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ORIGINAL ARTICLE

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Molecular characterization of a seed transmitted clavicipitaceous fungus occurring on dicotyledoneous plants (Convolvulaceae)

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Abstract Ergoline alkaloids (syn. ergot alkaloids) are constituents of clavicipitaceous fungi (Ascomycota) and of one particular dicotyledonous plant family, the Convolvulaceae. While the biology of fungal ergoline alkaloids is rather well understood, the evolutionary and biosynthetic origin of ergoline alkaloids within the family Convolvulaceae is unknown. To investigate the possible origin of ergoline alkaloids from a plant-associated fungus, 12 endophytic fungi and one epibiotic fungus were isolated from an ergoline alkaloid-containing Convolvulaceae plant, *Ipomoea asarifolia* Roem. & Schult. Phylogenetic trees constructed from 18S rDNA genes as well as internal transcribed spacer (ITS) revealed that the epibiotic fungus belongs to the family Clavicipitaceae (Ascomycota) whereas none of the endophytic fungi does. In vitro and in vivo cultivation on intact plants gave no evidence that the endophytic fungi are responsible for the accumulation of ergoline alkaloids in *I. asarifolia* whereas the epibiotic clavicipitaceous fungus very likely is equipped with the genetic material to synthesize these compounds. This fungus resisted in vitro and in vivo cultivation and is seed transmitted. Several observations strongly indicate that this plant-associated fungus and its hitherto unidentified relatives occurring on different Convolvulaceae plants are responsible for the isolated occurrence of ergoline alkaloids in Convolvulaceae. This is the first report of an ergot alkaloid producing clavicipitaceous fungus associated with a dicotyledonous plant.

Keywords Ergoline alkaloids · *Ipomoea* · *Turbina* · Plant–fungus association · Seed transmittance · Clavicipitaceae

Abbreviations ITS: Internal transcribed spacer · 18S rDNA: Small subunit ribosomal DNA · SSCP: Single strand conformation polymorphism

Data deposition: The sequences reported in this paper have been deposited in the GenBank (accession numbers are given in the text and in Fig. 3)

Dedicated to Dr. Dr. h. c. mult. Albert Hofmann, the great pioneer of ergot research, on the occasion of his 100th birthday

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Introduction

Ergoline alkaloids (syn. ergot alkaloids) are 3,4-substituted indole derivatives (Fig. 1) which are pharmaceutically important. They are produced by fungi belonging to the genera *Claviceps*, *Aspergillus* and *Penicillium*. The clavicipitaceous fungi form unique associations with monocotyledonous plants of the families Poaceae, Cyperaceae and Juncaceae. The fungi defend the plants against pests and confer drought resistance and fitness to their hosts (Gröger and Floss 1998; Clay and Schardl 2002; White et al. 2003).

It is well known, however, that ergoline alkaloids occur also in one particular dicotyledonous plant family, the Convolvulaceae. Alkaloid-containing species of this family belong to the genera *Ipomoea*, *Turbina*, *Argyreia* and *Strictocardia*. While our knowledge of fungal erg-

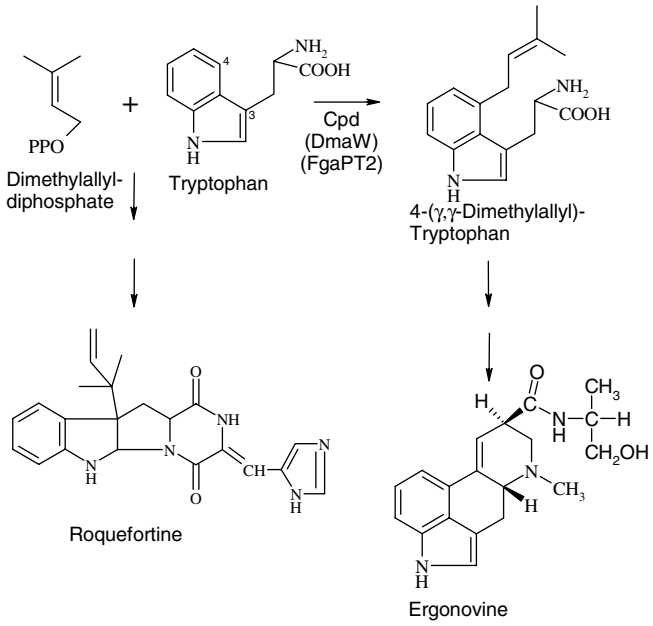


Fig. 1 Biosynthesis of roquefortine and an ergoline alkaloid (ergonovine) from 4-(γ,γ-dimethylallyl) diphosphate and tryptophan via 4-(γ,γdimethylallyl) tryptophan. The 4-(γ,γ-dimethylallyl) tryptophan synthase is encoded by the gene *cpd1* (*dmaW*, *fgaPT2*)

oline alkaloids associated with grasses is rather advanced (Gröger and Floss 1998; Clay and Schardl 2002), the evolutionary and biosynthetic origin of these alkaloids in dicotyledonous plants (Convolvulaceae) is unknown. The question as to the origin of these alkaloids is intriguing because they are present in taxonomically unrelated taxa (Convolvulaceae and Ascomycota). There may be different explanations for this phenomenon: (1) Formation of the alkaloids may have been repeatedly "invented" during evolution. (2) The genetic material responsible for the synthesis of alkaloids may have been acquired during evolution by one taxon from an unrelated taxon during a horizontal gene transfer. (3) The ability to produce ergoline alkaloids may be an ancestral trait that was lost in many lineages and retained in a selected few (fungi, Convolvulaceae). (4) It is conceivable that certain Convolvulaceae plants are infected by an alkaloid producing microorganism, possibly a fungus able to synthesize ergoline alkaloids.

Indeed, recent experiments have shown that alkaloids can be eliminated from *Ipomoea asarifolia* Roem. & Schult. (Convolvulaceae) plants by the treatment with certain fungicides, and this elimination occurs concomitantly with loss of a plant-associated fungus colonizing the upper leaf surface of the plant (epibiotic fungus). The elimination of alkaloids seems to be a rather specific process because the volatile oil components which are also present in the plant, are not removed during the fungicide treatment (Kucht et al. 2004).

The possibility that this fungus is clavicipitaceous and alkaloid producing would be unusual because fungi exhibiting these features were hitherto only reported to occur on monocotyledonous plants. Moreover, such a finding would give an explanation for the occurrence of hallucinogenic ergoline alkaloids in *Turbina* and *Ipomoea* plants (Hofmann and Tscherter 1960) called "ololiuqui" which are used by native Central American people in religious ceremonial practices.

Experiments described in the present paper were designed to investigate the nature of this leaf-associated fungus. Our observations extend the notion that this fungus is epibiotic as has been described for Poaceae plants colonized by *Balansia* and *Atkinsonella* species (Reddy et al. 1998), close relatives of leaf-associated fungus of our study. This fungus co-occurs with ergot alkaloids indicating that it may be responsible for the presence of alkaloids in the *Ipomoea* plants (Kucht et al. 2004). Moreover, the fungus is clavicipitaceous, seed transmitted and apparently carries the gene [called *cpd* (Tudzynski et al. 1999, 2001) or *dmaW* (Tsai et al. 1995; Wang et al. 2004; Coyle and Panaccione 2005) or *fgaPT2* (Unsöld and Li 2005)] responsible for the prenylation of tryptophan. The tryptophan prenylation is the pivotal step in ergoline alkaloid biosynthesis (Fig. 1; Gröger et al. 1963; Tsai et al. 1995; Gröger and Floss 1998; Tudzynski et al. 1999, 2001; Unsöld and Li 2005).

