Nonallelic Interactions Between het-c and a Polymorphic Locus, pin-c, Are Essential for Nonself Recognition and Programmed Cell Death in Neurospora crassa

Isao Kaneko,1 Karine Dementhon, Qijun Xiang2 and N. Louise Glass3

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720

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ABSTRACT

Nonself recognition in filamentous fungi is conferred by genetic differences at het (heterokaryon incompatibility) loci. When individuals that differ in het specificity undergo hyphal fusion, the heterokaryon undergoes a programmed cell death reaction or is highly unstable. In Neurospora crassa, three allelic specificities at the het-c locus are conferred by a highly polymorphic domain. This domain shows trans-species polymorphisms indicative of balancing selection, consistent with the role of het loci in nonself recognition. We determined that a locus closely linked to het-c, called pin-c (partner for incompatibility with het-c) was required for het-c nonself recognition and heterokaryon incompatibility (HI). The pin-c alleles in isolates that differ in het-c specificity were extremely polymorphic. Heterokaryon and transformation tests showed that nonself recognition was mediated by synergistic nonallelic interactions between het-c and pin-c, while allelic interactions at het-c increased the severity of the HI phenotype. The pin-c locus encodes a protein containing a HET domain; predicted proteins containing HET domains are frequent in filamentous ascomycete genomes. These data suggest that nonallelic interactions may be important in nonself recognition in filamentous fungi and that proteins containing a HET domain may be a key factor in these interactions.

SELF/NONSELF discrimination is a ubiquitous and essential function in both multicellular and microbrial species. In vertebrate species, genes involved in self/nonself recognition include the major histocompatibility complex (MHC) (Jones et al. 1998), consisting of >100 highly polymorphic loci. In plants, algae, and fungi, highly polymorphic loci have been implicated in nonself recognition during sexual reproduction. Alleles at both the MHC and the sexual nonself recognition loci are highly polymorphic and often show trans-species polymorphisms, a feature associated with loci subject to balancing selection (Figuerola et al. 1988; Charlesworth 1995; Klein et al. 1998; Badrane and May 1999). Balancing selection maintains polymorphism at loci in populations, often through multiple speciation events, which, under the neutral theory of evolution, should be lost either by genetic drift or directional selection.

In filamentous fungi, nonself recognition is also important during vegetative growth. A filamentous fungal colony is composed of a network of multinucleate hyphae that are formed via hyphal fusion (Glass et al. 2004). Hyphal fusion also occurs between different individuals, which results in a vegetative heterokaryon containing genetically different nuclei in a common cytoplasm. Nonself recognition and rejection of heterokaryon formation is regulated by het loci (for heterokaryon incompatibility; also called vic loci) (Saupé 2000; Glass and Kaneko 2003). Heterokaryon incompatibility (HI) reduces the risk of transmission of infectious cytoplasmic elements (Debets et al. 1994; Cortesi et al. 2001) and exploitation by aggressive genotypes (Debets and Griffiths 1998). In some cases, DNA polymorphisms associated with het allele specificity show trans-species polymorphisms (Wu et al. 1998), indicating that some of these loci are subject to balancing selection, an observation consistent with their role in mediating nonself recognition.

Two types of genetic systems, allelic and nonallelic, regulate HI in filamentous fungi (Saupé 2000; Glass and Kaneko 2003). In nonallelic systems, mostly characterized in Podospora anserina, HI is triggered by an interaction between specific alleles at two different het loci. In allelic systems, such as those described for numerous filamentous ascomycete species, including Neurospora crassa, nonself recognition is triggered by alternative allelic specificities at a single het locus.
In N. crassa, 11 allelic het loci have been identified by genetic analysis (Perkins 1988). For the het-c locus, isolates from populations fall into one of three allelic specificity groups (Myllyk 1976; Howlett et al. 1993; Saupe and Glass 1997), referred to as het-c<sup>OR</sup>, het-c<sup>u</sup>, or het-c<sup>aupe</sup> (herein referred to as het-c<sup>1</sup>, het-c<sup>2</sup>, and het-c<sup>3</sup>, respectively). Transformants, heterokaryons, or partial diploids containing het-c alleles of alternate specificity are asexual and show slow growth inhibition and hyphal compartmentation and death (Garnjobst and Wilson 1956; Perkins 1975; Myllyk 1976; Saupe and Glass 1997; Jacobson et al. 1998; Wu and Glass 2001). Allelic specificity at het-c is dependent upon an indel encoding 30–48 aa; swapping of this variable region between alleles switches het-c allelic specificity (Saupe and Glass 1997; Glass 2002). Co-immunoprecipitation experiments showed that a HET-C heterocomplex composed of alternative HET-C proteins is associated with HI, suggesting that a HET-C heterocomplex may play a role in nonself recognition (Sarkar et al. 2002).

