Molecular Characterization of \textit{tol}, a Mediator of Mating-Type-Associated Vegetative Incompatibility in \textit{Neurospora crassa}

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ABSTRACT

The mating-type locus in the haploid filamentous fungus, \textit{Neurospora crassa}, controls mating and sexual development. The fusion of reproductive structures of opposite mating type, A and a, is required to initiate sexual reproduction. However, the fusion of hyphae of opposite mating type during vegetative growth results in growth inhibition and cell death, a process that is mediated by the \textit{tol} locus. Mutations in \textit{tol} are recessive and suppress mating-type-associated heterokaryon incompatibility. In this study, we describe the cloning and characterization of \textit{tol}. The \textit{tol} gene encodes a putative 1011-amino-acid polypeptide with a coiled-coil domain and a leucine-rich repeat. Both regions are required for \textit{tol} activity. Repeat-induced point mutations in \textit{tol} result in mutants that are wild type during vegetative growth and sexual reproduction, but that allow opposite mating-type individuals to form a vigorous heterokaryon. Transcript analyses show that \textit{tol} mRNA is present during vegetative growth but absent during a cross. These data suggest that \textit{tol} transcription is repressed to allow the coexistence of opposite mating-type nuclei during the sexual reproductive phase. \textit{tol} is expressed in a mat A, mat a, A/a partial diploid and in a mating-type deletion strain, indicating that MAT A-1 and MAT a-1 are not absolutely required for transcription or repression of \textit{tol}. These data suggest that TOL may rather interact with MAT A-1 and/or MAT a-1 (or downstream products) to form a death-triggering complex.

ENTRY into the sexual cycle in the filamentous fungus, \textit{Neurospora crassa}, requires the fusion of reproductive structures of opposite mating types (A and a). Cell fusion results in the presence of opposite mating-type nuclei within a common cytoplasm. Proliferation of A and a nuclei occurs in the ascogenous hyphae before formation of the crozier, where opposite mating-type nuclei are partitioned (Raju 1980). Karyogamy between A and a nuclei occurs in the crozier and is immediately followed by meiosis and a single mitotic division to form eight linearly arranged ascospores. Over 200 progeny are typically formed from a single fertilization event. Mating-type function is required for both cell fusion and for postfertilization events, presumably during the proliferation and partitioning of opposite mating-type nuclei in the crozier (Griffiths and Delange 1978; Griffiths 1982; Glass and Lee 1992; Ferreira et al. 1998).

In addition to its role during the sexual cycle, the mating-type locus in \textit{N. crassa} has a function during vegetative growth. Although fusion of opposite mating-type reproductive structures is required for entry into the sexual cycle, fusion of opposite mating-type hyphae during vegetative growth results in growth inhibition and cell death (Beadle and Coonrath 1944; Garnjobst 1953). At least 10 additional genetic determinants called heterokaryon incompatibility \textit{(het}) loci also restrict formation of vegetative heterokaryons; genetic differences at these loci are not required for fertilization nor do they interfere with sexual reproduction (for review see Glass and Kuldau 1992; Leslie 1993).

The mat A and mat a sequences have been characterized and are composed of dissimilar sequences (Glass et al. 1990; Staben and Yanofsky 1990). The 5301-bp A idiomorph contains three open reading frames (ORFs), mat A-1, mat A-2, and mat A-3. The mat A-1 gene determines mating identity and also confers mating-type-associated heterokaryon incompatibility (Glass et al. 1990). Strains that contain null mutations in mat A-1 are both sterile and heterokaryon compatible with a strains (Griffiths 1982; Saupé et al. 1996). MAT A-1 displays a region of similarity to the mating-type transcriptional regulator, MATα1, of Saccharomyces cerevisiae (Glass et al. 1990). The mat A-2 and mat A-3 genes are not required for mating identity or heterokaryon incompatibility, but are required for postfertilization functions (Glass and Lee 1992; Ferreira et al. 1996).

The a idiomorph contains only one ORF, mat a-1. A functional mat a-1 is required for mating identity, heterokaryon incompatibility, and postfertilization...
functions (Staben and Yanofsky 1990). As with MAT A-1, mutations in mat a-1 result in strains that are sterile and that are able to form vigorous heterokaryons with either A or a strains (Griffiths and Delange 1978). An exceptional mat a-1 mutant, a₃₃, is fertile but heterokaryon compatible with A strains (Griffiths and Delange 1978). The a₃₃ mutant contains a missense mutation in the carboxyl-terminal region of mat a-1 (Staben and Yanofsky 1990). MAT a-1 has features in common with transcriptional regulators, specifically an HMG box. The HMG box of MAT a-1 has been shown to bind DNA and to be required for conferring mating identity, but not for heterokaryon incompatibility (Philley and Staben 1994).

