

# Biosynthesis and Accumulation of Ergoline Alkaloids in a Mutualistic Association between *Ipomoea asarifolia* (Convolvulaceae) and a Clavicipitalean Fungus<sup>1</sup>

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Ergoline alkaloids occur in taxonomically unrelated taxa, such as fungi, belonging to the phylum Ascomycetes and higher plants of the family Convolvulaceae. The disjointed occurrence can be explained by the observation that plant-associated epibiotic clavicipitalean fungi capable of synthesizing ergoline alkaloids colonize the adaxial leaf surface of certain Convolvulaceae plant species. The fungi are seed transmitted. Their capacity to synthesize ergoline alkaloids depends on the presence of an intact differentiated host plant (e.g. *Ipomoea asarifolia* or *Turbina corymbosa* [Convolvulaceae]). Here, we present independent proof that these fungi are equipped with genetic material responsible for ergoline alkaloid biosynthesis. The gene (*dmaW*) for the determinant step in ergoline alkaloid biosynthesis was shown to be part of a cluster involved in ergoline alkaloid formation. The *dmaW* gene was overexpressed in *Saccharomyces cerevisiae*, the encoded DmaW protein purified to homogeneity, and characterized. Neither the gene nor the biosynthetic capacity, however, was detectable in the intact *I. asarifolia* or the taxonomically related *T. corymbosa* host plants. Both plants, however, contained the ergoline alkaloids almost exclusively, whereas alkaloids are not detectable in the associated epibiotic fungi. This indicates that a transport system may exist translocating the alkaloids from the epibiotic fungus into the plant. The association between the fungus and the plant very likely is a symbiotum in which ergoline alkaloids play an essential role.

Ergoline (syn. ergot-) alkaloids are 3,4-substituted indole derivatives that exhibit remarkable and very diverse physiological activities. They are active ingredients in medications designed to treat migraine or Parkinson's disease or are used in childbirth and weaning (Groeger and Floss, 1998; Mutschler et al., 2001; Schardl et al., 2006). Ergoline alkaloids are also used as hallucinogens by native Central American people in religious ceremonial practices (Hofmann, 1961).

One of the more frequently investigated features of ergoline alkaloids is their ecological role as natural products synthesized by fungal endophytes or epibionts living in or on grasses. The alkaloids confer environmental tolerance, fitness, drought resistance, insecticidal activity, and feed deterrence to their monocotyledonous plants. Books (Rehacek and Sajdl, 1990; White et al., 2003; Hofmann, 2006) and many reviews (Bush et al., 1997; Groeger and Floss, 1998; Malinowski and Belesky, 2000; Tudzynski et al., 2001; Clay and

Schardl, 2002; Keller and Tudzynski, 2002; Schardl and Craven, 2003; Clay, 2004; Moon et al., 2004; Schardl et al., 2004, 2006; Tudzynski and Scheffer, 2004; Bacon and Lyons, 2005; Bischoff and White, 2005; Schardl and Leuchtmann, 2005) testify to the role of ergoline alkaloids in ecological, agronomical, genetic, biochemical, pharmacological, and anthropological research.

Another remarkable feature of ergoline alkaloids is their disjointed distribution in nature: They occur in ascomycetes belonging to the genera *Claviceps*, *Aspergillus*, and *Penicillium*, in the dicotyledonous plant families Convolvulaceae and Polygalaceae (Groeger and Floss, 1998), and were even isolated from an animal, a tunicate representing a *Eudistoma* species (Makarieva et al., 1999). The occurrence of ergoline alkaloids in taxonomically unrelated taxa was assumed to be due to a horizontal transfer of genes encoding the ergoline alkaloid biosynthetic pathway (Groeger and Floss, 1998; Tudzynski et al., 2001; Clay and Schardl, 2002; Fig. 1).

The disjointed distribution of natural products has not only been observed in the case of ergoline alkaloids, but also with other natural products, such as maytansinoids, which are bacterial products (Higashide et al., 1977), but occur also in plants belonging to the family Celastraceae (Kupchan et al., 1977). The distribution of maytansinoids in nature suggests that they are products of microorganisms (bacterium or fungus) that are

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associated with Celastraceae plants (Pullen et al., 2003; Cassidy et al., 2004). Indeed, plant-associated microorganisms are frequent producers of natural products (Gunatilaka, 2006).

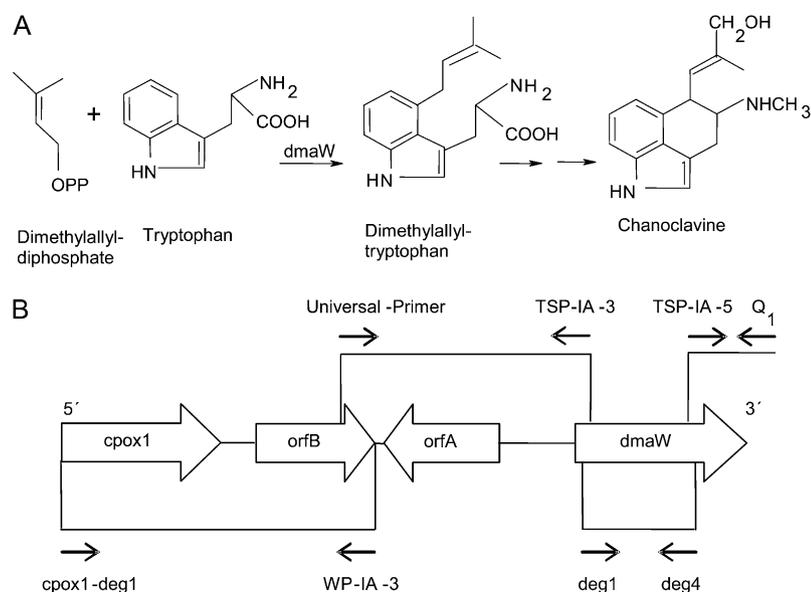
It is in line with these observations that *Ipomoea asarifolia* and related plant species belonging to the family Convolvulaceae are associated with a clavicipitalean fungus and contain ergoline alkaloids (Kucht et al., 2004; Steiner et al., 2006; Ahimsa-Mueller et al., 2007). Treatments of *I. asarifolia* (white blooming variety) and *Turbina corymbosa* (syn. *Rivea corymbosa*) with fungicides leads to the removal of epibiotic fungi and concomitant loss of ergoline alkaloids from the plants (Kucht et al., 2004). Sequencing of the nuclear ribosomal 18S rDNA and internal transcribed spacer showed that these fungi are members of the family Clavicipitaceae (Steiner et al., 2006; Ahimsa-Mueller et al., 2007). A taxonomically closely related fungus was also detected in ergoline alkaloid containing *Ipomoea violacea* plants (Ahimsa-Mueller et al., 2007). The fungi are seed transmitted and hitherto noncultivable in vitro. A fungus is also present in *I. asarifolia* callus and cell suspension cultures, which, however, contain no ergoline alkaloids. A plant regenerated from such a callus culture, however, does contain ergoline alkaloids and is colonized by the fungus (Steiner et al., 2006). The biosynthesis of ergoline alkaloids apparently depends on the presence of an intact host plant.

These observations indicate that the disjointed occurrence of ergoline alkaloids in nature is not due to a horizontal gene transfer that might have happened during evolution, but it has been shown (Kucht et al., 2004; Steiner et al., 2006; Ahimsa-Mueller et al., 2007) that ergoline alkaloid-producing clavicipitalean fungi do not only colonize monocotyledonous plants like grasses (compare books and reviews listed above), but also dicotyledonous plants such as Convolvulaceae.

Early tracer experiments demonstrated that ergoline alkaloids are derived from three primary precursors. These are L-Trp,  $\gamma,\gamma$ -dimethylallyl diphosphate, and the methyl group of Met. Extensive studies by various groups with putative precursors led to the reaction sequence (Fig. 1A), which starts with prenylation in the 4-position of Trp and conversion of the resulting dimethylallyl-Trp via its *N*-methyl derivative, chanoclavine, chanoclavine-1-aldehyde, agroclavine, elymoclavine, and paspalic acid to lysergic acid and its derivatives (Groeger and Floss, 1998; Fig. 1A). The determinant step in ergoline alkaloid biosynthesis is the prenylation of Trp, which is under control of phosphate and Trp (Robbers et al., 1972; Krupinski et al., 1976).