In an effort to identify additional genes required for het-c incompatibility, we identified mutants that suppressed het-e incompatibility. In this article, we describe the isolation and characterization of a pin-c (partner for incompatibility with het-c) mutant, which displays temperature-sensitive HI. The pin-c locus is closely linked to het-c and encodes alleles that are highly polymorphic in strains of alternate het-c specificity. The pin-c locus encodes a protein predicted to contain a HET domain. Five of the six molecularly characterized het interactions in filamentous ascomyceete species involve a protein containing a HET domain. Functional analysis of het-c and pin-c mutants showed that nonallelic interactions between het-c and pin-c are essential for nonself recognition and HI, while het-c allelic interactions contribute to the HI phenotype.

**MATERIALS AND METHODS**

**Strains and culture conditions:** All strains used in this study are listed in Table 1. The three het-c allelic specificities are referred to as het-c<sup>1</sup> (het-c<sup>u</sup>-type), het-c<sup>2</sup> (het-c<sup>OR</sup>-type), and het-c<sup>3</sup> (het-c<sup>aupe</sup>-type) (Saupe and Glass 1997). The corresponding pin-c alleles are referred to as pin-c<sup>1</sup>, pin-c<sup>2</sup>, and pin-c<sup>3</sup>. To construct mutants of het-c<sup>2</sup>, a 4-kbp fragment encoding the entire het-c<sup>2</sup> allele was introduced into C9-2 (Table 1). To construct mutants in pin-c<sup>1</sup> or pin-c<sup>2</sup>, a 1.5-kbp fragment from the 5′ region of pin-c<sup>1</sup> or a 1.5-kbp fragment from the 5′ region of pin-c<sup>2</sup> was introduced into RLM 57-30 or C9-2 (Table 1), respectively. All fragments were cloned into pCB1004, which confers hygromycin resistance (Carroll et al. 1994), and introduced into N. crassa strains via electroporation. Transformants were used in crosses to isolate mutants by repeat-induced point (RIP) mutation, a naturally occurring mutagenic process in N. crassa (Selker 1997). IK11-20 was a previously uncharacterized het-c<sup>1</sup> mutant (Saupe et al. 1996). Strains were grown on Vogel’s minimum medium (MM) (Vogel 1956) at 22° or 34°, with appropriate supplements added, if required.

**Heterokaryon tests:** Heterokaryon tests were performed as described (Xiang and Glass 2002). To compare growth rates, heterokaryons were cultured in race tubes at 22° or 34°. Data were collected from three independent experiments.

**Transformation:** Transformation of N. crassa conidia by electroporation was performed as described (Margolin et al. 1997). Constructs were made in pBM61 (Margolin et al. 1997) and were targeted to the his-3 locus by homologous recombination. Fifty transformants were picked for each DNA construct and growth rates and morphology were evaluated.

**Hyphal compartmentation and death assays:** Conidial suspensions of strains used for forcing heterokaryons were co-incubated onto minimal medium containing methionine (to reduce leakiness of the thr-2 marker) and incubated for 1 day to establish heterokaryotic growth. A small square of hyphae was transferred onto sterile cellophane on a MM plate containing methionine and 0.003% methylene blue, an indicator used to identify dead cells (Suzuki et al. 2000). After 1 day at 22° or 34°, the cellophane was removed from the plates, and the presence or absence of dead hyphal compartments was evaluated by microscopy. Wild-type colonies show ~1% cell death, while het-c incompatible colonies show 20–30% dead hyphal compartments, which are often clustered within the colony (Jacobson et al. 1998; Wu and Glass 2001; Xiang and Glass 2002).