Materials and methods

Plant material

I. asarifolia plants were either grown from 1-year-old seeds or derived from stem cuttings which were rooted. The plants were kept in the greenhouse and employed 5 months after start of the culture. The seeds were collected by Dr. E. Eich (Berlin, Germany) in Ecuador in December 1991. The plants were formerly identified as *Ipomoea piurensis* O'Donnel (Jenett-Siems et al. 1994) but are now known to belong to the species *I. asarifolia* (Desr.) Roem. & Schult (white-blooming form; Jenett-Siems et al. 2004). *Turbina corymbosa* (L.) Hall. F. [formerly *Rivea corymbosa* (L.) Raf.] seeds were obtained from "Rühlemans Kräuter und Duftpflanzen" (Horstedt, Germany). Plants devoid of both alkaloids and the epibiotic fungus IasaF13 were obtained from shoots of Folicur treated plants (Kucht et al. 2004). The shoots were rooted and the plants grown to full size within 5 weeks in the greenhouse.

Fungi

Balansia obtecta was obtained from Dr. C. L. Schardl (University of Kentucky, Lexington, KY, USA), *Claviceps purpurea* from Dr. Tudzynski (University of Münster, Germany), *Balansia cyperi*, *Neotyphodium coenophialum*, and *Atkinsonella hypoxylon* from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).

Identification of roquefortine

The fungus IasaF09 (*Penicillium roquefortii*) was grown in published media (Bacon 1985; Schulz et al. 1993) and the alkaloid fraction isolated from the media (Kucht et al. 2004). Roquefortine was identified by thin-layer (TLC) and high pressure liquid chromatography (HPLC) and by capillary electrophoresis using an authentic sample of roquefortine as reference. TLC on silica gel plates using ethyl acetate (75 ml), ethanol (96%, 10 ml), toluene (10 ml), ammonia (25%, 5 ml); Rf: 0.55. HPLC (column, RP 18), solvent: H_2O containing 5% acetonitrile; increasing gradient with a second solvent consisting of 5% H_2O in 95% acetonitrile; retention time 30 min. Capillary eletrophoresis was performed as described (Kucht et al. 2004).

Amplification of ITS, 18S rDNA and cpd1

DNA from plants was isolated using a modified method adapted from Dellaporta et al. (1983). DNA from fungi was isolated according to Cenis (1992). Polymerase chain reaction (PCR) was performed in a total volume of 50 µl. For PCR, the following published oligomers were used: Small subunit ribosomal DNA (18S rDNA): UF1 (forward) and S3 (reverse; Kappe et al. 1996); Internal transcribed spacer (ITS): ITS1F (forward) and ITS4 (reverse; White et al.1990; Gardes and Bruns 1993); cpd1: *deg*1 (forward) and *deg* 4 (reverse; Wang et al. 2004). The reaction mixture contained 500 µM of each dNTP (Eppendorf, Hamburg, Germany), 1 mM $MgCl_2$, 1 µM of each oligomer, 1× buffer (Eppendorf), 1 unit of Hot Master™ Taq DNA Polymerase (Eppendorf). Cycle conditions for amplification of 18S rDNA and ITS regions were as follows: initial denaturation at 98°C for 3 min, 27 cycles at 93°C for 1 min, 72°C for 2 min, and a final elongation for 10 min. Cycle conditions for amplification of a *cpd1* segment were as previously described by Wang et al. (2004). The PCR product was electrophoresed in 1% agarose gel. The positive band was extracted from the gel with the Qiaquick Gel Extraction Kit (Qiagen), and cloned into pBluescript IIKS (−) (Stratagene). When the presence of the fungus IasaF13 was checked by ITS sequencing in plants grown under sterile conditions, in callus or cell suspension cultures or in regenerated plants the experiment was repeated 6, 15, 9 or 5 times, respectively.

DNA sequencing

Sequencing reactions were carried out by the Sanger random chain terminator method with Big Dye® Terminator v 1.1 cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Both strands were sequenced using oligonuceotides T_3: AATTAACCCTCACTAAAGGG (forward) and T7: TAATACGACTACTATAGGG (reverse). Sequences were analysed with the software "Lasergene" (DNASTAR, Madison, USA).

Single strand conformation polymorphism

PCR for single strand conformation polymorphism (SSCP) was performed using forward oligomer ITS1F (Gardes and Bruns 1993) and reverse oligomer ITS2P (White et al. 1990). The reverse oligomer was phosphorylated at the 5′ end. Reaction mixtures (50 µl total volume) contained: 1× PCR buffer, 3 mM $MgCl_2$, 500 µM of each dNTP, 1 µM of each oligomer, 2.6 units Expand High Fidelity Polymerase (Roche, Mannheim, Germany).

Cycle conditions were as follows: Initial denaturation at 95°C for 2 min, 10 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and 25 cycles of 94°C for 1 min, 72°C for 1 min and additional 10 s cycle elongation for each successive cycle, the final elongation at 72°C for 7 min. As a control 5 µl of PCR product was electrophoresed in 1% agarose gel. The PCR products were purified using MinElute PCR purification kit (Qiagen) as described in the manufacturer's instructions. One-third of the purified PCR product was digested with 10 units of lambda exonuclease (Amersham Biosciences) to obtain single stranded DNA. Before loading onto the gels, the samples were denatured at 95°C for 5 min and were immediately cooled on ice. The samples were electrophoresed in a 10% polyacrylamide gel (21×21 cm^2, spacers 0.25 mm). The gels were run at room temperature at 10 W for 6 h. The positive bands were detected by silver staining following the modified method of Bassam et al. (1991).

Inoculation of intact plants with fungi

The epibiotic fungus IasaF13 was scraped off from young folded leaves whereas *Penicillium roquefortii* was grown on agar medium (Schulz et al. 1993) until sporulation occurred. Spores and mycelium (ca. 500 mg) were suspended in sterile water (8 ml). The suspension was injected into leaves with a syringe and in addition streaked onto the leaves. Ten leaves of each of four plants were inoculated. Harvest of the plants and analysis of alkaloids following Kucht et al. (2004) was carried out 26 weeks after inoculation.

Germination of seeds under sterile conditions

Seeds were kept in 96% ethanol for 5 min and subsequently in commercial bleach solution (13% NaOCl) for 15 min with gentle stirring. The seeds were washed three times in sterile water for 5 min with gentle stirring. The seeds were checked for any residual fungi by streaking on an agar malt medium allowing for fungal growth (Petrini et al. 1992). Contaminated seeds were discarded.

Germ free seeds were placed on half strength Murashige and Skoog medium (MS; Murashige and Skoog 1962) containing salts only. Three seeds were placed into one Erlenmeyer flask (300 ml) containing 50 ml agar medium. After 6–8 weeks, plants were transferred into Erlenmeyer flasks (2 l volume) containing 500 ml agar medium. The plants were harvested 8–10 weeks later. The Erlenmeyer flasks were kept in continuous white light, 48 µmol photons m^{-2} s^{-1} (GRO-LUX Sylvania F58W lamps; Sylvania, Stuttgart, Germany).

