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ORIGINAL ARTICLE

Differential gene expression in *Alternaria gaisen* exposed to dark and light

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Abstract Character states during sporulation have been used to segregate and describe many small-spored species of *Alternaria*, but some are not supported by published phylogenetic analyses. The conidiation response of *Alternaria gaisen* was characterized by selective subtractive hybridization of cDNA produced from cultures of *A. gaisen* grown either in total darkness or in total darkness followed by scarification and 24 h exposure to light. Transcripts or their translation products were identified using BLAST. Multiple transcripts with similarity to ORF-1 of the AM-toxin gene were obtained from the light library. L152 is a full reading frame EST in the light library whose ORF translation has similarity to the conserved domain aegerolysin (pfam06355). A set of 11 ex-type or representative isolates including *A. alternata, A. gaisen, A. yaliinficiens, A. arborescens, A. tenuissima and A. brassicicola* were resolved by UPGMA analysis of a partial genomic sequence (415–425 base pairs) of L152, but were not resolved by a similar analysis of ITS sequences. Furthermore, the resolved lineages of the L152 dataset were reflective of the diversity previously hypothesized by morphological evaluations of sporulation patterns. Although the ITS rDNA sequence region is generally accepted as the most likely candidate for fungal barcoding, the analysis of L152 sequences presented here resolved closely related species or species groups where other loci, including ITS, have not. Based on these results, the sequences of putative aegerolysin homologs were variable, parsimony-informative and warrant additional analyses with a broader isolate set including related genera and species.

Keywords *Alternata* · ITS · Aegerolysin · Hemolysis · Host specific toxins

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Introduction

Alternaria Nees is a cosmopolitan, anamorphic hyphomyceteous genus containing many species of economic importance, including saprophytes, phytopathogens, zoopathogens and as producers of mycotoxins and allergens. A recent class-level multigene phylogenetic analysis of the Dothideomycetes (Schoch et al. 2009) reaffirmed placement of *Alternaria* in the Pleosporales, a group heavily represented by phytopathogens. Disease reports and isolation records indicate discontinuous distribution of some phytopathogenic species of *Alternaria* (Farr and Rossman 2010; Simmons 2007) and in such cases these species may be considered exotic by an importing country and therefore subject to official regulation by that country (Anonymous 1995). The accuracy and effectiveness of phytosanitary regulatory activities are, from the outset, dependent upon correct identification of the pests of interest. In addition to being reflective of evolutionary history, a modern approach to taxonomy could include predictive biological information (e.g., biogeography, host ranges) that might not be reflected in some molecular analyses but be useful in managing phytosanitary issues (Roberts et al. 2000).

Alternaria taxonomy is discordant due to conflicting results from molecular and morphological analyses. Rossman (2007) described the rationale for selection of ITS for barcoding the

fungi but acknowledged that ITS does not work for some clearly recognizable species, and Seifert (2009) noted that additional barcode genes may be necessary for such fungi. Molecular studies using ITS rDNA (Kusaba and Tsuge 1995), protein coding housekeeping genes (Peever et al. 2004), and anonymous open reading frames (ORF; Andrew et al. 2009) sequence data, rDNA RFLPs (Kusaba and Tsuge 1994), and IGS restriction mapping (Hong et al. 2005) from the small-spored *Alternaria* have demonstrated a low level of variability that do not support most segregations resulting from morphological, chemotaxonomic, and DNA fingerprinting studies. Kusaba and Tsuge (1995) went as far as to conclude that the small-spored species are all intraspecific variants of one species, *A. alternata*. However, species such as *A. gaisen* and *A. yaliinficiens* that, prior to 1992 and 1995, respectively, were geographically limited to Asia (Kohmoto et al. 1992; Baudry et al. 1993; Roberts 2005) are supported in DNA fingerprint analyses and metabolite profiles that have supported morphological segregation of several species-groups and are generally congruent with morphological character states (Roberts et al. 2000; Hong et al. 2005; Peever et al. 1999; Andersen and Thrane 1996; Andersen et al. 2001, 2002). We used subtractive hybridization to isolate and characterize sporulation-associated expressed sequence tags (ESTs) produced at the onset of conidial chain formation in response to light exposure, which we determined is required for conidiation in *A. gaisen* RGR91.0166 but not in *A. alternata* RGR91.0102 (data not shown). Stable and discriminative variation in sporulation patterns and spore characteristics have been observed among *A. gaisen*, *A. longipes*, and the small-spored *Alternaria* species from citrus and was shown to be important in elucidating natural history information, especially host-specific pathogenicity (Simmons 1999a). Hong et al. (2006) suggested that finding more informative molecular loci may be necessary to fully resolve the taxonomy of *Alternaria* and rectify it with the observed morphological distinctions.

Simmons' methods (Simmons 2007) to grow and observe *Alternaria* for morphologic study prescribe growing them on low nutrient media under an 8/16-h cycle of fluorescent light and darkness. We reasoned that sampling mRNA populations during discriminative morphological development might provide access to previously unknown discriminative sequences for *Alternaria*. We initiated a search for candidate genes by noting the phenology of sporulation of *Alternaria* under controlled conditions, then used selective subtractive hybridization to identify and characterize mRNA transcripts harvested during early conidial chain formation. Here, we report the isolation and characterization of a set of differentially expressed transcripts from *A. gaisen* RGR91.0166 collected before and after 24 h exposure to light, and present a comparative preliminary UPGMA analysis based upon ITS and one class of transcripts with similarity to aegerolysin that are strongly upregulated by light exposure.

