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Structure and function of a mating-type gene from the homothallic species *Neurospora africana*

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Abstract The homothallic *Neurospora* species, *N. africana*, contains sequences that hybridize to the *A* but not to *a* mating-type sequences of the heterothallic species *N. crassa*. In this study, the *N. africana* mating-type gene, *mt A*-1, was cloned, sequenced and its function analyzed in *N. crassa*. Although *N. africana* does not mate in a heterothallic manner, its *mt A*-1 gene functions as a mating activator in *N. crassa*. In addition, the *N. africana mt A*-1 gene confers mating type-associated vegetative incompatibility in *N. crassa*. DNA sequence analysis shows that the *N. africana mt A*-1 open reading frame (ORF) is 93% identical to that of *N. crassa mt A*-1. The *mt A*-1 ORF of *N. africana* contains no stop codons and was detected as a cDNA which is processed in a similar manner to *mt A*-1 of *N. crassa*. By DNA blot and orthogonal field agarose gel electrophoretic analysis, it is shown that the composition and location of the mating-type locus and the organization of the mating-type chromosome of *N. africana* are similar to that of *N. crassa*.

Key words Mating type · Heterothallic · Homothallic *Neurospora* · Vegetative incompatibility

Introduction

Sexual reproduction in haploid filamentous fungi occurs either by heterothallic reproduction, requiring mating between compatible individuals, or by homothallic reproduction, requiring only a single genome (Blakeslee 1904). The genus *Neurospora* includes both heterothallic and homothallic species. Heterothallic species are composed of two mating populations *A* and *a* (Shear and Dodge 1927; Dodge 1935; Sansome 1946). Although mating occurs between sexual structures of opposite mating type, the mycelium of either *A* or *a* mating type is hermaphroditic. Mating is followed by the formation of perithecia, the macroscopic flask-shaped structures that enclose the haploid products of meiosis, the ascospores. Sexual development in homothallic species of *Neurospora* is morphologically similar to that of heterothallic species (Raju 1978), but differs in that haploid ascospore progeny are capable of initiating and completing the sexual cycle.

The sequences conferring *A* and *a* mating behaviour in the heterothallic species *N. crassa* have been cloned and characterized; these consist of dissimilar DNA sequences, termed idiomorphs, which are present at a single locus in haploid strains (Glass et al. 1988; 1990a; Staben and Yanofsky 1990; Metzenberg and Glass 1990). The sequences that flank the mating-type locus are almost identical between *A* and *a* strains. The *A* idiomorph is 5301 bp in length and the *a* idiomorph is 3235 bp long. Each strain contains only a single copy of either the *A* or *a* sequence in the haploid genome. A 1.2 kb region of the *A* idiomorph (*mt A*-1) has been shown to regulate events associated with mating and heterokaryon incompatibility (Glass et al. 1990a; Glass and Lee 1992). Analysis of the *mt A*-1 open reading frame (ORF) revealed a region of similarity to the *Saccharomyces cerevisiae* mating-type transcriptional activator MATα1. Similarly, a region has been identified in the *a* idiomorph (*mt a*-1 ORF) which contains a segment that shows similarity to a group of transcriptional regulators containing an HMG box DNA-binding region (Staben and Yanofsky 1990).

Both heterothallic and homothallic isolates have been described for the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*. Homothallism in these yeasts is based on the insertion of opposite mating-type cassettes from elsewhere in the genome into an expression locus, the mating-type locus (Nasmyth and Tatchell 1980; Strathern et al. 1980; Egel and Gutz 1981; Beach and Klar 1984). Therefore, homothallism in both *S. cerevisiae* and *S. pombe* reflects a process whereby a single

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cell gives rise to a population in which both mating types are present, and where mating proceeds as in heterothallic cell lines. In *S. cerevisiae*, the products of the **a** and α mating-type cassettes have been shown to encode transcriptional regulators that affect genes encoding mating-specific pheromones and receptors (Sprague et al. 1983; Bender and Sprague 1987; Tan et al. 1988) as well as genes involved in post-mating events (Kassir and Simchen 1976; Klar et al. 1979; Miller et al. 1985).

In an effort to elucidate the basis of homothallism in filamentous ascomycetes, homothallic members of the Sordariaceae were probed with the *A* and *a* mating-type sequences of *N. crassa* (Glass et al. 1990b). The species fell into two categories: those that contained single copy sequences similar to both *A* and *a* idiomorphs, and those that hybridized only to the *A* mating type. In no case were species found that were similar to the *a* mating type only. Thus, it seems that the molecular basis of homothallism in Sordariaceae does not function by a physical mating-type switching as it does in *S. cerevisiae* and *S. pombe*. To begin to understand the role of the mating-type locus in homothallic species of filamentous ascomycetes, we have examined the organization, regulation and function of the mating-type locus in detail in a representative of the *A* homothallic group, *N. africana* (Mahoney et al. 1969). We have cloned and sequenced the *N. africana* homologue of *mt A*-1 and examined its transcription as well as its ability to complement *N. crassa* mating-type mutants. A comparative analysis of mating-type sequences in heterothallic and homothallic species may provide information on the role of the mating-type products during mating and meiosis, as well as answering evolutionary questions concerning the relationship between these two types of sexual lifestyles.

