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

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ABSTRACT

Nonself recognition in filamentous fungi is conferred by genetic differences at *het* (*het*erokaryon 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* (**p**artner for **in**compatibility 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 microbial 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 (Figueroa *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 *het*erokaryon incompatibility; also called *vic* loci) (Saupe 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 (Saupe 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.

Sequence data for *pin-c1*, *pin-c2*, and *pin-c3* have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ309556, DQ309557, and DQ309558, respectively.

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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 (Mylyk 1976; Howlett *et al.* 1993; Saupe and Glass 1997), referred to as *het-c*^{OR}, *het-c*^{PA}, or *het-c*^{GR} (herein referred to as *het-c1*, *het-c2*, and *het-c3*, respectively). Transformants, heterokaryons, or partial diploids containing *het-c* alleles of alternate specificity are aconidial and show severe growth inhibition and hyphal compartmentation and death (Garnjobst and Wilson 1956; Perkins 1975; Mylyk 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; Wu and Glass 2001). 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-c* incompatibility. In this article, we describe the isolation and characterization of a *pin-c* (**p**artner for **in**compatibility 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 ascomycete 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.

TABLE 1

Strains used in this study

Strain	Genotype[a]	Origin
	het-c1/pin-c1 strains	
RLM 57-30	*cyh-1*; *pyr-4 A*	Gift from R. L. Metzenberg
FGSC 4564	*ad-3B cyh-1 a*^{m1}	FGSC
IK09-15	*thr-2*; *pan-2 A*	This study
IK09-51	*thr-2*; *arg-5 A*	This study
KD02-10	*his-3; pyr-4; pan-2 a*	This study
IK11-20	*het-c1*^{m20}; *nic-3 a*	This study
IR042	*pin-c1*^{m42} *pyr-4; pan-2 a*	This study
IR0128	*pin-c1*^{m128} *pyr-4 a*	This study
	het-c2/pin-c2 strains	
C9-2	*thr-2 a*	Smith *et al.* (2000)
C9-15	*thr-2 A*	Smith *et al.* (2000)
Xa-2	*arg-5*; *pan-2 a*	Xiang and Glass (2002)
IK09-2	*thr-2*; *pan-2 A*	This study
CJ44	Δ*het-c2* Δ*pin-c2 arg-5*; *pan-2 A*	Wu and Glass (2001)
C7-47	*het-c2*^{m47} *thr-2 a*	This study
KD06-15	*his-3; thr-2; pan-2 a*	This study
XK5	*pin-c2*^{m5} *arg-5*; *pan-2 A*	This study
XK108	*pin-c2*^{m108} *thr-2 arg-5 A*	This study
IRP66	*pin-c2*^{m66} *thr-2 A*	This study
IRP138	*pin-c2*^{m138} *thr-2 A*	This study
IRP153	*pin-c2*^{m153} *thr-2; pan-2 A*	This study
IRP163	*pin-c2*^{m163} *thr-2; pan-2 a*	This study
IRP216	*pin-c2*^{m216} *thr-2 a*	This study
IRP228	*pin-c2*^{m228} *thr-2*; *pan-2 a*	This study
	het-c3/pin-c3 strain	
FGSC 1945	Groveland WT *a*	FGSC

FGSC, Fungal Genetic Stock Center.

[a] All strains, except FGSC 1945, carry Oak Ridge alleles at all other *het* loci other than those designated in the table. Uppercase designation refers to mutant allele number.

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-c1* (*het-c*^{OR}-type), *het-c2* (*het-c*^{PA}-type), and *het-c3* (*het-c*^{GR}-type) (Saupe and Glass 1997). The corresponding *pin-c* alleles are referred to as *pin-c1*, *pin-c2*, and *pin-c3*. To construct mutants of *het-c2*, a 4-kbp fragment encoding the entire *het-c2* allele was introduced into C9-2 (Table 1). To construct mutants in *pin-c1* or *pin-c2*, a 1.5-kbp fragment from the 5′ region of *pin-c1* or a 1.5-kbp fragment from the 5′ region of *pin-c2* 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-c1* 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 coinoculated 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).

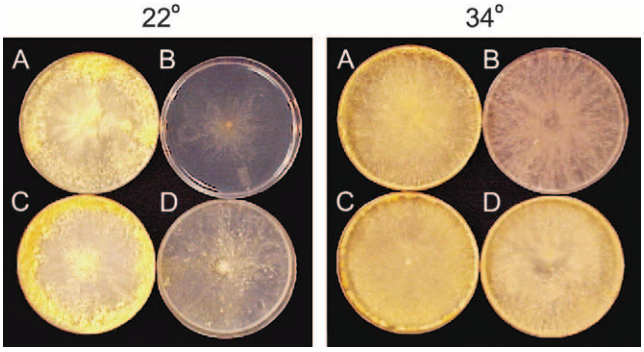

Figure 1.—Temperature-dependent *het-c* HI phenotype of the *pin-c* mutant, XK5. (A) A compatible heterokaryon IK09-51 (*het-c1 pin-c1*) + FGSC 4564 (*het-c1 pin-c1*). (B) An incompatible heterokaryon IK09-2 (*het-c2 pin-c2*) + FGSC 4564 (*het-c1 pin-c1*). (C) A XK5 (*het-c2 pin-c2*^{m}) + C9-15 (*het-c2 pin-c2*) heterokaryon with identical *het-c* specificity. (D) A XK5 (*het-c2 pin-c2*^{m}) + FGSC 4564 (*het-c1 pin-c1*) heterokaryon with alternate *het-c* specificity. All heterokaryons were incubated at 22° for 5 days (left) or 34° for 3 days (right).

