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Diverse interactions mediate asymmetric incompatibility by the *het-6* supergene complex in *Neurospora crassa*

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ABSTRACT

Heterokaryon incompatibility (HI) in filamentous fungi is a form of nonself recognition that operates during the vegetative phase of the life cycle. One HI gene complex in *Neurospora crassa*, the *het-6* locus, comprises two incompatibility genes, *het-6* and *un-24*, each having two allelic variants, Oak Ridge (OR) and Panama (PA). The *un-24* gene also encodes the large subunit of ribonucleotide reductase while *het-6* appears to be a member of a repetitive gene family with no other known function aside from HI. These two genes are in severe linkage disequilibrium such that only *un-24*^{OR} *het-6*^{OR} and *un-24*^{PA} *het-6*^{PA} haplotypes occur in nature. In this study we unravel several genetic interactions that govern the HI functions of this gene complex. We use novel *un-24*^{PA} *het-6*^{OR} strains and *het-6* deletion strains to demonstrate that nonallelic interactions occur between *un-24* and *het-6* and reveal an allelic incompatibility interaction between the OR and PA forms of *un-24* that is asymmetrically enhanced by the presence of *het-6*^{OR} or *het-6*^{PA}. We also show how two allelic forms of *vib-1*, a suppressor of *het-c*- and *mat*-associated incompatibility, differentially act as recessive suppressors of HI associated with nonallelic interactions between *un-24*^{PA} and *het-6*^{OR}. In contrast, *vib-1* is a dominant suppressor of HI associated with allelic differences at *un-24* and a dominant partial suppressor of the *un-24*^{OR} and *het-6*^{PA} nonallelic interaction. The range of suppressor activities is largely explained by an interesting differential effect on *het-6*^{OR} and *het-6*^{PA} transcript levels by VIB-1.

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1. Introduction

Nonself recognition systems are of fundamental biological importance, particularly in the filamentous fungi where conspecific individuals may grow in close proximity and readily initiate hyphal fusions (Glass and Kaneko, 2003). Proposed roles of fungal nonself recognition encompass parasitic and symbiotic relationships, territorial defense, and in reducing the transmission of infectious pathogenic elements such as parasitic organelles (Debets and Griffiths, 1998), viruses (Biella et al., 2002) and plasmids (Debets et al., 1994). *Neurospora crassa* is a model for the study of the genetic and biochemical bases of nonself recognition in fungi, as manifested by heterokaryon incompatibility (HI). In the HI process, heterokaryons (cells that contain genetically dissimilar nuclei) are unable to proliferate when the nuclei have allelic differences at heterokaryon incompatibility (*het*) loci. In *N. crassa*, 11 *het* loci have been identified; a difference at one or more of these loci in fusing strains results in hyphal compartmentalization, programmed cell death (PCD) and lysis of the cells in the region of fusion (Glass and Dementhon, 2006).

A developing theme in *N. crassa* and other filamentous ascomycetes is that *het* loci tend to operate as multi-gene complexes wherein one of the genes encodes a protein with a HET domain (Paoletti and Clavé, 2007). The HET domain is defined by three conserved amino acid blocks (Smith et al., 2000a; Micali and Smith, 2005) and has been identified within the predicted protein products of HI genes including *het-6*, *tol* and *pin-c* in *N. crassa*, and *het-d*, *het-e* and *het-r* in *Podospora anserina* (Smith et al., 2000a; Kaneko et al., 2006; Paoletti and Clavé, 2007; Chevanne et al., 2009). Different allelic forms of HET-domain genes are highly polymorphic. In *N. crassa*, for example, the three allelic forms of *pin-c* share as low as 62% identity at the nucleotide level (Kaneko et al., 2006) and the two forms of *het-6* share only 68% identity at the amino acid level (Micali and Smith, 2006).

