Nonself recognition during somatic growth is an essential and ubiquitous phenomenon in both prokaryotic and eukaryotic species. In filamentous fungi, nonself recognition is also important during vegetative growth. 

Hyphal fusion between genetically dissimilar individuals results in rejection of heterokaryon formation and in programmed cell death of the fusion compartment. In filamentous fungi, such as *Neurospora crassa*, nonself recognition and heterokaryon incompatibility (HI) are regulated by genetic differences at *het* loci. In *N. crassa*, mutations at the *vib-1* locus suppress nonself recognition and HI mediated by genetic differences at *het-c/pin-c, mat, and un-24/het-6*. *vib-1* is a homolog of *Saccharomyces cerevisiae NDT80*, which is a transcriptional activator of genes during meiosis. For this study, we determined that *vib-1* encodes a nuclear protein and showed that *VIB-1* localization varies during asexual reproduction and during HI. *vib-1* is required for the expression of genes involved in nonself recognition and HI, including *pin-c, tol, and het-6*; all of these genes encode proteins containing a HET domain. *vib-1* is also required for the production of downstream effectors associated with HI, including the production of extracellular proteases upon carbon and nitrogen starvation. Our data support a model in which mechanisms associated with starvation and nonself recognition/HI are interconnected. *VIB-1* is a major regulator of responses to nitrogen and carbon starvation and is essential for the expression of genes involved in nonself recognition and death in *N. crassa*.

Nonself recognition during somatic growth is an essential and ubiquitous phenomenon in both prokaryotic and eukaryotic species. In vertebrate species, self/nonself recognition relies on the major histocompatibility complex (MHC), which is an array of polymorphic genetic loci that generate proteins important in pathogen recognition and the activation of defense mechanisms (37). In filamentous fungi, nonself recognition is important during vegetative growth. Hyphal fusion between genetically dissimilar individuals results in rejection of heterokaryon formation and in programmed cell death of the fusion compartment (Fig. 1), an event analogous to nonself recognition following fusion in colonial marine invertebrates, such as *Hydractinia* and *Botryllus* (32, 73), and nonself recognition following death following fusion of plasmodia in the slime mold *Physarum polycephalum* (9). Nonself recognition during heterokaryon formation in filamentous fungi is regulated genetically by *het* loci (for heterokaryon incompatibility [HI]) (29, 70, 86). In the filamentous fungus *Neurospora crassa*, 11 *het* loci mediate nonself recognition and HI (28, 63); each *het* locus has two or three alternative allelic specificities. Since these *het* loci are effectively unlinked, the number of possible *het* genotypes within a segregating population is at least $2^{11}$ genotypes, thus forming an effective barrier to heterokaryon formation between genetically different isolates. HI in filamentous fungi has been shown to reduce the risk of transmission of pathogenic elements, such as infectious virus-like doubled-stranded RNAs (16, 18), and exploitation by aggressive genotypes (17). Similar selective pressures have been postulated to maintain diversity at nonself recognition loci in colonial ascidians, such as *Botryllus schlosseri* (78).

In *N. crassa*, the *het-c/pin-c* system is used as a model for understanding the molecular mechanisms of nonself recognition and HI and assessing the selection mechanisms for polymorphisms at *het* loci. Individuals within populations have one of three alternative specificities determined by the *het-c/pin-c* haplotype, i.e., *het-c1 pin-c1*, *het-c2 pin-c2*, or *het-c3 pin-c3* (38) (Fig. 1). The *het-c* locus encodes a plasma membrane protein (69); *het-c* allelic specificity is dependent upon a polymorphic region characterized by insertion/deletion which shows evidence of balancing selection (71, 88). The *pin-c* locus encodes a highly polymorphic protein containing a HET domain (38). The HET domain is an ~150-amino-acid region that is conserved among proteins involved in nonself recognition and HI in *N. crassa* and *Podospora anserina* (22, 76). Additional genes of unknown function that encode proteins with a HET domain are common in the genomes of filamentous ascomycete species (55 genes in *N. crassa*) (23). Nonallelic interactions between *het-c* and *pin-c* alleles of alternative specificities are essential for nonself recognition and HI (Fig. 1); allelic interactions of alternative *het-c* alleles increase the severity of the HI response (38). In addition to *het-c/pin-c* genetic interactions, physical interaction between HET-C1 and HET-C2 during HI has been shown by coimmunoprecipitation experiments (69). HI is associated with severe growth inhibition, repression of conidiation, and hyphal compartmentation and death, which is observed in forced heterokaryons, transformants, or partial diploids containing incompatibile *het-c/pin-c* haplotypes (Fig. 1).

A search for mutations that suppress *het-c/pin-c* HI identifi-
fied a locus called vib-1 (for vegetative incompatibility blocked) (91, 92). Strains containing mutations in vib-1 show deregulated conidiation and a slightly reduced growth rate compared to a wild-type strain with no allelic differences at het loci (right panel). het-c/pin-c nonallelic interactions are essential for HI (black arrows), while het-c allelic interactions increase the severity of the HI phenotype (dashed arrow) (38); only het-c1 pin-c1 and het-c2 pin-c2 interactions are shown. mat incompatibility requires the transcription factors mat A-1 and mat a-1 and the unlinked HET domain gene tol (60, 75). (B) Phenotypes of a het-c/pin-c-incompatible heterokaryon (left panel), a mat-incompatible heterokaryon (middle panel), and a compatible heterokaryon (right panel) after 3 days of growth at 25°C. Note the reduced growth and conidiation in the incompatible heterokaryons compared to those in the wild-type heterokaryon. (C) Confocal micrographs showing hyphal fusion and heterokaryon formation between two strains isogenic at all het loci (6103-4-3 het-c1 pin-c1 [ccg-1p–gfp] and RM1-01 het-c1 pin-c1 [ccg-1p–H1–dsRed]). First column, differential interference contrast (DIC) micrographs; second column, fluorescence micrographs showing cytoplasmic GFP fluorescence; third column, fluorescence micrographs showing nuclear dsRed fluorescence; fourth column, merged images from columns 2 and 3. Note the complete overlay of nuclear dsRed and cytoplasmic GFP fluorescence in the compatible fusion cell (top panels). In the incompatible compartmentalized fusion cell (bottom panels), the nuclear H1-dsRed signal is diffuse, presumably because of nuclear degradation (arrows). Bars = 10 μm.

