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Genetics

# Inactivation of the *Podospora anserina* vegetative incompatibility locus *het-c*, whose product resembles a glycolipid transfer protein, drastically impairs ascospore production

(heterocaryosis/filamentous ascomycete/sexual reproduction)

SVEN SAUPE*, CECILE DESCAMPS, BEATRICE TURCQ†, AND JOEL BÉGUERET

Laboratoire de Génétique, UPR 9026 Centre National de la Recherche Scientifique, Université de Bordeaux II, Avenue des Facultés, 33400 Talence, France

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**ABSTRACT** **The *het-c* locus contains different alleles that elicit nonallelic vegetative incompatibility through specific interactions with alleles of the unlinked loci *het-e* and *het-d*. The *het-c2* allele has been cloned. It encodes a 208-amino acid polypeptide that is similar to a glycolipid transfer protein purified from pig brain. Disruption of this gene drastically impairs ascospore production in homozygous crosses, and some mutants exhibit abnormal branching of apical hyphae. The protein encoded by *het-c2* is essential in the biology of the fungus and may be involved in cell-wall biosynthesis.**

Filamentous ascomycetes are naturally capable of forming heterokaryons by somatic fusion of hyphae from different strains. Genetically controlled impairment of heterokaryosis is referred to as vegetative or heterokaryon incompatibility. In most cases, heterokaryons cannot be formed between different geographical isolates of the same species. The genetic basis of vegetative incompatibility has been studied in various ascomycetes including *Neurospora crassa*, *Aspergillus nidulans*, and *Podospora anserina* (for reviews, see refs. 1 and 2). Vegetative incompatibility results from genetic differences at specific *het* loci. In most cases, heterokaryon incompatibility is controlled by alternate alleles at a single locus, but vegetative incompatibility systems involving interactions between nonallelic genes have also been described in *P. anserina* (3) and *Cryphonectria parasitica* (4).

Molecular analysis of *het* genes has been initiated in various species. The *mt a-1* and *mt A-1* genes of *N. crassa* elicit vegetative incompatibility between strains of opposite mating type (*a* and *A*) in addition to controlling the sexual functions of mating type. They encode dissimilar proteins that presumably regulate the transcription of a set of genes involved in mating and fructification (5, 6). How vegetative incompatibility is elicited by *mt a-1* and *mt A-1* has not yet been elucidated. Two incompatible alleles of the *het-s* locus of *P. anserina* have been cloned (7), and their gene product has been characterized (8). Strains containing a disrupted *het-s* locus retain a wild-type phenotype, suggesting that expression of this locus is not essential (9). The *het-C* and *het-6* incompatibility loci of *N. crassa* have also been cloned (G. Kuldau and M. Smith, personal communication). All these studies concern only allelic *het* genes. In the present study we report characterization of a nonallelic incompatibility gene, the *het-c2* gene of *P. anserina*.

In this species, the heterokaryotic cells formed by anastomosis of hyphae from strains that differ at a single *het* locus are rapidly destroyed by a lytic process (10). The incompatibility reaction can be easily observed at the macroscopic level. Incompatible strains grown on solid medium produce an abnormal contact named barrage (11). The genetic determination of vegetative incompatibility (3, 12) and the biochemical modifications that occur during the cell-death reaction elicited by interaction of the *het* genes (13) have both been studied. *het-c* is one of nine known *het* loci of *P. anserina*. This locus is involved in two nonallelic incompatibility systems: *het-c*/*het-e* and *het-c*/*het-d*. Each of these three loci is multiallelic. Each *het-c* allele is incompatible with a specific set of *het-e* and *het-d* alleles (Fig. 1).