Cloning of *pin-c2* and *pin-c3*: The region containing *pin-c2* was amplified from the *het-c2* strain C9-2 (Table 1) by inverse PCR. Genomic DNA was digested with *Xba*I and treated with T4 DNA ligase before being used as a template. PCR was performed with the *het-c2*-specific primers CP1 (5′-TTAGCAG ACGCAACCCTTGA-3′) and CP8 (5′-CGAACCTGGTCTGTC TTTCT-3′). The *pin-c3* allele was amplified from FGSC 1945 (Table 1) by PCR using CG1 (5′-GGGACGAGCATCGGAGGT ATTG-3′) and CP30 (5′-AAGTTCGTTCCGAATTGCCTCACC-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 CLUSTALw1.7 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 1C). 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-c2* strain of XK5) and RLM 57-30 (a *het-c1* 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 (Saupe *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-c*^{m} 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.

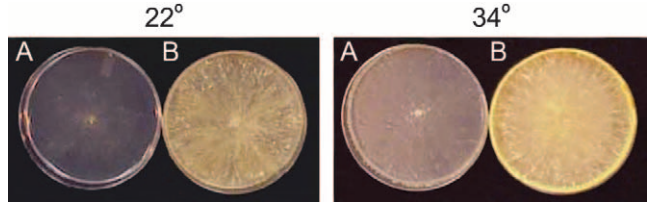

Figure 2.—Triheterokaryon tests with the *pin-c* mutants. (A) A XK5 (*het-c2 pin-c2*^{m}) + IK09-2 (*het-c2 pin-c2*) + IK09-51 (*het-c1 pin-c1*) triheterokaryon grown at 22° or 34° shows full complementation of HI at both temperatures. (B) By contrast, a XK108 (*het-c2 pin-c2*^{m}) + CJ44 (Δ*het-c2* Δ*pin-c2*) + IK09-15 (*het-c1 pin-c1*) triheterokaryon grown at 22° or 34° shows an identical phenotype to a heterokaryon between XK108 (*het-c2 pin-c2*^{m}) and IK09-15 (*het-c1 pin-c1*), *i.e.*, attenuated heterokaryon incompatibility at 22°, but full compatibility at 34°. Plates were incubated at 22° for 5 days (left) or 34° for 3 days (right).

***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 (Δ*het-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-c1* locus as probes showed that the deleted region in CJ44 included a locus centromere distal to *het-c*. An ORF (NCU03494) adjacent to *het-c1* 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