FIG. 1. Nonself recognition, heterokaryon incompatibility, and programmed cell death triggered by genetic interactions between alleles of different specificity at either the het-c/pin-c haplotype or the mat locus. (A) Representation of the genetic interactions in het-c/pin-c (left panel) and mat (center panel)-incompatible heterokaryons carrying nuclei of different genotypes (plain or dashed colors) in a common cytoplasm or in a wild-type strain with no allelic differences at het loci (right panel). het-c/pin-c nonallelic interactions are essential for HI (black arrows), while het-c allelic interactions increase the severity of the HI phenotype (dashed arrow) (38); only het-c1 pin-c1 and het-c2 pin-c2 interactions are shown. mat incompatibility requires the transcription factors mat A-1 and mat a-1 and the unlinked HET domain gene tol (60, 75). (B) Phenotypes of a het-c/pin-c-incompatible heterokaryon (left panel), a mat-incompatible heterokaryon (middle panel), and a compatible heterokaryon (right panel) after 3 days of growth at 25°C. Note the reduced growth and conidiation in the incompatible heterokaryons compared to those in the wild-type heterokaryon. (C) Confocal micrographs showing hyphal fusion and heterokaryon formation between two strains isogenic at all het loci (6103-4-3 het-c1 pin-c1 [ccg-1p–gfp] and RM1-01 het-c1 pin-c1 [ccg-1p–H1–dsRed]). First column, differential interference contrast (DIC) micrographs; second column, fluorescence micrographs showing cytoplasmic GFP fluorescence; third column, fluorescence micrographs showing nuclear dsRed fluorescence; fourth column, merged images from columns 2 and 3. Note the complete overlay of nuclear dsRed and cytoplasmic GFP fluorescence in the compatible fusion cell (top panels). In the incompatible compartmentalized fusion cell (bottom panels), the nuclear H1-dsRed signal is diffuse, presumably because of nuclear degradation (arrows). Bars = 10 μm.
Ndt80p, a transcriptional regulator of genes required for the completion of meiosis and sporulation (12, 13). In addition to vib-1, the N. crassa homolog, namely, NCU09915 and NCU04729, in Aspergillus nidulans, mutations in a homolog of NDT80, called xprG, decreased extracellular protease production in response to carbon and nitrogen starvation; it is unknown whether xprG is required for heterokaryon incompatibility in A. nidulans (40). In this study, we evaluated whether vib-1 encodes a nuclear protein and whether VIB-1 localization varies during vegetative growth or during HI. We assessed the expression of potential target genes of VIB-1, including het-c, pin-c, het-6, and tol, during both vegetative growth and HI and determined whether mutations in vib-1 affect protease production during nitrogen and carbon starvation. Our data support the model that VIB-1 is a transcriptional regulator during both vegetative growth and HI and that VIB-1 is essential for the expression of genes involved in nonsel recognition and death.

MATERIALS AND METHODS

Neurospora strains and growth conditions. The strains used in this study are listed in Table 1. The vib-1 deletion strains (FGSC 11308 and FGSC 11309) were constructed by the Neurospora Program Project Grant Consortium (15). Strains were grown on Vogel’s minimal medium (MM) (83) with added supplements when required. Crosses were performed on Westergaard’s medium (84). When strains carrying auxotrophic markers were used as females, the required supplement was added to the mating medium. Alternatively, a heterokaryon established between the auxotrophic strain and the helper strain FGSC 4564 was subsequently used as a female (62). Measurements of growth rates, heterokaryon tests, and hyphal death analysis were performed as described previously (38).

For conidial production, conidia were inoculated into slants containing 2 ml of solid Vogel’s medium and incubated at 25°C for 7 days under an alternating 12-h light-dark cycle. Conidia from slants were obtained by a tip filled with cheesecloth, washed twice with 1 ml of water, and counted using a hemacytometer. Three independent measurements were done for each strain.

Transformation of N. crassa strains. Transformation of N. crassa conidia by electroporation was performed as described previously (53), using a 1.5-kV voltage and 1-mm-gap cells (R. L. Metzenberg and K. Black, personal communication). pmF272 constructs were targeted to the his-3 locus by homologous recombination (24); transformants with pmF272 constructs become his-3 - . The correct integration of the different constructs at the his-3 locus in heterokaryotic transformants was confirmed by Southern blotting. To recover homokaryotic strains, transformants were crossed with a his-3 strain, and his - progeny were selected and subsequently screened for expression of integrated constructs.

Construction of vib-1 and het-c alleles. To construct the cgg-lp-vib-1 - allele, a fragment containing the vib-1 open reading frame (ORF) was obtained by PCR, using plasmid SAH4.8 (a 4-kbp fragment including vib-1) as a template and primers 5 ’ vib1XbaI (5 ’-CGCTCTAGATGCGAGTTTAGAGCTGAG-3 ’) and 3 ’ vib1XbaI (5 ’-CCGTCGACATGCGAGTTTAGAGCTGAG-3 ’).
3′-vibPac (5′-CCGCGGATCCCGTATCATATGAGCTG-3′) and 3′-vibPac (5′-GGCTC TAAGACCGGTATGTTAGTGGC-3′) as primers and SAH4.8 as the template. The 625-bp vib-1 product was cloned into the pBluescript SK+ vector (Stratagene), cut with HindIII and SpeI, end filled with Klenow DNA polymerase, and blunt end ligated into the HindIII-digested restriction site upstream of the AATG-2XFLAG sequence. The 3.5-kbp vib-1 ORF was amplified using forward (5′-GGATCC ATAGTTGAGCAATTGTCTTTGTCTGTCTTGC-3′) and reverse (5′-GGATCC TCTACGTTAAGGGTGTTTGTCTCTTGC-3′) primers and cloned into the BamH I and blunt end ligated into the SmaI site of the pOKE103 vector (87). All constructs were verified by DNA sequencing, and their functionality was confirmed in vivo.

Storage buffer containing 18% Ficoll, 0.5 mM CaCl2, 1.5% (wt/vol) sucrose, and 20 mM KH2PO4.