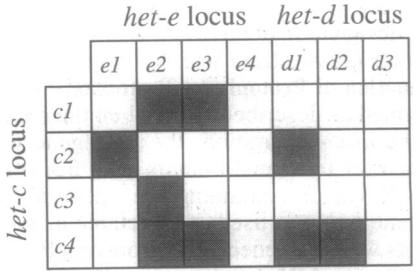


FIG. 1. Incompatible interactions between the different *het-c* alleles and alleles at *het-e* and *het-d* loci. Dark and white squares indicate incompatible and compatible combinations, respectively. This is a compilation of results from genetic analysis of 17 wild-type isolates (3). For each locus, the same designation has been attributed to the different alleles from wild-type strains that have the same specificity. *het-c1*, *het-c2*, *het-c3*, and *het-c4* designate the alleles present at the *het-c* locus in wild-type isolates A, s, H, and M, respectively. *het-e1*, *het-e2*, *het-e3*, and *het-e4* refer to the *het-e* alleles from strains A, C, F, and s; *het-d1*, *het-d2*, and *het-d3* designate alleles present at the *het-d* locus in isolates A, F, and s.

We have cloned the *het-c2* allele.‡ It encodes a 208-amino acid polypeptide that is similar to a glycolipid transfer protein. The *het-c2* gene product has been characterized as a 23-kDa protein. Mutant strains containing a disrupted *het-c* locus have been constructed. Their phenotype revealed that inactivation of *het-c* affects the production of ascospores. Some *het-c* mutations also disturb hyphal branching. This shows that *het-c* has a primary cellular function in addition to eliciting the incompatibility reaction.

## MATERIALS AND METHODS

***P. anserina* Strains.** *P. anserina* is a heterothallic ascomycete. Its life cycle and general methods for genetic anal-

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Abbreviations: GLTP, glycolipid transfer protein; ORF, open reading frame.

*Present address: Biotechnology Laboratory, Room 237, Wesbrook Building, University of British Columbia, Vancouver, BC Canada V6T 1W3.

†To whom reprint requests should be addressed.

‡The sequence reported in this paper has been deposited in the Genbank data base (accession no. U05236).

ysis have been described (14, 15). Compatibility between strains can be determined by confrontation on corn meal agar medium. Incompatibility results in the formation of a barrage, a dense and unpigmented line, in the region where the strains meet (11). The three *het* loci, *het-c*, *het-d*, and *het-e*, define two incompatibility systems: *het-c*/*het-e* and *het-c*/*het-d*. At each locus, alleles with different specificities have been identified. Genetic analysis of 17 wild-type strains has shown that the wild-type *het-c* alleles fall into four phenotypic classes (3). Specificity of each allele is defined by the spectrum of its incompatibility interactions with the different alleles of the antagonistic *het-e* and *het-d* loci (Fig. 1). All strains used were isogenic to wild-type isolate s (11), except that different alleles are present at the *het-c*, *het-e*, and *het-d* loci. The strains are designated by their Het-c, Het-e, and Het-d phenotypes. When the Het-e or Het-d phenotype of a strain is neutral (i.e., is compatible with all known *het-c* strains), the designation of this locus is omitted. For example, a *het-c2 het-d3 het-e4* strain will be designated as *het-c2*.

**Transformation of Protoplasts.** Protoplasts were prepared and transformed as described (16). When the recipient strain contained the *ura5-6* mutation, the *ura5* gene was used as selectable marker for transformants (17). For other strains, the pMOcosX vector containing the bacterial hygromycin resistance gene *hph* was used as selectable marker (18), and transformants were screened on hygromycin B at 100 μg/ml.

**cDNA Cloning and DNA Sequencing.** General methods for nucleic acid analysis and vector construction were as described (19). Total RNA was isolated from mycelium grown in liquid medium by a phenol extraction method, and poly(A)$^+$ RNA was purified as described (20). For intron mapping, single-stranded cDNA was synthetized from poly(A)$^+$ RNA, using the cDNA-Synthesis-Plus kit (Amersham) according to the manufacturer's protocol. The *het-c* cDNA was amplified by PCR using the *het-c*-specific primers SEQ5 (5′-CCCAGCTTATCTCGAGTTGAG-3′), positions 471–490, and INT1 (5′-CTTGTCGAGAGCAACAAGG-3′), positions 1204–1222. The amplified fragment was cloned in pBluescript II SK$^+$. All DNA sequences were determined on both strands using the dideoxynucleotide chain-termination reaction method (21) and synthetic oligonucleotides as primers.