A recessive mutation unlinked to the mating-type locus, tolerant (tol), suppresses mating-type-associated heterokaryon incompatibility such that tol A and tol a strains form a vigorous heterokaryon (Newmeyer 1970). Matting activity of these strains is unaffected and tol strains show normal fertility in crosses with an opposite mating-type strain. Attempts to identify additional suppressors of mating-type-associated incompatibility resulted only in the isolation of additional tol alleles (Veliani et al. 1994). The tol mutation apparently does not suppress incompatibility due to differences at other loci (Newmeyer 1970; Leslie and Yamashiro 1997).

This article reports the isolation and molecular characterization of the tol gene of N. crassa. The tol gene encodes a putative 1011-amino-acid (aa) polypeptide with a coiled-coil domain and a leucine-rich repeat. Mutants of tol obtained by repeat-induced point mutation (RIP; Selker 1990) lose their mating-type heterokaryon incompatibility function but are otherwise phenotypically normal during vegetative growth and sexual reproduction. Expression studies indicate that tol is not transcriptionally repressed or activated by MAT A-1 and/or MAT a-1 during vegetative growth but is apparently repressed during sexual development.

**MATERIALS AND METHODS**

**Strains, media, and culturing methods:** The N. crassa strains used in the study are listed in Table 1. Culturing and crossing, using Vogel’s (Vogel 1964) and Westergaard’s (Westergaard and Mitchell 1947) media, respectively, were performed as previously described, with modification (Davis and Deseres 1970; Perkins 1986). For heterokaryon tests, 2 µl conidial suspensions (10⁷/ml) of two strains containing different auxotrophic markers were cocultivated on vegetative growth media (Vogel 1964). A compatible heterokaryon forms a vigorously conidiating culture with 3 days of incubation with a mean growth rate of 7 cm/day. Mating-type incompatible heterokaryons are usually aconidial and have a growth rate of ~0.7 cm/day (Veliani et al. 1994). For perithecial RNA extraction, crosses were performed in petri dishes containing Westergaard medium layered with Miracloth membrane (Calbiochem, La Jolla, CA). Perithecia were scraped from the Miracloth and RNA was extracted as described below.

**Strain construction:** To introgress part of Mauriceville linkage group (LG) IV into T (IL → IIR) 39311 ser-3 A (Perkins and Barry 1977; RLM 04-08, Table 1), RLM 04-08 was crossed with FGSC (Fungal Genetics Stock Center, Kansas City, KS) strain 2226 (Mauriceville wild-type a). Numerous restriction fragment length polymorphisms (RFLPs) have been observed between Mauriceville strains (FGSC 2225 and 2226; Table 1) and the Oak Ridge background of standard laboratory strains. Genomic DNA from ser-3 A progeny were screened for Mauriceville LG IV by hybridization to cosmid G4:A9 (which contains trp-4, a LG IV locus closely linked to tol). A progeny, R5-28 (Table 1) had RFLP patterns identical to the FGSC 2226 parent when probed with G4:A9.

The strain NE-1 (his5 tol trp-4 A) was constructed by crossing his5 A (FGSC 456) with tol trp-4 A (FGSC 2337); a His- and Trp- progeny was selected. Crossing of NE-1 to R1-09 (un-3 a) gave R5-27 (un-3; his5 tol trp-4 A). The presence of the tol allele (N83) in R5-27 was confirmed by crossing with RLM 04-08 (Table 1); approximately half of the A/a partial diploid progeny displayed normal growth rates (see Figure 2). The strain R5-27 was subsequently crossed with a pan-2-containing strain (12-21-388) to give strains R4-71 and R4-72 (Table 1), which were used for transformation assays.

**Chromosome walk and physical mapping:** A Trp-4-containing cosmid was identified from the Orbach/Sachs pMOcosX genomic library (Orbach 1994; available from the FGSC) using a trp-4 clone (Voller and Yanofsky 1986) as a probe. A chromosome walk was performed in both directions using end fragments as probes for each step. Selected cosmids were physically mapped by RFLP analysis (Mitchell et al. 1985).

