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Gene Clusters for Insecticidal Loline Alkaloids in the Grass-Endophytic Fungus *Neotyphodium uncinatum*

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ABSTRACT

Loline alkaloids are produced by mutualistic fungi symbiotic with grasses, and they protect the host plants from insects. Here we identify in the fungal symbiont, *Neotyphodium uncinatum*, two homologous gene clusters (*LOL-1* and *LOL-2*) associated with loline-alkaloid production. Nine genes were identified in a 25-kb region of *LOL-1* and designated (in order) *lolF-1*, *lolC-1*, *lolD-1*, *lolO-1*, *lolA-1*, *lolU-1*, *lolP-1*, *lolT-1*, and *lolE-1*. *LOL-2* contained the homologs *lolC-2* through *lolE-2* in the same order and orientation. Also identified was *lolF-2*, but its possible linkage with either cluster was undetermined. Most *lol* genes were regulated in *N. uncinatum* and *N. coenophialum*, and all were expressed concomitantly with loline-alkaloid biosynthesis. A *lolC-2* RNA-interference (RNAi) construct was introduced into *N. uncinatum*, and in two independent transformants, RNAi significantly decreased *lolC* expression ($P < 0.01$) and loline-alkaloid accumulation in culture ($P < 0.001$) compared to vector-only controls, indicating involvement of *lolC* in biosynthesis of lolines. The predicted LolU protein has a DNA-binding site signature, and the relationships of other *lol*-gene products indicate that the pathway has evolved from various different primary and secondary biosynthesis pathways.

SEED-BORNE endophytic fungi—specifically the Epichloë species (asexual states, Neotyphodium species)—in symbiosis with cool-season grasses (Poaceae subfam. Poöideae) can impart to those grasses a variety of fitness enhancements including resistance to vertebrate and invertebrate herbivores, resistance to pathogens and parasites, enhanced phosphate uptake and nitrogen utilization, and increased tolerance of drought and heat (Bush *et al.* 1997; Malinowski and Belesky 2000; Schardl *et al.* 2004). Loline alkaloids produced by several Neotyphodium and Epichloë species are potent, broad-spectrum insecticides (Riedell *et al.* 1991; Dougherty *et al.* 1998; Wilkinson *et al.* 2000), have little or no antimammalian activities (Jackson *et al.* 1996), and sometimes accumulate in the plant to levels up to 20 mg g^{-1} plant dry weight (DW; Craven *et al.* 2001). These alkaloids have an unusual structure, comprising a saturated 1-aminopyrrolizidine-ring system, with a highly strained ether bridge between C-2 and C-7 (Petroski *et al.* 1989). Lolines are almost exclusively found in these grass-endophyte symbioses (Hartmann and Witte 1995; Bush *et al.* 1997); outside of the grasses they have been identified in only a few plant species in the families Fabaceae and Convolvulaceae (Hartmann and Witte 1995; Tofern *et al.* 1999). Genetic tests have confirmed linkage between capability of endophytes to produce lolines *in planta* and their bioprotective effects against aphids (Wilkinson *et al.* 2000). Improved survival under drought and/or competition of grass-endophyte symbiota possessing lolines has raised the possibility of additional roles of these alkaloids in host plant fitness and persistence (Malinowski and Belesky 2000; Schardl *et al.* 2004).

The biochemical pathway for the lolines is so far unknown. On the basis of certain structural similarities of lolines with plant pyrrolizidines (Hartmann and Witte 1995), Bush *et al.* (1993) proposed that lolines are produced by a pathway involving polyamines such as spermidine. However, results of precursor feeding studies have now ruled out this possibility and suggest that lolines are formed by a novel biosynthetic pathway from the amino acids L-proline and L-homoserine (Blankenship *et al.* 2005).

We have previously identified two genes, *lolA* and *lolC*, showing strong upregulation in loline-alkaloid-producing *Neotyphodium uncinatum* cultures and strict association with *in symbio* loline-alkaloid-production phenotypes among isolates of different endophyte species (Spiering *et al.* 2002). In this study we show that two

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gene clusters in the genome of *N. uncinatum* contain homologs of *lolA* and *lolC*, along with several additional genes whose predicted products showed relationships to enzymes typical of primary or secondary metabolic pathways. A functional test performed with RNA interference (RNAi) in *N. uncinatum* confirmed involvement of *lolC* in loline-alkaloid production. These findings provide first insight into the molecular genetics of loline-alkaloid production and constitute an important basis for detailed studies of the biosynthetic pathway and ecological roles of the lolines.

MATERIALS AND METHODS

Fungal strains and growth conditions: *N. uncinatum* CBS 102646 (deposited in Centraalbureau voor Schimmelculture) and *N. coenophialum* ATCC 62374 (deposited in the American Type Culture Collection) were grown in shake-cultures as described by Blankenship *et al.* (2001). Loline-alkaloid production by *N. uncinatum* was induced in minimal medium (MM; Blankenship *et al.* 2001) with 15 mm urea and 20 mm sucrose as nitrogen and carbon sources, respectively. To suppress loline-alkaloid production, the fungus was grown in complex medium (CM) consisting of potato dextrose broth (PDB; Difco, Detroit) diluted 1:1 with MM (Spiering *et al.* 2002). In all experiments, three replicate culture plates of each treatment were used for loline-alkaloid analysis. For *in symbio* experiments, *Lolium pratense* (*Festuca pratensis*) and *L. arundinaceum* (*F. arundinacea*) plants symbiotic with *N. uncinatum* CBS 102646 and *N. coenophialum* ATCC 90664, respectively, were grown in the greenhouse.

Bacterial strains and plasmids: The cosmid pMOCosX (Orbach 1994) and plasmid pCB1004 (Carroll *et al.* 1994) were used in genomic library construction and DNA cloning, respectively, in *Escherichia coli* strain XL1-Blue (Bullock *et al.* 1987). These plasmids contain the hygromycin B-phosphotransferase (*hph*) gene under control of constitutive promoters of the *cpc-1* gene in *Neurospora crassa* (pMOCosX) and the *trpC* gene in *Aspergillus nidulans* (pCB1004; *hph* cassette). Bacterial cultures were grown on LB plates or in LB medium with shaking (200 rpm) at 37° for 16 hr. Plasmid DNA was isolated from bacterial cultures by the method of Ahn *et al.* (2000).

Fungal genomic DNA isolation and genome walking: Fungal genomic DNA was isolated by the method of Al-Samarrai and Schmid (2000). To amplify long (>1 kb) genomic DNA segments, 5 ng of genomic DNA was amplified in 50-μl reaction volumes, by using the LA-PCR version 2.1 kit per manufacturer's instructions (Takara Shuzo, Otsu, Shiga, Japan). Reactions were performed in a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer, Boston). PCR conditions were 95° for 60 sec; 7 cycles of 95° for 20 sec and 70° for 8 min; 28 cycles of 95° for 20 sec and 67° for 8 min; and a final 16 min at 67°. To PCR walk into unknown genomic regions, the Universal Genome Walker kit (CLONTECH, Palo Alto, CA) and *N. uncinatum* or *N. coenophialum* genomic DNA were used and manufacturer's instructions were followed for generating genome-walker libraries. PCR primers for genome walking were designed to anneal to sites in known genomic DNA regions and synthesized by Integrated DNA Technologies (IDT; Coralville, IA). For gene-copy-specific genome-walking PCRs, a single nucleotide polymorphism (SNP) was incorporated into the terminal 3′-nucleotide position of each genome-walking primer. The amplified DNA fragments were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA) following the manufacturer's instructions and sequenced as described below.