**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLM 57-30</td>
<td>cyh-1; pcy-4 A</td>
<td>Gift from R. L. Metzenberg</td>
</tr>
<tr>
<td>FGSC 4564</td>
<td>ad-3B cyh-1 a&lt;sup&gt;−&lt;/sup&gt;</td>
<td>FGSC</td>
</tr>
<tr>
<td>IK09-15</td>
<td>thr-2; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>IK09-51</td>
<td>thr-2; arg-5 A</td>
<td>This study</td>
</tr>
<tr>
<td>KD02-10</td>
<td>his-3; pcy-4; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>IK11-20</td>
<td>het-c&lt;sup&gt;1&lt;/sup&gt; pcy-4 A</td>
<td>This study</td>
</tr>
<tr>
<td>IR042</td>
<td>pin-c&lt;sup&gt;1&lt;/sup&gt; arg-5; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>IR0128</td>
<td>pin-c&lt;sup&gt;1&lt;/sup&gt; arg-5; pcy-4 A</td>
<td>This study</td>
</tr>
<tr>
<td>C9-2</td>
<td>thr-2 a</td>
<td>Smith et al. (2000)</td>
</tr>
<tr>
<td>C9-15</td>
<td>thr-2 A</td>
<td>Smith et al. (2000)</td>
</tr>
<tr>
<td>Xa-2</td>
<td>arg-5; pan-2 a</td>
<td>Xiang and Glass (2002)</td>
</tr>
<tr>
<td>IK09-2</td>
<td>thr-2; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>CJ44</td>
<td>Δhet-c 2 Δpin-c&lt;sup&gt;2&lt;/sup&gt; arg-5; pan-2 A</td>
<td>Wu and Glass (2001)</td>
</tr>
<tr>
<td>C7-47</td>
<td>het-c&lt;sup&gt;2&lt;/sup&gt; thr-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>KD06-15</td>
<td>his-3; thr-2; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>XK5</td>
<td>pin-c&lt;sup&gt;3&lt;/sup&gt; arg-5; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>XK108</td>
<td>pin-c&lt;sup&gt;2&lt;/sup&gt; arg-5 A</td>
<td>This study</td>
</tr>
<tr>
<td>IRP66</td>
<td>pin-c&lt;sup&gt;2&lt;/sup&gt; thr-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>IRP138</td>
<td>pin-c&lt;sup&gt;2&lt;/sup&gt; thr-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>IRP153</td>
<td>pin-c&lt;sup&gt;2&lt;/sup&gt; thr-2; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>IRP163</td>
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<td>This study</td>
</tr>
<tr>
<td>IRP216</td>
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</tr>
<tr>
<td>IRP228</td>
<td>pin-c&lt;sup&gt;2&lt;/sup&gt; thr-2; pan-2 A</td>
<td>This study</td>
</tr>
<tr>
<td>FGSC 1945</td>
<td>Groveland WT a</td>
<td>FGSC</td>
</tr>
</tbody>
</table>

<sup>*</sup> All strains, except FGSC 1945, carry Oak Ridge alleles at all other het loci other than those designated in the table. Upper-case designation refers to mutant allele number.
Cloning of pin-c2 and pin-c3: The region containing pin-c2 was amplified from the het-c strain C9-2 (Table 1) by inverse PCR. Genomic DNA was digested with XbaI and treated with T4 DNA ligase before being used as a template. PCR was performed with the het-c2-specific primers CP1 (5' - TTAGCAG ACGCAAACCTTGA-3') and CP8 (5'-GAACAAGGTGTCGATGC TTTCCT-3'). The pin-c2 allele was amplified from FGSC 1945 (Table 1) by PCR using CG1 (5'-GGGACGAGCATCGGAGGT ATTG-3') and CP30 (5'-AAGTGGTCCGATGCCGTCACC-3').

Analysis of DNA sequences: Nucleotide sequences were determined at the DNA Sequencing Facility, University of California, Berkeley, California. Amino acid sequence alignments were created by CLUSTALw v1.7 with manual adjustments and shaded by MacBoxshade2.15.

RESULTS

A pin-c mutant shows temperature-dependent HI: Previously, a number of mutants that suppressed het-c HI were identified (Xiang and Glass 2002, 2004; Xiang 2003). One of these mutants (XK5) showed temperature-dependent HI. A typical het-c incompatible heterokaryon (IK09-2 + FGSC 4564) is aconidial and severely inhibited in its growth and displays hyphal death at both 22° and 34° (Figure 1B). A heterokaryon between XK5 (containing a het-c2 allele) and FGSC 4564 (containing a het-c1 allele) was fully compatible when grown at 34°, but was reduced in conidiation and showed inhibited growth and hyphal death at 22° (Figure 1D, left). A heterokaryon between XK5 and a het-c2 strain (C9-15) was fully compatible at both temperatures (Figure 1D). The mutation conferring temperature-dependent het-c HI, pin-c, mapped to the het-c region.

The compatible phenotype of the (XK5 + FGSC 4564) heterokaryon at 34° suggested that the mutation in XK5 was dominant at this temperature. However, Southern blot analyses showed that the region surrounding het-c was highly polymorphic between C9-2 (the parental het-c strain of XK5) and RLM 57-30 (a het-c strain) (data not shown). C9-2 is an introgressed strain that differs in het specificity only at het-c from strains of standard laboratory genetic background (Saupé et al. 1996), such as RLM 57-30. Since pin-c mapped to the het-c region, these observations suggested that the pin-c mutation might be specific for a het-c2 strain. To assess this possibility, a triheterokaryon was forced among a het-c2 pin-cm strain (XK5), a het-c1 strain (IK09-51), and a het-c2 strain (IK09-2). Unlike the (XK5 + FGSC 4564) heterokaryon, the triheterokaryon (XK5 + IK09-51 + IK09-2) showed a typical het-c incompatible phenotype at both 22° and 34° (Figure 2A). Thus, the inclusion of a het-c2 nucleus in a heterokaryon between XK5 and a het-c1 strain fully complemented the pin-c temperature-sensitive defect in XK5 (a het-c2 strain) and therefore the pin-c mutation in XK5 was actually recessive. These results indicated that het-c1 and het-c2 strains have strain-specific pin-c alleles.

