A Putative Rhamnogalacturonase Required for Sexual Development of Neurospora crassa

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ABSTRACT

In previous work, the asc-l (ascus development) gene of the filamentous fungus Neurospora crassa was identified as a gene expressed preferentially during the sexual cycle and shown to be essential for normal sexual development. The asc-l gene has been sequenced and further characterized. It contains two introns, the first of which is in-frame and inefficiently or differentially spliced. The predicted ASD-1 protein has extensive homology with rhamnogalacturonase B of Aspergillus aculeatus, which cleaves the backbone within the ramified hairy regions of pectin. In homozygous asc-l crosses, sexual development is initiated and large numbers of normal-sized asci are formed. Ascospore delineation does not occur, however, and no sexual progeny are produced. As most asc-l asci contain eight nuclei, the two meiotic divisions and subsequent mitotic division typical of normal crosses seem to occur, but the haploid nuclei are not partitioned into ascospores. In wild-type crosses, the ASD-1 protein is present in large amounts in croziers and young asci, but it is only faintly detectable in more mature asci containing developing ascospores. Models to explain the possible role of a rhamnogalacturonase in sexual development are presented.

Neurospora crassa is a heterothallic filamentous fungus that undergoes a complex pattern of sexual differentiation, including the formation of several types of specialized tissue that constitute the fruiting body or peritheciun. While many genes whose products are required for this process have been identified by mutation (PERKINS et al. 1982; RAJU 1992), little is known about the molecular structure of these genes or the functions of the encoded products. In previous work, we used subtractive hybridization to identify many genes expressed specifically during sexual development (NELSON and METZENBERG 1992); these genes were called sdo genes, for sexual development. Reverse genetics was used to disrupt many sdo genes, and mutants blocked specifically in the production of sexual progeny (ascospores) were isolated. Strains lacking a functional sdo-10 gene are specifically blocked in sexual development, and the gene was renamed asc-l to denote the role it plays in ascus development (NELSON and METZENBERG 1992). In homozygous asc-l crosses, asci are formed, but sexual development ceases thereafter, and no ascospore progeny are produced.

In plants, the pectic substances that make up the major matrix polysaccharides of cell walls have a backbone of alternating “smooth regions” (homogalacturonan) and “hairy regions” or rhamnogalacturonan (with arabinan, galactan and arabinogalactan side chains, or “hairs,” SCHOLS et al. 1990a). A number of polygalacturonases and pectin lyases that cleave within the smooth regions of pectin have been isolated and studied. Rhamnogalacturonases are defined as those enzymes that cleave within the hairy rhamnogalacturonan backbone (instead of hydrolyzing the arabinan, galactan or arabinogalactan side chains; SCHOLS et al. 1990a). Little is known about the functions of rhamnogalacturonases in the development of any organism.

In this paper we present evidence that the product of the N. crassa asc-l gene is a rhamnogalacturonase. Why should the loss of a rhamnogalacturonase cause such a dramatic defect in sexual development? There was no evidence (before this work) suggesting the presence of rhamnogalacturonans in fungi, but also no published reports of searches for their existence within this kingdom. While no direct evidence for the presence of rhamnogalacturonan-like molecules in N. crassa was sought or obtained, the dramatic defect seen when the putative rhamnogalacturonase (asc-l) gene was disrupted lends support to the theory that rhamnogalacturonan or related molecules are present at least within the fungal sexual structures, where they are key to normal sexual development.

MATERIALS AND METHODS

Strains: The following N. crassa wild-type and mutant strains were used: N. crassa wild-type 74A (74-OR23-IV A, FGSC 2489); A1111 sterile mutant (un-1 ad-3A nic-2 cyh-1 A1111; FGSC 4570); d1 sterile mutant (d1 ad-3B cyh-1; RLM 53-14);
isolated in
The conditions for growth of cultures for RNA preparations was grown on crossing plates covered with 
Vegetative RNA was isolated from the wild-type 74A strain grown under mating conditions, strains were grown in crossing medium plus 
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and any necessary supplements (WESTERGAARD and MITCHELL 1947). In growth of cultures for RNA preparations, conidia were inoculated at 10^6/ml. For RNAs from mating conditions, strains were grown in crossing medium plus 1% sucrose, 25 µg/ml ampicillin and any necessary supplements (WESTERGAARD and MITCHELL 1947).

