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RESEARCH ARTICLE

Identification of the *het-r* vegetative incompatibility gene of *Podospora anserina* as a member of the fast evolving *HNWD* gene family

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Abstract In fungi, vegetative incompatibility is a conspecific non-self recognition mechanism that restricts formation of viable heterokaryons when incompatible alleles of specific *het* loci interact. In *Podospora anserina*, three non-allelic incompatibility systems have been genetically defined involving interactions between *het-c* and *het-d, het-c* and *het-e*, *het-r* and *het-v*. *het-d* and *het-e* are paralogues belonging to the *HNWD* gene family that encode proteins of the STAND class. HET-D and HET-E proteins comprise an N-terminal HET effector domain, a central GTP binding site and a C-terminal WD repeat domain constituted of tandem repeats of highly conserved WD40 repeat units that define the specificity of alleles during incompatibility. The WD40 repeat units of the members of this *HNWD* family are undergoing concerted evolution. By combining genetic analysis and gain of function experiments, we demonstrate that an additional member of this family, *HNWD2*, corresponds to the *het-r* non-allelic incompatibility gene. As for *het-d* and *het-e*, allele specificity at the *het-r* locus is determined by the WD repeat domain. Natural isolates show allelic variation for *het-r*.

Sequence data reported here are available in the Genbank database under accession numbers FJ269240 and FJ269239 for *het-r* and *het-R*, respectively.

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Abbreviations

Het	Heterokaryon incompatibility gene
VI	Vegetative incompatibility
PCD	Programmed cell death

Introduction

All living organisms have developed mechanisms to recognize self from non-self, ranging from restriction modification systems in bacteria (Tock and Dryden 2005) to the highly refined immune system in mammals (Maizels 2005; Tock and Dryden 2005). In eukaryotes, the recognition systems usually rely on the same basic principle: the production of polymorphic proteins able to recognize variant non-self molecular markers in order to activate the appropriate cellular response [for reviews (Maizels 2005; Schwessinger and Zipfel 2008)]. Genes encoding for such recognition proteins are frequently members of gene families. Diversification of these recognition molecules is ensured by specialized molecular mechanisms such as somatic hypermutation and recombination (Maizels 2005; Mondragon-Palomino and Gaut 2005).

In filamentous fungi conspecific non-self recognition (recognition of different individuals within the same species) takes the form of so called vegetative incompatibility phenomena (VI). Fungi colonize their environment by developing a syncytial structure called the mycelium, a network of interconnected filaments. Individual filaments from the same species can fuse by anastomosis irrespective of their genetic backgrounds (Glass et al. 2004). In other words, genetically different mycelia can anastomose, and

the non-self recognition process takes place in the resulting heterokaryon [for review (Saupe et al. 2000; Glass and Dementhon 2006; Pinan-Lucarré et al. 2007)]. VI is genetically controlled by specific heterokaryon incompatibility loci (*het*), and expression of incompatible alleles in the same cytoplasm triggers a localized programmed cell death reaction (PCD) [for review (Pinan-Lucarré et al. 2007)]. The biological function of VI remains unclear but one of its consequences is to limit the horizontal propagation of deleterious cytoplasmic elements such as mycoviruses (Debets et al. 1994; Cortesi et al. 2001; Biella et al. 2002; Milgroom and Cortesi 2004).

het loci have been characterized in all fungal species where they have been sought, and generally, each species displays about half a dozen to a dozen such genes. So far, only a small set of *het* loci have been characterized at the molecular level and only in *Neurospora crassa* and *Podospora anserina*. These *het* loci share two characteristics: first, nearly all these systems involve a fungal specific protein domain termed the HET domain (Smith et al. 2000), that triggers a PCD identical to VI when expressed in *P. anserina* (Paoletti and Clavé 2007); second, *het* genes all appear to be extremely polymorphic. In a number of cases it could be shown that the highly polymorphic regions ensure recognition. Vegetative incompatibility systems can thus be viewed as modular systems that combine a recognition module (the highly polymorphic domains) to a death effector module (the HET domain) (Kaneko et al. 2006; Paoletti and Clavé 2007). In a number of cases there is now strong evidence that *het* genes are evolving rapidly (Saupe et al. 1996; Paoletti et al. 2007). These genes are under particular evolutionary regimens characterized by positive diversifying selection and/or balancing selection favouring diversification and maintenance of high levels of polymorphism (Saupe et al. 1995a, b; Wu et al. 1998; Paoletti et al. 2007).