Genomic DNA library construction and screening and cosmid sequencing: A partial *N. uncinatum* genomic DNA library was constructed in cosmid vector pMOCosX as previously described for construction of a genomic library from *Claviceps purpurea* (Wang *et al.* 2004). A total of 2613 primary clones were obtained and arrayed in 384-well microtiter plates. To identify cosmid clones containing *lolA* and *lolC* genes, plates were PCR screened by the method of Wang *et al.* (2004), with primers lolA-3′ and lolA-5′ (for detection of *lolA*) and primers lolC-3′a and lolC-5′a (for detection of *lolC*). To obtain random Tn7 insertions for sequencing, the TnsABC transposase/Tn7 transposon-based genome-priming system (GPS-1; New England Biolabs, Beverly, MA) was used following the manufacturer's instructions. GPS-tagged cosmids were electroporated into *E. coli* XL1-Blue, and colonies were selected on kanamycin and ampicillin. Approximately 200 independent GPS-tagged clones were obtained and partially sequenced. To obtain a contiguous sequence of the cosmid clone, sequences from all GPS-tagged clones were assembled using PhredPhrap and checked in Consed (University of Washington, http://www.phrap.org/). Gaps between contigs were closed by primer walking.

DNA sequencing: Sequencing of DNA was performed with the BigDye Version 3 Terminator cycle sequence kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 310 or 3100 DNA analyzer. PCR fragments of genomic DNA were sequenced with the PCR primers and with primers designed on sequences thereby obtained. For high-throughput sequencing of genomic library clones, the CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA) with the CEQ DTCS-Quick Start kit (Beckman Coulter) was used.

Identification of possible open reading frames and protein signature sites: To search for possible open reading frames (ORFs) in both strands of genomic sequence, with the search parameters for the *N. crassa* genome, genomic DNA sequences were entered into the FGENESH gene prediction program (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind; Salamov and Solovyev 2000). BLASTX similarity searches (Altschul *et al.* 1997) in the NCBI nr database (http://www.ncbi.nlm.nih.gov/BLAST/) were also performed to help identify coding regions. To search for highly significant matches, the BLASTX searches were first performed on long (>20 kb) contiguous sequences; to identify possible DNA regions having lower significant similarity ($E > 10^{-7}$) to known sequences masked by matches with higher similarity, BLASTX searches were repeated with smaller (2–3 kb) segments from the large contiguous sequences. On the basis of the gene-prediction and BLASTX search results, primers were designed on the sequence just outside of the ORFs predicted by FGENESH (~20–100 bp up- or downstream of the predicted 5′- and 3′-ends, respectively). To check expression of the predicted genes, the primers were used in reverse transcription PCR (RT-PCR) on RNA from loline-alkaloid-producing fungal cultures or endophyte-infected plant tissues. The cDNAs thus obtained were sequenced. cDNA 5′- and 3′-ends were amplified by rapid amplification of cDNA ends (RACE) methods (Schaefer 1995), using gene-specific primers (GSPs) oriented in the 5′- or 3′-direction and CLONTECH's universal PCR primer or 5′- or 3′-universal primer in the reverse transcription of RNA. Alternatively, cDNA ends were amplified by using the GSPs in combination with the vector primers tripleX 5′ amp or tripleX 3′ LD and cDNA from a previously constructed cDNA library (Spiering *et al.* 2002). Approximately 20 ng of total cDNA was used in pre-PCR with the GSPs in 20-μl reactions with thermal parameters of 95° for 60 sec and then 25 cycles of 95° for 20 sec, 64° for

20 sec, and 72° for 60 sec. The products were diluted 1:10 and 2 μl was used in 40-μl PCR reactions with a GSP and the corresponding RACE or vector primer (95° for 1 min; 5 cycles of 95° for 20 sec and 70° for 60 sec; and 27 cycles of 95° for 20 sec, 64° for 20 sec, and 72° for 60 sec). PCR products were purified and sequenced.

To identify conserved amino acid signature motifs, the protein sequences deduced from ORFs identified within the cDNA sequences were used in BLASTP and conserved domain (CD) similarity searches within the nr database and in searches of the Prosite database (http://us.expasy.org/prosite/).

Detection of *lol*-gene expression: RNA was extracted from fungal cultures and plant tissues and total cDNA was synthesized from total RNA as described previously (Spiering *et al.* 2002). Primers suffixed "cDNA" in supplementary Table 1 (at http://www.genetics.org/supplemental/) were used in PCR with the total cDNA as template. Diagnostic PCRs with *N. coenophialum* cDNA were performed with "cDNA" primers specific to the *LOL-2* genes. Approximately 10 ng of total cDNA was used in PCR with Takara's LA-PCR version 2.1 kit and the cDNA primers in 20-μl reactions with the following temperature regime: 95° for 60 sec; 7 cycles of 95° for 20 sec and 72° for 2 min; and 30 cycles of 95° for 20 sec, 64° for 20 sec, and 72° for 2 min. To test for possible contaminating genomic DNA, PCRs were performed on the RNA preparations used in the RT-PCR, using primers for detection of *lolA-1* and *seqX* (see supplementary Table 1 at http://www.genetics.org/supplemental/) and the same PCR conditions used for detection of cDNAs (see above). In addition, PCRs on total cDNA were performed repeatedly with *lolC-1* cDNA primers (see supplementary Table 1 at http://www.genetics.org/supplemental/), amplifying DNA regions spanning several introns. In none of the RNAs and cDNAs used in the gene expression profiling was any contaminating genomic DNA detected.

***lolC* RNAi construct:** A *lolC*-RNAi construct was created by PCR (LA-PCR version 2.1 kit) using 10 ng genomic DNA from *N. coenophialum* strain ATCC 62374 in 100-μl reactions (*N. coenophialum* has only a single copy of the *lolC* gene, and the DNA regions targeted for amplification were 100% identical to those in *LOL-2* in *N. uncinatum*). With primers RNAi 1 and RNAi 2 (see supplementary Table 1 at http://www.genetics.org/supplemental/), a DNA fragment was amplified (95° for 60 sec and then 35 cycles of 95° for 25 sec, 64° for 30 sec, and 72° for 90 sec) containing 769 bp promoter and 5′-untranslated regions (5′-UTRs) before the ATG of the *lolC* ORF, 98 bp of the first exon, and 54 bp of the first intron. In a second PCR (95° for 60 sec; 5 cycles of 95° for 25 sec, 62° for 30 sec, and 72° for 30 sec; and 30 cycles of 95° for 25 sec and 68° for 30 sec) with primers RNAi 3 and RNAi 4, a DNA fragment was generated containing 8 bp of *lolC* first intron forward sequence, followed by 98 bp of the first exon and 114 bp of the 5′-UTR of *lolC* in reverse-complement orientation. After PCR, both fragments were purified as described above, pooled, digested with *Xba*I (*Xba*I sites were built into primers RNAi 2 and RNAi 3), and ligated (Fast-Link ligation kit; Epicentre, Madison, WI) to give a 1.1-kb fragment. The fragment was digested with *Xho*I and *Kpn*I (sites built into the 5′-ends of primers RNAi 1 and RNAi 4, respectively), ligated into *Xho*I and *Kpn*I-cut pCB1004, and electroporated into *E. coli* XL1-Blue cells, and then transformants were selected on chloramphenicol. The construct, pKAES178 (Figure 1), was isolated with a QIAGEN Plasmid Midi kit. The integrity of the *lolC*-RNAi construct was checked by sequencing into the insert from flanking vector regions. Since sequencing of regions containing the first exon was hampered by secondary structures, diagnostic digests with different restriction enzymes and electrophoretic analysis of the restriction fragments was also performed to confirm that the desired construct had been obtained.

