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Genetic and epigenetic control of a nonself recognition complex in
N. crassa

By

Denis Lafontaine

**A dissertation
submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of Master of Science**

Carleton University

Ottawa, Ontario

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ISBN: 978-0-494-68617-1

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ABSTRACT

Heterokaryon incompatibility (HI) in filamentous fungi is a form of nonself recognition that operates during the vegetative phase of the life cycle. One HI complex in *Neurospora crassa*, the *het-6* locus, comprises two tightly linked incompatibility genes, *het-6* and *un-24*, each having two allelic variants, Oak Ridge (OR) and Panama (PA). The *un-24* gene also encodes the large subunit of ribonucleotide reductase while *het-6* appears to be a member of a repetitive gene family with no other known function aside from HI. These two genes are in severe linkage disequilibrium such that only *un-24*^{OR} *het-6*^{OR} and *un-24*^{PA} *het-6*^{PA} haplotypes occur in nature. In this study we constructed novel *un-24*^{PA} *het-6*^{OR} strains and *het-6* deletion strains. The *un-24*^{PA} *het-6*^{OR} strains are initially self-incompatible (SI), which demonstrates that nonallelic interactions occur between *un-24* and *het-6*. These SI strains escape from incompatibility in a predictable manner that is most likely due to epigenetic silencing and loss of function of *het-6*^{OR}. HI tests using *het-6* deletion strains reveals an allelic incompatibility interaction between OR and PA forms of *un-24*, which is asymmetrically enhanced by the presence of *het-6*^{OR} or *het-6*^{PA}. *vib-1*, a known suppressor of *het-c*- and *mat*-associated incompatibility, is shown to completely suppress HI associated with allelic differences at *un-24* and perhaps nonallelic interactions between *un-24*^{PA} and *het-6*^{OR}, but only partially suppresses incompatibility associated with nonallelic interactions between *un-24*^{OR} and *het-6*^{PA} incompatibility. We also identified a spontaneous suppressor mutation that maps within a region containing *vib-1* and present evidence that this suppressor is the result of a point mutation within *vib-1*. This study elucidates the control of the *het-6* locus by *vib-1*, and also suggests that mutations in *vib-1* compromise epigenetic silencing of HI.

ACKNOWLEDGMENTS

I must give my sincerest thanks to Dr. Myron Smith for his guidance and support throughout the research and literary aspects of my degree. I would also like to thank my advisory committee members Dr. John Vierula and Dr. Linda Bonen for their help and invaluable guidance along the road. I would also like to thank Shelley Hepworth and Owen Roland for being a part of my defense committee. My family and friends have played a large role and have always been there to encourage me throughout my life and academic career. I must acknowledge Melissa Begin for the preliminary het tests of incompatible strains possessing *vib-1*. I would also like to thank my lab mates Isabel Cruz, Rob Smith, Fuad Tanha, Stacie Lee-Jenkins, Adam Densmore, Kristin Duval, Mojgan Siahbazi, Tyler Black, Nicholas Mariani, and everyone that has come and gone in the Smith lab for their company and help with all things scientific.

TABLE OF CONTENTS

Abstract	iii
Acknowledgements	iv
Table of Contents	v
List of Tables	vi
List of Figures	vii
Glossary of Fungal Terms	viii
Abbreviations	viii
Genetic designations	iv
Protein designations	iv
Introduction	1
Nonself Recognition	1
Vegetative incompatibility in filamentous ascomycetes	2
Heterokaryon incompatibility during the <i>N. crassa</i> life cycle	3
Programmed cell death	9
Importance of studying heterokaryon incompatibility	10
Objective of this thesis	12
Materials and Methods	14
Strains and culture conditions	14
Molecular genetics methods	16
Transformation of <i>N. crassa</i>	20
Heterokaryon tests	20
Results	21
Replacement of <i>un-24^{ts}</i> with <i>un-24^{PA}</i>	21
Characterization of escape by <i>un-24^{PA} het-6^{OR}</i> strains	29
Mating of <i>un-24^{PA} het-6^{OR}</i> escape strains	34
<i>eib-1</i> is associated with a point mutation in <i>vib-1</i>	46
Characterization of strains having <i>het-6</i> deletions	49
Mutations in <i>vib-1</i> completely suppress HI associated with <i>un-24</i> and partially suppress <i>het-6</i> HI	56
<i>vib-1</i> and <i>eib-1</i> act synergistically to suppress HI associated <i>het-6^{OR}</i>	63
Discussion	70
<i>un-24</i> allelic incompatibility and asymmetric incompatibility associated with <i>het-6</i> .	70
Escape from <i>un-24^{PA} het-6^{OR}</i> nonallelic incompatibility.	72
Characteristics of <i>eib-1</i> and similarities with <i>vib-1</i> .	75
Suppression of <i>un-24 het-6</i> incompatibility by <i>vib-1</i> .	78
SUMMARY AND CONCLUSIONS	81
LITERATURE CITED	85
APPENDIX	90

LIST OF TABLES

Table 1: Strains used in this study	15
Table 2: Primers used in this study	17
Table 3: Summary of crosses using Type I and Type II escape strains	41
Table 4: Escape frequencies of heterokaryons possessing <i>vib-1</i>	62
Table 5: Summary of similarities and differences between <i>vib-1</i> and <i>eib-1</i>	76

LIST OF FIGURES

Figure 1: Alignment of Oak Ridge (OR) and Panama (PA) haplotypes in the <i>het-6</i> region of the left arm of linkage group II (LGII).	5
Figure 2: Strategy for the replacement of <i>un-24</i> ts with <i>un-24</i> PA in strains deficient in non-homologous end joining.	23
Figure 3: Ethidium bromide-stained polyacrylamide gel showing <i>Msp</i> I digest of the C-terminal portion of <i>un-24</i> from 12 strains recovered strains from the transformation of RecunPA into DLL1-26.	25
Figure 4: Sequence analysis reveals the extent of <i>un-24</i> replacements in representative strains obtained from the transformation of RecunPA into DLL1-26 (DLL-T2 strains) and DLL4-47 (DLL-T3 strains).	27
Figure 5: Escape from <i>un-24</i> ^{PA} and <i>het-6</i> ^{OR} nonallelic incompatibility reaction.	30
Figure 6: Two different <i>un-24</i> ^{PA} <i>het-6</i> ^{OR} escape forms have been identified.	32
Figure 7: Effects of temperature on the growth rate and morphology of Type I [DLL-T2j(0)] and Type II [DLL-T2k(0)] escape forms.	35
Figure 8: Characterization of the incompatibility function of Type I and Type II escape forms.	37
Figure 9: Type I escapes are compatible with <i>un-24</i> ^{OR} strains deleted for <i>het-6</i> ^{OR} , whereas Type II escapes are incompatible with all strains bearing <i>un-24</i> ^{OR} .	39
Figure 10: Map location of the <i>eib-1</i> mutation.	47
Figure 11: Sequencing of the <i>vib-1</i> gene in a Type II strain reveals a single G→A transition.	50
Figure 12: Alignment of conserved NDT80-like DNA binding domains in <i>Saccharomyces cerevisiae</i> NDT80 protein, a <i>N. crassa</i> NDT80 homolog and VIB-1.	52
Figure 13: Non-allelic and allelic incompatibility involving <i>het-6</i> and <i>un-24</i> .	54
Figure 14: Dominance of partial suppression of <i>un-24</i> <i>het-6</i> incompatibility by different <i>vib-1</i> mutations.	57
Figure 15: <i>vib-1</i> completely suppresses incompatibility associated with interactions between <i>un-24</i> heteroallelism but only partially suppresses HI-associated with nonallelic interactions between <i>un-24</i> and <i>het-6</i> .	59

Figure 16: The <i>vib-1</i> mutation only partially decreases the severity of HI associated with heterokaryon formation between a Type I <i>un-24</i> ^{PA} <i>het-6</i> ^{OR*} escape strain and an OR haplotype strain.	64
Figure 17: <i>vib-1</i> completely suppresses incompatibility associated with heterokaryon formation between a Type II <i>un-24</i> ^{PA} <i>het-6</i> ^{OR} escape strain and OR or PA haplotype strains.	66
Figure 18: <i>vib-1</i> behaves synergistically with the <i>eib-1</i> mutation to suppresses incompatibility associated with <i>het-6</i> ^{OR} but not <i>het-6</i> ^{PA} .	68
Figure 19: A model for HI associated with the <i>het-6</i> locus and suppression by <i>vib-1</i> .	82

Glossary of Fungal Terms

aconidial	without conidia
ascospore	sexual spore
ascus	ascospore-bearing cell found within a perithecium (sing. ascus)
conidia	asexual spore
conidiation	production of conidia
haplotype	a group of genes belonging to a specific allelic type
heterokaryon	two or more nuclear types occupy a hyphal compartment
homokaryon	one nuclear type occupies a hyphal compartment
hyphae	long and branching filamentous cell
mycelium	a hyphal mat or network
paracentric inversion	inversion polymorphism that does not include the centromere
perithecia	mating structure containing asci (singular ascus)

Abbreviations

aa	amino acids
°C	degrees Celcius
C-terminus	carboxyl terminal portion of a peptide
DNA	deoxyribonucleic acid
<i>het</i>	heterokaryon incompatibility loci or gene
HI	heterokaryon incompatibility
MHC	major histocompatibility complex
mm	millimeters
mg	milligrams
mu	map unit
MW	molecular weight
N-terminus	amino-terminal portion of a peptide
ng	nanogram
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PCD	programmed cell death
PCR	polymerase chain reaction
qPCR	quantitative reverse transcriptase PCR
RFLP	Restriction Fragment Length Polymorphism
RIP	repeat induced mutation
RNA	ribonucleic acid
SI	self-incompatibility
T _m	melting temperature
U	units
μg	microgram
μl	micro liter
UTR	untranslated region
<i>vic</i>	vegetative incompatibility loci or gene
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Genetic designations

OR	Oakridge alleles at all <i>het</i> loci
PA	<i>un-24</i> ^{PA} <i>het-6</i> ^{PA} and Oakridge at all other <i>het</i> loci
<i>un-24</i> ^{ts}	<i>un-24</i> ^{OR} temperature-sensitive mutant
<i>un-24</i> ^{OR}	Oakridge allelic form of <i>un-24</i>
<i>un-24</i> ^{PA}	Panama allelic form of <i>un-24</i>
<i>het-6</i> ^{OR}	Oakridge allelic form of <i>het-6</i>
<i>het-6</i> ^{PA}	Panama allelic form of <i>het-6</i>
<i>het-6</i> ^{OR*}	Potential modification of <i>het-6</i> ^{OR} after Type I escape
<i>vib-1</i>	“vegetative incompatibility blocked” suppressor
xxx-# ^Y	In general, gene abbreviations are lowercase, italicized and gene numbers are hyphenated. Uppercase superscript after the gene number designates allelic form.

Protein designations

UN-24 ^{OR}	protein product of <i>un-24</i> ^{OR}
UN-24 ^{PA}	protein product of <i>un-24</i> ^{PA}
HET-6 ^{OR}	predicted protein product of <i>het-6</i> ^{OR}
HET-6 ^{PA}	predicted protein product of <i>het-6</i> ^{PA}
HET-6 ^{OR*}	Potential modification of HET-6 ^{OR} after Type I escape
VIB-1	protein product of <i>vib-1</i>
XXX-# ^Y	In general, protein abbreviations are uppercase and gene numbers are hyphenated. Uppercase superscript after the gene number designates allelic form.

INTRODUCTION

Nonself recognition. The ability to discriminate self from nonself has evolved independently numerous times through evolutionary history and seems to be a necessary feature of living organisms. Somatic or vegetative incompatibility is widespread among protists, animals, plants and fungi (De Boer et al., 1995). Restriction enzyme systems in bacteria, for example, function as prokaryotic nonself recognition systems that recognize and destroy foreign DNA entering the cell (Tock & Dryden, 2005). There are several interesting emerging themes among eukaryotic nonself recognition systems. In eukaryotes, most nonself recognition systems rely on polymorphic loci that are under balancing selection. The loci mediating these systems are often organized in tightly linked gene complexes that are under intense diversifying selection, the result of which is thought to reduce recombination between genes and prevent disruption of adaptive gene complexes (reviewed in Nasrallah, 2002; Nasrallah, 2005). Examples of such nonself recognition gene complexes include the organization of NKRP1 family natural killer receptors and C-type lectin related ligands in mice (Iizuka et al., 2003), in self-incompatibility systems in numerous flowering plant species (reviewed in Charlesworth et al., 2005) and in histocompatibility complexes in the invertebrate *Hydractinia symbiolongicarpus* (Cadavid et al., 2004). In vertebrates, an array of polymorphic genetic loci, termed the major histocompatibility complex (MHC), generate proteins necessary for pathogen recognition and the activation of defense mechanisms (Kumánovics et al., 2003). These MHC systems are also under balancing selection, which promotes the persistence of allelic types in the population. Therefore, the diversity being generated within the genes in these complexes is thought to mediate the recognition function of the

effector genes (Paoletti & Clavé, 2007) and balancing selection ensures the maintenance of diverse allelic forms within the population.

Vegetative incompatibility in filamentous ascomycetes. Nonspecific recognition systems are particularly critical for maintaining biological integrity in organisms that are able to form genetic chimeras such as protists, sponges, tunicates, ascidians and filamentous fungi (Paoletti et al., 2007). That different genetic individuals can fuse to form chimeras make these organisms vulnerable to somatic cell parasitism, germline invasion and/or resource plundering (Debets et al., 1998). In filamentous ascomycetes, nonspecific recognition systems are thought to reduce the risk of exploitation by other more aggressive genotypes and also reduce the risk of transmission of infectious pathogenic elements such as mycoviruses and plasmids (Debets et al., 1994; Debets et al., 1998; Biella et al., 2002). In the case of filamentous fungi, hyphal fusion can occur between two distinct individuals of the same species, to result in a heterokaryon in which two genetically dissimilar nuclei occupy a common cytoplasm (reviewed in Glass et al., 2000). The formation of such heterokaryons may allow one nucleus to complement the deficiencies of the other by conferring, for example, certain nutritional competencies or antibiotic resistance. However, vegetative growth of a heterokaryon may be greatly inhibited, and may lead to hyphal compartmentation and lysis of the incompatible hyphal chamber. This form of nonspecific recognition is termed heterokaryon incompatibility (HI) and results in a form of programmed cell death (PCD). HI systems seem to be restricted to filamentous fungi (Pinan-Lucarre et al., 2007), and investigation of HI has only been undertaken in a limited number of fungal species, namely *Neurospora crassa*, *Podospora*

anserina, and *Cryphonectria parasitica*. Three conserved regions have been identified among several *het* loci involved in HI (Smith et al., 2000b). These three regions form the HET domain which has been found in the predicted protein products of the HI genes *het-6*, *tol* (Smith et al., 2000b) and *pin-c* (Kaneko et al., 2006) in *N. crassa*, and *het-d* and *het-e* in *Podospora anserina* (Paoletti & Clavé, 2007). The HET domain is associated with nearly all known HI systems, either encoded in the *het* gene itself or in another interacting protein product (Glass & Dementhon, 2006). In *P. anserina*, overexpression of *het-e* elicits induction of PCD and mutations in the conserved regions of the HET domain of *het-e* have also been found to suppress the lethality of the incompatibility associated with interactions between *het-c* and *het-e* (Paoletti & Clavé, 2007). Another hallmark of HI is the formation of protein heterocomplexes as seen with HET-C (Sarkar et al., 2002) and UN-24 in *N. crassa* (Wellman et al., unpublished; Reviewed in Smith, 2009) and with HET-S (Reis et al., 2002) in *P. anserina*. These observations have led to the proposal of a model in which polymorphisms of *het* genes mediate nonself recognition through the formation of a heterocomplex of the protein that does not contain the HET domain whereas the HET domain mediates PCD. Therefore, both allelic and nonallelic interactions mediate HI. Allelic interactions occur between alternate forms of the same gene whereas nonallelic interactions occur between specific alleles of different genes. As we will see, there is often asymmetry in the degree of PCD elicited by different allelic combinations of the interacting components.