The enzyme (DmaW or DMATS; i.e. 4-[ $\gamma,\gamma$ -dimethylallyl]Trp synthase) was first detected by Heinsteins et al. (1971), purified to apparent homogeneity by Lee et al. (1976) and Gebler and Poulter (1992), and crystallized by Cress et al. (1981). The gene (dmaW) encoding this enzyme was cloned from *Claviceps purpurea* and *Claviceps fusiformis* (Tsai et al., 1995; Tudzynski et al., 1999), *Neotyphodium* Lp1 (Wang et al., 2004), *Neotyphodium lolii* (Fleetwood et al., 2007), and *Aspergillus fumigatus* (Coyle and Panaccione, 2005; Unsoeld and Li, 2005). The sequence information obtained was used to analyze the adjacent DNA, revealing that dmaW is part of a gene cluster (Tudzynski et al., 2001). This cluster has been extended by Haarmann et al. (2005). Assignment of individual genes in this cluster is under way (Haarmann et al., 2006; Fleetwood et al., 2007).

PCR experiments revealed that a dmaW gene is also present in clavicipitalean fungi associated with the *I. asarifolia* plant (Steiner et al., 2006) and taxonomically related Convolvulaceae (Ahimsa-Mueller et al., 2007). We here provide independent proof that this is actually the case and that the respective gene (dmaW) encodes a physiologically active 4-( $\gamma,\gamma$ -dimethylallyl)Trp syn-



**Figure 1.** Role of the 4-( $\gamma,\gamma$ -dimethylallyl)Trp synthase gene (dmaW) in the biosynthesis of ergoline alkaloids (e.g. chanoclavine; A); arrangement of genes (orfA, orfB, cpox-1) associated with dmaW in the fungus IAsaF13, an epibiont living on the *I. asarifolia* plant (B). Positions of oligonucleotides employed for chromosome walking are indicated.

thase (DMATS, DmaW). Thus, the synthesis of ergoline alkaloids takes place in the fungus, whereas the biosynthetic capacity is not detectable in the plant. By contrast, ergoline alkaloids accumulate in the plant material and, if at all, only traces are detectable in the epibiotic fungus, suggesting that the mutualistic system investigated here very likely is a symbiotum (vide infra).

## RESULTS

### Presence of Ergoline Alkaloids in Leaves and Nondetectability in Mycelium

Individual ergoline alkaloids present in *I. asarifolia* and *T. corymbosa* plants associated with fungal mycelium were identified by a HPLC system connected to a mass spectrometer as described previously (Steiner et al., 2006). The following alkaloids were detected in both *I. asarifolia* and *T. corymbosa*: chanoclavine (Fig. 1A), lysergic acid- $\alpha$ -hydroxyethylamide and its isomer, lysergic acid amide and its isomer, and ergonovine. In addition *T. corymbosa* contains agroclavine and elymoclavine.

The fungi, which are provisionally named IasaF13 (present on *I. asarifolia*) and TcorF01 (present on *T. corymbosa*), are visible to the naked eye on the adaxial leaf surface (Ahimsa-Mueller et al., 2007). Attempts to locate the fungi within the plant tissue by microscopic techniques were not successful. A similar case was reported for *Ipomoea batatas* colonized by *Fusarium lateritium* (Clark, 1994).

From young leaves of *I. asarifolia* and *T. corymbosa*, respectively, the fungal mycelium was removed by two different techniques as outlined in "Materials and Methods." Ultrasonic treatment of leaves separated the mycelium almost completely from the leaf surface as shown by microscopic inspection (Fig. 2). Alternatively, the mycelium was collected from the leaf with the aid of a spatula. Isolation of the basic fraction from both the untreated and treated leaf material, as well as the harvested fungal mycelium, revealed that the alkaloids were almost completely recovered from the leaves, but that the epibiotic mycelium was devoid of alkaloids (Table I). Residual amounts of alkaloids detected in the mycelium after collecting the fungus from the leaves with a spatula may have been due to the mechanical impact of the spatula on the leaf surface (Table I). The alkaloids were identified by thin-layer chromatography (TLC) and by HPLC-mass spectrometry (MS; Steiner et al., 2006).

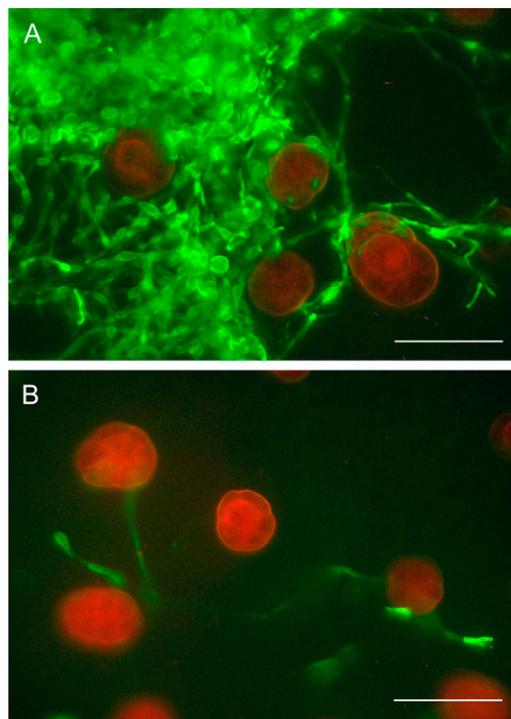
### Presence of Genes Responsible for Ergoline Alkaloid Biosynthesis in the Epibiotic Mycelium and Nondetectability of These Genes in the Leaves

Because the alkaloids are present in the leaves, the plant material was investigated for the presence of the 4-( $\gamma,\gamma$ -dimethylallyl)Trp synthase gene (dmaW) encoding the determinant step in ergoline alkaloid bio-

synthesis (vide supra; Fig. 1, A and B). Because the dmaW gene was found to be part of a cluster responsible for ergoline alkaloid biosynthesis in clavicipitalean fungi (compare Haarmann et al., 2005, 2006; Fleetwood et al., 2007), the genetic material on the chromosome adjacent to this gene was also analyzed. We have previously demonstrated (Kucht et al., 2004) that alkaloids and the clavicipitalean fungi can be removed from *I. asarifolia* and *T. corymbosa* by treatment of the plant with systemic azole fungicides. Shoots of these plants rooted in garden soil grew into well-developed plants within a few weeks. They were devoid of alkaloids and the epibiotic fungus.

For the analysis of the genetic material, we used these fungus-free plants as a source of genomic plant DNA, which was employed as a template in PCRs with degenerate oligonucleotides (Wang et al., 2004) targeted to the dmaW gene. The degenerate oligonucleotides deg1 and deg2 (forward) and deg3 and deg4 (reverse) were used in four different combinations (deg1/deg3; deg1/deg4; deg2/deg3; deg2/deg4). The results of these experiments, however, all turned out to be negative. There was no indication suggesting that the dmaW gene was present in either plant species.

We therefore turned to the fungi IasaF13 and TcorF01 collected from the leaf surface and found by PCR (deg1/deg4) a DNA sequence of the expected



**Figure 2.** Fungal colonization of the adaxial leaf surface of *I. asarifolia* by the fungus IasaF13 (A) and leaf surface after ultrasonic treatment that removes most of the hyphae (B). Secretory glands stained red with Nile red. Hyphae stained green with Oregon Green-labeled wheat germ agglutinin (bars = 50  $\mu$ m).

length (>900 bp), which exhibited high similarity (76% for IasaF13 and 73% for TcorF01) to the corresponding dmaW sequence of *C. purpurea* (AJ011963). The similarity between both partial dmaW genes found in IasaF13 and TcorF01 was 93%. The partial dmaW sequence amplified from IasaF13 was now extended by chromosome walking to the 5'- as well as to the 3'-end as described in "Materials and Methods." The complete DmaW protein in IasaF13 (AAZ20613) comprises 456 amino acids and has a molecular mass of 52.4 kD. This compares well with the dmaW gene in *C. purpurea* (CAB39314) showing 448 amino acids and 51.68 kD. The cDNA of the dmaW gene (DQ121453) in IasaF13 has 73% similarity to that of *C. purpurea*. The intron/exon structure of all seven dmaW genes known up to now is compared in Table II. Interestingly, the length of exon 2 is conserved to 122 bp in each case.