For plasma membrane fractions, incompatible and compatible cultures were harvested after 36 and 48 h at 4°C with a mortar and pestle. Ground mycelia were resuspended in 20 mM HEPES-KOH, pH 7.5, 250 mM sucrose, 5 mM Mg(OAc)2, 2 mM EDTA, 2 mM diethiothreitol, 140 mM NaCl, 10% glycerol, 0.5% polyvinylpyrrolidone, and EDTA-free protease cocktail inhibitor (Roche). The homogenate was filtered through 10 layers of cheesecloth and centrifuged at 5,000 × g to yield crude extract. The crude extract was centrifuged for 90 min at 100,000 × g to yield cytosolic and membrane fractions. The cytosolic fractions were dialyzed (3-kDa cutoff) against 50 mM HEPES-KOH buffer, pH 7.5, and concentrated in a 50-m1 Amicon ultrafiltration cell (3-kDa cutoff) at 55 (ml/min). The membrane fractions were resuspended in the above buffer without sucrose and layered onto a 35 to 40% discontinuous sucrose gradient. The gradient was centrifuged for 2 h at 100,000 × g in an SW41 rotor, and material corresponding to the plasma membrane was collected and diluted in the above buffer without sucrose and glycerol. All fractions were frozen and stored at −80°C.

**Antibodies, immunoprecipitations, and immunoblotting.** Fungal tissue from cellular fractions was harvested at 4°C for 6 h with either anti-FLAG (1:1,000 dilution; Sigma Aldrich) or anti-HA (clone 12CA5; Roche) (1:1,000 dilution) antibodies. Protein G-agarose beads (50 μl; Roche) were added, and immunoprecipitation was carried out according to the manufacturer’s instructions. The maximum stringency of the salt wash was 750 mM. The resulting pellet was subject to electroelution on 4% to 20% HEPES-sodium dodecyl sulfate-polyacrylamide gels. Western analyses of nuclear and cytoplasmic fractions were performed using anti-FLAG M2 antibody (Sigma Aldrich) and anti-HA (clone 12CA5; Upstate Biotechnology) antibodies per the manufacturers’ instructions.

**Light and fluorescence microscopy.** For light or fluorescence microscopy, hyphae or conidia were transferred to a microscope slide and covered with 1× Vogel’s medium (83) and a cover slide. Analyses were performed using a Zeiss Axioskop II microscope equipped with an Endow GFP filter block (excitation, HQ470/40; emission, HQ525/50) and a Hamamatsu digital camera. For quantitative real-time fluorescence microscopy, micrographs were taken using an Axioskop II microscope equipped with an Endow GFP ByPass filter set (excitation, HQ470/40; emission, HQ525/50) and a Hamamatsu digital camera.

**Results**

**VIB-1 is a nucleus-localized protein.** Mutations in vib-1 suppress het-c/pin-c+ HI and also lead to deregulation of condia formation during vegetative growth, but they do not affect meiosis (91). We predicted that VIB-1 would localize to nuclei and that localization may be regulated during asexual reproduction and/or during HI. We constructed a full-length vib-1+ allele tagged with the sequence for GFP at the C terminus under the regulation of the vib-1 native promoter (vib-1+−vib-1− −gfp+). This construct was targeted to the his-3 locus in a vib-1 mutant strain (KD02-44), and homokaryotic progeny were obtained by crossing transformants to KD02-39 (Table 1). Homokaryotic vib-1+ (vib-1+−vib-1− −gfp+) progeny showed wild-type

**Organellar Fractionation.** For isolation of nuclei, transformants containing vib-1+ or vib-1− were transferred to a microscope slide and covered with 1× Vogel’s medium each. The culture was harvested, frozen in liquid nitrogen, and ground into a fine paste, and homogenized by 10 strokes with a Dounce pestle. The lysate was spun at 4,000 rpm in a JA-20 rotor for 8 min. The supernatant was transferred to tubes and centrifuged at 20,000 rpm in an SW41 rotor for 30 min. The resulting pellet was homogenized and stored in a nuclear storage buffer containing 18% Ficoll, 0.5 mM CaCl2, 1.5% (wt/vol) sucrose, and 20 mM KH2PO4.

For plasma membrane fractions, incompatible and compatible cultures were harvested after 36 and 48 h at 4°C with a mortar and pestle. Ground mycelia were resuspended in 20 mM HEPES-KOH, pH 7.5, 250 mM sucrose, 5 mM Mg(OAc)2, 2 mM EDTA, 2 mM diethiothreitol, 140 mM NaCl, 10% glycerol, 0.5% polyvinylpyrrolidone, and EDTA-free protease cocktail inhibitor (Roche). The homogenate was filtered through 10 layers of cheesecloth and centrifuged at 5,000 × g to yield crude extract. The crude extract was centrifuged for 90 min at 100,000 × g to yield cytosolic and membrane fractions. The cytosolic fractions were dialyzed (3-kDa cutoff) against 50 mM HEPES-KOH buffer, pH 7.5, and concentrated in a 50-m1 Amicon ultrafiltration cell (3-kDa cutoff) at 55 (ml/min). The membrane fractions were resuspended in the above buffer without sucrose and layered onto a 35 to 40% discontinuous sucrose gradient. The gradient was centrifuged for 2 h at 100,000 × g in an SW41 rotor, and material corresponding to the plasma membrane was collected and diluted in the above buffer without sucrose and glycerol. All fractions were frozen and stored at −80°C.

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growth and conidiation and were fully restored for \textit{het-c-pin-c} HI (data not shown).

We assessed the localization of VIB-1–GFP during vegetative growth and asexual reproduction. During vegetative growth, \textit{N. crassa} colonies grow as an interconnected network of multinucleate hyphae (30); over 28 different cell types are associated with the life cycle of \textit{N. crassa} (4). In a colony, leading hyphae are associated with growth at the periphery of the fungal colony, while large trunk (\textasciitilde 20 \mu m) and thinner branch/fusion (\textasciitilde 5 \mu m) hyphae make up the colony interior (34). In the vib-1 (\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) strain (444-7-1) (Table 1), VIB-1–GFP localized to nuclei, as shown by subsequent staining with the DNA-specific stain DAPI, in leading, trunk, and branch/fusion hyphae within the colony (Fig. 2A to C); there was no apparent differential localization of VIB-1–GFP to a subset of nuclei in the different types of vegetative hyphae.