Callus formation

In order to grow a callus from germfree plant material a surface-sterilized (*vide infra*) piece of the stem (1 cm) was placed on the same MS medium which, however, also contained agar (8.0 g l^{-1}), sucrose (20.0 g l^{-1}), benzylaminopurine (2.0 mg l^{-1}) and indoleacetic acid (1.4 mg l^{-1}). Before autoclaving the medium was adjusted to pH 5.7–5.8. Surface sterilization was carried out by immersing the plant material in an aqueous solution of 0.1% $HgCl_2$ for up to 15 min and rinsing in water for three times.

Regeneration of plants

The piece of stem and the callus originating from this piece of stem were placed into liquid MS medium containing sucrose (20.0 g l^{-1}) and benzylaminopurine at a concentration of 0.01 mg l^{-1}. A shoot formed within 2–3 weeks. It was cut-off and placed into agar (8 g l^{-1}) medium containing half-strength MS salt medium without sucrose and without hormones. The shoot formed roots within 2 weeks.

Construction of phylogenetic trees

Alignment assembly and phylogenetic analyses were done with the MEGA3 software package (Kumar et al. 2004). The new sequences of the fungal isolates were used to retrieve similar sequences from the databases in sensitive (low complexity filter off, minimal word size) BLAST searches (Altschul et al. 1990) at the NCBI server http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, which were included in the alignments. Additional entries from less closely related fungi were added to highlight phylogenetic distance for clarity. Alignments were constructed with different gap creation and extension penalties to exclude a significant influence of ambiguous ITS alignment regions on reliably identified nodes. The resulting alignments of the 18S rDNA (900 nucleotide positions), the ITS (826 nucleotide positions) and the dmaW homologuous (505 amino acid positions) were used independently for phylogenetic analyses. Phylogenetic trees were constructed with the neighbour-joining method using Kimura-2-parameter distances for nucleotides and Poisson corrected amino acid distances with the pairwise gap deletion option. Node significance was evaluated with 10,000 bootstrap random addition replicates.

Identification of ergoline alkaloids

Alkaloids extracted from plant material (Kucht et al. 2004) were identified by comparison with authentic samples using a HPLC-MS system. HPLC analysis was performed using an Agilent (Hewlett-Packard, Palo Alto, USA) series 1100 instrument equipped with a binary mobile phase delivery system, an autosampler with a vacuum degasser, and a diode-array detector which was set from 275 to 315 nm. Absorptions at individual wavelengths within this range were summed up (see Fig. 6). The alkaloids were separated by a 125×2.0 mm^2 5 µm, Nucleodur®-PYRAMID C_{18} column from Macherey-Nagel (Dueren, Germany). The column was connected to an API2000 LC/MS/MS system (Applied Biosystem/MDS SCIEX) with a TIS source. Alkaloids were eluted from the column with a solution of 2 mM ammonium acetate in 70% H_2O mixed with a solution of 2 mM ammonium acetate in 30% methanol. The mixture was kept constant for 4 min increasing to 100% methanolic solution within 20 min, which was kept constant for 10 min and reduced to 30% methanolic solution within 2 min. The flow rate was 0.25 ml min^{-1} throughout and the entire system was controlled with Analyst 1.3 software.

Light microscopy

For microscopic investigations, a Leitz DMRB photomicroscope (Leica, Bensheim, Germany) equipped with Normaski interference contrast and epifluorescence was used. Fungal structures were visualized with calcofluor (Fluorescence Brightner 28, Sigma) which binds to polysaccharides with ß-glycosidic bonds. Fresh specimens were mounted on a slide in a drop of 10 µg/ml calcofluor before observation. The filter combination giving blue fluorescence of the fungal cell walls used was filter block A (BP 340–380 nm, beam splitter 400 nm, LP 425 nm).

Scanning electron microsopy

Leaf samples were fixed in 2.5 glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.3 for 24 h (Karnovsky 1965). After rinsing with distilled water, specimens were dehydrated through a graded series of ethanol and critical point dried from CO_2 in eight cycles according to Svitkina et al. (1984) using a Balzers CPD 030 (BAL-TEC, Schalksmühlen, Germany). Dried specimens were mounted on aluminium sample holders and sputter coated with 2 nm platinum/

palladium in a HR 208 coating device (Cressington, Watford, UK). Scanning electron microsopy (SEM) was performed using an XL 30 SFEG (Philips, Eindhoven, The Netherlands) equipped with a through lens secondary electron detector.

Results

Previous experiments suggested that a fungus on the upper leaf surface of *I. asarifolia* plants was responsible for the presence of ergot alkaloids. This fungus forms colonies on the upper leaf surface (Fig. 2). The hyphae often encircle oil glands (Kucht et al. 2004). In order to identify and characterize this fungus, culturable fungi were isolated from *I. asarifolia* plants using a published procedure (Petrini et al. 1992). Nineteen different fungal isolates were obtained from leaves, stems and flowers of plants kept in the greenhouse.

It was to be expected that some fungi were repeatedly isolated. Therefore, all fungal isolates were grown in vitro and the DNA extracted from the mycelium. In every case, the DNA was subjected to PCR in which part of the small subunit rDNA gene (18S rDNA) was amplified using fungus-specific oligomers ($UF_1$and S_3; Kappe et al. 1996).

Comparison of the sequences of the amplified DNA stretches showed that the isolates belonged to 12 different fungi which were provisionally named IasaF01 to IasaF12. Database homology searches of the amplified sequences at the NCBI server using BLAST (Altschul et al. 1990) gave a first hint as to the possible taxonomic position of the fungal isolates. These fungal species may or may not be identical to our isolated fungi, however, they certainly bear a close taxonomic relationship to our isolates. Among the isolated fungi three belong to the genus *Penicillium* (Fig. 3), with *Penicillium roquefortii* known to be a producer of roquefortine (Fig. 1) and ergoline alkaloids (Scott et al. 1976). Thin-layer, HPLC and capillary electrophoresis showed that this strain (IasaF09) actually is a producer of roquefortine and of minor components which stain positive with van Urk's reagent. This reaction indicates the possible presence of indole derivatives including ergoline alkaloids. Incubation of each strain IasaF01 to IasaF12 in liquid medium (Bacon 1985; Schulz et al. 1993) and TLC analysis of the medium and the mycelium did not give any evidence, however, that ergoline alkaloids were present in strains other than IasaF09.

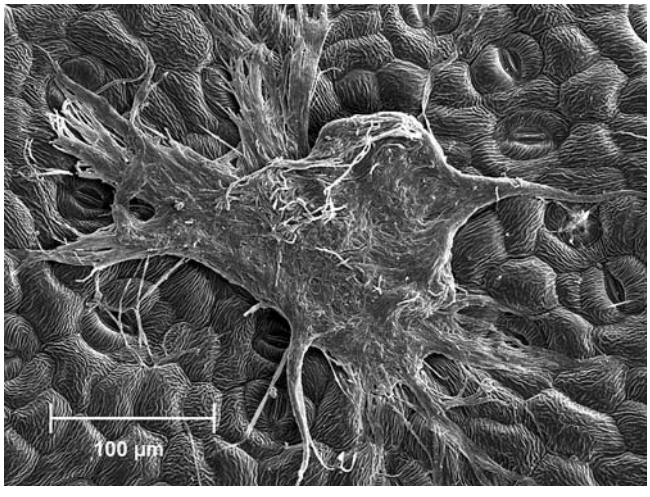


Fig. 2 SEM picture of a fungal colony on the upper leaf surface of an *I. asarifolia* plant. *Bar* = 100 µm

While all fungi IasaF01 to IasaF12 were endophytic as judged from the isolation procedure (Petrini et al. 1992), microscopic inspection of the leaf surface had previously shown that an epibiotic fungus is associated with the leaf surface (Fig. 2). This fungus occurs notably on the surface of young leaves which are not yet unfolded. The fungus which apparently is epibiotic can be scraped off from the leaf surface with a spatula and was designated IasaF13. Attempts to grow this fungus on 15 different agar media designed for fungal growth were unsuccessful, however, even when the media contained leaf homogenates of the host plant.