**Immunoblotting.** Rabbit antibodies were raised to the C-terminal portion of the *het-c* open reading frame (ORF) expressed as a glutathione *S* transferase–*het-c* fusion protein in pGEX plasmid (22). The 174-bp *Eco*RI–*Hin*dIII fragment, coding for amino acids 115–172, was cloned in the *Eco*RI and *Hin*dIII sites of the pGEX A plasmid and expressed in *Escherichia coli*. The fusion protein was electroeluted after SDS/PAGE of the insoluble fraction of bacterial proteins. Proteins were extracted from mycelia as described (9). Procedures for SDS/PAGE and immunoblotting were as described (23).

## RESULTS

**Cloning the *het-c2* Allele.** Previous genetic studies revealed that the different wild-type *het-c* alleles are codominant in heterokaryotic strains. This offered the opportunity to clone any *het-c* allele by expression. The *het-c2* allele was isolated by phenotypic expression upon transformation in a strain containing the *het-c3* allele. It was cloned using the sib-selection procedure (24). A library of the genomic DNA of the *het-c2* strain in the pHC79-ura5 cosmid (7) was used to transform a *het-c3 ura5-6* strain. About 4500 prototrophic transformants from 10 cosmid pools were obtained and tested for incompatibility with the *het-e1* strain. One transformant was found to be incompatible with the tester. Three rounds of sib-selection were used to isolate the cosmid that conferred the Het-c2 phenotype. It contained a genomic DNA insert of

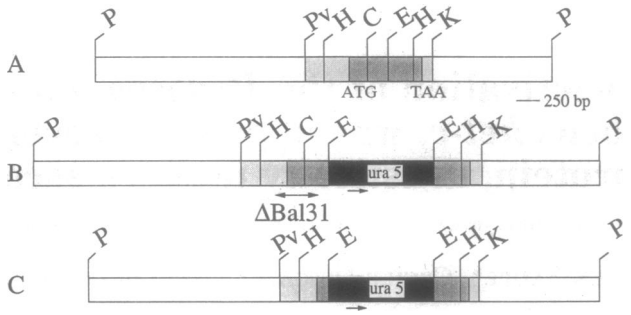


FIG. 2. Physical map of *het-c2* and derived constructions. (*A*) Map of the cloned *Pst* I fragment; the stippled box shows the sequenced region. Initiation and stop codons of the ORF are marked. (*B*) Map of the insert cloned in pCura to disrupt *het-c2*. The *ura5* gene is shown as a black box. The arrow under *ura5* gives orientation of its transcription. (*C*) Map of the insert in pCuraΔ used to disrupt *het-c2*. The region of pCura deleted using Bal-31 is marked by the double-headed arrow under the pCura map. P, *Pst* I; Pv, *Pvu* II; H, *Hin*dIII; C, *Cla* I; E, *Eco*RI; K, *Kpn* I.

≈40 kbp. Genetic analysis of six *het-c2* transformants has shown that cosmid integration occurred at the *het-c* locus in four transformants. The frequent targeting to this locus is strong evidence that the cosmid contained the *het-c* locus. The *het-c2* gene was subcloned as a 5.2-kbp *Pst* I fragment (Fig. 2*A*); a 1.6-kbp *Pvu* II–*Kpn* I fragment was sufficient to confer the Het-c2 phenotype.