pin-c encodes a predicted protein containing a HET domain: Previously, a mutant (CJ44) that contained a deletion that spanned the het-c2 locus was identified (Wu and Glass 2001; Xiang and Glass 2004). The exact size of the deletion in CJ44 could not be determined because of polymorphisms in the region surrounding het-c and an unknown rearrangement at the breakpoint (Xiang and Glass 2004). When CJ44 (Δhet-c2) was used in the triheterokaryon assay (CJ44 + XK108 + IK09-15), the temperature-dependent HI phenotype of a pin-c mutant (XK108) was not complemented (Figure 2B). These results indicated that the deleted region in CJ44 included the pin-c locus. Analyses using DNA fragments surrounding the het-c locus as probes showed that the deleted region in CJ44 included a locus centromere distal to het-c. An ORF (NCU03494) adjacent to het-c is predicted to encode a protein with a HET domain (PFAM06985) (Figure 3) (http://www.broad.mit.edu/annotation/fungi/neurospora/). The HET domain is composed of three blocks of high amino acid
Figure 3.—Amino acid sequence alignment of PIN-C1, PIN-C2, and PIN-C3. The three conserved regions of the HET domain identified by alignment of 78 HET domain proteins (PFAM06985); regions that define the HET domain (I, II, and III) are underlined. The amino acid substitution Leu410Pro in the pin-c2m allele in X5 is indicated with lowercase "p." Solid background indicates amino acid identity, shaded boxes indicate conserved aa substitutions, and dots indicate deletion events.
The predicted protein sequences of \textit{pin-c1}, \textit{pin-c2}, and \textit{pin-c3} are 884, 989, and 938 aa, respectively; \textit{pin-c1}/\textit{pin-c2} shows 56\% aa identity, \textit{pin-c1}/\textit{pin-c3} shows 47\% aa identity, and \textit{pin-c2}/\textit{pin-c3} shows 67\% aa identity (Figure 3). The three regions of the HET domain were highly conserved (\textit{pin-c1}/\textit{pin-c2}: 81\% aa identity; \textit{pin-c1}/\textit{pin-c3}: 81\% aa identity; \textit{pin-c2}/\textit{pin-c3}: 98\% aa identity; Figure 4). An AG-rich insertion in the predicted N-terminal region of \textit{pin-c2} results in 10 repeats of G(K/E)XV (Figure 3). Pairwise comparison between the \textit{pin-c} alleles showed that \textit{pin-c2} was more similar to \textit{pin-c3} in the HET domain and C-terminal region, but was more similar to \textit{pin-c1} in the N-terminal region. These data suggested that the \textit{pin-c2} allele was generated by recombination within the conserved HET domain between \textit{pin-c1} and \textit{pin-c3} (Figure 4).

\textit{pin-c2} loss-of-function mutations confer temperature-dependent \textit{het-c} HI, but \textit{pin-c1} mutations do not: Strains containing \textit{pin-c1} and \textit{pin-c2} loss-of-function mutations were generated by RIP mutation (Selker 1997). Isogenic \textit{het-c3} \textit{pin-c3} strains were not available for heterokaryon tests and so the function of \textit{pin-c3} was evaluated by transformation (see below). Two independent \textit{pin-c1} mutants that carried stop codons at aa positions Q56 and W280 (IRO42 and IRO128, respectively; Table 1) were identified. Three independent \textit{pin-c2} mutants were recovered that carried stop codons at aa position W32 (IRP138, IRP216, IRP228; Table 1), plus three additional mutants with a stop codon at W316 (IRP66), at Q151 (IRP163), or at W291 (IRP153; Table 1). These \textit{pin-c1}\textsuperscript{a} and \textit{pin-c2}\textsuperscript{a} strains were indistinguishable in vegetative growth and sexual reproduction from wild type. However, similar to the XK5 mutant, all six \textit{pin-c2}\textsuperscript{a} mutants showed temperature-dependent \textit{het-c} incompatibility when paired in a heterokaryon with a \textit{het-c1} strain. These \textit{(het-c1 \textit{pin-c1} + \textit{het-c2} \textit{pin-c2})} heterokaryons showed attenuated HI at 22\% but were fully compatible at 34\% (11 cm/day) (Figure 5, A and B, open circles; Table 2). These results show that the amino acid substitution (L410P) in \textit{pin-c} in the XK5 mutant was a loss-of-function or hypomorphic mutation and indicate that the conserved HET domain region in PIN-C2 is essential for function. By contrast, the phenotype of a \textit{(het-c1 \textit{pin-c1}\textsuperscript{a} + \textit{het-c2} \textit{pin-c2})} heterokaryon was similar in phenotype to a wild-type incompatible heterokaryon at 34\% (Figure 5, A and B, solid circles), although it showed attenuated HI at 22\% (Table 2).