Preparation of DNA and RNA: Genomic *N. crassa* DNA was isolated as described by STEVENS and METZENBERG (1982). RNA isolation from *N. crassa* cultures was as described by 1977. Polyadenylated RNA was isolated by chromatography on oligo(dT)-cellulose (AVIV and LEDER 1972). The conditions for growth of cultures for RNA preparations are described above.

Northern (RNA) and Southern hybridization blots: Northern blot procedures were those of FOURNEY et al. (1988). Hybridization conditions were as previously described (NELSON et al. 1989). Southern hybridizations were performed as described by SAMBROOK et al. (1989). Probes were labeled using the random priming method (FENNEBerg and VOGELSTEIN 1983). The control *an* probe (glutamate dehydrogenase; KINNARO et al. 1982) that was used in Northern hybridizations was joined to 1.4-kbp EcoRI-BamHI fragment from pB9II (MANN et al. 1988).

DNA sequencing: Single-stranded template DNA was obtained from the derivatives of the Amersham M13 mp18 and mp19 vectors, per the manufacturer's suggestions. Genomic sequences were determined (by sequencing both strands, by sequencing the same strand more than once, or both) by the University of Wisconsin Biotechnology Center using an Applied Biosystems Model 370A Automated DNA Sequencing System. Ambiguities in that sequence were resolved using the chain-termination method of SANGER et al. (1977), with the U.S. Biochemical Sequenase Version 2.0 Kit. The nucleotide sequences of the *asd-1* cDNA and the *asd-1* (A1) mutant allele were determined using an Applied Biosystems Model 377 Automated Sequencer in the Molecular Biology Facility (RMI; NSF-supported) in the Department of Biology at the University of New Mexico.

dNA isolation and analysis: A cDNA library was constructed using the Uni-ZAP XR vector system according to manufacturer's instructions (Stratagene). The host strain [asd-1] was used for plating excised phagemids, and the host strain XL1-Blue MRF used for other manipulations. The ExAssist helper phage was used for excision of the pBluescript phagemid from the Uni-ZAP XR vector. The library was screened using a 1.4-kbp *BamHI* genomic fragment internal to the *asd-1* gene.

To examine splicing of the first *asd-1* intron, reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were carried out using these primers: 5'AAAATCACCACGGCACCACATCG 3' (nucleotides 1353-1374 in Figure 1) and 5'CAGCGAAAGGACAGTATGTCG 3' (complement to nucleotides 1839-1860). The Invitrogen DNA Cycle Kit was used to amplify across the intron using 0.8 µg of poly(A)-

Nucleotide sequence accession number: The *asd-1* sequence has been registered with GenBank under the accession number U70861.

DNA and protein sequence analyses: The DNA sequence of the *asd-1* gene and its derived protein sequence were compared with the DNA and protein databases available through the National Center for Biotechnology Information, using the BLAST algorithm (ALTSCHUL et al. 1990). The DNASIS programs were used for other analyses.

Sequence analysis of an *asd-1* mutant strain: A 1.4-kbp fragment of the *asd-1* genomic sequence was PCR-amplified from the *asd-1* (A1) strain. Approximately 0.5-1 μg of genomic DNA was amplified with AmpliTaq DNA polymerase (Perkin Elmer) and 3.5 pmol of each primer [5'CGTCTCTGTCAGGCCAAGCTCG 3' (nucleotides 730-747 in Figure 1) and 5'GCAGGGCATGAGATCTG 3' (complement to nucleotides 2137-2154)]. Reactions were subjected to 35 cycles of denaturation at 94° for 1 min, annealing at 55° for 2 min and extension at 72° for 7 min. The PCR product was electrophoresed on a 1.5% agarose gel to verify its size. The 1.4-kbp fragment was purified using the GeneClean Kit (BIO 101 Inc.). Nested primers were then used to sequence within the 1.4-kbp fragment of the *asd-1* (A1) allele.

Antibody production and purification: Based on the predicted ASD-1 protein sequence, a region of probable high antigenicity was identified using the method of HOPP and WOODS (1981). A peptide 12 amino acids long (Figure 1) was synthesized by Quality Controlled Biochemicals, Inc., with bovine serum albumin as the carrier. The adjuvant TierMax (Vaxcel, Inc.) was used to stimulate antiserum production in a rabbit; immunizations were according to HERBERT (1978).