The 1.6-kbp *Pvu* II–*Kpn* I fragment was completely sequenced on both strands (Fig. 3). This fragment contains an ORF beginning with an ATG at position 530 and terminating

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AAGCTTTATGCAATATCGAAGGTGAAAACGAGACGAATCAGCTGGCAGGATGGGGTGCTT      60
CCTCAGCTGCCCTGTCCCAGCGAGAAGTGACTGCGCATCATTCGACCTTTCCTGCCCATG     120
ACCTTTTTTTTTGTGCCCGAGGGCTCTTTGAGGGTTGGAAGATAGCAGTGCTTGTTTGATT    180
TGAACCCCGTATCTCTCCTTTCAAAAACAAGTGCCCATGCGACGGCGATGTCCGCATTTG     240
CCAAGAGATTGGTCGCAACATCACACCTTCACCTTCAGACACACGTGATGGATGCTCACC     300
TTCACCAAGCCAACGGTGAAGCGGCTGCGGGGGCGGGGTAACTCCCTGTCCTGTTAGGGG     360
CGAGGGCGGGGCAGCGGCTAGTGTAGCGGTTAGGGGTCCGTCGGGCATGTTCCAGCTCCC     420
GTCCAAGGCTCAATTGACCGCCTCGTGTTCCATGTTCCTGCACCGAACCCCCAGCTTATC     480

TCGAGTTGAGTCCAACCTTCAACCAACCTTCACCATCCAAAACACCACCATGGCCGCCGC     540
                                                  M  A  A  A       4

CGCCGTTGTCCAAATCCCCGCCGGGGCTACCTTCCTTGAGACCTTCAAGAAGTCCTTCGT     600
  A  V  V  Q  I  P  A  G  A  T  F  L  E  T  F  K  K  S  F  V      24

TGATGTCCCAATCGATGCTGAGAAGGGCAATGCCATCTCCACCGCCGAGTTCCTTGAGGC     660
  D  V  P  I  D  A  E  K  G  N  A  I  S  T  A  E  F  L  E  A      44

GGCCGAGTCCTTGACCACCATGTTCGATGTGCTCGGCTCCATCGCCTTCTCCCCCGTCAA     720
  A  E  S  L  T  T  M  F  D  V  L  G  S  I  A  F  S  P  V  K      64

GACGGATATGTTGGGCAACGTCGAGGTGCGCTGCTATTATACCTGTTGGTTAGGACGTCA     780
  T  D  M  L  G  N  V  E                                          72

GCTAACCACCCTTTCCTGCTAGAAAATTCGCAAGCGCATGCTTGCCGCCCCCCTCGAGTC     840
                        K  I  R  K  R  M  L  A  A  P  L  E  S      85

CCAGAACATCCAGGATCTTGTGAGGAACGAGCTCAAGACCAAAAGCCATACCGCGACGGA     900
  Q  N  I  Q  D  L  V  R  N  E  L  K  T  K  S  H  T  A  T  E     105

GGGGTTGCTGTGGCTGGTCAGGTGAGGGCGCCTTGATCTGTTCCATAGCCAAGTCAAGTC     960
  G  L  L  W  L  V  R                                            112

ACTGACAGTGGTCTGTGACAGGGTCTCGAATTCACATGCATTGCTCTTAGCAAGAACAT    1020
                        G  L  E  F  T  C  I  A  L  S  K  N  I     125

CGGCTCAACAGAGGAGCTCGCCGACTCCTTCCGCGGGTCTTACAGGGTGACTCTCAAGCC    1080
  G  S  T  E  E  L  A  D  S  F  R  G  S  Y  R  V  T  L  K  P     145

ACACCACAGCTTCCTGGTGAAGCCCATCTTCAGCGCCGCCATGAGCGCGTGCCCATACCG    1140
  H  H  S  F  L  V  K  P  I  F  S  A  A  M  S  A  C  P  Y  R     165

CAAGGACTTCTACGCCAAGCTTGGTGACGACGAGCAAAAGGTTCAAGAGGAGCTTCGCGA    1200
  K  D  F  Y  A  K  L  G  D  D  E  Q  K  V  Q  E  E  L  R  E     185

ATACCTTGTTGCTCTCGACAAGATCGTCAACATTCTCAAGAGATTCTTGGAGAGCAAGGA    1260
  Y  L  V  A  L  D  K  I  V  N  I  L  K  R  F  L  E  S  K  E     205