In *P. anserina* the *het-c*/*het-d* and *het-c*/*het-e* and *het-r*/*het-v* are non-allelic VI systems, where co-expression of incompatible alleles of different loci results in VI [Fig. 1; (Saupe et al. 2000; Glass and Dementhon 2006)]. In appropriate crosses, these non-allelic systems will produce F1 progeny that harbour incompatible alleles at these loci. Soon after germination of such spores growth stops and the PCD will develop, resulting in a characteristic Self Incompatible (SI) phenotype. These three non-allelic systems share two essential properties. First, they can be suppressed by a combination of the same mutations in genes *mod-A* and *mod-B*, pointing to the existence of a common cellular mechanism in all three systems (Belcour and Bernet 1969; Bernet 1971). Second, these incompatibility systems appear genetically unstable. When *het-c*/*het-d*, *het-c*/*het-e* or *het-r*/*het-v* SI spores are produced, escape from cell death by mutation in *het-d*, *het-e* or *het-r* is frequent (Labarère

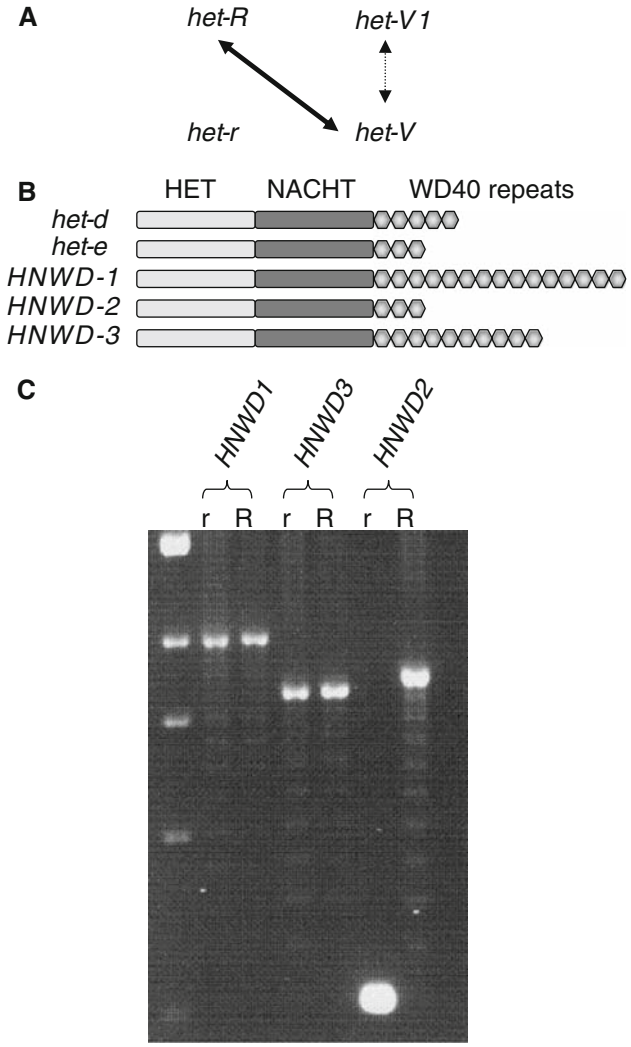

Fig. 1 Incompatible interactions and alleles at *HNWD* loci. **a** The non-allelic incompatibility between *het-R* and *het-V* alleles is represented by a *plain arrow*. The *het-V* allele is also involved in an allelic incompatibility reaction with *het-V1* as represented by the *dashed arrow*. **b** Schematic representation of the *HNWD* gene structure of the sequenced S strain. **c** PCR amplification of the WD repeat domains of the *HNWD1*, *HNWD2* and *HNWD3* loci from strains *het-R het-V1* and *het-r het-V* strains, labeled *R* and *r*, respectively

1978), pointing at some sort of genetic instability in these genes. Three of these non-allelic incompatibility genes have been characterized. *het-c* encodes for a glycolipid transfer protein (Saupe et al. 1994). *het-d* (Saupe et al. 1995a, b) and *het-e* (Espagne et al. 2002) are paralogues, and analysis of the recently sequenced *P. anserina* genome (Espagne et al. 2008) revealed that they belong to a gene family termed *HNWD* containing five members [Fig. 1; (Paoletti et al. 2007)]. Members of this family share a N-terminal HET domain, a central NACHT domain with a GTP binding site essential for VI (Espagne et al. 1997; Koonin and Aravind 2000), and a C-terminal WD domain made of a variable number of tandemly repeated WD40

unit sequences (Paoletti et al. 2007). The sequence of the WD domain of *het-d* and *het-e* defines the specificity of interaction with *het-C* alleles (Saupe et al. 1995a, b; Espagne et al. 2002). We have previously reported that a combination of original evolutionary mechanisms governs the generation of extensive polymorphism at loci of the *HNWD* family. Repeat units are constantly exchanged both within and between members of the gene family. Specific positions at the protein/protein interaction surface of the WD repeat domain are under positive diversifying selection. Diversification of *het-d* and *het-e* is thus ensured by high mutation supply, followed by reshuffling of the repeats and positive selection for favourable variants (Paoletti et al. 2007).