Yet another common theme is that each HET-domain protein is proposed to trigger HI through a nonallelic interaction with a second non-HET-domain protein that, in turn, may have a function in addition to HI (Kaneko et al., 2006). For example, in *P. anserina* the HET-domain proteins of *het-E* and *het-D* (Espagne et al., 2002) interact with HET-C, a glycolipid transfer protein (Mattjus et al., 2003). In *N. crassa*, *pin-c* encodes a HET-domain protein that interacts with HET-C, a glycine-rich plasma membrane protein in this species (Sarkar et al., 2002). Reminiscent of MHC class I genes (reviewed in Kumánovics et al., 2003) in vertebrates and

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self-recognition genes in plants (reviewed in Charlesworth et al., 2005), these fungal HI gene complexes can be tightly linked as is the case for the *het-c*|*pin-c* (Kaneko et al., 2006) and *un-24*|*het-6* (Smith et al., 2000a) gene complexes, or they can be unlinked and segregate independently to give rise to self-incompatible (SI) progeny, as in the case of the *het-E*|*-D*|*-C* system in *P. anserina*.

The *het-6* locus of *N. crassa* is of particular interest because it comprises two incompatibility genes that are locked into a supergene complex. This supergene occurs in one of two possible forms, *un-24*^{OR} *het-6*^{OR} or *un-24*^{PA} *het-6*^{PA}, representing the Oak Ridge (OR) and Panama (PA) haplotypes, respectively. No additional functional alleles have been identified for either gene (Mir-Rashed et al., 2000). It was proposed that the OR and PA supergenes are maintained as distinct haplotypes through intergenic sequence divergence associated with a paracentric inversion that prevents recombination in the region (Micali and Smith, 2006). The absence of recombinant forms (e.g. *un-24*^{PA} *het-6*^{OR}) precludes studies on basic features of this complex, such as whether the incompatibility functions are mediated by allelic or nonallelic interactions.

Within the complex, *het-6* encodes a HET-domain protein that has no known function aside from nonself recognition. The *un-24* gene encodes the large subunit of ribonucleotide reductase (RNR), which is a highly conserved protein necessary for *de novo* deoxyribonucleotide synthesis (Smith et al., 2000b). The N-terminal of UN-24 is evolutionarily conserved across biological kingdoms, whereas the C-terminal region of the *N. crassa* protein is unique compared to that of other organisms (Micali and Smith, 2005). Interestingly, this C-terminal region differs strikingly between OR and PA allelic forms and thus may mediate the nonself recognition properties of this protein (Micali and Smith, 2006).

Other factors modulate heterokaryon incompatibility in addition to nonself recognition genes. For example, the HI system is apparently turned off by an unknown mechanism when filamentous ascomycetes enter the sexual cycle (Shiu and Glass, 1999). It is of interest in this regard that mutations in the *vib-1*$^{+}$ gene suppress incompatibility associated with differences at *het-c*, mating-type and *het-e* in *N. crassa* (Xiang and Glass, 2004a). VIB-1^{+} is a putative transcription factor that possesses predicted DNA binding and nuclear localization domains. It has sequence similarity to NDT80, a transcription factor involved in regulating meiosis in *Saccharomyces cerevisiae* (Xu et al., 1995; Hepworth et al., 1998), and it was demonstrated that VIB-1^{+} is required for the transcription of *het-6*, *pin-c* and *tol* in *N. crassa* (Dementhon et al., 2006). VIB-1^{+} is also similar to the *xprG* (*phoG*) protein product in *Aspergillus nidulans*, which is a putative transcription factor involved in the positive regulation of phosphate-non-repressible acid phosphatase activity (MacRae et al., 1993) as well as the production of extracellular proteases that are expressed upon carbon, nitrogen or sulfur starvation (Katz et al., 2006). In fact, VIB-1^{+} was shown to be a positive regulator of phosphate-non-repressible acid phosphatase activity, and it is known to be differentially localized during the cell cycle of *N. crassa* (Dementhon et al., 2006). These observations suggest that *vib-1*$^{+}$ may have multiple functions, some of which may be unrelated to HI.