HI during the *N. crassa* life cycle. Several fungal HI systems characterized to date involve nonallelic interactions between a gene encoding a HET domain protein and

another gene that does not. Both genes possess allelic forms that are highly polymorphic. In some cases, the genes that interact with the HET domain proteins have functions other than HI. The *het-c* gene in *P. anserina* is a putative glycolipid transfer protein and displays nonallelic HI with the *het* domain genes *het-e* and *het-d* (Espagne et al., 2002). Similarly, the *het-c* gene in *N. crassa* encodes a putative plasma membrane protein and interacts with the closely linked *pin-c* gene which encodes a HET domain protein (Kaneko et al., 2006). In *N. crassa* the mating-type locus also acts as a HI factor during vegetative growth (Garnjobst & Wilson, 1956). The mating-type factors MAT-A and MAT-a complex together in the presence of TOL, a protein that encodes a HET domain, to trigger PCD (Jacobson, 1992; Vellani et al., 1994; Leslie & Yamashiro, 1997).

Finally, similar interactions appear to govern HI associated with *het-6*. The *het-6* locus contains two genes each having incompatibility function. Both genes have two allelic forms, Oak Ridge (OR) and Panama (PA). The two genes are in severe linkage disequilibrium, occurring in nature only as the *un-24^{OR} het-6^{OR}* or *un-24^{PA} het-6^{PA}* haplotypes. Characterization of OR and PA allelic forms at *het-6* has revealed a paracentric inversion polymorphism (Figure 1) and extreme sequence divergence between these genes and the inversion break points. This sequence divergence is thought to prevent recombination and to account for the observed linkage disequilibrium between *un-24* and *het-6* (Micali & Smith, 2006). The *het-6* gene encodes a HET domain protein and amino acid sequences of the OR and PA forms share only 68% identity. The *un-24* gene encodes the large subunit of ribonucleotide reductase RNR, which is a highly conserved protein necessary for *de novo* deoxyribonucleotide synthesis (Jordan & Reichard, 1998). In *N. crassa*, *un-24* also has incompatibility function that involves

Figure 1: Alignment of Oak Ridge (OR) and Panama (PA) haplotypes in the *het-6* region of the left arm of linkage group II (LGIIL). Arrows indicate extent and orientation of incompatibility genes. Inverted segment is indicated by parentheses and dotted lines; centromere is represented as a circle. Figure adapted from Micali & Smith (2006).

allelic interaction between the OR and PA forms (Smith et al., 2000b). The N-terminal coding sequence in *un-24* is highly conserved, whereas the OR and PA allelic forms are highly divergent in the C-terminal region, a peptide portion that seems to be unique to *N. crassa* (Micali & Smith, 2005). *un-24* incompatibility function involves an asymmetric interaction between the Oak Ridge (OR) and Panama (PA) allelic forms. Transformation of *un-24*^{OR} into a PA-background strain results in severe incompatibility, characterized by death, whereas the transformation of *un-24*^{PA} into an OR-background strain gives rise to colonies having a spidery morphology that grow slowly (Micali & Smith, 2006). These spidery *un-24*^{OR} *het-6*^{OR}::*un-24*^{PA} self-incompatible colonies escape to near wild-type growth rates after 4-7 days. This PA-specific incompatibility can be reproduced by transformations using a vector containing only the C-terminal portion of *un-24*^{PA} (Wellman et al., unpublished; Reviewed in Smith, 2009). *un-24*^{OR} *het-6*^{OR}::*un-24*^{PA} self-incompatibility is associated with UN-24 aggregation, which was initially thought to occur through aberrant cysteine bridges in the C-terminus of UN-24^{PA} and the catalytic site of UN-24^{OR} (Wellman et al., unpublished; Reviewed in Smith, 2009). However, the UN-24 aggregate does not appear to break down under reducing conditions, suggesting that aggregation does not occur through disulfide bond formation, but rather through some sort of non-reduceable cross-linkage that is, nevertheless, driven by redox-reactive cysteines in the C-terminus. Escape from self-incompatibility is correlated with disaggregation or degradation of the UN-24 aggregate. Ribonucleotide reduction can become the rate-limiting step in DNA synthesis which makes RNR a key enzyme in the regulation of cell division and cell proliferation. The importance of this enzyme makes this HI function a unique and very interesting evolutionary adaptation.

To date, two suppressors of HI have been identified. Mutations in the *tol* gene, which encodes a HET domain protein, abolish HI associated with differences at the mating-type locus only (Vellani et al., 1994; Leslie & Yamashiro, 1997). Mutations in *vib-1* have been shown to suppress incompatibility associated with differences at the *het-c* locus and partially suppress HI associated with allelic differences at the mating-type and *het-6* loci (Xiang & Glass, 2004; M.M. Begin, unpublished). VIB-1 is a putative transcription factor that possesses a predicted DNA binding domain and nuclear localization sequence. VIB-1 has sequence similarity to NDT80, which is a transcription factor involved in regulating meiosis in *Saccharomyces cerevisiae* (Xu et al., 1995). VIB-1 also shares considerable identity with the *xprG* (*phoG*) protein product in *A. nidulans*, which encodes a putative transcription factor involved in the positive regulation of phosphate-non-repressible acid phosphatase activity (MacRae et al., 1993) as well as the production of extracellular proteases upon carbon, nitrogen or sulfur starvation (Katz et al., 2006). It was recently demonstrated that VIB-1 is required for the transcription of *het-6*, *pin-c* and *tol* (Dementhon et al., 2006). VIB-1 is also involved in the positive regulation of phosphate-non-repressible acid phosphatase activity, and is known to be differentially localized during the cell cycle of *N. crassa* (Dementhon et al., 2006). These observations indicate that *vib-1* is involved in functions in addition to HI.

There are several interesting aspects to HI in *N. crassa*. The genetic organization of *het* genes into linked gene complexes as seen with the *het-c* and *het-6* loci is reminiscent of the previously mentioned self/nonself-recognition systems in plants and vertebrates. Another interesting aspect of HI in *N. crassa* is that cytogamy of strains bearing the opposite mating type is required for entry into the sexual cycle; however,

strains bearing opposite mating types cannot coexist as a heterokaryon during vegetative growth. Moreover, strains bearing opposite mating types as well as differences at single or multiple *het* loci are able to form heterokaryons and display normal fertility during the sexual cycle. This suggests the presence of a system that differentially regulates the incompatibility function of *het* genes during the sexual and vegetative life cycles. This process is reminiscent of immunological tolerance of the embryo in placental mammals. In cattle, complete shutdown of MHC class I expression in trophoblast cells is required during early pregnancy, to ensure normal fetal development and fetal survival (Davies et al., 2004). Further discovery and characterization of genes that suppress HI may, therefore, be very beneficial to understand genetic and molecular mechanisms by which HI is regulated.

Programmed cell death. PCD is generally defined as a cascade of signalling events that lead to the demise of the cell. Temperature-dependent incompatibility systems have been characterized in *P. anserina* (reviewed in Pinan-Lucarré et al., 2007) and *N. crassa* (Kaneko et al., 2006), and are being used as model systems to investigate PCD in filamentous fungi. Recent studies in *N. crassa* indicate that PCD associated with *het-c/pin-c* incompatibility involves the production of Reactive Oxygen Species (ROS) as well as the upregulation of genes involved in the production of ROS such as NADPH oxidase and ROS scavengers such as glutaredoxin, and cytochrome c (Hutchison et al., 2009). This observation is reminiscent of apoptosis in yeast and higher eukaryotes (reviewed in Chandra et al., 2000; Einsenberg et al., 2007). *N. crassa* does possess homologs of genes involved in apoptosis such as metacaspases, cytochrome c and

apoptosis inducing factor, however, these genes are not necessary for PCD mediated by *het-c/pin-c* incompatibility (Hutchison et al., 2009). PCD by incompatibility in *P. anserina* is characterized by the upregulation of genes involved in autophagy (Pinan-Lucarré et al., 2003; Dementhon et al., 2004), formation of large vacuoles (Pinan-Lucarré et al., 2003), and lysis, all of which are characteristics of autophagy-dependent (Type II) PCD. The use of *atg-1* and *atg-8* mutants demonstrated that cell death and vacuolization mediated by incompatibility is not suppressed in the absence of autophagy, in fact the indications are that autophagy has a prosurvival function against cell death by incompatibility (Pinan-Lucarré et al., 2005). As previously mentioned, one of the hallmarks of HI is the formation of protein heterocomplexes. It is therefore possible that autophagy may rescue cells from incompatibility by sequestering and degrading these protein aggregates.

Importance of studying heterokaryon incompatibility. Fungi are heterotrophic organisms and therefore depend on their environment for the uptake of nutrients. Saprophytic fungi acquire their nutrients from dead organic material whereas pathogenic fungi do so at least partially from other living organisms. Fungal pathogens are known for all plants and animals, including humans, and can therefore lead to severe health and economic problems. The study of fungal nonself recognition systems may lead to treatment and/or prevention strategies for fungal parasitism. For example, the parasitic fungus *C. parasitica* is the causal agent for chestnut blight which is responsible for the decimation of the chestnut tree in North America. Efforts to control the spread of *C. parasitica* using viruses that cause hypovirulence have been thwarted because the virus is

not transmitted effectively between vegetatively incompatible strains of *C. parasitica*. Therefore, the HI system in this fungus is of particular interest since it is directly related to biological control strategies of chestnut blight using fungal viruses (Kubisak & Milgroom, 2006; Biella et al., 2002; Cortesi et al., 2001).

The HET-s system in *P. anserina* has opened doors into characterization of fungal prions, which may prove beneficial to our understanding about other prions such as that associated with Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (mad cow disease). The use of temperature-dependent HI systems in *P. anserina* and *N. crassa* has led to the investigation of PCD associated with HI in these organisms. As previously described, *N. crassa* does possess homologues of proteins involved in apoptosis and induces ROS production during PCD, but there are many differences between this type of PCD and PCD/apoptosis in higher eukaryotes. The study of HI-mediated PCD in filamentous fungi may lead to the discovery of novel PCD pathways that are conserved in higher organisms. On the other hand, the differences between PCD pathways in fungi and higher organisms can be exploited as targets for the development of new antifungals.

N. crassa possesses a haploid life cycle, which makes the genetics of this organism relatively tractable. Sequencing and extensive mapping of the *N. crassa* genome, along with a vast number of available mutants and gene deletions make this organism a powerful genetic model. HI in filamentous fungi displays many similarities to self/nonself recognition systems in other organisms and is therefore an interesting model that can be used to investigate molecular mechanisms of recognition and evolution of different recognition systems. HI in *N. crassa* is also differentially regulated during the vegetative and sexual cycles, which is reminiscent of processes in higher organisms. As

discussed earlier, the discovery and characterization of novel mutations that suppress HI may help to unravel genetic and biochemical mechanisms involved in the control of this phenomenon. Dissection of these systems may reveal homology and shared evolutionary relationships with similar systems in other organisms.

Objective of this thesis. My M.Sc research explores whether or not there are functional interactions between *het-6* and *un-24*. The obstacle to this work has been creating strains that have alternate allelic forms at the *un-24* and *het-6* loci; only two haplotypes, *un-24*^{OR} *het-6*^{OR} and *un-24*^{PA} *het-6*^{PA}, have been found in surveys of over 200 strains from nature (Mir-Rashed et al., 2000; Smith et al., 2000b). The generation of the recombinant haplotypes, *un-24*^{PA} *het-6*^{OR} or *un-24*^{OR} *het-6*^{PA} has so far been unsuccessful using traditional crossing methods due to inversion and sequence polymorphism that prevent homologous recombination between the two linked genes (Figure 1; Micali & Smith, 2006). Recently, the efficient generation of gene replacements has become possible in *N. crassa* using recipient strains deficient in non-homologous end joining due to deletions in one or both *mus-51* and *mus-52* (Ninomiya et al., 2004; Colot et al., 2006). The use of Δ *mus-51/52* strains allows the insertion of a cassette containing a selectable marker and subsequent deletion or replacement of any given locus in the *N. crassa* genome. I crossed a Δ *mus* strain with a strain containing a temperature sensitive mutation at *un-24*^{OR} (*un-24*^{ts}) and have selected for both of these traits in the resulting progeny. I also produced a cassette containing *un-24*^{PA} coding region with flanks homologous to that of the *un-24*^{OR} 5' and 3'UTRs. Transformation of this cassette into a strain containing Δ *mus::bar* and *un-24*^{ts} resulted in transformants that are able to grow above the restrictive temperature.

Herein, the resulting novel *un-24*^{PA} *het-6*^{OR} haplotype will be used to elucidate allelic and nonallelic interactions of the *het-6* locus as well as HI suppression.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study are listed in Table 1.

Routine culture of *N. crassa* was on Vogel's minimal medium containing 1.5% sucrose, 1.5% agar and supplements as required (Davis & DeSerres, 1970) at 30°C unless otherwise specified. Crosses were performed on plates containing Synthetic Crossing Medium (SCM) with supplements (Davis & DeSerres, 1970) where conidial or mycelia suspensions were spotted at opposite edges of the plate and allowed to grow at room temperature (21-25°C) under natural light. Spot tests were used to screen strains for nutritional markers or resistance to antibiotics and were performed by inoculating conidial suspensions onto Vogels medium containing 0.8% sorbose and 0.4% sucrose and supplements. Phosphinothricin (PPT) was purified from Finale® (Farnam Co.) using a one step extraction with 1-butanol (Hays & Selker, 2000) and concentration used in the spot tests was determined by spotting *bar*⁺ and *bar*⁻ (resistant to PPT) strains onto varying concentrations of the purified PPT. A ratio of 1: 250 (v/v) of purified PPT in Vogel's medium was shown to effectively inhibit the growth of *bar*⁻ strains and allow growth of *bar*⁺ strains, and was used in subsequent experiments. The hygromycin B (HygB, Roche, Laval, Quebec) concentration used in selection plates and genotyping experiments was 15-20 mg per 100 ml of Vogel's medium. The *un-24*^{ts} mutation was screened by incubating strains at 39°C. C8c-164 and the KU disruption strain FGSC 9720 were crossed and the resulting progeny (DLL1) were screened for *un-24*^{ts} as well as resistance to PPT (indicating the Δ *mus-52::bar* knockout). The DLL3-15 strain was crossed to DLL1-24, and ascospores were plated onto medium containing HygB (indicating the Δ *het-6::hph* knockout) and all progeny were screened for *un-24*^{ts} using spot tests. From a