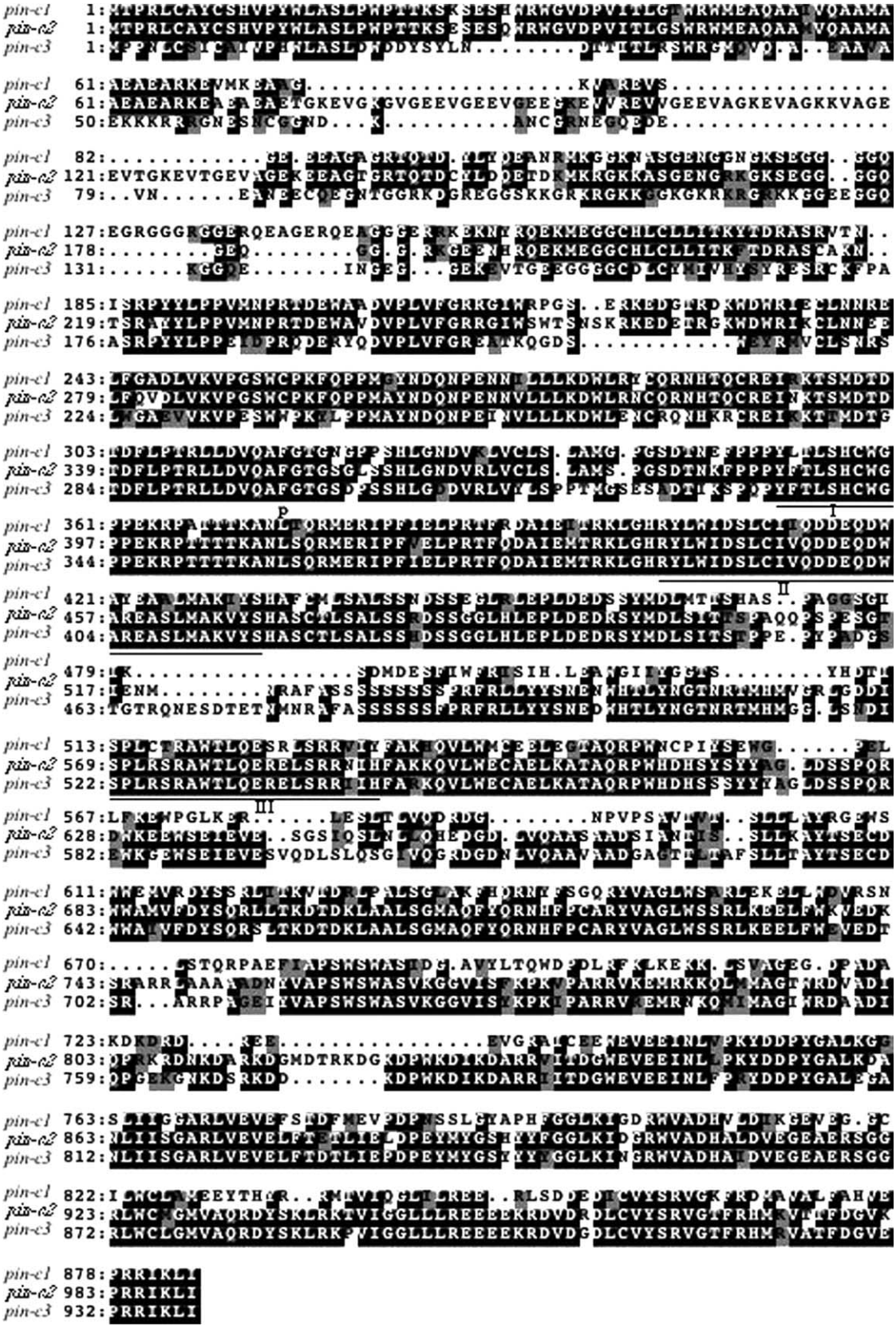

FIGURE 3.—Amino acid sequence alignment of PIN-C1, PIN-C2, and PIN-C3. The three conserved regions of the HET domain identified by alignment of 78 HET domain proteins (PFAM06985); regions that define the HET domain (I, II, and III) are underlined. The amino acid substitution Leu410Pro in the *pin-c2*^{m} allele in XK5 is indicated with lowercase "p." Solid background indicates amino acid identity, shaded boxes indicate conserved aa substitutions, and dots indicate deletion events.

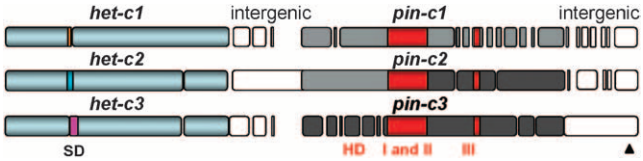

Figure 4.—Representation of *het-c1/pin-c1*, *het-c2/pin-c2*, and *het-c3/pin-c3* haplotypes. The specificity domain (SD) of *het-c* (Saupe and Glass 1997) is represented by different colors. Outside the SD, the conserved *het-c* sequence is shown in blue. A comparison of nucleotide sequences of the three alternate *pin-c* alleles show numerous indels, as represented by open spaces. The HET domain (HD, in red) is highly conserved. The gray or black shading indicates polymorphisms in the *pin-c* sequence outside of the HD. The *pin-c2* allele shows similarity to *pin-c1* in the 5′ region (gray region) and similarity to *pin-c3* in the 3′ region (black region). A high level of nucleotide identity among the three *het-c* haplotypes is restored in the intergenic region 3′ of *pin-c* (arrowhead).

similarity within an ~200-aa region and is conserved among proteins involved in HI in both *N. crassa* and *P. anserina* (Espagne *et al.* 2002).

NCU03494 was recovered from the *het-c2* strain C9-2 by inverse PCR. To test whether NCU03494 from a *het-c2* strain could complement the temperature-sensitive HI phenotype of XK5, we introduced NCU03494 from *het-c1* and *het-c2* strains into the *pin-c* mutant XK5 via transformation. The introduction of NCU03494 from a *het-c1* strain (FGSC 2489) into XK5 failed to restore HI at 34°. By contrast, when NCU03494 from a *het-c2* strain (C9-2) was introduced into XK5, most transformants showed the restoration of HI at 34° (data not shown), indicating that NCU03494 encodes *pin-c*. These results showed that *pin-c* from a *het-c1* strain is functionally different from *pin-c* from a *het-c2* strain. The alleles of NCU03494 in *het-c1* and *het-c2* strains were therefore designated as *pin-c1* and *pin-c2*, respectively.

The predicted *pin-c1* and *pin-c2* polypeptides are rich in tryptophan residues (2.99–3.73%) (Tompa 2002), but otherwise have no defining features, other than the presence of a HET domain. Sequence comparison of *pin-c2* from XK5 and C9-2 showed a single-nucleotide exchange, T to C, which changed a Leu 410 to a Pro codon within the predicted HET domain region (Figure 3).

The *pin-c* locus encodes highly polymorphic alleles: Three functionally alternate alleles have been characterized at *het-c*, *het-c1*, *het-c2*, and *het-c3* (Saupe and Glass 1997; Wu and Glass 2001). We therefore isolated the *pin-c* allele from a *het-c3* strain (FGSC 1945 Table 1) by using primers to conserved regions outside of *pin-c*. The *pin-c1*, *pin-c2*, and *pin-c3* alleles were highly polymorphic; *pin-c1/pin-c2* showed 67% nt identity, *pin-c1/pin-c3* showed 62% nt identity, and *pin-c2/pin-c3* showed 76% nt identity. In addition to nucleotide diversity at *pin-c*, a total of 46 indels ranging from 1 to 86 bp in length occurred between the *pin-c* alleles (Figure 4). Some indels resulted in frameshift mutations, which were compensated by downstream indels to restore the *pin-c* ORF. All three *pin-c* alleles contained a single intron with splice sites at an identical location, although the length of the intron was variable: 217, 192, and 110 bp in *pin-c1*, *pin-c2*, and *pin-c3*, respectively. Transcripts of *pin-c1* and *pin-c2* were detected by RT–PCR and cDNA sequence and RFLP analysis confirmed the presence and splice sites of the predicted intron.

The predicted protein sequences of *pin-c1*, *pin-c2*, and *pin-c3* are 884, 989, and 938 aa, respectively; *pin-c1/pin-c2* shows 56% aa identity, *pin-c1/pin-c3* shows 47% aa identity, and *pin-c2/pin-c3* shows 67% aa identity (Figure 3). The three regions of the HET domain were highly conserved (*pin-c1/pin-c2*: 81% aa identity; *pin-c1/pin-c3*: 81% aa identity; *pin-c2/pin-c3*: 98% aa identity; Figure 4). An AG-rich insertion in the predicted N-terminal region of *pin-c2* results in 10 repeats of G(K/E)XV (Figure 3). Pairwise comparison between the *pin-c* alleles showed that *pin-c2* was more similar to *pin-c3* in the HET domain and C-terminal region, but was more similar to *pin-c1* in the N-terminal region. These data suggested that the *pin-c2* allele was generated by recombination within the conserved HET domain between *pin-c1* and *pin-c3* (Figure 4).