Herein, we have explored the hypothesis that additional members of the *HNWD* gene family might also display a function in heterokaryon incompatibility. We report that indeed the *HNWD2* gene is allelic to the previously genetically characterized *het-r* heterokaryon incompatibility locus. As other *het* genes *het-r* appears to be polymorphic in natural isolates.

Materials and methods

Strains, incompatibility relationships and media

Crosses are performed either by confronting strains of opposite mating type on a plate, the perithecia then developing in the contact zone, or by fertilising a female strain with a suspension of conidia washed off the male isolate with sterile water. *P. anserina* produces mature perithecia enclosing mostly asci with four binucleated spores, and occasionally asci containing five spores, two of which being homokaryotic. Note that the genome of the S strain of *P. anserina* was sequenced (Espagne et al. 2008). The S strain is expected to be nearly isogenic to the s strain except for the *het-s* incompatibility locus, and behaves as the *het-r het-V* strain as far as incompatibility at the *het-r* and *het-v* loci is concerned. The media and growth conditions have already been described elsewhere (http://podospora.igmors.u-psud.fr/). Other strains used in this study are listed in Table 2.

Nucleic acid manipulation and nomenclature

Routine genomic DNA extractions were performed as described by (Lecellier and Silar 1994), or using the DNeasy Plant Mini Kit (Qiagen) when high quality DNA was necessary. PCR was performed using the Expand Long Template PCR System (Roche), according to the manufacturer recommendations. Time for DNA extension was adjusted according to the length of the fragment to amplify. Electrophoresis of PCR products was performed on 0.8% agarose gels. PCR products were gel purified and cloned either in the pCR-XL-TOPO vector (Invitrogen) or in the pGEM-T vector (Promega). For WD repeat sequences, we systematically sequenced cloned DNA fragments amplified from two independent PCR reactions. The oligonucleotides used for PCR and/or sequencing are listed in Table 1.

Plasmid construction and transformation of *Podospora*

The *HNWD2* locus from a *het-R* strain was amplified with the oligonucleotides C and D, and cloned in the pCR-XL-TOPO vector. Then the locus was cloned as a 5.1-kb *Kpn*I-*Xba*I fragment into the pCB1004 vector (Carroll et al. 1994) containing the selectable *hph* marker, and named pCB-HNWD2. The pCB-HNWD2 vector was used to transform protoplasts from a het-*r het-V1* strain. Preparation and transformation of protoplasts were performed as previously described (Bergès and Barreau 1989), and transformants were screened on hygromycin B at 100 μg/ml.

Results

HNWD2 shows length polymorphism between *het-r* and *het-R* strains

The reference *P. anserina* isolate is called *s*, and bears the *het-r* allele at the *het-r* locus and the *het-V* allele at the *het-v* locus and will be called *het-r het-V* strain from now on. The *het-R het-V1* strain bears the *het-R* allele at the *het-r* locus, and the *het-V1* allele at the *het-v* locus (Labarère 1973). The incompatibility interactions involving the *het-r* and *het-v* loci are depicted on Fig. 1. Basically, the *het-V* allele is incompatible with the *het-V1* allele in an allelic incompatibility reaction, and is also incompatible with the *het-R* allele in a non-allelic interaction. In the progeny of a cross between the *het-R het-V*1 and the *het-r het-V* strains, one recovers F1 progeny harbouring the *het-R* and *het-V* incompatible alleles in the same nucleus. As the *het-R het-V* non-allelic incompatibility is thermosensitive, these SI F1 progeny can grow as wild type at 32°, but display the SI phenotype when transferred to the non-permissive temperature (26°). The same loci are also involved in sexual incompatibility as a cross ♀*het-R het-V1* × ♂*het-r het-V* is sterile, while the reciprocal cross is fertile (Labarère et al. 1974).

Based on the shared properties between the *het-c*/*het-d*, *het-c*/*het-e* and *het-r*/*het-v* non-allelic incompatibility systems (suppression by the same mutations and genetic instability), we hypothesized that *het-r* might be allelic to one of the *HNWD* gene family members (Fig. 1). We reasoned that as for the *het-d* and *het-e* genes, differences in the alleles of the *het-r* locus might lie in the number of WD40 repeats of the WD domain (Saupe et al. 1995a, 1995b; Espagne et al.