Materials and methods

Strains and culture conditions

N. africana strain 1740 was obtained from the Fungal Genetic Stock Center (FGSC) and grown in either solid or liquid media containing modified Westergaard's salts (Glass et al. 1990b) with 2% fructose. *N. crassa* strains (see below) were grown in either solid or liquid Vogel's medium (Vogel 1964) with added nutritional supplements or in Westergaard's medium (Westergaard and Mitchell 1947). *Escherichia coli* strain NM522 (Gough and Murray 1983) was used as a recipient for DNA transformations (Sambrook et al. 1989).

DNA hybridizations

N. africana genomic DNA was isolated from 5-day stationary cultures grown in liquid medium and purified by the method of Berlin and Yanofsky (1985). DNA was digested with restriction enzymes (Boehringer-Mannheim, Laval, Québec; Promega, Madison, Wis.) and subsequently subjected to electrophoresis in an 0.8% agarose gel and transferred to Hybond membrane (Amersham, Oakville, Ontario) according to the manufacturer's procedures. The *N. crassa A* idiomorph probes (Fig. 1) were generated by digestion of cloned fragments and purified from agarose gels with Geneclean II (Bio 101, La Jolla, Calif.). DNA probes were labelled with α-[^{32}P]dCTP (Amersham) by the random primer method (T7 Quick Prime; Pharmacia, Baie d' Urfe, Québec), and hybridized to membranes overnight at 65°C as previously described (Sambrook et al. 1989). Membranes were washed at 60°C in 0.1% SDS and 0.1% SSC and exposed to Kodak X-OMAT film at −70°C. *E. coli* colony hybridizations were according to Sambrook et al. (1989).

Orthogonal field agarose gel electrophoresis (OFAGE)

Neurospora spheroplasts were liberated with Novozym 234 (batch 3835; Novo Industries, Wilton, Conn.) from cultures grown at 30°C in liquid media by the method of Royer and Yamashiro (1992) . Spheroplasts were suspended in 0.6% low melting point agarose (Bio-Rad, Mississauga, Ontario) in 1 M sorbitol, 50 mM EDTA (pH 8.0) with 2 mg/ml proteinase K (Boehringer Mannheim) at a concentration of 1×10^8 spheroplasts/ml and subsequently treated as in Orbach et al. (1988). OFAGE (2015 Pulsaphore unit with 2301 Macrodrive1; Pharmacia-LKB) was in 15 × 15 cm 0.6% agarose gels (SeaKem, FMC BioProducts, Rockland, Me.) 0.5 × TBE (Sambrook et al. 1989) at 8°C. Pulse time was 45 min, at 50 V for a duration of 7 days. Transfer of DNA to Hybond (Amersham) membranes and DNA hybridization were as described above.

DNA amplifications and the polymerase chain reaction

Oligonucleotides for the DNA amplification of genomic and cDNA copies of *mt A*-1 were synthesized with an Applied BioSystems 380A DNA synthesizer (Nucleic Acid-Protein Service Unit, Biotechnology Laboratory, University of British Columbia). DNA amplifications were performed using a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's conditions on a Model 480 Perkin-Elmer DNA thermal cycler.

DNA sequencing

DNA sequencing of the *N. africana mt A*-1 was performed by dideoxy chain-termination (Sanger et al. 1977) on a Model S2 sequencing apparatus (BRL Life Technologies, Gaithersburg, Md.). Subclones of the *N. africana A* idiomorph were constructed in pGEM3Zf plasmids (Promega, Madison, Wis.). Oligonucleotides used for DNA sequencing were synthesized as above and were based on the *N. crassa mt A*-1 sequence. DNA and protein sequence analyses and comparisons were performed using the programs available from the Wisconsin Genetics Computer Group (Devereux et al. 1984).

Transformation and mating assays

Competent spheroplasts of *N. crassa* were prepared according to the method of Schweizer et al. (1981). The *N. africana mt A*-1 gene was co-transformed into *N. crassa* strains *ad*-3*B*, *cyh*-1 a^{m1} (FGSC 4564) and *un*-3, *ad*-3*A*, *cyh*-1 A^{m64} (FGSC 4573) with pBC1 (Vollmer and Yanofsky 1986), which encodes resistance to benomyl (Dupont NeMours). Mating assays were as described previously (Glass et al. 1988). The *N. crassa* strains *qa*-2; *aro*-9 *A* and *qa*-2; *aro*-9; *nic*-3 *a* were used as transformation recipients for heterokaryon incompatibility assays.

RNA and cDNA isolation

Total RNA was extracted from *N. africana* by the guanidinium-HCl method of Logemann et al. (1987). The RNA was subjected to RNAase-free DNAse I treatment (Pharmacia). RNA electrophoresis was according to Forney et al. (1988). Construction of cDNAs from total RNA was performed with a First-Strand cDNA Synthesis kit (Pharmacia).