GGCCAAGTGGTAAACGGTGGGGAAAACCATTTTCGGGGGACGCAAAAAGCATACGCATCT    1320
  A  K  W  -                                                      208

TATACGGGATAATACCTACCCATGCCACACACATGCAATTCTCACTCAAATTGCCAGAGC    1380
TCAGCTCAGGTCCCAGCTCTCAGCTCTCAGCTCCCAGTCACTGCCGACCCTCACCACCGG    1440
CTGTGCTCCGCTCTATGTAGTCTCCACTAGATAGGAGCTCAAACATGGTACCATCCCCCA    1500
CCATCAACTACACC                                                  1514
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FIG. 3. Nucleotide sequence of *het-c2* and flanking regions and amino acid sequence of the predicted polypeptide. Intron sequences are printed in italics. Boundaries and putative lariat sequences are underlined.

with a TAA at position 1271 and encoding a putative 208-amino acid polypeptide. The context of the *het-c2* translation initiation codon (CCACCATGGC) matches the filamentous fungi consensus sequence [TCA(C/A)(A/C)ATG(G/T)C] (25).

The *het-c* ORF is interrupted by two putative introns. A sequence similar to the internal branchpoint consensus sequence of fungi, [(T/A)(G/A)CT(A/G)AC] (25), is present in the first intron but not in the second. The 3′ acceptor sites match the [(A/C)(C/T)AG:G] consensus, whereas the 5′ intron donor sites only contain the G:GT sequence of the G:GTA(Y/A)GT(T/C) consensus. To confirm the existence and the positions of these introns, poly(A)$^+$ RNA was isolated from the *het-c2* strain, and single-stranded cDNA was synthesized using oligo(dT) as a primer. The *het-c2* cDNA was amplified by PCR with primers specific for the 5′ and 3′ ends of the ORF. Cloning and sequencing of the PCR product confirmed the presence of the introns at the expected positions. The first intron is 57 bp long and extends from position 746 to 802. The second is 60 bp long from position 922 to 981.

A data base search revealed that the HET-C2 polypeptide was homologous to a glycolipid transfer protein (GLTP) purified from pig brain (26). Both polypeptides are 208 amino acids long, and they display 30% identity and 37% similarity (Fig. 4). Similarity is distributed all along the sequence. The GLTP has been purified from pig brain using a glycolipid transfer assay. The GLTP transfers different glycolipids, including both glycosphingolipids and glyceroglycolipids, between membranes (28).

**Expression of mRNA and Protein from the *het-c* Locus.** Northern blot analysis of poly(A)$^+$ RNA, using the 1.1-kbp *Hin*dIII fragment as a probe, showed that the *het-c2* transcript is a 1.3-kb RNA. The level of *het-c* mRNA was comparable to that of the *ura5* gene, used as a control to probe the same blot (data not shown).

Antibodies were produced to the C-terminal region of the polypeptide, from amino acid positions 115 to 172. These detected a 23-kDa protein in crude extracts of the wild-type *het-c2* strain (Fig. 5), in good agreement with the size of the putative polypeptide encoded by tl.e *het-c2* gene. This is strong evidence that this 23-kDa polypeptide is the protein encoded by the *het-c* locus.

**Disruption of *het-c2*.** *het-c2* was disrupted with the objective of analyzing the phenotype of mutant strains in which the *het-c* locus is inactive and obtaining information on the function of this locus in the biology of the fungus. Two plasmids, pCura and pCuraΔ, were constructed for disruption of *het-c2*. The gene was disrupted *in vitro* by insertion of the *ura5* gene of *P. anserina* (20) at the *Eco*RI site of the *het-c2* allele (Fig. 2*B*). The resulting plasmid, pCura, was used to transform a *het-c2 ura5-6* strain. The incompatibility phenotypes of 440 prototrophic transformants were tested by confrontation with a strain containing the *het-e1* allele. One