***pin-c2* loss-of-function mutations confer temperature-dependent *het-c* HI, but *pin-c1* mutations do not:** Strains containing *pin-c1* and *pin-c2* loss-of-function mutations were generated by RIP mutation (Selker 1997). Isogenic *het-c3 pin-c3* strains were not available for heterokaryon tests and so the function of *pin-c3* was evaluated by transformation (see below). Two independent *pin-c1* mutants that carried stop codons at aa positions Q56 and W280 (IRO42 and IRO128, respectively; Table 1) were identified. Three independent *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 *pin-c1*^{m} and *pin-c2*^{m} strains were indistinguishable in vegetative growth and sexual reproduction from wild type. However, similar to the XK5 mutant, all six *pin-c2*^{m} mutants showed temperature-dependent *het-c* incompatibility when paired in a heterokaryon with a *het-c1* strain. These (*het-c1 pin-c1* + *het-c2 pin-c2*^{m}) 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 *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 (*het-c1 pin-c1*^{m} + *het-c2 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 *het-c* or *pin-c* do not confer HI: The heterokaryon results presented above suggested that either *pin-c* functions as an independent *het* locus or nonallelic interactions between *het-c* and *pin-c* are

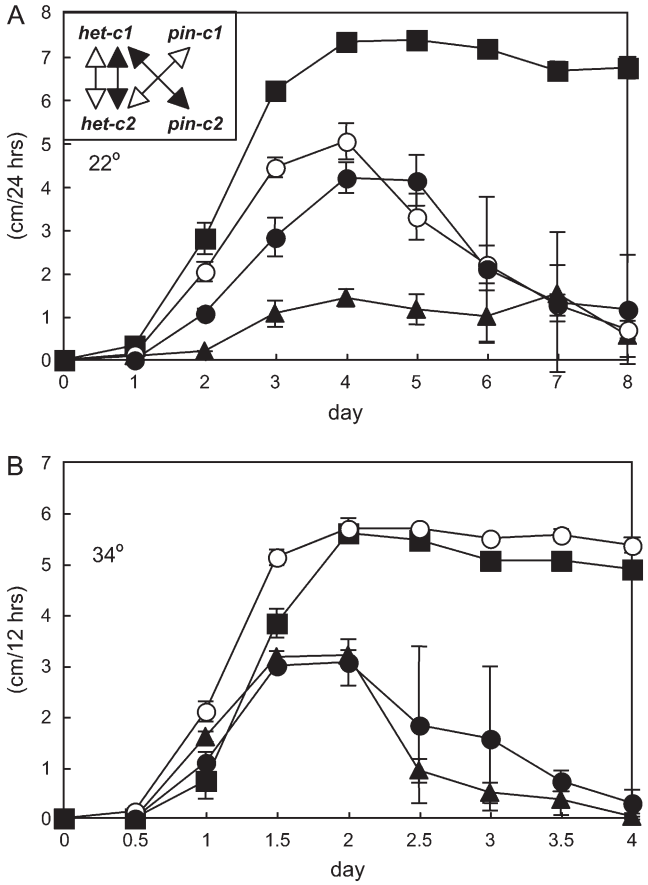

FIGURE 5.—HI phenotype of *pin-c1* and *pin-c2* mutants at 22° (A) or 34° (B). (A) A RLM 57-30 (*het-c1 pin-c1*) + FGSC 4564 (*het-c1 pin-c1*) heterokaryon (■) is fully compatible (~7 cm/day). A FGSC 4564 (*het-c1 pin-c1*) + C9-2 (*het-c2 pin-c2*) heterokaryon (▲) is incompatible (~1 cm/day). A FGSC 4564 (*het-c1 pin-c1*) + IRP228 (*het-c2 pin-c2*^{m}) heterokaryon (○) initially grew at ~5 cm/day, followed by growth arrest (~1 cm/day). An IRO42 (*het-c1 pin-c1*^{m}) and C9-2 (*het-c2 pin-c2*) heterokaryon (●) initially grew at ~4 cm/day, followed by growth arrest. (B) The (RLM 57-30 + FGSC 4564) (■) heterokaryon grew at ~11 cm/day, while the (FGSC 4564 + C9-2) heterokaryon (▲) grew at ~6 cm/day and underwent growth arrest. The (FGSC 4564 + IRP228) heterokaryon (○) was fully compatible (~11 cm/day), while the (IRO42 + C9-2) heterokaryon (●) showed a phenotype similar to an incompatible control. Predicted *het-c1/het-c2* allelic and *het-c1/pin-c2* or *het-c2/pin-c1* nonallelic interactions in the heterokaryons are indicated in the inset in A.

TABLE 2

Genotype and phenotype of heterokaryons

Heterokaryon[a]	22°	34°
het-c1 pin-c1 ↕ ↕ *het-c2 pin-c2*	HI[b]	HI
het-c1 pin-c1^m ↕ *het-c2 pin-c2*^{m}	C[c]	C
het-c1^m *pin-c1* ↕ *het-c2*^{m} *pin-c2*	C	C
het-c1 pin-c1^m ↘ ↙ *het-c2*^{m} *pin-c2*	Attenuated HI	Attenuated HI
het-c1^m *pin-c1* ↘ ↙ *het-c2 pin-c2*^{m}	Attenuated HI	C
het-c1 pin-c1 ↕ ↘ *het-c2 pin-c2*^{m}	Attenuated HI (arrest)[d]	C
het-c1 pin-c1^m ↘ ↘ *het-c2 pin-c2*	Attenuated HI (arrest)[d]	HI
het-c1 pin-c1 ↙ ↕ *het-c2*^{m} *pin-c2*	Attenuated HI	Attenuated HI
het-c1^m *pin-c1* ↙ ↕ *het-c2 pin-c2*	Attenuated HI	C
het-c1 pin-c1 *het-c2*^{m} *pin-c2*^{m}	C	C

[a] Arrows indicate functional interactions.
[b] Heterokaryons showed typical HI.
[c] Heterokaryons showed a fully compatible phenotype (C).
[d] Heterokaryons showed attenuated HI, but subsequently underwent growth arrest.

required for nonself recognition and HI. To differentiate these two possibilities, we constructed strains that contained mutations at *het-c* and/or *pin-c* and assessed their phenotype in pairwise combinations via heterokaryon tests.