Table 1 Primers used to PCR amplify and/or sequence the *HNWD2* locus

Name	Sequence	Sense	Location (base position)[a]	Use
A	GCACCGGTTGGCAGTCTGG	Forward	Upstream from WD domain (+2388 to +2406)	Analysis of WD domain size, WD domain cloning
B	CCAGGCCCTTCTCGTGTTAGG	Reverse	Terminator (+3150 to +3170)	Analysis of WD domain size, WD domain cloning
C	CCTGGAGCACCTCCTCC	Forward	Promoter (−771 to −755)	Locus cloning, HET domain sequencing
D	CATGGGAGATGCTAGAATTCC	Reverse	Terminator (+3199 to +3219)	Locus cloning
E	TACGAACTCGACAGCTACTT	Forward	Promoter (−190 to −171)	Verification of locus transformation, HET domain sequencing
F	GAACTCCTATAGCCTTCGAG	Reverse	Downstream from WD domain (+2871 to 2890)	Verification of locus transformation, WD domain sequencing
G	GAATGCATGCCTTCAGACG	Forward	Upstream from WD domain (+2474 to 2492)	WD domain sequencing
H	CTCGAAGGCTATAGGAGTTC	Forward	Downstream from WD domain (+2871 to 2890)	Sequencing of the end of the locus
I	CTGATCCAGCTAGGTTCTTCC	Reverse	Upstream from WD domain (+2426 to +2446)	NACHT domain and intron sequencing
J	AAGTAGCTGTCGAGTTCGTA	Reverse	Promoter (−190 to −171)	Promoter sequencing
K	GATAGAGGTCTTCCCTGAAG	Reverse	Downstream from HET domain (+534 to +553)	HET domain sequencing
L	AGCTCTTCAGGGAAGACCTC	Forward	Downstream from HET domain (+530 to +549)	NACHT domain sequencing
M	CTCGCTATTCGGTATCTTTGACG	Forward	Upstream from NACHT domain (+626 to +648)	NACHT domain sequencing
N	GATCCAGACTTGAAGGAAAC	Forward	NACHT domain (+1170 to +1189)	NACHT domain and intron sequencing
O	GTTTGGGTAGGTCGATAACG	Reverse	NACHT domain (+1220 to +1239)	HET and NACHT domains sequencing
P	GATCTTGTCTTCTCGGATGG	Reverse	NACHT domain (+1801 to +1820)	NACHT domain sequencing
Q	CATCAGTCAGCCAAGGACTA	Forward	NACHT domain (+1830 to +1849)	Intron sequencing

[a] Location of primers corresponds to their position in the *HNWD2* locus (compared to start codon) from the *P. anserina* genome sequence of the S strain

2002). Therefore, we PCR amplified and compared the WD domains of the *HNWD1*, *HNWD2* and *HNWD3* loci between the *het-R het-V1* and *het-r het-V* strains. As shown in Fig. 1, alleles at the *HNWD1* and *HNWD3* loci result in amplicons of identical size, suggesting that these genes encode the same number of WD40 repeats in both isolates. The size of the *HNWD1* WD repeat amplicon suggests presence of twelve repeats, while *HNWD3* would encode ten WD40 repeats. In contrast, the length of the PCR products corresponding to the WD40 repeat domain from the *HNWD2* locus was different between the *het-R het-V1* and the *het-r het-V* strains. The amplification produced a fragment of about 500 bp from the *het-r het-V* strain, the expected size for a WD domain made of only one WD40 repeat, while a fragment of about 1.8 kbp was amplified from the *het-R het-V1* strain corresponding to the expected size for a WD domain made of 11 WD40 repeats.

Genetic linkage between *HNWD2* size polymorphism and *het-r*

We analysed the genetic linkage between length polymorphism at the *HNWD2* locus and the *het-R* and *het-r* phenotypes in incompatibility. A *het-R het-V1* strain was crossed with a *het-r het-V* strain and the phenotypes of the F1 progeny were determined by their aspects after germination to identify (SI) strains, and by barrage testing for the other progeny. Note that this approach allows to identify *het-R het-V* [(SI) strains] and *het-r het-V* strains with certainty, but does not allow to discriminate strains with the *het-R het-V1* genotype from strains with a *het-r het-V1* genotype because of the allelic incompatibility reaction between *het-V* and *het-V1* alleles (Fig. 1). We thus focused our analysis on a set of 20 *het-R het-V* and 20 *het-r het-V* progeny. Amplifications of the WD repeat region of the *HNWD2* locus were performed for these progeny. All the progeny of the *het-r het-V* genotype as well as the *het-r het-V* parental strain produced the small 500-bp amplicon. All the progeny of the *het-R het-V* genotype as well as the *het-R het-V*1 parental strain produced the large 1.8-kbp amplicon. We conclude from this experiment that the *HNWD2* locus is genetically linked to the *het-r* incompatibility locus which further suggested that *HNWD2* could be allelic to *het-r*.