Allelic interactions at \textit{het-c} or \textit{pin-c} do not confer HI: The heterokaryon results presented above suggested that either \textit{pin-c} functions as an independent \textit{het} locus or nonallelic interactions between \textit{het-c} and \textit{pin-c} are
required for nonself recognition and HI. To differentiate these two possibilities, we constructed strains that contained mutations at \( \text{het-c} \) and/or \( \text{pin-c} \) and assessed their phenotype in pairwise combinations via heterokaryon tests.

To test whether \( \text{het-c} \) allelic interactions were sufficient for HI, we assessed the phenotype of heterokaryons carrying mutations at \( \text{pin-c} \). Surprisingly, a \( \text{(het-c1 pin-c1}\text{1}\) heterokaryon was fully compatible and lacked hyphal death at both 22\(^\circ\) (Figure 6A, open circles) and 34\(^\circ\) (Table 2). These data indicate that allelic interactions between alternative \( \text{het-c} \) alleles were not sufficient to confer nonself recognition and HI. Thus, the \( \text{het-c} \) locus is not an allelic \( \text{het} \) locus in \( \text{N. crassa} \).

To test whether \( \text{pin-c} \) functions as an independent \( \text{het} \) locus, a \( \text{(het-c1m pin-c1}\text{1}} \) heterokaryon was

### Table 2: Genotype and phenotype of heterokaryons

<table>
<thead>
<tr>
<th>Heterokaryon*</th>
<th>22(^\circ)</th>
<th>34(^\circ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{het-c1 pin-c1} )</td>
<td>HI(^\circ)</td>
<td>HI</td>
</tr>
<tr>
<td>( \text{het-c2 pin-c2} )</td>
<td>C(^\circ)</td>
<td>C</td>
</tr>
<tr>
<td>( \text{het-c1}\text{1}\text{m pin-c1} )</td>
<td>C(^\circ)</td>
<td>C</td>
</tr>
<tr>
<td>( \text{het-c2 pin-c2m} )</td>
<td>Attenuated HI</td>
<td>Attenuated HI</td>
</tr>
<tr>
<td>( \text{het-c1m pin-c1} )</td>
<td>Attenuated HI</td>
<td>Attenuated HI</td>
</tr>
<tr>
<td>( \text{het-c2m pin-c2} )</td>
<td>Attenuated HI</td>
<td>C</td>
</tr>
<tr>
<td>( \text{het-c1 pin-c1} )</td>
<td>Attenuated HI (arrest)(^\dagger)</td>
<td>C</td>
</tr>
<tr>
<td>( \text{het-c2 pin-c2m} )</td>
<td>Attenuated HI (arrest)(^\dagger)</td>
<td>HI</td>
</tr>
<tr>
<td>( \text{het-c1 pin-c1} )</td>
<td>Attenuated HI</td>
<td>Attenuated HI</td>
</tr>
<tr>
<td>( \text{het-c2 pin-c2} )</td>
<td>Attenuated HI</td>
<td>C</td>
</tr>
<tr>
<td>( \text{het-c1m pin-c1} )</td>
<td>Attenuated HI</td>
<td>C</td>
</tr>
<tr>
<td>( \text{het-c2m pin-c2m} )</td>
<td>Attenuated HI</td>
<td>C</td>
</tr>
</tbody>
</table>

*Arrows indicate functional interactions.
\(^\dagger\)Heterokaryons showed typical HI.
\(^\dagger\)Heterokaryons showed a fully compatible phenotype (C).
\(^\dagger\)Heterokaryons showed attenuated HI, but subsequently underwent growth arrest.
constructed. Similar to the pin-c" heterokaryon above, the het-c" heterokaryon showed full compatibility and lacked hyphal death at both temperatures (Figure 6A, solid circles; Table 2). These data indicate that pin-c also does not function as an independent allelic het locus in *N. crassa*.

**Nonallelic interactions between het-c and pin-c are essential for het-c HI:** The results presented above indicate that nonallelic interactions between het-c and pin-c must be required for nonself recognition and HI in *N. crassa*. This hypothesis was evaluated using the following heterokaryons: (*het-c1 pin-c1" + het-c2" pin-c2") for het-c1/pin-c2 nonallelic interaction and (*het-c1" pin-c1 + het-c2 pin-c2") for het-c2/pin-c1 nonallelic interaction. Both of these heterokaryons showed hyphal death and growth rate reduction to ~5 cm/day at 22° (Figure 6B, solid and open circles, respectively). Unlike a het-c incompatible heterokaryon (Figure 6B, triangles), severe growth inhibition and growth arrest did not occur in heterokaryons with only one het-c/pin-c nonallelic interaction (Table 2).

As expected, a (*het-c1" pin-c1 + het-c2 pin-c2") heterokaryon was fully compatible at 34° and displayed a temperature-dependent HI phenotype identical to the original XK5 mutant (Figure 6C, open circles). These data indicate that the genetic interaction between het-c2 and pin-c1 is thermosensitive (Table 2). By contrast, the (*het-c1 pin-c1" + het-c2" pin-c2") heterokaryon showed attenuated HI with a reduction in growth rate to ~5 cm/day at 34° (Figure 6C, solid circles).