In \textit{N. crassa}, asexual spore production is initiated in the colony interior by the production of aerial hyphae, which form the asexual spore-producing structures, termed conidiophores, and asexual spores, termed conidia. Mutations in \textit{vib-1} result in inappropriate temporal and spatial conidiation across a fungal colony (91). Although a \textit{vib-1} mutant (KD02-44) (Table 1) showed deregulation of conidiation, the number of conidia produced by the \textit{vib-1} mutant was similar to that for a wild-type strain (Fig. 3A and B). However, aerial hypha extension was substantially reduced in the \textit{vib-1} mutant (\textasciitilde 1 cm/day until day 3) compared to that in a wild-type strain (\textasciitilde 2 cm/day until day 3) (Fig. 3C and D). We therefore predicted that VIB-1 activity may be regulated during asexual development. Nuclear GFP fluorescence was observed in nuclei of mature conidia in strain 444-7-1 (\textit{vib-1}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}), but only cytoplasmic GFP fluorescence was observed in the conidiophores and in immature conidia (Fig. 2D and E). These data suggest that differential localization of VIB-1 in vegetative hyphae versus conidiophores may be an important cue for correct spatial and temporal regulation of asexual reproduction.

Overexpression of VIB-1–GFP resulted in a reduction in conidiation but did not recapitulate HI. We predicted that similar to the absence of \textit{vib-1}, overexpression of \textit{vib-1} would perturb the process of conidiation. We therefore constructed a \textit{vib-1}\textasciitilde-gfp\textsuperscript{+} allele regulated by either a \textit{ccg-1} or \textit{gpd} promoter; both promoters show strong expression in vegetative hyphae (25, 67). By real-time quantitative RT-PCR, strain 153-7-5 (\textit{gpd}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) showed a fourfold increase in \textit{vib-1} transcript level compared to that in a wild-type strain (data not shown). As expected, the intensity of GFP nuclear fluorescence in vegetative hyphae of strains 153-7-5 (\textit{his-3}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) and 151-3-6 (\textit{his-3}\textit{ccg-1}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) was substantially more intense than when \textit{vib-1}\textasciitilde-gfp\textsuperscript{+} was regulated by its own promoter. As with the native \textit{vib-1} promoter constructs, VIB-1–GFP fluorescence was observed in nuclei of mature conidia in 153-7-5 and 151-3-6 but was cytoplasmically localized in conidiophores and immature conidia. We evaluated the numbers of conidia produced in strains 151-3-6 (\textit{his-3}\textit{ccg-1}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) and 153-7-5 (\textit{his-3}\textit{gpd}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) compared to that for a wild-type strain (FGSC 2489). Both 151-3-6 and 153-7-5 produced fewer conidia than the wild type (Fig. 3B), although overexpression of \textit{vib-1} did not affect the length of aerial hyphae (data not shown).

Mutations in \textit{vib-1} suppress \textit{het-c-pin-c} HI. HI is associated with severe growth inhibition, repression of conidiation, and hyphal compartmentation and death in strains that are heterozygous for a \textit{het} locus (29, 70) (Fig. 1). We therefore assessed whether overexpression of \textit{vib-1} might recapitulate phenotypes associated with HI. Strains overexpressing \textit{vib-1} (\textit{ccg-1}\textit{vib-1}\textit{gfp} and \textit{gpd}\textit{vib-1}\textasciitilde-gfp\textsuperscript{+}) (151-3-6 and 153-7-5, respectively) (Table 1) showed a “flat” phenotype and reduced conidiation (Fig. 3A and B) but had an identical growth rate to that of a wild-type strain and lacked hyphal death (data not shown). These data indicate that overexpression of \textit{vib-1} is not sufficient to trigger phenotypes associated with HI.

VIB-1–GFP localization to nuclei increases during heterokaryon incompatibility. Mutations in \textit{vib-1} suppress phenotypic aspects associated with \textit{het-c-pin-c} HI, including growth inhibition, repression of conidiation, and hyphal death. We predicted that transcription of \textit{vib-1} and/or localization of VIB-1 during HI might also be a regulated process. We therefore assessed VIB-1 localization during \textit{het-c-pin-c} HI by comparing VIB-1–GFP fluorescence in a compatible heterokaryon (444-7-1 + FGSC 4564) to that in a \textit{het-c-pin-c}-incompatible heterokaryon (444-7-1 + C9-15) (Table 1). The VIB-1–GFP nuclear signal in the compatible heterokaryon (444-7-1 + FGSC 4564) was uniform across vegetative hyphae. However, in the aconidial, incompatible heterokaryon (444-7-1 + C9-15), the VIB-1–GFP signal in nuclei within some compartments in vegetative hyphae showed increased intensity (Fig. 4A and B). We quantified the intensity of the GFP fluorescence and determined that the nuclear VIB-1–GFP fluorescence signal in hyphal compartments of the incompatible heterokaryon (444-7-1 + C9-15) was approximately 10 times above that in nuclei of the compatible heterokaryon (444-7-1 + FGSC 4564) (Fig. 4D). We confirmed by cell fractionation and immunoblotting that VIB-1–GFP localized primarily to nuclei in both \textit{het-c-pin-c}-compatible and -incompatible transformants (Fig. 4E).
In WT hyphae, the fluorescence intensity of VIB-1–GFP in nuclei of vegetative hyphae was uniform across a colony (Fig. 2). In contrast, in the \textit{het-c/pin-c}-incompatible heterokaryon, not all hyphal compartments showed an increased intensity of VIB-1–GFP nuclear fluorescence. As shown previously, approximately 30% of the hyphal compartments in a \textit{het-c/pin-c}-incompatible heterokaryon, partial diploid, or transformant are dead (36, 87, 91), which can be visualized by using vital dyes, such as Evan’s or methylene blue. We therefore evaluated whether increased VIB-1–GFP nuclear fluorescence was correlated with dead hyphal compartments in the incompatible heterokaryon (444-7-1 + C9-15). Approximately 15% of the total number of hyphal compartments showed increased VIB-1–GFP nuclear fluorescence in an incompatible heterokaryon. The hyphal compartments that showed increased VIB-1–GFP nuclear fluorescence surrounded dead hyphal compartments, as evaluated by vital dye staining (Fig. 4C), and often these adjacent compartments subsequently died. These data suggest that a signal from a dying hyphal compartment is transmitted to adjacent compartments to increase localization of VIB-1 to nuclei, which may subsequently result in the death of these compartments. The increase in nuclear localization of VIB-1 during HI is specific, for no increase in the fluorescence intensity of H1-GFP in nuclei was observed in incompatible heterokaryons or during hyphal fusion between incompatible hyphae (Fig. 1).