Materials and methods

Growth, observation and harvest of *Alternaria* isolates

Cultures used in this study are given in Table 1, and were maintained as agar blocks held in refrigerated water tubes. All cultures were grown and maintained on potato carrot agar (PCA) made by the method of Simmons (2007). Observations of conidiophore and conidium development were made using a stereomicroscope at ×50 magnification. For the dark-exposed RNA, *A. gaisen* was grown on Amersham Hybond-N nylon membranes on PCA plates inoculated with a mycelial agar plug and incubated in total darkness at 23°C and 21% RH. After 4 days, the mycelium was harvested by scraping and then suspended in liquid nitrogen. The plates were returned to the incubator and held for an additional 24 h but under continuous fluorescent light, then the conidiophores and conidia produced were scraped and processed as described.

RNA extraction

mRNA was extracted from crushed, frozen mycelium with a Qiagen RNeasy Plant mini-kit (Qiagen Sciences, MD, USA) per the manufacturer's instructions. All RNA extracts were treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) following the manufacturer's instructions.

Observation of morphologic response

Cultures of *A. gaisen* were observed using stereomicroscopy at ×50 after both 4 days of incubation in the dark and again after scarification and an additional 24 h of growth under constant light. Cultural characters and developmental status of conidiophores and conidia were noted.

Selective subtractive hybridization (SSH)

All kits were used according to the manufacturer's instructions except where noted. First strand cDNA was synthesized from total RNA from both the dark- and light-exposed tissues using the Super SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) and purified using the Clontech NucleoSpin Extract II kit. First strand cDNA was amplified by long distance PCR and cleaned using a QIAquick PCR purification kit (Qiagen) in lieu of the phenol/chloroform cleanup protocol in the manufacturer's instructions, then digested with RsaI (New England Biolabs, Ipswich, MA, USA). Differentially-expressed ESTs from *A. gaisen* were

Table 1 Isolates of *Alternaria* spp. studied

Specific epithet	RGR Accession[a]	Host / Substrate	Origin	Species group[b] / other referring numbers[a]
gaisen	91.0125[f]	*Pyrus pyrifolia*	JP	2 / EGS 90.1310
gaisen[d]	91.0166[f]	*P. pyrifolia*	JP	2 / EGS 90.0512, ATCC 90611, CBS 632.93
'mali [e]	87.0010[g]	*Malus domestica*	JP	2 / ATCC 44899, NRBC (IFO) 8984
'mali [e]	87.0031[g]	*M. domestica*	JP	2 / ATCC 42096, NRBC (IFO) 0-159
mali[e]	98.0382	*M. domestica*	US	5 / EGS 38.029, ATCC 13963, CBS 106.24
arborescens[c]	99.0128	*Lycopersicon esculentum*	US	3 / EGS 39.128
arborescens-group[d]	91.0117	*P. pyrifolia*	JP	3 / EGS 90.1071
alternata[c]	91.0102	*Arachis hypogaea*	IN	4 / EGS 34.0160, ATCC 66981, CBS 916.96 (epitype)
alternata[d]	91.0128	*Datura metel*	IN	4 / EGS 34.0390, ATCC 66982
tenuissima[d]	96.0028	*Dianthus* sp.	UK	5 / EGS 34.015
yaliinficiens[c]	01.0204	*P. bretschneideri*	CN	* / CBS 121547, EGS 50.048
yaliinficiens[d]	03.0055	*P. bretschneideri*	CN	* / EGS 41.163
brassicicola[d]	96.0070	*Brassica oleracea*	UK	* / EGS 42.002

[a] RGR isolates are accessioned into the first author's culture collection at the U.S. Department of Agriculture, Tree Fruit Research Laboratory, Wenatchee, WA. EGS numbers refer to accessions to the private collection of E.G. Simmons. *ATCC* American Type Culture Collection, Rockville, MD; *BPI* U.S. National Fungus Collection, Beltsville, MD; *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *NRBC* National Biological Resource Center, Kisarazu-shi, Japan

[b] Species-group as defined by sporulation pattern (Simmons 1993); * sporulation pattern not treated by Simmons (1993)

[c] Ex-type culture

[d] Representative culture

[e] *mali A. mali* fide Roberts (36); '*mali*' '*A. alternata* apple pathotype'

[f] Produces AK-toxin (Simmons and Roberts 1993; Roberts 2005)

[g] Produces AM-toxin (Roberts 2005)

obtained by subtractive hybridization of cDNA using a Clontech PCR-Select cDNA Subtraction kit. All clones were screened for cDNA inserts by PCR using NewSP6 and T7 sequencing primers (Eurofins MWG Operon, Huntsville, AL, USA). A Clontech PCR-Select Differential Screening Kit and virtual Northern analysis were used to confirm the differential expression of ESTs (Table 3). A reverse subtraction, conducted as above but with tester and driver strains reversed, was also completed. ESTs with confirmed specificity were maintained in a light or dark cDNA library for *A. gaisen*.

cDNA insert-bearing plasmids were recovered using an Invitrogen S.N.A.P. Miniprep Kit (Invitrogen, Carlsbad, CA, USA) and sequenced using a Beckman GenomeLab Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) with primers NewSP6 (Operon) and pUC/M13 forward (Promega) and analyzed using a Beckman CEQ8000 DNA sequencer. The forward and reverse sequences were screened for vector sequences then aligned and edited to give consensus sequences. GenBank was queried for homologous sequences in BLAST (Altschul et al. 1990) using blastn, blastx or ORF finder/blastp queries sequentially. Blastn searches of the nt/nr, est_others, and wgs databases were made in preferential order. Blastx queries of non-redundant protein (nr) sequences used the default nucleotide translation.