**Transformation assay and subcloning of tol:** Spheroplast preparation and transformation were performed as previously described (Schweizer et al. 1981; Vollmer and Yanofsky 1986) with the exception of selecting for resistance to hygromycin B (250 units/ml; Calbiochem). Cosmids in the walk were assayed for Tol⁺ activity by cotransfomring the cosmids (conferring hygromycin resistance) with a mat A-1-containing pOKE103 construct (pOKE103 has a pan-2 selectable marker; Gro et al. unpublished results) into strain R4-71 (his5 tol trp-4; pan-2 a) and strain R4-72 (ade3; trp-4 cot-1; pan-2 A) spheroplasts. The tol gene was further subcloned into the hygromycin-resistant vector (pCB1004; Carroll et al. 1994). Homokaryotic transformants were isolated according to Ebbelle and Sachs (1990).

**Nucleic acid isolation, DNA hybridization, and reverse transcriptase PCR analysis:** Standard molecular biology procedures were used throughout (Sambrook et al. 1989). Genomic DNA isolation from Neurospora was adapted from Oakley et al. (1987). Total RNA was extracted according to Logemann et al. (1987) and enriched for poly(A)+ using the OligoTex mRNA kit (Qiagen, Chatsworth, CA). Gel electrophoresis and nucleic acids transferred to Nylon filters (Schleicher and Schuell, Keene, NH) were performed according to manufacturer’s specifications. [α-³²P]dCTP-labeled probes (Amer sham, Oakville, ON) were generated from digested DNA using the T7 QuickPrime Kit (Pharmacia, Baie d’Urfe, Quebec). For reverse transcriptase (RT)-PCR, cDNAs were synthesized by the Not I-d(T)₅ primer using a procedure from the First Strand cDNA Synthesis Kit (Pharmacia). PCRs of DNA and cDNA were performed using primers tol 13 (5’GGGCGGAG TATAGGAGG 3’; bases 641 to 657) and tol 11 (5’CCAGCAG TGGCTCAGC 3’; bases 1144 to 1129). The tol⁺ mutant allele (N83) was amplified from strain R5-27 DNA using primers tol ATG1 (5’ CCTGGGCTCACCATTGC 3’; base 50 to -34) and tol 3-end (5’ CGGCGGATCTCTTCTG 3’; base 2894 to 2877). The tol cDNA and tol⁺ (N83) PCR products were cloned into the PCRII vector using a TA cloning kit (Invitrogen, San Diego) and subjected to DNA sequencing. The entire DNA sequence of two different tol⁺ (N83) clones was determined and the mutation point was confirmed from three additional subclones.
TABLE 1
Neurospora crassa strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
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<tr>
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<td>FGSC 456</td>
</tr>
<tr>
<td>R1-09</td>
<td>un-3 a</td>
<td>R. L. Metzenberg</td>
</tr>
<tr>
<td>—</td>
<td>tol trp-4 a</td>
<td>FGSC 2337</td>
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<tr>
<td>—</td>
<td>tol a</td>
<td>FGSC 4142</td>
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<td>NE-1</td>
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<tr>
<td>MV 1c-a</td>
<td>Mauricville WT a</td>
<td>FGSC 2226</td>
</tr>
<tr>
<td>MV 1c-A</td>
<td>Mauricville WT A</td>
<td>FGSC 2225</td>
</tr>
<tr>
<td>RLM 04-08</td>
<td>T(II → IIR) 39311 ser-3 A</td>
<td>R. L. Metzenberg</td>
</tr>
<tr>
<td>R5-28</td>
<td>T(II → IIR) 39311 ser-3 A</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>(Mauricville background on LG IV)</td>
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<tr>
<td>R5-27</td>
<td>un-3; his-5 tol trp-4 a</td>
<td>This study</td>
</tr>
<tr>
<td>12-21-388</td>
<td>ad-3B al-2; cot-1; pan-2 A</td>
<td>A. J. F. Griffiths</td>
</tr>
<tr>
<td>R4-71</td>
<td>his-5 tol trp-4; pan-2 a</td>
<td>This study</td>
</tr>
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<td>R4-72</td>
<td>ad-3B; trp-4 cot-1; pan-2 A</td>
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<td>74-OR23-IVA</td>
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<td>FGSC 2489</td>
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<td>A. J. F. Griffiths</td>
</tr>
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<td>A. J. F. Griffiths</td>
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<td>A. J. F. Griffiths</td>
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<td>FGSC 988</td>
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<td>This study</td>
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DNA sequence analyses: A 6.9-kb EcoRI tol-containing construct was subcloned into overlapping fragments suitable for DNA sequencing. DNA sequences were determined for both strands using the ABI (Mississauga, ON) automated Taq Dye-Deoxy Terminator cycle method at the NAPS unit, Biotechnology Laboratory, University of British Columbia. Computer sequence analyses for protein and DNA were done using the MacVector/AssemblyLIGN software (International Biotechnologies, New Haven, CT) and GCG package available from the Wisconsin Genetics Computer Group (Devereux et al. 1985) and all mapped to trp-4.