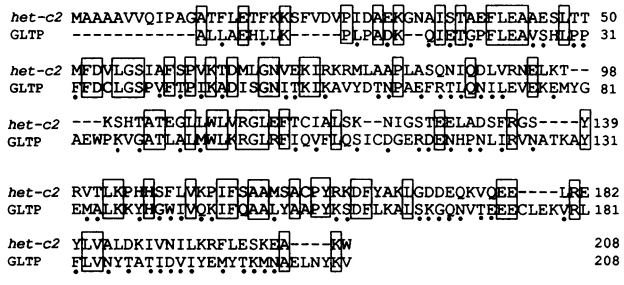


FIG. 4. Sequence alignment of the proteins encoded by *het-c2* and GLTP from pig brain. The computation was done at the National Center for Biotechnology Information using the BLAST Network Service (27). Identical amino acid residues are boxed, dots show positions of conservative differences, and hyphens mark gaps.

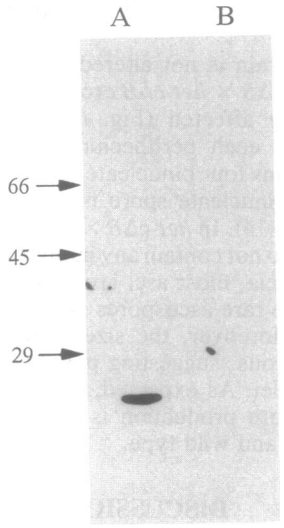


FIG. 5. Immunoblot analysis of proteins extracted from strains containing wild-type *het-c2* (*A*) and disrupted *het-cΔB* (*B*) alleles. Markers used for gel calibration were bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase from bovine erythrocytes (29 kDa).

transformant, *het-cΔA*, had lost the Het-c2 phenotype. Disruption of *het-c2* in this strain was confirmed by Southern blot analysis, which showed that the *het-c2* allele had been replaced by the disrupted copy (data not shown). This strain exhibits a null incompatibility phenotype, being compatible with strains containing any allele at the *het-e* and *het-d* loci. Northern blot analysis of RNA from this mutant showed the presence of a 0.9-kb mRNA encoded by the *het-c* locus (data not shown). This truncated RNA was expressed approximately at the same level as the 1.3-kb *het-c2* mRNA and is transcribed from the 5′ end of the disrupted gene.

To overcome the possible expression of a truncated protein in the *het-cΔA* strain, we constructed another mutant strain in which expression of the *het-c* locus cannot occur. About 500 bp at the 5′ end of the *het-c* gene were deleted from the pCura plasmid. This plasmid was first digested with *Cla* I then with Bal-31. After ligation, the deleted plasmids were recovered in *E. coli* and sequenced. The pCuraΔ plasmid in which the deletion overlaps the ATG and extends from position 186 to 734 was retained (Fig. 2*C*). This plasmid was used to transform a *het-c2 ura5-6* strain. Among 750 prototrophic transformants, one had lost the Het-c2 phenotype and was compatible with strains containing the different *het-e* and *het-d* alleles. Disruption of the *het-c* locus in this *het-cΔB* strain was confirmed by Southern blot analysis; no *het-c* mRNA could be detected on Northern blots, and the 23-kDa polypeptide was missing (Fig. 5).

The phenotypes of these two mutant strains were examined. The *het-cΔA* strain exhibits an altered vegetative phenotype. On solid medium, it has a colonial phenotype, and its radial growth is reduced by ≈20% compared with the *het-c2* wild-type strain. Microscopic observations showed that branching of apical hyphae is notably increased in the *het-cΔA* strain. This phenotype is recessive; ectopic integration of the pCura plasmid in transformants does not result in a colonial phenotype.

Many colonial mutants have been described in *N. crassa*, most of which are affected in cell-wall or membrane composition (29). The *het-cΔA* mutant is also more sensitive than wild type to components that are known to affect cell-wall synthesis, such as 2-deoxyglucose (30). For instance, on a medium containing 2-deoxyglucose at 2 g/liter, the radial growth rate of wild type is reduced by 15%, whereas that of

the mutant is reduced by 40% compared with growth on the control medium.