Analysis of L152 and ITS5

Primers L152-F1 and L152-R1 were designed from the distal bases of L152 (Table 2). The L152 primers were used in PCR reactions to produce amplicons from genomic DNA of a selected set of ex-type cultures or representative cultures representing sporulation pattern groups 2–5 Simmons (Simmons 1993; Table 1) and *A. yaliinficiens* (Roberts 2005). The L152 amplicons were sequenced as described, aligned and edited to 415–425 bp using default parameters in MUSCLE (Edgar 2004). ITS sequences obtained using ITS4 and ITS5 primers (White et al. 1990; Table 2) were aligned and trimmed to about 500 bp. Similarity matrices were calculated for L152 and ITS in BioNumerics 6.1 from which a UPGMA dendrogram was constructed for each sequence type. Analyses of L152 and ITS sequences used the default cost table, no correction and 0% gap penalty for alignments. Cluster analysis was by UPGMA, with 1,000 bootstrap analyses and 100% open gap cost, 0% unit gap cost, minimum match sequence=2, maximum number of gaps=9.

Hemolysis assay

Cultures of *A. gaisen* were grown in YG medium (Haffie et al. 1985) and incubated at 200*g* and 25C for 24–48 h.

Table 2 Primers, primer sequences, related Genbank accessions, expected size, and PCR cycling parameters for AK-toxins I and II, AM-toxins I and II, L126, L152, and ITS

Primer [a]	5'-Sequence-3'	GenBank	BP	Denaturation [b]	Annealing	Extension [c]	Cycles
AKT-1F	GCT CGA CTG GCC TCA AAA GC	AB015351	350	94 C / 1m 94 C / 30s	65 C / 30s	72 C / 1m 72 C / 7m	40
AKT-1R	AGA ACC AGG CGC ATC GGT TA						
AKT-2F	GCC GGG AGA GAC GAG GAA AG	AB015352	549				
AKT-2R	CAT GCG ACC TCA ATC GAT CG						
AMT-LinF1	TAT CGC CTG GCC ACC TAC GC	AF184074.1	500	95 C / 5m 94 C / 30s		72 C / 45s 72 C / 7m	30
AMT-LinR	TGG CCA CGA CAA CCC ACA TA						
AMT-2f2	GTT GCA GAA TCG CAA ACT CA	AB525200.1	653	95 C / 1m 95 C / 30s	57 C / 30s	72 C / 1m 72 C / 7m	40
AMT-2r2	GGC TCT TGG TCT CAA ATC CA						
L126-F1	CAT CGA ACT CTG CTC CGA TAG	GW873072	161	95 C / 5m 94 C / 30s		72 C / 1m 72 C / 5m	40
L126-R1	CCG AGG AGG TGA TTC ACA TTA						
L152-F1	GCA CCA GAG TCC TGA TTC CA	GW574213	446	94 C / 5m 94 C / 30s	59 C / 30s		
L152-R1	GTC AAA AAT GCT GAA GCC ATG						
ITS-4	TCC TCC GCT TAT TGA TAT GC	-	~ 500	94 C / 1 m	60 C / 90s	72 C / 2m 72 C / 5m	35
ITS-5	GGA AGT AAA AGT CGT AAC AAG G						

[a] AMT-LinF1 and AMT-LinR for AMT-1 are from Johnson et al. (2000); ITS-4 and ITS-5 are from White et al. (1990). All other primer sets are first reported here

[b] In each box with two entries, the upper line is the initial denaturation temperature and duration; the lower line is denaturation conditions during the indicated number of cycles

[c] In each box with two entries, the upper line is the extension temperature and duration during the indicated number of cycles; the lower line is final extension temperature and duration

Mycelium was decanted through Miracloth, rinsed with distilled water, and then dehydrated in two changes of 200 ml of acetone. The acetone was decanted and the defatted mycelium was frozen, lyophilized, finely ground, then 1.9 g powdered mycelium was extracted with agitation for 24 h at 25°C in 100 ml modified (phenol omitted) Coca's solution ($NaHCO_3$, 3 g; NaCl, 9 g; phenol, 5 g; H_20, 1 L) (Paris et al. 1990). The extract was centrifuged at 5,000*g* for 1 h at 4°C and the supernatant was collected, frozen and lyophilized in a tared centrifuge tube. The dry extract was reconstituted to a 10% extract by weight by addition of an appropriate volume of 0.05 M TRIS-HCl, pH 8.0. About 100 ml of reconstituted extract was placed into wells cut into sheep red blood cell agar (SRBC; Teknova, CA, USA) and the plates were held at 37°C for 24 h. A control well received only TRIS-HCL. A positive assay for hemolytic activity was determined as a zone of clearing around the point of application of the extract caused by lysis of the embedded red blood cells.

Results

Morphologic observations

Radial growth of dark-grown mycelium was ca 40 mm diam after 4 days. A sparse growth of funiculose hyphal elements developed vertically from the mycelium. Nearly all sporulation observed was directly upon the agar block and from aerial mycelium that grew from the plug. Conidia were in short, unbranched chains, generally 5 or less per chain. Conidiophores developed synchronously from the membrane surface after light exposure, nearly all of which exhibited a single, hyaline conidium, but occasionally chains of up to three conidia were present.

