Molecular and Functional Analyses of Incompatibility Genes at het-6 in a Population of Neurospora crassa

N. Mir-Rashed,* D. J. Jacobson,† M. R. Dehghany,* O. C. Micali,* and M. L. Smith*

*Biology Department, Carleton University, Ottawa, Ontario K1S 5B6, Canada; and †Department of Biology, Stanford University, Stanford, California 94305

Accepted for publication July 10, 2000

Mir-Rashed, N., Jacobson, D. J., Dehghany, M. R., Micali, O. C., and Smith, M. L. 2000. Molecular and functional analyses of incompatibility genes at het-6 in a population of Neurospora crassa. Fungal Genetics and Biology 30, 197–205. Two closely linked genes, un-24 and het-6, associated with the het-6 heterokaryon incompatibility functional haplotype were examined in 40 Neurospora crassa strains from a Louisiana sugarcane field. Partial diploid analyses were used to determine that half of the strains were functionally Oak Ridge (OR) and half were non-OR and indistinguishable from the standard Panama (PA) form. PCR-based markers were developed to identify polymorphisms within both un-24 and het-6. Two common forms of each gene occur based on these molecular markers. Rare forms of both un-24 and het-6 were identified as variants of the non-OR form by a DNA transformation assay. The heterokaryon incompatibility function of haplotypes, based on partial diploid analyses, was perfectly correlated with the PCR-based markers at both loci. This correlation indicates that the two loci are in severe linkage disequilibrium in this population sample and may act as an incompatibility gene complex. Southern hybridizations using OR- and PA-derived cloned probes from the region that spans un-24 and het-6 showed that the absence of recombination in this ∼25-kbp region is associated with low levels of overall sequence identity between the PA and OR forms.

Index Descriptors: fungi; non-self-recognition; recombination; disequilibrium; gene complex.

Non-self-recognition in filamentous fungi occurs during both the asexual and sexual stages of the life cycle (reviews in: Glass and Kuldau, 1992; Leslie, 1993; Bégueret et al., 1994; Coppin et al., 1997; Worrall, 1997). One well-studied form of non-self-recognition is the heterokaryon incompatibility system in Neurospora crassa. This system is controlled by at least 10 heterokaryon incompatibility (het) loci and the mating-type locus (mat) (Perkins, 1988). A difference at any one of these loci prevents the stable growth of heterokaryotic hyphae during the vegetative phase of the life cycle but does not interfere with the limited heterokaryosis associated with sexual reproduction. Het gene differences in N. crassa restrict transmission of cytoplasmic elements between individuals (Debets et al., 1994) and prevent parasitism of unfertilized strains by nuclei of the same mating type (Debets and Griffiths, 1998). These potentially important functions would suggest that allelic variation at het loci would be maintained within populations by balancing selection (review in Klein et al., 1998; Hartl et al., 1975; Nauta and Hoekstra, 1994). Recently, the occurrence of balancing selection at the het-c locus in N. crassa was supported by observations that allelic forms of het-c are maintained across genera within the Sordariaceae, that relatively large numbers of nonsynonymous base substitutions occur within the het-c specificity region, and that approximately equal numbers of...
RFLPs, indicative of the three allelic forms of het-c, occurred in a field population of N. crassa from Louisiana (Wu et al., 1998). Based on the balancing selection model, we would expect (1) approximately equal frequency of allelic forms at other het loci and (2) linkage disequilibrium at independently functioning het loci in field populations.