The *het-cΔB* strain is not altered for vegetative growth. However, in *het-cΔB* × *het-cΔB* crosses, ascospore production is drastically affected (Fig. 6). In crosses between wild-type strains, each perithecium produces ≈100 asci. Each ascus contains four binucleate spores (except for some asci in which a binucleate spore is replaced by two small uninucleate ones) (14). In *het-cΔB* × *het-cΔB* crosses, ≈80% of the perithecia do not contain any mature ascospores. In the remaining perithecia, most asci are aborted, and the maturation stage of the rare ascospores is not synchronous as in wild-type asci. Moreover, the size of these ascospores is highly heterogeneous, suggesting possible defects in distribution of the nuclei. As expected, this phenotype is recessive, and ascospore production is not affected in crosses between *het-cΔB* and wild type.

## DISCUSSION

Heterokaryon incompatibility is a very common phenomenon in filamentous ascomycetes. Most often, it results in a lethal reaction that is elicited by the coexpression of incompatibility genes in a single cell. Molecular mechanisms leading to cell death remain unknown and, except for *mt a-1* and *mt A-1* in *N. crassa*, it has not been determined whether prevention of heterokaryon formation is the only function of these genes; further work is necessary to provide information on the function of the *het* genes. Here, we have described the cloning and characterization of a *het* gene involved in nonallelic vegetative incompatibility.

Data base searches revealed that the protein encoded by *het-c2* has homology with a GLTP from pig brain (26). This protein is responsible for transport of specific glycolipids from donor to acceptor membranes. The precise biological function of GLTP is unknown, and it is the only GLTP sequenced. GLTP is a cytosolic protein, whereas glycolipids are mainly located on the luminal face of the Golgi cisternae (31). The polypeptide encoded by *het-c2* displays a putative amphipathic α-helix (position 176–193). Involvement of such structures in lipid binding has been demonstrated for other proteins (32). It is likely, based on sequence similarities and presence of amphipathic α-helix, that the *het-c2* gene product is involved in lipid transfer. Several classes of lipid transfer proteins have been described, phospholipid transfer proteins, GLTPs, and nonspecific lipid transfer proteins (33). Information on the physiological function of lipid transfer proteins has come from the study of *sec14* mutants of *Saccharomyces cerevisaie*. *SEC14* encodes a phosphatidylcholine and phosphatidylinositol transfer protein that is essential for transport from the Golgi complex. Null *sec14* mutations are lethal (34). Recently, implication of a lipid transfer protein in a signal-transduction system involving phospholipase C has been described. Mammalian phosphatidylinositol transfer protein (termed PI-TP), is believed required to ensure substrate supply for phospholipase C. It has been proposed that phosphatidylinositol transfer activity is regulated by phosphorylation of PI-TP and indirectly modulates phospholipase C activity and secretion (35, 36).

