEAHNSNMRHRPIGVGVQGLADAF LALRMPFSDAASKLNIQIFETIYHAA
*****

541 LTASCQLAKEQGPYATYEGSPVSQGILQYDMWNVTPTNLWDWTALKADIKKYGVRNSLLL
541 LTASCQLAKEQGPYATYEGSPVSQGILQYDMWNVTPTNLWDWTALKADIKKYGVRNSLLL
*****

601 APMPASTSQILGNNECFEPYTSNIYQRRVLAGEFQVVNPWLLRDLVEMGLWSDAMKNRI
601 APMPASTSQILGNNECFEPYTSNIYQRRVLAGEFQVVNPWLLRDLVEMGLWSDAMKNRI
*****

661 IAEGGSIQNIQSIPNDIKALYKTVWEISQRTIVKMAADRGAFIDQSQSLNIHMREPTMGK
661 IAEGGSIQNIQSIPNDIKALYKTVWEISQRTIVKMAADRGAFIDQSQSLNIHMREPTMGK
*****

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721 ITSMHFAGWKMGGLKTGMYLRTQAAAQPIQFTVDQEALRATDDRVAPAHSGLKKRSPPAG
721 ITSMHFAGWKMGGLKTGMYLRTQAAAQPIQFTVDQEALRATDDRVAPAHSGLKKRSPPAG
*****

781 TYTSIVLRENTSGPRPYAQTGVSGTSTPIGTRDVPTPASTPPPTEVPETLVQSDNRPRPL
781 TYTSIVLRENTSGPRPYAQTGVSGTSTPIGTRDVPTPASTPPPAEDSETLVQSDNRPRPL
***** * *****

841 VSPAKSAGFKADLPEPESPKALATDPIVKTEDIGSPLLERKEGQNEVDDEDSQERDENIY
841 VSPAKNAGFKADLPEPESPKALATDPIVKTEAISSRELEMKEGQSEDKDEESKEREEDIY
***** * * ** * * * * *

901 SNAPLSEQQVAACAWNPGADPSSCEMCSG
901 SD-----EVVSCPYPVPGADPSSCVMCSG
* * * *****

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The upper aqueous phase containing RNA was carefully removed. Each sample was supplemented with 0.3 mL of 100% ethanol and mixed by inversion. To pellet DNA the samples were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant containing protein was retained. 1.5 mL of isopropanol was added and samples were incubated at room temperature for 10 minutes. To pellet the protein, samples were centrifuged at 12000 x g for 10 minutes at 4°C. The supernatant was removed and the protein pellets were washed three times in a solution containing 0.3 M guanidine hydrochloride in 95% (v/v) ethanol. During each wash, 2 mL of the solution was incubated with the pellet at room temperature for 20 minutes, samples were centrifuged at 7500 x g for 5 minutes at 4°C, and the supernatant was decanted. The protein pellet was vortexed in 2 mL of ethanol, incubated for 20 minutes at room temperature and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was decanted and the pellet was vacuum dried using a Savant Speed Vac Concentrator. The protein pellets were dissolved in 1% sodium dodecyl sulphate (SDS). Insoluble material was sedimented by centrifuging at 10000 x g for 10 minutes at 4°C and retaining the supernatant. Samples were stored at -20°C.

20 µg or 10 µg of total protein extract was run on a 0.75 mm 8% acrylamide gel (0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 8% (w/v) acrylamide/bisacrylamide, 0.05% (w/v) ammonium persulphate (APS), 0.0005% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED)) with a 3% acrylamide stacking gel (0.125 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, 0.1% (w/v) SDS, 3% (w/v) acrylamide/bisacrylamide, 0.05% (w/v) ammonium persulphate (APS), 0.001% (v/v) TEMED). The protein samples were combined with protein loading buffer (0.125 M Tris HCl, pH 6.8; 20% (v/v) glycerol; 2.5% (w/v) SDS; 10% (v/v) β-mercaptoethanol;

0.0025% (w/v) bromophenol blue) in an approximate 1:4 ratio and boiled for four minutes. The samples were loaded into the wells of the stacking gel and electrophoresed at 120 volts for one hour in electrophoresis buffer (5 g Tris base [Tris{hydroxymethyl}aminomethane], 14.4 g glycine, 1 g SDS per litre, pH 8.3).