RESULTS

Cloning of tol: The tol locus is on the right arm of LG IV and is flanked by trp-4 (~1 map unit) and his-5 (2 to 6 map units; Figure 1). A cosmid containing trp-4 (G4:A9) was identified from a genomic library using the trp-4 gene (Vollmer and Yanofsky 1986) as a probe. A chromosome walk was initiated from G4:A9 in both directions. Twelve overlapping cosmids spanning a region of 350 kb around the trp-4 locus were isolated (Figure 1). The physical location of selected cosmids was determined using RFLP mapping (Metzenberg et al. 1985) and all mapped to trp-4.

To determine the orientation of the walk from trp-4, we analyzed progeny that contained crossovers between his-5, tol, and trp-4 from a cross between R5-27 (un-3; his-5 tol trp-4 a; Oak Ridge background) and MV1c-A (FGSC 2225; Mauricville background). Genomic DNA
The tol locus was bracketed by genetic crossovers and RFLP analysis of progeny. (A) Crossovers between tol and trp-4 were detected in progeny 43-9 and 43-31 (his-5+ tol+ trp-4); hybrid RFLP patterns that differed from both parental strains were observed when genomic DNA was probed with X14:C2. (B) Recombination events between his-5 and tol were selected for by isolating A/a partial diploid progeny that were his-5+, tol+, and trp-4. RFLP analysis of genomic DNA from A/a tol trp-4 progeny identified crossover points in the overlapping region of cosmids G13:C8 and X25:D7. When probed with G13:C8, eight progeny (T4, T5, T10, T15, T18, T21, T35, and T37) displayed the R5-28 (MV LG IV) pattern and one progeny (T11) displayed a hybrid pattern, indicating that the tol locus resided between G13:C8 and X14:C2 in the cosmid walk.

was isolated from His+ Trp- progeny and probed with cosmids shown in Figure 1. Recombination points in two his-5+ tol+ trp-4 progeny (43-9 and 43-31) were identified when cosmid X14:C2 was used as a probe (Figure 2A). These results indicated that cosmids directed toward G13:C8 were oriented toward the tol locus, and that the tol locus was centromere proximal to X14:C2 (Figure 1).

To bracket the tol locus within the cosmid walk, we needed to detect recombination points that were centromere proximal to tol, i.e., between tol and his-5. A cross was performed between a R5-28 (T(IL → IIR) 39311 ser-3 A; MV LG IV) × R5-27 (un-3; his-5 tol trp-4 a) (Figure 2B). The R5-28 strain contains an insertional translocation in which the left arm of LG I (which in-
cludes ser-3, un-3, and mat) is inserted into the right arm of LG II (Perkins and Barry 1977). Progeny were selected that displayed wild-type growth in medium lacking serine at 30° (selection for A/a tol partial diploid progeny; Figure 2B) and that contained tryptophan but lacked histidine (selection for progeny with recombination between his5 and tol). Genomic DNA from 68 A/a tol trp-4 progeny were screened for RFLP differences from the R5-27 parent when probed with the cosmid centromere proximal to G4:A9. Fifty-nine progeny contained RFLPs that were identical to the R5-27 parent, indicating that in these progeny the recombination point between his5 and tol lay centromere proximal to the cosmid identified in the walk. In genomic DNA from eight progeny, an R5-28 pattern was identified when G13:C8 was used as a probe, but an R5-27 pattern was observed when probed with X25:D7 (Figure 2B). These data indicated that the recombination point between his5 and tol in these progeny occurred between G13:C8 and X25:D7, and that tol must be centromere distal to the crossover point. When genomic DNA from progeny T11 was probed with G13:C8, a hybrid RFLP pattern was identified, consistent with the interpretation that the tol locus resided between G13:C8 and G4:A9.