Seeds were also investigated for the presence of fungi because seeds of Convolvulaceae are known to contain alkaloids (Gröger and Floss 1998). No fungus, however, grew within 8 weeks at 20°C from surface-sterilized seeds which were crushed after sterilization and put onto an agar medium (Bacon 1985; Schulz et al. 1993) allowing for fungal growth. This, however, does not mean that no fungus is present in the seeds (*vide infra*).

After 18S rDNA gene amplification of strains IasaF01 to IasaF13 a phylogenetic tree was constructed (Fig. 3a) in which all fungi isolated from *I. asarifolia* are represented. In addition, sequencing data obtained from authentic clavicipitaceous fungi (*Balansia cyperi*, *Balansia obtecta*, *Atkinsonella hypoxylon*, *Claviceps purpurea*, *Neotyphodium coenophialum*, *Epichloë festucae*) were also entered. The phylogenetic tree (Fig. 3a) shows that our non-culturable strain (IasaF13) occurring on the leaf surface (Fig. 2) is related to representatives of the Clavicipitaceae family. It was desirable to confirm this observation. We therefore analysed the ITS which provide a better phylogenetic fine resolution for closely related taxa. These data are shown in Fig. 3b. In this case, clustering of our strain IasaF13 with authentic clavicipitaceous fungi was seen.

These experiments indicated that there were two possible candidates responsible for the presence of ergoline alkaloids in the *I. asarifolia* plant, *P. roquefortii* (IasaF09) and the non-culturable epibiotic clavicipitaceous fungus IasaF13.

P. roquefortii is a fungus known to produce roquefortine, a toxic diketopiperazine with an indole moiety and ergoline alkaloids such as isofumigaclavine A (Scott et al. 1976). In vitro culture of isolate IasaF09 (*P. roquefortii*) and analysis of its media and mycelium confirmed the presence of roquefortine and additional van Urk positive compounds.

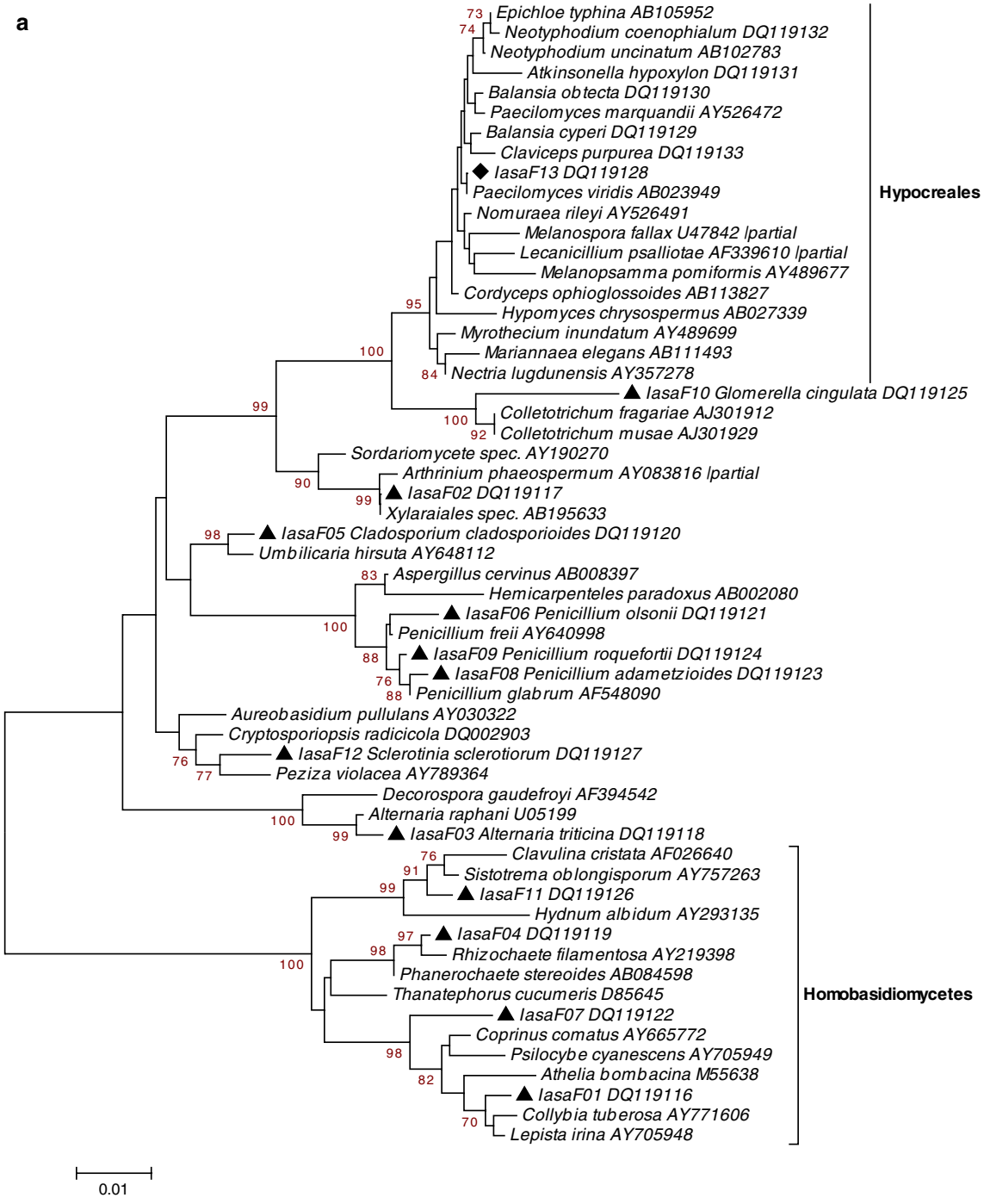
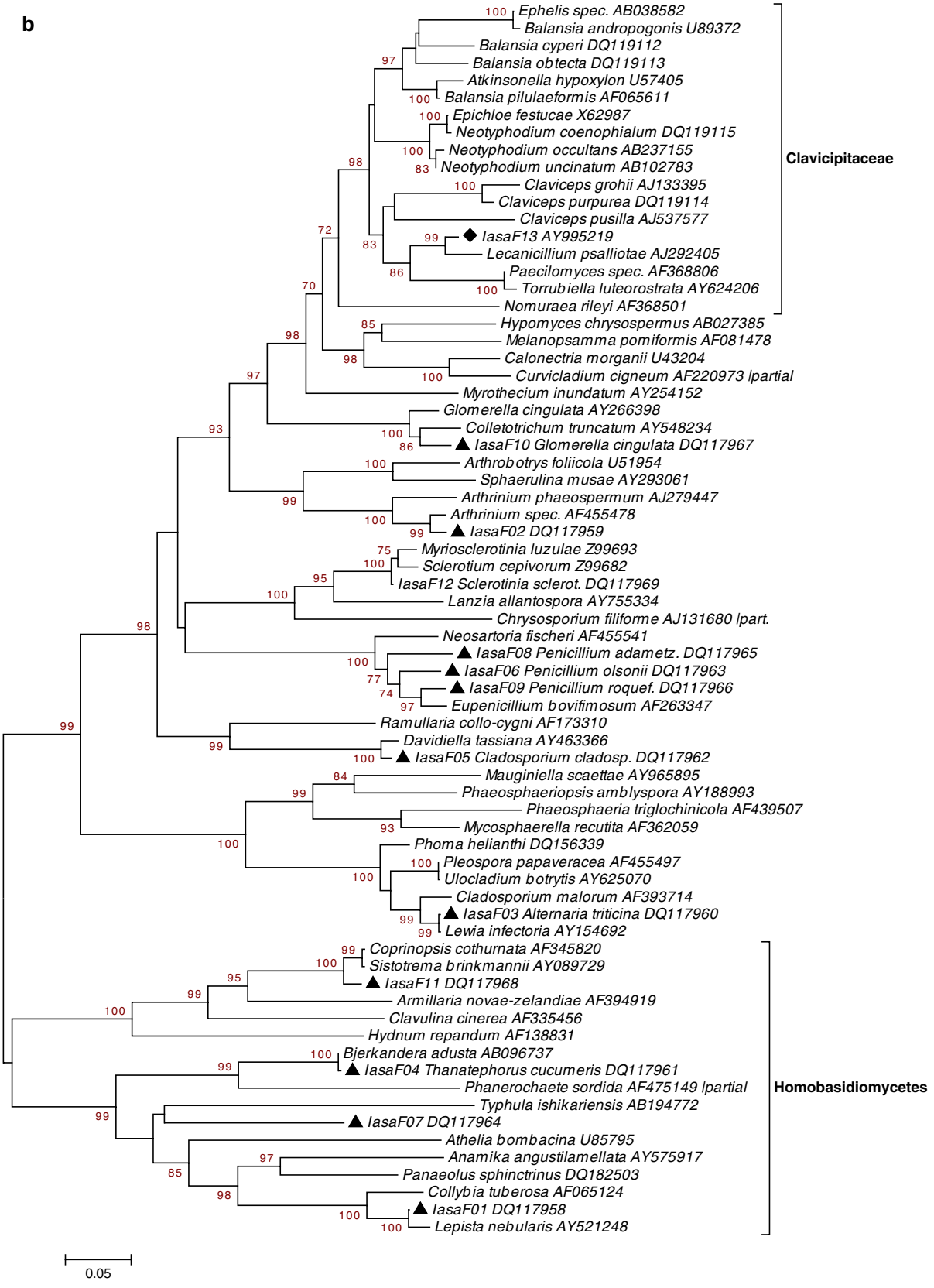