The above heterokaryon results showed that het-c/pin-c nonallelic interactions are essential for HI in *N. crassa*. However, the phenotype of the incompatible heterokaryons carrying only one nonallelic het-c/pin-c combination was much less severe than that of a typical wild-type het-c-incompatible heterokaryon (Figure 6, B and C). These data suggest that het-c or pin-c allelic interactions contribute to the phenotype of het-c HI when nonallelic interactions are present or that the het-c/pin-c interactions act synergistically or both.

**het-c, but not pin-c, allelic interactions contribute to het-c HI when het-c/pin-c nonallelic interactions are functional:** To evaluate the possibility that pin-c allelic interactions may be important for HI when het-c/pin-c nonallelic interactions were functional, we compared the phenotype of the following heterokaryons: (*het-c1 pin-c1" + het-c2" pin-c2") for het-c1/pin-c2 nonallelic interaction only (Figure 6B, solid circles) and (*het-c1 pin-c1 + het-c2" pin-c2") for het-c1/pin-c2 nonallelic interaction plus pin-c allelic interaction (Figure 6D, solid circles). Both heterokaryons were identical in phenotype. Similarly, a (*het-c1" pin-c1 + het-c2 pin-c2") heterokaryon (Figure 6B, open circles) was identical in phenotype to a (*het-c1" pin-c1 + het-c2 pin-c2") heterokaryon (Figure 6D, open circles). These data indicate that pin-c allelic interactions do not contribute to HI when het-c/pin-c nonallelic interactions are functional.

To determine whether het-c allelic interactions contribute to HI when het-c/pin-c nonallelic interactions...
are functional, we compared the phenotype of the following heterokaryons: \((\text{het-c}1 \ \text{pin-c}1^m + \ \text{het-c}2^m \ \text{pin-c}2)\) for \text{het-c}/\text{pin-c} interaction only (Figure 6B, solid circles) to \((\text{het-c}1 \ \text{pin-c}1^m + \ \text{het-c}2 \ \text{pin-c}2)\) for \text{het-c}/\text{pin-c} and \text{het-c} allelic interactions (Figure 5A, solid circles). Heterokaryons carrying both \text{het-c} allelic and \text{het-c}/\text{pin-c} nonallelic interactions were initially similar in phenotype to heterokaryons carrying only a \text{het-c}/\text{pin-c} nonallelic interaction (growth rate of \(\sim 4\) cm/day). However, the \((\text{het-c}1 \ \text{pin-c}1^m + \ \text{het-c}2 \ \text{pin-c}2)\) heterokaryon, which carried both \text{het-c}/\text{pin-c} nonallelic and \text{het-c} allelic interactions, subsequently underwent growth rate reduction (to \(\sim 1\) cm/day) and growth arrest (Figure 5A, solid circles). Similarly, a heterokaryon with both \text{het-c} allelic and \text{het-c}/\text{pin-c} nonallelic interactions \((\text{het-c}1 \ \text{pin-c}1 + \ \text{het-c}2 \ \text{pin-c}2^m)\) (Figure 5A, open circles) underwent growth rate reduction to \(\sim 1\) cm/day and growth arrest, unlike a heterokaryon containing only \text{het-c}/\text{pin-c} nonallelic interaction \((\text{het-c}1^m \ \text{pin-c}1 + \ \text{het-c}2 \ \text{pin-c}2)\) (Figure 6B, open circles). These data indicate that both \text{het-c} allelic and \text{het-c}/\text{pin-c} nonallelic interactions contribute to the \text{het-c}/\text{HI} phenotype (Table 2) and that their interactions are synergistic, rather than additive.

**Alternate pin-c alleles function to induce nonself recognition and heterokaryon incompatibility:** The above heterokaryon tests indicated that nonself recognition and HI are mediated by nonallelic interactions between \text{het-c} and \text{pin-c}. To assess the function of \text{pin-c}3, we performed transformation experiments in \text{het-c}1 \ \text{pin-c}1 and \text{het-c}2 \ \text{pin-c}2 strains.

The three alternate \text{pin-c} alleles were targeted to the \text{his-3} locus via transformation into KD02-10 (a \text{het-c}1 \ \text{pin-c}1 strain; Table 1). The KD02-10 (\text{pin-c}1) transformants all showed a compatible phenotype (\(\sim 7.5\) cm/day). By contrast, a significant portion of the KD02-10 (\text{pin-c}2) and KD02-10 (\text{pin-c}3) transformants (\(\sim 20\%\)) showed variable conidiation and a growth rate reduction to \(\sim 5\) cm/day (at \(22^\circ\)). Similarly, \(\sim 16\%\) of KD06-15 (\text{het-c}2 \ \text{pin-c}2) transformants carrying \text{pin-c}3 showed reduced growth (\(\sim 3.5\) cm/day) with variable conidiation. These results indicated that \text{pin-c}3 was functional in inducing HI and were consistent with the phenotype of heterokaryons carrying only one \text{het-c}/\text{pin-c} nonallelic interaction (see Figure 6, B and D). By contrast, a more typical, severe \text{het-c} incompatible phenotype was observed when \text{het-c}1 was introduced into KD06-15 (\(\sim 1\) cm/day) or when \text{het-c}2 was introduced into KD02-10 (\(\sim 3\) cm/day). Such transformants have both \text{het-c}/\text{pin-c} nonallelic and \text{het-c} allelic interactions contributing to the HI phenotype. These transformation data were consistent with \text{het-c} allelic interactions increasing the severity of the incompatibility phenotype when \text{het-c}/\text{pin-c} nonallelic interactions are functional.