Subtractive hybridizations

Differentially-expressed transcripts in both libraries (Table 3) were most similar to sequences of related Dothideomycetous taxa; *Pyrenophora tritici-repentis, Phaeosphaeria nodorum,* and *Cochliobolus heterostrophus* and anamorphic Dothideomycetes. A total of 184 transcripts were differentially expressed and are listed by putative function in Table 3. Differential expression was confirmed by virtual northern analysis, but a small number were either not differentially expressed or were not expressed in the expected library. Of the transcripts, 72% returned either No Hit (29/184), best hits to whole genome shotgun sequences (WGS) with unknown functionality (35/184), or best hits to hypothetical proteins (68/184) with no putative function. Some ESTs present in both libraries but upregulated in the dark were similar to genes with conidiation-associated regulatory function (D282, D150, D188, L40). Other conidiation-associated ESTs (L152, L182, L3) were strongly upregulated after light exposure.

The most abundant class of transcripts showed similarity to a hypothetical protein in the AM-toxin gene cluster and was strongly upregulated after light exposure (Table 3). L126 represents 30 transcripts, each with about 100 bp that are highly similar to bases 4,959–4,860 of the "AMT genes region" (AB525200.1). Twenty-one additional transcripts

Table 3 Inferred GO identifiers, inferred identities of sequences with similarity to EST clones, dbEST Genbank accession numbers, transcript sizes, *e*-values and expression profiles for differentially-expressed transcripts from *Alternaria gaisen* RGR91.0166

Inferred GO Identifier[a]	Inferred identity and Genbank accession of similar sequences[b]	Clone[c] / GenBank Accession no.	Size (bp)[d] / no.	*e*-value	Expression in[e]	
					Light	Dark
MOLECULAR FUNCTION						
Expression in light						
0016868: phosphotransferase activity, 0000287: magnesium ion binding activity	Phosphoglucomutase, XM_001938863	L3 / GT030099	222 / 14	1*e*-08	++++	+
0003735: structural constituent, ribosome	18S small subunit ribosomal RNA gene, partial, DQ678031	L45 / GR979335	300	6*e*-147	++++	+
Expression in dark						
0016491: oxidoreductase activity	Maleylacetate reductase, XM_001941551.1	D248 / GR979507	429	7*e*-41	-	++++
	NADH dehydrogenase subunit 2, YP_001427406.1	D147 / GR979496	512	1*e*-77	-	++++
	NADH dehydrogenase subunit 1, YP_001427399.1	D237 / GR979506	613	1*e*-27	++	++++
	NADH-ubiquinone oxidoreductase chain 1, YP_001427399.1	L19 / GR979334	379	3*e*-60	++	++++
	Voltage-gated K^+ channel subunit β-1, XM_001938287.1	D282 / GR979510	233	6*e*-42	++	++++
0046872: metal ion binding activity	Maleylacetate reductase, XM_001941551.1	D248 / GR979507	429	7*e*-41	-	++++
0008137: NADH dehydrogenase activity	NADH-ubiquinone oxidoreductase chain 1, YP_001427399.1	L19 / GR979334	379	3*e*-60	++	++++
	NADH dehydrogenase subunit 2, YP_001427406.1	D147 / GR979496	512	1*e*-77	-	++++
0005216: ion channel activity	Voltage-gated K^+ channel subunit β-1, XM_001938287.1	D282 / GR979510	233	6*e*-42	++	++++
0015078: H^+ transmembrane transport	Mitochondrial ATPase subunit 6, X13439.1	L80 / GW574203	476	4*e*-48	+	++++
0016811: hydrolase activity	Formamidase FmdS, XM_001932127.1	D135 / GW574227	666	1*e*-97	+	++++
0004672: protein kinase activity	PKc-like protein kinase (catalytic domain), XM_001938526.1	D150 / GW574228	357	1*e*-10	++	++++
0008080: N-acetyltransferase activity	GCN5-related N-acetyltransferase, XM_001938526.1	D252 / GR979508	654	1*e*-38	++	++++
0005515: protein binding activity	HP, (IBR Domain), XP_001791469.1	D9 / GR979512	420	2*e*-37	++	++++