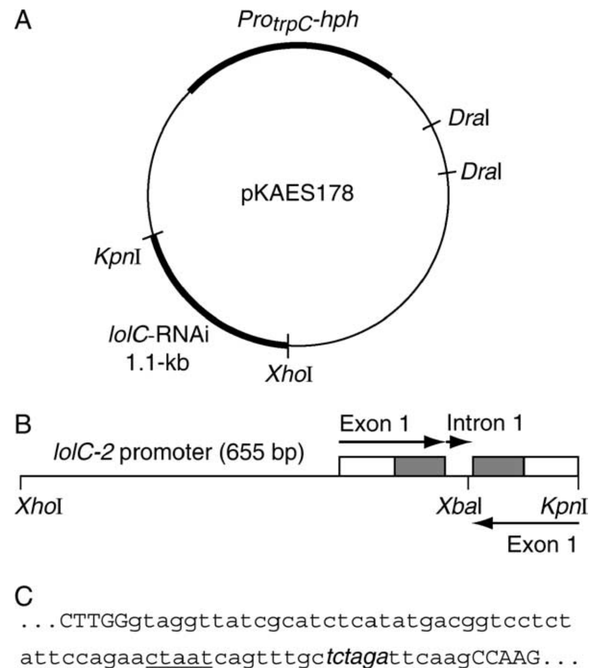

Figure 1.—Construct for RNAi of *lolC* genes. (A) Map of the plasmid construct. (B) Map of the *lolC* sequences in the construct. Open and shaded boxes are, respectively, the 5′-UTR and coding sequences in exon 1. (C) Sequence of the *lolC* first intron (lowercase letters) between the complementary first exon sequences (uppercase letters). Underlined sequence indicates the putative intron branch point, and italicized sequence indicates the *Xba*I site introduced for cloning.

Fungal transformation: Protoplasts were prepared from a 7-day-old half-strength PDB culture of *N. uncinatum* CBS 102646. The mycelium was treated for 4 hr at 30° with 7 mg ml^{-1} Novozyme 234 (Novo Industri AS, Bagsvaerd, Denmark) and 3 mg ml^{-1} Glucanex (Novo Industri AS) with bovine serum albumin (Sigma, St. Louis) added at 5 mg ml^{-1}. The protoplasts were harvested and then electroporated with 5 μg of *Dra*I-linearized pKAES178 or pCB1004 (vector-only control) as described by Tsai *et al.* (1992). After electroporation, the protoplasts were mixed with 4 ml of regeneration medium (Panaccione *et al.* 2001) and plated onto regeneration medium plates with 80 μg ml^{-1} hygromycin B (Calbiochem; San Diego). After ∼6 weeks of growth at 22°, viable fungal colonies were transferred onto potato dextrose agar (PDA) with 80 μg ml^{-1} hygromycin B for sporulation and then single-spore isolated three times on the same medium.

Loline-alkaloid analysis: Loline-alkaloid extraction from freeze-dried culture filtrates and quantification by gas chromatography were performed as described by Blankenship *et al.* (2001). Reported is the sum of all lolines produced in culture, *e.g.*, loline, *N*-acetylnorloline, and *N*-formylloline. The lower limit of detection in the assay was 10 μg $(g\ DW)^{-1}$.

Quantification of *lolC* expression: Primers and probes for TaqMan real-time PCR were designed with Primer Express software (Applied Biosystems) following the manufacturer's specifications. Primers and probe searches were performed on *lolC-1* and *lolC-2* sequences. Sequences contained within pKAES178 were excluded from this search. Primers lolC forw, lolC-1 rev, and lolC-2 rev (see supplementary Table 1 at http://www.genetics.org/supplemental/) were designed for amplification of cDNA from the *lolC* orthologs. To specifically discriminate between expression of *lolC-1* and *lolC-2* in the real-time

PCR assay, TaqMan probes specific to *lolC-1* (lolC-1 RT-PCR probe) or *lolC-2* (lolC-2 RT-PCR probe) were designed to span two SNPs for 3′-labeling with a minor groove binding (MGB) nonfluorescent quencher (dihydrocyclopyrroloindole tripeptide; Kutyavin *et al.* 2000). As a quantification standard, the coding sequence of the β-tubulin (*tub2*) gene in *N. uncinatum* (L06946) was used to design the PCR primers, tub2 forw and tub2 rev, and the fluorescently labeled *tub2* RT-PCR probe (see supplementary Table 1 at http://www.genetics.org/supplemental/). TaqMan probes 5′-labeled with 6-fluorescein (6-FAM) reporter and 3′-labeled with MGB were synthesized by Applied Biosystems, and forward and reverse primers were synthesized by IDT. Real-time PCR was performed with the TaqMan One-Step RT-PCR Master Mix reagents kit (Applied Biosystems). Reactions (25 μl) were set up in duplicate in 96-optical-well plates, and PCR was performed in an Applied Biosystems PRISM 7700 (PCR cycling: 48° for 30 min, 95° for 10 min, and then 40 cycles of 95° for 15 sec and 60° for 60 sec). Effects of varying primer and probe concentrations (in the range of 0.2 and 0.9 μm for primers and 0.2 and 0.4 μm for probes) were tested on *N. uncinatum* total RNA (5 ng $reaction^{-1}$), and no effects were detected. Therefore, 0.4 μm of each primer and 0.2 μm probe was used together with 50 ng (fungal) or 100 ng (plant-fungus symbiotum) total RNA in each reaction. The threshold cycle number (C_T) for the fungal *tub2* gene was used to correct for differences in RNA concentration between samples. The average coefficient of variation of the C_T in duplicate measurements was <1% for all genes tested. To relate C_T to template concentration, standards for each *lolC* ortholog were generated by PCR (95° for 9 min and then 35 cycles of 95° for 25 sec, 64° for 30 sec, and 72° for 30 sec) with primer lolC forw and lolC-1 rev or lolC-2 rev, using purified *lolC-1* and *lolC-2* cDNA as template and AmpliTaq Gold enzyme (Applied Biosystems). The products were purified as described above, quantified in a fluorometer (Hoefer; Amersham Pharmacia Biotech, Piscataway, NJ), and used as standards in real-time PCR to generate standard curves ($r^2 > 0.99$) in the same C_T range as the RNA samples. Specificity was tested by using primers and probe of *lolC-1* or *lolC-2* on the matching or mismatching *lolC* DNA standard at concentrations ranging from 0.5 to 500 fg $reaction^{-1}$. Differences in C_T between matching and mismatching probe-standard combinations showed >10-fold higher specificity of the matching combinations, indicating that each assay was highly discriminative in the quantification of *lolC-1* or *lolC-2* expression.

RESULTS

Gene clusters associated with loline-alkaloid production: We previously identified *lolC* cDNA and cDNA clones of two *lolA* homologs, *lolA-1* and *lolA-2*, whose expression correlated with loline-alkaloid production (Spiering *et al.* 2002). Secondary metabolite pathways are often clustered in fungal genomes (Zhang *et al.* 2004), so we hypothesized that *lolA* and *lolC* may also be clustered. Long-range PCR with primers, lolA-3′ and lolC-5′a (see supplementary Table 1 at http://www.genetics.org/supplemental/), gave an 8-kb product. This product was purified and sequenced, verifying the presence of *lolA* and *lolC* sequences at the ends. Submission of the 8-kb sequence to BLASTX search of the nr database revealed two additional genes, *lolD* and *lolO*, having similarities to genes for ornithine decarboxylase and oxidoreductases, respectively (Table 1).

A partial genomic library of *N. uncinatum* was screened for *lolA*, and a single positive clone was identified and sequenced. The cosmid was a chimeric clone that included a 10,138-bp insert with *lolA* sequence within a region of 95% identity to the 8-kb LA-PCR fragment. Thus, the LA-PCR fragment and the cosmid insert were derived from similar but distinct gene clusters, which we designated *LOL-1* and *LOL-2*, respectively (Figure 2). Additional sequences from the two clusters were determined by genome walking into unknown regions with primers specific to *LOL-1* and *LOL-2*. Genome walking was stopped when several PCR attempts with different primer sets failed to yield distinct products. A total of 25 kb was sequenced from *LOL-1* and 16 kb was sequenced from *LOL-2*.

Surprisingly, fragments generated by attempted genome walking from *lolC-1* exhibited single-nucleotide polymorphisms in sequencing traces, suggesting that some product was also generated from the *LOL-2* cluster in the same PCR reactions. These sequences revealed two alleles of another likely gene, designated *lolF*. Primers were designed for locus-specific amplification of the *lolF* to *lolC* regions, but product was obtained only for *lolF-1* to *lolC-1*. Nevertheless, the inferred *lolF-2* region was further sequenced by primer walking until, again, no specific PCR products were obtained. Although *lolF-2* and *lolC-2* were not definitively joined in one contig, we have tentatively assigned them to the same locus, *LOL-2*, as a working hypothesis (Figure 2).