Table 1: Strains used in this study

Strain ^a	Genotype ^b	Origin
Strains used for crosses and transformation		
FGSC 9720	<i>his-3; Δmus-52::bar A</i>	Colot <i>et al.</i> (2006)
C8c-164	<i>un-24^{ts}; trp-1; inl a</i>	Smith <i>et al.</i> (2000a)
FGSC 12442	<i>Δhet-6::hph A</i>	Colot <i>et al.</i> (2006)
DLL1-24	<i>un-24^{ts}; Δmus-52::bar; inl his-3 A</i>	FGSC 9720 X C8c-164
DLL1-26	<i>un-24^{ts}; Δmus-52::bar; inl a</i>	FGSC 9720 X C8c-164
DLL3-15	<i>Δhet-6::hph; trp-1 a</i>	FGSC 12442 X C8c-164
DLL4-47	<i>un-24^{ts} Δhet-6::hph; Δmus-52::bar; inl a</i>	DLL3-15 X DLL1-24
DLL8-16	<i>un-24^{ts} Δhet-6::hph; Δmus-52::bar; inl A</i>	DLL4-47 X FGSC 9720
RLM57-30	<i>(cyh-1) pyr-4 A</i>	Smith <i>et al.</i> (1996)
6-3	<i>ad3B arg-1; het-6^{PA}; inl A</i>	M.L. Smith
FGSC 11309	<i>Δvib-1::hph A</i>	Colot <i>et al.</i> (2006)
DLL2-17	<i>un-24^{ts}; trp-1; Δvib-1::hph inl a</i>	C8c-164 X FGSC 11309
DLL2-18	<i>un-24^{ts}; trp-1; Δvib-1::hph A</i>	
<i>un-24 het-6</i> incompatibility tester strains		
A1	<i>ad3A nic-2 (cyh-1) a</i>	M.L. Smith
FGSC 4568	<i>ad3B a^{m33}</i>	Griffiths (1982)
C2(2)-1	<i>het-6^{PA} thr-2 a</i>	Smith <i>et al.</i> (1996)
6-18	<i>het-6^{PA}; inl a</i>	M.L. Smith
DLL3-15	<i>Δhet-6::hph; trp-1 a</i>	FGSC 12442X C8c-164
DLL2-45	<i>trp-1; Δvib-1::hph a</i>	C8c-164 X FGSC 11309
DLL9-11	<i>ad3B arg-1; het-6^{PA}; Δvib-1::hph inl a</i>	6-3 X DLL2-17
vib	<i>thr-2; vib-1; pan-2 a</i>	N.L. Glass
MMB-14	<i>ad3B arg-1; het-6^{PA}; vib-1 a</i>	vib X 6-3
vΔ6-1	<i>Δhet-6::hph; vib-1; pan-2 a</i>	FGSC 12442 X vib
D15	<i>Δhet-6::hph pan-2 a</i>	FGSC 12442 X vib
Mating type tester strains		
FGSC 4317	<i>fl A</i>	<i>fl A</i> fluffy tester
FGSC 4347	<i>fl a</i>	<i>fl a</i> fluffy tester
<i>un-24</i> replacement strains^c		
DLL-T2j(0)	<i>un-24^{PA} het-6^{OR}; Δmus-52::bar; inl a</i>	Type I escape of DLL1-26::RecunPA
DLL-T2k(0)	<i>un-24^{PA} het-6^{OR}; Δmus-52::bar+; inl eib-1 a</i>	Type II escape of DLL1-26::RecunPA
<i>un-24^{PA} het-6::hph</i> strains		
DLL14-8	<i>un-24^{ts} Δhet-6::hph; Δmus-52::bar; inl eib-1 A</i>	DLL-T2k(0)X DLL8-16
DLL14-9	<i>un-24^{ts} Δhet-6::hph; Δmus-52::bar; inl eib-1 A</i>	DLL-T2k(0)X DLL8-16
DLL14-13	<i>un-24^{ts} Δhet-6::hph; Δmus-52::bar; inl eib-1 A</i>	DLL-T2k(0)X DLL8-16
DLL15-1	<i>un-24^{PA} Δhet-6::hph; Δmus-52::bar; inl eib-1 a</i>	DLL-T2k(0)X DLL8-16
DLL15-2	<i>un-24^{PA} Δhet-6::hph; Δmus-52::bar; inl A</i>	DLL-T2k(0)X DLL8-16
DLL15-6	<i>un-24^{PA} Δhet-6::hph; Δmus-52::bar; inl A</i>	DLL-T2k(0)X DLL8-16

^a FGSC, Fungal Genetics Stock Center, University of Kansas Medical Center.

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

^c These strains have complete replacement of *un-24^{ts}* with *un-24^{PA}*. These and strains with partial replacement are further described in Figure 2.

screen of 220 progeny from this cross, only one had a *un-24^{ts} Δhet-6::hph* genotype indicating a linkage of approximately 0.25 mu between *un-24^{OR}* and *het-6^{OR}*.

Molecular genetics methods. The primers used for the construction of the *un-24* replacement cassette are found in Table 2. The 3' flank (2kb) fragment downstream of the *un-24^{OR}* coding region was amplified from the G8:G1 cosmid (Smith et al., 1996) using the primers 3F2991Hyb (5' half is *un-24^{PA}*-specific and 3' half is *un-24^{OR}*-specific) and sm3F5130ORrv (*un-24^{OR}*-specific). The resulting PCR product was purified using GenElute™ PCR Clean-up kit (Sigma, Oakville, Ontario), digested with *DpnI* (New England Biolabs, Pickering, Ontario) and added as a primer/template along with the pA3 clone (Micali & Smith, 2006) as the primary template in a second PCR reaction using the primers sm5F840PA (*un-24^{OR}* and *un-24^{PA}*-specific) and sm3F5130ORrv primers (*un-24^{OR}*-specific). The resulting 6 kb product was cloned into pCR2.1TOPO (Invitrogen, Burlington, Ontario), transformed into competent Top10 *E. coli* cells (Invitrogen) and plated onto LB broth medium containing 50 µg ampicillin and 25 µg kanamycin per ml of medium. 40 µl of 40 mg/ml X-Gal in dimethylformamide (DMF) was spread onto the solidified agar to facilitate blue/white screening of inserts. Plasmids were extracted from insertion-positive (white) transformants using the Promega Wizard® miniprep system (Fischer Scientific, Nepean, Ontario), digested using *EcoRI* (New England Biolabs) and insert size was checked following agarose gel electrophoresis. Subsequently, cloned inserts were sequenced at the Genome Québec Innovation Centre. The resulting DNA construct possesses 840 bp of the highly conserved 5' flank, the *un-24^{PA}* coding region and a *un-24^{OR}*-specific non-coding 3' flanking region. This 6 kb fragment was named

Table 2: Primers used in this study

Primer name	Primer Sequence 5'→ 3'
RECunPA construction and <i>un-24</i> sequencing	
sm5F840fw	ACGGCGCTGTGAATATCTGTGA
3F2991HYBfw	GCCTGCCTGGTATGGTGCATGAAGCTTATGGTCAAAAATG
3F2991HYBrv	CATTTTGGACCATAAGCTTCATGCACCATAACCAGGCAGGC
sm3F5130ORrv	TTCCGAGGGTAGCTGTCTGTCT
sm3F3969ORrv	AAGAAGCCCTATCCATGCTGCTGA
3F1182rev	GTTCTGCTCAACACGCTTCA
3F1092fwd	AAGAACCACGGTAAGGAAGAGG
Mapping primers	
5-55- <i>MspI</i> fw	ATTCCTCCTTCTCGTCCTCCT
5-55- <i>MspI</i> rv	TTGTCTGGTCGAACCATACCT
5-91- <i>Tsp509I</i> fw	CGCAACTGCACCAAGGCTCTC
5-91- <i>Tsp509I</i> rv	TGAGAGCCGTAGCCGATGAGA
5-94- <i>DpnII</i> fw	GGATCTGGTTATGGCTGATGC
5-94- <i>DpnII</i> rv	GGCGGTTACTAACTTGTGGAC
5-95- <i>DpnII</i> fw	ATATCCGACTAGGGAGCGTG
5-95- <i>DpnII</i> rv	CAGGGGAAACAAGGGCATGA
5-98- <i>Tsp509I</i> fw	AAACCCATACCGAGGAGGA
5-98- <i>Tsp509I</i> rv	GCGTGGTCTGTGACATCTACA

RECunPA and was used in subsequent transformations to replace the functionally OR form of *un-24^{ts}* with a functionally PA form of *un-24*.

DNA extractions from *N. crassa* was performed by homogenizing 100-200 mg of mycelial tissue in 1 ml of TENS buffer [100 mM Tris-HCl (pH 8), 100 mM NaCl, 100 mM EDTA (pH 8), and 2% (w/v) SDS] at 30 Hz for 6 minutes using a Retsch MM 301 homogenizer. The homogenized tissue was then centrifuged at 12000 xg for 10 minutes and 1/6 volume of 3 M sodium acetate was added to the resulting supernatant and the mixture was incubated on ice for at least 15 minutes with occasional mixing and then centrifuged at 12000 xg for 10 minutes. The supernatant was then combined with one volume of isopropanol and incubated at -20°C overnight. The resulting precipitate was centrifuged at 12000 xg for 10 minutes, dried at 60°C for 20 minutes and resuspended in 150 µl of ddH₂O. To check for insertion of *un-24^{PA}* into *un-24^{ts}* (OR) strains, DNA from each of the RECunPA transformants was amplified using the 3F1092fwd and sm3F3969ORrv primer set. The forward primer lies within the *un-24* ORF approximately 2 kb upstream of the stop codon and is not allele specific. The reverse primer lies approximately 100 bp downstream of the stop codon outside of the *un-24* ORF and is OR specific. This primer set will amplify endogenous *un-24^{ts}* as well as any endogenous insertion of RECunPA. The resulting 2 kb fragment was digested with *MspI* restriction enzyme and then subject to electrophoresis on a 16 cm X 20 cm polyacrylamide gel (4% stacking gel and 10% separating gel) at 40 V for 24 hrs. The gel was stained in ethidium bromide and photographed over a UV light source with an AlphaImager (Cell Biosciences, Santa Clara, California). DNA from selected transformants was also amplified using the sm5F840fw and sm3F3969ORrv primer set for verification by

sequencing using the sm5F840fw, 3F1182rv, 3F1092 and sm3F3969ORrv primers. The forward primer lies outside of the *un-24* ORF approximately 840 bp from the start codon and is not allele specific. The reverse primer lies approximately 100 bp from the stop codon outside of the *un-24* ORF and is OR specific. These primers will also amplify endogenous *un-24^{ts}* as well as any *un-24^{ts}* replacements. The resulting 4 kb amplification products were purified and sequenced. The concentration and quality of all DNA extractions, plasmid preparations and purified PCR products was determined using a Nanodrop ND-1000 (V3.7.1 software) spectrophotometer. PCR amplification for cloning purposes was performed using IPProofTM high fidelity DNA polymerase (BioRad, Mississauga, Ontario). PCR for diagnostic and mapping purposes employed Taq DNA Polymerase (Bioshop, Burlington, Ontario). All PCRs were done using a Biometra T-gradient thermocycler (Biometra, Germany). Oligonucleotides were synthesized by Sigma.

Mapping of *N. crassa* was performed using the primers for the cleaved amplified polymorphic sequence (CAPS) found in Table 2. The Type II escape strain was crossed to the FGSC 2225 Mauriceville strain and resulting SI and Type II progeny were identified and DNA was extracted as described by Oakley et al. (1987). DNA from selected progeny, DLL1-26 and FGSC 2225 were PCR amplified using primer sets for each CAPS marker and digested with the appropriate restriction endonuclease (given in primer names) according to manufacturer's (New England Biolabs) specifications. Polymorphisms for each of the progeny were scored as Oak Ridge (OR) or Mauriceville (MO) according to the RFLP patterns of DLL1-26 and FGSC 2225, respectively.

Transformation of *N. crassa*. Transformation of the RECunPA into *N. crassa* DLL1-26 and DLL4-47 strains was done as described previously (Royer & Yamashiro, 1992; Smith et al., 2000b) by adding 1-2 µg of the vector DNA to a 100 µl aliquot of spheroplasts at $1-2 \times 10^8$ cells/ml in 1 M Sorbitol. Transformation plates were incubated at 39°C and transformants were observed after 3-5 days, transferred to Vogel's agar medium and enriched into homokaryotic cultures by serially subculturing hyphal tip fragments four or five times and growing them at 39°C. The resulting strains were named DLL-T2 (for DLL1-26 transformants) and DLL-T3 (for DLL4-47 transformants).

Heterokaryon tests. The incompatibility function of the strains constructed in this study was examined using heterokaryon tests (het tests), as described in Smith et al. (1996). The test is done by co-inoculating two strains bearing complementary auxotrophic markers onto medium that will not support growth by either strain alone. If the strains are identical at all *het* loci, a heterokaryon will form and grow at wildtype rates on the medium. If the strains bear differences at one or more *het* loci, growth of the resulting heterokaryon is slow or completely inhibited. All photos of fungal colonies were taken using a Canon Powershot SD450 camera 2.68 +/- 0.04 days after inoculation, with the exception of photos in Figure 7, some of which were taken 7 days after inoculation, as indicated. All strains used for heterokaryon tests in this study were mating type *a* and all growth rates represent the maximum radial growth rates measured on each plate.

RESULTS

Replacement of *un-24^{ts}* with *un-24^{PA}*. Strain C8c-164 possesses *un-24^{ts}*, a temperature sensitive mutation of *un-24^{OR}*, and is able to grow at 30°C but not 39°C. The FGSC 9720 strain possesses the $\Delta mus-52::bar+$ deletion which confers resistance to phosphinothricin. Mutations in either *mus-51* or *mus-52* abolish non-homologous end-joining DNA repair and greatly increase the frequency of homologous insertion of transformed DNA (Ninomiya et al., 2004). Use of Δmus strains thus has allowed the directed insertion of markers and has led to the development a high-throughput gene deletion strategy in *N. crassa*. C8c-164 was crossed with FGSC 9720 and single spore progeny were selected (DLL1-24 and DLL1-26) that had the *un-24^{ts}* mutation and the $\Delta mus-52::bar+$ deletion. Similarly, DLL1-24 was crossed to the DLL3-15 strain and selected for *un-24^{ts}*, $\Delta mus-52::bar+$ and $\Delta het-6^{OR}::hph$. From a total of 217 progeny screened, only one (DLL4-47) was hygromycin resistant and unable to grow at restrictive temperature. DLL4-47 presumably arose through meiotic recombination between *un-24^{ts}* and $\Delta het-6$.

Since OR and PA forms of *un-24* have low sequence identity in the 3' region of the gene, a vector possessing the *un-24^{PA}* coding region as well as 1-2 kb flanks that are homologous to the *un-24^{OR}* haplotype form was constructed for use in the replacement of *un-24*. The pCR2.1 subclone of this construct will be referred to as pRecunPA whereas the PCR-amplified linear construct will be referred to as RecunPA. The idea behind this approach takes advantage of the loss of non-homologous end joining in Δmus strains and thus increased frequency of insertion via homologous recombination and also uses the *un-24^{ts}* mutation as a selectable marker for replacement of *un-24^{PA}*. To replace *un-24^{ts}* with *un-24^{PA}*, $\Delta mus-52$ strains that carry *un-24^{ts}* (DLL1-26 and DLL4-47) were

transformed with a PCR product containing the wild type *un-24*^{PA} (RECunPA) and selected at a restrictive temperature (Figure 2). Strains DLL4-47 and DLL1-26 were used as recipients for these transformations. DLL1-26 is identical to DLL4-47 except that it has *het-6*^{OR} deleted and thus serves as an ideal control in case there are interactions between *un-24*^{PA} and *het-6*^{OR} that influence the viability of transformants. Relatively few DLL1-26::RecunPA transformants able to grow at restrictive temperature were obtained [1.47 +/- 0.39 transformants/1-2 µg of DNA (*n* = 15 plates)]. About, 10.1 +/- 1.98 transformants/1-2 µg of DNA (*n* = 15 plates) were recovered when DLL4-47 was used as the recipient strain. DNA was extracted from a total 22 RecunPA transformants and these were analyzed for replacement of the C-terminal region of *un-24* using *MspI* RFLP patterns and DNA sequencing. The *MspI* digests of the C-terminal portion of *un-24* from DLL1-26 (DLL-T2) and DLL4-47 (DLL-T3) transformants showed that at least 3 transformants have full or partial conversion of the endogenous *un-24*^{ts} into its PA counterpart (Figure 3). The *un-24* genes from each of the 22 transformants (9 from DLL1-26, 13 from the DLL4-47) were PCR amplified and subjected to DNA sequencing. Sequencing results showed that the recovered strains display varying degrees of *un-24* replacement (Figure 4). Surprisingly, 11 of 13 sequenced transformants recovered from the transformation of RECunPA into a *un-24*^{ts} Δ *het-6* haplotype strain (DLL4-47) retain all three *un-24*^{ts} mutations and yet were able to grow at restrictive temperature. On the other hand, only 3 of 9 transformants of RecunPA into a *un-24*^{ts} *het-6*^{OR} strain (DLL1-26) that were able to grow at 39°C were found to possess the *un-24*^{ts} mutations. The mechanism by which these strains grow at restrictive temperature is unknown but may be due to a *un-24*^{ts} suppressor mutation (Kotierk, 2004).