Serum samples were collected before immunization and at various times after immunization; the 90 day postimmunization serum was used in the immunolocalization experiments and Western blots. Antibody titers were measured via ELISA (HARLOW and LANE 1988). The SulfoLink Kit (Pierce) was used to purify the ASD-1 antibodies following manufacturer's instructions, except that a batch method was used in which 6 ml of
was collected by filtration and immediately frozen and ground.

**Western blot analysis:** N. crassa wild-type and asd-1 mutant strains [asd-1 (A1) A, asd-1 (A2) a, asd-1 (A3) A, asd-1 (A4) a and asd-1 (A5) A] were inoculated (10⁶/ml) in crossing medium, 1% sucrose and 25 µg/ml ampicillin. The cultures were grown in the light without shaking at 25°C for 48 hr. The tissue was then resuspended in PEB (50 mM Tris-HCl pH 7.5, 5 mM EDTA) and 1 g of zirconia beads (0.5 mm, from Sigma) on ice. Phenylmethylsulfonyl fluoride was added to 1 mM and the entire mixture was homogenized for 4 min. Following homogenization, the samples were centrifuged and the supernatant recovered. A Bradford protein assay (Bio-Rad #500-0006) was used to determine protein concentration. Proteins (10 µg) were fractionated on a 7.5% SDS-polyacrylamide gel and transferred to Immobilon-P (Millipore) by electrobolting. Blots were incubated with purified ASD-1 antibodies, diluted 1:2000 in TBS (50 mM Tris-HC1 pH 7.4, 130 mM NaCl) with 0.5% Tween 20. Blots were washed three times for 10 min in TBS and visualized using the ECL chemiluminescence detection kit (Amersham) as specified by the manufacturer.

**Sample preparation for microscopy:** Seven days after fertilization, perithecia were cleaned of mycelial debris and the asci squeezed into 4% paraformaldehyde and incubated for 1 hr at room temperature. Following fixation, the paraformaldehyde was removed and asci washed with PBS (all washes were in PBS, repeated three times) followed by a 1-hr incubation in 1% Triton-X. The asci were incubated, washed for 1 hr in primary antibody (1:100 in PBS), washed again, and incubated for 2 hr with a rhodamine-phycoerythrin-conjugated goat anti-rabbit secondary antibody (Sigma) diluted 1:10 in PBS. In double-staining experiments, the DNA-specific stain mithramycin (Slater 1978) (Sigma) was applied with the secondary antibody. After antibody treatment, the tissue was washed in PBS and the asci were suspended in the anti-fading agent DABCO (triethylenediamine, 100 mg/ml with 90% glycerol and 10% PBS) before mounting on a slide. A coverslip was placed over the preparation and sealed with nail polish.

**Confocal microscopy:** Preparations were examined with a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad Labs., Inc., Hercules, CA) fitted with a krypton-argon laser and a Nikon Diaphot microscope (Garden City, NY). Fluorescent images were recorded using the laser’s 488 nm line to excite the fluorochrome. Between five and 15 serial fluorescent images were taken at 1-µm intervals and superimposed digitally. Bright field images were obtained using the system’s transmitted light detector.

**RESULTS**

**Sequence analysis of the asd-1 gene:** In previous work, a 1.4-kbp BamHI fragment internal to the asd-1 4.4 gene was cloned into pMSN1 and used to generate repeat-induced point (RIP) mutations (Selker and Garrett 1988) in the asd-1 gene (Nelson and Metzenberg 1992). The resulting mutant strains have dramatic defects in sexual development (below). Sequences from cosmids 12.7B (Vollmer and Yanofsky 1986) corresponding to the asd-1 gene and its flanking regions were subcloned into the multiple cloning sites of M13mp18 or M13mp19, and the sequence of the asd-1 gene was determined.

The genomic sequence containing the asd-1 gene and the deduced amino acid sequence of the asd-1 protein are shown in Figure 1. The predicted open reading frame begins with an ATG codon at position 485 and terminates with a UAA codon at position 2166. Two introns are present within the asd-1 gene, and the first intron is differentially spliced, as demonstrated by cDNA sequence analysis and RT-PCR experiments (below). These introns contain typical 5' and 3' consensus sequences (Bruchez et al. 1993a), indicated in Figure 1. The first intron also has the lariat consensus sequence CTRAG, which the second intron lacks; however, the sequence CTAAT, present 11 nucleotides upstream from the splice site of the second intron, might serve as a lariat sequence.