	Actin-3, XM_001934300	L306 / GT030100	232	3*e*-59	++	++++
0005524: ATP binding activity	Actin-3, XM_001934300	L306 / GT030100	232	3*e*-59	++	++++
0008236: serine-type peptidase activity	CHP, (Peptidase family S41), XP_001934517.1	D2 / GR979501	167	1*e*-06	-	++++
0004112: cyclic-nucleotide phosphodiesterase	CHP, (cyclic phosphodiesterase-like), XP_001931106.1	D188 / GR979500	770	3e-70	++	++++
0008270: zinc ion binding activity	HP, (IBR Domain), XP_001791469.1	D9 / GR979512	420	2*e*-37	++	++++
	18S small subunit ribosomal RNA gene, partial, AY642518.1	D3 / GW574219	302	1*e*-32	++	++++
	12S small subunit ribosomal RNA gene, partial, FJ190610.1	L39 / GT030103	612	0.0	++	++++
0005215 : transporter activity	HP, (PRT2; di-/tri-peptide permease), XP_383523.1	D20 / GR979502	359	9*e*-47	+	++++
0003735: structural constituent, ribosome	18S small subunit ribosomal RNA gene, partial, AY642518.1	D3 / GW574219	302	1*e*-32	++	++++
	12S small subunit ribosomal RNA gene, partial, FJ190610.1	L39 / GT030103	612	0.0	++	++++
BIOLOGICAL PROCESS						
Expression in light						
0055114: oxidation reduction	NADP-dependent malic enzyme, XM_001932142.1	L95 / GW574205	274	7*e*-05	++++	+
0006412 : translation	18S small subunit ribosomal RNA gene, partial, DQ678031	L45 / GR979335	300	0.0	++++	+
0005975: carbohydrate metabolic process	Phosphoglucomutase, XM_001938863	L3 / GT030099	222 / 14	1*e*-08	++++	+
0006108: malate metabolic process	NADP-dependent malic enzyme, XM_001932142.1	L95 / GW574205	274	7*e*-05	++++	+
0030582: fruiting body development, 0019835: cytolysis	Asp-hemolysin (aegerolysin), XP_748379.1	L182 / GW574214	193 / 3	6*e*-11	++++	+
	Aegerolysin (pfam06355), XP_001823125	L152 / GW574213	485 / 8	2*e*-22	++++	+
Expression in dark						
0055114: oxidation reduction	Maleylacetate reductase, XM_001941551.1	D248 / GR979507	429	7*e*-41	-	++++
	NADH dehydrogenase subunit 1, YP_001427399.1	D237 / GR979506	613	1*e*-27	++	++++
	NADH-ubiquinone oxidoreductase chain 1, YP_001427399.1	L19 / GR979334	379	3*e*-60	++	++++
	Voltage-gated K^+-channel subunit β-1, XM_001938287.1	D282 / GR979510	233	6*e*-42	++	++++
0003993 : acid phosphatase activity	CHP, (histidine phosphatase domain), XP_002376536.1	D22 / GW574221	384	6*e*-31	++	++++
0016070: RNA metabolic process	CHP, (cyclic phosphodiesterase-like), XP_001931106.1	D188 / GR979500	770	3e-70	++	++++
0009975 : cyclase activity	HP, (cyclase, pfam04199) XM_001800183.1	D154 / GR979497	246	3*e*-46	-	++++
0006857 : oligopeptide transport	HP, (PRT2; di-/tri-peptide permease), XP_383523.1	D20 / GR979502	359	9*e*-47	+	++++
0006412 : translation	12S small subunit ribosomal RNA gene, partial, FJ190610.1	L39 / GT030103	612	0.0	++	++++
	18S small subunit ribosomal RNA gene, partial, AY642518.1	D3 / GW574219	302	1*e*-32	++	++++
0005975: carbohydrate metabolic process	HP, WSC domain, AY618901.2	D85 / GW574224	293	2*e*-06	++	++++
0006417 : regulation of translation	Translation initiation inhibitor, XM_001937030.1	L40 / GW574200	199	2*e*-18	++	++++
0042773: ATP-coupled electron transport	NADH dehydrogenase subunit 2, YP_001427406.1	D147 / GR979496	512	1*e*-77	-	++++
0006810: transport	Mitochondrial ATPase subunit 6, X13439.1	L80 / GW574203	476	4*e*-48	+	++++
0008152: metabolic process	Formamidase FmdS, XM_001932127.1	D135 / GW574227	666	1*e*-97	+	++++
0006468: protein/AA phosphorylation	PKc-like protein kinase, XM_001938526.1	D150 / GW574228	357	1*e*-10	++	++++
0009228: thiamine biosynthetic process	Putative thiazole synthase THI4, BN001302.1	D231 / GW574215	296	6*e*-15	+	++++