During vegetative growth, \textit{vib-1} is required for the expression of \textit{pin-c}, \textit{het-6}, and \textit{tol}, but not \textit{het-c}. It is possible that mutations at \textit{vib-1} suppress \textit{het-c/pin-c} incompatibility because downstream death effectors are lacking or that \textit{vib-1} is required
FIG. 4. VIB-1-GFP localization to nuclei increases during HI. VIB-1-GFP localization was assessed in a compatible (444-7-1 plus FGSC 4564) (A) or incompatible (444-7-1 plus C9-15) (B and C) heterokaryon grown for 2 days at 20°C. Top panels in panels A, B, and C are DIC micrographs. For panel C, hyphae were stained with methylene blue, a stain that is taken up by dead hyphal compartments, which are therefore dark. Bottom panels show GFP fluorescence. Note that in the bottom part of panel C the dead hyphal compartments either lack GFP fluorescence (long arrow) or have a diffuse GFP signal (short arrow), while adjacent compartments show intense nuclear GFP signal. (D) Quantification of the intensities of GFP fluorescence in the cytoplasm and nuclei of the compatible (white bars) and incompatible (black bars) heterokaryons described above. Each value is the average of four independent experiments, with five measurements each. (E) A vib-1 pan-2 strain (X61-20) was complemented with the N-terminally epitope-tagged 2XFLAG-vib-1 construct. Total proteins (10 μg) from nuclear (N) and cytoplasmic (C) extracts were immunoblotted from a 4 to 20% HEPES-sodium dodecyl sulfate-polyacrylamide gel and probed with anti-FLAG antibodies. The same blot was probed with anti-tubulin and anti-histone H4 antibodies to check for the integrity of the cell fractions. Lanes 1 and 2, X61-20 (2XFLAG-vib-1; het-c2); lanes 3 and 4, X61-20 (2XFLAG-vib-1'); lanes 5 and 6, X61-20 (vib-1; het-c2); lanes 7 and 8, X61-20(pOKE103; pCB1004).

for the transcription of het-c and/or pin-c. We therefore evaluated by quantitative RT-PCR the hypothesis that VIB-1 is required for the expression of the het-c and pin-c genes. We also included tol and het-6 in our analysis because mutations in vib-1 also suppress mat and het-6 incompatibility (90; Smith, personal communication). mat incompatibility requires mat A-1, mat a-1, and tol, while het-6 incompatibility is mediated by un-24 (encoding ribonucleotide reductase) and het-6 (56, 76). pin-c, tol, and het-6 all encode predicted proteins containing the conserved ~150-amino-acid HET domain (38). For these experiments, we chose to use a strain deleted for vib-1 (FGSC 11309 [Table 1]) (15); we first evaluated the effect of a full deletion of vib-1 in FGSC 11309 on both mat and het-c HI.

A compatible heterokaryon grows 8 cm/day at 25°C and shows 2% hyphal death, which is associated with the older sections of the colony. A het-c-incompatible heterokaryon (FGSC 4564 + JH11) grew ~2 cm/day and showed 30% hyphal death throughout the colony, similar to previous results (36, 52, 87). A mat-incompatible heterokaryon (JH1 + C9-2) grew ~1 cm/day and showed 34% hyphal death. In contrast, a Δvib-1 heterokaryon suppressed for het-c HI (KD13-33 + KD13-01) or a Δvib-1 heterokaryon suppressed for mat HI (KD13-01 + KD13-23) exhibited a growth rate and conidiation pattern identical to those of a Δvib-1 compatible heterokaryon (KD13-21 + KD13-51) (~7 cm/day). However, a reduced percentage of hyphal death was still observed in both the Δvib-1 heterokaryon suppressed for het-c HI (14%) and the Δvib-1 heterokaryon suppressed for mat HI (7%). These data indicate that loss-of-function mutations in vib-1 fully suppress growth inhibition and the repression of conidiation associated with mat and het-c/pin-c HI but only partially suppress hyphal death, indicating that other unknown factors also play a role.

To determine if mutations in vib-1 affect transcriptional levels of het-c, pin-c, het-6, and tol, we assessed the relative expression levels of these genes during vegetative growth in a wild-type strain versus the Δvib-1 mutant. The expression levels of the control gene, act-1 (actin), were comparable in the Δvib-1 mutant (FGSC 11309), the wild type (FGSC 2489), and a vib-1 overexpression strain (153-7-5). As shown in Fig. 5, the expression of het-c was slightly increased in the Δvib-1 mutant (FGSC 11309) compared to that in a wild-type strain (FGSC 2489). However, the expression of pin-c, tol, and het-6 was almost undetectable in the Δvib-1 mutant, indicating that vib-1 is required for the expression of these HET domain genes during vegetative growth. We also evaluated whether overexpression of vib-1 (153-7-5) affected the transcription of het-c or pin-c; both genes showed a slight increase in expression level compared to that in the wild-type strain (~1.5-fold) (data not shown).

During HI, het-c expression is elevated. Our expression data suggested that mutations in vib-1 suppress het-c/pin-c, mat, and het-6 HI because VIB-1 is required for the expression of these genes. To further test this hypothesis, we evaluated the expression of het-c, pin-c, tol, and het-6 during het-c and mat incompatibility compared to the expression of these genes in Δvib-1.
heterokaryons suppressed for HI. The endogenous control gene, act-1, showed a slight decrease in relative expression level in both incompatible het-c and mat heterokaryons (C_T for het-c HI, 15.8 [standard deviation (SD) = 0.136]; C_T for mat HI, 16.61 [SD = 0.476]) compared to that in the WT (C_T, 14.11 [SD = 0.06]). Previous studies have shown that during HI a general decrease in transcription occurs, including the expression of many housekeeping genes (6, 7, 44), which was also reflected in our data. Therefore, the differences observed are conservative estimates of changes in relative gene expression levels.