Next and upstream of the 5'-end of the dmaW gene, open reading frames (ORFs) were found with a similarity (amino acid level) of 61% (1,000 bp; EU091292) to orfA, 74% (1,079 bp; EU121852) to orfB, and 73% (1,380 bp; EU121853) to cpox-1 of *C. purpurea* (Tudzynski et al., 2001; Fig. 1B), indicating that the dmaW gene in IasaF13 is also part of a cluster encoding genes of ergoline alkaloid biosynthesis. This cluster is also found in *C. purpurea* (Haarmann et al., 2005), *A. fumigatus* (Coyle and Panaccione, 2005; Unsoeld and Li, 2005), and *N. lolii* (Fleetwood et al., 2007).

#### Biosynthesis of Ergoline Alkaloids in the Epibiotic Fungus and Nondetectability of the Biosynthetic Process in the Plant Tissue

The plants *I. asarifolia* and *T. corymbosa* were investigated for their ability to synthesize ergoline alkaloids. The plants (colonized by their respective fungus IasaF13 or TcorF01) were fed with isotopically labeled [5,5-<sup>2</sup>H<sub>2</sub>]1-deoxy-D-xylulose ([d<sub>2</sub>]DOX) and [2,2-<sup>2</sup>H<sub>2</sub>]mevalonic acid lactone ([d<sub>2</sub>]MVA) for 48 h or 1 week. Both compounds are precursors of isopentenyl diphosphate and dimethylallyl diphosphate (DMAPP), which supply the isoprene moiety of ergoline alkaloids (Groeger et al., 1963; Heinstein et al., 1971; Tsai et al. 1995). The pathway to the phosphor-

ylated hemiterpenes is located either in plastids (DOX pathway) or in the cytosol (MVA pathway). Both pathways are involved in the biosynthesis of different terpenoid natural products, but sesquiterpenes are an exception in that their terpenoid skeletons may be derived from either the DOX or the MVA pathway or simultaneously from both (Piel et al., 1998; Stanjek et al., 1999; Laule et al., 2003; Page et al., 2004; Dudareva et al., 2005; Flügge and Gao, 2005). During the incubation experiment discussed here, we have not only analyzed ergoline alkaloids for a possible isotopic deuterium enrichment, but also (*E*)- $\beta$ -caryophyllene, a sesquiterpene known to be a product of the *I. asarifolia* plant but not the associated fungus IasaF13 (Kucht et al., 2004). The expected incorporation pattern of deuterium labels from [d<sub>2</sub>]DOX and [d<sub>2</sub>]MVA into (*E*)- $\beta$ -caryophyllene and chanoclavine are shown in Figure 3. Mass spectroscopic analysis of (*E*)- $\beta$ -caryophyllene from our labeling experiment (Fig. 4) was in agreement with published data (Hampel et al., 2005). (*E*)- $\beta$ -caryophyllene is strongly labeled from both precursors with [d<sub>2</sub>]DOX giving an enrichment in the (*E*)- $\beta$ -caryophyllene molecular ion of 22.7% (*m/z* 210.4) or even 62.5% (*m/z* 210.6) from [d<sub>2</sub>]MVA. The molecular ion of natural (*E*)- $\beta$ -caryophyllene is *m/z* 204.4 (Fig. 4).

This experiment showed that the proffered precursors [d<sub>2</sub>]DOX and [d<sub>2</sub>]MVA were taken up by the plant and metabolized. In spite of this, however, the ergoline alkaloids chanoclavine, lysergic acid amide, and iso-lysergic acid amide were devoid of any deuterium. These experiments were carried out in July 2004 (48-h feeding time) and repeated in December 2004 (48-h feeding time) and May 2005 (1-week feeding time) with the same result regardless of whether *I. asarifolia* or *T. corymbosa* were used as experimental systems.

During biosynthesis of ergoline alkaloids, DMAPP is involved in the prenylation of Trp (Fig. 1A). In a follow-up experiment, we fed 3-[<sup>14</sup>C]L-Trp to *I. asarifolia* shoots during a 48-h experiment. Also, in this case, no incorporation of labeled precursor into ergoline alkaloids was observed.

We therefore concluded that it is not the plant that is the producer of alkaloids, but the associated clavicipitalean fungus and that the experimental conditions

**Table I.** Quantitative estimation of ergoline alkaloids in leaf material and in epibiotic fungal mycelium

The mycelium was removed from the leaf material either by ultrasonic treatment or by spatula. For the analysis, either 10 g of leaf material or mycelium collected from 10 g of leaf material was analyzed. The amount of alkaloids was determined by van Urks' method as described by Kucht et al. (2004). Alkaloids are given as micrograms ergonovine maleate equivalents per gram plant material (fresh weight). The data represent the mean of six independent experiments.

Plant Species	Leaves		Mycelium	
	Before Ultrasonic Treatment	After Ultrasonic Treatment	Collected after Ultrasonic Treatment	Collected by Spatula
<i>I. asarifolia</i>	21.6	20.5	0.0	0.25
<i>T. corymbosa</i>	18.8	18.0	0.0	0.37

did not allow the precursors to reach the site of synthesis within the epibiotic plant-associated fungus.

The fact that we found genes necessary for ergoline alkaloid biosynthesis (vide supra), including the gene for the pivotal step in ergoline alkaloid biosynthesis in the *IasaF13* fungus, supports this view.

To corroborate our conclusions, the *dmaW* gene detected in *IasaF13* was probed for its activity after overexpression in yeast (*Saccharomyces cerevisiae*) following published procedures that were used to overexpress and characterize the *DmaW* enzyme from *A. fumigatus* (Unsoeld and Li, 2005). The *DmaW* enzyme (200  $\mu\text{g}$ ) from *IasaF13* was obtained from 1 L of culture broth and purified to apparent homogeneity as a His-tagged protein. The protein was incubated with  $\gamma,\gamma$ -dimethylallyl diphosphate and L-Trp and the reaction mixture was analyzed by HPLC. A product was formed as indicated by a peak with a retention time of 18 min. Formation of this peak depended on the presence of DMAPP, L-Trp, and a recombinant enzyme. Heat-denatured enzyme did not give this peak. The product 4-( $\gamma,\gamma$ -dimethylallyl)Trp was identified by  $^1\text{H-NMR}$  and MS. The spectroscopic properties of the product were identical to those previously reported after incubation with *DmaW* enzyme from *A. fumigatus* (Unsoeld and Li, 2005). The *DmaW* enzyme from *IasaF13* followed Michaelis-Menten kinetics. The  $K_m$  for L-Trp was 25  $\mu\text{M}$  ( $V_{\max}$  2.029  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ; Lineweaver-Burk) or 12  $\mu\text{M}$  ( $V_{\max}$  1.695  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ; Hanes-Woolf) and for DMAPP 27  $\mu\text{M}$  ( $V_{\max}$  0.317  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ; Lineweaver-Burk) or 30  $\mu\text{M}$  ( $V_{\max}$  0.297  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ; Hanes-Woolf), respectively. The activity of the *DmaW* enzyme could be modulated by addition of different metal ions (5 mM); however, the enzyme was also active in the absence of any metal and even in the presence of EDTA: no additives (100%),  $\text{Ca}^{2+}$  (153%), EDTA (84%),  $\text{Co}^{2+}$  (79%),  $\text{Mg}^{2+}$  (76%),  $\text{Mn}^{2+}$  (66%),  $\text{Na}^+$  (66%),  $\text{K}^+$  (64%),  $\text{Fe}^{2+}$  (35%),  $\text{Zn}^{2+}$  (22%), and  $\text{Cu}^{2+}$  (14%).

When the substrate specificity of the enzyme was tested, L-Trp exhibited as expected the highest activity (100%). The activity dropped in the following order when Trp was replaced with Trp derivatives with an altered side chain: L- $\beta$ -homo-Trp (47.5%),  $N_\alpha$ -methyl-L-Trp (40.2%), indole-3-propionic acid (16.5%), L-Trp

hydroxamate (12.0%), L-indole-3-lactic acid (5.6%), L-Trp methyl ester (4.2%), D-Trp (1.7%), *N*-acetyl-DL-Trp (0.8%), tryptamine (<0.2%), indole-3-butyric acid (<0.2%), and indole-3-acetic acid (<0.2%).