In this study we use gene replacements to overcome the linkage disequilibrium that characterizes the *het-6* gene complex and to create strains that bear novel *un-24 het-6* allelic combinations. This allowed us to study interactions that occur within the *un-24 het-6* gene complex during HI. We demonstrate that both allelic (*un-24*) and nonallelic (*un-24* with *het-6*) interactions occur. We further use *het-6* deletion strains to show that allelic specificity at *het-6* is responsible for the heretofore unexplained but commonly observed characteristic of asymmetric incompatibility (Wilson et al., 1961; Williams and Wilson, 1966; Micali and Smith, 2006). We also formally demonstrate that mutations in *vib-1* suppress incompatibility associated with allelic differences at *un-24* and suppress nonallelic incompatibility between *un-24* and *het-6* in an asymmetric fashion through the differential control of transcription of *het-6* alleles.

2. Material and methods

2.1. Strains and culture conditions

Details of strains used in this study are provided in Table 1. Genetic naming conventions used in this paper follow those of Perkins (1999). Routine culture of *N. crassa* was on Vogel's minimal medium containing 1.5% sucrose, 1.5% agar and supplements as required (Davis and De Serres, 1970) at 30 °C unless otherwise specified. Crosses were performed on plates containing Synthetic Crossing Medium (SCM) with supplements (Davis and De Serres, 1970) where conidial or mycelial suspensions were spotted at opposite edges of the plate and allowed to grow together at room temperature (21–25 °C) under natural light. Spot tests were used to screen strains for nutritional markers or resistance to antibiotics and were performed by inoculating conidial suspensions onto Vogel's medium containing 0.8% sorbose and 0.4% sucrose and supplements. Phosphinothricin (PPT) was purified from Finale® (Farnam Co., Phoenix, Arizona) using a one step extraction with 1-butanol (Hays and Selker, 2000) and was used at a ratio of 1: 250 (v/v) in Vogel's medium. Hygromycin B (HygB, Roche, Laval, Quebec) was used in selection plates and genotyping experiments at 15–20 mg per 100 ml of Vogel's medium.

2.2. un-24 allele substitution and vib-1 introgression

The primers used for construction of the *un-24* replacement cassette are described in Supplemental Table S1. Source sequences of genes are reported in GenBank as follows: *un-24*^{PA} (DQ525966.1), *un-24*^{OR} (AF171697.1), *het-6*^{PA} (AF208542.1) and *het-6*^{OR} (AF206700.1). A 2 kbp fragment extending downstream from the 3′ end of *un-24*^{OR} was amplified from the G8:G1 cosmid (Smith et al., 1996) using the primers 3F2991HYBfw (5′ half is *un-24*^{PA}-specific and 3′ half is *un-24*^{OR}-specific) and sm3F5130ORrv (*un-24*^{OR} -specific). The resulting PCR product was purified using GenElute PCR Clean-up kit (Sigma, Oakville, Ontario), digested with *Dpn*I (New England Biolabs, Pickering, Ontario) to remove any G8:G1 template and added as a primer/template along with the pA3 clone (contains *un-24*^{PA}; Micali and Smith, 2006) as the primary template in a second PCR reaction using the primers sm5F840fw (anneals to upstream regions of both *un-24*^{OR} and *un-24*^{PA}) and sm3F5130ORrv. The resulting 6 kbp product, named RECunPA, was inserted into pCR2.1TOPO (Invitrogen, Burlington, Ontario) and transformed into competent Top10 *E. coli* cells (Invitrogen). Plasmids were extracted by Promega Wizard miniprep (Fischer Scientific, Nepean, Ontario) and verified by DNA sequencing (Genome Québec Innovation Centre, Montreal, PQ). RECunPA contains 840 bp of 5′ un-translated region (UTR) of *un-24*^{PA}, which has only a few sequence differences to the 5′ UTR of *un-24*^{OR}, the 3 kbp *un-24*^{PA} coding region, and 2 kbp of the *un-24*^{OR}-specific 3′ UTR. RECunPA was used in subsequent transformations to replace *un-24*^{ts}, a temperature sensitive mutant form of *un-24*^{OR} (Smith et al., 2000b), with the functionally PA form, *un-24*^{PA}.

2.3. Transformation of N. crassa

Transformation of the RECunPA into the *N. crassa* DLL1-26 strain was done as described previously (Royer and Yamashiro, 1992; Smith et al., 2000a). Transformation plates were incubated at 39 °C for 3–5 days to select for replacement of *un-24*^{ts} and transformed colonies were individually subcultured to Vogel's agar

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