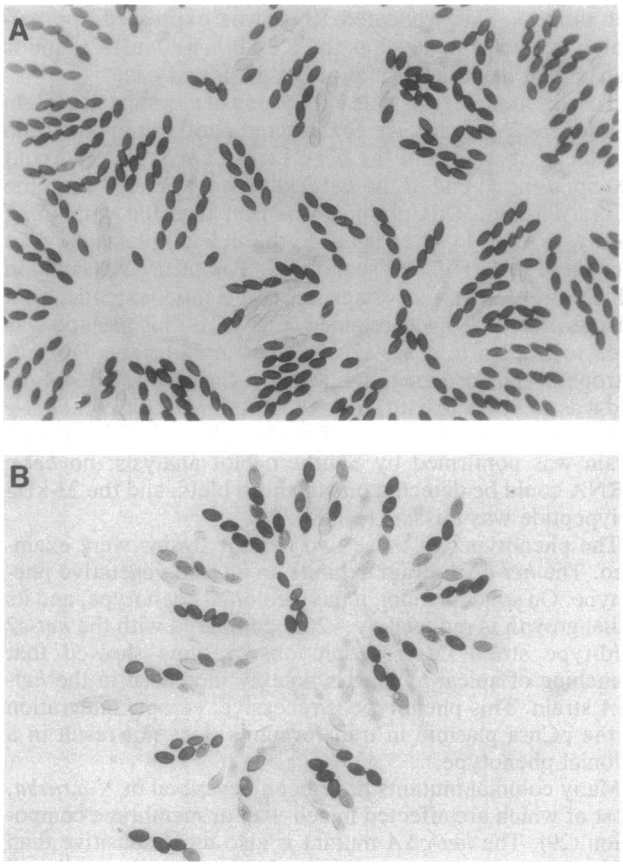


FIG. 6. Asci produced on corn meal agar by perithecia from crosses between wild-type strains (*A*) and from *het-cΔB* × *het-cΔB* (*B*). (×60.)

*het-cΔB*, a mutant strain in which *het-c* has been inactivated by gene disruption, is drastically impaired for ascospore production. *het-cΔB* × *het-cΔB* crosses are barren. Also, partial disruption of *het-c2* (*het-cΔA*) confers a colonial phenotype characterized by increased branching of apical hyphae. This recessive phenotype may be due to the expression of a truncated protein that disturbs the pathway in which the protein encoded by *het-c2* is involved. This strain is also characterized by an increased sensitivity to substances such as 2-deoxyglucose that affect cell-wall biosynthesis.

Numerous colonial mutants have been described in *N. crassa*, most of which are affected in cell-wall or membrane composition (29). The phenotype of the *Podospora het-c* mutants suggests that expression of *het-c* is required to maintain normal membrane composition and vesicle transport within the cell. The phenotypes associated with *het-c2* mutations could reflect a defect in cell-wall material deposition at hyphal tips or on ascospore membranes, resulting from an alteration of vesicular transport. A relationship has been established between the sec14p phospholipid transfer protein and the actin cytoskeleton (37). Cytoskeleton integrity is required for intracellular movements that are necessary for vesicular transport, hyphal tip growth, and ascospore formation (38). So, it cannot be excluded that *het-cΔA* and *het-cΔB* mutants are affected in cytoskeleton organization. How coexpression of the proteins encoded by *het-c2* and by the antagonistic alleles at *het-d* and *het-e* triggers cell death remains unclear. Analysis of *het-e*, which has now been cloned (S.S. and B.T., unpublished results), could help in understanding this aspect.

The significance of heterokaryon incompatibility in the biology of fungi is an open question. Different hypotheses have been proposed (2). Incompatibility genes could have been selected through evolution to limit heterokaryosis and prevent horizontal transfer of cytoplasmic genetic elements such as viruses, mitochondrial mutations, or factors that affect virulence of fungal pathogens. Alternatively, *het* genes may have primary cellular functions, and vegetative incompatibility could be the consequence of genetic divergence in genes coding for some homo- or heteromultimeric proteins. The coexpression in heterokaryotic cells of genes that have diverged through evolution might alter the structure and function of the protein complexes, leading to lethal disorders (8). In *P. anserina* it has been proposed that *het* genes and

genes that suppress heterokaryon incompatibility may encode components of a multigenic complex involved in the regulation of the development of aerial hyphae and female organs (39, 40). *het-c* provides evidence that a vegetative incompatibility gene has an essential function, as its inactivation leads to drastic impairment in the production of ascospores. Several *het-c* alleles have now been cloned. Preliminary sequence data (S.S. and B.T., unpublished results) show that the proteins encoded by alleles with different specificities contain a limited number of amino acid differences. These results support the hypothesis that vegetative incompatibility originated by genetic divergence in genes with primary cellular functions. Such a conclusion is also supported by the demonstration in *P. anserina* (41) that nonallelic incompatibility can result from single mutations in two genes different from the *het* genes identified in wild-type isolates.

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