Figure 2: Strategy for the replacement of *un-24*^{ts} with *un-24*^{PA} in strains deficient in non-homologous end joining. The thin segments represent the *un-24* 5' and 3' UTRs, whereas the thick segments represent the *un-24* coding region and the arrow heads designate the direction of transcription of the *un-24* ORF. The solid grey segments represent areas that are conserved between Oak Ridge (OR) and Panama (PA) allelic forms. The dashed and grey segments represent areas that are PA-specific, whereas the dashed black segments represent areas that are OR-specific. The location of the three transition mutations associated with *un-24*^{ts} are designated ts. **a)** Areas where meiotic recombination can occur are indicated by X (crossover symbols). A paracentric inversion polymorphism and extreme sequence divergence at the 3' end of *un-24* is thought to prevent homologous meiotic recombination between the OR and PA haplotypes and results in the observed extreme linkage disequilibrium of *un-24* and *het-6*. **b)** This thesis uses *mus-51/mus-52* deletion strains transformed with the RecunPA construct to replace the temperature sensitive mutation *un-24*^{ts} with the wildtype (wt) version of *un-24*^{PA}.

a) Meiotic recombination

b) Transformation

Figure 3: Ethidium bromide-stained polyacrylamide gel showing *MspI* digest of the C-terminal portion of *un-24* from C8c-164 (*un-24*^{OR}), 6-18 (*un-24*^{PA}) and 12 strains recovered from the transformation of RecunPA into DLL1-26. Lanes marked with M contain the 1 kb size standards. Lanes containing the *MspI* digests of the *un-24* C-terminal portion from DLL-T2j, DLL-T2k and DLL-T2f display RFLP patterns that are diagnostic for *un-24*^{PA} and are therefore candidate replacement strains. All other lanes contain the *MspI* digests of *un-24* C-termini that are OR-specific.

Figure 4: Sequence analysis reveals the extent of *un-24* replacements in representative strains obtained from the transformation of RecunPA into DLL1-26 (DLL-T2 strains) and DLL4-47 (DLL-T3 strains). The thick grey and black segments represent the *un-24*^{PA} and *un-24*^{OR} coding regions, respectively. The arrow heads designate the direction of transcription of the *un-24* ORF. The thin grey and thin black line segments represent, respectively, the *un-24*^{PA} and *un-24*^{OR} 5' and 3' UTRs. The DLL-T2j and DLL-T2k transformants initially possessed a self-incompatible (SI) phenotype, but escaped to near-wildtype form at about 5-7 days after subculture. The incompatibility of each transformant was inferred using heterokaryon compatibility tests. *RecunPA designates the *un-24*^{PA} replacement construct (~2 kb of the 3' flank is not represented in this figure) which does not encode the *het-6* gene, therefore *het-6* incompatibility function is not applicable (N/A) in this case. The C8c-164 strain possesses three G→A mutations in *un-24* that confer inability to grow at 39°C, whereas other strains such as C9-2 and C2(2)-1 display wildtype (wt) growth and morphology at 39°C. A 1 kb scale bar is provided at bottom.

Strains DLL-T2k and DLL-2j were found to have the complete conversion of the *un-24^{ts}* coding region into *un-24^{PA}*; however the two strains differ slightly in the extent of the 5' non-coding region replaced (Figure 4). Both of these strains initially exhibited a self-incompatible phenotype as described below in more detail. Other strains (e.g. DLL-T2F, -T3t and -T2a) had partial replacements of *un-24^{ts}*, resulting in chimeric forms of *un-24*. Of note, most of the *un-24* coding region in the DLL-T2f strain is PA-specific (-103bp to 2821bp relative to the start codon) and only 193bp in the 3'-most region is OR-specific. Based on het tests, the DLL-T2f strain is OR-compatible. This verifies that incompatibility specificity is encoded within 193bp of the 3' end of the *un-24^{OR}* gene (Haidari, 2002). This 3' region that is highly variable between OR and PA forms.

Characterization of escape by *un-24^{PA} het-6^{OR}* strains. The DLL-T2j and DLL-T2k strains both initially displayed a SI phenotype and both undergo escape after 5-8 days after subculture (Figure 5). The DLL-T2j and DLL-T2k strains could be maintained in a pre-escape state by storage of the SI form on silica gel or by refrigeration and subculturing every week or so. The DLL-T2j/T2k self-incompatibles were set up on Vogel's medium and replicates of each strain were allowed to escape independently. The escape process seems to alter the growth characteristics and incompatibility function of the strain into one of two states; these two states were designated as Type I and Type II, as described in Figure 6. Type I escape occurs far more frequently (37/38) than the Type II escape (1 of the DLL-T2k escapes). Type I escapes occur at day 4-6 after subculture of the *un-24^{PA} het-6^{OR}* SI strain, have a wildtype growth rate, produce abundant conidia, and have a wildtype appearance at 30°C and at 39°C. At 30°C, Type II escapes produce

Figure 5: Escape from *un-24*^{PA} and *het-6*^{OR} nonallelic incompatibility reaction. **a)**

Nonallelic interactions between *un-24*^{PA} and *het-6*^{OR} result in SI strains that are slow growing, have a spidery morphology and produce few conidia. Escape (E) is usually initially evident as a rapid growing sector that arises 4-6 days after subculture of an SI colony. Subsequently, Type I escaped cultures (shown) grow at about 10X the rate of SI colonies and conidiate similar to wildtype. **b)** Mean growth rates ($\pm$ SE) of *un-24*^{PA} *het-6*^{OR} strains DLL-T2J ($n = 8$) and DLL-T2K ($n = 9$) were measured daily after subculture of SI colonies to Vogel's medium. All escape forms represented in this figure are Type I escapes. Escape to the Type I form occurs consistently between day 4 and day 6, as evident by a sudden increase in growth rate.

Figure 6: Two different *un-24*^{PA} *het-6*^{OR} escape forms have been identified. Individual subcultures of both the DLL-T2J and DLL-T2K self-incompatibles were allowed to escape independently. The most frequent type of escape results in Type I escape strains [e.g. DLL-T2J(0)] that display near-wildtype growth rates and morphology. A single Type II escape [e.g. DLL-T2K(0)] was recovered; the Type II escape form grows slower than wildtype colonies and has an aberrant morphology. Mean growth rates (+/- SE, at 30°C) are presented ($n > 13$) at top. Pictures were taken of cultures grown at 30°C for 64 hours after inoculation on Vogel's medium.

Post-escape <i>un-24^{PA} het-6^{OR}</i> forms				
	Type I		Type II	
Growth Rate (mm/hr)
Morphology after 2.68 days at 30°C
Frequency of occurrence (n=38)	97%		3%	

conidia but display a slower growth rate than typical for wildtype. At 39°C, Type II escapes display a reduction in growth rate, have a pronounced ragged colony margin and undergo growth arrest when the colony margin approaches the edge of the plate (Figure 7). Growth of these arrested colonies resumes upon transfer to fresh medium of inoculum from the colony margin and incubation at either 30°C or 39°C. Later crossing experiments revealed that this Type II phenotype at 39°C is accentuated in strains that also possess the *inl* mutation. Type II escape strains also display a distinctive brown and aconidial phenotype when grown at 39°C on Vogel's medium containing 0.8% sorbose and 0.4% sucrose. Heterokaryon compatibility tests indicate that Type I escapes are fully compatible with *un-24^{PA} het-6^{PA}* strains and incompatible with *un-24^{OR} het-6^{OR}* strains. Type II escapes are incompatible with both *un-24^{PA} het-6^{PA}* and *un-24^{OR} het-6^{OR}* strains (Figure 8). Heterokaryon formation between a Type I escape strain and a strain bearing *un-24^{OR} Δhet-6* are fully compatible. However, Type II escape strains are heterokaryon incompatible with strains bearing *un-24^{OR} Δhet-6* (Figure 9). The above characteristics of Type I escapes are consistent with a modification of *het-6^{OR}* function. This putative modified *het-6^{OR}* form is denoted subsequently as *het-6^{OR*}*.

Mating of *un-24^{PA} het-6^{OR}* escape strains. Key crosses described in this section are summarized in Table 3. Replicate crosses were made with Type I (*un-24^{PA} het-6^{OR*}*) escape strains and *mat A* strains carrying an OR (RLM57-30, not shown), *un-24^{ts} Δhet-6* (DLL8-16, cross 1), PA (6-3, cross 2), or *un-24^{ts} het-6^{OR}; Δvib-1* (DLL2-18, cross 3) haplotype. Most crosses with each of these four *mat A* strains were fertile and produced perithecia (80 of 91 crosses) and ascospore germination at frequencies were > 87 % for

Figure 7: Effects of temperature on the growth rate and morphology of Type I [DLL-T2j(0)] and Type II [DLL-T2k(0)] escape forms. **a)** Pictures are of Type I (left) and Type II (right) escape cultures grown at 39°C for 168 hours. **b)** Growth rates are presented of Type I (DLL-T2j(0), $n = 8$) and Type II (DLL-T2k(0), $n = 9$) *un-24^{PA} het-6^{OR}* escape strain subcultures grown in race tubes and measured daily at 30°C and 39°C.

a)

b)

Figure 8: Characterization of the incompatibility function of Type I and Type II

escape forms. A compatible heterokaryon formed by the fusion of two PA (*un-24^{PA} het-6^{PA}*, not shown) strains or two OR (*un-24^{OR} het-6^{OR}*) strains (A1 + FGSC 4568 are shown) display wildtype morphology and growth rate, whereas an incompatible heterokaryon formed through the fusion of OR and PA haplotype strains [A1 + 6(18) are shown] displays complete inhibition of growth. Type I escape strains [e.g. DLL-T2J(0)] are heterokaryon compatible with *un-24^{PA} het-6^{PA}* [C2(2)-1] and incompatible *un-24^{OR} het-6^{OR}* (A1) strains. Type II escape strain, DLL-T2k(0), is heterokaryon incompatible with *un-24^{PA} het-6^{PA}* [C2(2)-1] as well as with *un-24^{OR} het-6^{OR}* (A1) haplotype strains. Mean growth rates of heterokaryons (+/- SE, $n = 3$, at 30°C) are plotted across the top. Pictures are of heterokaryons grown at 30°C for 64 hours after inoculation on Vogel's minimal medium. At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis. The dashed red arrows indicate functional interactions that are inferred to be suppressed in Type I escape strains and the wavy red arrows indicate functional interactions that are inferred to be partially suppressed in Type II escape strains.

OR and PA haplotype controls			Type I escape		Type II escape	
Heterokaryon	OR + OR	OR + PA	Type I + OR	Type I + PA	Type II + OR	Type II + PA
Growth Rate (mm/hr)	3 2.5 2 1.5 1 0.5 0	3 2.5 2 1.5 1 0.5 0	3 2.5 2 1.5 1 0.5 0	3 2.5 2 1.5 1 0.5 0	3 2.5 2 1.5 1 0.5 0	3 2.5 2 1.5 1 0.5 0
Morphology after 2.68 days at 30°C
Functional interaction

Figure 9: Type I escapes are compatible with *un-24*^{OR} strains deleted for *het-6*^{OR}, whereas Type II escapes are incompatible with all strains bearing *un-24*^{OR}. Heterokaryon formation between a Type I [DLL-T2J(0)] strain and a *un-24*^{OR} *het-6*^{OR} strain (A1) results in heterokaryon incompatibility. However, the Type I escape strain forms a compatible heterokaryon with strains possessing the *un-24*^{OR} Δ *het-6* haplotype (DLL3-15). Heterokaryon incompatibility occurs when a Type II strain [DLL-T2K(0)] is paired with either *un-24*^{OR} *het-6*^{OR} (A1) or *un-24*^{OR} Δ *het-6* (DLL3-15) haplotype strains. Mean growth rates ($\pm$ SE, $n = 3$, at 30°C) are plotted across the top. Pictures in the middle panel are of heterokaryons grown at 30°C for 64 hours after inoculation on Vogel's minimal medium. At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis, the dashed red arrows indicate functional interactions that are inferred to be suppressed in Type I escape strains and the wavy red arrows indicate functional interactions that are inferred to be partially suppressed in Type II escape strains.

Table 3: Summary of crosses using Type I and Type II escape strains

Cross #	Parents		Relevant Genotype ¹		Progeny phenotypes and proportions ²			Inferred Ratio (wt:TI:TII)	Ascospore germination (%) ⁴
	<i>mat a</i>	<i>mat A</i>	<i>mat a</i>	<i>mat A</i>	wildtype	SI→Type I	Type II ³		
1	T2J(0)	DLL8-16	<i>un^{PA}Δ6^{OR*}</i> (i.e. TI)	<i>mat A</i>	25/25	0	0	1:0:0	87
2	T2J(0)	6-3	<i>un^{PA}Δ6^{OR*}</i> (i.e. TI)	<i>un^{PA}Δ6^{PA}</i>	24/24	0	0	1:0:0	92
3	T2J(0)	DLL2-18	<i>un^{PA}Δ6^{OR*}</i> (i.e. TI)	<i>un^{IS}Δ6^{OR*}, vib</i>	51/51	0	0	1:0:0	30
4	T2K(0)	DLL8-16	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	<i>un^{IS}Δ6</i>	11/18	4/18	3/18	2:1:1	92
5	T2K(0)	FGSC 2225	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	<i>un^{PA}Δ6^{PA}</i>	31/58	16/58	11/58	2:1:1	45
6	T2J(0)	DLL14-1	<i>un^{PA}Δ6^{OR*}</i> (i.e. TI)	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	9/27	12/27	6/27	1:2:1	13
7	DLL14-5	DLL14-6	<i>un^{OR}Δ6</i>	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	7/15	4/15	4/15	2:1:1	3
8	DLL14-10	DLL14-6	<i>un^{IS}Δ6</i>	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	6/16	5/16	5/16	1:1:1	11
9	T2J(0)	DLL15-2	<i>un^{PA}Δ6^{OR*}</i> (i.e. TI)	<i>un^{PA}Δ6</i>	31/31	0	0	1:0:0	57
10	DLL15-1	DLL14-15	<i>un^{PA}Δ6^{OR*}, eib</i>	<i>un^{PA}Δ6^{OR*}</i> (i.e. TI)	16/23	0	7/23	2:0:1	95
11	DLL23-12	DLL8-16	<i>un^{PA}Δ6^{OR*}, eib</i>	<i>un^{IS}Δ6</i>	nt	0	nt	nt	95
12	T2K(0)	DLL2-18	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	<i>un^{IS}Δ6^{OR*}, vib</i>	25/48	0	23/48	1:0:1	74
13	T2K(0)	DLL14-6	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	Does not form perithecia				
14	DLL15-1	DLL14-6	<i>un^{PA}Δ6^{OR*}, eib</i>	<i>un^{PA}Δ6^{OR*}, eib</i> (i.e. TII)	Does not form perithecia				

¹Refers to the genotype that is relevant to *un-24-het-6* function. Complete genotypes can be found in Table 1. The presence of *eib-1* (*eib*) in strains bearing the *un^{OR}Δ6* haplotype was determined through conidiation pattern (see text).

²Profuse conidiation phenotypes associated with *eib-1* in wildtype-background strains as referred to in the text, are categorized in this Table as wildtype for simplicity. "SI→Type I" designates germings that were initially SI and escape to Type I escape form within 4-7 days.

³The strains presented in this column from Cross #10 are Type II-like strains characterized by lack of conidiation and brown coloration at 39°C. These strains do not, however, display aberrant morphology at 39°C characteristic of Type II escape strains.

⁴Percent of germinating ascospores was determined from at least one trial. Spores were heat activated at 60°C for 55 minutes and incubated overnight at room temperature. Percent of germinating ascospores was determined by examining at least 75 ascospores at 20 x magnification under the dissecting microscope.

all crosses producing perithecia. Type I escape strains were observed to be male and female fertile ($n=80$ crosses, in pairings with the above 4 *mat A* strains). Germinating ascospores were picked from these crosses involving Type I escape strains and all resulting progeny (>75 in total) had wildtype growth rates and conidiated. Therefore, Type I escape strains display a normal sexual cycle and, as evident from crosses 1, 2, 3 and 9 (Table 3), the Type I-associated *het-6*^{OR*} form is heritable and appears to cosegregate with the *het-6* locus.