Approximately 74% of the DNA sequences in *LOL-1* and *LOL-2*, including all inferred genes (described below), gave significant alignment with each other, averaging 93% identity between aligned sequences. The *LOL-1* and *LOL-2* clusters had 46% and 49% G + C content, respectively.

Identification of genes in *LOL-1* and *LOL-2*: Open reading frames (ORFs) in *LOL-1* and *LOL-2* (Table 1; Figure 2) were identified by gene-prediction searches with the FGENESH program. To test whether the identified ORFs corresponded to expressed genes, primers specific to each putative ORF were designed (all primers suffixed "cDNA" in supplementary Table 1 at http://www.genetics.org/supplemental/) and used in PCR with total cDNA from loline-alkaloid-producing *N. uncinatum* cultures (Figure 3A). All of the ORFs predicted by FGENESH were expressed.

The *LOL-1* cluster had an arrangement of four pairs of divergently transcribed genes, with only one unpaired gene, *lolD* (Figure 2). *LOL-2* exhibited the identical order and orientations of genes as in *LOL-1*, with the possible exception of *lolF-2*, whose linkage with *lolC-2* was unconfirmed. Near *lolF-2* was a putative tenth gene, designated *seqX*, which was also identified by FGENESH, and was transcribed under loline-alkaloid-producing conditions (Figure 3, A and B). No homolog of *seqX* was identified in or near *LOL-1*.

Complete cDNA sequences, including the 5′- and

TABLE 1

Sizes of the predicted *lol*-gene products, relationships to known enzymes, and protein signature patterns

Gene[a]	Predicted function	Size[b]	Closest enzyme match[c] (GenBank accession no.)	Identity[d]	*E*-value	Signature patterns in predicted proteins (Prosite or pfam pattern)[e]
lolF-1	FAD-containing monooxygenase	540	1,2-Cyclopentanone monooxygenase (CAD10798)	35 (482)	7*e*-89	VIVVGAGFSGILAV (pfam00743.11, FAD-containing monooxygenases; probable FAD-binding site)
lolF-2	FAD-containing monooxygenase	540	1,2-Cyclopentanone monooxygenase (CAD10798)	35 (482)	2*e*-89	AIVVGAGFSGILAV (see *lolF-1*)
lolC-1	γ-type PLP enzyme	473	*O*-Acetylhomoserine (thiol)-lyase (P50125)	54 (431)	1*e*-126	DIVVHSATKWIGGHG (Prosite PS00868, γ-type pyridoxal phosphate (PLP) enzymes; PLP-attachment site)
lolC-2	γ-type PLP enzyme	473	*O*-Acetylhomoserine (thiol)-lyase (XP381593)	53 (426)	1*e*-124	DIVVHSATKWIGGHG (see *lolC-1*)
lolD-1	PLP enzyme/ decarboxylase	420	Ornithine decarboxylase (CAC80209)	35 (401)	3*e*-69	FAVKSSYDRRLIQTLATCG (Prosite PS00878, α-type PLP enzymes; decarboxylases; family 2 PLP-attachment site) ARRVGLNPTVLDIGGGYT (Prosite PS00879, family 2 signature 2)
lolD-2	PLP enzyme/ decarboxylase	415	Ornithine decarboxylase (P27121)	36 (428)	6*e*-69	FAVKSSYDRRLIQTLATCG (Prosite PS00878; see *lolD-1*) ARQVGLNPTVL DIGGGYT (Prosite PS00879 see *lolD-1*)
lolO-1	Oxidoreductase/ dioxygenase	362	Probable oxidoreductase[f] (NP248837)	25 (359)	2*e*-21	ND
lolO-2	Oxidoreductase/ dioxygenase	362	Probable oxidoreductase[f] (NP248837)	25 (359)	2*e*-23	ND
lolA-1	Amino acid binding	209	Aspartate kinase (KIBYD; C-terminal domain)	28 (160)	7*e*-08	ND
lolA-2	Amino acid binding	210	Aspartate kinase (KIBYD; C-terminal domain)	28 (160)	5*e*-08	ND
lolU-1	Possible DNA-binding protein	495	*A. nidulans* predicted protein (EAA61586)	21 (484)	7*e*-04	WTRSEDGSL (Prosite PS00037, Myb transcription factor; DNA-binding domain repeat signature 1)
lolU-2	Possible DNA-binding protein	506	*A. nidulans* predicted protein (EAA61586)	20 (489)	5*e*-04	WTTSEDGTL (see *lolU-1*)
lolP-1	P450 monooxygenase	496	Pisatin demethylase (Q12645)	28 (477)	1*e*-47	FGLGRWQCAG (Prosite PS00086, cytochromes P450; cysteine heme-iron ligand signature)
lolP-2[g]	P450 monooxygenase	184	Pisatin demethylase (Q12645)	28 (152)	1*e*-12	FGLGRWQCAG (see *lolP-1*)
lolT-1	Class V-aminotransferase PLP-enzyme	454	Isopenicillin N epimerase (P18549)	24 (388)	1*e*-13	PDFFVSDCHKWLFVPRPCAV (Prosite PS00595, α-type PLP enzymes: aminotransferases and related enzymes; class-V PLP-attachment site)
lolT-2	Class V-aminotransferase PLP-enzyme	464	Isopenicillin N epimerase (P18549)	25 (400)	2*e*-13	PDFFVSDCHKWLFVPRPCAF (see *lolT-1*)
lolE-1	Epoxidase/hydroxylase	256	Epoxidase subunit A (BAA75924)	39 (242)	2*e*-42	ND
lolE-2	Epoxidase/hydroxylase	256	Epoxidase subunit A (BAA75924)	39 (242)	2*e*-42	ND

[a] The letters in the gene designations were assigned according to the predicted functions of known proteins related to the *lol*-gene products: *lolF*, *F*AD-containing monooxygenases; *lolC*, *C*YSD (homocysteine synthase) in *A. nidulans*; *lolD*, ornithine *d*ecarboxylase; *lolO*, *o*xidoreductases; *lolA*, *a*spartate kinase; *lolU*, *u*nknown (no significant match); *lolP*, *P*450 monooxygenases; *lolT*, amino *t*ransferases; and *lolE*, *e*poxidase.

[b] In amino acids; predicted on the basis of longest contiguous ORF in cDNAs and FGENESH HMM-based gene structure predictions (*N. crassa*) at http://www.softberry.com.

[c] Including only genetically or functionally characterized activities/enzymes, unless indicated otherwise.

[d] Identity (%) with best match determined by BLASTP; number of aligned amino acids is given in parentheses (note: number of aligned amino acids can be greater than the number in *lol*-gene products due to gaps in the BLASTP alignment).

[e] Functionally conserved amino acids in the signature patterns are underlined. ND, none detected.

[f] The predicted *lolO* gene products gave several highly significant matches to putative/probable oxidoreductases/dioxygenases; matches to enzymes with known activity were less significant and included gibberellin 7-oxidase (T09683; $E < 2e\text{-}14$) and isopenicillin N synthetase (P05326; $E < 1e\text{-}04$).

[g] The *lolP-2* gene appeared to be truncated due to a 469-bp intragenic deletion (determined by comparison with *lolP-1* and *lolP* in *N. coenophialum*).

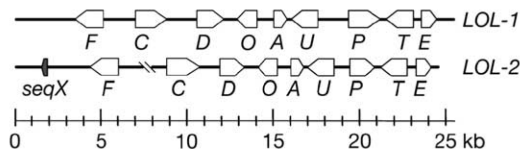

Figure 2.—Two orthologous gene clusters, *LOL-1* and *LOL-2*, associated with loline-alkaloid production in *N. uncinatum*. Inferred genes are indicated by arrows, indicating direction of transcription. The *lol* genes are indicated as *F* (*lolF*), *C* (*lolC*), etc. The predicted products of the *lol* genes and similarities to known enzymes are listed in Table 1. Linkage of *lolF-2* and *lolC-2* is hypothesized but unconfirmed.