To test whether *het-c* allelic interactions were sufficient for HI, we assessed the phenotype of heterokaryons carrying mutations at *pin-c*. Surprisingly, a (*het-c1 pin-c1*^{m} + *het-c2 pin-c2*^{m}) heterokaryon was fully compatible and lacked hyphal death at both 22° (Figure 6A, open circles) and 34° (Table 2). These data indicate that allelic interactions between alternative *het-c* alleles were not sufficient to confer nonself recognition and HI. Thus, the *het-c* locus is not an allelic *het* locus in *N. crassa*.

To test whether *pin-c* functions as an independent *het* locus, a (*het-c1*^{m} *pin-c1* + *het-c2*^{m} *pin-c2*) heterokaryon was

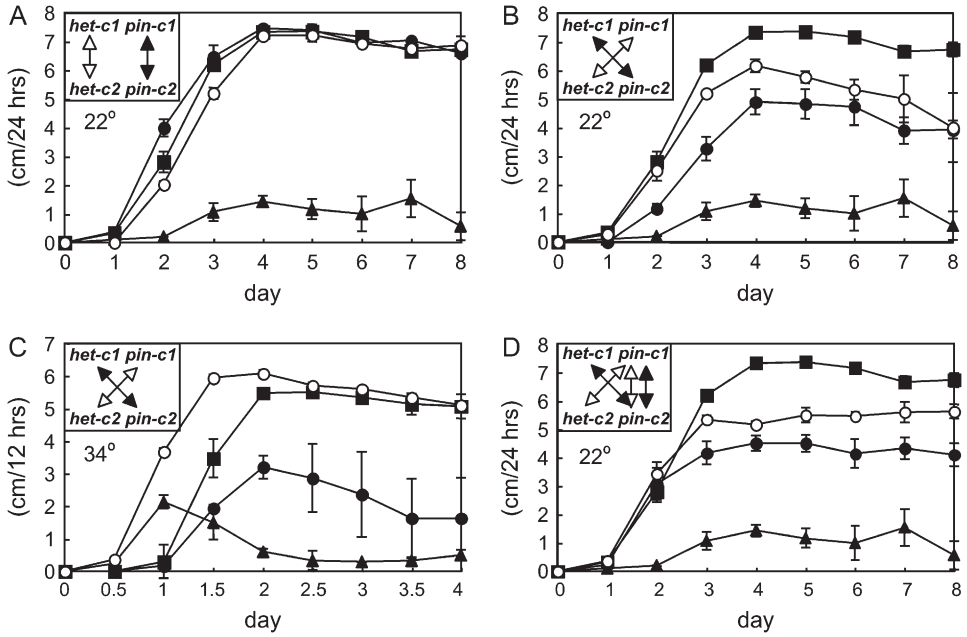

FIGURE 6.—HI phenotype of heterokaryons having *het-c* or *pin-c* allelic or *het-c/pin-c* nonallelic interactions at 22° (A, B, and D) or 34° (C). A compatible heterokaryon (RLM 57-30 + FGSC 4564) (■) and incompatible heterokaryon (FGSC 4564 + C9-2) (▲) were used as controls. (A) A (IRO42 + IRP216) heterokaryon (○) with a *het-c* allelic interaction only (*het-c1 pin-c1*^{m} + *het-c2 pin-c2*^{m}) was fully compatible. A IK11-20 + C7-47 heterokaryon (●) with a *pin-c* allelic interaction only (*het-c1*^{m} *pin-c1* + *het-c2*^{m} *pin-c2*) was fully compatible. (B) A (IRO42 + C7-47) heterokaryon (●) with a *het-c1/pin-c2* nonallelic interaction (*het-c1 pin-c1*^{m} + *het-c2*^{m} *pin-c2*) grew at ~5 cm/day. A (IK11-20 + IRP228) heterokaryon (○) with a *het-c2/pin-c1* nonallelic interaction (*het-c1*^{m} *pin-c1* + *het-c2 pin-c2*^{m}) grew at ~5.5 cm/day. (C) A (IRO42 + C7-47) heterokaryon (●) with a *het-c1/pin-c2* nonallelic interaction grew at ~5 cm/day. The (IK11-20 + IRP228) heterokaryon (○) with a *het-c2/pin-c1* nonallelic interaction (*het-c1*^{m} *pin-c1* + *het-c2 pin-c2*^{m}) was fully compatible (~11.5 cm/day). (D) A (FGSC 4564 + C7-47) heterokaryon (●) containing *het-c1/pin-c2* and *pin-c1/pin-c2* interactions was identical in phenotype to a heterokaryon containing only a *het-c1/pin-c2* interaction (IRO42 + C7-47) (B). A (IK11-20 + Xa-2) heterokaryon (○) with *het-c2/pin-c1* and *pin-c1/pin-c2* interactions was identical in phenotype to (IK11-20 + IRP228) (B). Predicted *het-c* or *pin-c* allelic or *het-c/pin-c* nonallelic interactions are shown in the insets as solid and open arrows, which correspond to solid or open circles in A–D.