Type II *un-24*^{PA} *het-6*^{OR} escape strains were also crossed to OR (RLM57-30, not shown), *un-24*^{ts} Δ *het-6* (DLL8-16, cross 4), PA (FGSC 2225, cross 5) and *un-24*^{ts} *het-6*^{OR}; Δ *vib-1* (DLL2-18, cross 12) haplotype strains (Table 3). Most crosses with these parental combinations were female fertile and produced perithecia (8 of 12 crosses) and had subsequent ascospore germination frequencies of $\geq 92\%$ ($n = 2$ different crosses for each strain pair). However, crosses between mating-type compatible Type II escape strains do not form perithecia ($n = 4$, e.g. cross 13). Germinating single ascospores from a cross between a Type II escape and a strain with the *un-24*^{ts} Δ *het-6* haplotype (cross 4, Table 3) were subcultured without selection. The eighteen progeny from this cross (DLL14) could be classified into one of four different phenotypic categories: 1) 4 progeny were SI and undergo Type I escape, 2) 3 progeny were Type II-like 3) 6 progeny were *un-24*^{ts} Δ *het-6* with wildtype conidiation and 4) 5 were *un-24*^{ts} Δ *het-6* with profuse conidiation (note, these last two categories are designated wildtype in Table 3). The following inferences can be made from this phenotypic ratio: A) The SI form that is concealed in Type II escapes (i.e. *un-24*^{PA} *het-6*^{OR}) is transmitted through sexual crosses;

B) the Type II factor segregates independent of *het-6*; and C) the Type II factor in a non-SI background results in profuse conidiation.

Germinating single ascospores from cross # 4 were also dually selected for hygromycin resistance and the ability to grow at 39°C. The 5 progeny resulting from this double selection presumably possessed a *un-24^{PA} Δhet-6* haplotype and were classified into two different categories: 1) 3 progeny were wildtype and 2) 2 progeny produced profuse conidia, indicating the presence of the Type II factor. The absence of SI and Type II progeny in strains possessing the *un-24^{PA} Δhet-6* haplotype, is another observation that further validates that SI arises from nonallelic interactions between *un-24^{PA}* and *het-6^{OR}*. These ratios suggests that Type II escape is due to a suppressor mutation (*eib-1*, for escape from incompatibility blocked) that segregates independently of the *het-6* locus in a Mendelian fashion, partially relieves *un-24^{PA} het-6^{OR}* nonallelic incompatibility and prevents escape of the *un-24^{PA} het-6^{OR}* strains to a Type I state. Profuse conidiation has also been associated with the *het* suppressor *vib-1* (vegetative incompatibility blocked; Xiang & Glass., 2004a) which is an observation that will be addressed later. Nevertheless, profuse conidiation may be used here as a phenotypic marker associated with the presence of the *eib-1* mutation in non- *un-24^{PA} het-6^{OR}* strains.

Several approaches were taken to further validate the existence of *eib-1* and to investigate the effects of this mutation on the Type I escape. As mentioned before, approximately 50% of *un-24^{ts} Δhet-6* progeny from DLL-T2k(0) x DLL8-16 (cross 4, Table 3) exhibited profuse conidiation. These profuse conidiation strains will subsequently be referred to as hyperconidiating *un-24 Δhet-6* strains and inferred to carry *eib-1* along with a *het-6* haplotype other than *un-24^{PA} het-6^{OR}*. This inference was tested

in the following way. If profuse conidiation is a phenotypic marker for strains that possess *eib-1* along with a non-*un-24*^{PA} *het-6*^{OR} haplotype, then 50% of the progeny from a cross between a hyperconidiating *un-24* Δ *het-6* strain and a Type II *un-24*^{PA} *het-6*^{OR} strain would be hyperconidiating *un-24* Δ *het-6*, and the other 50% would be Type II *un-24*^{PA} *het-6*^{OR} (i.e. all progeny would possess *eib-1*). However, crosses between hyperconidiating *un-24* Δ *het-6* strains and a Type II *un-24*^{PA} *het-6*^{OR} strains ($n = 6$ crosses, e.g. cross 14) do not produce perithecia. Hyperconidiating *un-24* Δ *het-6* strains are, however, fertile in crosses with strains that do not possess the putative *eib-1* mutation (i.e. non- hyperconidiating; $n = 6$, e.g. cross 10). In addition, the wildtype *un-24* Δ *het-6* progeny from cross 4 produce perithecia with both Type I *un-24*^{PA} *het-6*^{OR*} ($n = 9$, e.g. cross 9) and Type II *un-24*^{PA} *het-6*^{OR} strains ($n = 9$, e.g. crosses 7 and 8). These results are consistent with the observation that crosses between Type II escape strains of opposite mating type do not form perithecia. This further suggests that the *eib-1* gene has a role during mating. Disruption of mating function in hyperconidiating *un-24* Δ *het-6* strains also supports the idea that the hyperconidiating phenotype is a marker for the *eib-1* mutation in strains with non-*un-24*^{PA} *het-6*^{OR} haplotypes.

Mutations in *eib-1* seem to prevent Type I escape (i.e. from *het-6*^{OR} to *het-6*^{OR*}) as demonstrated by the appearance of both SI and Type II-like progeny in crosses involving *un-24*^{PA} *het-6*^{OR} Type II escapes (crosses 4, 5, 6, 7, and 8, Table 3). This observation could indicate that the *eib-1* mutation may also reverse the Type I modification (i.e. *het-6*^{OR*} to *het-6*^{OR}). To test this hypothesis, a fluffy *un-24*^{PA} Δ *het-6* (*eib-1*) strain was crossed to a Type I *un-24*^{PA} *het-6*^{OR*} escape strain (cross 10, Table 3). Relevant markers for twenty three progeny from this cross were determined as follows: 7

un-24^{PA} Δhet-6, 6 *un-24^{PA} het-6^{OR*}*, 7 brown flat that grow at 39°C (*un-24^{PA} het-6^{OR*}*) and 3 that were hyperconidiating (*un-24 Δhet-6*). Note that the screening for hygromycin B resistance was used to indicate the *un-24^{PA} Δhet-6* haplotype, while hygromycin sensitivity indicated the *un-24^{PA} het-6^{OR}* genotype. The 7 progeny that were brown and flat at 39°C, but wildtype at 30°C are inferred to be *het-6^{OR*}*; *eib-1*. One brown-at-39°C strain was crossed to a *un-24^{IS} Δhet-6* strain (cross 11, Table 3) and of progeny examined, none were SI. This indicates that *het-6^{OR*}* (Type I escape form) is maintained in strains that also carry *eib-1*. If the *eib-1* mutation was to reverse the putative Type I modification (i.e. *het-6^{OR*} → het-6^{OR}*), we would expect to see about ¼ SI progeny that subsequently undergo Type I escape. The absence of SI progeny from this cross indicates that the *eib-1* mutation cannot reverse the Type I escape form (*het-6^{OR*}*) once it is in place.

In summary, crossing experiments have demonstrated that the Type I modification is a heritable trait that cosegregates with the *het-6* locus. The Type II phenotype was shown to segregate independently of the *het-6* locus, suggesting that Type II escape is due to a mutation which we have named *eib-1*. *eib-1* has demonstrated the ability to prevent the Type I modification, but does not reverse it once it is in place. Strains possessing *eib-1* along with a non-*un-24^{PA} het-6^{OR}* haplotype display a profuse conidiation phenotype, perhaps indicating involvement of *eib-1* in conidiation. Interestingly, crosses between strains that both possess the *eib-1* mutation do not produce perithecia, suggesting that *eib-1* may also play a role during mating. Taken together, these various crosses have revealed that Type I escape is consistent with a modification of *het-6* or HET-6 and that Type II escape is due to a mutation in a gene involved in HI, mating and conidiation.

***eib-1* is associated with a point mutation in *vib-1*.** Mapping of the *eib-1* mutation was made possible through the use of a PCR/RFLP-based technique termed cleaved amplified polymorphic sequence (CAPS) that exploits the use of the highly polymorphic Mauriceville strains (Kotierk & Smith, 2004; Jin et al., 2007). The Type II *un-24^{PA} het-6^{OR}* escape strain [DLL-T2K(0), which carries the *inl* marker] was crossed to a Mauriceville strain (FGSC 2225; cross 5, Table 3) and single spore progeny from this cross were subcultured and allowed to grow at 39°C. Fifty-one Type II-like and SI (that escape to a Type I state) progeny were selected and spotted onto 0.8% sorbose plates to determine the genotype at *inl*. It was observed that 23 of 27 Type II-like progeny and 4 of the 24 Type I escape progeny possessed *inl*, evidence that *eib-1* is linked to *inl* on Linkage Group V (LGV) (2-tailed, 2 x 2, Fisher Exact, $p < 0.001$, 95% confidence). Several CAPS markers were designed [or adapted from Jin et al (2007)] and DNA was extracted from each of the 36 Type II-like *inl⁺* and 37 of the Type I escapes from the DLL-T2K(0) X FGSC 2225 cross to further examine the position of *eib-1* within LGV. A Summary of the CAPS mapping is found in Figure 10. These results show that *eib-1* is located within a region of approximately 300 Kb on LGV that also contains *vib-1*. Interestingly, a cross between a Type II [DLL-T2k(0)] and a *un-24^{ts} het-6^{OR}; Δ*vib-1** strain (DLL2-18) (cross 12, Table 3), does not yield any SI progeny ($n = 48$), but rather yields an inferred 1:1 ratio of wt (*un-24^{ts} het-6^{OR}*) and Type II-like (*un-24^{PA} het-6^{OR}*) progeny. This observation indicates that *eib-1* and *vib-1* not only map to the same region in the genome, but also display similar phenotypes when expressed with the *un-24^{PA} het-6^{OR}* haplotype. Therefore, there is a possibility that Type II escape (i.e. the *eib-1*

Figure 10. Map location of the *eib-1* mutation. **a)** Crossing initially revealed that *eib-1* was linked to the auxotrophic marker *inl* on LGV. A Type II escape strain (OR) was then crossed to a Mauriceville (MO) strain. A PCR/RFLP-based method for genetic mapping in *N. crassa* developed by Kotierk and Smith (2004), has allowed us to determine **b)** the position of the *eib-1* mutation within a 300 kb region on LGV between 5-95 and 5-98 that also contains *vib-1*.

a)

Strain	Escape type	Marker						
		5-55	5-91(<i>ib-2</i>)	5-94	5-95	<i>elb-1</i> ²	5-98	<i>int</i> ³
DLL24a-1	Type II	nt ¹	OR	OR	OR	-	MO	+
DLL24a-30	Type II	OR	OR	nt	OR	-	MO	+
DLL24a-32	Type II	OR	OR	OR	OR	-	MO	+
DLL24a-33	Type II	OR	OR	OR	OR	-	MO	+
DLL24b-34	Type I	OR	MO	MO	MO	+	MO	+
DLL24b-49	Type I	OR	MO	MO	MO	+	MO	+
DLL24b-30	Type I	OR	OR	OR	OR	+	MO	-
DLL24b-4	Type I	OR	OR	MO	MO	+	MO	+
DLL24b-7	Type I	OR	nt	MO	MO	+	MO	+
DLL24b-9	Type I	OR	MO	MO	MO	+	MO	+
DLL24b-3	Type I	OR	MO	MO	MO	+	MO	+
DLL24b-41	Type I	MO	MO	MO	MO	+	MO	-
DLL24b-48	Type I	MO	MO	MO	MO	+	MO	-

¹ nt = not tested² “₂” is the OR allele, “₊” is the wt allele in the Mauriceville strain³ “₂” is the OR allele, “₊” is the allele from the Mauriceville parent

b)

mutation) is due to a mutation in *vib-1*. The *vib-1* genes from FGSC 2225 (Mauriceville), DLL14-15 (Oak Ridge) and DLL24a-1 (Type II) were sequenced and alignments revealed that a single G→A point mutation occurs in the *vib-1* gene of a Type II strain as seen in Figure 11. This point mutation alters codon GGC to GAC and is predicted to cause an amino acid change from a glycine to aspartic acid at position 188 in VIB-1. This non-conservative substitution is positioned within the predicted NTD80-like DNA binding domain. Figure 12 shows that the predicted NDT80 domain encoded by *vib-1* possesses homology to the NDT80 protein in *Saccharomyces cerevisiae* as well as a NDT80-like protein in *N. crassa* especially within the C-terminal-most amino acids (270-400) of the predicted DNA binding domain. Taken together, these results present the possibility that Type II escape is caused by a single point mutation in *vib-1*. This mutation is located within an area of relatively low identity but may nevertheless have deleterious effects to VIB-1 function.

Characterization of strains having *het-6* deletions. Germinating ascospores from a cross between Type II escape strain and a strain with a *un-24^{ts} Δhet-6* haplotype (cross 4, Table 3) were allowed to germinate on medium containing hygromycin B at 39°C. Three progeny bearing *un-24^{PA} Δhet-6* (and wildtype conidation) were recovered using this double selection, each of which display wildtype growth rates. This *un-24^{PA} Δhet-6* along with *un-24^{OR} Δhet-6* strains were subjected to heterokaryon tests (Figure 13). Strains bearing *un-24^{PA} Δhet-6* are compatible with PA-haplotype strains and are heterokaryon incompatible when paired with *un-24^{OR} het-6^{OR}* haplotype strains. *un-24^{OR} Δhet-6* strains are compatible with OR-haplotype strains and severely incompatible when paired

Figure 11: Sequencing of the *vib-1* gene in a Type II strain reveals a single G→A transition. This mutation is found at position 686 in the DNA sequence shown and results in an amino acid change from glycine to aspartic acid at position 188 within the predicted Ndt80 DNA binding domain (underlined). The DNA sequence of the *vib-1* gene is numbered on the left and the predicted amino acid sequence is numbered on the left. Two predicted introns are italicized.