3′-ends, were obtained by RACE for both *lolO* gene homologs. The 5′-end of *lolE-2* cDNA was likewise mapped by RACE, and sequence containing its full-length ORF was obtained. cDNA sequences including the complete putative ORFs were also obtained for *lolC-2*, *lolD-1*, *lolD-2*, *lolE-1*, *lolF-2*, *lolP-1*, *lolP-2*, *lolT-2*, *lolU-1*, and *lolU-2*. The cDNA sequence for *lolF-1* was nearly complete except for the 3′-end of its ORF. No cDNA sequence was obtained from *lolT-1*, due to apparently very low expression of this gene homolog both *in planta* and in culture (Figure 3). The *seqX* sequence was predicted by FGE NESH to encode a gene, and was transcribed, but the cDNA contained several small ORFs, suggesting that it may be an expressed pseudogene. Complete cDNA sequences of *lolA-1, lolA-2*, and *lolC-1* were determined in the previous study (Spiering *et al.* 2002).

The *lolD-1* ORF appeared to be ∼15 bp longer than the *lolD-2* ORF, due to sequence differences near the 3′-ends. The *lolP* homologs showed an even more dramatic difference, namely, a 469-bp deletion within *lolP-2* that shifted the reading frame such that a stop codon truncated its ORF to 555 bp (the *lolP-1* ORF was 1491 bp long). A further indication that *lolP-2* had a deletion was that the *lolP* sequence in *N. coenophialum* had high similarity (>99% identity) with *lolP-2*, but encoded an ORF identical in length to that of *lolP-1*.

Most *lol* genes contained between one and five introns, and in all homologous pairs the genes had identical intron positions. Genes *lolU-1* and *lolU-2* apparently had no introns. Comparisons of the cDNA sequences with gene structures predicted by FGENESH identified some concordances and differences. FGENESH correctly assigned the ATG start codon in >80% of the genes. Moreover, FGENESH predicted the exact locations of 52% of the exons (total number, 54) and 58% of introns (total number, 36). All of the introns had 5′-GT and 3′-AG splice junctions common to most spliceosomal introns in Neotyphodium and Epichloë species [an exception being the 5′-GC intron boundary in one of the β-tubulin gene introns (Byrd *et al.* 1990)].

Expression of *lol* genes: Loline-alkaloid production in *N. uncinatum* cultures can be controlled by culture conditions (Blankenship *et al.* 2001; Spiering *et al.* 2002).

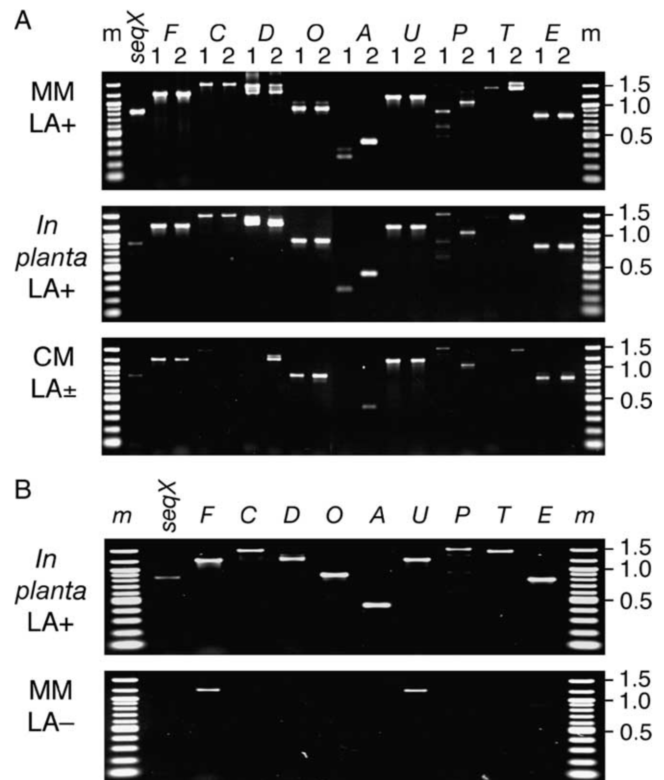

Figure 3.—Expression of the *lol* genes in (A) *N. uncinatum* and (B) *N. coenophialum*. Expression was monitored for both fungi in minimal medium (MM) and *in planta*; expression in *N. uncinatum* was also assessed in complex medium (CM). The *lol* genes analyzed are indicated above each lane as *F* (*lolF*), *C* (*lolC*), etc., and for the *N. uncinatum* genes the ortholog of each was analyzed separately as indicated by numeral 1 or 2 above each lane. Molecular sizes of some marker (m) bands are indicated in kilobases. Loline-alkaloid expression was detected *in planta* (+); in culture, lolines were detected at either high (>100 mg liter^{-1}; +) or low (∼10 mg liter^{-1}; ±) levels or not detected (−).

To determine whether the *LOL*-cluster genes show coordinated expression under loline-alkaloid-inducing or suppressing conditions, we extracted RNA from these cultures and from *N. uncinatum*-symbiotic plants for use in reverse-transcription PCR with the cDNA primers (see supplementary Table 1 at http://www.genetics.org/supplemental/). Expression of all genes from both *LOL* clusters was detected in loline-alkaloid-producing cultures (total lolines >100 mg liter^{-1} culture medium), as well as *in planta* (Figure 3A), as was expression of *seqX*. Reverse-transcription PCR of *lolT-1* sequence consistently gave low amounts of product, suggesting lower expression levels from *lolT-1* compared with the other genes. Several *lol* genes, especially *lolC, lolA*, and *lolT*, appeared to be expressed at lower levels or unexpressed in cultures low (≤10 mg liter^{-1}) in lolines.

Amplified cDNA products from *lolP-1* and *lolP-2* differed in size (Figure 3A), due to the 469-bp deletion in the *lolP-2* sequence described above. In RT-PCR analyses

of some *lol*-gene transcripts, multiple products were observed or there were differences in product sizes from different RNA samples (most prominently observed for *lolP-1*, but also for *lolT-2* and *lolD*). Sequencing of these bands suggested alternative splicing; *e.g.*, in some *lolT-2* cDNAs only the second of the two introns in *lolT-2* had been spliced out.

We also profiled *lol*-gene expression in *N. coenophialum*, an endophyte of tall fescue (*L. arundinaceum*). This endophyte contained sequences highly similar (~99% identity) to *LOL-2* in *N. uncinatum*, but lacked *LOL-1*. Although *N. coenophialum* did not produce lolines under the MM culture conditions that induced *N. uncinatum* to produce lolines (Blankenship *et al.* 2001), plants symbiotic with *N. coenophialum* accumulated lolines. Expression of all *lol* genes was detected in tall fescue plants with *N. coenophialum*, but only *lolF* and *lolU* transcripts were detectable in *N. coenophialum* cultures (Figure 3B). Thus, loline-alkaloid production was associated with expression of the *lol* genes in both *N. uncinatum* and *N. coenophialum*.

Relationships of predicted *lol* genes to known metabolic genes: The predicted LolC, LolD, and LolT sequences gave highly significant BLASTP matches to pyridoxal phosphate (PLP)-containing enzymes involved in amino acid metabolism/interconversion and secondary metabolite pathways (Table 1; Figure 4). All of the predicted PLP enzymes had a lysine residue for PLP binding within a conserved signature region. In addition, LolD had a substrate-binding signature site typical of ornithine decarboxylase.