constructed. Similar to the *pin-c*^{m} heterokaryon above, the *het-c*^{m} 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*^{m} + *het-c2*^{m} *pin-c2*) for *het-c1/pin-c2* nonallelic interaction and (*het-c1*^{m} *pin-c1* + *het-c2 pin-c2*^{m}) 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*^{m} *pin-c1* + *het-c2 pin-c2*^{m}) 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*^{m} + *het-c2*^{m} *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*^{m} + *het-c2*^{m} *pin-c2*) for *het-c1/pin-c2* nonallelic interaction only (Figure 6B, solid circles) and (*het-c1 pin-c1* + *het-c2*^{m} *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*^{m} *pin-c1* + *het-c2 pin-c2*^{m}) heterokaryon (Figure 6B, open circles) was identical in phenotype to a (*het-c1*^{m} *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: (*het-c1 pin-c1*^{m} + *het-c2*^{m} *pin-c2*) for *het-c1/pin-c2* interaction only (Figure 6B, solid circles) to (*het-c1 pin-c1*^{m} + *het-c2 pin-c2*) for *het-c1/pin-c2* and *het-c* allelic interactions (Figure 5A, solid circles). Heterokaryons carrying both *het-c* allelic and *het-c1/pin-c2* nonallelic interactions were initially similar in phenotype to heterokaryons carrying only a *het-c1/pin-c2* nonallelic interaction (growth rate of ~4 cm/day). However, the (*het-c1 pin-c1*^{m} + *het-c2 pin-c2*) heterokaryon, which carried both *het-c/pin-c* nonallelic and *het-c* allelic interactions, subsequently underwent growth rate reduction (to ~1 cm/day) and growth arrest (Figure 5A, solid circles). Similarly, a heterokaryon with both *het-c* allelic and *het-c2/pin-c1* nonallelic interactions (*het-c1 pin-c1* + *het-c2 pin-c2*^{m}) (Figure 5A, open circles) underwent growth rate reduction to ~1 cm/day and growth arrest, unlike a heterokaryon containing only *het-c2/pin-c1* non-allelic interaction (*het-c1*^{m} *pin-c1* + *het-c2 pin-c2*^{m}) (Figure 6B, open circles). These data indicate that both *het-c* allelic and *het-c/pin-c* nonallelic interactions contribute to the *het-c* 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 *het-c* and *pin-c*. To assess the function of *pin-c3*, we performed transformation experiments in *het-c1 pin-c1* and *het-c2 pin-c2* strains.

The three alternate *pin-c* alleles were targeted to the *his-3* locus via transformation into KD02-10 (a *het-c1 pin-c1* strain; Table 1). The KD02-10 (*pin-c1*) transformants all showed a compatible phenotype (~7.5 cm/day). By contrast, a significant portion of the KD02-10 (*pin-c2*) and KD02-10 (*pin-c3*) transformants (~20%) showed variable conidiation and a growth rate reduction to ~5 cm/day (at 22°). Similarly, ~16% of KD06-15 (*het-c2 pin-c2*) transformants carrying *pin-c3* showed reduced growth (~3.5 cm/day) with variable conidiation. These results indicated that *pin-c3* was functional in inducing HI and were consistent with the phenotype of heterokaryons carrying only one *het-c/pin-c* nonallelic interaction (see Figure 6, B and D). By contrast, a more typical, severe *het-c* incompatible phenotype was observed when *het-c1* was introduced into KD06-15 (~1 cm/day) or when *het-c2* was introduced into KD02-10 (~3 cm/day). Such transformants have both *het-c/pin-c* nonallelic and *het-c* allelic interactions contributing to the HI phenotype. These transformation data were consistent with *het-c* allelic interactions increasing the severity of the incompatibility phenotype when *het-c/pin-c* nonallelic interactions are functional.

DISCUSSION

In this article, we demonstrate that *het-c* nonself recognition and HI require nonallelic interactions between *het-c* and the closely linked locus, *pin-c*, providing the first definitive molecular and genetic proof that an allelic *het* locus functions in a nonallelic manner. Each of the three *het-c* specificities is represented by a specific haplotype with a particular genetic constitution at both *het-c* and *pin-c*. Nonallelic interactions at *het-c* have not been reported, primarily due to the temperature-sensitive nature of the *het-c2/pin-c1* interaction and decreased severity in HI in strains with only one functional *het-c/pin-*c nonallelic interaction. Our analyses showed that a typical *het-c* HI phenotype requires three synergistic interactions: two nonallelic interactions between *het-c* and *pin-*c, which are essential for nonself recognition and HI, and an allelic interaction at *het-c*, which increases the severity of the HI phenotype.

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

The *pin-c* locus encodes a HET domain protein. Remarkably, five of the six molecularly characterized *het* interactions in filamentous ascomycete species involve genes encoding predicted proteins with HET domains. These include *mat* incompatibility in *N. crassa*, which requires the HET domain protein TOL (Shiu and Glass 1999) and *het-6* incompatibility; the *het-6* locus encodes a HET domain protein (Smith *et al.* 2000). In *P. anserina*, *het-e* and *het-d*, involved in nonallelic interactions with *het-c*, encode HET domain proteins (Espagne *et al.* 2002). The only molecularly characterized *het* interaction that may not involve a HET domain protein is the *het-s* locus, which encodes a prion (Coustou *et al.* 1997). We predict that most, if not all, *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 *N. crassa*, the *un-24* locus encodes a ribonucleotide reductase (Smith *et al.* 2000), the *mat* locus encodes transcription factors (Glass *et al.* 1990; Staben and Yanofsky 1990), and the *het-c* locus encodes a plasma membrane protein (Sarkar *et al.* 2002), while in *P. anserina*, the *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 multiallelic 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 *S*-locus receptor kinase (SRK) and its ligand *S*-locus 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 *S*-locus 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 *S*-locus are ~40% identical at the amino acid level (Ioerger *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 *trans*-species polymorphisms (Ioerger *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) in the genus Neurospora and its close relatives, Sordaria and Gelasinospora. We predict that polymorphisms regulating 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.