Results

Structure of the mating-type locus of *N. africana*

In a previous study, it was determined that N. *africana* harboured sequences similar to the *A* idiomorph of *N. crassa*, although the extent of conservation between the two idiomorphs was not determined (Glass et al. 1990b). In the present study, a series of probes spanning the entire *N. crassa A* idiomorph (Fig. 1, A2-A6) was hybridized to *N. africana* genomic DNA. All of the *N. crassa A* idiomorph probes hybridized to *N. africana* genomic DNA. The presence of identically sized bands when adjacent *A*-specific probes were used indicated contiguity of the *A*-hybridizing sequences in *N. africana* (Fig. 2A, B, lanes B-D; additional data not shown). Based on the hybridization data with the *N. crassa* probes, *N. africana* contains sequences that are similar in composition and organization to the entire 5301 *A* idiomorph of *N. crassa*.

The *A* and *a* idiomorphs of *N. crassa* are dissimilar in sequence, although sequences flanking the mating-type locus are nearly identical (Glass et al. 1990a). To determine if the *A* idiomorph resides at a locus similar in flank DNA composition to that of *N. crassa*, centromere-proximal and centromere-distal sequences of the *N. crassa* mating-type locus (Fig. 1) were used as probes against genomic DNA of *N. africana*. The centromere distal probe of *N. crassa* (Fig. 1, LF1) hybridized to *N. africana* DNA and showed similar hybridization patterns to those detected with internal *A* idiomorph probes (Fig. 2A, lanes B-D; Fig. 2B, lanes B-D), showing that the *A* idiomorph of *N. africana* is linked to the centromere-distal flank sequences. In contrast, a probe that contains 2 kb of DNA centromere-proximal to the mating-type locus (Fig. 1, RF1) failed to hybridize to sequences in the *N. africana* genome (Fig. 2C, lanes B-D). In *N. crassa*, sequences in the centromere-proximal region of the *A* idiomorph are thought to encode an *A* mating-specific pheromone (T. Randall and R.L. Metzenberg, personal communication).

To determine the extent of variability between *N. crassa* and *N. africana* in the centromere proximal flank, a probe that included 12 kb of centromere-proximal flank sequence (Fig. 1, RF2) was hybridized to *N. africana* genomic DNA. Within the *N. africana* genome, the 12 kb RF2 probe hybridized to fragments of DNA that totalled only 6 kb (Fig. 2D, lanes C,D), suggesting that at least 6 kb of centromere-proximal flank region of *N. africana* may be divergent from that of *N. crassa*. The linkage of the RF2 probe to the *N. africana A* idiomorph could not be unequivocally established using a variety of restriction enzymes.

Previous cytological studies with *N. africana* showed that seven chromosomes are present and that events associated with meiosis and ascospore formation are very similar to those in *N. crassa* (Raju 1978; 1980). We

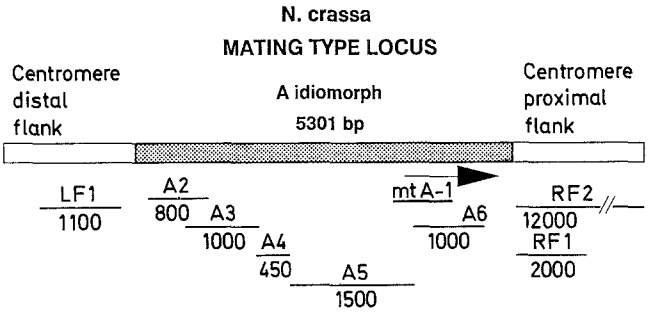

Fig. 1 The mating-type locus of *Neurospora crassa* showing fragments of the *A* idiomorph used as probes to determine the relatedness to the *A* locus of *N. africana*. Fragments A2–A6 are internal to the *A* idiomorph. LF1, RF1 and RF2 are cloned portions of the centromere-distal and centromere-proximal region, respectively, that flank the mating-type locus. The open reading frame, *mt A*-1, specifies mating behaviour and heterokaryon incompatibility in *N. crassa*. Size of the cloned insert DNA is given below each probe in bp