In this study, we examined heterokaryon incompatibility at het-6 using a functional assay and molecular genetic markers within a population sample of N. crassa from Louisiana. Mlyk (1975) identified het-6 on the left arm of linkage group II (LG II; Fig. 1) by partial diploid analyses using the translocation strains T(II → VI)P2869 and T(II → III)AR18. The het-6 heterokaryon incompatibility phenotype is associated with at least two closely linked genes within the 270-kbp translocated region of LG II (Smith et al., 2000b). The two genes have been named un-24 and het-6 and together form the haplotype we will refer to as the het-6 region. Both genes possess at least two allelic variants, Oak Ridge (OR) and Panama (PA), named for the wild-type strain of origin. Superscripts of these symbols are used to denote wild-type alleles at a locus. The Oak Ridge haplotype will be referred to as OR and the Panama haplotype as PA. Un-24 encodes the large subunit of ribonucleotide reductase (Smith et al., 2000a), while het-6 OR putatively encodes a protein of 680 residues of unique sequence (Smith et al., 2000b). DNA transformations with the OR allele of either gene (het-6 OR or un-24 OR) into PA, but not OR, spheroplasts results in a loss of viability of transformants (Smith et al., 2000b).

Our objectives in this study were threefold. The first was to develop molecular markers to easily discriminate alleles at un-24 and het-6. The second was to examine the relationship between these molecular markers and incompatibility function. The third was to estimate the number and frequencies of alleles at un-24 and het-6 and to determine whether combinations of alleles at the two loci provide additional heterokaryon incompatibility specificity.

**METHODS AND MATERIALS**

**Strains, Media, and Culture Conditions**

A sample of 54 Neurospora isolates was collected as masses of conidia from separate distinct colonies on burned sugarcane stubble in a 5-ha field near Franklin, Louisiana. Each isolate was cultured, purified, and identified to species using standard methods (Perkins and Turner, 1988) and has been deposited in the Perkins collections (Nos. P4448–P4501), which is curated at the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, KS). Forty of the 54 isolates were identified as N. crassa (Table 1). The het-c locus was determined for 36 of these strains previously as Oak Ridge, Panama, or Groveland, by correlation of RFLP patterns to known reference strains (Wu et al., 1998). The het-c locus is ~12 MU centromere proximal to het-6 on Linkage Group II (Fig. 1). Other strains used in this study were het-6 OR standard strains, RLM 58-18 and C2(2)-1 (Smith et al., 1996), het-6 OR standard strains, 74-OR23-1VA (FGSC 2489) and C9-2 (Saupe et al., 1996), the RFLP mapping strains (FGSC Nos. 2225, 4450–4488), and translocation strains T(II → VI)P2869 (FGSC 1828A and 1829a) and T(II → III)AR18 (FGSC 2643A and 2644a). N. crassa strains were cultured with either solid or liquid Vogel’s medium and crosses were performed using Synthetic Cross Medium and standard methods (Davis and de Serres, 1970; Perkins, 1986). For partial diploid analyses, a wild strain and the translocated strain of opposite mating type were co-inoculated to a 13 × 100 mm tube containing 2.5 ml Synthetic Cross Medium. Crosses were incubated at 25°C for at least 4 weeks to allow ascospores to shoot and mature. For each cross, 100 ascospores were plated in an equally spaced grid pattern on a single 9-cm-diameter petri dish containing minimal medium with sorbose (1%), fructose (0.05%), and glucose (0.05%) substituted for sucrose to restrict colony size and supplemented with tyrosine (0.5 g/L) and phenylalanine (0.2 g/L) to encourage inhibited partial diploid progeny to produce brown pigment. The entire plate was placed at 60°C for 40–45 min, incubated for 2 days at 34°C, and examined under a dissecting microscope (magnification up to ×60) to assess germination, growth, and colony morphology. Wild-type versus inhibited morphology was confirmed after an additional 1–4 days incubation at room temperature (22–24°C). Only inhibited colonies developed brown pigment, usually after 3 to 4 days growth on this medium.

**DNA Isolation**

Mycelia grown in liquid medium were harvested by vacuum filtration, washed two times with 0.9% NaCl, dried under vacuum overnight, and pulverized to a fine powder using a glass rod. Neurospora DNA extractions of powdered mycelium were by the method of Oakley et al. (1987). Relative concentrations of genomic DNAs were
determined by visual approximation after agarose gel electrophoresis and staining with ethidium bromide over a long-wavelength ultraviolet light source.