The *HNWD2* allele confers *het-R* phenotypes

To confirm allelism between *HNWD2* and *het-r*, we set out to demonstrate that the *HNWD2* allele from the *het-R* strain carries the information to confer the *het-R* phenotype. The entire *HNWD2* gene was amplified from the genomic DNA of a *het-R* strain with primers C and D (Table 1), cloned and transformed in a *het-r het-V*1 strain. As the recipient strain is incompatible with the *het-r het-V* tester strain because of the allelic *het-V het-V1* incompatibility reaction (Fig. 1), integration of an active *het-R* allele in the recipient strain will not alter the barrage phenotype of the strain. However, integration of a functional *het-R* allele is expected to confer a female sterility in crosses with a *het-V* strain used as male parent (Labarère et al. 1974). Consequently, 107 transformants were used as females in fertility tests with both *a het-r het-V* and a *het-R het-V*1 strain as the male parent. Strains expressing a *het-R* transgene are expected to be fertile when crossed with a *het-R het-V*1 male but sterile when crossed to a *het-r het-V* male. The majority of the transformants were fertile with both parents. However, one transformant (S1) was fertile when crossed with the *het-R het-V1* strain but sterile with the *het-r het-V* strain, while two transformants (S2 and S3) were sterile when crossed with both parents (Fig. 2). These transformants were further characterized at the molecular level. For the three of them, PCR amplification with primers E and F specific of the *HNWD2* locus yielded two bands, one corresponding to *HNWD2* allele of the recipient strain, and one corresponding to transformed ectopic *HNWD2* allele (Fig. 2) confirming that the entire transgene was integrated in these transformants.

These three transformants were individually crossed with the *het-r het-V* strain. For the three crosses, the formation of asci in the perithecia appeared abnormal to various degrees with generally few mature asci containing four spores (Fig. 3). We were nonetheless able to isolate a

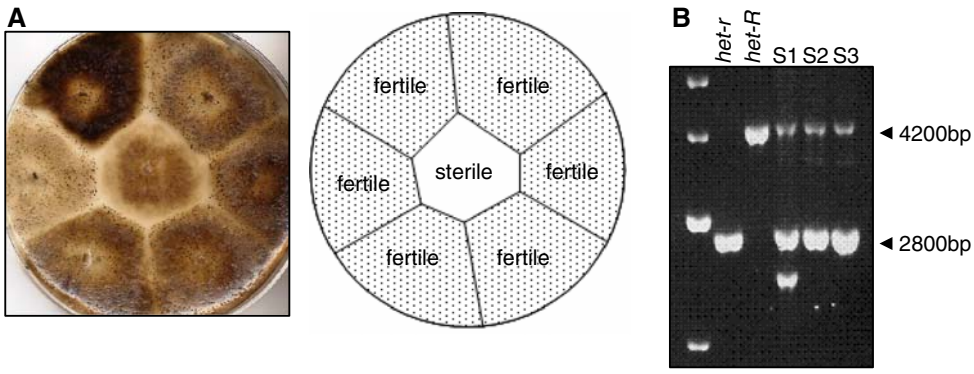

Fig. 2 Selection of *HNWD2* transformants. **a** *het-r het-V1* strains transformed with the *HNWD2* gene were assessed for their fertility with a *het-r het-V* male fertilizer strain. **b** PCR amplification of the *HNWD2* locus from *het-r het-V* and *het-R het-V1* control strains and from three sterile *HNWD2* transformants S1–S3

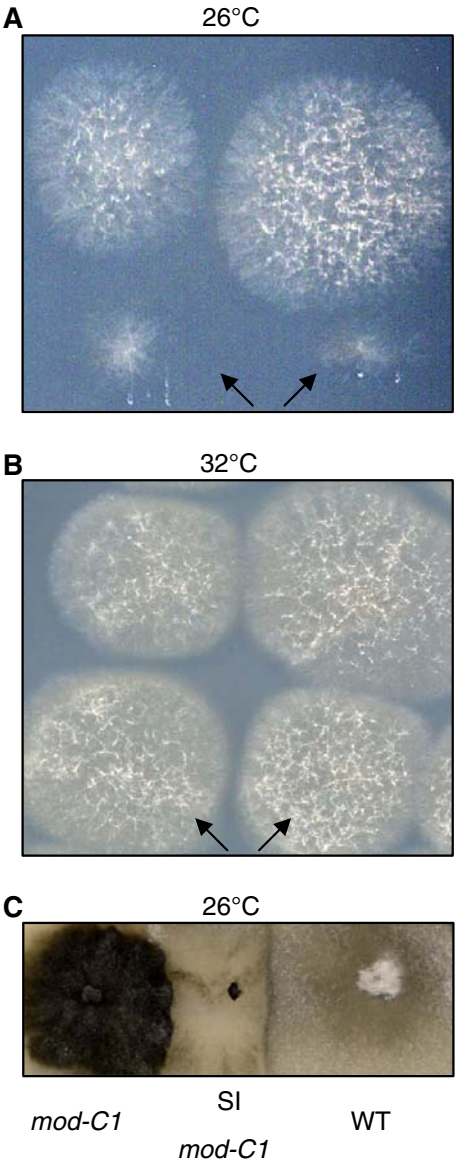

Fig. 3 *het-R* phenotype in incompatibility conferred by the *HNWD2* gene. F1 spores from a cross between *HNWD2* transformants and a *het-r het-V* strain were seeded and grown at **a** 26°C and **b** 32°C. Two spores display clear SI phenotypes (*arrowheads*) as opposed to two self compatible spores. **c** F1 progeny of the cross between the S1 transformant and the *het-r het-V mod-C1* strain displaying a WT phenotype, a SIM phenotype associated to suppression by *mod-C1* of the incompatibility between the ectopic *HNWD2* and *het-V* genes, and a *mod-C1* phenotype