Potential CAAT and TATA box sequences in the promoter region of asd-1 are noted in Figure 1; however, the spacing between these sequences is unlike that noted in other N. crassa promoters that contain both boxes (Bruchez et al. 1993a). The putative asd-1 CAAT and TATA boxes are separated by ~280 bp, compared to fewer than 90 bp for characterized N. crassa promoters. Many N. crassa genes contain only a CAAT or a TATA box, not both, so only one of these elements may be functional in the asd-1 promoter. A good N. crassa Kozak sequence, or consensus sequence for the initiation of translation (Bruchez et al. 1993b), is present at the proposed asd-1 start codon (underlined): CAA-CATGG.

**Expression of the asd-1 gene:** As previously observed (Nelson and Metzenberg 1992), the asd-1 transcript was abundant in wild-type strains grown under crossing conditions (floating mycelial mats, 2 or 3 days old). The expression was extremely low (nearly undetectable) in wild-type strains grown in vegetative (Vogel) medium for 13 hr, or in shaken crossing medium (shaking is thought to prevent formation of the female sexual structure). The A and a mating type products of N. crassa are thought to encode transcriptional regulatory proteins (Glass et al. 1990; Staben and Yanofsky 1990). The asd-1 transcript was barely detectable in the A⁢M⁢α⁢t⁢e⁢r⁢i⁢n⁢e⁢r⁢y⁢m⁢a⁢l⁢e⁢mal⁢e⁢f⁢e⁢r⁢i⁢l⁢i⁢t⁢y⁢ strain (Griffiths 1982) grown under conditions that promote crossing in fertile strains (Nelson and Metzenberg 1992).

Additional Northern analyses were done to more precisely define the window of expression of the asd-1 gene, and to examine its expression in mutant strains (Figure 2). The a⁢M⁢α⁢t⁢e⁢r⁢i⁢n⁢e⁢r⁢y⁢m⁢a⁢l⁢e⁢mal⁢e⁢f⁢e⁢r⁢i⁢l⁢i⁢t⁢y strain. The product of the asd-1 gene must be functional in both the male and female parent to allow normal sexual development; when one parent harbors a dysfunctional asd-1 gene, sexual development stops shortly after fertilization (Johnson 1979). The wc-1 and wc-2 mutants were also examined; the wc (white
collar) genes encode products required for all of the blue light-induced phenomena of *N. crassa*, including the formation of the female reproductive structure and the phototropism of the perithecial beaks (DEGLI-INNOCENTI et al. 1983; HARDING and MELLES 1983). The *we-1* gene was recently characterized and shown to encode a zinc finger protein with a putative transcriptional activation domain (BALLARO et al. 1996). The expression of the *asd-1* transcript varied in cultures grown under crossing conditions, perhaps reflecting limited windows of expression of this gene (Figure 2A). The *asd-1* transcript was seen to accumulate to similar levels in wild-type, *fmf-1*, *we-1* and *we-2* strains; expression levels were very low in the *A^m14* strain. The apparent overexpression of *asd-1* in the *a^d1* sterile mutant may reflect an aberration based on the fluctuating (normal) expression of the *asd-1* gene. Similar differences in levels of the *asd-1* transcript in wild type (*74A*), the *fmf-1* mutant and a forced heterokaryotic strain grown under crossing conditions were observed in initial experiments (NELSON and METZENBERG 1992), but contains CAAT (underlined). The amino acids absent when the first intron is spliced are italicized. The 12-residue peptide used to raise antibodies to the ASD-1 protein is shown in bold. The site of polyadenylation is indicated in bold and labeled. The GenBank accession number for the *asd-1* sequence is U70861.