**DNA Transformations**

Transformation of spheroplasts derived from macroconidia was by the method of Royer and Yamashiro (1992). The strains C9-2 and C2(2)-1 were used in transformation experiments as het-6<sup>OR</sup> and het-6<sup>PA</sup> functional haplotype standards, respectively. Approximately 1 μg of DNA of various constructs of the hygromycin-resistance vector pCB1004 (Carroll et al., 1994) was mixed with 100 μl of a spheroplast suspension (about 8.0 × 10<sup>7</sup> spheroplasts/mL). Transformants were selected on medium with a final concentration of 250 U/mL.

**RFLP Analysis**

Genomic DNA was digested separately with EcoRI and HindIII (Gibco/BRL, Burlington, Ontario), fractionated by electrophoresis in 0.7% agarose with a 1 kbp standard, and transferred to nylon membranes (Hybond N, Amersham, UK) by the method of Southern (1975). DNA probes were labeled with [α-<sup>32</sup>P]dCTP by nick translation (Bethesda Research Laboratories, Gaithersbourg, MD) or by the random primer method using T7 Quick Prime (Pharmacia, Baie d’Urfe, Quebec). Prehybridization and hybridization conditions were according to the membrane manufacturer’s recommended procedure. Autoradiography was performed with Kodak Bio-Max-1 film at −80°C for 1 to 4 days. Probe DNAs included p8AS-1, pEC016M, and pA3 (Fig. 2). p8AS-1 has a ∼2.4-kbp Apal/Smal insert that contains het-6<sup>OR</sup> (Smith et al., 2000b). pEC016M has a ∼4.8-kbp EcoRI/Mbol insert that contains un-24<sup>OR</sup> (Smith et al., 2000a). pA3 was prepared for this study by cloning a ∼11-kbp HindIII fragment from strain RLM58-18 (het-6<sup>PA</sup> standard strain) into the vector pUC118 (Sambrook et al., 1989). pA3 contains all of un-24<sup>PA</sup> and about 1.7 kbp of the 5′ end (∼84%) of the het-6<sup>PA</sup> coding region.

**Polymerase Chain Reaction (PCR) Amplification**

Oligonucleotides used in PCR reactions were synthesized (Molecular Genetics Laboratories at Carleton University, Biology Department) based originally on the DNA sequence of Oak Ridge alleles at het-6 (GenBank Accession No. AF206700) or un-24 (GenBank Accession No. AF171697). The het-6<sup>PA</sup> sequence was subsequently determined (GenBank Accession No. AF208542; Smith et al., 2000b). In addition, the sequence of 72.8 kbp of OR background DNA that includes un-24 and het-6 was recently made available at MIPS (contig b2a19, Munich Information Center for Protein Sequences, N. crassa genome project, http://www.mips.biochem.mpg.de/desc/neurospora/). Combinations of primer pairs and restriction enzymes were tested with standard OR (74-OR23-1V) and PA (RLM58-18) strains to identify PCR-based markers at both loci. For allele typing at het-6, primers 6VP3 (5′-CGGTATACTGTTCAGCT-3′) and 6VP5 (5′-CCCCGT-AAGCAGAGGTCC-3′) were used (Fig. 2). For un-24, primers 6JP6 (5′-GTCGGGGCTTTAACGCGT-3′) and 6JP11 (5′-CTCCGGTAAAGGTCTGCC-3′) were used.