number of homokaryotic spores from each cross. For each cross we found F1 progeny that displayed a clear SI phenotype at 26°. Growth of these F1 progeny was restored when transferred to 32°C as expected for the *het-R/het-V* incompatibility system. We conclude that the *HNWD2* allele amplified from the *het-R het-V1* strain confers the thermosensitive *het-R* activity in incompatibility. The different female sterility phenotypes with a *het-V* male of the transformants might be due to variation in the expression levels of the transgenes. The transformants were also crossed with a *het-r het-V mod-C1* strain. *mod-C1* is a mutation that suppresses the *het-R het-V* incompatibility reaction leading to easily identified SIM phenotype characterized by a mycelium devoid of any aerial filaments but with a more intense pigmentation (Labarère and Bernet 1977). These crosses again produced few mature perithecia and we could only isolate a limited number of spores. However, from the cross with the S1 transformant, we recovered one progeny that displayed a SIM phenotype (Fig. 3). The hygromycin resistance phenotype of this strain strongly confirms presence of the ectopic *HNWD2* allele. This is consistent with *mod-C1* suppressing the incompatibility reaction conferred by the *HNWD2* gene amplified from the *het-R* strain.

The low frequency of transformants leading to expression of the *het-R* phenotype is comparable to what was observed during the cloning of the *het-e1* allele, when transformations with cosmids bearing a functional *het-e1* allele yielded only about 5% of transformants with the *het-E* phenotype (Saupe, unpublished). This low yield of active transformants with members of the *HNWD* gene family might be due to rearrangements in the repeat region of these genes during the transformation procedure. In addition, the sterility phenotype of transformants as females is expected to be recessive. Indeed heterokaryotic transformants (the majority of transformants) associating transformed and untransformed nuclei will appear fertile through production of perithecia from untransformed nuclei. Our screen thus allows for the identification of *het-R* activity in homokaryotic transformants only.

Mutants of *het-r* are affected at the *HNWD2* locus

As already reported, SI strains spontaneously give rise to mutant sectors that escape cell death through mutations in *het-d*, *het-e* or *het-r* [for review (Saupe 2000)]. We have isolated two such mutants (H9 and M10) that resumed growth from a SI *het-R het-V* strain maintained at 26°C, the non-permissive temperature. When the mutant strains H9 and M10 are confronted to a *het-r het-V1* tester strain a barrage is observed indicating that incompatibility between the *het-V* and *het-V1* loci is functional. In contrast, no barrage is observed when the mutant strains are confronted to the *het-r het-V* strain (Fig. 4). These observations indicate that the mutant strains have maintained active alleles at the *het-v* locus but both harbour an inactive *het-r* allele. This was confirmed in female sterility tests. Both mutant strains indeed produced perithecia filled with mature spores when fertilised with male *het-r het-V* conidia (data not shown). As genetic instability is often associated to repeated sequences (Dudas and Chovanec 2004), we analysed the size of the WD repeat domain of the *het-r* locus in the mutants H9 and M10. As shown in Fig. 4, the *het-r* locus

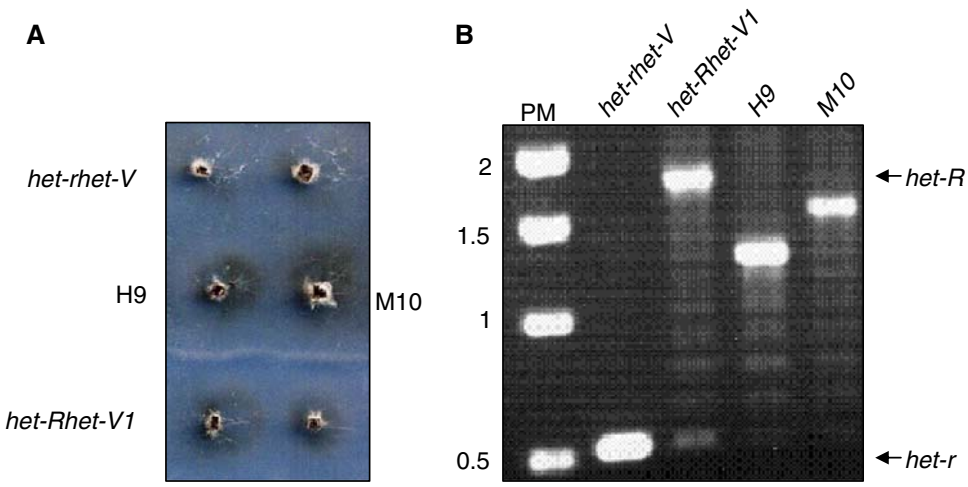

Fig. 4 Mutants H9 and M10 of *het-r* are affected in the WD repeat domain of *HNWD2*. **a** Incompatibility tests against the *het-r het-V* and *het-R het-V1* tester strains. The barrage indicated by an *arrowhead* indicates incompatible interactions. **b** PCR amplification of the WD repeat domain of the *HNWD2* locus from the *het-r het-V* and *het-R het-V1* parental strains and from the mutant strains H9 and M10