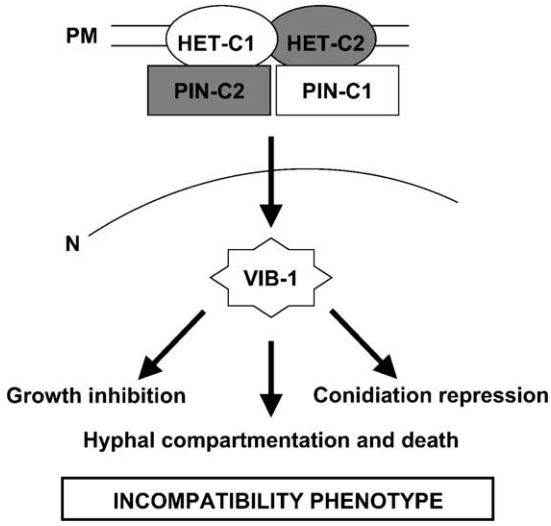

Figure 7.—Model for heterokaryon incompatibility mediated by genetic differences at *het-c/pin-c*. When a hyphal compartment is heterozygous for *het-c* and *pin-c* (for example, during a hyphal fusion event or in a partial diploid constructed via a cross or by transformation), nonself recognition is 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* do not completely suppress HI.

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).

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

Awadalla, P., and D. Charlesworth, 1999 Recombination and selection at Brassica self-incompatibility loci. Genetics **152:** 413–425.

Badrane, H., and G. May, 1999 The divergence-homogenization duality in the evolution of the *b1* mating type gene of *Coprinus cinereus*. Mol. Biol. Evol. **16:** 975–986.

Carroll, A. M., J. A. Sweigard and B. Valent, 1994 Improved vectors for selecting resistance to hygromycin. Fungal Genet. Newsl. **41:** 22.

Charlesworth, D., 1995 Multi-allelic self-incompatibility polymorphisms in plants. BioEssays **17:** 31–38.

Cortesi, P., C. E. McCulloch, H. Y. Song, H. Q. Lin and M. G. Milgroom, 2001 Genetic control of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*. Genetics **159:** 107–118.

Coustou, V., C. Deleu, S. Saupe and J. Bégueret, 1997 The protein product of the *het-s* heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog. Proc. Natl. Acad. Sci. USA **94:** 9773–9778.

Cullen, M., J. Noble, H. Erlich, K. Thorpe, S. Beck *et al.*, 1997 Characterization of recombination in the HLA class II region. Am. J. Hum. Genet. **60:** 397–407.

Debets, A. J. M., and A. J. F. Griffiths, 1998 Polymorphism of *het*-genes prevents resource plundering in *Neurospora crassa*. Mycol. Res. **102:** 1343–1349.

Debets, F., X. Yang and A. J. F. Griffiths, 1994 Vegetative incompatibility in *Neurospora*: its effect on horizontal transfer of mitochondrial plasmids and senescence in natural populations. Curr. Genet. **26:** 113–119.

Espagne, E., P. Balhadere, M.-L. Penin, C. Barreau and B. Turcq, 2002 HET-E and HET-D belong to a new subfamily of WD40 proteins involved in vegetative incompatibility specificity in the fungus *Podospora anserina*. Genetics **161:** 71–81.

Figueroa, F., E. Gunther and J. Klein, 1988 MHC polymorphisms pre-dating speciation. Nature **335:** 265–271.

Fobis-Loisy, I., C. Miege and T. Gaude, 2004 Molecular evolution of the *S* locus controlling mating in the *Brassicaceae*. Plant Biol. **6:** 109–118.

Garnjobst, L., and J. F. Wilson, 1956 Heterocaryosis and protoplasmic incompatibility in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **42:** 613–618.

Glass, N. L., and I. Kaneko, 2003 Fatal attraction: nonself recognition and heterokaryon incompatibility in filamentous fungi. Eukaryot. Cell **2:** 1–8.

Glass, N. L., J. Grotelueschen and R. L. Metzenberg, 1990 *Neurospora crassa A* mating-type region. Proc. Natl. Acad. Sci. USA **87:** 4912–4916.

Glass, N. L., C. Rasmussen, M. G. Roca and N. D. Read, 2004 Hyphal homing, fusion and mycelial interconnectedness. Trends Microbiol. **12:** 135–141.

Howlett, B., J. F. Leslie and D. D. Perkins, 1993 Putative multiple alleles at the vegetative (heterokaryon) incompatibility loci *het-c* and *het-8* in *Neurospora crassa*. Fungal Genet. Newsl. **40:** 40–42.

Ioerger, T. R., A. G. Clark and T. H. Kao, 1990 Polymorphism at the self-incompatibility locus in the Solanaceae predates speciation. Proc. Natl. Acad. Sci. USA **87:** 9732–9735.

Jacobson, D. J., K. Beurkens and K. L. Klomparens, 1998 Microscopic and ultrastructural examination of vegetative incompatibility in partial diploids heterozygous at *het* loci in *Neurospora crassa*. Fungal Genet. Biol. **23:** 45–56.

Jacobson, D. J., A. J. Powell, J. R. Dettman, G. S. Saenz, M. M. Barton *et al.*, 2004 Neurospora in temperate forests of western North America. Mycologia **96:** 66–74.

Jones, E. Y., J. Tormo, S. W. Reid and D. I. Stuart, 1998 Recognition surfaces of MHC class I. Immunol. Rev. **163:** 121–128.

Kahmann, R., C. Basse and M. Feldbrügge, 1999 Fungal-plant signalling in the *Ustilago maydis*-maize pathosystem. Curr. Opin. Microbiol. **2:** 647–650.