For het-6, PCR was carried out in a final reaction volume of 50 μl and included about 100 ng N. crassa genomic DNA, 200 μM each dNTP, 10 μM each of primers, 1.5 mM MgCl₂, 1.25 units Taq DNA polymerase, and 1× Taq buffer (Gibco BRL, Burlington, ON). The PCR program was 30 cycles of: 15 s at 95°C (denaturation), 30 s at 48°C (annealing), and 2 min/1 kbp DNA at 72°C (extension). Amplified products were analyzed by gel electrophoresis in 1.5% agarose, 1× TAE (0.04 M Tris–borate, 1 mM EDTA) or 0.04 M Tris–acetate, 1 mM EDTA, with and without digestion with Mbol restriction enzyme.

For un-24, the PCR reaction volume was 50 μl and the conditions were 300 nM each primer, 200 μM each dNTP, 2.27 units Expand DNA polymerase mix (Boehringer Mannheim, Laval, QC), 1× Expand reaction buffer, 1.5 mM MgCl₂, and between 60 and 200 ng of N. crassa genomic DNA. The same cycle parameters were used for un-24 as described for het-6 except the annealing temperature was changed from 48°C to 58°C. About 2 μg of each un-24 PCR product was digested with 20 units of Spl restriction endonuclease and subjected to electrophoresis in 0.75 × 20 × 20 cm, 10% nondenaturing polyacrylamide gels containing 10% glycerol, 0.5% agarose, and 1× TBE (0.095 M Tris–borate, 2 mM EDTA, pH 8.0). Electrophoresis conditions were 200 V, 15 mA for 10 min, then 180 V, 14 mA for 1 h, followed by 140 V, 10 mA for 2 days, all at 4°C while recirculating the buffer. After electrophoresis the gels were stained with ethidium bromide and photographed.

**RESULTS**

**Partial Diploid Analyses**

The translocation strains, hereafter referred to as T(AR18) and T(P2869), are known to produce partial dip-
loids for the het-6-region (Mylyk, 1975). The translocated segment in T(P2869) has a right breakpoint that is within 5.6 kbp of the right breakpoint of T(AR18) (Smith and Glass, 1996) but extends distally beyond the left breakpoint of T(AR18) toward the left telomere region of LG II (Fig. 1; Perkins et al., 1982). Both T(P2869) and T(AR18) are OR for the het-6 region (Mylyk, 1975). One third of the viable progeny from a cross involving either translocation-bearing strain and a normal sequence strain will have the LG II region of the translocation duplicated. If the parent wild-type and translocation strains differ in the het-6 region, progeny carrying this duplication will be self-incompatible, i.e., inhibited growth accompanied by a brown discoloration (Mylyk, 1975; Perkins, 1975).

In the first round of partial diploid analyses the 40 Louisiana strains were crossed to T(P2869) and progeny were examined for self-incompatibility. Twenty Louisiana wild-type strains were observed to produce 20–42% inhibited progeny. It was tentatively concluded from this that alleles in the het-6 region of each of these wild-type strains were functionally different from the OR alleles in T(P2869). However, since T(P2869) duplicates a large segment of LG IIL, it is possible that inhibited progeny may be caused by heterozygosity not only in the het-6 region, but also at any as yet undescribed het loci centromere distal to the het-6 region. To confirm that the het-6 region was responsible for the observed inhibited progeny, the 20 non-Oak Ridge isolates were crossed with the insertional translocation T(AR18) which duplicates a much smaller LG IIL segment around the het-6 region. All partial diploids involving T(AR18) (DpAR18) initially have an inhibited, colonial phenotype even when homozygous at het-6. However, DpAR18 that are heterozygous at the het-6 region are more inhibited and produce brown pigment as they age. This difference could not be identified on the sorbose plate. Therefore, each inhibited colony was transferred from the sorbose plate to a tube of complete medium and incubated at 25°C. Classifying the inhibited phenotype required that DpAR18 cultures be observed daily, for up to 7 days, and compared to DpAR18 cultures of both homozygous and heterozygous het-6 haplotypes obtained from crosses with OR and PA standard strains. For all 20 strains, inhibited duplication progeny were produced in crosses to both T(P2869) and T(AR18), confirming that no loci centromere distal to T(AR18) were responsible for the inhibited phenotype. From this it was inferred that 20 strains carried the OR haplotype and the remaining 20 strains carried a non-OR haplotype in the het-6 region (Table 1, where strains designated as functionally non-OR are indistinguishable from the PA standard strain based on this partial diploid assay).