After incubation with Trp having variously substituted indole moieties, the following activities were observed: 7-methyl-DL-Trp (26.3%), 5-hydroxy-L-Trp (20.9%), 6-methyl-DL-Trp (9.8%), 5-methyl-DL-Trp (5.3%), and serotonin (2.2%). The identity of the enzymic products was confirmed by electrospray ionization-MS (negative and positive mode) in every single case as described by Steffan et al. (2007).

## DISCUSSION

The disjointed occurrence of ergoline alkaloids in taxonomically unrelated organisms, such as ascomycetes, and a higher plant family, the Convolvulaceae, was until recently explained by one of the following assumptions (Steiner et al., 2006). (1) It was thought that, during evolution, genetic material encoding ergoline alkaloid biosynthetic genes was transferred horizontally from one organism, possibly a fungus, to a higher plant family, the Convolvulaceae (Groeger and Floss, 1998; Tudzynski et al., 2001; Clay and Schardl, 2002). (2) Another possibility was that the biosynthetic pathway leading to ergoline alkaloids was repeatedly invented during evolution (Steiner et al., 2006). (3) The idea that a fungus capable of synthesizing ergoline alkaloids might colonize the higher plants was also discussed, although this latter idea had been abandoned (Hofmann, 2006).

We have shown in this and previous publications (Kucht et al., 2004; Steiner et al., 2006; Ahimsa-Mueller et al., 2007) that there is no compelling evidence for a horizontal gene transfer or a repeated invention during evolution of the biosynthetic pathway leading to ergoline alkaloids. In our hands, investigation of *I. asarifolia* and related Convolvulaceae plants by molecular biological as well as microscopic techniques showed instead that a clavicipitalean epibiotic fungus colonized the plant (Fig. 2; for images, see also Kucht et al., 2004; Steiner et al., 2006; Ahimsa-Mueller et al., 2007). This fungus is always found when ergoline

**Table II.** Structure of the *dmaW* gene encoding the 4-( $\gamma,\gamma$ -dimethylallyl)Trp synthase (*DmaW*) in *IasaF13* and *TcorF01* as compared to known *dmaW* genes in different ergoline alkaloid-producing fungi

The length of the DNA sequences is given in base pairs.

Fungus (Accession No.)	Genomic Sequence	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	cDNA
<i>IasaF13</i> (accession no. DQ121453)	1,486	1,169	58	122	57	80	1,371
<i>TcorF01</i> (DQ121454)	1,483	1,169	55	122	57	80	1,371
<i>C. purpurea</i> (accession no. AJ011963)	1,466	1,166	65	122	54	59	1,347
<i>C. fusiformis</i> (accession no. L39640)	1,504	1,187	74	122	62	59	1,368
<i>Balansia obtecta</i> (accession no. AY262013)	1,513	1,166	59	122	56	110	1,398
<i>Neotyphodium coenophialum</i> (accession no. AY259838)	1,480	1,172	64	122	69	53	1,347
<i>A. fumigatus</i> (accession no. AY775787)	1,512	1,151	61	122	74	104	1,377

alkaloids are detectable (Kucht et al., 2004; Steiner et al., 2006; Ahimsa-Mueller et al., 2007).

The experiments described in this article are in agreement with these conclusions. They provide independent proof that the clavicipitalean fungus IasaF13 is responsible for the presence of ergoline alkaloids in *I. asarifolia* and that a closely related clavicipitalean fungus occurs on *T. corymbosa* (Ahimsa-Mueller et al., 2007). The fungus IasaF13 harvested from *I. asarifolia* harbors a *dmaW* gene that is part of a cluster also found in ergoline alkaloid-producing fungi other than IasaF13 (Tsai et al., 1995; Tudzynski et al., 1999; Coyle and Panaccione, 2005; Haarmann et al., 2005; Unsoeld and Li, 2005). Four genes of this cluster in IasaF13 were sequenced and turned out to exhibit high similarity to genes known to be responsible for ergoline alkaloid biosynthesis in *C. purpurea* (Tudzynski et al., 2001; Haarmann et al., 2005) and *A. fumigatus* (Coyle and Panaccione, 2005; Unsoeld and Li, 2005). The order of these genes and their orientation in the cluster are identical to the cluster described for *C. purpurea* (Tudzynski et al., 2001), but are different from that of *A. fumigatus* (Unsoeld and Li, 2005) and *N. lolii* (Fleetwood et al., 2007). Moreover, all *dmaW* genes known up to now, including the IasaF13 gene, have one thing in common: They all lack the prenyl diphosphate binding site (ND)DXXD (Liang et al., 2002; Kremer et al., 2007) and thus are members of a new class of prenyl transferases. The *dmaW* gene in IasaF13 has the same intron/exon structure known from other *dmaW* genes (Table II) with exon II comprising 122 bp in each case.

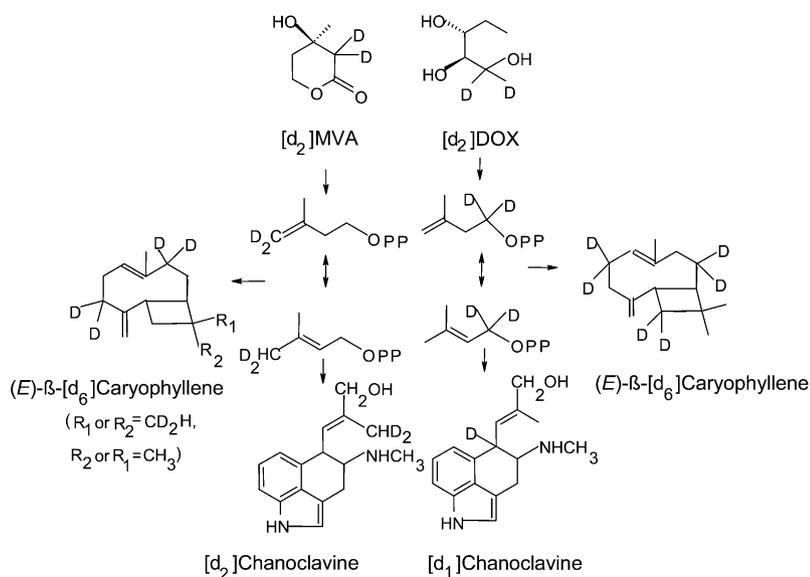
The *dmaW* genes in both *I. asarifolia* and *T. corymbosa* were isolated by two different techniques. These are PCR and reverse genetics starting from RNA and leading to an intronless cDNA. The RNA was isolated from both epibiotic fungi IasaF13 and TcorF1. This shows that the epibiotic fungi do not only harbor, but also transcribe, the *dmaW* gene. The properties of the encoded DmaW enzyme compare favorably to those

observed for the corresponding enzymes in the ergoline alkaloid-producing fungi *A. fumigatus* (Unsoeld and Li, 2005; Steffan et al., 2007) and *Claviceps* species (Heinstein et al., 1971; Lee et al., 1976; Cress et al., 1981; Gebler and Poulter, 1992) as far as  $M_r$ , kinetic data, influence of various ions on the enzyme activity, and substrate specificity are concerned. It is therefore evident that the *dmaW* gene in the fungus IasaF13 encodes a functioning DmaW enzyme capable of catalyzing the pathway-specific step initiating ergoline alkaloid biosynthesis.