Fig. 3 Neighbour joining phylogeny derived from the 18S (**a**) and ITS (**b**) nucleotide data sets and the dmaW protein sequences (**c**) Bootstrap support for nodes is indicated only where at least 70%. Accessions starting with DQ11 or DQ12 are new sequences determined in the course of this study, others were selected from the public databases and are denoted as partial when not available for the full alignment length (**a**, **c**) or when the ITS sequences do not extend into the 18S or 28S rRNA (**b**), respectively. The fungal isolates IasaF01–F12 are highlighted with a *triangle*, IasaF13 with a *diamond*. Sequences of the new fungal isolates IasaF01–IasaF13 are supplemented with a species name when a respective identical ITS sequence was identified in the database. The respective clades of homobasidiomycete sequences (Hymenomycetes, Basidiomycota) were used to root the tree topologies for the remaining fungal sequences in (**a**) and (**b**). The distantly related sirD proteins encoding tyrosyl dimethlyallyl transferases were used to root the phylogeny of dmaW homologues (**c**). Several protein sequence entries with similarity to dmaW proteins are predictions from genomic sequences and may be subject to refinement upon cDNA analyses in the future

The isolated fungi IasaF09 (*P. roquefortii*) and IasaF13 were also characterized by PCR-SSCP as shown in Fig. 4. Comparison of the DNA of the isolated fungi (IasaF09 and IasaF13) with that of the total DNA obtained from the intact plant shows (Fig. 4) that both fungi (IasaF09 and IasaF13) are present but that fungus


b
Ephelis spec. AB038582
Balansia andropogonis U89372
Balansia cyperi DQ119112
Balansia obtecta DQ119113
Atkinsonella hypoxylon U57405
Balansia pilulaeformis AF065611
Epichloe festucae X62987
Neotyphodium coenophialum DQ119115
Neotyphodium occultans AB237155
Neotyphodium uncinatum AB102783
Claviceps grohii AJ133395
Claviceps purpurea DQ119114
Claviceps pusilla AJ537577
IasaF13 AY995219
Lecanicillium psalliotae AJ292405
Paecilomyces spec. AF368806
Torrubiella luteorostrata AY624206
Nomuraea rileyi AF368501
Clavicipitaceae
Hypomyces chrysospermus AB027385
Melanopsamma pomiformis AF081478
Calonectria morganii U43204
Curvicladium cigneum AF220973 |partial
Myrothecium inundatum AY254152
Glomerella cingulata AY266398
Colletotrichum truncatum AY548234
IasaF10 Glomerella cingulata DQ117967
Arthrobotrys foliicola U51954
Sphaerulina musae AY293061
Arthrinium phaeospermum AJ279447
Arthrinium spec. AF455478
IasaF02 DQ117959
Myriosclerotinia luzulae Z99693
Sclerotium cepivorum Z99682
IasaF12 Sclerotinia sclerot. DQ117969
Lanzia allantospora AY755334
Chrysosporium filiforme AJ131680 |part.
Neosartoria fischeri AF455541
IasaF08 Penicillium adametz. DQ117965
IasaF06 Penicillium olsonii DQ117963
IasaF09 Penicillium roquef. DQ117966
Eupenicillium bovifimosum AF263347
Ramullaria collo-cygni AF173310
Davidiella tassiana AY463366
IasaF05 Cladosporium cladosp. DQ117962
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Phaeosphaeria triglochinicola AF439507
Mycosphaerella recutita AF362059
Phoma helianthi DQ156339
Pleospora papaveracea AF455497
Ulocladium botrytis AY625070
Cladosporium malorum AF393714
IasaF03 Alternaria triticina DQ117960
Lewia infectoria AY154692
Coprinopsis cothurnata AF345820
Sistotrema brinkmannii AY089729
IasaF11 DQ117968
Armillaria novae-zelandiae AF394919
Clavulina cinerea AF335456
Hydnum repandum AF138831
Bjerkandera adusta AB096737
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Athelia bombacina U85795
Anamika angustilamellata AY575917
Panaeolus sphinctrinus DQ182503
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Lepista nebularis AY521248
Homobasidiomycetes
0.05

Fig. 3 (Contd.)