**RFLPs in the un-24-het-6 Region**

Genomic DNAs of 30 N. crassa samples from Louisiana and the OR and PA standard strains were digested with HindIII. HindIII does not cut within the het-6.OR gene. The digested DNAs were subjected to electrophoresis, Southern blotted, and then hybridized with a radiolabeled insert from p8AS-1, containing the entire het-6.OR open reading frame (Smith et al., 2000b; Fig. 2). Fragments that

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Strain and mat&lt;sup&gt;a&lt;/sup&gt;</th>
<th>het-6 function&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PCR-RFLP&lt;sup&gt;c&lt;/sup&gt;</th>
<th>un-24</th>
<th>het-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Louisiana strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4448a, P4449a, P452a,</td>
<td>Non-OR</td>
<td>PA</td>
<td>OR</td>
<td>OR</td>
</tr>
<tr>
<td>P4468a, P4469a, P476a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P448a, P4486a, P449a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4490a, P4493a, P4494a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4498a, P4500a, P4501a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4450a, P4451a, P4453a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4454a, P4455a, P4456a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4459a, P4463a, P4464a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4465a, P4467a, P4470a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4483a, P4484a, P4487a,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4491a, P4496a, P4499a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4471a, P4472a, P4497a</td>
<td>Non-OR</td>
<td>D</td>
<td>PA</td>
<td>D</td>
</tr>
<tr>
<td>P4479a</td>
<td>Non-OR</td>
<td>D</td>
<td>PA</td>
<td>D</td>
</tr>
<tr>
<td>Standard strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLM 58-18a</td>
<td>PA</td>
<td>PA</td>
<td>PA</td>
<td>PA</td>
</tr>
<tr>
<td>74-OR 23-1VA</td>
<td>OR</td>
<td>OR</td>
<td>OR</td>
<td>OR</td>
</tr>
</tbody>
</table>

<sup>a</sup> mat, mating-type locus.

<sup>b</sup> Based on partial diploid analysis, strains that are not OR at het-6 are designated “non-OR”; these are indistinguishable from functionally PA forms.

<sup>c</sup> PCR-RFLP designated as OR-like, PA-like, or “D” for different (see Figs. 5 and 6).
hybridized to the het-6<sup>OR</sup> probe were detected in 17 of these 30 isolates and in the het-6<sup>OR</sup> standard strain (Table 1). However, all 32 strains (including both standard strains) hybridized to the N. crassa pan-2<sup>1</sup> gene probe excised from pOKE103 (a gift from J. Grotelueschen and R. Metzenberg) (Fig. 3). The 13 Louisiana strains and the PA standard strain that did not hybridize to the het-6<sup>OR</sup> probe were functionally PA in partial diploid tests. All of the strains that hybridized to the het-6<sup>OR</sup> probe were functionally OR, and all but two had a 4.2-kbp fragment hybridizing to the het-6<sup>OR</sup> probe. The exceptions were two functionally OR strains (P4454 and P4456) that had a fragment of about 3.3 kbp hybridizing to the het-6<sup>OR</sup> DNA probe.

To examine RFLPs associated with un-24, DNAs from N. crassa strains were digested with EcoRI, blotted, and hybridized with the 4.8-kbp insert of pEco16M that includes un-24<sup>OR</sup> (Smith et al., 2000a). There are no EcoRI recognition sites within this 4.8-kbp insert. Hybridization patterns with pEco16M were examined in 28 Louisiana strains and the 2 standard strains. Each strain yielded a single hybridizing fragment, either of 9.7 kbp (3 non-OR strains and 17 functionally OR) or 11 kbp (9 non-OR strains, including the PA standard strain, RLM58-18, and the OR standard strain 74-OR23-1VA).