Four genes were predicted to encode enzymes for oxidation or oxygenation reactions (Table 1). One, *lolP*, was predicted to encode a cytochrome P450 enzyme, having a heme-iron-binding motif. LolF showed similarity to FAD-containing monooxygenases, including the putative FAD-binding site in the N-terminal segment. The *lolO* and *lolE* products showed relationships to non-heme-iron oxidoreductases. Alignment of LolO with isopenicillin N synthetase from *A. nidulans* (GenBank accession no. P05326) indicated conservation of the 2-His-1-carboxylate facial triad motif (His^{222}, Asp^{224}, His^{280}) implicated in metal (iron) binding (Roach *et al.* 1997; Costas *et al.* 2004). The LolE sequences gave significant similarities with a fungal epoxidase and several dioxygenases/ hydroxylases. CD searches with LolE indicated significant similarity (*E*-value = 7*e*-16) to domains (PhyH; pfam05721.3) of phytanoyl-CoA dioxygenase. Alignment of LolE with the pfam05721.3 consensus also indicated a likely 2-His-1-carboxylate facial triad (Figure 4C).

There was no significant match of LolU with known enzymes, but Prosite searches identified a potential DNA-binding site in LolU (Table 1), suggesting that it may be a transcription activation or regulatory protein. No BLASTP or CD match was identified for *seqX*.

Structures of the *lol* genes were compared with those of the known genes to which they were related. In *lolC*, the numbers of exons and introns were identical to its closest match, the *O*-acetylhomoserine (thiol)-lyase (*cysD*) gene from *A. nidulans*, and all of the intron positions appeared to be identical between *lolC* and *cysD* (Figure 4A). Similarly, the first intron in *lolD* was at the same position as the intron in two fungal genes for ornithine decarboxylase (*odc*), although *lolD* had two additional introns not present in the known fungal *odc* genes (Figure 4B). In *lolE*, the intron was at the same position as the second intron in its closest match, a fungal epoxidase gene (Figure 4C). Comparison of *lolP* with one of its closest matches, *PDA* (L20976, encoding pisatin demethylase), indicated that the genes differed in the numbers of introns and exons; *lolP* had four exons, whereas *PDA* had five exons, and amino acid sequences did not align at the intron-exon boundaries.

RNA interference of *lolC*: A *lolC*-RNAi construct, pKAES178 (see Figure 1), was introduced by transformation into *N. uncinatum* protoplasts, and empty vector was introduced into another batch of protoplasts to generate vector-only transformants as controls. Among the protoplasts transformed with the *lolC*-RNAi construct, two independent hygromycin-resistant transformants were obtained (designated NUMS1 and NUMS2). PCR with combinations of vector- and *lolC*-specific primers verified genomic integration of the *lolC*-RNAi construct (data not shown). The presence of both insert-vector junctions in the *lolC*-RNAi transformants suggested that the constructs were integrated at ectopic positions.

Expression of the *lolC* genes, growth, and loline-alkaloid production by the *lolC*-RNAi transformants and two vector-only control transformants (designated NUMS3 and NUMS4) were assessed in loline-alkaloid-inducing cultures. The *lolC*-RNAi transformants and vector-only controls showed no detectable difference in growth (DW accumulation per culture volume). Expression of both *lolC-1* and *lolC-2* was quantified by real-time PCR, and expression of the β-tubulin gene (*tub2*) was measured as a standard to correct for differences in mRNA concentration between reactions (very similar results were obtained whether *lolC* expression was normalized to *tub2* or to total RNA in each reaction). Transformants with the same construct (*lolC*-RNAi or vector) showed similar levels of *lolC* expression and loline-alkaloid production, so their data were combined for statistical analysis. Expression of both *lolC* homologs in the *lolC*-RNAi transformants was ~25% of *lolC* expression in the vector-only controls (significant at $P < 0.05$ for *lolC-1* and $P < 0.01$ for *lolC-2*, Mann-Whitney *U*-test; Figure 5A). In cultures of both *lolC*-RNAi transformants, loline-alkaloid levels were significantly lower than the levels in the two vector-only cultures (~50%; $P < 0.001$, *t*-test; Figure 5B). Thus, introduction of the *lolC*-RNAi construct into *N. uncinatum* significantly decreased expression of both *lolC* homologs and significantly decreased production of lolines.

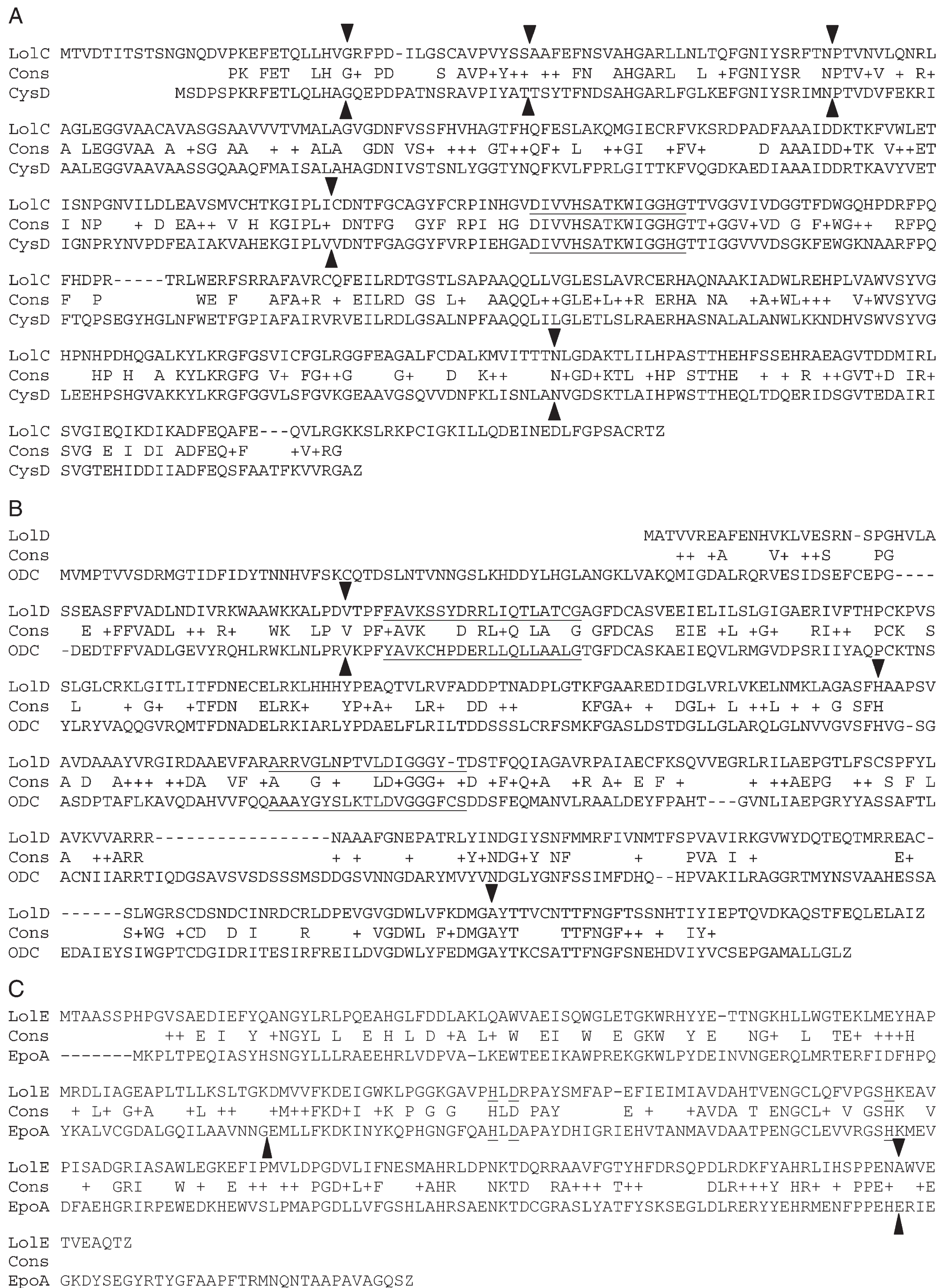

FIGURE 4.—Intron locations in relation to amino acid sequences of putative *lol*-gene products and paralogues. Indicated are alignments of amino acid sequences deduced from (A) *lolC-1*, (B) *lolD-1*, and (C) *lolE-1* cDNAs with protein sequences of their closest putative paralogues (the corresponding *LOL-2* orthologs gave very similar alignments with both paralogues). The putative paralogues of the *lol*-gene products were *O*-acetylhomoserine (thiol)-lyase (CysD) from *A. nidulans* (U19394), ornithine decarboxylase (ODC) from *N. crassa* (BX842618), and epoxidase subunit A from *Penicillium decumbens* (D73371). Locations of introns are indicated as solid triangles. Triangles between letters indicate introns between codons, and those above or below letters indicate introns within the corresponding codons. Sequences similar to protein signatures in Prosite (see Table 1) are underlined. Conserved residues in LolE-1 (His[125], Asp[127], His[162]), possibly forming a 2-His-1-carboxylate facial triad typical of many non-heme-iron(II) enzymes (COSTAS *et al.* 2004), are underlined in C.