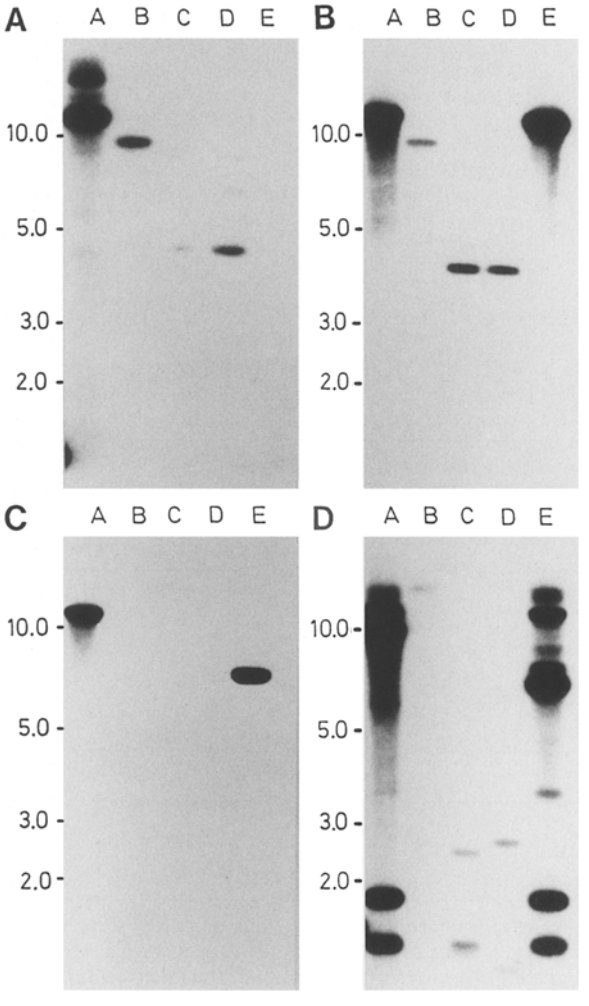

Fig. 2 **A–D** Autoradiographs from hybridization of *N. africana* genomic DNA to probes made from fragments of *N. crassa* (Fig. 1): A5, 1.5 kb (**A**), LF1, 1.1 kb (**B**), RF1, 2.0 kb (**C**) and RF2, 12 kb (**D**). Lanes contain restriction enzyme digests of genomic DNA with (A) *N. crassa A*, *Bam*HI; (B) *N. africana*, *Bgl*II; (C) *N. africana*, *Bam*HI; (D) *N. africana*, *Hin*dIII; (E) *N. crassa a*, *Bam*HI. The multiple bands observed in **D**, lanes A and E reflect the hybridization of the 12 kb probe to *N. crassa* genomic DNA

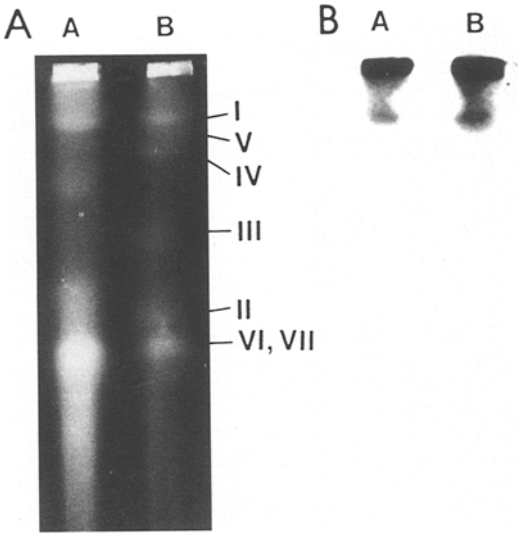

Fig. 3 **A** Ethidium bromide-stained orthagonal field agarose gel of *N. africana* (lane A) and *N. crassa* (lane B, strain C2(3)-46 A, Oak Ridge background). Linkage group designations for *N. crassa* at right of **A** follow those of Orbach et al. (1988). **B** Hybridization between blot of gel in **A** and probe A6 (Fig. 1). Independent measurements of migration distance for the largest three chromosomes of *N. africana* were performed on additional orthagonal field agarose gels (Table 1) and indicate that the mating-type chromosome of *N. africana* is approximately 1 Mb smaller than LGI of *N. crassa*

Table 1 Migration distance for the three largest *Neurospora africana* chromosomes and locations of hybridization signals with four *N. crassa* LGI markers (*nt* not tested)

Agarose gels		
Chromosome[a]	Migration distance[b] (mm)	
	a	b
1	9.5	10
2	12	12
3	14	14
Autoradiographs		
Probe[c]		
a5	Nil[d]	Nil[d]
A7	12	nt
X23:B:9	nt	12
pcy-20	12.5	nt

[a] Chromosome-sized bands were numbered in order of increasing migration distance in orthagonal field agarose gels

[b] Migration distances in two lanes, a and b, measured from the top of the loading well to the centre of the band

[c] Probes a5 and A7 are specific to *mt-a* and *mt-A*, respectively. Cosmid X23:B:9 from the Orbach/Sachs library (Fungal Genetics Stock Center) was mapped to the right telomere region of *N. crassa* LGI by RFLP patterns (Metzenberg et al. 1985; Metzenberg and Grotelueschen 1992). Plasmid pcy-20 (gift of A. Lambowitz) is located centromere-distal to the mating-type locus of *N. crassa*, on the left of LGI

[d] nil, no hybridization signal

performed karyotypic analyses of the *N. africana* genome to establish whether similar chromosomes contain the mating-type locus in *N. africana* and *N. crassa*. As shown in Fig. 3, the electrophoretic karyotypes of the seven chromosomes of *N. africana* and *N. crassa* are similar, although not identical. In *N. crassa*, the mating-type locus is on the largest 10.3 Mb chromosome (Orbach et al. 1988; Orbach 1992; Fig. 3). In *N. africana*, the *A* idiomorph probe hybridized to the second largest chromosome, which is about 1 Mb smaller than linkage group I (LGI) of *N. crassa* (Table 1). Although the mating-type chromosome in *N. africana* is smaller than LGI of *N. crassa*, two additional probes from LGI (X23:B:9 and pCY-20) produce hybridization signals at the same position as the mating-type chromosomes of *N. africana* (Table 1). This result suggests that the mating-type chromosomes of *N. crassa* and *N. africana* are similar in composition, but that a portion of *N. africana* LGI may either be present on another chromosome, or be absent altogether.