Proteins within the gel were transferred electrophoretically onto Trans-Blot[®] Transfer Medium pure nitrocellulose (Bio-Rad, Mississauga, ON). The blotting cassette was arranged in the following order from cathode to anode: brillo pad, 3 layers of Whatman[®] filter paper, nitrocellulose membrane, gel, 3 layers of Whatman[®] filter paper, and brillo pad. Proteins were transferred overnight at 35 volts in transfer buffer (25 mM Tris base, 192 mM glycine, 20% (w/v) methanol).

The nitrocellulose was separated from the gel, washed in water four times, in Ponceau Red (0.5% Ponceau Red (w/v) (Sigma, Oakville, ON), 3% (v/v) trichloroacetic acid) for 10 minutes and rinsed in water. The membrane was washed for 10 minutes in phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ per litre, pH 7.4), and 3 times for 10 minutes in TBST (tris-buffered saline (8 g NaCl, 0.2 g KCl, 3 g tris base per litre, pH 7.4) with 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween[®] 20)). To block non-specific binding of the 1[°] and 2[°] antibodies, the membranes were placed into 5% (w/v) skim milk powder in TBST for 2 hours.

To test the 8-week production bleed samples, 20 µg and/or 10 µg of strain C9-2 or C2(2)-1 proteins were probed with three different primary antibody conditions: (1) pre-immunization serum (1:10 dilution), (2) 8 week production bleed (1:1000 dilution), and (3) no primary antibody. The membranes were incubated for 1 hour in 1[°] antibody diluted in 1% (w/v) skim milk powder in TBST and washed 8 times for 5 minutes in

TBST. To visualize binding of the 1° antibody, membranes were incubated in a 2° goat anti-rabbit (H+L) horse radish peroxidase (HRP) conjugated antibody (Promega, Madison WI). The 2° antibody was diluted 1:5000 in TBST and incubated with the membranes for 1 hour. The membranes were washed 8 times for 5 minutes in TBST. The secondary antibody was visualized using Lumi-Light^{PLUS} Western Blotting Substrate (Roche, Basel, Switzerland). The Lumi-Light^{PLUS} stable peroxide solution and the Lumi-Light^{PLUS} luminol/enhancer solutions were equilibrated to room temperature, mixed in equal proportions, and spread evenly over the membrane. The membranes were exposed to Kodak Bio-Max MS film.

The final Rabbit A and Rabbit B antisera were commercially purified by immunoaffinity chromatography using a Protein G ligand (BioCarta, San Diego, CA). IgG antibodies have a high affinity for Protein G and were used to isolate IgG antibodies from the rabbit serum. The antibodies were diluted in PBS containing NaN₃. The enzyme-linked immunosorbent assay (ELISA) was conducted to ensure that the UN-24 antibody was present in a 1:16000 dilution of the antisera (BioCarta, San Diego, CA). Immunoblotting was conducted to test the specificity and titer of the purified antibodies. C9-2 protein was extracted by crushing the tissue in first-dimension sample buffer (FDSB; 9.5 M urea, 2.0% (w/v) Triton[®] X-100, 5% (v/v) β-mercaptoethanol, 1.6% Bio-Lyte[®] 5/7 ampholyte (Bio-Rad Laboratories, Hercules, CA), 0.4% Bio-Lyte[®] 3/10 ampholyte (Bio-Rad Laboratories, Hercules, CA)), centrifuging 5000 RPM for 10 minutes at 4°C, and retaining the supernatant. Immunoblotting was performed as described above except that 1:500 and 1:1000 dilutions of the Protein G purified antibodies from rabbit A and rabbit B were used as 1° antibodies.