The construct pA3 contains a 11-kbp insert of PA-background DNA. By probing this construct with 6JP11/6JP6 and 6VP3/6VP5 PCR products from un-24<sup>PA</sup> and het-6<sup>PA</sup>, respectively, we determined that pA3 contains sequences from both these genes. RFLP mapping data and the MIPS sequence data were used to generate maps of the un-24-het-6 region for the OR and PA standard strains 74-OR23-1VA and RLM58-18, respectively (Fig. 2). From these maps it is evident that there are major structural differences between the two forms. The distance between un-24<sup>PA</sup> and het-6<sup>PA</sup> is ~19 kbp, while only ~5.2 kbp separate un-24<sup>PA</sup> and het-6<sup>PA</sup>. In addition, a low
level of sequence identity was evident between the OR and PA forms of this region based on Southern analysis. In Fig. 4, where pA3 was used to probe RFLP mapping strains, het-6<sup>PA</sup> strains exhibit the expected ~11-kbp fragment, while only a ~1.7-kbp fragment is evident in het-6<sup>OR</sup> strains, aside from a second band of just over 2 kbp which appeared in nearly all lanes of this autoradiograph. RFLP mapping of this polymorphism verified that sequences hybridizing to pA3 in het-6<sup>PA</sup> strains segregate opposite het-6<sup>OR</sup> (Metzenberg and Grotelueschen, 1993).

**PCR-RFLP Analysis of het-6 and un-24**

To develop molecular markers that distinguish alleles of un-24 and het-6 based on internal sequence characteristics we used a PCR-based method. Initially to differentiate between the PA and OR standard strains, two primer sets and restriction enzymes were selected for PCR-RFLP allele typing of het-6<sup>(6VP3/6VP5 PCR products cut with MboI)</sup> and un-24<sup>(6JP6/6JP11 PCR products cut with MspI)</sup>. The positions of primer pairs in het-6<sup>PA</sup> and un-24<sup>PA</sup> are given in Fig. 2.

The 6VP3/6VP5 PCR amplification products were approximately 1.0 kbp in size for all 40 N. crassa strains tested. MboI digestion patterns of these PCR products could be placed into one of two general classes which corresponded to either the PA standard strain (a fragment just under 1.0 kbp) or the OR standard strain (two fragments each ~0.5 kbp) (Fig. 5). het-6<sup>OR</sup> contains four MboI sites in the region flanked by 6VP3 and 6VP5 (Fig. 2). Based on DNA sequence we expect that the two larger fragments (each ~0.5 kbp) would be visible and the remaining three fragments would be too small to detect by the electrophoresis conditions used. The larger fragment size of the het-6<sup>PA</sup> class is due to the absence of all four MboI sites found in the OR form and a unique site at about 960 bp in the 1020-bp PCR product. Among the 40 Louisiana strains, 19 had the 960 bp het-6<sup>PA</sup> fragment and 20 had the 0.5 kbp het-6<sup>OR</sup> fragments. Strain P4479 had an additional variant of 800 and 200 bp fragments (Fig. 5). This pattern, designated “D” for “different” in Table 1, is interpreted to represent a het-6<sup>PA</sup>-like PCR product with an additional MboI site.

The size of un-24 6JP6/6JP11 PCR products was ~1.5 kbp for all strains. PCR products of un-24 digested with MspI gave distinct patterns for the PA and OR standard strains (Fig. 6). Ad-
four MspI fragments add up to 1355 bp, we infer that additional fragments of less than about 100 bp were not visible using these conditions. Among the 40 isolates, 16 had fragment patterns indistinguishable from un-24\textsuperscript{PA}, and 20 were identical to un-24\textsuperscript{OR}. Four strains (P4471, P4472, P4479, and P4497) produced a fragment pattern that differed from those of both un-24\textsuperscript{RA} and un-24\textsuperscript{OR} (Fig. 6); this pattern is designated “D” for “different” in Table 1.