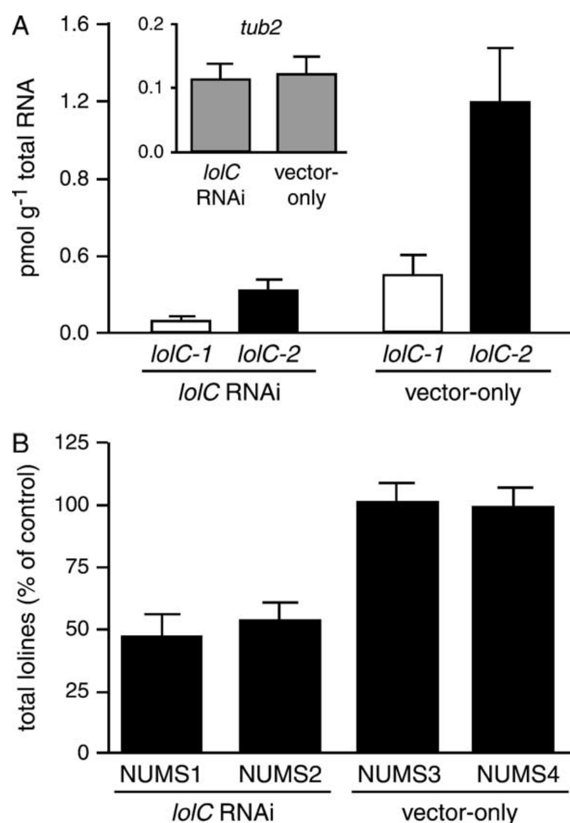

FIGURE 5.—(A) Expression of *lolC* and (B) loline-alkaloid production in *N. uncinatum* transformed with pKAES178 or empty vector (vector-only). The graphs show the means of five independent experiments; expression of the housekeeping gene, *tub2*, in the *lolC*-RNAi and vector-only transformants is indicated in A. Loline-alkaloid production (based on total lolines per gram fungal DW) is expressed as percentage of the mean in the vector-only controls in each experiment. Error bars indicate the standard error of the mean.

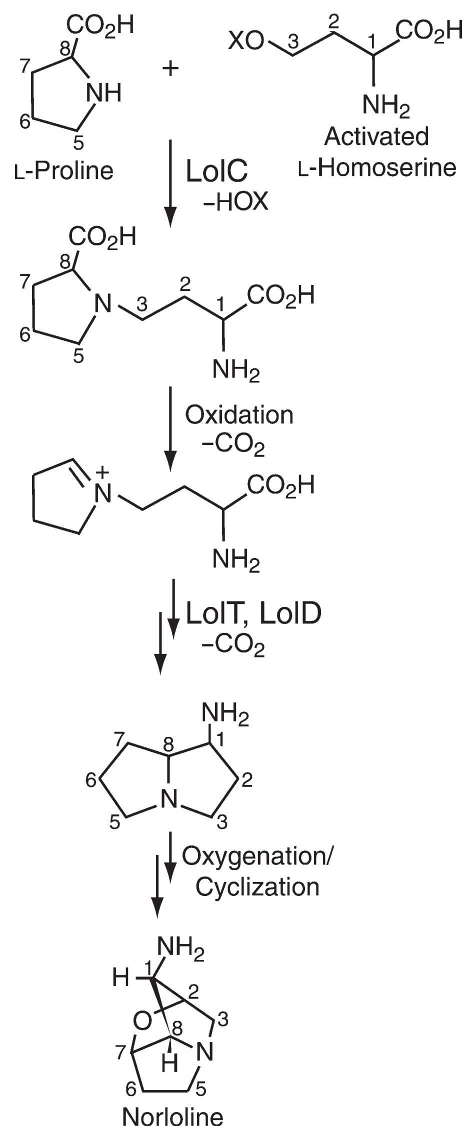

FIGURE 6.—Summary of a possible biosynthetic route to norloline, with hypothesized roles of some *lol*-gene products indicated.

***lol*-gene presence in loline-alkaloid-producing endophytes:** We used diagnostic PCR to test several endophytes with known alkaloid profiles (SPIERING *et al.* 2002) for orthologs of *lolF* and *lolE*, the genes at the ends of the known *LOL-1* sequence. The PCR primers used in this test were not allele specific, but were predicted to anneal to the *lolF* or *lolE* sequences from both *N. uncinatum* clusters. Both genes were detected in all loline-alkaloid producers tested, namely, *N. coenophialum* ATCC 90664, *N. siegelii* ATCC 74483, *Epichloë festucae* CBS 102475, and *E. festucae* × *E. typhina* isolate Tf18. There was no indication of *lolE* or *lolF* in any of the nonproducers tested, namely, *E. festucae* CBS 102477, *E. typhina* ATCC 200736, *N. lolii* isolate 138, and *N. lolii* × *E. typhina* isolate Lp1 (data not shown).

In tests with two different sets of PCR primers, *seqX* sequences were identified in the loline producers, *N. coenophialum* ATCC 90664, *E. festucae* × *E. typhina* isolate Tf18, and a loline nonproducer, Neotyphodium sp. 269 (MYA2503), but not in the loline producers *E. festucae* CBS 102475 and *N. siegelii* ATCC 74483.

DISCUSSION

We identified nine genes associated with loline biosynthesis by the fungal endophyte, *N. uncinatum*, and obtained experimental evidence that most or all of these genes encode enzymes or regulatory proteins for loline-alkaloid biosynthesis. Two homologs of each gene were present in *N. uncinatum*, and (with the possible exception of *lolF*) the homologs were arranged similarly in the two clusters, *LOL-1* and *LOL-2*. Among the *LOL*-cluster genes were *lolA* and *lolC*, associated with loline-alkaloid production in a prior study (SPIERING *et al.* 2002). Here, we have shown that knocking down *lolC* expression in *N. uncinatum* significantly decreases loline-alkaloid production in culture, providing direct evidence for involvement of *lolC* in loline-alkaloid biosynthesis. Furthermore, all nine *lol* genes are expressed in

N. uncinatum under culture conditions conducive to loline-alkaloid production and *in planta*, whereas expression of many *lol* genes is apparently reduced under culture conditions with low loline levels. The association was strengthened by investigating *N. coenophialum*, which expressed lolines and all *lol* genes *in planta*, but expressed no lolines and only *lolU* and *lolF* in culture. Endophyte isolates differing in their capabilities to produce lolines were screened for orthologs of *lolF* and *lolE*—the genes located at the 5′- and 3′-ends of the known *LOL-1* sequence—and orthologs of these genes were detectable only in the loline-alkaloid producers.

Roles of the *lol* genes in loline-alkaloid biosynthesis were suggested by the putative functions of their products: LolF, LolO, LolP, and LolE were predicted to carry out redox reactions; LolC, LolD, and LolT were predicted to possess a PLP cofactor and, therefore, to act on primary amine-containing substrates; LolU was a possible regulatory protein; and the role of LolA was unclear except that it likely binds amino acids.