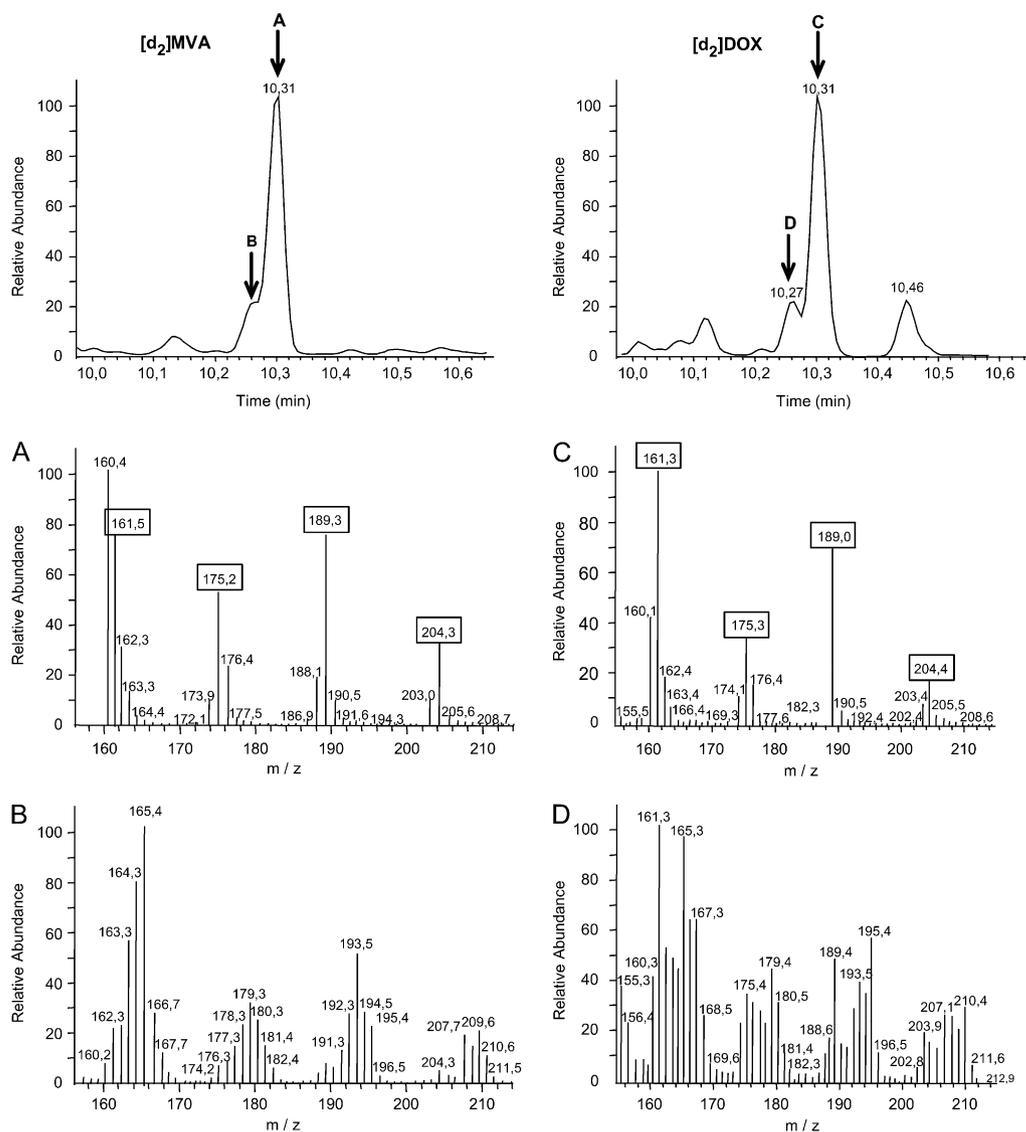
These observations leave little doubt that the plant is not the site of ergoline alkaloid biosynthesis, but that the plant-associated epibiotic fungi IasaF13 and TcorF01 are responsible for the biosynthesis of ergoline alkaloids, whereas these natural products accumulate almost exclusively within the *I. asarifolia* and *T. corymbosa* plants.

## CONCLUSION

Biosynthesis of ergoline alkaloids in the fungus and accumulation of these alkaloids in the plant leaves suggest that a transport system should exist that translocates the alkaloids from the fungus into the leaves. Translocation of carbohydrates, including an unknown compound, the structure of which remained undefined, is known to occur from fungus to host and vice versa in a fungal/plant association comprising *Myriogenospora atramentosa* (Clavicipitaceae) and *Paspalum notatum* (bahiagrass; Smith et al., 1985). In this system, movement of substances takes place through the apparently intact cuticle. The transport of ergoline alkaloids into the plants makes sense from an ecological point of view because the alkaloids are known to improve drought tolerance (Arechavaleta et al., 1992; Faeth and Fagan, 2002), stimulate growth (Faeth and Fagan, 2002), and confer fitness and herbivore deter-



**Figure 3.** Expected deuterium labeling of chanoclavine and (E)-β-caryophyllene after administration of [d<sub>2</sub>]MVA or [d<sub>2</sub>]DOX to *I. asarifolia* or *T. corymbosa* plants.



**Figure 4.** Observed incorporation of  $[d_2]$ MVA and  $[d_2]$ DOX into (*E*)- $\beta$ -caryophyllene. HPLC profile and MS spectra of unlabeled (A and C) and labeled (B and D) (*E*)- $\beta$ -caryophyllene. Headspace volatile (*E*)- $\beta$ -caryophyllene was collected during 48 h. The whole experiment was carried out three times using both *I. asarifolia* and *T. corymbosa* plants.

rence to the host plant (Bacon and Siegel, 1988; Bush et al., 1997). The fungi, in turn, receive nutrients, protection, reproduction, and dissemination (Malinowski and Belesky, 2000; Clay, 2004; Schardl et al., 2006). We conclude that the association between the *I. asarifolia* and *T. corymbosa* plants and their respective fungi IasaF13 and TcorF01 very likely benefits both plant and fungus and consequently should be considered a symbiotum.

## MATERIALS AND METHODS

### Plant Material

The plant material has been described previously (Steiner et al., 2006).

### Localization of Ergoline Alkaloids

To localize the ergoline alkaloids in either the fungal mycelium or in the plant tissue, both organisms were separated by two different techniques. The fungus (500  $\mu$ g) was collected from the upper surface of young leaf buds (10 g) using a small spatula. Alternatively, the leaf material (10 g) covered with mycelium was immersed in water (200 mL) and submitted to treatment in a small laboratory ultrasonic water bath. Separation of fungal hyphae and leaf material was followed by microscopic inspection of leaves and found to be almost complete after 8-min treatment (Fig. 2). For microscopic inspection of successful removal of fungal material from the leaf surface, samples were stained simultaneously with Nile Red- (Sigma) and Oregon Green-labeled wheat germ agglutinin (Invitrogen) and examined with a Leitz DMRB photomicroscope (Leica) equipped for epifluorescence with filter combination 490/15, 500, BP 525/20 according to Kucht et al. (2004).

The plant material was removed from the water and the water containing the hyphae was centrifuged (11,000g, 10 min). The plant material before and after ultrasonic treatment was analyzed for ergoline alkaloids and the alkaloids were identified as described (Kucht et al., 2004). After sonication

and centrifugation, the aqueous supernatant was devoid of any ergoline alkaloids.

### Application of Labeled Precursors to *Ipomoea asarifolia* and *Turbina corymbosa* Cuttings

Several shoots with a total weight of 9 g were cut and immediately placed into a vial containing an aqueous solution (4 mL) of 3-<sup>14</sup>C]-Trp (0.37 MBq; specific activity 1.97 MBq  $\mu\text{M}^{-1}$ ). The plant cuttings were kept at room temperature in a fume hood. The vials were refilled with water after uptake of the labeled Trp had been completed 9 h after the start of feeding. The alkaloids were extracted 48 h after the start of the experiment, and the alkaloids were separated by TLC (Kucht et al., 2004) and examined for their radioactivity by scintillation counting.

[d<sub>2</sub>]MVA (4 mg in 2 mL of water) and [d<sub>2</sub>]DOX (4 mg in 2 mL of water) were fed to cuttings of *I. asarifolia* (8–9 g) and *T. corymbosa* (8–9 g) during 48 h or 1 week. The plants were enclosed in desiccators for headspace analysis of (*E*)- $\beta$ -caryophyllene as described by Mithoefer et al. (2005). The setups were exposed to a day (10 h, 4,000 lux)/night (14 h) cycle. Ergoline alkaloids were isolated from the plant material immediately after termination of the feeding procedure and the alkaloids separated by TLC as described (Kucht et al., 2004).