Table 3 (continued)

Inferred GO Identifier[a]	Inferred identity and Genbank accession of similar sequences[b]	Clone[c] / GenBank Accession no.	Size (bp)[d] / no.	*e*-value	Expression in[e]	
					Light	Dark
0043581: mycelium development	GCN5-related N-acetyltransferase, XP_001938940	D252 / GR979508	654	1*e*-38	++	++++
0055085: transmembrane transport	Voltage-gated K^+ channel subunit β-1, XM_001938287.1	D282 / GR979510	233	6*e*-42	++	++++
0006508: proteolysis	CHP (Peptidase family S41), XP_001934517.1	D2 / GR979501	167	1*e*-06	-	++++
CELLULAR COMPONENT						
Expression in light						
0022627 : cytosolic ribosomal SSU	18S small subunit ribosomal RNA gene, partial, DQ678031	L45 / GR979335	300	0.0	++++	+
Expression in dark						
0005739: mitochondrion	NADH dehydrogenase subunit 1, YP_001427399.1	D237 / GR979506	613	1*e*-27	++	++++
	NADH dehydrogenase subunit 2, YP_001427406.1	D147 / GR979496	512	1*e*-77	-	++++
	Mitochondrial ATPase subunit 6, X13439.1	L80 / GW574203	476	4*e*-48	+	++++
	NADH-ubiquinone oxidoreductase chain 1, YP_001427399.1	L19 / GR979334	379	3*e*-60	++	++++
	Putative thiazole synthase THI4, BN001302.1	D231 / GW574215	296	6*e*-15	+	++++
0005763 : mitochondrial ribosomal SSU	12S small subunit ribosomal RNA gene, partial, FJ190610.1	L39 / GT030103	612	0.0	++	++++
0016021: integral to membrane	NADH dehydrogenase subunit 1, YP_001427399.1	D248 / GR979507	429	7*e*-41	-	++++
	NADH-ubiquinone oxidoreductase chain 1, YP_001427399.1	L19 / GR979334	379	3*e*-60	++	++++
0005856 : cytoskeleton	Actin-3, XM_001934300	L306 / GT030100	232	3*e*-59	++	++++
0022627 : cytosolic ribosomal SSU	Translation initiation inhibitor, XM_001937030.1	L40 / GW574200	199	2*e*-18	++	++++
HYPOTHETICAL PROTEINS						
	HP, AMT gene region, AB525200.1, *A. alternata* apple pathotype	L172 / GT030094	294	2*e*-45	++++	-
	HP, AMT gene region, (ORF-1, +/−), AB525200.1, '*A. mali'*	L126 / GW873072	290 / 30	8*e*-37	++++	+
	HP, AMT gene region, (ORF-1, +/+), AB525200.1, '*A. mali*'	L217 / GT030096	321 / 21	4*e*-37	++++	+
	HP, WSC domain, *Coccidioides immitis,* XP_001240679.1	L136 / GT030091	442 / 2	1*e*-15	++++	-
	HP, *Gibberella zeae,* XP_383564.1	L343 / GR979518	327	9*e*-25	++++	+
	HP, *Botryotinia fuckeliana,* XP_001548827.1	L201 / GR979514	431 / 3	9*e*-28	++++	+
	PP, *Pyrenophora tritici-repentis,* XP_001940196.1	L259 / GR979515	145	2*e*-07	++++	+
	HP, *Gibberella. zeae* PH-1, XM_390261.1	L111 / GW574207	377 / 2	6*e*-06	++++	+
	HP, *Phaeosphaeria nodorum* SN15, XM_001794956.1	L42 / GW574201	224	2*e*-12	++++	+++
	HP, *Pyrenophora tritici-repentis,* XM_001941086.1	D175 / GR979499	236	3*e*-26	+	++++
	HP, *Pyrenophora tritici-repentis,* XM_001933238.1	D96 / GR979513	535	9*e*-08	++	++++
	HP, *Phaeosphaeria nodorum* SN15, XP_001803363.1	D165 / GR979498	184	4*e*-07	++	++++
	CHP, *Debaryomyces hansenii*, CAG86465.2	D243 / GT066289	879 / 2	4*e*-05	+++	++++
	PP, *Pyrenophora tritici-repentis* Pt-1 C-BFP, XP_001937710	L78 / GR979339	294	3*e*-05	+++	++++
UNKNOWN FUNCTION						
	WGS, *A. brassicicola,* ACIW01000018.1	L56 / GT030104	211	2*e*-14	++++	-
	WGS, *A. brassicicola,* ACIW01000017.1	L8 / GT030106	127 / 18	2*e*-09	++++	+
	WGS, *A. brassicicola,* ACIW01002438.1	L61 / GR979336	240	2*e*-28	++++	+
	WGS, *A. brassicicola,* ACIW01002860.1	L261 / GT030098	146 / 7	3*e*-23	++++	+
	Cryptic EST, *A. brassicicola,* DN479599.1	D6 / GR979511	201	5*e*-06	-	++++
	Cryptic cDNA clone, *Cordyceps militaris,* GR225938.1	D263 / GR979509	721	2*e*-15	+	++++
	WGS, *A. brassicicola,* ACIW01000731.1	D32 / GW574222	175	7*e*-20	+	++++
	WGS, *P.tritici-repentis* Pt-1 C-BFP, AAXI01000469.1	D100 / GW574225	230	4*e*-29	+	++++
	WGS, *A. brassicicola* ACIW01000357.1	D115 / GW574226	147	1*e*-16	++	++++
	Cryptic sequence, *Cochliobolus heterostrophus,* FK683551.1	D235 / GR979505	326	1*e*-62	++	++++
	Subtracted cDNA clone, *Lentinula edodes,* DC878656.1	D327 / GT066291	182	1*e*-07	++	++++
	mRNA, *A. brassicicola,* DN475937.1	D172 / GW574230	162	2*e*-42	++	++++
NO HIT						
	-	L75 / GT030105	191 / 4	-	++++	-
	-	L274 / GW574217	136	-	++++	-
	-	L243 / GW574215	154	-	++++	-
	-	L17 / GT030093	168	-	++++	+
	-	L31 / GT030101	201	-	++++	+
	-	L81 / GT030107	355 /2	-	++++	+
	-	L117 / GT030090	143	-	++++	+
	-	L119 / GW574208	119	-	++++	+
	-	L134 / GW574209	86	-	++++	+
	-	L163 / GW574232	173	-	++++	+

Table 3 (continued)

Inferred GO Identifier[a]	Inferred identity and Genbank accession of similar sequences[b]	Clone[c] / GenBank Accession no.	Size (bp)[d] / no.	*e*-value	Expression in[e]	
					Light	Dark
	-	L166 / GT030092	98	-	++++	+
	-	L169 / GW815602	185	-	++++	+
	-	L213 / GT030095	374 / 2	-	++++	+
	-	L239 / GT030097	170	-	++++	+
	-	L313 / GR979517	182	-	++++	+
	-	L350 / GT030102	165 / 3	-	++++	+
	-	L226 / GT030110	221	-	+	++++
	-	D4 / GW574220	251	-	+	++++
	-	D286 / GT066290	359	-	+	++++
	-	L151 / GW574212	318	-	++	++++
	-	D47 / GW574223	287	-	++	++++
	-	D26 /GT066292	422	-	+++	++++