amplicon of the WD repeat domain are about 1.4 and 1.6 kbp in size in the H9 and M10 mutant isolates, corresponding to WD repeat domains containing è and 9 WD repeat units, respectively. It thus appears that the mutations that occurred in these two mutants correspond to deletions of WD repeat units of *HNWD2*, likely similar to "pop out" mutations described for many repeat sequences (Dudas and Chovanec 2004).

From the above two experiments, we conclude that the *HNWD2* allele isolated from a *het-R* strain is both necessary and sufficient to confer the *het-R* activity, in other words that *HNWD2* is allelic to the *het-R* incompatibility gene.

WD40 repeat domain defines allelic specificity of *het-r* and *het-R* alleles

We sequenced the entire *HNWD2* allele conferring the *het-R* activity and compared it to the *HNWD2* locus identified in the *P. anserina* genome sequence of the S strain (Espagne et al. 2008). The sequenced S strain is of the *het-r* genotype. Interestingly, differences between both alleles lie only in the WD repeat domain, the remaining of the sequences being identical. This indicates that as for the *het-d* and *het-e* loci, allelic specificity is determined by the WD repeat domain sequence and length variation. We also sequenced the WD40 repeat domain of the *het-r* locus from the s strain we used in our analysis and found that it encodes for a unique WD40 unit sequence. A schematic representation of the different *het-r* alleles is presented in Fig. 5. The variation of the number of WD40 repeat sequences is best explained by the fact that these repeated sequences of all members of the *HNWD* gene family are evolving in a concerted manner (Paoletti et al. 2007), a mechanism known to lead to variations in the number of repeated units.

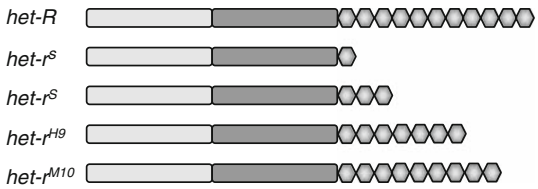

Fig. 5 Schematic representation of the *het-r* alleles. *het-R* corresponds to the active allele, *het-r*$_s$ to the inactive allele present in the *s* strain and *het-r*$_S$ to the inactive allele present in the sequenced *S* strain, and H9 and M10 to the alleles present in the mutant strains selected

The *het-r* locus is polymorphic in natural isolates

We have analysed the size of the WD domain of the *het-r* locus in a sample of ten natural isolates of *P. anserina* randomly chosen from a collection already described (van der Gaag et al. 2000; van Diepeningen et al. 2008) or retrieved from the CBS (Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands), thereby extending our previous analysis of natural isolates from France (Paoletti et al. 2007). The number of WD40 repeats was deduced from the size of the WD repeat domain amplicons, and are reported in Table 2. As expected the size of the WD repeat domain displays extensive polymorphism with the number of WD repeats ranging from one (as for the inactive *het-r* allele) to 14. Surprisingly however, we observe now that six out of ten isolates possess a larger WD repeat domain (more than ten repeats) while our previous analysis revealed that six out of seven isolates had small WD repeat domains of two to four WD40 repeats (Paoletti et al. 2007). Finally, note that we observed two amplicons corresponding to WD repeat domains containing 11 and 14 WD40 repeat units in the strain CBS333.63, indicating that this strain is heteroallelic at the *het-R* locus.

Table 2 Number of WD40 repeats at the *het-R* locus in natural isolates of *P. anserina* from Wageningen, The Netherlands (Wa-numbers), and from Central African Republic and Argentina (CBS253.71 and CBS333.63, respectively)

	Isolates														
	Wa2	Wa3	Wa5	Wa6	Wa20	Wa21	Wa25	*CBS253.71*	*CBS333.33*	D[a]	E[a]	M[a]	S[a]	Y[a]	Z[a]
Number of repeats	1	10	11	1	12	11	11	11	11 + 14	4	4	11	3	4	4

[a] Indicates isolates from France previously analysed by Paoletti et al. (2007)