**DISCUSSION**

In this article, we demonstrate that \text{het-c} nonself recognition and HI require nonallelic interactions between \text{het-c} and the closely linked locus, \text{pin-c}, providing the first definitive molecular and genetic proof that an allelic \text{het} functions in a nonallelic manner. Each of the three \text{het-c} specificities is represented by a specific haplotype with a particular genetic constitution at both \text{het-c} and \text{pin-c}. Nonallelic interactions at \text{het-c} have not been reported, primarily due to the temperature-sensitive nature of the \text{het-c}/\text{pin-c} interaction and decreased severity in HI in strains with only one functional \text{het-c}/\text{pin-c} nonallelic interaction. Our analyses showed that a typical \text{het-c} HI phenotype requires three synergistic interactions: two nonallelic interactions between \text{het-c} and \text{pin-c}, which are essential for nonself recognition and HI, and an allelic interaction at \text{het-c}, which increases the severity of the HI phenotype.

The data presented in this article indicate that nonself recognition and HI at the \text{het-c} locus are mediated by nonallelic interactions between \text{het-c} and \text{pin-c}. Molecular characterization of two other allelic \text{het} loci in \text{N. crassa} (\text{mat} and \text{het-6}) indicates that nonallelic interactions may be the norm for nonself recognition and HI. The genes required for \text{mat} incompatibility, \text{mat} A-1 and \text{mat} a-1, encode evolutionarily unrelated transcription factors (Glass et al. 1990; Starren and Yanofsky 1990). The \text{het-6} haplotype is composed of \text{un-24} and \text{het-6}, allele-specific polymorphisms at \text{un-24} and \text{het-6} show severe linkage disequilibrium, suggesting that nonallelic interactions between \text{un-24} and \text{het-6} play a role in \text{het-6} incompatibility (Mir-Rashed et al. 2000). These observations suggest that, similar to \text{mat}, \text{het-c}, and \text{het-6}, interactions at other allelic \text{het} loci in \text{N. crassa} may also involve nonallelic interactions between closely linked genes.

The \text{pin-c} locus encodes a HET domain protein. Remarkably, five of the six molecularly characterized \text{het} interactions in filamentous ascomycete species involve genes encoding predicted proteins with HET domains. These include \text{mat} incompatibility in \text{N. crassa}, which requires the \text{HET} domain protein \text{TOL} (Shu and Glass 1999) and \text{het-6} incompatibility; the \text{het-6} locus encodes a HET domain protein (Smith et al. 2000). In \text{P. anserina}, \text{het-c} and \text{het-d}, involved in nonallelic interactions with \text{het-c}, encode HET domain proteins (Espagne et al. 2002). The only molecularly characterized \text{het} interaction that may not involve a HET domain protein is the \text{het-s} locus, which encodes a prion (Coustou et al. 1997). We predict that most, if not all, \text{het} interactions in filamentous ascomycete species require a HET domain gene and that nonself recognition requires nonallelic interactions between a HET domain gene and a second gene, whose nature can vary. For example, in \text{N. crassa}, the \text{un-24} locus encodes a ribonucleotide reductase (Smith et al. 2000), the \text{mat} locus encodes transcription factors (Glass et al. 1990; Starren and Yanofsky 1990), and the \text{het-c} locus encodes a plasma membrane protein (Sarkar et al. 2002), while in \text{P. anserina}, the \text{het-c} locus encodes a glycolipid transfer protein (Mattjus et al. 2003).
Predicted HET domain genes are common in the genomes of filamentous ascomycete species, with ~50 predicted HET domain proteins in *N. crassa*, >150 in *Stagonospora nodorum*, 38 in *Magnaporthe grisea*, but only 9 in *Aspergillus nidulans*. Genes encoding HET domain proteins are apparently absent from the genomes of the ascomycete yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the basidiomycete species *Ustilago maydis*, *Cryptococcus neoformans*, and *Coprinus cinereus*. Predicted HET domain proteins are otherwise dissimilar outside of the HET domain and may contain other functional regions, such as a NACHT, acetyltransferase, or protein kinase domains. The NACHT domain (PFAM: PF05729) is common in apoptosis proteins and includes an ~350-aa nucleoside triphosphatase (NTPase) domain. Preliminary DNA sequence analysis indicates that some of the predicted *N. crassa* HET domain genes show a level of polymorphism similar to that of *pin-c*, suggesting that some of these HET domain genes may also function in nonself recognition and HI in *N. crassa*.