[a] GO annotation are not available for hypothetical proteins with no known function, whole genome shotgun read sequences with no known function, or those transcripts for which there was no similarity in BLAST searches

[b] *WGS* whole genome shotgun sequence, *CHP* conserved hypothetical protein, *PP* predicted protein, *HP* hypothetical protein

[c] Clone numbers in bold are referenced in the body of the paper

[d] Where followed by a forward slash and a second number, the first number represents the number of base pairs for the longest transcript among similar clones before removal of a poly-A tail, if present. The second number represents the number of clones with the same or highly similar sequences

[e] For each virtual Northern, relative densitometric values were assigned to each lane, with the darkest area of the two lanes were arbitrarily assigned the value 100 (++++), and the densitometric values of the lighter lane were measured and expressed as a percentage in reference to the darkest lane and binned as follows: +++ = 67–99, ++ = 34–66, + = 1–33, - =0

represented by L217 are complimentary to the region spanned by L126. L172 is similar to a putative intron at positions 97,086–97,350 on the (−) strand of AB525200.1. No transcript in either library was similar to an AK-toxin gene or homolog, except some similarity was seen with the AF-toxin biosynthesis gene cluster (AB179766).

Eleven transcripts are represented by L152, which encodes a 402 bp (−1) reading frame whose translation product is a 133aa hypothetical protein with similarity to aegerolysin, a conserved domain (pfam06355). L152-F1 and -R1 primers gave a 445-bp product from genomic DNA of *A. gaisen* RGR91.0166.

Hemolysis assay

Hemolytic activity of Coca's extracts of powdered mycelium from *A. gaisen* RGR91.0166 was confirmed by a zone of SRBC lysis ca 5 mm wide around the wells after 24 h at 37°C. The TRIS negative controls did not produce a zone of SRBC lysis.

Sequence analysis of L152 and ITS-5

L152 primers produced a single amplicon from genomic DNA from 11 *Alternaria* isolates representing described sporulation phenotypes 2–5 (Table 1, Simmons 1993), *A. yaliinficiens* and *A. brassicicola*. *A. arborescens* and an unnamed *arborescens*-group isolate RGR91.0117 each produced two amplicons, the smaller of which was the expected size and was sequenced. Phylograms from the UPGMA analysis of ITS-5 and L152 are presented in Fig. 1, and the MUSCLE alignments are available for download at www.TreeBASE.org (SN10790; Sanderson et al. 1994). The ITS alignment revealed 0 parsimony informative characters and the analysis showed all ten representatives of groups 2–5 and *A. yaliinficiens* in a single unresolved clade. The alignment matrix of derivative L152 sequences contained 102 parsimony informative characters (25% of total characters) and resolved the small-spored species into two primary clades with 99% support. The uppermost clade resolved as three terminal clades corresponding to *A. alternata*, *A. arborescens*, and *A. tenuissima*, but with low bootstrap support for the *A. tenuissima* group. The lowermost clade resolved *A. gaisen* and *A. yaliinficiens* with high bootstrap support.

Discussion

Stable and discriminative phenotypic expression in small-spored *Alternaria* is most obvious in the morphologic variations of sporulation pattern and spore characters, and for *A. gaisen*, *A. longipes* and the small-spored *Alternaria* species from citrus, have been shown experimentally to be predictive of important biological characters, especially host-specific pathogenicity (Simmons 1999a, 1999b;

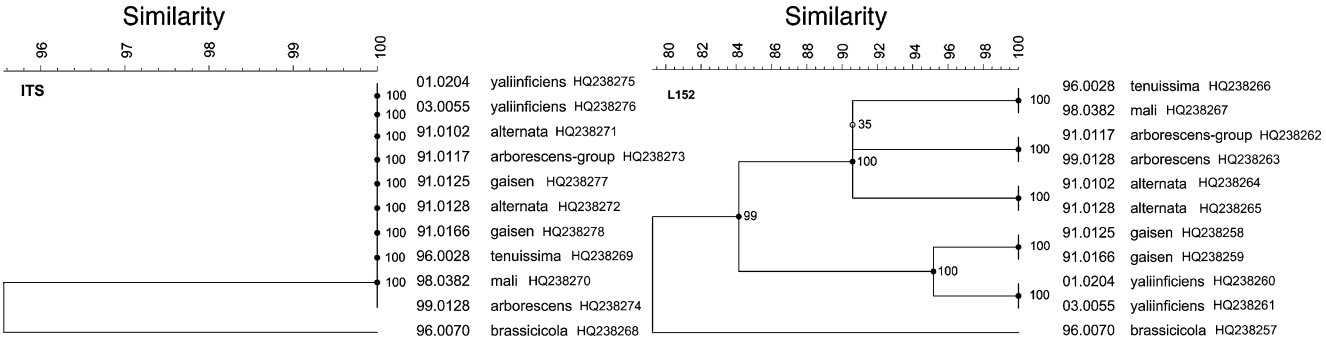

Fig. 1 UPGMA analyses of ITS4-ITS5 (ITS) and a partial sequence (415–425 bp) of a hypothetical protein with similarity to aegerolysin (L152, GW574213). *Numbers* at nodes are bootstrap values from 1,000 replications. GenBank accession numbers for each sequence are given after the specific epithet or group designation

Simmons and Roberts 1993). The synchronous conidiophores and conidia produced after light exposure were used to isolate and characterize sporulation-associated ESTs produced at the onset of conidial chain formation in response to light exposure, which we determined is required for conidiation in *A. gaisen* RGR91.0166 but not in *A. alternata* RGR91.0102 (data not shown).