**Figure 1.**—The *asd-1* genomic nucleotide sequence and deduced protein product. Possible CAAT and TATA boxes are labeled and indicated in bold. The nonsense mutation at codon 107 (CAG to TAG) identified in the *asd-1* (A1) allele is indicated with an asterisk and labeled. The two intron sequences are italicized, and the splice junctions and internal consensus sequences are underlined; the second intron lacks a CTRAC sequence within the lariat consensus (BRUCHEZ et al. 1993a), but contains CAAT (underlined). The amino acids absent when the first intron is spliced are italicized. The 12-residue peptide used to raise antibodies to the ASD-1 protein is shown in bold. The site of polyadenylation is indicated in bold and labeled. The GenBank accession number for the *asd-1* sequence is U70861.
At the conclusion of the shaking (MATERIALS AND METHODS). In repeated RT-
RNA was isolated from the designated strains grown under conditions in which the culture would have exhausted many of the available nutrients (24 hr of growth or stationary phase). The asd-1 transcript accumulated to significant and similar levels in 74A grown under crossing conditions or in Vogel medium for 12 hr (exponential vegetative growth) or 24 hr (stationary phase). See MATERIALS AND METHODS for detailed strain descriptions and culture conditions. The positions of the ~2.0 kb asd-1 and 1.8-kb am transcripts are shown.

(NELSON and METZENBERG 1992). In the Northern hybridizations shown in Figure 2B, this analysis was extended to include a wild-type culture grown in Vogel (vegetative) medium for a longer time, to a point at which the culture would have exhausted many of the available nutrients (24 hr of growth or stationary phase). The asd-1 transcript accumulated to significant and similar levels in 74A grown under crossing conditions or starved vegetative conditions, and was not detected among the transcripts from exponential vegetative conditions. The significance of asd-1 expression late in vegetative growth is unclear, since asd-1 mutants have not been shown to exhibit any vegetative growth defects.

Analysis of asd-1 cDNAs: A single asd-1 cDNA was isolated from a library made using perithecial mRNAs; the site of polyadenylation is indicated in Figure 1. This cDNA was derived from an mRNA in which the first intron (which is in-frame) had not been spliced, while the second intron had been removed. Since the cDNA insert was truncated at the 5' end, it did not yield information about the transcriptional start of the asd-1 gene. At the 3' end, the open reading frame was followed by a noncoding trailer of 190 bp.

Splicing of the first asd-1 intron was examined in a population of mRNAs prepared from an unmated wild-type strain (74A) grown in crossing medium without shaking (MATERIALS AND METHODS). In repeated RT-PCR experiments, a fragment of ~200 bp was consistently amplified, indicating that the first intron had been spliced; no band corresponding to unspliced product was detected (not shown). In PCR experiments using the same set of primers and genomic N. crassa DNA, a fragment of ~310 bp was amplified (this band would contain the 108-bp intron in addition to the flanking sequences). However, it was unspliced in the sole asd-1 cDNA isolated from a perithecial mRNA preparation. We conclude, therefore, that the first asd-1 intron is inefficiently or differentially spliced. Differential or perhaps inefficient splicing of introns has been noted for other N. crassa genes (FOSSA et al. 1995; SAUPE et al. 1996). The rhgB cDNA of Aspergillus aculeatus (KOFOD et al. 1994) was examined for intron consensus sequences typical of A. nidulans (GURR et al. 1987), but such sequences were not found, suggesting that the sequences corresponding to the differentially spliced intron of asd-1 may never be spliced in rhgB.

The ASD-1 protein: The predicted ASD-1 protein is a hydrophilic polypeptide of 53,949 or 58,008 Da, if the first intron is spliced or not spliced, respectively. The larger (540 residue) polypeptide has 65% identity with rhamnogalacturonase B (RGase B) of A. aculeatus, which is encoded by the rhgB gene (KOFOD et al. 1994); the extent of identity is shown in Figure 3. In recent work, rhamnogalacturonase B was shown to be a rhamnogalacturonan α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronide lyase (or RG-lyase), which non-hydrolytically cleaves the linkage between a rhamnose and an α-D-galacturonic acid in the backbone of the pectin hairy regions (MUTTER et al. 1996).

It is not known how the two potential ASD-1 proteins, encoded by the longer mRNA (first intron not spliced) or the shorter mRNA (both introns spliced), might differ in function. Since the first intron is in-frame, its splicing would result in a protein that is shorter by 36 amino acids, but the residues encoded after the first intron would remain unchanged. The residues encoded by the first intron of the asd-1 gene are highly conserved in the rhgB protein (89% identity), but their role, if any, in the function of that protein is unknown.