### MS Analysis of Volatiles and Alkaloids

The collected volatiles were eluted from the charcoal traps with dichloromethane (2  $\times$  20  $\mu\text{L}$ ) containing *n*-bromodecane (100 ng  $\mu\text{L}^{-1}$ ) as an internal standard. Samples were analyzed on a Thermo TRACE GC 2000 connected to a TRACE MS equipped with an ECTM-5 capillary column (15-m  $\times$  0.25-mm i.d., 0.25- $\mu\text{m}$  film; Alltech). Injection port, 220°C; transfer line, 280°C; injection volume, 1  $\mu\text{L}$  (split ratio 1:10); ionization energy, 70 eV. Compounds were eluted under programmed conditions starting from 40°C (2-min hold) and ramped up at 10°C  $\text{min}^{-1}$  to 200°C, followed by 30°C  $\text{min}^{-1}$  to 280°C. Helium at a flow rate of 1.5 mL  $\text{min}^{-1}$  served as a carrier gas. The headspace volatile (*E*)- $\beta$ -caryophyllene was identified by comparing its mass spectrum with authentic reference material.

Prior to gas chromatography-MS analysis, the alkaloids were silylated by treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (2 h, 60°C). The compounds were separated on the ECTM-5 capillary column (see above) by programmed elution from 120°C to 280°C at 10°C  $\text{min}^{-1}$ . The data were analyzed using Finnigan Xcalibur 1.2 software.

### Strains, Plasmids, and Cultivation Conditions

Vector pYES2/NTC and yeast (*Saccharomyces cerevisiae*) INVSc1 were purchased from Invitrogen and maintained on yeast extract/peptone dextrose medium (Ausubel et al., 1996).

Transformants were cultivated in liquid or solid minimal medium lacking uracil containing 0.67% yeast nitrogen base (without amino acids but with ammonium sulfate), 2% carbon source (e.g. Glc or raffinose), 0.01% each of adenine, Arg, Cys, Leu, Lys, Thr, and Trp, and 0.005% each of Asp, His, Ile, Met, Phe, Pro, Ser, Tyr, and Val and 2% agar for plates.

### PCR of Plant DNA

Attempts to detect the dmaW gene in plant material (*I. asarifolia* and *T. corymbosa*) devoid of fungi (Kucht et al., 2004) were carried out by PCR using the HotMaster Taq DNA polymerase (Eppendorf) following the supplier's instructions. The template DNA (up to 200 ng) was extracted (Dellaporta et al., 1983) and incubated with oligonucleotides (50  $\mu\text{M}$ ), dNTPs (25 mM), buffer (5  $\mu\text{L}$ , 10-fold concentrated), MgCl<sub>2</sub> (25 mM, 2  $\mu\text{L}$ ), HotMaster Taq DNA polymerase (0.5–1.5 units), and water (ad 50  $\mu\text{L}$ ). Oligonucleotides targeted to the dmaW gene were deg1, deg3, deg4 (Wang et al., 2004), and deg2 (gift from Dr. Tudzynski, Muenster, Germany; GGNATHHTYAATCAYAT). The forward (deg1, deg2) and reverse (deg3, deg4) primers were employed in the combinations deg1/deg3, deg1/deg4, deg2/deg3, and deg2/deg4. Cycle conditions for amplification of the dmaW (cpd1, fgaPT2) gene were as described by Wang et al. (2004). The PCR products were electrophoresed in 1% agarose gel.

### Sequencing of Fungal Genes (dmaW, orfA, orfB, cpox1) from Iasaf13 and TcorF01

Fungal mycelium (500  $\mu\text{g}$ ) was collected with a spatula from leaf buds of *I. asarifolia* and *T. corymbosa* and DNA extracted according to Cenis (1992). The

DNA was used as a template during partial amplification of the dmaW gene using primers deg1 and deg4 and the High Fidelity PCR system (Roche). Conditions were as described by Wang et al. (2004) and described above. The PCR product was electrophoresed in a 1% agarose gel. Positive bands were extracted from the gel with the Qiaquick gel extraction kit (Qiagen) and cloned into pBluescriptII KS(-) (Stratagene). Sequencing has been described (Steiner et al., 2006). The partial dmaW gene consisted of more than 900 bp. This DNA stretch, however, did not yet comprise the 5'- and the 3'-end of the dmaW gene.

Subsequently the 5'-end of the dmaW gene was amplified using the DNA Walking SpeedUp premix kit (Seegene) as published by Hwang et al. (2003). Target-specific primers (TSPs) were TSP-1A-1 (TAGAGTTGGAGCAAT-TCAAGC), TSP-1A-2 (CTCAGCTCAAAGGGGATGC), and TSP-1A-3 (CCC-TTGTCCTCGGATAG). This approach gave an approximately 2,000-bp amplification product including the 5'-end.

The 3'-end of the dmaW gene was amplified using 3'-RACE-RCR as published by Frohmann et al. (1988). RNA (2.8  $\mu\text{g}$ ) was isolated from fungal mycelium (50 mg) using the E.Z.N.A. Fungal RNA kit (PEQLAB). TSPs were TSP-IA-4 (CTTGACATGGTTCGCGAAC; one amplification) and TSP-IA-5 (TCTGGAATCTGCTCGAAATGCC; two amplifications) and Q1 as a reverse primer following the method of Dieffenbach and Dveksler (1995). The resulting putative dmaW gene had a length of 1,371 bp (without introns). This was now confirmed using cDNA obtained from the RACE-PCR experiment and primers for the 5'-end (GTCAAAGTCCGAGCAACCAT) and the 3'-end (TGCAAACCTGACGCATTTTC).

Eventually genomic DNA from fungal mycelium and the following primers were employed to determine the sequences of the introns: Q4 (CAGGGAGATTGATCTTTTGG) and TSP-IA-4 (CTTGACATGGTTCGCGA-AAC). This gave an approximately 590-bp stretch, which was cloned and sequenced. Two introns with a length of 58 and 57 bp were found. The accession number of the complete dmaW gene is DQ 121 453.

The dmaW gene from the epibiotic clavicipitaceous fungus living on *T. corymbosa* was essentially sequenced in the same way. The gene had a length of 1,483 bp and two introns comprising 55 and 57 bp. The GenBank accession number is DQ 121 454.

For amplification of a partial cpox1 gene, a degenerate primer (cpox1-deg1) was constructed from the known sequence of the cpox1 gene in *Claviceps purpurea* (Tudzynski et al., 1999) and by comparison with conserved published sequences of *Aspergillus fumigatus* (accession no. XM\_751049), *Aspergillus nidulans* (accession no. EAA61522), *Aspergillus oryzae* (accession no. BAB13480), and *Neurospora crassa* (accession no. AABX01000271). The degenerate forward primer had the sequence ATHAARAAYACNGNCAYGA. The reverse primer WP-IA-3 (TCCAAGCCATTCTACCTTC) was derived from the now known sequence 5' upstream of the dmaW gene. The amplification protocol was designed for long DNA sequences: denaturing (5 min, 90°C), denaturing (45 s, 94°C), annealing (45 s, 49.5°C), extension (2.5 min; 35 cycles), extension (5 min, 72°C), and cooling at 4°C. Incubation of the primers with genomic DNA from Iasaf13 gave a 2,729-bp DNA sequence with a 74% similarity to the cpox gene from *C. purpurea*. A *Bam*HI/*Eco*RI restriction fragment carrying the dmaW gene was ligated into the vector pYES2/NTC (Invitrogen) giving plasmid pAM-16.

### Overproduction and Purification of DmaW

The pAM16 was introduced into yeast INVSc1 by electroporation (Ausubel et al., 1996). Recombinant clones were selected and maintained on solid minimal medium lacking uracil in 1 M aqueous sorbitol solution. For gene expression, the cells were grown in 500-mL Erlenmeyer flasks containing 250 mL of liquid minimal medium (containing 2% Glc) at 30°C and 200 rpm for 24 h. Then the cells were washed with the induction medium containing 1% raffinose and 2% Gal instead of Glc and cultivated in 500 mL of the same medium (in a 1,000-mL Erlenmeyer flask) for a further 16 h before harvest by centrifugation (5 min, 1,500g, 4°C). Total protein was obtained after breaking the cells in a mortar using liquid nitrogen according to Ausubel et al. (1996). After resuspension of 1 volume cell paste in 2 volumes storage buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), the mixture was stirred on ice for 5 min. After centrifugation (6,000g, 15 min, 4°C), the crude protein extract was obtained as supernatant. One-step purification of the recombinant His<sub>6</sub>-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer's instructions. The protein was eluted with 250 mM imidazole in 50 mM NaH<sub>2</sub>PO<sub>4</sub> and 300 mM NaCl, pH 8.0. The buffer of the protein fraction was changed to 50 mM Tris-HCl (pH 7.5) containing 15% glycerol by passing through a Sephadex

G25 column, which had been equilibrated with the same buffer. The protein was then aliquoted and stored frozen at  $-80^{\circ}\text{C}$  for enzyme assays.