The most striking result from evaluating expression levels of het-c, pin-c, tol, het-6, and vib-1 during HI was a dramatic increase in relative expression of het-c during both het-c and mat incompatibility (Fig. 6A and B); het-c showed a 12- to 18-fold increase in expression level relative to that in the WT. An increase in relative expression of the HET domain genes pin-c and het-6 during either het-c or mat incompatibility was not observed, although a modest increase in tol expression (~3-fold) during mat incompatibility was apparent (Fig. 6B). The relative expression level of vib-1 also showed a slight increase during HI.

We then assessed the expression of het-c, pin-c, tol, and het-6 in Δvib-1 heterokaryons suppressed for HI. The act-1 expression level in the Δvib-1 heterokaryon suppressed for het-c HI (KD13-01 + KD13-33) was very similar to act-1 expression levels in a wild-type strain (FGSC 2489) and a Δvib-1 compatible heterokaryon (KD13-21 + KD13-51). As shown in Fig. 6A, the relative expression level of het-c was also elevated in the Δvib-1 heterokaryon suppressed for het-c HI (KD13-01 + KD13-33), similar to the case for the het-c-incompatible heterokaryon. As with the Δvib-1 mutant, the expression of tol and het-6 was virtually undetectable in the (KD13-01 + KD13-33) heterokaryon. Surprisingly, the pin-c transcript was detected in the Δvib-1 heterokaryon suppressed for het-c HI (KD13-01 + KD13-33), at a relative expression level similar to those detected in both the wild-type strain and the het-c-incompatible heterokaryon (FGSC 4564 + JH1). These data indicate that during het-c HI, a transcriptional regulator other than VIB-1 can induce the expression of pin-c. Since the (het-c1 pin-c1; Δvib-1 + het-c2 pin-c2; Δvib-1) heterokaryon is almost completely suppressed for HI, these data indicate that additional downstream functions mediated by vib-1 are required for het-c/pin-c HI.

For comparison to the Δvib-1 heterokaryon suppressed for het-c HI, we also evaluated the expression of het-c, pin-c, tol, and het-6 in a Δvib-1 heterokaryon that was suppressed for mat HI (KD13-01 + KD13-23). The act-1 expression level in the (KD13-01 + KD13-23) heterokaryon was similar to that in the mat-incompatible heterokaryon (C9-2 + JH1) (C_T for mat HI, 16.61 [SD = 0.476]; C_T for suppressed Δvib-1 mat HI, 15.91 [SD = 0.044]). As shown in Fig. 6B, the relative expression level of het-c was also elevated in the Δvib-1 heterokaryon suppressed for mat HI (KD13-01 + KD13-23), as observed in the het-c- and mat-incompatible heterokaryons and the Δvib-1 heterokaryon suppressed for het-c HI. However, tol, pin-c, and het-6 were virtually undetectable in the (KD13-01 + KD13-23) heterokaryon (Fig. 6B).

Mutations in vib-1 reduce extracellular protease production.

In A. nidulans, mutations in a homolog of vib-1 (NDT80),

![FIG. 6. vib-1 regulates HET domain genes and is required for additional downstream functions during HI. Gene expression was evaluated by quantitative RT-PCR for incompatible heterokaryons, with or without vib-1. (A) Comparison of relative gene expression levels during het-c/pin-c incompatibility. For het-c HI (het-c/pin-c incompatible heterokaryons, RNAs were isolated from het-c/pin-c incompatible heterokaryons (FGSC 4564 [het-c1 pin-c1 Δm1] plus JH1 [het-c2 pin-c2 A]) (Δm1), vib-1 strain), and vib-1 mat HI (heterokaryon carrying incompatible het-c/pin-c haplotypes and a deletion of vib-1 [KD13-33 [het-c1 pin-c1; Δvib-1 A] plus KD13-1 [het-c2 pin-c2; Δvib-1 a]], This heterokaryon was morphologically indistinguishable from the compatible Δvib-1 FH described above. The WT strain is FGSC 2489. (B) Comparison of relative gene expression levels during mat incompatibility. For mat HI (heterokaryon incompatibility), RNAs were isolated from a mat incompatible heterokaryon (C9-2 [het-c2 pin-c2 a] plus JH1 [het-c2 pin-c2 A]), For the WT FH, RNAs were isolated from a compatible heterokaryon which carried a deletion of vib-1 (KD13-21 [het-c1 pin-c1; Δvib-1 A] plus KD13-31 [het-c1 pin-c1; Δvib-1 A]; Δm1 strains are null for mat incompatibility [31]). For the Δvib-1 forced heterokaryon (FH), RNAs were isolated from a compatible heterokaryon carrying the vib-1 mutation (KD13-21 [het-c1 pin-c1; Δvib-1 A] plus KD13-51 [het-c1 pin-c1; Δvib-1 A]). For the Δvib-1 het-c FH, RNAs were isolated from a heterokaryon carrying incompatible het-c/pin-c haplotypes and a deletion of vib-1. For the Δvib-1 mat FH, RNAs were isolated from a compatible heterokaryon which also carried a deletion of vib-1 (KD13-21 [het-c2 pin-c2; Δvib-1 a] plus KD13-23 [het-c2 pin-c2; Δvib-1 A]). This heterokaryon was morphologically indistinguishable from the compatible Δvib-1 FH described above. For the WT sample, expression of the act-1 (actin) gene was used for normalization of relative expression levels among the different samples. The asterisk indicates that the melting curve showed that the signal obtained from the Δvib-1 mat FH sample does not correspond to the vib-1 transcript but to primer dimer amplification.](image-url)
called xprG, resulted in mutants that showed a drastic decrease in extracellular protease production in response to carbon starvation and a reduction in protease production upon nitrogen and sulfur starvation (40). xprG shows comparable similarities to both vib-I and NCU04729, while NCU09915 is most similar to NdIt80p. In P. anserina, heterokaryon incompatibility has been shown to be associated with proteolytic activity (3, 45, 61) and the response to starvation (20, 64, 68). We therefore evaluated whether mutations in vib-I also affect extracellular protease production.