The only extended stretch of hydrophobic residues in ASD-1 occurs at the amino-terminus, where the first 29 residues have average hydropathies indicating that they could constitute a signal peptide (KYTE and DOOLITTLE 1982). A postulated signal peptidase cleavage site is located between Ala-24 and Phe-25. Cleavage at this site would yield ASD-1 polypeptides of 480 or 516 residues, depending on splicing of the first (differentially spliced) intron. However, the amino-terminal positively charged residues found in most signal peptides (VONHEJNE 1990) are not present near the amino terminus of the proposed ASD-1 polypeptide. Also, immunolocalization experiments (below) showed that the ASD-1 protein was abundant in croziers and young asci, suggesting that it is at best inefficiently transported out of the ascus. The predicted ASD-1 protein lacks the putative N-linked glycosylation site (characteristic of many exported proteins) present at residue 350 in the rhgB protein of A. aculeatus (KOFOD et al. 1994).
Structure of the asd-1 gene in a RIP-generated mutant strain: The structure of the asd-1 gene was determined for one of the mutant strains, containing the asd-1 (A1) allele. The centrally located 1.4-kbp BamHI fragment of asd-1, which had been subjected to RIP-generated mutagenesis (Nelson and Metzenberg 1992), was amplified from the asd-1 (A1) A strain and sequenced. Thirty-two GC to AT transition mutations were detected for one of the mutant strains, containing the asd-1 allele. The centrally located 4-kbp BamHI fragment of this strain was purified using ASD-1 peptide-coupled beads (Materi- als and Methods). The purified ASD-1 antibodies recognized many bands in both wild-type and mutant strains (not shown), so the antibodies were used to characterize the ASD-1 polyclonal antibodies and to detect the ASD-1 protein in wild-type and mutant strains (grown under crossing conditions). These protoperithecia were fertilized efficiently when crossed to either wild-type or mutant strains, containing the A1, A2, A3, A4 and A5 asd-1 alleles were examined for production of the ASD-1 protein. As shown in Figure 4, the asd-1 (A1) A strain, which contains a nonsense mutation at codon 107, lacked a band at that position (no proteins were recognized by the purified ASD-1 antibodies). In the four other asd-1 mutants examined, bands of ~65 kDa were recognized; the amounts of ASD-1 protein detected in these mutant strains varied from much less to more than that detected in the wild-type strain. In these four mutants, the RIP process may have generated missense but not nonsense mutations, resulting in the production of non-functional but non-functional ASD-1 protein.

Confocal microscopic analysis of sexual development in the asd-1 mutant: The asd-1 mutant strain had normal vegetative growth, but sexual development was highly aberrant when both parents harbored the asd-1 mutation (Nelson and Metzenberg 1992). When an asd-1 strain was the female parent, normal-sized protoperithecia were formed. When an asd-1 strain was the male parent, normal-sized protoperithecia were formed. These protoperithecia were fertilized efficiently when crossed to either wild-type or asd-1 strains, and early sexual development appeared normal. The perithecia enlarged, accumulated melanin, and formed ascogenous tissue. However, in homozygous asd-1 crosses, the outer development of the fruit-
The lanes include wild type and the five strain cated by their allele numbers (A1, A2, A3, A4 and A5). The position of the marker (bovine serum albumin, of apparent molecular weight 68 kDa) is indicated with an arrow.

**Figure 4.** Western blot analysis of wild-type and *asd-1* mutant strains. Crude extracts were prepared from the wild-type strain 74A and five independently isolated *asd-1* mutants grown under crossing conditions (MATERIALS AND METHODS). The lanes include wild type and the five *asd-1* extracts, indicated by their allele numbers (A1, A2, A3, A4 and A5). The position of the marker (bovine serum albumin, of apparent molecular weight 68 kDa) is indicated with an arrow.

ing body stopped when only short beaks had been formed, unlike the long beaks typical of wild-type crosses (not shown). In these homozygous *asd-1* crosses, the asci that formed were the same length as mature asci from wild-type crosses (~200 μm long), and the number of asci per peritheium was similar in *asd-1* and wild-type crosses (~200 asci per fructifying body). However, the shape of the *asd-1* asci was quite irregular and the apical pore through which ascospores are normally ejected did not form (NELSON and METZENBERG 1992).

To more precisely define the defects in sexual development caused by mutations in the *asd-1* gene, we used confocal microscopy to visualize the nuclei and to localize the ASD-1 protein within the developing sexual tissue (below). In a normal strain, the diploid nucleus within the immature ascus undergoes two meiotic divisions and a subsequent mitotic division to yield eight haploid nuclei that are partitioned into eight ascospores. The products of meiosis are arranged linearly within the ordered ascus, and ascospore delineation occurs soon after the nuclear divisions.