1 ACTAGACAAAACCTATCCTCCAACCTAAGCGACTCGAAGGGTTTTTTTCTGTGAAGGAAACCTTCGGGCAATCTTGGAGGAGTGACAGCT
 91 GCCCATGTGAGCGACAGCAACATACACGGTCAGTATGGCAGAGTTGAGAGCTGAGACTCAGCACGAGGCATCTGCCCAACTACGGGAA
 m a e l r a e t q h g g i w p n y g n 19
 181 TCCGGTGCAGATGAATACTGGTCGGTACAACACTCAAGAGTCTTCGGTACCAGTAGGATCGGCGGCTTCTTACATCTTGTACGCCCTCG
 p v q m n t g r y n t q e s s v p v g s a a s s h l v r p r 49
 271 CAGTCGTGAGCACAGTGGACTATCACAACGCACCATACCATCATGGACGGCCTGCCAGGAGGATGGCGATGGCTACGAGAGATATCC
 s r q h t m d y h n a p y h h g r p a q e d g d g y e r y p 79
 361 ACACCCTTCGTTGATGAACATCCCCAGCATCACCACCGCATGAAGCGCAGCTACTCCAGGTGACCAAACCTCCCTATACGGAATGGT
 h p s l m n i p s i t t g m k r s y s q v d q t p y t e m v 109
 451 CCAAGATCTCCGCGACGACTACAAGCCGCCATGAATCAGGACGAGAAGCTACTCTCATTCAAGAAGGTTGGCGACAAACATACCATCGT
 q d l r d d y k p a m n h d q k l l s f k k v g d k h t i v 139
 541 CGACCACAAAGGGCGGATTACGAAATCGAAATCGAAGCTCAACTTCACGGCATGTTCTTTCTGTGGAGTTTCCTCGCCTGGCGACGG
 d h k g r i h e i e i e a q l h g m f f l s e f p s p g d g 169
 A
 631 CAATGTCTTGAACGCCGAGTTGACTTGCTACCGCCGCAACCTGTTCAAATCAGCGCAATATCTGCTTTCCTCAAATACCGTTGTCTGT
 n v l n a e l t c y r r n l f q i s g n i c f p q i p l s v 199
 D
 721 CATGCTAGAAACGGCGGAGACCAGCCAGATCAAGAACATGGAAGTTACTATATCGGCAATCGAATCTGTCGACGCCACCCTGTGCGCCT
 m l e t g e t s q i k n m e v t i s a i e s v d g h p v r l 229
 811 AATCGTCATTCCCTGGAAGACGCCCCCAACCTCACCAGAAGTCAACCAAGCTCCCGATCAAGAACCTCCATCAC'TCCTCTGATTCC
 i v i p w k t p p p n s p e v n q a p d q e p p s l p l i p 259
 901 CTGGTCCGAAGAGGAGGAAGACAATGGCGGAGATCACTACGCTATCTATCCAAATGGGTGGCGTCGACTTCAATTAGGATGTAAGCATC
 w s e e e e d n g g d h y a i y p i g w R R L Q F R I 286
 991 ACTTCAGAACCACTCACTCCACATTTCGTTATGTTCAATAGCTGATGCGTTCTCTATCTAGCGCAACGCCAACACCGTAGACGGA
 A T A N N G R R 294
 1081 AGGAATTACAGCAACACTTCGTCCTACACTTGAAGCTCCATGGAACCCCTAGCGAATGGTACGAAACTTGTGCTTTCGGAGCTTACCACTG
 K E l q q h f v l h l k l h g t l a n g t k l v l s e l t t 324
 1171 CGCCGATTGTTGTCCGAGGAAGAAGCCCGCGGAATTTCCAAGCGAGAAGGAGATCCCGCTGTTGGGATCTAGCGCTGGTTCAAGAGGAC
 a p i v v r g r s p r n f q a r k e i p l l g s s a g s r g 354
 1261 AGACACTCGTGAAACCGGTCACTCAATGTTGCGCAAGCTGTGCGCTTAACAAGCCTCCCTATGATTTCGAGACCCCGTGTCTCGAGCA
 q t l v e t g h s i v a q a v a l n k p p y d s r p r v s s 384
 1351 TGGATTGCCCCAGAACAGCGTTACGTTTCACGTCTGCGAAGCAGATGCCCCAGAGTCTATGCAAAATGCGTTGCAAGTCAAGTCAACAAAA
 m d l p r t a f t f t s a k q m p q s p m q m r s n 410
 1441 TTTCAGATACGGAGATGTGGCATATTCAAGGCAGTGATGCTAGCTTCACTAGGGTCTGACTTGTGTAGAGGAAACGATACGTGATGCT
 1531 AACATAGTTCAGCAGTTCTTACCCACAAAGTTGGAACCCATCTAGTCAGGTGTCGATGCCACACAACCCAGGATCGACTTCTTACCCGAC
 s y p t s w n p s s q v s m p h n p g s t s y p t 435
 1621 AACATCGATGGCCGGCCCCGAGCCCTATCCTAAAATGCCCTTTCTGGAGCGCCAGCTACACGGCCGAGCCCCAAGAGATGCCTATCCA
 t s m a g p e p y p k m p l s g a p s y t a e p q e m p i q 465
 1711 GCAGACCTCCATGCCTTCTATGCAACTCTCTATGGTAGCCCAAGACCAACCAACCGTCGGCCCCCATCCGAACACAATACGCAACTTACGC
 q t s m p s m q l s m v a q d q q p s a p i r t q y a t y a 495
 1801 ATCGGCACCTCCGCCGATCTTTGTTGCCGAGCACAGCGGACAGCTCGCTTAACGTGCCGCGCTATGTGGACAGCAATCCCCGTCCTTC
 s a p p p h l s l p s t a d s s l n v p r y v d s n p r p s 525
 1891 AAAGAGCCCTCGCCACGGTAGCCATGGCTCACTCACGAACGAGACGGCCTCGGGCGAGTATCGCTATGGCCCCCTTCGTACCTTGGCAA
 k s p r h g s h g s l t n e t a s g e y r y g p p s y l g n 555
 1981 CAGCTCAAGTGATATAAGTCCCCAATCGCAACATCATCCGCTACGTGAGTGAGGAGCAGGCGGGGCGTGTGCGGGCGCTTACGGAAC
 s s s d i s p q s q h h p p t s g a g a g g a s s g a y g t 585
 2071 ACCATCTCAAGAAGGAGGTGCCTCTGCCCCAGCGTCTGCGCTACGAGTGGCGCTCCGCCGCGTATTACTTCCCCCATCTCAGTCTCTG
 p s q e g g a s a p a s a p t s a a p p r d y f p p s q s w 615
 2161 GACCAGCACTGCCGGTGAGGGCAGACTTCATCGTACACGAACGAGGTGACAGGTCTATTCTTCTTACAGGTGTGAAACGGAGCC
 t s t a g e g q t s s y t n g q d r s y s f p t g v k t e p 645
 2251 GCACTCGCAGCCTTCGCACTCGGGTGCACAGTGCCAGCGGTGTATGGCAACAACCACTATGCATGGAACGCCACGTAAA
 h s q p s h s g a p v p g v y g n n h y a w n a t * 670

Figure 12: Alignment of conserved NDT80-like DNA binding domains in *Saccharomyces cerevisiae* NDT80 protein, a *N. crassa* NDT80 homolog and VIB-1. This alignment was generated using ClustalW (EBI) and Boxshade. Black boxes indicate amino acid identity, grey boxes indicate conservative amino acid substitution and non-shaded areas indicate non-conservative differences. The glycine residue (G) shown in red is predicted to occur as an aspartic acid (D) in *vib-1* of the strain DLL-T2k(0) (Type II escape).

NDT80_SACCE 1 MNEEN--DDVFCDDVSKYDELTEOEDT---PVIQTQNEED---GIDSNYDTR-----
 NDT80_NEUCR 7 SNAIPSPESOLSURESYANTPTERRIHHTER---SPSFGGHTERRPSPSPSPATVNTIR-----
 VIB-1_NEUCR 5 RASTQGGIWEYGNPQONNGGNNPSSIVGSAASHVRPRSOQTIDYHNAHYHGPAQEDGDGYERYPHPSLM

NDT80_SACCE 53 ----UKIAPETILAFKVGPPFETRRYCPVVESHTETDDRRIPTREREDHITSEVVGKPNTEILS---DETAN
 NDT80_NEUCR 67 ----LDITYGSKTPNIPDELSETECGVLSGGPOITPVAETSGI--KEFPATQOTCERNNVSCC---SSS---
 VIB-1_NEUCR 85 NIPSITTEMKESYSVDOITSEYELQELRDDKPMNNDOGLSFKVGDKHTIVHKGRHEIEIEAQHGMFTLEFP

NDT80_SACCE 125 CDITTFKSSFDLLIEDSVESRLRQNFARKKKKNDD--DTEINQCHAAETVSPQFCVSCPVSSEIKHTIIRE
 NDT80_NEUCR 136 --LTPSPLETHQFOTGSPPYIVYGSAMSISAVAS--NOTPPLVCHPPEVSSVAKKEVRLCKAQOOTHLS
 VIB-1_NEUCR 165 SPCGGLNAGITCYRRNLEISCNICSPCPUSVLWGTQKKNV--ISAIESVDCHEVNITISNKTSPPSSEV

NDT80_SACCE 204 SNVRNITMKKYDSTFHLRHCHNYEEVVSLLSYPEDSOKVRRVSVASSISVKKPSQ-QIKESSEVVGTV
 NDT80_NEUCR 213 S-----LSSNEGSLSSRYEBQPIQQQSPITEHTETTCQKATNKKRP-AAQV-YHVELW
 VIB-1_NEUCR 245 N-----QAPDOEPSPPLIPSETEEDNCGDHVATPTG--RELTRERATLREKELQHSVVKCHET

NDT80_SACCE 283 VCPDFFHENPGIPYDELALKNGSKGMVYQENEPSTHFFSS--ASSOETVRP--SVN--SNTKRMPSMA--
 NDT80_NEUCR 276 AVESQGAEN-----VKKVAYRESKNTVTFEGBNERNRFGTS--GEFGCAGTGGYSSVILG
 VIB-1_NEUCR 311 PANCK-----VVSSEPIAAIV--RERETAR--EPIPLGSAASREILVETESTIAA

NDT80_SACCE 363 DNCSCLNARPSKRSKVAEAEENSEASISPIISR---
 NDT80_NEUCR 339 GSD--Y--GTIMLPGGFLOFEDPFGYGARHHHEJ---
 VIB-1_NEUCR 369 VLN--KPYDS--PRVSS--DLLETAIFGSAQ--POS

Figure 13: Non-allelic and allelic incompatibility involving *het-6* and *un-24*. Mean growth rates of heterokaryons ($\pm$ SE, $n \geq 3$, at 30°C) are plotted across the top. In the middle panel, pictures are of cultures grown at 30°C for 64 hours on Vogel's medium. The positive controls in the left panel shows that strains bearing *un-24*^{PA} Δ *het-6* (DLL15-6) are heterokaryon compatible with strains of *un-24*^{PA} *het-6*^{PA} haplotype [C2(2)-1], and strains bearing *un-24*^{OR} Δ *het-6* (DLL3-15) are heterokaryon compatible with a strain of *un-24*^{OR} *het-6*^{OR} haplotype (A1). Asymmetric non-allelic affects of *het-6* on *un-24*-associated incompatibility are evident with *het-6*^{PA} eliciting a stronger incompatibility reaction than *het-6*^{OR} in heterokaryons that are incompatible at *un-24* (middle panel). In this panel, strains bearing *un-24*^{OR} Δ *het-6* (DLL3-15) are severely incompatible with strains bearing *un-24*^{PA} *het-6*^{PA} [C2(2)-1] while strains bearing *un-24*^{PA} Δ *het-6* (DLL15-6) are moderately incompatible when paired with strains bearing the *un-24*^{OR} *het-6*^{OR} haplotype. In the right panel, a weak incompatibility is observed in heterokaryons formed by *het-6* deletion strains that differ only at *un-24*. Heterokaryons formed between strains that are genotypically *un-24*^{PA} Δ *het-6* (DLL15-6) and *un-24*^{OR} Δ *het-6* (DLL3-15) grow at ~1 mm/hr ("pre", pictured far right) for approximately two days after which sectors appear that grow at ~2 mm/hr. At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis.

Positive Controls		$\Delta het-6$ Heterokaryon Tests			
Heterokaryon	PA + PA $\Delta 6$	OR + OR $\Delta 6$	OR + PA $\Delta 6$	PA + OR $\Delta 6$	OR $\Delta 6$ + PA $\Delta 6$
Growth Rate (mm/hr)					
Morphology after 2.68 days at 30°C					
Functional interaction					

with strains bearing the *un-24*^{PA} *het-6*^{PA} haplotype. Heterokaryons formed between a *un-24*^{PA} Δ *het-6* haplotype strain and a *un-24*^{OR} Δ *het-6* haplotype strain grow very poorly for approximately two days after which escape occurs ($n = 4$). The escaped heterokaryons display wildtype morphology and growth rates. These results show that *het-6*^{PA} elicits a stronger incompatibility reaction than does *het-6*^{OR} in heterokaryons wherein different alleles of *un-24* occur. It also shows that an allelic difference at *un-24* alone elicits a weak incompatibility that is overcome quickly by escape.

Mutations in *vib-1* completely suppress HI associated with *un-24* and partially suppress *het-6* HI. The effect of the HI suppressor mutation *vib-1* was investigated for its ability to suppress incompatibility associated with the *het-6* locus. A *vib-1* deletion mutation (Δ *vib-1::hph*) was first compared to a RIP-induced mutation (*vib-1*^{m32}). The premise of this experiment was to examine suppression of *un-24* *het-6* incompatibility by the *vib-1*^{m32} mutation (which putatively encodes a truncated version of *vib-1*) as well as a complete *vib-1* deletion to determine whether or not both mutation types have a similar function. Figure 14 demonstrates that both the RIP-induced mutation and complete deletion are dominant over *vib-1*⁺. However, the *vib-1*^{m32} mutation is a stronger suppressor of *un-24* *het-6* incompatibility compared to the complete deletion. For this reason, subsequent analysis of the effects of *vib-1* was undertaken using the stronger suppressor allele, *vib-1*^{m32}. It should also be noted that mutations in *vib-1* only partially suppress HI associated with *un-24* and *het-6* differences (Figure 15). Such heterokaryons that are partially suppressed are characterized as having a slow growth rate that is intermediate between compatible and fully incompatible heterokaryons and they produce

Figure 14: Dominance of partial suppression of *un-24* and *het-6* incompatibility functions by *vib-1* mutations. The black bars represent the growth rates of heterokaryons without mutations in *vib-1* (*vib-1*⁺). The grey and white bars represent the growth rates of heterokaryons possessing a RIP-induced mutation of *vib-1* (*vib-1*^{m32}) and a complete deletion of *vib-1* (Δ *vib-1::hph*), respectively. Pre-escape (where applicable) mean growth rates of heterokaryons (+/- SE, *n* ≥ 3, at 30°C) are plotted. OR designates the *un-24*^{OR} *het-6*^{OR} haplotype and PA designates *un-24*^{PA} *het-6*^{PA}. The strains used are A1 (OR), C2(2)-1 (PA), DLL2-45 (OR Δ *vib-1::hph*), DLL9-11 (PA Δ *vib-1::hph*), *vib* (OR *vib-1*) and MMB-14 (PA *vib-1*). The PA haplotype strain used in the positive control at left is 6-18.

Figure 15: *vib-1* completely suppresses incompatibility associated with interactions between *un-24* heteroallelism but only partially suppresses HI-associated with nonallelic interactions between *un-24* and *het-6*. The *vib-1* mutation completely suppresses allelic incompatibility reactions associated with differences at *un-24* (left panel), but is inferred to only partially suppress HI interactions involving differences at *un-24* when either the OR (middle panel) or (right panel) PA form of *het-6* is expressed. Pre-escape (where applicable) mean growth rates of heterokaryons ($\pm$ SE, $n \geq 3$, at 30°C) are plotted across the top. The strains used are A1 (OR), 6-18 (PA), DLL3-15 (OR Δ 6), DLL15-6 (PA Δ 6), *vib* (OR *vib-1*), MMB-14 (PA *vib-1*) and v Δ 6-1 (OR Δ 6 *vib-1*). In the middle panel, pictures are of cultures grown at 30°C for 64 hours on Vogel's medium. At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis. Flat arrowheads designate HI interactions that are blocked by the *vib-1* mutation.

Heterokaryon	ORAΔ6 + PAAΔ6 heterokaryon tests		PAAΔ6 heterokaryon tests		ORAΔ6 heterokaryon tests	
	PAAΔ6 + ORΔ6	PAAΔ6 + ORΔ6 vib	PAAΔ6 + OR	PAAΔ6 + OR vib	ORAΔ6 + PA	ORAΔ6 + PA vib
Growth Rate (mm/hr)
Morphology after 2.68 days at 30°C
Functional interaction

aconidiate colonies with a very diffuse mycelium. Another hallmark of partial suppression by mutations in *vib-1* is the increased likelihood of escape from HI when compared to heterokaryons that do not possess *vib-1*, especially when the mutation is in the PA haplotype nucleus. Five of six OR (A1) + PA *vib-1* (MMB-14) heterokaryons were observed to escape 57.8+/-5.82 hours ($n=5$) after inoculation. However, only one of five OR *vib-1* (vib) + PA; *vib-1* (MMB-14) heterokaryons were observed to escape and none of six OR *vib-1* (vib) + PA (6-18) heterokaryons were observed to escape during more than 10 days of observation. These results are in contrast to zero escapes observed out of more than 100 PA [6-18 or C2(3)-41] + OR (A1 or RLM57-30) heterokaryons examined over the course of these studies. This demonstrates that escape frequencies are much higher when heterokaryons possess *vib-1* especially as a single copy in the PA haplotype nucleus.