**Incompatibility Function of un-24\textsuperscript{D} and het-6\textsuperscript{D}**

Whether un-24\textsuperscript{D} and het-6\textsuperscript{D} function as non-OR alleles was tested by DNA transformation assays. Spheroplasts of strains P4471 un-24\textsuperscript{D} het-6\textsuperscript{D} a and P4479 un-24\textsuperscript{D} het-6\textsuperscript{D} a were transformed separately with each of three plasmids; “plasmid 1” contained the hyg\textsuperscript{R} selectable marker alone, “plasmid 2” contained hyg\textsuperscript{R} and un-24\textsuperscript{OR}, and “plasmid 3” contained hyg\textsuperscript{R} and het-6\textsuperscript{OR}. As controls, each plasmid was also transformed into the standard strains, C9-2 (un-24\textsuperscript{OR} het-6\textsuperscript{D} a) and C2(2)-1 (un-24\textsuperscript{RA} het-6\textsuperscript{D} a). Transformation of P4471, P4479, and C2(2)-1 behaved similarly in these transformation assays; each yielded 100–200 colonies/\textmu g of DNA. This transformation assay indicates that strains carrying either un-24\textsuperscript{D} or het-6\textsuperscript{D} are incompatible with the OR-haplotype, and are therefore indistinguishable from the PA alleles at these loci. Since un-24\textsuperscript{RA} and het-6\textsuperscript{RA} do not have incompatibility activity in transformations (Smith et al., 2000b), and transformations of the PA haplotype for this region are not available, we can not unambiguously determine whether un-24\textsuperscript{D} and het-6\textsuperscript{D} are compatible with un-24\textsuperscript{RA} and het-6\textsuperscript{PA}, respectively.

**Recombination Frequencies in the het-6 Region**

het-6 and un-24 appear to be inherited as a block; all strains identified by PCR-RFLP as un-24\textsuperscript{RA} or un-24\textsuperscript{D} were also het-6\textsuperscript{RA} or het-6\textsuperscript{D} and functionally non-OR, and all strains identified as het-6\textsuperscript{OR} were also un-24\textsuperscript{OR} and functionally OR. Restriction enzyme maps indicate that un-24 and het-6 are separated by \(\sim 19\) and \(\sim 5.2\) kbp in OR and PA strains, respectively (Fig. 2). It is unlikely that the physical proximity of these genes, alone, accounts for the observed linkage disequilibrium. In a laboratory cross (Smith et al., 2000b), recombination frequencies in the 30-kbp centromere distal and 40-kbp centromere proximal regions of the un-24–het-6 gene pair were \(\sim 0.1\) MU/kbp. We expect, therefore, that the genetic distance between un-24 and het-6 is in the range of 0.5 to 2 MU. In our population sample recombination events within about 2.3 kbp between un-24 and a centromere distal EcoRI site may explain the departure from perfect association between RFLP and PCR-RFLP markers in our population sample (above). The un-24\textsuperscript{OR} PCR-RFLP fragment is associated with a \(-11\)-kbp EcoRI RFLP fragment in 8 strains and the un-24\textsuperscript{OR} PCR-RFLP fragment with a \(-9.7\)-kbp EcoRI RFLP fragment in 17 strains. Three strains (P4471, P4481, and P4494) have a \(-9.7\)-kbp RFLP fragment associated with the un-24\textsuperscript{RA} or un-24\textsuperscript{D} PCR-RFLP fragments.