The DNA-specific stain mithramycin was used to detect the nuclei within homozygous *asd-1* and wild-type asci (SLATER 1978). In homozygous *asd-1* crosses, development usually proceeded through the first and second meiotic divisions and the subsequent mitotic division to yield eight haploid nuclei that are partitioned into eight ascospores. The products of meiosis are arranged linearly within the ordered ascus, and ascospore delineation occurs soon after the nuclear divisions.

![Confocal microscopic analysis of ascus development in crosses homozygous for the *asd-1* mutation (A1 allele), 7 days postfertilization. (A) Bright field image showing aberrant ascus morphology and absence of spore production. (B) Fluorescent image showing the nuclei (evident as bright dots) within the asci shown in A, stained with the DNA-specific stain mithramycin. The diffuse fluorescent material within the “kink” in the leftmost ascus may help the viewer to visualize the nuclear distribution within the respective asci. The asci are ~200 μm long, like wild-type asci, but the morphology of the asci and the distribution of nuclei within the asci are grossly aberrant.](image)

The volumes of the more mature (weakly stained) asci were only slightly greater than those of many strongly stained younger asci, suggesting that the weaker staining of more mature asci was not due to a dilution effect. Also, the DNA-specific stain mithramycin stained the nuclei contained within the younger and the more mature asci to the same extent. No dramatic subcellular localization of the *asd-1* protein within the asci was evident, although in some preparations the nuclei were more densely stained than surrounding regions.

**Vegetative growth and osmotic sensitivity of vegetative and sexual tissue:** The vegetative growth patterns of *asd-1* mutant strains were examined using Vogel medium plus sorbose, glucose and fructose (colon-forming conditions) at various temperatures (DAVIS and DESSERT 1970), and found to be indistinguishable from those of wild type. Also, vegetative growth of the *asd-1* strains on Vogel medium plus 0.1, 1 or 10% glucose was not distinguishable from that of wild type. *A. nidulans* can use polygalacturonic acid as sole carbon source (DEAN and TIMBERLAKE 1989); wild-type and *asd-1* mutant *N. crassa* strains were tested for growth on 1% polyg-
alacturonic acid, and the strains demonstrated equal (weak) growth.

The osmotic sensitivity of asd-1 strains was examined, since if a rhamnogalacturonan-like molecule is present in *N. crassa*, the inability to break the molecule down might result in altered osmotic sensitivity. Vegetative growth on 7.5 and 10% sorbitol and 4% NaCl at 25° and 37° was the same in wild-type and asd-1 strains, suggesting a lack of osmotic sensitivity. The asci from homozygous asd-1 crosses were also tested for osmotic sensitivity. Wild-type and asd-1 asci were squeezed from 7-day-old fruiting bodies into water, 10% sorbitol or 4% NaCl and observed from 5 min to 6 hr after treatment. No differences were detected between wild-type and asd-1 asci.

Figure 6.—Localization of the ASD-1 protein in wild-type asci, 7 days postfertilization. Ascogenous tissue was incubated with ASD-1 antibody followed by a rhodamine-phycoerythrin-conjugated goat anti-rabbit secondary antibody. (A) Young to fairly mature wild-type asci. Abundant staining with the ASD-1 antibody is evident in the young asci (in which there is no evidence of ascospore delineation). The scale bar indicates 25 μm. (B) Two croziers (hook-shaped cells in the center) and young asci, showing abundant staining with the ASD-1 antibody. There is little staining in the partial ascus containing delineated ascospores, of which three are visible (at right). Scale bar, 10 μm.

DISCUSSION

The *asd-1* gene of *N. crassa* plays an essential role in the process of sexual development, where it is required not for the initiation of the sexual process, but fairly late in development, for the production of ascospores. This gene was cloned and characterized, and the predicted ASD-1 product was shown to have extensive homology with rhamnogalacturonase B (RGase B) of *A. aculeatus* (KOFOD et al. 1994). The work presented here is the first to suggest a role for rhamnogalacturonase activity in the sexual development of a fungus.