Discussion

The analysis of the recently sequenced genome of *P. anserina* (Espagne et al. 2008) allowed us to identify the *HNWD* gene family to which the two vegetative incompatibility genes *het-d* and *het-e* belong (Paoletti et al. 2007). Members of this gene family encode for the fungal specific HET domain responsible for the cell death reaction associated to vegetative incompatibility (Paoletti and Clavé 2007), as well as a WD repeat domain whose repeated units undergo concerted evolution (Paoletti et al. 2007). We now report that *HNWD2* is also an incompatibility gene as it corresponds to the long identified *het-r* gene (Labarère 1973). In addition, phylogenetic analysis of the members of this gene family based on the HET and NACHT domain sequences reveals that confirmed VI genes are interspersed with other *HNWD* genes (Fig. 6). These observations provide evidence that all *HNWD* genes might be involved in incompatibility. These genes could have escaped genetic identification as VI genes because the limited population of wild-type isolates which were analysed did not include the proper combinations of alleles. Indeed, it has been demonstrated in *Cryphonectria parasitica* that VI genes in a population are not necessarily active in another population (Milgroom and Cortesi 1999). It is possible that active *HNWD* alleles are absent from the analysed population. Alternatively, our population could lack the incompatible partners (*het-c*, *het-v* or others) necessary to reveal *HNWD* gene activities in incompatibility. One would expect that extending the genetic characterization of additional natural isolates would unravel the proper combinations of alleles to detect incompatibility at the remaining *HNWD* loci. In that respect, it is relevant to note that mutagenesis experiments have lead to the creation of new incompatibility genes independent from *het-d*, *het-e* or *het-r* but that shared properties with these three *HNWD* genes (Delettre and Bernet 1976). These observations would be readily explained by modifications of the remaining *HNWD* through rearrangements of the WD repeat sequences leading to incompatible combinations with the resident *het-c* or *het-v* alleles.

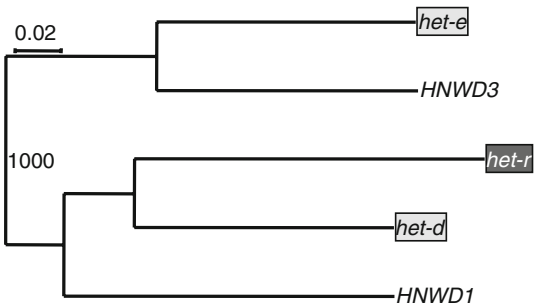

Fig. 6 Neighbor-joining phylogenetic tree constructed with DNA sequences of the *HNWD* gene family members encoding for the HET and NACHT domains. The WD40 repeat sequences were excluded from the alignment. The *light gray shading* indicates family members involved in incompatibility reactions with *het-C* and the *dark gray shading* indicate incompatibility with *het-V*

As for *het-d* and *het-e*, specificity of *het-r* alleles is defined by the WD repeat sequence (Espagne et al. 2002). WD repeat domains adopt a β propeller structure usually including seven WD repeat units (Smith et al. 1999). It is thus likely that the *het-r* alleles we identified, encoding for one or three WD40 repeat sequences only, lead to non functional WD repeat domains thereby preventing recognition of the incompatible HET-V partner. However, determination of the specificity of WD repeat domains appears more complex than a mere problem of number of repeats. Indeed all known *het-D* and *het-E* alleles active in incompatibility encode for at least ten WD40 repeats while alleles with ten or more repeats are not necessarily active in the process (Espagne et al. 2002). The sequence of WD40 repeat units is thus also important in specificity determination. It thus appears possible that different alleles might display the *het-R* phenotype defined as an incompatible reaction with *het-V*. We also confirm that variation of the number of WD40 repeat units and thus variation in the sequence of this WD40 repeat domain at the *het-R* locus is frequent in wild isolates as was already observed (Paoletti et al. 2007). Interestingly however, in our previous report analysing isolates from France we found that most isolates displayed a small number of WD40 repeats at the *het-R* (then called *HNWD2*) locus. We now analysed isolates from other populations and find that seven of these natural isolates display ten or more repeats, suggesting that in these isolates the *het-R* gene might be functional. It would seem that activity of *het-R* in incompatibility varies in different populations as already observed for some incompatibility genes in *Cryphonectria parasitica* (Milgroom and Cortesi 1999). Variation of the number of repeats at the *HNWD* loci in the

natural population as well in the two mutants of the *het-R* gene that we selected here also suggests that the repeated nature of the WD40 repeat sequences is at the origin of the genetic instability of *HNWD* loci.

Interestingly *het-d* and *het-e* interact with *het-c*, a unique gene in *P. anserina* genome encoding a glycolipid transfer protein (Saupe et al. 1995a, b), while *het-R* interacts with *het-V* (Labarère 1973), a gene that has not been characterized yet, indicating that HNWD proteins are able to recognize different partners. Because of the possibility of exchange of WD40 repeats between loci, it might well be possible that some *het-r* alleles will be able to recognize *het-c* alleles, and reciprocally that *het-d* and *het-e* alleles will be able to recognize *het-v* alleles.

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