Regarding the detection of transcripts with some similarity to AM-toxin synthetase, homologs of biosynthetic genes involved in toxin biosynthesis are thought to be shared among those pathotypes whose toxins share a common moiety (Tanaka et al. 1999; Masunaka et al. 2005) and AM-toxin is thought to be limited to a single lineage of '*A. alternata* apple pathotype' (Johnson et al. 2000; Bushley and Turgeon 2010), not *A. gaisen*. AM-toxin and AK-toxin have different moieties, so the presence of a genomic sequence in *A. gaisen* with similarity to an AM-toxin gene was unexpected. We confirmed the absence of AK-toxin homologs or transcripts by PCR from genomic DNA of two isolates of '*A. alternata* apple pathotype' and *A. alternata*, and confirmed the absence of AM-toxin transcripts in *A. gaisen* and *A. alternata*. AK-toxin production by *A. gaisen* RGR91.0166 was reported by Simmons and Roberts (1993) based upon excised leaf assays and confirmed by Roberts (2005) using PCR, who also reported the presence of AM-toxin PCR transcripts in two common reference strains of '*A. alternata* apple pathotype', RGR87.0010 and RGR87.0031. None of the other isolates listed in Table 1 produced amplicons with primers for either AK- or AM-toxins except *A. brassicicola*, which was not tested (Roberts 2005). The detection of L126 PCR products from RGR87.0010 but not RGR87.0031, both of which are AM-toxin producers, is inconsistent with L126 being an AM-toxin homolog. AM-toxin synthetase is part of a large and diverse family of exclusively fungal non-ribosomal peptide synthetase (NRPS) genes with multiple domains (Bushley and Turgeon 2010). L126 may instead be an uncharacterized NRPS with partial similarity to one of the AM-toxin gene domains and expressed in response to conidiation-conducive stimuli. We did not detect L126-related sequences in *A. alternata* and one strain of '*A. alternata* apple pathotype', so we do not consider this transcript a candidate for phylogenetic study.

Similarity of transcripts represented by L152 to the aegerolysin domain is notable because it is not represented amongst the unigenes occurring in the Pleosporales, even though the domain is conserved in bacteria and fungi. The distribution, function and role of fungal hemolysins were recently reviewed by Berne et al. (2009), who posited that aegerolysin was present in the ancestral lineage that gave rise to both Ascomycetes and Basidiomycetes. The predicted protein size from the L152 EST is similar to that of other fungal aegerolysins (Berne et al. 2009). Hemolytic activity of Coca's extracts of *A. gaisen* RGR91.0166 was confirmed and demonstrates at least one of the functional associations for aegerolysin (0019835: cytolysis). Although L152 was first detected from the phytopathogen *A. gaisen*, the presence of putative aegerolysin homologs in both phytopathogenic and non-phytopathogenic species (Fig. 1) argues against its involvement in pathogenesis.

The sequence analysis of a putative aegerolysin homolog resolved several species of *Alternaria* that some have considered conspecific with *A. alternata* (Kusaba and Tsuge 1995; Peever et al. 2004). The relatively invariable ITS sequence for these species, which has been used to support the synonymy of *A. gaisen* and *A. alternata,* did not resolve these same isolates. With such a limited taxon sample we hesitate to draw broad conclusions or predictions about the utility and resolution of the putative aegerolysin from *Alternaria* for large-scale phylogenetic analyses. A complete genomic sequence of L152 and flanking regions may inform the design of competent sequencing primers and allow use of the putative aegerolysin L152 for expanded phylogenetic analyses, and efforts to obtain the complete L152 genomic sequence

have begun. Even with such a limited isolate set, however, it is clear that even the partial-sequence analysis in this paper was parsimony-informative and provided well-supported segregation of *A. alternata* from several species or groups with which it is often considered conspecific in the current mycological literature. Furthermore, because we have detected the putative aegerolysin homolog from species such as *A. yaliinficiens* and *A. mali* (Roberts 1924) that do not produce the extracellular toxins associated with the various "pathotypes" of *A. alternata*, we deduce that the putative aegerolysin gene does not reside on one of the conditionally-disposable chromosomes known to carry the genes responsible and is therefore likely to be present in all small-spored *Alternaria* spp., not just the toxin producers.

Identifying genes expressed during discriminative developmental processes such as sporulation in *Alternaria* spp. did result in discovery of discriminative gene sequences such as L152, and is proof of concept that such discriminative sequences do exist in small-spored *Alternaria* spp. Such work should continue and be expanded, as there are surely other discriminative gene sequences to be discovered and utilized. Among the ESTs isolated in this study were several with similarity to genes with known regulatory function and have been functionally associated with sporulation; phosphoglucomutase (L3), Pka (protein kinase, D150), and a translation initiation inhibitor-like protein (L40). All should be considered for additional study. Additionally, inclusion of other developmental stages in time-course studies may reveal genes with different patterns of temporal expression such as hemolysin, of which there are apparently several forms that are expressed at different developmental stages (Pires et al. 2009).

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