The Tol+ activity of contiguous cosmids between G13:C8 and G4:A9 was assayed by cotransforming each cosmid with pOKEmat A-1 into R4-71 (his5 tol trp-4: pan-2 a) and R4-72 (ad-3B: trp-4 cot-1: pan-2 A) spheroplasts. Transformants were selected for both hygromycin resistance (cosmid marker) and for growth in the absence of panthothenic acid (pOKEmat A-1 marker). Transformants fail to regenerate following the induction of mating-type-associated incompatibility (Glass et al. 1990) and therefore a cosmid with Tol+ activity should exhibit significantly lower transformation frequencies when introduced with mat A-1 into tol a spheroplasts as compared to A spheroplasts. Only cosmid X25:D7 (plus mat A-1) exhibited a significant reduction in transformation frequency in a tol background. Further subcloning identified a 6.9-kb EcoRI fragment from X25:D7 that caused a 20- to 30-fold reduction in transformation frequencies when introduced into tol a spheroplasts as compared to A spheroplasts.

**Molecular characterization of tol:** DNA sequence determination of a 4.2-kb SalI-NsiI construct within the 6.9-kb EcoRI fragment revealed an ORF of 3127 bp interrupted by a putative intron of 94 bp. The 5' and 3' and internal sites of the tol intron fit intron splicing consensus sequences (Brueck et al. 1993; Figure 3). The sequence of a tol cDNA spanning the intron site was characterized and confirmed the presence of the intron in the genomic tol sequence. DNA sequences surrounding the tol ATG start codon were in good agreement with the consensus for N. crassa (Edelman and Staben 1994). A sequence matching the transcription initiation consensus for N. crassa was found at position −324 (TCATCANC; Brueck et al. 1993) and a CAAT motif (Bucher 1990; Chen and Kinsey 1995) was identified at position −413, 85 bp away from the proposed transcription start site (Figure 3). Three pairs of short perfect repeats (CGCGCCCA, TTTGGTG, and GAGAAGTCTA) were found 5′ to the proposed CAAT box.

Translation of the tol ORF identified a 1011-aa polypeptide (Figure 3) with a calculated M, of 113,712. TOL has a predicted isolectric point of 4.67 and is made up of 40% nonpolar and 26% charged residues. The carboxyl-terminal portion of TOL (aa position 837-1011) is rather hydrophilic, composed of 30% nonpolar and 30% charged residues. BLAST (Altschul et al. 1990) and FASTA algorithm (Pearson and Lipman 1988) searches did not reveal significant similarity between TOL and any other protein sequence present in protein databases. However, a heptad repeat structure was identified from aa position 177-211 (Figure 4, A and B), as predicted by the COILS program (Lupas et al. 1991). A coiled-coil is an α-helical bundle that is thought to wind into a superhelix, with the hydrophobic residues (a and d) forming the hydrophobic packing interface (for review see Lupas 1996). Coiled-coil domains play structural roles in numerous fibrous proteins (Pauling and Corey 1951; Crick 1953) and are also a dimerization motif in the leucine-zipper class of transcription factors (Landschulz et al. 1988). The putative coiled-coil domain in TOL had five heptad repeats and contained mostly hydrophobic residues in positions a and d with some charged residues in positions b, c, e, f, and g. Occasional polar residues in the core (a and d, such as in the case of TOL) favor dimerization over trimer- or tetramerization, because they can still be partly solvated (Harbury et al. 1993; Lumb and Kim 1995).

A single putative leucine-rich repeat (LRR) was found in the carboxyl-terminal portion of TOL (position 804-823; Figure 4A). LRRs, which have been found in numerous proteins, are thought to mediate protein-protein interactions (for reviews, see Kobe and Deisenhofer 1994; Buchanan and Gay 1996). Each LRR contributes an exposed β-sheet that could participate in strong interactions with other proteins. The LRR in TOL is predicted to form a β-sheet by both the Chou-Fasman (Chou and Fasman 1978) and the Robson-Garnier algorithms (Garnier et al. 1978). Although LRRs are usually present in tandem arrays, at least three proteins with a single LRR have been reported: Rev (HIV-1 nuclear regulatory protein; Malim et al. 1989), GPIβ (human platelet glycoprotein Ib β subunit; Lopez et al. 1988), and GPIX (human platelet glycoprotein IX; Hickey et al. 1989). These single LRRs are thought to be functionally important (Malim et al. 1989; Noris et al. 1997).

**Functional analysis of tol constructs:** Several tol deletion and frameshift constructs were obtained to determine what portions of TOL were important for function (Figure 5). A +1 frameshift mutation at the XhoI site (aa position 50) abolished Tol+ activity, confirming that the 1011-aa ORF encoded TOL. Deletion of the LRR
portion also abolished Tol\textsuperscript{+} activity. However, an internal deletion of 139 aa in the region before the LRR did not affect TOL function. N-terminal deletion constructs indicated that a region between aa position 98 and 531, which includes the coiled-coil domain, was also essential.