In N. crassa, extracellular protease (de novo) biosynthesis requires both catabolite derepression (nutrient limitation) and induction by an exogenous protein present in the growth medium (49). As shown in Fig. 7, extracellular protease production was nearly abolished in the Δvib-I mutant (FGSC 11309) in response to both nitrogen and carbon starvation compared to that in the wild-type strain (FGSC 2489) (P < 0.001). The reduction in extracellular protease production by mutations in vib-I was more severe than that observed in the A. nidulans xprG mutant, especially in response to nitrogen starvation. These data indicate that in addition to being necessary for pin-c, tol, and het-6 expression, functional vib-I is required for the production of extracellular proteases in response to both nitrogen and carbon starvation.

vib-I is not required for HET-C heterocomplex formation.

Our quantitative RT-PCR results indicated that vib-I is not required for expression of het-c. Previously, we determined that the formation of a HET-C1/HET-C2 heterocomplex was associated with nonself recognition and HI; the pin-c locus is not required for HET-C heterocomplex formation (38, 69). Thus, we evaluated whether functional vib-I is required for HET-C heterocomplex formation, possibly by regulating the expression of an unknown gene required for HET-C1/HET-C2 heterocomplex formation. We constructed FLAG epitope-tagged het-c1 in a vector encoding hygromycin resistance (het-c1–FLAG) (10) and an HA epitope-tagged het-c2 allele in a pan-2" vector (het-c2–HA) (87); both constructs were regulated by the native het-c promoter. These constructs were co-transformed into a het-c2 pin-c2; pan-2 mutant (G30) (Table 1) and a het-c2 pin-c2; vib-1; pan-2 mutant (G11) (Table 1). The het-c2 pin-c2; pan-2 (het-c1–FLAG; het-c2–HA) transformants showed a typical het-c incompatibility phenotype (~1 cm/day). The het-c2 pin-c2; vib-1; pan-2 (het-c1–FLAG; het-c2–HA) transformants grew significantly better than incompatible transformants, although not quite as well as vector controls. Anti-FLAG antibodies were used for immunoprecipitation of plasma membrane fractions, which were subsequently subjected to immunoblot analysis using anti-HA antibodies. Consistent with previous results (69), HET-C1–FLAG and HET-C2–HA coimmunoprecipitated from plasma membrane fractions from incompatible transformants (G30 [het-c1–FLAG; het-c2–HA]) (Fig. 8, lane 6), while HET-C1–FLAG and HET-C2–HA were undetectable in compatible transformants (Fig. 8, lane 1, and data not shown). In the vib-I (het-c1–FLAG; het-c2–HA) transformants, both HET-C1–FLAG and HET-C2–HA also coimmunoprecipitated from plasma membrane fractions (Fig. 8, lane 5). These data indicate that functional vib-I is not required for HET-C heterocomplex formation.
DISCUSSION

VIB-1 is differentially localized during vegetative growth and HI. In this paper, we determined that vib-1 encodes a nuclear protein that shows dynamic localization during both asexual differentiation and HI. During vegetative growth, VIB-1–GFP localized to all nuclei in hyphae. This observation is in contrast to the case for a number of transcriptional regulators that show differential nuclear localization during nutrient depletion (81) or developmental processes (1, 2) or for proteins/mRNA that show differential localization throughout a fungal colony (79, 82). Although VIB-1–GFP was observed in nuclei of all vegetative hyphae, it was cytoplasmically localized in conidiophores and immature conidia. The process of conidiation in N. crassa occurs in response to desiccation or nutrient starvation and is a developmentally regulated process (77). The reappearance of VIB-1–GFP fluorescence in nuclei was associated with mature conidia, which is correlated with the formation of the double septum. Dynamic localization of VIB-1–GFP was observed when vib-1 was under the regulation of native and heterologous promoters, indicating that differential localization of VIB-1–GFP is regulated posttranscriptionally. The vib-1 mutant shows reduced aerial hypha extension and inappropriate conidiation, indicating that VIB-1 is important in the coordination of sporulation, perhaps by affecting developmental programs that respond to starvation.

During HI, an increase in VIB-1–GFP nuclear fluorescence was observed in hyphal compartments surrounding dead/dying hyphal compartments. Hyphal fusion between compatible hyphae is associated with the movement of organelles/nuclei through incomplete septa; large alterations in cytoplasmic flow in fusion hyphae are often observed (34). However, in fusion cells between incompatible strains containing alternative het-c/pin-c alleles, these incomplete septa are rapidly plugged (29) (Fig. 1). Death of the hyphal fusion compartment is apparent at ~20 min postfusion and is characterized by permeabilization of the plasma membrane, the presence of terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive nuclei, and the formation of large vesicles, which subsequently lyse (26, 29, 36, 52, 87). Overexpression of vib-1 did not recapitulate HI, indicating that factors in addition to VIB-1 are required to signal and mediate death in N. crassa following nonself recognition.

An increase in het-c expression is associated with heterokaryon incompatibility. A striking result from this study was the observed increase in expression of het-c under conditions of both mat and het-c HI and in Δvib-1 heterokaryons suppressed for mat and het-c HI. Mutants deleted for het-c have no vegetative or sexual phenotype other than the fact that they will form compatible heterokaryons with strains, regardless of their het-c/pin-c haplotype (72). Preliminary data suggest that het-c, pin-c, tol, and het-6 and vib-1 are induced by treatment with 3-aminotriazole, which mimics histidine starvation, but not by rapamycin, which inactivates the TOR kinase and which is associated with nutrient sensing in S. cerevisiae (66). The identification of the regulators of het-c expression upon nonself recognition may reveal players involved in HI in addition to vib-1.