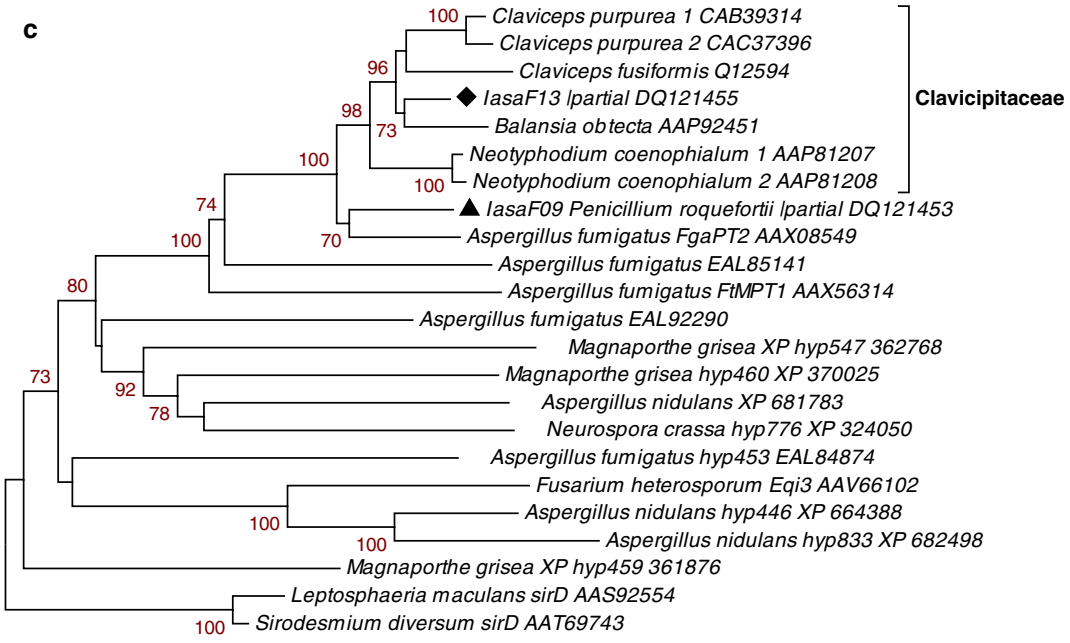

Fig. 3 (Contd.)

IasaF13 clearly predominates among all the plant-associated fungi suggesting that it is identical to the abundantly visible fungus attached to the upper leaf surface (Fig. 2). In contrast, an *Ipomoea* plant devoid of both fungi and alkaloids does not show the pattern of bands observed for both isolated fungi IasaF09 and IasaF13 (Fig. 4).

If one of the isolated fungi is a producer of ergoline alkaloids in the *Ipomoea* plant, it should contain genes responsible for ergoline alkaloid biosynthesis. Oligonucleotides (deg1 and deg4) targeted to conserved regions of the dmaW (or cpd) dimethylallyl-tryptophan-synthase gene (Wang et al. 2004), known to be involved in the introduction of a dimethylallyl residue into tryptophan (Fig. 1) were synthesized and employed in a PCR reaction with DNA from the epibiotic fungus IasaF13 as a template. A PCR product of 939 bp was obtained and its deduced protein sequence showed very high similarity with the available dmaW sequences of Clavicipitaceaen fungi, being most similar to a *Balansia obtecta* homologue with 76% identical amino acids (Fig. 3c). The clavicipitaceous fungi live on grasses and are known producers of ergoline alkaloids. The same experiment was carried out with DNA isolated from IasaF09 (P. *roquefortii*) revealing a dmaW homologue with 63% sequence identity to an *Aspergillus fumigatus* sequence. Hence, a dimethylallyl-tryptophan-synthase appears also to be present in IasaF09 (P. *roquefortii*), however, the corresponding gene in the epibiotic fungus IasaF13 is expectedly much more closely related to the genes detected in Clavicipitaceae. A phylogenetic analysis of the Cpd1 protein sequences, also including several predicted protein sequences form different fungi, indicates several distant homologues in *Aspergillus* and more recent, independent gene duplications in the *Clavicipitaceae*, at least in *Claviceps purpurea* and *Neotyphodium coenophialum* (Fig. 3c).

Subsequently both candidate fungi were used to inoculate *I. asarifolia* plants free of alkaloids and fungi. The fungus IasaF09 (*P. roquefortii*) was grown on a defined solid medium, a mixture of hyphae and spores was suspended in water following a method used by Latch and Christensen (1985) and the suspension injected into leaves with a syringe and in addition spread onto leaves of the *I. asarifolia* plant. The inoculated plants were kept in the greenhouse. Microscopic examination of the plants 6, 18 and 26 weeks after inoculation showed that the fungus was well established on the plant. Analysis of the plant 26 weeks after inoculation did not show the presence of roquefortine or of any ergoline alkaloid. Thus, *P. roquefortii* appears not to be the candidate fungus responsible for the accumulation of ergoline alkaloids in *I. asarifolia*.

The same experiment was carried out with hyphae of IasaF13 isolated from the unfolded leaves of an *I. asarifolia* plant and spread onto and injected into *I. asarifolia* leaves of a plant devoid of alkaloids and fungi. As opposed to the experiment with *P. roquefortii*, however, no fungal growth was observed. This observation was not unexpected (see later).

The inability to establish the epibiotic fungus IasaF13 on *I. asarifolia* was also experienced in the so-called "attachment experiment", in which a normal plant and a plant devoid of fungi and alkaloids were kept in close contact in a cylindrical plastic glass container in the green house with the upper leaf surfaces of both plants attached to each other. After 18 weeks no spread of fungal hyphae to the plant devoid of IasaF13 was observed. In addition, this plant did not contain any alkaloids.

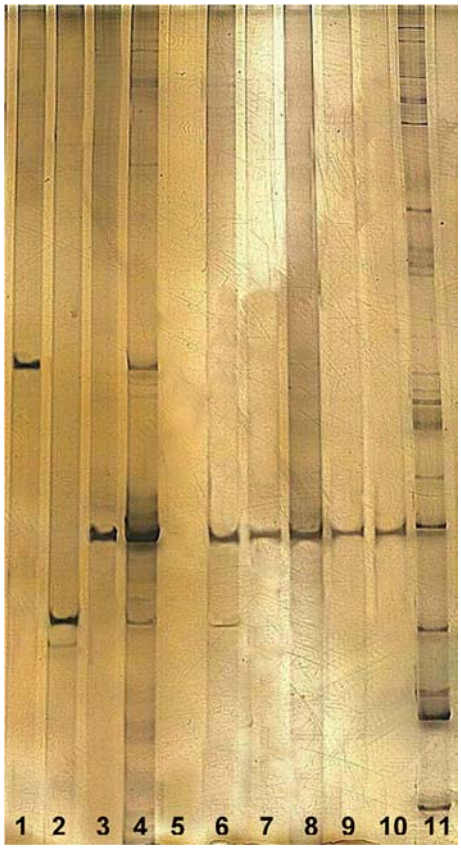

Fig. 4 Single strand conformation polymorphism (SSCP) of fungal DNA from IasaF02 (*lane 1*), IasaF09, i.e. *P. roquefortii* (*lane 2*), IasaF13 (*lane 3*), total DNA extracted from leaves of an alkaloid-containing *I. asarifolia* plant (*lane 4*), total DNA extracted from an *I. asarifolia* plant without alkaloids and without IasaF13 (*lane 5*), total DNA extracted from seeds of *I. asarifolia* (*lane 6*), total DNA extracted from a plant grown from surface-sterilized seeds under germfree conditions (*lane 7*), total DNA extracted from an *I. asarifolia* plant regenerated from a callus culture under sterile conditions (*lane 8*), total DNA extracted from an *I. asarifolia* callus culture (*lane 9*), and total DNA extracted from an *I. asarifolia* cell suspension culture (*lane 10*). Molecular weight marker (*lane 11*)

Spread of fungal hyphae and presence of ergoline alkaloids, however, were observed when externally sterilized seeds were germinated in a sterile environment on an artificial agar medium in a closed Erlenmeyer flask. Plants grown under these conditions contained both fungus IasaF13 and alkaloids indicating that *I. asarifolia* seeds harbour fungal propagules of IasaF13 that were spread to the growing plant. Indeed, SSCP (Fig. 4) and microscopic investigation of seeds showed that seeds clearly contain the fungus IasaF13. Evidently, this fungus is spread to the shoot of the plant during growth (Fig. 4). Moreover, the plantlets contained the full spectrum of alkaloids (TLC, HPLC-MS) known to be present in the untreated intact plant.