**Complementation of the tol mutant and DNA sequence analysis of the tol\textsuperscript{+} allele:** The original tol mutation was identified in an A/a partial diploid strain that had escaped from inhibited wild-type growth rates (Newmeyer 1970). The mutation was subsequently mapped to LG IV, near trp-4. To confirm that we cloned tol, we performed complementation experiments with the tol\textsuperscript{−} mutant and determined the DNA sequence of the tol\textsuperscript{−} allele (N83).

Two pOKE103-tol\textsuperscript{+} constructs, SalI-EcoRI (5.1 kb; pOKE-SE5.1) and EcoRI-EcoRI (6.9 kb; pOKE-EE6.9; Figure 5), plus a vector control (pOKE103), were transformed into tol a spheroplasts (strain R4-71). Homokaryotic pan-2\textsuperscript{+} tol a (pOKE103, pOKE-SE5.1, or pOKE-EE6.9) transformants were isolated and subjected to heterokaryon tests with tol\textsuperscript{−} A (I-20-26) and tol\textsuperscript{−} A (I-20-41) testers. As expected, homokaryotic pan-2\textsuperscript{+} tol a transforms containing only the vector (pOKE103) formed a vigorous heterokaryon with the tol a tester (I-20-41). In contrast, the tol\textsuperscript{−} A tester (I-20-26) indicated that a region between aa position 98 and 531, which includes the coiled-coil domain, was also essential.

The original tol mutation, E858 (GAA) → Stop (TAA) is underlined in bold and italicized. The GenBank accession number for this sequence is AF085183.
Figure 4.—Structural features identified in TOL. (A) The amino acid sequence of the coiled-coil domain (position 177-211) in TOL is shown with a schematic heptad position a-g underneath. The leucine-rich repeat (LRR; aa position 804-823) is shown and the consensus for Arabidopsis thaliana RPS2 (Mindrinos et al. 1994) is depicted underneath. For the LRR, an “X” stands for an arbitrary amino acid while an “a” stands for an aliphatic amino acid. (B) A helical wheel representation of the coiled-coil domain in TOL. The hydrophobic residues of the heptad repeats are in bold while charged residues are underlined.

Isolation of tol mutants by RIP mutation: The mutation in the original tol- allele (N83) occurred near the carboxyl-terminus of TOL, and therefore it is possible that TOL retains partial function. We therefore attempted to obtain additional tol mutants by RIP mutation (Selker 1990). RIP is a mechanism in Neurospora that causes duplicated sequences to undergo multiple G-C to A-T transition mutations during the sexual cycle. A 2.7-kb EcoRV fragment (cloned into pCB1004) that contained an internal portion of tol (position 3-2735; Figure 3) was transformed into strain A2 (ad-3A nic-2 cyh-1 a; Table 1). A hygromycin-resistant homokaryotic RIP mutants segregated with the tol locus, tol-43 a (as a female) were crossed separately with FGSC male and tol-83 a (as a female) were crossed separately with FGSC male to determine that the tol sequence in the tol-RIP strains showed restriction site changes (Figure 6B), consistent with the presence of transition mutations that are characteristic of sequences that have undergone RIP (Selker 1990).

Expression analysis of tol by RT-PCR: Transcripts of tol were not detectable by RNA hybridization analysis; therefore the expression of tol was analyzed by RT-PCR. Primers spanning the intron (tol 11 and tol 13) were as tol-RIP strains (tol-43, tol-83, tol-95, tol-106, and tol-135).

To confirm that the suppressor phenotype in the tol-RIP mutants segregated with the tol locus, tol-43 a (as a male) and tol-83 a (as a female) were crossed separately to his-5 A. Of the 30 His+ progeny tested from the two crosses, all but one formed vigorous heterokaryons with both tol a (I-10-1) and tol A (I-20-41) strains (3.3% recombination), indicating that the new mutations in tol-43 and tol-83 were closely linked to the his-5 (and hence tol) locus. By Southern blot and RFLP analysis, it was determined that the tol sequence in the tol-RIP strains showed restriction site changes (Figure 6B), consistent with the presence of transition mutations that are characteristic of sequences that have undergone RIP (Selker 1990).