Cloning and characterization of *mt A*-1 of *N. africana*

In N. *crassa*, an ORF in the *A* idiomorph, *mt A*-1, confers mating specificity during the sexual cycle and heterokaryon incompatibility between opposite mating-types during vegetative growth (Glass et al. 1990a). DNA hybridization studies indicate that the *A* sequence is conserved in *N. africana*, and therefore we wanted to determine whether the region also encodes functional products. A portion of the *N. africana A* sequence was cloned by making a subgenomic library in *E. coli* and colonies containing the *A* sequence were identified by hybridization to *N. crassa mt A*-1. A 3 kb clone was recovered that was subsequently shown to be lacking approximately 2 kb of centromere-distal *A* sequence. The DNA sequence of *N. africana mt A*-1 was determined from subclones of the 3 kb fragment; approximately 200 bp of *mt A*-1 at the 5' end of the gene was missing. Oligonucleotide primers were synthesized that corresponded to the 3' end of the *mt A*-1 of *N. crassa*, and the remainder of *N. africana mt A*-1 was amplified by PCR and sequenced.

DNA sequence analysis of the *N. africana mt A*-1 revealed an ORF encoding a 286-amino acid polypeptide (Fig. 4). DNA sequence comparison revealed that the *N. africana mt A*-1 is 91% identical to *mt A*-1 of *N. crassa*. A comparison of the *N. africana mt A*-1 ORF revealed 88% amino acid identity with the *N. crassa mt A*-1 ORF. Thirty-four amino acids differ between the two ORFs and these are clustered in the carboxyl-terminal portions of the ORFs (Fig. 5); most are conservative amino acid differences. In addition, a 6-bp deletion resulting in the absence of two amino acids (Arg and Gly; Fig. 5) occurs in the amino-terminal portion of the *N. africana mt A*-1 ORF relative to the *N. crassa mt A*-1 ORF. In the region of similarity to *S. cerevisiae* MATα1, the *N. crassa* and *N. africana mt A*-1 ORFs are almost

Fig. 4 DNA sequence of *mt A*-1 of *N. africana*. The 5' and 3' splice junctions of the 58 bp intron are *double underlined*. Deduced amino acid sequence of *mt A*-1 is shown below the DNA sequence

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1 ccccctcccgccccccctcggtcgtcaagtgaagggagagagaagccgccccacccaaaatt
  aaccaatcaacccccatgtcttctatttaagaatgcccagttcatcatttccaccttcac
  tcaaacttgccgccacatttcctcgaatcatcaatcttccaaccaaaagctcggctgcac
  ttcttcaagtgttaaacgctctccaatcaagaattcaaccgccagaaacacgatgtcgtg
                                                        MetSerCy
                                                         1
  cgtcgatcaaatcgtcaagacgttcgccgacctcactgagggtgatcgtgaagcggcaat
  sValAspGlnIleValLysThrPheAlaAspLeuThrGluGlyAspArgGluAlaAlaMe
                                  30
  gagagctttctcaatgatgatgcgcaccgaacctgttcgccaaaccccgcggcaaagaa
  tArgAlaPheSerMetMetMetArgThrGluProValArgGlnThrProAlaAlaLysLy

  gaaggtcaacggcttcatgagtttcagatgtaagtcaaatctggatcaatcttgttgaaa
  sLysValAsnGlyPheMetSerPheArgS
                                52
  atccattctaattgccttttatttcagcgtactattccccgctcttctctcagctcccgc
                                  erTyrTyrSerProLeuPheSerGlnLeuProG