Using the previously mentioned *het-6* deletion strains, I was able to investigate whether or not heteroallelism at *vib-1* suppresses non-allelic ($un-24^{PA}-het-6^{OR}$ and $un-24^{OR}-het-6^{PA}$) and allelic ($un-24^{PA}-un-24^{OR}$) incompatibility functions. *vib-1* completely suppresses HI associated with allelic differences at *un-24*, but only partially suppresses incompatibility when strains also bear either *het-6^{OR}* or *het-6^{PA}* (Figure 15). The growth characteristics associated with partial suppression of HI by *vib-1* in $un-24^{OR} \Delta het-6 + un-24^{PA} het-6^{PA} vib-1$ heterokaryons is reminiscent of OR + PA *vib-1* heterokaryons. Both heterokaryons are initially slow growing, aconidiate, exhibiting very diffuse mycelia and frequently undergo escape. These comparisons of escape characteristics are summarized in Table 4. Three out of four $un-24^{OR} \Delta het-6 + un-24^{PA} het-6^{PA} vib-1$ heterokaryons were observed to escape as opposed to zero escapes observed out of more than fifteen

Table 4: Escape frequencies of heterokaryons with and without *vib-1*

Genotype of Heterokayon ¹		Frequency for occurrence of escape ²	Time of escape (hours +/- SE) ³
OR	PA	0/ 100	N/A
OR	PA; <i>vib-1</i>	5/6	57.8 +/- 5.82
OR; <i>vib-1</i>	PA	0/6	N/A
OR; <i>vib-1</i>	PA; <i>vib-1</i>	1/5	24.5
<i>un-24</i> ^{OR} Δ <i>het-6</i>	PA	0/15	N/A
<i>un-24</i> ^{OR} Δ <i>het-6</i>	PA; <i>vib-1</i>	3/4	61.67 +/- 9.17
<i>un-24</i> ^{PA} Δ <i>het-6</i>	OR	0/3	N/A
<i>un-24</i> ^{PA} Δ <i>het-6</i>	OR; <i>vib-1</i>	1/3	64
<i>un-24</i> ^{PA} <i>het-6</i> ^{OR*} (i.e. TI)	OR	0/51	N/A
<i>un-24</i> ^{PA} <i>het-6</i> ^{OR*} (i.e. TI)	OR; <i>vib-1</i>	3/4	66.75 +/- 14.4

¹A genotype of OR and PA represent *un-24*^{OR} *het-6*^{OR} and *un-24*^{PA} *het-6*^{PA} haplotypes, respectively

²Observations were made up to at least day 10

³Not Applicable (N/A)

un-24^{OR} Δ *het-6* + *un-24*^{PA} *het-6*^{PA} heterokaryons observed over a period greater than 10 days. The presence of the *vib-1* mutation also increased the likelihood of escape from HI in a *un-24*^{PA} Δ *het-6* + *un-24*^{OR} *het-6*^{OR} *vib-1* heterokaryon (Table 4). Partial suppression of HI by *vib-1* is also observed in the heterokaryon Type I *un-24*^{PA} *het-6*^{OR*} + *un-24*^{OR} *het-6*^{OR} (Figure 16) which was reminiscent of the partial suppression of HI in the previously mentioned *un-24*^{PA} Δ *het-6* + *un-24*^{OR} *het-6*^{OR} heterokaryon. Both heterokaryons display a very subtle increase in growth rate and both are more likely to undergo escape from HI when possessing *vib-1* (Table 4). These results suggest that *vib-1* is a dominant partial suppressor of *het-6*-associated HI and completely suppresses *un-24* allelic incompatibility. A slight asymmetry of suppression is also observed depending in which nucleus *vib-1* is found and may perhaps suggest *cis*-acting properties of *vib-1*. *vib-1* also increases the frequency of escape from *het-6* HI, especially when *vib-1* is found in the PA nucleus. These results suggest that *vib-1* may control escape from HI in a *cis*-acting fashion.

***vib-1* and *eib-1* act synergistically to suppress HI associated *het-6*^{OR}.** The first evidence of synergy between *vib-1* and *eib-1* was observed in heterokaryon incompatibility tests involving the original Type II mutant against OR and PA tester strains possessing *vib-1* (Figure 17). These tests showed that *vib-1* was able to suppress incompatibility associated with Type II escape strain. It was found that *eib-1*, alone, does not suppress HI associated with interactions at the *het-6* locus, but does when combined with *vib-1* (Figure 18). These observations suggest a genetic interaction between *eib-1* and *vib-1* that is involved in the regulation of *het-6*^{OR} incompatibility function.

Figure 16: The *vib-1* mutation only partially decreases the severity of HI associated with heterokaryon formation between a Type I *un-24*^{PA} *het-6*^{OR*} escape strains and an OR haplotype strain. Pre-escape (where applicable) mean growth rates of heterokaryons (+/- SE, $n \geq 3$, at 30°C) are plotted across the top. The strains used are A1 (OR), *vib* (OR *vib-1*) and Type I [DLL-T2j(0)]. In the middle panel, pictures are of cultures grown at 30°C for 64 hours on Vogel's medium. At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis, the dashed red arrows indicate functional interactions that are inferred to be suppressed in Type I escape strains. Flat arrowheads designate HI interactions that are blocked by the *vib-1* mutations.

Type I escape		
Heterokaryon	Type I + OR	Type I + OR vib
Growth Rate (mm/hr)
Morphology after 2.68 days at 30°C
Functional interaction

Figure 17: *vib-1* completely suppresses incompatibility associated with heterokaryon formation between a Type II *un-24*^{PA} *het-6*^{OR} escape strain and OR or PA haplotype strains. Mean growth rates of heterokaryons (+/- SE, $n \geq 3$, at 30°C) are plotted across the top. The strains used are A1 (OR), C2(2)-1 (PA), *vib* (OR *vib-1*), MMB-14 (PA *vib-1*) and Type II [DLL-T2k(0)]. In the middle panel, pictures are of cultures grown at 30°C for 64 hours on Vogel's medium. At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis, and the wavy red arrows indicate functional interactions that are inferred to be partially suppressed in Type II escape strains. Flat arrowheads designate HI interactions that are suppressed by the *vib-1* mutation.

Type II escape				
Heterokaryon	Type II + OR	Type II + OR vib	Type II + PA	Type II + PA vib
Growth Rate (mm/hr)	3 2.5 2 1.5 1 0.5 0
Morphology after 2.68 days at 30°C
Functional interaction	<i>im-24^{PA}</i> <i>het-6^{OR}</i>	<i>im-24^{PA}</i> <i>het-6^{OR}</i>	<i>im-24^{PA}</i> <i>het-6^{PA}</i>	<i>im-24^{PA}</i> <i>het-6^{PA}</i>

Figure 18: *vib-1* behaves synergistically with the *eib-1* mutation to suppress incompatibility associated with *het-6*^{OR} but not *het-6*^{PA}. Mean growth rates of heterokaryons (+/- SE, $n \geq 3$, at 30°C) are plotted across the top. The strains used are A1 (OR), 6(18), DLL14-8 (*un-24*^{OR} Δ *het-6*; *eib-1*), DLL14-9 (*un-24*^{OR} Δ *het-6*; *eib-1*), DLL14-13 (*un-24*^{OR} Δ *het-6*; *eib-1*), C2(2)-1 (PA, only used for heterokaryon tests against *un-24*^{OR} Δ *het-6*; *eib-1* strains), *vib* (OR *vib-1*) and MMB-14 (PA *vib-1*). At bottom, the red arrows indicate putative functional interactions that mediate incompatibility during heterokaryosis, flat arrowheads designate HI interactions that are suppressed by the *vib-1* and/or *eib-1* mutations. **a)** Moderate incompatibility is observed in *un-24*^{PA} Δ *het-6* + *un-24*^{OR} *het-6*^{OR} heterokaryons, and when these heterokaryons contain either mutations in *eib-1* or *vib-1*. Complete suppression of incompatibility is observed when *un-24*^{PA} Δ *het-6* + *un-24*^{OR} *het-6*^{OR} heterokaryons contain mutations in *eib-1* and *vib-1*. **b)** Severe HI is observed when *un-24*^{PA} *het-6*^{PA} strains are paired with *un-24*^{OR} Δ *het-6* strains with or without *eib-1*. However, HI is only partially suppressed when *un-24*^{PA} *het-6*^{PA}; *vib-1* strains are paired with *un-24*^{OR} Δ *het-6* strains with or without *eib-1*. **c)** Weak incompatibility is observed in *un-24*^{PA} Δ *het-6* + *un-24*^{OR} Δ *het-6* heterokaryons, and also in the same heterokaryons containing a mutation in *eib-1*. Complete suppression of *un-24*^{OR}-*un-24*^{PA} incompatibility is observed when *un-24*^{PA} Δ *het-6* + *un-24*^{OR} Δ *het-6* heterokaryon contain mutations in *vib-1*, but not *eib-1*.

DISCUSSION

***un-24* allelic incompatibility and asymmetric incompatibility associated with *het-6*.**

The heterokaryon tests performed with the *het-6* deletion strains in this study indicate that *het-6*^{PA} elicits a stronger incompatibility reaction than *het-6*^{OR} in strains that are also incompatible due to allelic differences at *un-24*. Asymmetric HI interactions associated with the *het-6* locus were first identified by Micali & Smith (2006) using transformation assays. It was demonstrated that transformation of *un-24*^{OR} into a PA haplotype strain caused severe incompatibility characterized by the complete absence of growth by transformants, whereas the transformation of *un-24*^{PA} into an OR haplotype strain produced viable transformants most of which are phenotypically SI. These observations are consistent with the results obtained from the heterokaryon tests performed in this study using the *het-6* deletion strains. Asymmetric interactions are common in HI systems and were also observed in the *het-c/pin-c* nonself recognition loci in *N. crassa* which shares many similarities to the *un-24 het-6* nonself recognition complex (Kaneke et al., 2006). Both systems involve two tightly-linked genes, one of which contains a HET domain. Incompatibility is associated with heterocomplex formation between products of the non-HET domain genes. Both systems involve nonallelic interactions between alleles of the two linked genes. Moreover, PA-specific *het* genes seem to elicit stronger incompatibility than their OR-specific counterparts in heterokaryons that are also incompatible due to interactions associated with allelic forms of the non-HET gene. The observed asymmetry within these incompatibility loci may reflect the ability of the PA allele *het* gene products to form more stable heterocomplexes or elicit a more pronounced PCD response than the products encoded by the OR *het* genes. Another, less plausible,

explanation for the observed asymmetry in HI systems is based on the background of tester strains used for HI studies. The PA haplotype strains used in this study (and also in the *het-c pin-c* studies) have PA alleles of a single *het* locus in an otherwise completely OR strain. The asymmetry observed in these incompatibility systems may be attributed to lack of, or improper regulation of PA-specific *het* loci in OR background strains. The *het-c* and *het-6* systems do differ in the degree of incompatibility they elicit through allelic interactions. Allelic differences at *het-c* and at *pin-c* do not elicit HI. However, allelic differences at *het-c* are found to contribute to HI in heterokaryons incompatible due to nonallelic differences between *het-c* and *pin-c*. As will be discussed further, this is not the case for allelic interactions at *un-24* where no phenotypic differences are observed between SI and escape from self-incompatibility in *un-24^{OR} het-6^{OR}::un-24^{PA}* partial diploids and the *un-24^{PA} het-6^{OR}* strains constructed in this study. However, het tests have demonstrated weak allelic interactions that are relatively easily overcome when *un-24^{OR} Δhet-6* and *un-24^{PA} Δhet-6* haplotype strains are forced into a heterokaryon. Interestingly, heterokaryon formation between otherwise compatible *C. parasitica* strains co-expressing OR and PA forms of the *N. crassa un-24* alleles leads to abnormal and sectoring growth (M.L. Smith, unpublished). These results validate that interactions between *un-24^{OR}* and *un-24^{PA}* result in abnormal growth when co-expressed in a heterokaryon. Since RNR is an essential gene, the existence of an allelic interaction between *het-6^{OR}* and *het-6^{PA}* is very difficult to assess without the inclusion of *un-24*. It may be possible to obtain a replacement of *un-24*, using the method described in this study, to replace the native *un-24^{ts}* coding sequence with the coding region of a RNR1 gene from a related organism such as *C. parasitica*, which does not elicit incompatibility.

Such experiments are ongoing and may help determine if any allelic incompatibility is associated with heteroallelism at *het-6*.

Escape from *un-24*^{PA} *het-6*^{OR} nonallelic incompatibility. Escape from incompatibility has been examined in heterokaryons (Pittenger, 1964), translocation partial diploids (Smith et al., 1996) and in incompatible transformants (Micali & Smith, 2006). In SI transformants and partial diploids, escape has been associated with inactivation of the ectopic or endogenous duplicated allele through mutational events that range from large deletions (Smith et al., 1996) to point mutations (Saupe et al., 1996; Coustou et al., 1999). However, these and other mutational events are very stochastic in occurrence whereas the occurrence of Type I escape observed in this study is extremely predictable in timing and outcome. An argument could be made that transposons are involved in Type I escape. Some transposable elements are known to be activated upon various stresses in fungi such as in *Aspergillus nidulans* (Ogasawara et al., 2009) and *Ophiostoma ulmi* (Bouvet et al., 2008) so that timed and directed insertion of a mobile element as a mechanism for escape is not a far stretch. Micali and Smith (2006) had reported the presence of a repetitive sequence within the *het-6* locus of the PA haplotype, which may indicate the remnants of such a mobile element. Given that Type I escape strains behave somewhat like Δ *het-6* strains (but not identical, as discussed below) may indicate that Type I escapes involves transposon-mediated insertional mutagenesis of *het-6*. The existence of such a mutation-generating system may even provide an explanation for the repetitive nature and diversification of HET-domain nonself recognition genes in *N. crassa*. Sequencing of the *het-6* genes from several Type I escapes is underway to test this

hypothesis. One may also propose that the HET-6^{OR*} protein behaves as a prion.

However, the non-infectious nature of the putative modified HET-6^{OR*} is not consistent with the idea that escape is due to *het-6*^{OR} having prion-like properties.

Documentation (Micali & Smith, 2006) of escape from incompatibility associated with ectopic introduction of *un-24*^{PA} into an OR haplotype strain (to obtain a *un-24*^{OR} *het-6*^{OR::un-24}^{PA} SI strain) is strikingly similar to SI and Type I escape observed in this study. This *un-24*-specific incompatibility can be reproduced by transformations using a vector containing only the C-terminal portion of *un-24*^{PA} gene. These findings parallel the results in this study where the DLL-T2f strain is OR-compatible and possesses a chimeric *un-24* gene that is completely PA with the exception of ~200 bp of OR sequence at the 3' end. Self-incompatibility caused by co-expression of different *un-24* alleles is associated with UN-24 aggregation, which is thought to occur through a non-reduceable cross-linkage mediated by cysteines in the C-terminus of UN-24^{PA} (Wellman et al., unpublished; Reviewed in Smith, 2009). Escape from self-incompatibility is correlated with the disappearance of the UN-24 aggregate. The transcript levels of *un-24*^{PA} and *un-24*^{OR} have been shown to remain relatively constant before and after escape by *un-24*^{OR} *het-6*^{OR::un-24}^{PA} strains (M. Siahbazi, 2008), ruling out differential expression of the alleles or deletion of one of the two alleles as an escape mechanism. Furthermore, escape from *un-24*^{OR} *het-6*^{OR::un-24}^{PA} incompatibility was observed when *qde-2* (quelling defective) and *dim-2* (deficient in DNA methylation) spheroplasts are transformed with *un-24*^{PA}. This would appear to rule out the involvement of gene silencing mechanisms as a means of escape. Self-incompatibility may also be overcome by mutations in suppressor loci such as *vib-1* (Xiang & Glass, 2002; Xiang & Glass, 2004; Dementhon et

al., 2006). Sequencing of the *vib-1* gene from a Type I escape strain revealed no observable mutations within the *vib-1* ORF or within 1 kb of the upstream non-coding region. Moreover, quantitative real-time RT-PCR (qPCR) has shown that *vib-1* transcript levels are not reduced after escape from *un-24^{OR} het-6^{OR}::un-24^{PA}* SI (Appendix 1). Taken together, there are striking phenotypic similarities between the escape process and SIs generated when comparing *un-24^{PA} het-6^{OR}* SI and *un-24^{OR} het-6^{OR}::un-24^{PA}* SI. Since the same interaction (*un-24^{PA} het-6^{OR}*) presumably mediates both SI and escape types, we can therefore assume that the mechanisms of escape in both systems are extremely similar and both can unequivocally be used to infer SI and escape associated with nonallelic interactions between *un-24^{PA}* and *het-6^{OR}*.