Expression analysis of tol by RT-PCR: Transcripts of tol were not detectable by RNA hybridization analysis; therefore the expression of tol was analyzed by RT-PCR. Primers spanning the intron (tol 11 and tol 13) were
used to amplify tol mRNA from vegetatively growing cultures. Figure 7 shows that a tol cDNA could be detected from mRNA from A, a, and a mating-type deletion strain grown in vegetative and crossing media (V and C; Figure 7). These data indicated that neither mating-type constitution nor growth conditions materially affected the expression of tol. A tol cDNA could also be detected in an A/a tol partial diploid strain, indicating that the presence of both mat A and mat a in the same nucleus did not significantly alter activation or repression of tol. Expression of tol was also detectable in both an incompatible (A + a inc.) and compatible heterokaryon (A + a<sup>113</sup>); the a<sup>113</sup> strain forms compatible heterokaryons with A strains, but sexual function is not affected (Griffths and Delange 1978).

Following fertilization, opposite mating-type nuclei coexist and divide within a common cytoplasm. Therefore, mating-type-associated incompatibility mediated by tol must be suppressed during sexual reproduction. To determine if tol is transcriptionally repressed during a cross, thus allowing the coexistence of opposite mating-type nuclei, we analyzed the presence of the tol cDNA by RT-PCR in mRNA preparations from perithecia at 3, 5, 6, and 9 days postfertilization. Although a cDNA for mat A-2 could easily be detected by RT-PCR from perithecial RNA preparations for all time points, a tol cDNA could not be detected at any time point postfertilization (cross; Figure 7 and data not shown).

**DISCUSSION**

This study describes the cloning, characterization, and expression analysis of tol, a mediator of mating-type-associated incompatibility in *N. crassa*. The tol locus is the only molecularly characterized mediator of allelic incompatibility from fungi. Nonallelic suppressors of incompatibility have been characterized in *Podospora anserina* and encode proteins thought to be involved in developmental processes and signal transduction (Loubradou et al. 1997; Barreau et al. 1998). In *N. crassa*, it is not clear how tol, mat a-1, and mat A-1 function to trigger incompatibility during vegetative growth. One hypothesis is that, in an A + a heterokaryon or A/a partial diploid, MAT a-1 and MAT A-1 form a heterodimer and this complex acts as a transcription regulator to activate or repress the expression of tol (Glass and Staben 1990), which would then trigger incompatibility. Our studies show that the expression of tol occurs in an A, a, and even in a mat a strain. These data indicate that MAT A-1 and MAT a-1 are not required for either repression or activation of tol, although we cannot rule out the possibility that the mating-type polypeptides may modulate tol expression levels. The expression of tol was also detected in an A/a tol partial diploid and an A + a incompatible heterokaryon, indicating that if a MAT A-1/MAT a-1 heterodimer is formed, it does not materially affect the expression of tol.

On the basis of the data presented in this article, a second more plausible possibility for the mechanism of mating-type-associated incompatibility can be formulated. In our current hypothesis, TOL physically interacts with the MAT A-1/MAT a-1 complex or their downstream products to trigger incompatibility. The 1011-aa TOL protein possesses a putative coiled-coil domain and a LRR, both of which are thought to mediate protein-protein interactions. Putative LRRs are found in both MAT A-1 and MAT a-1 (P. K. T. Shiu, unpublished results), and alteration of these motifs affects the capacity of MAT A-1 or MAT a-1 to induce incompatibility.
Molecular Characterization of tol

Figure 7.—Expression of tol as analyzed by RT-PCR. Primers spanning the intron (tol 13 and tol 11) were used to amplify tol cDNA from different mRNA preparations. The size of the genomic tol product is 504 bp with these primers, while the tol cDNA product is 410 bp. Strains used are as follow: 740-023-1VA (A), OR8-1a (a), RLM44-02 (ΔmatA), D25 (A/tol), I-20-26 + 1-I-51 (A+a), and I-20-26 + R2-11 (A+a^m3). mRNA was isolated from the above cultures grown either in vegetative medium (Vogel 1964; V) or crossing medium (Westergaard and Mitchell 1947; C) or from perithecial (cross). A tol cDNA could be detected in mRNA from all strains, with the exception of perithecial mRNA. The quality of the perithecial mRNA samples was checked by the amplification of a mat A-2 mRNA (using primers rI.1 and 2423-2406; Ferreira et al. 1996); mat A-2 is expressed at low levels throughout the sexual cycle (data not shown). DNA size markers (1-kb ladder) are given on the left.

A 4-aa deletion in the carboxyl terminus of MAT a-1 abolished incompatibility but not DNA binding or mating activity (Phillip and Staben 1994). These data suggest that for MAT a-1, mating (which probably involves transcriptional activation) and vegetative incompatibility are mediated by biochemically distinct mechanisms.