Kämper, J., M. Reichmann, T. Romeis, M. Bölker and R. Kahmann, 1995 Multiallelic recognition: nonself-dependent dimerization of the bE and bW homeodomain proteins in *Ustilago maydis*. Cell **81:** 73–83.

Klein, J., A. Sato, S. Nagl and C. O'hUigin, 1998 Molecular trans-species polymorphism. Annu. Rev. Ecol. Syst. **29:** 1–21.

Kwok, W. W., S. Kovats, P. Thurtle and G. T. Nepom, 1993 HLA-DQ allelic polymorphisms constrain patterns of class II heterodimer formation. J. Immunol. **150:** 2263–2272.

Margolin, B. S., M. Freitag and E. U. Selker, 1997 Improved plasmids for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet. Newsl. **44:** 34–36.

Mattjus, P., B. Turcq, H. M. Pike, J. G. Molotkovsky and R. E. Brown, 2003 Glycolipid intermembrane transfer is accelerated by HET-C2, a filamentous fungus gene product involved in cell-cell incompatibility response. Biochemistry **42:** 535–542.

May, G., F. Shaw, H. Badrane and X. Vekemans, 1999 The signature of balancing selection: fungal mating compatibility gene evolution. Proc. Natl. Acad. Sci. USA **96:** 9172–9177.

Mir-Rashed, N., D. J. Jacobson, M. R. Dehghany, O. C. Micali and M. L. Smith, 2000 Molecular and functional analyses of incompatibility genes at *het-6* in a population of *Neurospora crassa*. Fungal Genet. Biol. **30:** 197–205.

Mylyk, O. M., 1976 Heteromorphism for heterokaryon incompatibility genes in natural populations of *Neurospora crassa*. Genetics **83:** 275–284.

Nasrallah, J. B., 2002 Recognition and rejection of self in plant reproduction. Science **296:** 305–308.

Perkins, D. D., 1975 The use of duplication-generating rearrangements for studying heterokaryon incompatibility genes in Neurospora. Genetics **80:** 87–105.

Perkins, D. D., 1988 Main features of vegetative incompatibility in *Neurospora crassa*. Fungal Genet. Newsl. **35:** 44–46.

Sarkar, S., G. Iyer, J. Wu and N. L. Glass, 2002 Nonself recognition is mediated by HET-C heterocomplex formation during vegetative incompatibility. EMBO J. **21:** 4841–4850.

Saupe, S. J., 2000 Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. Microbiol. Mol. Biol. Rev. **64:** 489–502.

Saupe, S. J., and N. L. Glass, 1997 Allelic specificity at the *het-c* heterokaryon incompatibility locus of *Neurospora crassa* is determined by a highly variable domain. Genetics **146:** 1299–1309.

Saupe, S. J., G. A. Kuldau, M. L. Smith and N. L. Glass, 1996 The product of the *het-C* heterokaryon incompatibility gene of *Neurospora crassa* has characteristics of a glycine-rich cell wall protein. Genetics **143:** 1589–1600.

Selker, E. U., 1997 Epigenetic phenomena in filamentous fungi: Useful paradigms or repeat-induced confusion? Trends Genet. **13:** 296–301.

Shiu, P. K., and N. L. Glass, 1999 Molecular characterization of *tol*, a mediator of mating-type-associated vegetative incompatibility in *Neurospora crassa*. Genetics **151:** 545–555.

Smith, M. L., O. C. Micali, S. P. Hubbard, N. Mir-Rashed, D. J. Jacobson *et al.*, 2000 Vegetative incompatibility in the *het-6* region of *Neurospora crassa* is mediated by two linked genes. Genetics **155:** 1095–1104.

Staben, C., and C. Yanofsky, 1990 *Neurospora crassa a* mating-type region. Proc. Natl. Acad. Sci. USA **87:** 4917–4921.

Suzuki, C., M. Kawano, T. Kashiwagi, Y. Arata, T. Kawasumi *et al.*, 2000 Lethal effect of the expression of a killer gene SMK1 in *Saccharomyces cerevisiae*. Protein Eng. **13:** 73–76.

Tompa, P., 2002 Intrinsically unstructured proteins. Trends Biochem. Sci. **27:** 527.

Vogel, H. J., 1956 A convenient growth medium. Microbiol. Genet. Bull. **13:** 42–46.

Watanabe, M., A. Ito, Y. Takada, C. Ninomiya, T. Kakizaki *et al.*, 2000 Highly divergent sequences of the pollen self-incompatibility (*S*) gene in class-I *S* haplotypes of *Brassica campestris* (syn. *rapa*) L. FEBS Lett. **473:** 139–144.

Wu, J., and N. L. Glass, 2001 Identification of specificity determinants and generation of alleles with novel specificity at the *het-c* heterokaryon incompatibility locus of *Neurospora crassa*. Mol. Cell. Biol. **21:** 1045–1057.

Wu, J., S. J. Saupe and N. L. Glass, 1998 Evidence for balancing selection operating at the *het-c* heterokaryon incompatibility locus in a group of filamentous fungi. Proc. Natl. Acad. Sci. USA **95:** 12398–12403.

Xiang, Q., 2003 Genetic analysis of hyphal fusion and heterokaryon incompatibility in *Neurospora crassa*. Ph.D. Thesis, University of California, Berkeley, CA.

Xiang, Q., and N. L. Glass, 2002 Identification of *vib-1*, a locus involved in vegetative incompatibility mediated by *het-c* in *Neurospora crassa*. Genetics **162:** 89–101.

Xiang, Q., and N. L. Glass, 2004 Chromosome rearrangements in isolates that escape from *het-c* heterokaryon incompatibility in *Neurospora crassa*. Curr. Genetics **44:** 329–338.

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