  agaaggagagatcacccttcatgaccattctctggcagcacgatcccttccacaacgaat
  lnLysGluArgSerProPheMetThrIleLeuTrpGlnHisAspProPheHisAsnGluT
                     70
  ggaatttcatgtgctcggtgtattcgtcgatccgcacctaccttgagcaggagaaagtta
  rpAsnPheMetCysSerValTyrSerSerIleArgThrTyrLeuGluGlnGluLysValT
                     90
  ccctgcaactctggattcactatcgtgtccgccatctgggagtgattatccgcgacaact
  hrLeuGlnLeuTrpIleHisTyrArgValArgHisLeuGlyValIleIleArgAspAsnT
                    110
  acatggcatcgtttggctggaacctcgtccagctgcccaacggcactcacgacctcgagc
  yrMetAlaSerPheGlyTrpAsnLeuValGlnLeuProAsnGlyThrHisAspLeuGluA
                    130
  gcaccgctcttcctttggttcagcataacctccagcccatgaacggcctatgcctgttca
  rgThrAlaLeuProLeuValGlnHisAsnLeuGlnProMetAsnGlyLeuCysLeuPheT
                    150
  ccaagtgcctcgagagcggattgcctcttgccaatcctcaccctgtcatcgccaagcttt
  hrLysCysLeuGluSerGlyLeuProLeuAlaAsnProHisProValIleAlaLysLeuS
                    170
  cagatcctagctacgacatgatctggttcaacaagcgtcctcaccgtcagcagggacacg
  erAspProSerTyrAspMetIleTrpPheAsnLysArgProHisArgGlnGlnGlyHisA
                    190
  ccggccaaacttacaattctgaacttggagtgtcggcgctcttcccttgcaatcacgcag
  laGlyGlnThrTyrAsnSerGluLeuGlyValSerAlaLeuPheProCysAsnHisAlaV
                    210
  tcgctgcagcggtcgatggcatcaccgaccttcctctctcccattggcttcagcagggag
  alAlaAlaAlaValAspGlyIleThrAspLeuProLeuSerHisTrpLeuGlnGlnGlyA
                    230
  atttcggcaccgaggccggattctcacctcagtttgagaccttgttggattcgatccttg
  spPheGlyThrGluAlaGlyPheSerProGlnPheGluThrLeuLeuAspSerIleLeuG
                    250
  agaatggaaacgcctctatcaatgacccctacaatatggctcttggtatgggtgttccca
  luAsnGlyAsnAlaSerIleAsnAspProTyrAsnMetAlaLeuGlyMetGlyValProM
                    270
  tgatgggttag                                                  1150
  etMetGlyEnd
       286
```

identical. There is only a single amino acid difference between the two in this region (Fig. 5; amino acids 40–72).

An intron is predicted within the *N. africana mt A*-1 in a position identical to that of the *N. crassa mt A*-1 intron. The *N. africana mt A*-1 intron is 55 bp in length and 78% identical in DNA sequence to the 56 bp *N. crassa mt A*-1 intron. The intron 5' consensus sequences differ by 1 bp, while the 3' consensus splice sites are identical.

Mating behaviour of *N. crassa* transformants containing *N. africana mt A*-1

It is difficult to assess the function of mt *A*-1 in *N. africana* due to the inability to cross different strains and a lack of mutants and transformation protocols. The mating function of *N. crassa mt A*-1 homologue is assayed by introducing it into sterile *N. crassa* A^{m64} and a^{m1} mutants (Griffiths and DeLange 1978; Griffiths 1982). The A^{m64} mutant contains a frameshift mutation in *mt A*-1 that renders it sterile and heterokaryon compatible (Glass et al. 1990a); the sterile and heterokaryon compatible a^{m1} mutant contains a 200 bp insertion in the *mt a*-1 ORF (Staben and Yanofsky 1990). The introduction by ectopic insertion of the *N. crassa mt A*-1 into

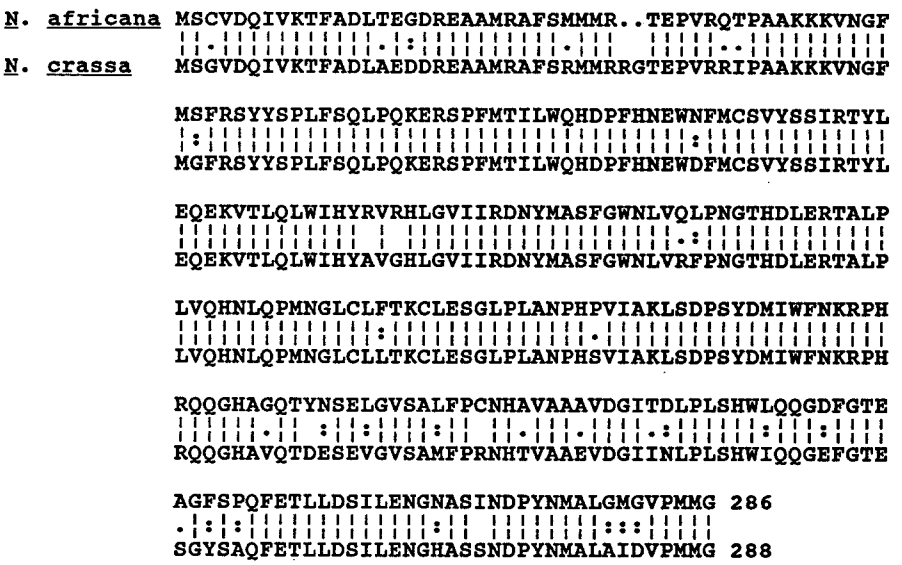

Fig. 5 Comparison of the deduced amino acid sequence of the *N. crassa mt A*-1 polypeptide and the *N. africana mt A*-1 polypeptide. Identical amino acids are indicated by *vertical lines*; conservative amino acids are indicated by either *one* (.) or *two dots* (:), depending on the degree of similarity; *blank spaces* indicate non-conservative amino acid differences between the two polypeptides. The two-amino acid deletion in *N. africana* is denoted by *horizontal dots* (..)

either a^{m1} or A^{m64} spheroplasts enables the transformants to mate as an *A* strain, although ascospore formation is only restored by gene replacement at the mating-type locus (Glass et al. 1988; 1990a).

The mating function of *N. africana mt A*-1 was assayed by introducing it into a^{m1} and A^{m64} mutants by co-transformation with a vector specifying benomyl resistance (Vollmer and Yanofsky 1986). The benomyl-resistant transformants were then replica-plated onto *A* and *a* tester plates to assay for mating activity. In all of the experiments, approximately 20% of a^{m1} and A^{m64} benomyl-resistant transformants were capable of mating as an *A* strain and forming perithecia in crosses with an *a* strain. This percentage of *N. africana mt A*-1 co-transformants that mate is consistent with control co-transformations with *N. crassa mt A*-1. The development of the perithecia was similar to a^{m1} and A^{m64} transformants bearing the *N. crassa mt A*-1 and also to wild type for 3–4 days post-fertilization. After this point, further development of the perithecia was arrested. This phenotype is similar to that observed when *N. crassa mt A*-1 transformants are assayed for mating activity. The examination of perithecial contents revealed only the presence of paraphyses (sterile hyphae); no asci or ascospores were observed. The a^{m1} and A^{m64} mutants transformed with vector alone did not induce perithecial formation when crossed with either an *A* or *a* strain.