Due to a high AT content and several repeat sequences within the DNA flanking the clusters, we have so far been unable to walk outward from *LOL-1* or *LOL-2*. However, there was some evidence that *lolF* and *lolE* homologs might be at the ends of the regions uniquely possessed by loline-alkaloid-producing endophytes. Preliminary results of long-range PCR suggest that *lolE* might be located next to an acetamidase gene, which is also present in loline-alkaloid nonproducers (M. J. Spiering, H. H. Wilkinson and C. L. Schardl, unpublished data). This gene arrangement is also indicated in *E. festucae* (H. H. Wilkinson and B. L. Kutil, unpublished data). Also, the putative gene or pseudogene that we designated *seqX*, located near *lolF-2*, was not detected in a loline-alkaloid-producing isolate of *E. festucae* and in the loline producer *N. siegelii*. Therefore, at present we consider it a reasonable hypothesis that *lolF* and *lolE* demarcate the boundaries of the *LOL* clusters, but further studies will be required to test this.

Clustering of secondary-metabolism genes is very common in fungi (Zhang *et al.* 2004). Such pathways often involve activities similar to those predicted for some *lol*-gene products—especially monooxygenases, oxidoreductases, and dioxygenases—though PLP enzymes are much less common in secondary product pathways. The sequence relationships of LolC to biosynthetic enzymes are of particular interest in light of feeding studies that have identified loline-alkaloid precursors (Blankenship *et al.* 2005). LolC includes a conserved PLP-binding site and has highly significant similarity to *O*-acetylhomoserine (thiol)-lyase (homocysteine synthase), which is involved in methionine biosynthesis (Sienko *et al.* 1998). A further indication of this relationship was the correspondence of all five intron positions in *lolC* with those in the *A. nidulans cysD* gene encoding this enzyme. However, expression of the *lolC* ORF in an *A. nidulans cysD*$^-$ mutant did not restore methionine prototrophy (M. J. Spiering and C. L. Schardl, unpublished results), suggesting that the activity of LolC differs from homocysteine synthase in terms of substrate specificity or catalytic activity. Homocysteine synthase uses activated L-homoserine as a substrate in PLP-mediated γ-substitutions (Sienko *et al.* 1998; Steegborn *et al.* 1999). The amino acids L-proline and L-homoserine have been identified as loline-alkaloid precursors, and observations by Blankenship *et al.* (2005) raise a possibility that an early step in the loline pathway might be a novel biochemical reaction in which the L-proline amine is condensed with the 4-carbon of L-homoserine by a LolC-catalyzed γ-substitution (Figure 6).

Reasonable conjectures can be made about the roles of other *lol*-gene products (Figure 6). One of the oxidizing enzymes (LolE, LolF, LolO, or LolP) might oxidatively decarboxylate the L-proline moiety, generating a pyrrolinium ion, a reasonable intermediate in A-ring formation. One of the PLP enzymes (LolD or LolT) could act on the L-homoserine-derived primary amine to facilitate decarboxylation, and the other could promote cyclization to complete the pyrrolizidine ring system. Other oxidases/oxygenases would be involved in ether bridge formation, although the strained ether linkage characteristic of lolines is highly unusual, and there appears to be no known precedent to suggest the mechanism of its formation. In total it is conceivable that the simplest loline alkaloid, norloline, could be synthesized with the three predicted PLP enzymes plus oxidases/oxygenases sufficient for six electron transfers. We predict that 8–12 electron transfers would be catalyzed by LolF, LolP, LolO, and LolE. Therefore, we hypothesize that the products of the *lol* genes identified in this study may be sufficient for biosynthesis of the entire loline-alkaloid three-ring structure.

The *lolU* gene product is a candidate *lol* regulatory protein, since it has a potential DNA-binding site, but further studies will be required to test this possibility. *lolU* was also expressed in nonproducing cultures when most other *lol* genes were not expressed (Figure 3). Thus, if LolU is involved in transcriptional regulation of the *lol* genes, it may require activation or deactivation (*e.g.*, via phosphorylation) in a regulatory cascade.

The possible role of LolA is also of interest, considering that it has similarity only to the C-terminal domain of aspartate kinases, excluding the kinase active site (Spiering *et al.* 2002). The C-terminal domain likely contains an allosteric binding site for amino acids produced from aspartate (Arévalo-Rodríguez *et al.* 1999). In fungi, L-homoserine is an intermediate in the pathway from aspartate 4-phosphate to L-threonine, L-methionine, and L-isoleucine. We speculate that LolA might interact with aspartate kinase to prevent feedback inhibition of its activity by these end products (Arévalo-Rodríguez *et al.* 1999; Azevedo and Lea 2001) and thereby promote production of L-homoserine from L-aspartate.

The gene orders in *LOL-1* and *LOL-2* and their orien-

tations were highly conserved between the two clusters, and most genes showed head-to-head arrangements with one of their proximal neighbors, suggesting that genes located next to each other may share 5′-regulatory sequences as has been shown for some clustered genes in other systems (Liu and Xiao 1997). However, not all of the proximal neighbors showed identical patterns of expression. For example, although *lolU* was expressed in *N. coenophialum* in culture, expression of its 5′ neighbor, *lolP*, was not detected, suggesting more complex regulation for at least some of the *lol* genes.

There are two possible reasons for the presence of two *LOL* clusters in *N. uncinatum*: duplication of an ancient *LOL* cluster in *N. uncinatum* or inheritance of the clusters from two ancestors. Like many Neotyphodium species, *N. uncinatum* is a heteroploid interspecific hybrid (Craven *et al.* 2001). We consider its hybrid origin the most plausible reason, on the basis of phylogenetic analyses of the *lolC* intron sequences in different loline-producing endophyte species and isolates (M. J. Spiering and C. L. Schardl, unpublished results), suggesting that *N. uncinatum* has inherited *lolC* from its two likely ancestors, *E. typhina* and *E. bromicola* (Craven *et al.* 2001).

Functional tests of genes in the Neotyphodium species can be conducted by marker-exchange mutagenesis (Panaccione *et al.* 2001; Wang *et al.* 2004), but because of their slow growth each transformation experiment takes several months, frequencies of knockouts are low (<1%), and repeated knock-outs would be required for *N. uncinatum.* In an attempt to knock out *lolO* in *N. uncinatum,* PCR screening of >350 hygromycin-resistant *N. uncinatum* transformants indicated no replacement of *lolO* (M. J. Spiering and C. L. Schardl, unpublished observations). Yet, *N. uncinatum* is the only endophyte species so far known to produce lolines in culture, and in other species time-consuming reintroduction into plants would be required. Given these considerations, we used the alternative approach of gene silencing by RNAi, performed for the first time with a mutualistic fungus. A potential disadvantage of RNAi is that it usually reduces but does not completely abolish expression of the target gene (Smith *et al.* 2000; Ullu *et al.* 2002). In the *lolC*-RNAi transformants, expression of both *lolC-1* and *lolC-2* was significantly reduced to ∼25%, and total lolines were also significantly reduced to ∼50% of the vector-only controls. The lack of strict correspondence between the relative levels of *lolC* mRNA and the relative amounts of lolines produced was unsurprising. Enzyme expression is regulated at various steps of transcription, translation, and enzyme modification, and in a multiple-enzyme metabolic process other regulated steps and kinetic effects that would lead to such a difference are likely. Nevertheless, the observed effect of reduced *lolC* expression on loline-alkaloid production indicates that LolC is involved in biosynthesis of lolines.

Although it is likely that the effect of the *lolC*-RNAi construct was dsRNA-mediated gene silencing, other effects are conceivable. In particular, because the *lolC-2* promoter was used for expression—on the basis of the very high level of *lolC* expression in loline-alkaloid-producing cultures (Spiering *et al.* 2002)—it is possible that competition for regulatory factors between native and introduced promoters might also have influenced *lolC* expression in the *lolC*-RNAi transformants.

In summary, the RNAi results indicating a role for LolC, ample precedence in fungi for clustering of genes for biosynthetic pathways, expression studies indicating that all *LOL*-cluster genes are expressed under conditions of loline-alkaloid production, and the presence of the cluster genes in all loline-alkaloid producers, all indicate that the genes identified in the *LOL-1* and *LOL-2* clusters encode biosynthetic and regulatory functions for the lolines.

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