Nonallelic interactions between linked genes are a common theme among nonself recognition systems. In the MHC, the class II loci DQA1 and DQB1 are multi-allelic and code for polypeptides that form a heterodimer (*Kwok et al. 1993*). These two loci are ~20 kbp apart, but behave as a single genetic unit (*Cullen et al. 1997*). In the Brassicaceae, self-incompatibility relies on nonallelic interactions between the *Slocus* receptor kinase (SRK) and its ligand *Slocus* cysteine-rich protein (SCR) (*Nasrallah 2002*). In the basidiomycete *U. maydis*, formation of the infectious dikaryon requires an interaction between two closely linked loci, bE and bW (*Kahmann et al. 1999*). Similar to other nonself recognition systems (*Cullen et al. 1997; Awadalla and Charlesworth 1999*), a *het-c* haplotype may be maintained either by the suppression of recombination or by lethality of recombinant progeny. In the Brassicaceae, to maintain the linkage between alleles within a given S-haplotype, recombination is suppressed in the *Slocus* region, although recombination has occurred in the evolutionary history of these alleles (*Awadalla and Charlesworth 1999*). A comparison of *pin-c1*, *pin-c2*, and *pin-c3* alleles suggests that the *pin-c2* allele arose due to recombination between *pin-c1* and *pin-c3*. Further analysis of *het-c/pin-c* interactions will provide an exceptional model to explore the role of recombination vs. mutation in the evolution of matched allelic polymorphisms.

Another feature that *het-c* HI shares with other nonself recognition systems is that of extreme polymorphism. Similarity between the highly polymorphic SCR alleles in *Brassica campestris* rarely exceeds 30% (*Watanabe et al. 2000; Fobis-Loisy et al. 2004*), while in the Solanaceae, alleles at the *Slocus* are ~40% identical at the amino acid level (*Joerger et al. 1990*). In *U. maydis*, the N-terminal domain of the multiallelic bE and bW polypeptides is highly variable (*Kämper et al. 1995*). The *pin-c* alleles are extremely polymorphic and contain numerous indels and amino acid substitutions. Polymorphisms at nonself recognition loci are maintained by balancing selection, which often leads to transspecies polymorphisms (*Joerger et al. 1990; Klein et al. 1998; May et al. 1999*). Allelic polymorphisms that predate speciation occur at *het-c* (*Wu et al. 1998; Jacobson et al. 2004*). HI is mediated by allelic specificity at *het-c* and *pin-c* will be in severe linkage disequilibrium in *N. crassa* populations, that polymorphisms at *pin-c* will be maintained in other Neurospora species, and that the evolutionary history of *het-c* and *pin-c* polymorphisms will show congruence.

Nonself recognition is often mediated by protein-protein interactions. For example, nonself recognition in *U. maydis* is mediated by formation of a bE and bW heterocomplex, which occurs only if they are derived from different haplotypes (*Kämper et al. 1995*). Previously, we proposed that a HET-C heterocomplex is required for nonself recognition and HI (*Sarkar et al. 2002*). Our data show that genetic differences at *pin-c* are required for nonself recognition, suggesting that PIN-C may also physically interact with HET-C (Figure 7).

**Figure 7.**—Model for heterokaryon incompatibility mediated by genetic differences at *het-c/pin-c*. When a hyphal compartment is heterozygous for *het-c* and *pin-c* (for example, during a hyphal fusion event or in a partial diploid constructed via a cross or by transformation), nonself recognition is mediated by interactions between HET-C and PIN-C encoded by alternative haplotypes. The recognition event is predicted to occur at the plasma membrane because HET-C is a plasma membrane protein (*Sarkar et al. 2002*) and PIN-C is predicted to reside in the cytoplasm. The nonself recognition signal is amplified by HET-C1/PIN-C2, HET-C2/PIN-C1, and HET-C1/HET-C2 interactions. The signal is transduced to VIB-1, which is predicted to encode a transcription factor (*Xiang and Glass 2002*). VIB-1 is required for the repression of conidiation, growth inhibition, and hyphal compartmentation and death. Other factors are also important, because mutations at *vib-1* fail to completely suppress HI.
The temperature-sensitive nature of the het-c2/pin-c1 interaction supports this hypothesis. Nonself recognition mediated by an interaction between PIN-C and the HET-C heterocomplex might trigger entry into the programmed cell death pathway (Figure 7). Further characterization of HET-C/PIN-C interaction in N. crassa provides an excellent model to unravel molecular mechanisms associated with nonself recognition and programmed cell death in filamentous fungi, the evolution of nonself recognition systems, and the function of HET domain proteins.

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LITERATURE CITED


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