In a similar experiment, a surface-sterilized piece of a stem was placed on an agar medium. After 2–4 weeks a callus was formed. Stem and callus were then transferred into a liquid medium. Shoots regenerated from the plant material. The shoots of these plantlets were cut-off and the cuttings placed into solid agar medium. Roots developed within 2 weeks . Microscopic inspection, SSCP and chemical analysis showed that both fungus IasaF13 and the complete spectrum of alkaloids were again present. We conclude that both seeds and plant cell cultures of the *I. asarifolia* plant contain the clavicipitaceous fungus and that this fungus is involved in the accumulation of alkaloids in *I. asarifolia*.

Absence and presence of fungus IasaF13 was also investigated microscopically, by SSCP and also by sequencing of DNA after PCR amplification using oligomers (ITS1F and ITS4) (White et al. 1990; Gardes and Bruns 1993) targeted to the ITS region. These experiments fully confirmed the presence of IasaF13 in the intact control plant, seeds, plants grown under sterile conditions from seeds, regenerated plants, plant callus and cell suspension cultures. The ITS sequencing was repeated 35 times (cf. Materials and methods). Each time the sequence of IasaF13 but never that of IasaF09 (*P. roquefortii*) was found. The fungal ITS sequence again was not found in plants which are devoid of alkaloids (see Fig. 4). Whenever ITS and SSCP (Fig. 4) were positive with respect to the presence of fungus IasaF13 microscopic inspection of the plant material confirmed the presence of this fungus, including the plant cell culture material (Fig. 5) and in spite of the fact that plant cell cultures are considered to be sterile.

An epibiotic fungus was also detected on another plant species, *Turbina corymbosa* (L.) Hall. This plant also belongs to the family Convolvulaceae and contains ergoline alkaloids. *T. corymbosa* and *I. asarifolia* are indigenous to Central or South America, respectively. The epibiotic fungus from *T. corymbosa* was submitted to 18S rDNA (DQ127 278) and ITS analysis (AY995219). It turned out that the sequences were 100% identical when compared with those of the epibiotic fungus IasaF13 on *I. asarifolia*. This result was obtained independently in the laboratories of U.S. and E.L.

The alkaloid spectrum of aerial parts of both plant species was investigated quantitatively and qualitatively

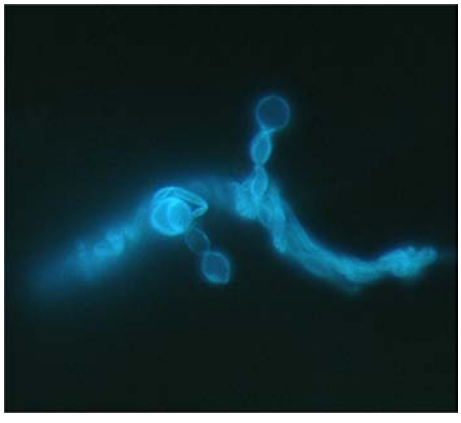

Fig. 5 Fungal cells present in an *I. asarifolia* callus culture visualized after staining with calcofluor (magnification 1000×)

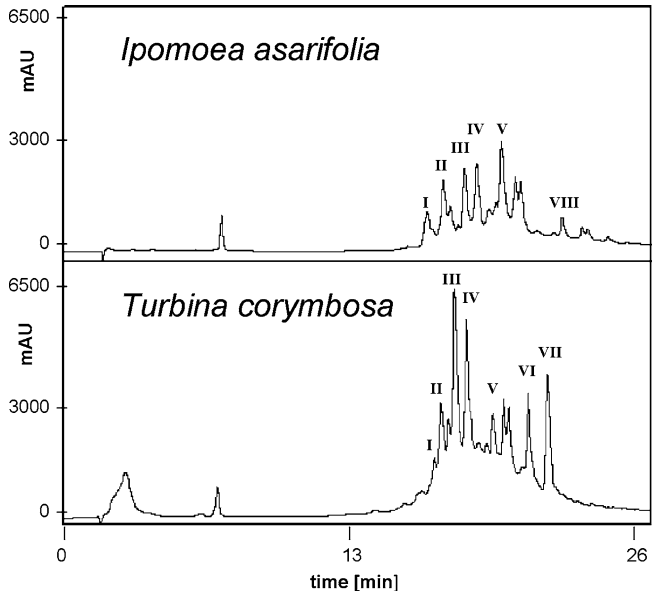

Fig. 6 HPLC traces of the alkaloid fractions from *T. corymbosa* and *I.asarifolia*. Alkaloids: I chanoclavine, II lysergic acid amide, III lysergic acid α-hydroxyethylamide, IV isolysergic acid α-hydroxyethylamide, V isolysergic acid amide, VI elymoclavine, VII agroclavine, VIII ergonovine. The HPLC trace does not reveal the presence of VIII in *T. corymbosa*. The mass spectrum, however, is more sensitive and shows the presence of this alkaloid beyond doubt. Alkaloids were identified by comparison of their UV spectrum (diode array detection between 275 and 315 nm) and mass spectrum with authentic samples. Ergobalansine, which is a constituent of *I. asarifolia* (Jenett-Siems et al. 1994, 2004) remained unidentified due to a lack of authentic material

using a high pressure liquid chromatograph connected to a mass spectrometer (Fig. 6). The compounds were identified by comparison with authentic standards. Both plants contain chanoclavine, lysergic acid α-hydroxyethyl amide (including its isoform), lysergic acid amide (including its isoform) and ergonovine. In addition, elymoclavine and agroclavine are present in *T. corymbosa* but were not detectable in *I. asarifolia* (Fig. 6). The total amount of alkaloids in the *T. corymbosa* plant amounted to roughly twice as much as found in the *I. asarifolia* plant (Fig. 6). The latter contained 7.0 µg alkaloids expressed as ergonovine per gram fresh weight (Kucht et al. 2004) whereas the former contained 14.6 µg alkaloids per gram fresh weight expressed as ergonovine.

Discussion

Twelve culturable fungi and one unculturable fungus were isolated from the *I. asarifolia* plant. Phylogenetic analysis of these organisms resulted in essentially congruent observations for the 18S rDNA and the ITS data set with respect to confidently identified nodes (Fig. 3a, b). In three cases, sequences from the new isolates have identical counterparts in the databases both for 18 SrDNA and for ITS, respectively: IasaF05 (*Cladosporium cladosporioides*) Iasa F10 (*Glomerella cingulata*, anamorph: *Colletotrichum gloeosporioides*) and IasaF12 (*Sclerotinia sclerotiorum*). Isolates IasaF01, IasaF04, IasaF07 and IasaF11 clearly fall into the Homobasidiomycetes, and are related to available Agaricales (*Collybia*, *Lepista*) or Aphyllophorales (*Athelia*, *Phanerochaete*, *Sistotrema*) sequence entries, respectively. Generally, the ITS data set (Fig. 3b) provides better phylogenetic fine resolution for closely related taxa. The ITS sequence of isolate IasaF04 is identical to the corresponding sequence of *Thanatephorus cucumeris* (anamorph: *Rhizoctonia solani*). Sequences from isolates IasaF06, IsaF08 and IasaF09 are identical to those of different *Penicillium* species. Isolate IasaF13 is clearly identified as a member of the family Clavicipitaceae (Hypocreales). Other genera and those of the sister families in the Hypocreales, the Bionectriaceae (*Myrothecium*), Ceratostomataceae (*Melanospora*), Hypocreaceae (*Hypomyces*), Nectriaceae (*Calonectria*) and Niessliaceae (*Melanopsamma*) branch more distantly.