The a^{m1} and A^{m64} transformants bearing *N. africana mt A*-1 were plated individually onto mating medium (Westergaard and Mitchell 1947) and examined for homothallic behaviour. Although the transformants formed numerous male (conidia) and female (protoperithecia) mating structures, no perithecia were formed under these conditions.

Heterokaryon incompatibility function of *N. crassa* transformants containing *N. africana mt A*-1

In addition to mating, the N. *crassa A* idiomorph also confers heterokaryon incompatibility during vegetative growth (Beadle and Coonradt 1944; Garnjobst 1953). The heterokaryon incompatibility function has been localized to *mt A*-1 by mutational analysis (Griffiths and DeLange 1978; Griffiths 1982). All of the A^{m} sterile mutants are also heterokaryon compatible and contain frameshift mutations within *mt A*-1 (Glass et al. 1990a). The heterokaryon incompatibility function of *mt A*-1 can be assayed by the introduction of *mt A*-1 into *A* and *a* spheroplasts by DNA-mediated transformation. The transformation efficiency is 20-fold lower when *N. crassa mt A*-1 is introduced into *a* spheroplasts relative to *A* spheroplasts. The introduced *mt A*-1 sequences are deleted or rearranged in the surviving *a* transformants (Glass et al. 1988; N. L. Glass, unpublished results) and do not confer *A* mating activity.

It was, therefore, of interest to determine whether *N. africana mt A*-1 confers heterokaryon incompatibility in *N. crassa*, as it does *A* mating activity. The *N. africana mt A*-1 was first cloned into a vector containing the *qa*-2 (Naf*A*1.qa2) gene and subsequently introduced into *A* and *a* strains bearing *qa*-2 and *aro*-9 mutations. Similar transformation efficiencies were observed when Naf*A*1.qa2 or the qa-2 vector were introduced into *N. crassa A* spheroplasts. In contrast, transformation efficiencies were 12-fold lower when Naf*A*1.qa2 was introduced into *a* spheroplasts as compared to the *qa*-2 vector alone. The surviving transformants with the *a* spheroplasts were tested for *A* fertility by replica-plating onto *a* mating-type testers; perithecia were not formed on any of the plates.

RNA analyses of *N. africana mt A*-1

Although N. *africana mt A*-1 confers both mating and heterokaryon incompatibility in *N. crassa*, the above experiments do not demonstrate whether or not *mt A*-1 functions in *N. africana*. To obtain direct evidence for *mt A*-1 transcription in *N. africana*, we performed mRNA analyses of *mt A*-1. Total RNA was isolated from sta-

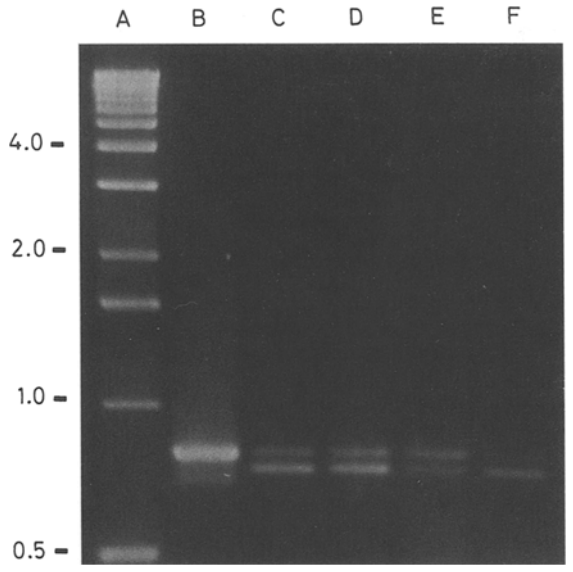

Fig. 6 Reverse PCR analysis of the *N. africana mt A*-1 transcript. **A** molecular weight markers; **B** *N. africana mt A*-1 amplified from genomic DNA; **C** *N. africana mt A*-1 cDNA detected by reverse polymerase chain reaction from total RNA isolated from a 3-day-old culture; **D** *N. africana mt A*-1 cDNA detected from a 5-day-old culture; **E** *N. africana mt A*-1 cDNA detected from a 6-day-old culture; **F** *N. crassa mt A*-1 cDNA. The 5' primer used for amplification precedes the intron by 48 bp and the 3' primer used for amplification spans the *mt A*-1 stop codon

tionary liquid cultures of *N. africana* grown at 25°C at 3, 5 and 6 days after inoculation. Using reverse transcriptase and random primers, cDNAs were constructed prior to PCR using *mt A*-1 specific primers. A cDNA copy of *mt A*-1 could be detected at all three time points, although the level of the cDNA was less in RNA isolated from the 6-day culture (Fig. 6). In *N. africana*, perithecial formation began at 3 days after inoculation at the periphery of the culture. By day 6, the mycelium was covered with developing black perithecia. Ascospore ejection from the perithecia typically occurs between days 8 and 10 in *N. africana*.

Discussion

In this study, we have examined the structure of the *A* mating-type locus in *N. africana* and have begun a functional analysis of its *mt A*-1 ORF. Through Southern blot analyses, we have shown that the *A* sequence of *N. africana* is similar in size and composition to the *A* idiomorph of *N. crassa*. Based on DNA-DNA hybridizations to OFAGE gels, the *A* locus of *N. africana* and the mating-type locus of *N. crassa* are present on chromosomes of similar composition and size in the two species. Furthermore, the composition of the centromere-distal mating-type flank sequences is similar in *N. crassa* and *N. africana*. These results show that the composition and location (as defined by flanking sequences) of the *A* locus and the organization of the chromosome containing the mating-type locus have been conserved between the heterothallic species *N. crassa* and the homothallic species *N. africana*.