Some mat genes have cellular functions other than to limit heterokaryosis (for review, see Béguer et al. 1994). The original tol mutant and our tol-RIP strains exhibit normal vegetative growth and sexual reproduction. However, the tol mutation has been shown to suppress the phenotype of an fmf-1 mutant (Johnson 1979), which is sterile as both a male and a female in crosses to a wild-type partner. Fertility is restored when an fmf-1 tol strain is crossed to an fmf-1^+ tol strain. Our expression analysis of tol is consistent with the hypothesis that tol activity is suppressed during sexual development to allow the coexistence of opposite mating-type nuclei in the ascogenous hyphae. It is possible that FMF-1 regulates tol either directly or indirectly, and thus may play a role in the repression of tol transcription. Presumably, the misexpression of tol during the sexual cycle would result in mating-type-associated incompatibility and block further sexual development. The presence of short repetitive sequences in the promoter of tol may provide putative binding sites for such regulators.

Some species within the genus Neurospora do not exhibit mating-type-associated incompatibility. This fact is presumably due to the presence of tol mutations within these species that suppress mating-type-associated incompatibility. In the heterothallic species N. sitophila, isogenic strains that differ only in mating type will form a vigorous heterokaryon. However, when mat A and mat a from N. sitophila were introgressed into N. crassa, mating-type-associated incompatibility was observed (Perkins 1977). In the pseudohomothallic species N. tetrasperma, opposite-mating-type nuclei normally reside in a common cytoplasm. Similar to the results observed with N. sitophila, mat A and mat a from N. tetrasperma displayed mating-type incompatibility when introgressed into N. crassa (Metzenberg and Ahlgren 1973). The introgression of tol^+ from N. crassa into N. tetrasperma induced mating-type-associated incompatibility and disrupted the pseudohomothallic nature of the species (Jacobson 1992). All of these data argue that tol is the major mediator of mating-type-associated incompatibility. The evolutionary history and selection for or against mating-type-associated incompatibility in the different species within the genus Neurospora remain an enigma. The molecular characterization of tol from N. crassa and related species will provide the necessary tools to address these questions.

Both allelic and nonallelic incompatibility systems have been described for a number of filamentous fungi, and it is believed that heterokaryon (or vegetative, somatic, or heterogenic) incompatibility is a universal phenomenon among filamentous ascomycetes and basidiomycetes (Glass and Kuldau 1992; Leslie 1993; Béguer et al. 1994; Esser and Blaich 1994; Worrall 1997). It has been proposed that vegetative incompatibility may protect an individual from the transfer of deleterious cytoplasmic factors (Cat en 1972). Cytoplasmic transfer of hypovirulence-associated dsRNA virus in Cryphonectria parasitica and detrimental KalDNA plasmids in N. crassa is reduced by vegetative incompatibility.
Vegetative incompatibility mediated by mating type could be selected for by two mechanisms. The first mechanism is that incompatibility mediated by mating type could promote outbreeding by eliminating the possibility of heterokaryon formation with opposite mating-type siblings. However, in an outbreeding population, the formation of vegetative heterokaryons is also excluded by differences at any of 10 additional het loci in N. crassa; N. crassa populations are highly polymorphic for het loci (Mylyk 1976). A second mechanism is that mat A-1 and mat a-1 evolved solely for sexual reproduction, but that molecular divergence of tol resulted in mating-type-associated incompatibility during vegetative growth. Because the mating-type locus is always polymorphic in populations in a heterothallic species like N. crassa, mating-type-associated incompatibility would be an efficient way to restrict heterokaryon formation with 50% of the population, even in the absence of polymorphisms at other het loci.

Mating-type-associated incompatibility has been reported in other fungal species, such as Aspergillus fumigatus (A. N. Bist is, personal communication), Aspergillus heterothallicus (Kwon and Raper 1967), and Sordaria brevicolis (J. Bond, personal communication). It is possible that the phenomenon of mating-type-associated incompatibility is even more widespread in filamentous ascomycetes but cannot be assessed because of the lack of isogenic strains that differ only at the mating-type locus. The characterization of the three major components of mating-type-associated incompatibility, mat A-1, mat a-1, and tol, will provide tools to analyze the molecular mechanism of mating-type-associated incompatibility in filamentous fungi and will facilitate the challenge of delineating the molecular mechanism of growth inhibition and cell death that is a characteristic feature of this phenomenon.

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