VIB-1 is required for the expression of HET domain genes. Consistent with its predicted function as a transcription factor, vib-1 is required for the expression of the HET domain genes pin-c, tol, and het-6. Mutations in vib-1 suppress het-c/pin-c and mat HI, partially suppress het-6 HI, and increase the recovery of het-e- and het-δ-incompatible partial diploid progeny (90). These data indicate that VIB-1 is a global mediator of fungal HI in Neurospora. A search of 500-bp promoter segments of the pin-c, het-6, and tol genes for common motifs identified a 12-bp consensus sequence (CTAC/GG/CA/CT/AC/GC/AC AC/T [E value = 3.10e -18]). Interestingly, this consensus sequence was enriched in the set of 55 predicted HET domain genes (P = 0.004 by the two-tailed Fisher test). A number of these HET domain genes are polymorphic among isolates of Neurospora (N. L. Glass, unpublished results), which is a characteristic of cloned het loci (27, 70). The closest paralog of vib-1, NCU04729, also contained the 12-bp consensus sequence, but NCU09915, the paralog with the highest similarity to NDT80, did not. These data suggest that NCU04729 might also be involved in HI and extracellular protease production.

Five of the six molecularly characterized het interactions involve predicted proteins that share a common ~150-amino-acid HET domain (Pfam06985), including the proteins encoded by N. crassa pin-c, tol, and het-6 and P. anserina het-D and het-E (22, 76). No function for these genes is known other than their role in HI. Our model for HI is that nonself recognition is mediated by a HET domain-containing protein interacting with another protein partner whose function can be diverse (e.g., transcription factors, such as those encoded by mat A-1 and mat a-1; plasma membrane proteins, such as that encoded by het-c, or ribonucleotide reductases, such as that encoded by ur-24) (Fig. 9). Predicted HET domain genes are specific to and common among filamentous ascomycete genomes (23). We hypothesize that proteins containing this domain might be recruited to a cell death signal transduction pathway and interact with downstream partners to trigger a common cellular response, mediated partly by VIB-1, to cause HI.

VIB-1 shows similarity to the DNA binding domain of Ndt80p; Ndt80p is involved in the activation of genes expressed during the middle stages of meiosis and the sporulation pathway (12, 13, 93). The crystal structure of Ndt80 bound to the middle sporulation element showed that Ndt80 is a member of the immunoglobulin-fold family of transcription factors (46, 57); other members include p53, STAT, and NF-κB. The residues implicated in DNA binding are conserved in NCU09915 (putative NDT80 ortholog [46]), but not all of them are conserved in VIB-1. In Candida albicans, an ortholog of NDT80 is required for the expression of CDR1, an ATP efflux pump gene (11); mutations in CaNDT80 abolished the induction of CDR1 expression by antifungal agents. These data suggest that transcriptional regulatory networks regulated by homologs of NDT80 have diverged among the fungi.

Regulation of extracellular protease production by vib-1. In both N. crassa and A. nidulans, the production of extracellular proteases is subject to regulation by carbon, nitrogen, and sulfur availability and pH. In N. crassa, two or three extracellular protease types (acid proteases, an alkaline protease, and/or a neutral protease) are secreted in response to nitrogen, sulfur, or carbon starvation (14, 20, 48, 49). Derepression of an alkaline protease (48) is blocked in N. crassa strains carrying loss-of-function mutations in nit-2 (an ortholog of A. nidulans
areA) and cys-3 (33). The nit-2 and cys-3 loci encode the major transcriptional activators of nitrogen and sulfur acquisition genes, respectively (54, 55).

In A. nidulans, areA mutants have abolished production of extracellular proteases in response to nitrogen limitation (39). Mutations in xprG abolish extracellular protease production in response to carbon starvation and reduce extracellular protease production in response to nitrogen starvation (40). By epistasis experiments, it was shown that areA acts downstream of xprG (40). Dynamic nuclear accumulation of AreA was observed in response to nitrogen starvation (81), suggesting that XprG may function in regulating nuclear localization of AreA in A. nidulans.

Although the xprG mutant was impaired in extracellular protease production in response to carbon and nitrogen starvation, the secretion of other extracellular enzymes, such as amylases and xylanases, was not affected (40). In N. crassa, NCU04729 and vib-1 are equally similar to xprG. Our results indicate that mutations in vib-1 impair the secretion of extracellular proteases in response to both carbon and nitrogen starvation, a phenotype similar but not identical to that for xprG mutants. Secreted proteases are important in industrial fungi (51) and during pathogenesis of plant, other fungal, and human pathogens by filamentous fungi (43, 51); in C. albicans, aspartyl proteases are secreted during infection and are implicated in adherence and the disease process (59). It is possible that the vib-1 orthologs in these fungi play an important role in the regulation of extracellular protease activity that could be manipulated for industrial enzyme production or an understanding of mechanisms associated with pathogenesis.

Link between starvation, vib-1 function, and death by heterokaryon incompatibility. The data presented in this paper suggest a link between starvation and HI. Previous work with P. anserina has shown a connection between the cellular response to nutrient starvation and HI (8, 19). These studies suggested that autophagy, which is regulated by TOR in S. cerevisiae, may be associated with HI in P. anserina (65). However, inactivation of the P. anserina orthologs of S. cerevisiae ATG1 (a kinase involved in the early stage of autophagy) and ATG8 (required for autophagosome formation) suppressed autophagy but accelerated cell death associated with HI (64). These observations suggest that within the fungal mycelium, both death-inducing and survival signal transduction mechanisms are simultaneously activated during HI (27). Heterokaryon incompatibility is also associated with proteolytic activity in P. anserina (3, 45, 61). In A. nidulans, caspase-like activities were associated with asexual sporulation (80), and in carbon-depleted cultures of both A. nidulans and the human pathogen Aspergillus fumigatus, apoptotic phenotypes characterized by TUNEL and annexin V staining were observed (21, 58). TUNEL-positive nuclei are also associated with het-c/pin-c HI (52). These observations suggest a link between signal transduction mechanisms associated with starvation, apoptosis, and HI, which may be mediated by VIB-1. In S. cerevisiae, a number of interconnected signaling pathways are regulated in response to nutrient sensing/starvation, including the protein kinase A, Snf1, TOR, Pho85, Gcn2, and Pas pathways (85). It will be interesting to determine the relationship of VIB-1 to these nutrient-sensing signal transduction pathways and their possible role during HI.

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