The mating-type region that is most divergent between *N. crassa* and *N. africana* occurs in the centromere-proximal flank sequences. A hybridization signal was not observed in *N. africana* when portions of the 2–6 kb centromere-proximal flank of *N. crassa* were used as probes. The composition of this region in *N. africana* is unknown. However, the centromere-proximal mating-type flank sequences are known to be variable in hybridization to genomes of other heterothallic *Neurospora* species (T. Randall and R. Metzenberg, personal communication) as well as in the homothallic species *N. terricola* (N. Beatty and N. L. Glass, unpublished results). Interactions leading to fertilization of opposite mating types in *N. crassa* are thought to be mediated by the production of mating type-specific, diffusible pheromones (Bistis 1981; 1983). A transcript encoding a putative fungal pheromone has been detected in this region of *N. crassa* (T. Randall and R. Metzenberg, personal communication). It is possible that the centromere-proximal flank sequences diverge in different heterothallic *Neurospora* species, which presumably utilize different pheromones, as well as in homothallic *Neurospora* species that do not mate.

The conserved structure of the *N. africana* mating-type locus suggests that there may be functional constraints on change within the region. This is further supported by sequence analysis and investigations into function of *mt A*-1. First, *N. crassa* and *N. africana* exhibit 91% sequence identity for the ORF. As in *N. crassa*, the *N. africana mt A*-1 intron is excised during mRNA processing and stop codons or frameshift mutations are not found in the *mt A*-1 ORF. Most of the differences between the *N. africana mt A*-1 and the *N. crassa mt A*-1 reside in the carboxyl-terminal portion of the two ORFs. The carboxyl-terminal portion of the *N. crassa mt A*-1 ORF has been implicated in conferring mating activity and heterokaryon incompatibility (Glass et al. 1990a). The alterations in this region in *N. africana mt A*-1 are not sufficient to abolish these functions when assayed in *N. crassa*. Two amino acids in the amino-terminal portion of *N. africana mt A*-1 are absent as compared to *N. crassa mt A*-1 (amino acids 32,33). The significance of this difference is unknown, but may prove useful in examining the evolutionary relationship between homothallic species of *Neurospora* or in the dissection of functional properties of *mt A*-1.

We addressed the question of whether the *mt A*-1 of *N. africana* encodes a functional polypeptide by assaying mating and heterokaryon incompatibility in *N. crassa*. Both the mating and heterokaryon incompatibility phenotype conferred by *N. africana mt A*-1 were indistinguishable from that conferred by *N. crassa mt A*-1. This is in spite of the fact that *N. africana* does not mate and cannot form heterokaryons with an opposite mating-type strain because only one mating-type sequence (*A*) is present in the genome of the species. Significantly,

the introduction of *N. africana mt A*-1 into *N. crassa* did not confer homothallism. This result suggests that differences elsewhere in the genomes of *N. africana* and *N. crassa* are important in defining sexual behaviour. Previous attempts to make *N. crassa* exhibit homothallic behaviour have also not been successful. The transformation of the *A* idiomorph into *a* strains that contain a mutation, *tol*, a suppressor of mating-type-associated heterokaryon incompatibility (Newmeyer 1970), does not result in the recovery of homothallic isolates (N. L. Glass, unpublished results). This result suggests that the evolution of a homothallic isolate from a heterothallic ancestor is a multi-step process, but that the functions of the mating type *A*-1 sequence are retained.

N. africana is not capable of mating in a heterothallic manner, at least in part because it lacks the male structures necessary for the mating process (Mahoney et al. 1969; Perkins and Turner 1988) as well as female receptive hyphae (G. N. Bistis, personal communication). The *N. africana A* locus may function during outbreeding in nature following hyphal fusion with another individual; whether this actually occurs is unknown. Alternatively, it may be required for processes in the sexual cycle other than mating. In *N. crassa*, the amino-terminal region of *mt A*-1 has been shown by mutational analyses to be involved in post-fertilization functions (Stenberg et al. 1993; L. Stenberg, N. L. Glass and A. J. F. Griffiths, unpublished results); this region is almost identical to *N. africana mt A*-1 (amino acids 40–72, Fig. 5). In addition, a second region of the *N. crassa A* idiomorph other than *mt A*-1 is required for post-fertilization events (Glass and Lee 1992). It is possible that products from the entire *A* locus are required for proper functioning of the sexual cycle in *N. africana*. Alternatively, the *N. africana A* locus may not function during sexual development, but may be a vestige of an heterothallic ancestral state. This last possibility seems unlikely given the functional and structural similarities of the *N. crassa* and *N. africana mt A*-1 genes revealed in this study.

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