The major matrix polysaccharides of the cell walls of dicotyledonous plants are pectic compounds consisting of alternating smooth regions (pectin) and hairy regions (rhamnogalacturonan; SCHOLS et al. 1990b). Rhamnogalacturonan I (RG-I) is a major component of the primary cell walls of plants, where it is thought to play a central role in determining the structure and functions of the cell walls (O’NEILL et al. 1990). While the detailed structures of RG-I are still being deciphered, they are known to consist of a family of polysaccharides with a linear backbone of repeating diglycosyl units (alternating galacturonic acid and rhamnose; McNEIL et al. 1980; SCHOLS and VORAGEN 1994). In different members of the RG-I family, various numbers of the rhamnosyl residues are branched (often 50% or more), with side chains consisting of heterogeneous oligoglycosyl units (largely arabinosyl and galactosyl; LEROUGE et al. 1993).

While several enzymes that cleave the side chains from the RG-I backbone have been described, only two types of enzymes that cleave within the backbone have been identified (RGase A and RGase B; KOFOD et al. 1994). The rhamnogalacturonase class of enzymes was first described in 1990 (SCHOLS et al. 1990a). RGase A is an endoβ-hydrolase, while RGase B is an endoα-hydrolase (AZADI et al. 1995). The only RG-lyase described in the literature is that of A. aculeatus; pectin and pectate lyases are much more common, and are often present in multiple isoforms (MUTTER et al. 1996). It should be noted that lyases, unlike hydrolases, result in formation of a double bond in the ring at the newly generated nonreducing end of the reaction product, not a hydroxyl group, and the energy of the glycosidic bond can be expected to be conserved in the product. This reaction product is, therefore, potentially able to react with a suitable functional group of another molecule without the requirement for outside activation.

Three hypotheses might be entertained to explain the role of a putative RGase in the sexual development of *N. crassa*. These are, essentially, that RG prevents sexual development, that RG is essential for sexual development but must be reshaped, and that RG breakdown products are essential for sexual development. In the first model, the intact rhamnogalacturonan would prevent the movement of nuclei to their proper posi-
tions in the developing ascus, perhaps simply by virtue of its viscosity. ASD-1 protein would be needed in sexual development for the depolymerization of this pectin-like substance. In the second model, we assume again that *N. crassa* contains RG-I or rhamnogalacturonan-like molecules, but that the remodeling of those molecules (partial cleavage and perhaps eventual repolymerization in a different form) might be an essential part of the morphogenesis that occurs as the ascospores are formed in the developing asci. In *asd-1* or RG-lyase mutants, the RG-I-like molecules could not be remodeled, and the result would be the observed aberrantly shaped asci and aborted development. Support for the first two models comes from the immunological detection of large amounts of the ASD-1 protein in young wild-type asci, and the apparent gradual disappearance of this protein as asci mature. That the protein is localized primarily inside the asci suggests that its site of action is internal.

In the third hypothesis, the ASD-1 protein would cleave RG-I-like molecules located outside the asci, and this cleavage would generate RG fragments that serve some function, perhaps as signalling molecules to coordinate and/or control sexual development. The ability of polysaccharides to generate signalling molecules called "oligosaccharins" is established, and RG fragments might be involved in many plant processes, including morphogenesis, wound signaling and phytoalexin elicitation (ALDINGTON et al. 1991). The presence of a potential signal peptide that could direct localization of the ASD-1 protein outside the asci lends support to this theory.

The cell wall-degrading enzymes produced by fungi may be required for the penetration of plant tissues by pathogenic fungi, and in the saprophytic fungi like *Neurospora* might serve to release carbon sources necessary for growth. In this work, we present data that at least one putative cell wall-degrading enzyme, encoded by the *N. crassa asd-1* gene, is required for the process of sexual development. Since degradation of pectin requires the sequential action of several enzymes, it seems possible that mutations in genes encoding other pectinolytic enzymes would cause a phenotype like that observed in the *asd-1* mutant strains. Three extracellular pectinases of *N. crassa* (polygalacturonase, pectin lyase and pectate lyase) have been detected; the constitutive polygalacturonase activity present in the developing asci. In *asd-1* or RG-lyase mutants, the RGI-like molecules could not be remodelled, and the result would be the observed aberrantly shaped asci and aborted development. Support for the first two models comes from the immunological detection of large amounts of the ASD-1 protein in young wild-type asci, and the apparent gradual disappearance of this protein as asci mature. That the protein is localized primarily inside the asci suggests that its site of action is internal.

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