## Enzymatic Reactions

All of the enzyme assays contained 50 mM Tris-HCl (pH 7.5) and 5 mM  $\text{CaCl}_2$ . They differed from each other by incubation volumes, substrate concentrations, amount of DmaW (DMATS), and incubation times. The reaction mixtures of the standard assay for determination of the substrate specificity (100  $\mu\text{L}$ ) contained 1 mM Trp or derivatives, 1 mM DMAPP, and 1  $\mu\text{g}$  of purified DmaW. After incubation for 1 h at  $30^{\circ}\text{C}$ , the reaction was stopped with 10  $\mu\text{L}$  of TCA (1.5 M). After removal of the protein by centrifugation (15,000g, 10 min,  $4^{\circ}\text{C}$ ), the enzymatic products were analyzed on an HPLC and a LC-MS system (described below). Two independent assays were carried out for quantification. The assay for the isolation of the enzymatic product for structural elucidation contained 1 mM Trp, 2 mM DMAPP, and 20  $\mu\text{g}$  of purified DmaW and was incubated for 16 h. The assays for determination of the kinetic parameters of L-Trp (100  $\mu\text{L}$ ) contained 0 to 0.2 mM L-Trp, 1 mM DMAPP, and 0.32  $\mu\text{g}$  of DmaW. The assays for determination of the kinetic parameters of DMAPP (100  $\mu\text{L}$ ) contained 0 to 0.5 mM DMAPP, 1 mM L-Trp, and 0.32  $\mu\text{g}$  of DmaW. Incubation was 20 min at  $30^{\circ}\text{C}$ .

## HPLC Analysis

Reaction mixtures containing the DmaW protein were analyzed on an Agilent (Hewlett-Packard) HPLC series 1100 instrument by using an Eclipse XBD-C18 column ( $4.6 \times 150$  mm, 5  $\mu\text{m}$ ; Agilent) at a flow rate of 1  $\text{mL min}^{-1}$ . Water (solvent A) and acetonitrile (solvent B), each containing 0.5% trifluoroacetic acid, were used as solvents. A gradient was run from 0% to 70% B in 20 min. After washing with 100% solvent B for 4 min, the column was equilibrated with 100% solvent A for 6 min. The substances were detected with a photo diode array detector.

## Spectroscopic Analysis

The enzymatic products were analyzed by positive and negative electrospray ionization-MS with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled to an Agilent series 1100 HPLC instrument equipped with a RP18 column ( $2 \times 250$  mm, 5  $\mu\text{m}$ ). For separation, the column was run with 10% solvent B (methanol) in solvent A (water, each containing 0.1% HCOOH) for 5 min, followed by a gradient from 10% to 100% B over 30 min. After washing with 100% B, the column was equilibrated with 10% B for 10 min. The flow rate was 0.2  $\text{mL min}^{-1}$ .

The NMR spectrum of 4-( $\gamma,\gamma$ -dimethylallyl)Trp was taken on an Avance DRX 500 spectrometer (Bruker) using 0.1 M DCl as solvent.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ011963, AAZ20613, CAB39314, DQ121453, EU091292, EU121852, EU121853, DQ121454, L39640, AY262013, AY259838, AY775787, XM\_751049, EAA61522, BAB13480, and AABX01000271.

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## LITERATURE CITED

- Ahimsa-Mueller MA, Markert A, Hellwig S, Knoop V, Steiner U, Drewke C, Leistner E (2007) Clavicipitaceous fungi associated with ergoline alkaloid-containing Convolvulaceae. *J Nat Prod* **70**: 1955–1960
- Archavaleta M, Bacon CW, Plattner RD, Hoveland CS, Radcliffe DE (1992) Accumulation of ergopeptine alkaloids in symbiotic tall fescue under deficits of soil water and nitrogen fertilizer. *Appl Environ Microbiol* **58**: 857–861
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1996) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York
- Bacon CW, Lyons P (2005) Ecological fitness factors for fungi within the Balansieae and Clavicipitaceae. In J Dighton, JF White, P Oudemans, eds, *The Fungal Community, Its Organisation and Role in the Ecosystem*, Ed 3. CRC Taylor & Francis, Boca Raton, FL, pp 519–532
- Bacon CW, Siegel MR (1988) Endophyte parasitism of tall fescue. *J Prod Agric* **1**: 45–55
- Bischoff JE, White JF Jr (2005) Evolutionary development of the Clavicipitaceae. In J Dighton, JF White, P Oudemans, eds, *The Fungal Community, Its Organisation and Role in the Ecosystem*, Ed 3. CRC Taylor & Francis, Boca Raton, FL, pp 505–518
- Bush LP, Wilkinson HH, Schardl CL (1997) Bioprotective alkaloids of grass-fungal endophyte symbiosis. *Plant Physiol* **114**: 1–7
- Cassady JM, Chan KK, Floss HG, Leistner E (2004) Recent development in the maytansinoid antitumor agents. *Chem Pharm Bull (Tokyo)* **52**: 1–26
- Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res* **20**: 2380
- Clark CA (1994) The chlorotic leaf distortion pathogen, *Fusarium lateritium*, cross protects sweet potato against *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *batatas*. *Biol Control* **4**: 59–61
- Clay K (2004) Fungi and the food of the gods. *Nature* **427**: 401–402
- Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am Nat (Suppl)* **160**: S99–S127
- Coyle CM, Panaccione DG (2005) An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. *Appl Environ Microbiol* **71**: 3112–3118
- Cress WA, Chayet LT, Rilling HC (1981) Crystallization and partial characterization of dimethylallyl pyrophosphate: L-tryptophan dimethylallyl transferase from *Claviceps* sp. SD58. *J Biol Chem* **256**: 10917–10923
- Dellaporta SL, Wool J, Hicks JB (1983) A plant miniprep: version 2. *Plant Mol Biol Rep* **1**: 19–21
- Dieffenbach CW, Dveksler CS (1995) *PCR Primer: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, Boland W, Gershenzon J (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proc Natl Acad Sci USA* **102**: 933–938
- Faeth SH, Fagan WF (2002) Fungal endophytes: common host plant symbionts but uncommon mutualists. *Integr Comp Biol* **42**: 360–368
- Fleetwood DJ, Scott B, Lane GA, Tanaka A, Johnson RD (2007) A complex ergovaline gene cluster in *Epichloe* endophytes of grasses. *Appl Environ Microbiol* **73**: 2571–2579
- Flügge UI, Gao W (2005) Transport of isoprenoid intermediates across chloroplast envelope membranes. *Plant Biol* **7**: 91–97
- Frohmann MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNA from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* **85**: 8998–9002
- Gebler JC, Poulter CD (1992) Purification and characterization of dimethylallyl tryptophan synthase from *Claviceps purpurea*. *Arch Biochem Biophys* **296**: 308–313
- Groeger D, Floss HG (1998) Biochemistry of ergot alkaloids—achievements and challenges. In GA Cordell, ed, *The Alkaloids—Chemistry and Biology*, Vol 50. Academic Press, New York, pp 171–218
- Groeger D, Mothes K, Floss HG, Weygand F (1963) Zur Biogenese von Ergolin-Derivaten in *Ipomoea rubro-coerulea* Hook. *Z Naturforsch* **18b**: 1123–1124
- Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J Nat Prod* **69**: 509–526
- Haarmann T, Machado C, Luebbe Y, Correia T, Schardl CL, Panaccione DG, Tudzynski P (2005) The ergot alkaloid gene cluster in *Claviceps purpurea*: extension of the cluster sequence and intra species evolution. *Phytochemistry* **66**: 1312–1320
- Haarmann T, Ortel I, Tudzynski P, Keller U (2006) Identification of the cytochrome P450 monooxygenase that bridges the clavine and ergoline alkaloid pathways. *ChemBioChem* **7**: 645–652
- Hampel D, Mosandl A, Wuest M (2005) Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythryl phosphate pathways. *Phytochemistry* **66**: 305–311
- Heinstein PF, Lee SL, Floss HG (1971) Isolation of dimethylallylpyrophosphate: tryptophan dimethylallyltransferase from the ergot fungus (*Claviceps* spec.). *Biochem Biophys Res Commun* **44**: 1244–1251
- Higashide E, Asai M, Ootsu K, Tanida S, Kozay Y, Hasegawa T, Kishi T, Sogino Y, Yoneda M (1977) Ansamitocin, a group of novel maytansinoid antibiotics with anti-tumor properties from *Nocardia*. *Nature* **270**: 721–722