Progeny from a cross involving Type I escapes do not yield SI progeny, meaning that the mechanism of Type I escape is a heritable trait that cosegregates with the *het-6* locus. Since *un-24* is a gene required for DNA replication and cell proliferation in *N. crassa*, expression of *un-24^{PA}* is essential and any modification or differential regulation of this gene would have serious phenotypic effects. The *het-6* gene does not seem to have any function other than during incompatibility and is a likely target for regulation and/or modification that could potentially lead to escape involving non-allelic interaction between *un-24^{PA}* and *het-6^{OR}*. Heterokaryon tests demonstrate that Type I escape strains have completely lost *het-6* incompatibility function. The Type I modification also seems to suppress *un-24* allelic incompatibility. These results demonstrate that Type I escape strains have a subtle difference in incompatibility activity from *un-24^{PA} Δhet-6* strains, suggesting that escape occurs through mechanisms more complex than simply the down-regulation or silencing of *het-6^{OR}*. Loss of *het-6^{OR}* incompatibility function may also

occur indirectly through the regulation of a suppressor of HI such as *vib-1*, a putative transcription factor responsible for the expression of *het* genes (Dementhon et al., 2006) which may also have means to regulate *un-24* allelic interactions. This study has demonstrated that the *eib-1* mutation is able to prevent Type I escape from *un-24^{PA} het-6^{OR}* SI. As we will discuss below, *eib-1* appears to be associated with a mutation in *vib-1* which indicates that escape may occur through regulation of HI via control by the putative VIB-1 transcription factor.

Characteristics of *eib-1* and similarities with *vib-1*. The *eib-1* mutation was initially found to partially suppress HI associated with nonallelic interactions between *un-24^{PA}* and *het-6^{OR}*. The presence of SI progeny in crosses involving the Type II escape strain suggests that the *eib-1* mutation prevents Type I escape from *un-24^{PA} het-6^{OR}* incompatibility. This result suggests that the *eib-1* gene plays a key role in Type I escape. Strains bearing the *eib-1* mutation were crossed to a Type I escape *un-24^{PA} het-6^{OR*}* strain and produced *un-24^{PA} het-6^{OR*}* progeny that were brown and aconidiate at 39°C (cross 10, Table 3) and inferred to be *un-24^{PA} het-6^{OR*}; eib-1*. Unlike Type II escape strains, these *un-24^{PA} het-6^{OR*}; eib-1* progeny are unable to generate SI progeny that undergo Type I escape. These results suggest that the *eib-1* mutation is required for Type I escape to occur, but cannot completely reverse the Type I modification once it is in place.

The *eib-1* gene shares many differences and similarities with *vib-1* which are summarized in Table 5. It was demonstrated in this thesis that mutations in *vib-1* partially suppresses HI associated with differences at the *het-6* locus (i.e. in heterokaryons bearing

Table 5: Summary of similarities and differences between *vib-1* and *eib-1*

Characteristic	<i>vib-1</i>	<i>eib-1</i>
Suppression of <i>un-24</i> allelic HI interaction	Yes	No
Suppression of Type I escape from <i>un-24</i> ^{PA} <i>het-6</i> ^{OR} HI interaction	Yes, Temperature dependent aberrant morphology	Yes, Temperature dependent aberrant morphology
Partial suppression of HI in a <i>un-24</i> ^{OR} Δ <i>het-6</i> + PA haplotype heterokaryon	Yes	No
Control of Type I <i>un-24</i> ^{PA} <i>het-6</i> ^{OR} escape	nt	Yes
Mating abnormalities	No	Yes

OR and PA haplotypes at *het-6*) . Although the *eib-1* mutation has not yet been tested for its ability to suppress HI associated with heterokaryon formation between OR and PA haplotype strains, this mutation does not partially suppress incompatibility associated with heterokaryon formation between *un-24*^{OR} Δ *het-6* and PA haplotype strains, which distinguishes it from *vib-1*. This study has also demonstrated that the presence of *vib-1* abolishes the weak incompatibility associated with interactions between *un-24* allelic forms, which is another difference between the two mutations. Moreover, mating abnormalities as seen when both strains in the mating pair possess the *eib-1*, have not been observed or reported with mutations in *vib-1*. Nonetheless, the single point mutation in *vib-1* associated with the *eib-1* mutation and the fact that the Type II phenotype maps within a 300 kb region that also contains *vib-1* is consistent with the idea that *eib-1* is a novel mutation in the *vib-1* gene. This G→A transition causes an amino acid change at position 188 in the predicted VIB-1 protein and is located within the predicted DNA binding domain (Figures 11 and 12). This mutation is found within a portion of the DNA binding domain that is not relatively well conserved with NDT80, but would correspond to a Glycine to Aspartic acid substitution positioned within one of seven Ig-fold subdomains (Lamoureux et al., 2002), which make up the core structure of the NDT80 protein. This single amino acid substitution has the potential compromise structural integrity of VIB-1. Another piece of evidence suggesting that *eib-1* is in fact a mutation in *vib-1*, is that *un-24*^{PA} *het-6*^{OR}; *vib-1* progeny resulting from a *un-24*^{PA} *het-6*^{OR}; *eib-1* X *un-24*^{IS} *het-6*^{OR}; *vib-1* cross, display a temperature dependent phenotype identical to Type II *un-24*^{PA} *het-6*^{OR}; *eib-1* escape strains. Het tests using the *het-6* deletion strains produced in this study were not examined when both strains contain mutations in *vib-1*.

However, if the amino acid substitution observed in the predicted VIB-1 protein associated with the *eib-1* mutation results in loss or altered VIB-1 function, then the proposed synergy or genetic interaction between *eib-1* and *vib-1* during the suppression of *het-6*^{OR}, in fact indicates that the absence of a functional *vib-1* in both nuclear types completely suppresses *het-6*^{OR}, but not *het-6*^{PA} incompatibility function. Additional *het* tests using *het-6* deletion strains possessing *vib-1* mutations, along with tests to see whether or not reversion occurs when *eib-1* is replaced with *vib-1*⁺ are in progress with an aim to clarify the identity of *eib-1*. Taken together, although *eib-1* has qualities that distinguish it from *vib-1*, the *eib-1* mutation may well be a novel mutation in the *vib-1* gene.

Suppression of *un-24 het-6* incompatibility by *vib-1*. Mutations in *vib-1* have previously been shown to suppress incompatibility due to differences at the *het-6* (M. Begin, unpublished), the *het-c* (Xiang & Glass, 2002) and the *mat* locus (Xiang & Glass, 2004). *vib-1* also increases the likelihood of escape of *het-8*^{OR}/*het-8*^{PA} partial diploid progeny (Xiang & Glass, 2004), which parallels the findings observed in this study. The *vib-1* gene is thought to encode a putative transcription factor that is involved in the expression of the *het* genes *het-6*, *pin-c* and *tol* (Dementhon et al., 2006). VIB-1 possesses a predicted DNA binding domain similar to the domain of NDT80, a transcription factor involved in regulating meiosis in *Saccharomyces cerevisiae* (Xu et al., 1995). RT-qPCR analysis has revealed that *vib-1* is required for expression of *het* genes such as *het-6*, *tol* and *het-c*, and a predicted 12 bp target-DNA consensus sequence has been identified within the upstream regions in each of these genes (Dementhon et al.,

2006). These observations suggest that VIB-1 participates in HI by actively binding to the upstream elements of *het* gene DNA to regulate their transcription. The dominance of *vib-1* mutations during HI at the *het-6* locus is therefore puzzling since the wildtype form of VIB-1 should complement the mutant in heterokaryons bearing both forms of *vib-1*. The *vib-1^{m32}* mutant encodes a predicted truncated *vib-1* allele and displays greater suppression of HI than the complete deletion. This observation is interesting and suggests that the *vib-1^{m32}* mutant allele does not result in complete loss of function, and that its presence in an incompatible heterokaryon may somehow influence increased suppression of HI. If both mutants result in loss of *vib-1* function, they should theoretically display the same degree of suppression, which is not the case. This *vib-1^{m32}* mutant allele possesses approximately 60% of the predicted DNA-Binding domain, which may indicate that this mutation does not result in total loss of *vib-1* function and may somehow further inhibit *het* gene expression. For both *vib-1* alleles, suppression of HI is greatest when *vib-1* is present in the OR-background strain. This suggests that *vib-1* is perhaps a *cis*-acting element. This asymmetry in suppression of incompatibility is extremely evident in heterokaryon tests using complete deletions of *vib-1* (Figure 14). Incompatible heterokaryons possessing the *vib-1* mutation are also very likely to undergo escape from HI, and this also occurs in a very asymmetric fashion. To date the nature of these observed asymmetries due to *vib-1* suppression remain unclear, but may involve the previously mentioned differential regulation of *het-6* allelic forms by VIB-1.

As previously discussed, *eib-1* maps to a region that also includes *vib-1*. A point mutation was discovered in *vib-1* of Type II escape strains. The point mutation causes a single amino acid substitution in the NDT80 binding domain of the predicted VIB-1

protein. Assuming that this mutation disrupts VIB-1 function, the synergy observed between *vib-1* and *eib-1* during the suppression of *het-6*^{OR} but not *het-6*^{PA} HI is rather due to the absence of a functional *vib-1* gene. These inferred results would suggest that *vib-1* is required for *het-6*^{OR} but not *het-6*^{PA} incompatibility function. Suppression of *un-24* allelic interactions by *vib-1* is not explained by control of *het-6* and may occur through other means, nevertheless mediated by *vib-1*. Taken together, further identification of *eib-1* and the construction of *het-6* deletion strains bearing *vib-1* will reinforce the results obtained in this study and help elucidate genetic mechanisms by which VIB-1 controls *het-6*.

SUMMARY AND CONCLUSIONS

A series of models explaining the major findings in this paper were constructed and are found in Figure 19. The construction of *het-6* deletion strains bearing *un-24*^{OR} as well as *un-24*^{PA} has enabled us to make inferences about the incompatibility function of *het-6*^{OR} and *het-6*^{PA} when expressed in heterokaryons also incompatible with allelic differences at *un-24* (Figure 19a). This study has demonstrated allelic interactions at *un-24* that are quickly overcome by escape suggesting that this is a weak incompatibility reaction that is relatively easily conquered. The use of *het-6* deletion strains also indicate asymmetric incompatibility associated with *het-6*, where *het-6*^{PA} elicits a stronger incompatibility reaction than *het-6*^{OR} in strains that are also incompatible due to allelic differences at *un-24*. Nonallelic interaction between *un-24*^{PA} and *het-6*^{OR} is initially characterized by a SI phenotype after which strains escape from incompatibility in a very predictable manner (Figure 19b). The *eib-1* mutation (inferred to be *vib-1*) partially suppresses nonallelic interactions associated with *un-24*^{PA} *het-6*^{OR} SI and also prevents Type I escape. Furthermore, escape of self-incompatible *un-24*^{PA/OR} *het-6*^{OR} partial diploids has not demonstrated to be associated with a decrease in *vib-1* transcript level (Lafontaine, unpublished), suggesting that Type I escape may occur through altered regulation of *het* genes including of *het-6*^{OR} through translational or post-translational modification in *vib-1* mRNA, or VIB-1 protein levels, respectively. Suppression of incompatibility associated with the *het-6* locus by *vib-1* has demonstrated asymmetries in the degree of suppressed incompatibility and frequency of escape depending on the nucleus bearing the *vib-1* mutation. As mentioned previously, the nature of the observed asymmetries of *vib-1* suppression remain unclear but may involve differential regulation

Figure 19: A model for *un-24 het-6* HI and suppression by *vib-1*. **a)** This thesis has demonstrated non-allelic incompatible interaction between *un-24*^{PA} and *het-6*^{OR}. In addition, allelic incompatibility of *un-24* was documented. A weak incompatibility is associated with *un-24* heteroallelism that is greatly accentuated with the expression of *het-6*. Moreover, *het-6*^{PA} elicits a stronger incompatibility (thick diagonal line) reaction than *het-6*^{OR} (thin diagonal line) in heterokaryons also incompatible due to allelic differences at *un-24*. **b)** Escape to a Type I state is extremely predictable and associated with loss of *het-6*^{OR} incompatibility function. We infer from this that the Type I escape process involves a modification of *het-6*^{OR} or HET-6^{OR}, rather than mutation, which would be much more stochastic in occurrence. Since there are indications that the predicted glycine to aspartic acid amino acid change observed in the *vib-1* gene of Type II strains is responsible for the Type II *un-24*^{PA} *het-6*^{OR} escape phenotype, we assume here that the *eib-1* marker is due to a mutation in *vib-1* and plays a key role in Type I escape. **c)** The synergy between *vib-1* and *eib-1* during suppression of *het-6*^{OR} may suggest that expression of *het-6*^{OR} is completely abolished when both nuclei bear mutations in the putative transcription factor *vib-1*. **d)** However, *het-6*^{PA} incompatibility is only partially suppressed in an incompatible heterokaryon bearing *vib-1* mutations in both nuclei, suggesting that *vib-1* only partially controls the expression of *het-6*^{PA}.

of the *het-6* alleles by the putative VIB-1 transcription factor. It was demonstrated that *vib-1*⁺/*vib-1*^{m32} heteroallelism was sufficient to abolish incompatibility associated with allelic differences at *un-24*, but could only partially suppress incompatibility associated with either allele of *het-6*. A heterokaryon bearing *vib-1* mutations in both nuclear type (*vib-1*^{m32}/*eib-1*) completely abolishes HI associated with *het-6*^{OR} but not *het-6*^{PA}. Although mechanisms by which *vib-1* suppresses *un-24* allelic incompatibility are yet unknown, the findings reported in this study suggest that the predicted VIB-1 transcription factor is required for incompatibility associated with *het-6*^{OR} (Figure 19c) but not *het-6*^{PA} incompatibility (Figure 19d), which indicates differential regulation of the *het-6* alleles by VIB-1.

This study has demonstrated allelic and nonallelic interactions that occur within the *het-6* nonself gene complex and has also identified possible differential regulation of this gene complex by the putative *vib-1* transcription factor. The models developed in this study would explain the partial suppression of HI associated with the *het-6* locus by *vib-1* and may also help explain the asymmetrical nature of nonallelic interactions between *un-24* and *het-6*. This thesis proposes that *vib-1* is a mediator of nonself recognition and also has a pro-survival role in mediating escape from incompatibility resulting from nonallelic interactions between *un-24*^{PA} and *het-6*^{OR}.

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APPENDIX 1

Summary of *vib-1* transcript quantitation from cDNA libraries of *un-24^{OR} het-6^{OR}::un-24^{PA}* partial diploids before and after escape from SI. The RT-qPCR reactions were run on a Roto-gene RG-3000 thermocycler and amplification was monitored using SyBR green (BioRad Cat# 170-8880). Values are given as Critical threshold values (Ct), are normalized against *un-24^{OR}* transcript levels and are also given without normalization. Primers were designed to span the junction site of two exons found in each gene and effectively minimized the amplification of contaminating genomic DNA.

RT-qPCR of *vib-1* mRNA expression

Query	Transformants Assayed	Normalized against	AVG pre/post (Ct)	AVG pre-post (Ct)	Paired t-test
<i>vib-1</i>	5	<i>un-24</i>	1.28 +/- 0.20	6.45 +/- 4.50	0.0004224
	13	none	1.09 +/- 0.1	2.34 +/- 2.69	0.0009933

Primer sets used for PCR

Primer set	Primer	Sequence	Tm* (°C)	Target mRNA	Amplicon size
<i>vib-1</i>	vib-1splice	5' TTGTGGGGTAAGAATTCGAACGCA	71.7	<i>vib-1</i>	242bp
	vib-1FWD	5' CCGCGGAATTTCCAAGCGAGAAA	74.9		
<i>un-24^{OR}</i>	6JP-OR2	5' CGGGATTCCAGGCGCAAGCAG	72.3	<i>un-24^{OR}</i>	294bp
	un-24FWDB	5' ACGGATCCTCCCACTGAGGTTCC	76.6		