**DISCUSSION**

The objectives of this study were to investigate genetic variation and distribution of alleles at un-24 and het-6 based on molecular markers in conjunction with a heterokaryon incompatibility functional assay based on partial diploid analyses. This is the first study to use a population genetics approach to compare both molecular and functional polymorphisms at a heterokaryon incompatibility locus. A previous study by Mylyk (1976) used analysis of partial diploids (Perkins, 1975) to ascertain incompatibility alleles at six unlinked loci, het-5, het-6, het-7, het-8, het-9, and het-10. His analysis of 15 isolates from three different Louisiana populations of *N. crassa* revealed that all but 2 of these strains contained at least one allelic difference at the loci studied. Recently, Wu et al. (1998) used restriction fragment patterns to infer that three alleles at het-c were in approximately equal frequency in the same Louisiana *N. crassa* population sample examined in this study. We have revealed that this population is also balanced in its representation of two functionally distinct forms of the het-6 haplotypes: of the 40 strains examined by partial diploids analysis, 20 were functionally OR and 20 were non-OR, or PA-like. Equal allelic frequency such as this could occur through chance or could indicate that the two forms are maintained in the population by means of a frequency-dependent selection mechanism, as suggested for het-c.

We found that functional allelism, based on partial diploid tests, was invariably correlated with the PCR-RFLP alleles at both un-24 and het-6 in this population sample. This corroborates data from transformation-based assays that both un-24 and het-6 contribute to heterokaryon incompatibility function associated with the het-6 region (Smith et al., 2000b). Of four possible allelic combinations,
however, only un-24GR het-6GR and un-24PA het-6PA were observed. The two other combinations in the population sample are expected under a model of frequency-dependent selection if un-24 and het-6 function independently. This model would predict that strains with rare combinations of alleles at distinct het loci would have a selective advantage and that each combination would tend toward approximately equal frequencies. This is clearly not the case. However, linkage equilibrium in the Louisiana population is, in fact, evident for the het-6-haplotype and the three het-c alleles (Wu et al., 1998; N. L. Glass, pers. comm.). The het-6-het-c combinations PA-OR, OR-OR, PA-PA, OR-PA, PA-GR, and OR-GR occur in 7, 7, 7, 4, and 4 strains, respectively.

Taken together, our data suggest that un-24 and het-6 act as an incompatibility complex. This is the only example, of which we are aware, where two closely linked genes apparently contribute to a single heterokaryon incompatibility phenotype. Persistence of this incompatibility complex could be due to suppression of recombination in the region or to nonviability of recombinant progeny. In support of the former, there are significant sequence differences between the two forms that may prevent homologous recombination (Figs. 2–4). The distance between un-24 and het-6 is about 19 kbp in the OR form compared to 5.2 kbp in the PA form. Restriction site maps also differ significantly in the region between the two genes. Finally, overall DNA sequence identity between the two forms is low; aside from un-24, there is little or no cross-hybridization between the OR and PA DNAs in the region covered by pA3. It is interesting that at least four predicted genes are located between un-24OR and het-6OR (MIPS database, http://www.mips.biochem.mpg.de/desc/neurospora), including the essential regulatory gene cyd-3. The lack of sequence identity between OR and PA strains in the region covered by pA3 suggests that the genes located between un-24OR and het-6OR are either highly dissimilar or are located elsewhere in strains carrying the PA form. We are now using this population-based information to characterize the genetic structure of the het-6PA region, to define the extent of this recombination block, and to identify PA-specific incompatibility factors in the region.

The molecular markers developed for un-24 and het-6 in this study provide a rapid means of evaluating allelic constitution of the het-6 haplotype in population samples of N. crassa. Examining the distribution of these markers in the Louisiana population provides unique insights into the molecular basis of het-6 incompatibility function. Similar approaches would be useful in understanding non-self-recognition systems in general.

ACKNOWLEDGMENTS

This research was funded by a grant to M. L. Smith from the Natural Sciences and Engineering Research Council of Canada. D. J. Jacobson is supported, in part, by NSF Grant MCB-9728675, awarded to David D. Perkins. We also acknowledge the technical assistance of R. Troiano.

REFERENCES


Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.