Thus, both trees distinguish between fungi belonging to the family of Clavicipitaceae and those which do not. In both phylogenetic trees IasaF13 groups together with ergoline alkaloid-producing clavicipitaceous fungi. This is an important observation because it strongly suggests that the nonculturable epibiotic fungus IasaF13 (Fig. 2) is responsible for the production of ergoline alkaloids as is the case for clavicipitaceous fungi occurring on plants belonging to grasses. Epibiotic clavicipitaceous fungi are also found within the genus *Balansia* (Reddy et al. 1998), relatives of our epibiotic strain IasaF13 (Fig. 3b).

As expected, the fungus IasaF13 has a gene with significant similarity to the gene encoding a protein responsible for catalysing the synthesis of 4-(γ, γ-dimethylallyl)tryptophan (Tsai et al. 1995; Tudzynski et al. 1999, 2001; Unsöld and Li 2005) a precursor of ergoline alkaloids. This gene is present in *C. purpurea* (Tudzynski et al. 1999, 2001), *C. fusiformis* (Wang et al. 2004), *Neotyphodium* sp. isolate Lp1 (Wang et al. 2004), and *Aspergillus fumigatus* (Unsöld and Li 2005) and is known to be responsible for the first committed step in ergoline alkaloid biosynthesis.

At present we cannot fully exclude the possibility that *P. roquefortii* contributes to the spectrum of ergoline alkaloids in *I. asarifolia* but we did not find any evidence that supports this view:

1. Alkaloids occurring in *I. asarifolia* are chanoclavine-I, elymoclavine, lysergic acid amide, isolysergic acid amide (Kucht et al. 2004) as well as ergobalansine and ergobalansinine (Jenett-Siems et al. 1994). Isofumigaclavine A, the ergoline alkaloid present in *P. roquefortii* (Scott et al. 1976), has so far not been reported to be a constituent of *I. asarifolia*.
2. Inoculation of *I. asarifolia* plants with *P. roquefortii* showed that the fungus was well established on the plant which, however, was devoid of ergoline alkaloids and roquefortine.

3. Growth of *I. asarifolia* plants (either from seeds or after regeneration) in a sterile environment gave a full spectrum of alkaloids.
4. These plants as well as the plant cell cultures contained the ITS sequence of the clavicipitaceous fungal isolate IasaF13 alone: Among 36 (see Materials and methods) cloned ITS sequences none was identical to that of IasaF09 (*P. roquefortii*) but all were identical to those of IasaF13.
5. Throughout these experiments, it was found that the fungus IasaF13 was always detected when alkaloids were present: Thus, treatment of the plant with systemic fungicides "Folicur" and "Pronto Plus" gave plants devoid of both fungus and alkaloids. Treatment of the plants with fungicides "Benomyl" and "Switch" neither removed the fungus IasaF13 nor the alkaloids (Kucht et al. 2004).

The fungus *P. roquefortii* may be a non-specific associate of *I. asarifolia*. It has been isolated from various substrates such as soil samples (Ohomo et al. 1975) and from livestock feed (Boysen 1999). The fungus grows even on cheese (Carlile et al. 2001). It is conceivable that *P. roquefortii* is horizontally transmitted as is often the case among endophytes (Arnold et al. 2003).

The fact that the clavicipitaceous fungus IasaF13 is non-culturable in defined media shows that it heavily depends on the plant for growth and vegetative reproduction and that there is a highly specific interaction between both organisms. It is remarkable that the fungus never spread to *I. asarifolia* plants devoid of fungus and alkaloids although the plant carrying the fungus was kept in the immediate neighbourhood in the same green house. This is in line with the result of the "attachment-experiment" in which no inoculation of the plant devoid of fungi was observed (*vide supra*) although both plants were kept in close contact in an environment of high humidity. Our observations are in agreement with results from experiments on clavicipitaceous fungi colonizing grasses: Infection of host plants with asexual propagules (conidia and mycelia) is very difficult and unlikely to occur in nature (Gentile et al. 2005). Thus, the fungus IasaF13 behaves in a similar way experienced for clavicipitaceous fungi living on Poaceae plants, including the fact that they are often seed transmitted (Clay and Schardl 2002).

Plant callus and cell suspension cultures are believed to be sterile. This, however, was not experienced with the *I. asarifolia* cell culture derived from surface-sterilized stems. Microscopic inspection (Fig. 5), SSCP analysis and ITS sequencing of DNA obtained from a callus and a cell suspension culture of *I. asarifolia* showed that the fungus was also present in cell cultures. Fungal hyphae typically consisting of up to 20 compartments which stained with calcofluor were clearly and microscopically visible (Fig. 5). Thus, during establishment of the cell culture, the process of surface sterilization of a stem segment of the plant does not remove the fungus IasaF13 which even in cell cultures is able to live in association with the plant cells (cf. Figs. 4, 5). The presence of fungal hyphae in the cultured cells is not visible to the naked eye and plant cells seem to grow unaffected by the fungus. This may indicate that plant cells and the fungus keep each other in check during a balanced growth.

Intensive studies using different culture media, however, did not give any indication that undifferentiated cultured plant cells contained any trace of ergoline alkaloids (Kucht et al. 2004). Alkaloids and fungal colonies (Fig. 2) appeared only during the regeneration process (Fig. 4). This shows that for the successful production of ergoline alkaloids the fungus IasaF13 and a morphologically differentiated *I. asarifolia* plant are essential.

We observed that a fungus like IasaF13 is not only present on *I. asarifolia* but also on *T. corymbosa*. Again, the epibiotic fungus can be removed by fungicide treatment. Loss of the fungus from the plant again (Kucht et al. 2004) occurs concomitantly with elimination of ergoline alkaloids (data not shown). The fungus on *T. corymbosa* is identical to the epibiotic fungus IasaF13 as far as ITS and 18S rDNA sequences are concerned. The alkaloid spectrum of both plants, however, differs qualitatively (Fig. 6) and quantitatively (Fig. 6 and Results). This is in agreement with data obtained from experiments with *Neotyphodium lolii*, an endophyte of the grass *Lolium perenne*: Although it is clearly the fungus that is responsible for the synthesis of alkaloids, accumulation of alkaloids is affected and modulated by the plant genotype (Lane et al. 2000; Spiering et al. 2005).

We conclude that the presence of alkaloids in the family Convolvulaceae very likely is not due to a horizontal gene transfer which occurred during evolution, or a repeated "invention" of the same biosynthetic pathway in two different taxa Ascomycota and Convolvulaceae, or due to an ancestral trait that was eliminated during evolution in most taxa except a few but rather that clavicipitaceous fungi not only colonize monocotelydonous plants such as Poaceae but also dicotyledonous plants belonging to the family Convolvulaceae.

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