The purified antibodies from rabbit B were purified further using immunoaffinity chromatography with the UN-24 peptide as the ligand. The peptide was resynthesized with an additional C-terminus cysteine residue (BioCarta, San Diego, CA). The SulfoLink[®] Kit (Pierce Biotechnology, Rockford, IL) was used to covalently link the sulfhydryl group on the terminal cysteine residue to the iodoacetyl group on the SulfoLink[®] Coupling Gel. A 2 mL gel bed was made by adding 4 mL of gel slurry to a 5 mL column plugged with a porous disk. The storage solution (10 mM ethylenediaminetetracetic acid disodium salt (EDTA-Na), 0.05% NaN₃, 50% (v/v) glycerol) was drained from the column, reducing the gel to 2 mL. To equilibrate the gel to a 20% (v/v) dimethyl sulfoxide (DMSO) solution, the column was washed with 4 mL 5% (v/v) DMSO in SulfoLink[®] Coupling Buffer (50 mM Tris, 5mM EDTA-Na, pH 8.5), 4 mL 10% (v/v) DMSO in Coupling Buffer, and 7 mL 20% (v/v) DMSO in SulfoLink[®] Coupling Buffer.

1.5-2 mg of peptide was suspended in 2 mL of 20% DMSO Coupling Buffer (column capacity is 1 mg peptide per 1 mL of Coupling Gel). Ellman's Reagent (5,5'-dithio-*bis*-(2-nitrobenzoic acid); Pierce Biotechnology) was used to determine if free sulfhydryls were available on the peptide. In the presence of sulfhydryls Ellman's Reagent splits into two molecules. One covalently bonds with sulfhydryls and the other produces a yellow colour. First a standard curve was constructed of the relationship between cysteine concentration and absorbance at 412 nm wavelength. A 0 mM to 1.5 mM range of L-cysteine·HCl dissolved in coupling buffer containing 20% (v/v) DMSO was prepared in 0.25 mM increments. 250 µL of each cysteine dilution was incubated for 15 minutes at room temperature with 50 µL of Ellman's Reagent and 2.5 mL of Reaction

Buffer (0.1 M Na_2HPO_4 , pH 8, containing 1 mM ethylenediaminetetracetic acid (EDTA)). The absorbance at 412 nm was measured using a Novaspec II (Amersham Pharmacia Biotech, Piscataway, NJ) spectrophotometer. The peptide solution was incubated with Ellman's Reagent as described above. The absorption at 412 nm was used to extrapolate the concentration of free sulfhydryls from the standard curve.

The peptide solution was added to the column and the column was rocked end over end at room temperature for 15 minutes. The column was incubated at room temperature for 30 minutes without mixing. The solution was drained from the column and retained to measure the unbound peptide concentration. The column was equilibrated to 0% (v/v) DMSO Coupling Buffer by washing with 2.5 mL of 10% (v/v) DMSO Coupling Buffer, 2.5 mL of 5% (v/v) DMSO Coupling Buffer, and 5 mL of 0% (v/v) DMSO Coupling Buffer. To prepare the column for storage the column was washed with 5 mL of PBS, 0.05% NaN_3 . With the column bottom capped an additional 2 mL of PBS, 0.05% NaN_3 was added. The column was stored at 4°C. To measure coupling efficiency the peptide concentration was tested before and after coupling to the column by measuring the absorbance at 230 nm and 260 nm with a Hewlett Packard (Palo Alto, CA) model 8452A diode array spectrophotometer. Protein concentration (in $\mu\text{g/mL}$) was calculated as $183 (A_{230}) - 75.8 (A_{260})$ where A_{230} and A_{260} are the absorbance at 230 nm and 260 nm, respectively (Kalb & Bernlohr, 1977).

The peptide-linked column was used to further purify the G protein purified antibodies from rabbit B. The column was washed with 6 mL of Coupling Buffer to wash the storage buffer from the column. To block the free iodoacetyl groups on the Coupling Gel, 2 mL of 0.05 M L-cysteine·HCl was added to the column, the column was

mixed end-over-end at room temperature for 15 minutes and incubated without mixing for 30 minutes at room temperature. The cysteine solution was expelled and the column was washed with 10 mL SulfoLink[®] Wash Solution (1.0 M NaCl, 0.05% NaN₃). To prevent the gel from drying out a porous disk was inserted just above the gel bed.

The gel was equilibrated with 6 mL PBS (the buffer in which the antibody was diluted). To the column 1.5 mL of G protein purified antibodies from rabbit B was added and allowed to enter into the gel bed. 0.2 mL of PBS was allowed to enter into the gel bed and then the column bottom was capped. An additional 0.5 mL of PBS was added and the column was incubated 1 hour at room temperature. To remove unbound antibodies the column was washed with 12 mL of PBS. The antibody was eluted with 8 mL of 100 mM glycine, pH 3. 0.5 mL fractions were collected and each was neutralized with 35 μ L of 1M Tris-HCl pH 8.5. The protein concentration in fractions was measured using a Beckman Coulter DU[®]-65 spectrophotometer (Mississauga, ON). Protein concentration (in μ g/mL) was calculated as $1552 (A_{280}) - 757.3 (A_{260})$ where A_{280} and A_{260} are the absorbance at 280 nm and 260 nm, respectively. The absorbance values at 280 nm and 260 nm were corrected for a background absorbance at 320 nm (Warburg and Christian, 1942).

The peptide affinity-purified antiserum antibody was tested using immunoblotting as described above. C9-2 protein was extracted by grinding mycelium in cold 1 mM Tris-HCl pH 7.2, centrifuging 1000 x g for 10 minutes at 4°C, and retaining the supernatant. 1 mM phenyl methyl sulfonyl fluoride (PMSF) was added as a protease inhibitor. 1:50, 1:100, 1:500, and 1:1000 dilutions of the purified antibody were used as primary antibodies.

RESULTS

A polyclonal anti-UN-24 peptide antibody was raised commercially in two rabbits. Immunoblotting was used to test the specificity and titer of the pre-immunization, eight-week post-immunization, and final serum. The anti-UN-24 antibody was expected to give a band of approximately 100 kDa; UN-24 Oak Ridge and UN-24 Panama are estimated as 103.8 kDa and 103.2 kDa, respectively. When a 1:10 dilution of the rabbit pre-serum was used as the 1° antibody in immunoblotting, three bands between 70-50 kDa for rabbit A and two 120 kDa and 100 kDa band for rabbit B were observed (Figure B.2). A 1:1000 dilution of 8-week post-immunization serum from rabbits A and B yielded a many bands ranging between 55 kDa and greater than 220 kDa. The negative control (no 1° antibody) did not produce banding (Figure B.3). Protein G purified antibodies from the final serum of rabbits A and B produced a prominent 45 kDa band. A faint band of approximately 100 kDa was seen with the 1:500 dilution of rabbit A G Protein purified antibodies (Figure B.4).

Immunnoaffinity chromatography with the UN-24 peptide as the ligand was used to isolate anti-UN-24 antibodies from other IgG antibodies produced by rabbit B. The 10.36 g/L peptide solution (measured using spectrophotometry) used in ligation contained 0.25 mM of free sulfhydryl groups available to bind the peptide to the gel (assayed using Ellman's reagent). The concentration of peptide that did not couple to the column and flowed out of the column was measured as 11.66 g/L, which was more than the starting peptide concentration. The UN-24 peptide coupled column was used to purify anti-UN-24 antibodies from the rabbit B antibody solution. The concentration of

Figure B.2. Immunoblot of C2(2)-1 protein probed with a 1:10 dilution of rabbit pre-immunization serum. Production rabbit and amount of C2(2)-1 protein are given above the blot.

Figure B.3. Immunoblot of 20 µg C9-2 protein probed with a 1:1000 dilution of an eight week production bleed from two rabbits immunized with an UN-24 peptide. As a negative control, one blot was not probed with a primary antibody.

Figure B.4. Immunoblot of 20 μ g C9-2 protein probed with a Protein G purified UN-24 antibody. The production rabbit and primary antibody dilution are given above the blot.

protein in elution fractions was negligible except for the second fraction with 1.19 ng/ μ L of protein. When the second fraction was used as a 1 $^{\circ}$ antibody in immunoblotting, no bands were observed (Figure B.5).

Figure B.5. Immunoblot of 20 μ g of C9-2 protein probed with a rabbit B peptide affinity purified anti-UN-24 antibody. The dilutions of primary antibody are given above the blot.