- Hofmann A (1961) Die Wirkstoffe der mexikanischen Zauberdroge "Ololiuqui". *Planta Med* 9: 354–367
- Hofmann A (2006) LSD-mein Sorgenkind, die Entdeckung einer Wunderdroge, Ed 12. Deutscher Taschenbuchverlag, Munich
- Hwang IT, Kim YL, Kim SH, Kwak CI, Gu YY, Chun JY (2003) Annealing control primer system for improving specificity of PCR amplification. *Biotechniques* 35: 1180–1184
- Keller U, Tudzynski P (2002) Ergot alkaloids. In HD Osiewacz, ed, *The Mycota*, Vol X. Springer, Berlin, pp 157–181
- Kremer A, Westrich L, Li SM (2007) A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization. *Microbiology* 153: 3409–3416
- Krupinski VM, Robbers JE, Floss HG (1976) Physiological study of ergot: induction of alkaloid synthesis by tryptophan at the enzymic level. *J Bacteriol* 125: 158–165
- Kucht S, Gross J, Hussein Y, Grothe T, Keller U, Basar S, Koenig WA, Steiner U, Leistner E (2004) Elimination of ergoline alkaloids following treatment of *Ipomoea asarifolia* (Convolvulaceae) with fungicides. *Planta* 219: 619–625
- Kupchan SM, Komoda Y, Branfman AR, Sneden AT, Court WA, Thomas GJ, Hintz HPJ, Smith RM, Karim A, Howie GA, et al (1977) The maytansinoids. Isolation, structural elucidation, and chemical interrelation of novel ansa macrolides. *J Org Chem* 42: 349–357
- Laule O, Fürholz A, Chang HS, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange BM (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 100: 6866–6871
- Lee SL, Floss HG, Heinstein P (1976) Purification and properties of dimethylallylpyrophosphate: tryptophan dimethylallyl transferase, the first enzyme of ergot alkaloid biosynthesis in *Claviceps* sp. *Arch Biochem Biophys* 77: 84–94
- Liang PH, Ko TP, Wang AH (2002) Structure, mechanism and function of prenyltransferases. *Eur J Biochem* 269: 3339–3354
- Makariewa TN, Ilyin SG, Stonik VA, Lyssenko KA, Denisenko VA (1999) Picobin, the first ergoline marine alkaloid from the Far-Eastern ascidian *Eudistoma* sp. *Tetrahedron Lett* 40: 1591–1594
- Malinowski DP, Belesky DP (2000) Adaptations of endophyte-infected cool season grasses to environmental stresses. *Crop Sci* 40: 923–940
- Mithoefer A, Wanner G, Boland W (2005) Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol* 137: 1160–1168
- Moon CD, Craven KD, Leuchtmann A, Clement SL, Schardl CL (2004) Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. *Mol Ecol* 13: 1455–1467
- Mutschler E, Geisslinger G, Kroemer HK, Schaefer-Korting M (2001) *Arzneimittelwirkungen- Lehrbuch der Pharmakologie und Toxikologie*, Ed 8. Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart
- Page JE, Hause G, Raschke M, Gao W, Schmidt J, Zenk MH, Kutchan M (2004) Functional analysis of the final steps of the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway to isoprenoids in plants using virus-induced gene silencing. *Plant Physiol* 134: 1401–1413
- Piel J, Donath J, Bandemer K, Boland W (1998) Mevalonat-unabhängige Biosynthese terpenoider Duftstoffe in Pflanzen: Induzierter und konstitutiv emittierter Duft. *Angew Chem* 110: 2622–2625
- Pullen CB, Schmitz P, Hoffmann D, Meurer K, Boettcher T, von Bamberg D, Pereira AM, de Castro França S, Hauser M, Geertsema H, et al (2003) Occurrence and non-detectability of maytansinoids in individual plants of the genera *Maytenus* and *Putterlickia*. *Phytochemistry* 62: 377–387
- Rehacek Z, Sajdl P (1990) Ergot Alkaloids, Chemistry, Biological Effects, Biotechnology. Academia, Prague
- Robbers JE, Robertson IW, Hornemann KM, Jindra J, Floss HG (1972) Physiological studies on ergot: further studies on the induction of alkaloid synthesis by tryptophan and its inhibition by phosphate. *J Bacteriol* 112: 791–796
- Schardl CL, Craven KD (2003) Interspecific hybridisation in plant-associated fungi and oomycetes: a review. *Mol Ecol* 12: 2861–2873
- Schardl C, Leuchtmann A (2005) The epichloe endophytes of grasses and the symbiotic continuum. In J Dighton, JF White, P Oudemans, eds, *The Fungal Community, Its Organisation and Role in the Ecosystems*, Ed 3. CRC Taylor & Francis, Boca Raton, FL, pp 475–503
- Schardl CF, Pannacione DG, Tudzynski P (2006) Ergot alkaloids-biology and molecular biology. In GA Cordell, ed, *The Alkaloids: Chemistry and Biology*, Vol 63. Academic Press, New York, pp 45–86
- Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbiosis of grasses with seedborne fungal endophytes. *Annu Rev Plant Biol* 55: 315–340
- Smith KT, Bacon CW, Luttrell ES (1985) Reciprocal translocation of carbohydrates between host and fungus in Bahiagrass infected with *Myriogenospora atramentosa*. *Phytopathology* 75: 407–411
- Stanjek V, Piel J, Boland W (1999) Biosynthesis of furanocoumarins: mevalonate-independent prenylation of umbelliferone in *Apium graveolens* (Apiaceae). *Phytochemistry* 50: 1141–1145
- Steffan N, Unsoeld IA, Li SM (2007) Chemoenzymatic synthesis of prenylated indole derivatives using a 4-dimethylallyltryptophan synthase from *Aspergillus fumigatus*. *ChemBioChem* 8: 1298–1307
- Steiner U, Ahimsa-Mueller MA, Markert A, Kucht S, Gross J, Kauf N, Kuzma M, Zych M, Lamshoef M, Furmanowa M, et al (2006) Molecular characterisation of a seed transmitted clavicipitaceous fungus occurring on dicotyledonous plants (Convolvulaceae). *Planta* 224: 533–544
- Tsai HF, Wang H, Gebler JC, Poulter CD, Schardl CL (1995) The *Claviceps purpurea* gene encoding dimethylallyltryptophan synthase, the committed step for ergot alkaloid biosynthesis. *Biochem Biophys Res Commun* 216: 119–125
- Tudzynski P, Correia T, Keller U (2001) Biotechnology and genetics of ergot alkaloids. *Appl Microbiol Biotechnol* 57: 593–605
- Tudzynski P, Hoelter K, Correia T, Arntz C, Grammel N, Keller U (1999) Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. *Mol Gen Genet* 261: 133–141
- Tudzynski P, Scheffer J (2004) *Claviceps purpurea*: molecular aspects of a unique pathogenic lifestyle. *Mol Plant Pathol* 5: 377–388
- Unsoeld IA, Li SM (2005) Overproduction, purification and characterization of FgaPT2, a dimethylallyltryptophan synthase from *Aspergillus fumigatus*. *Microbiology* 151: 1499–1505
- Wang J, Machado C, Pannacione DG, Tsai HF, Schardl CL (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. *Fungal Genet Biol* 41: 189–198
- White JF Jr, Bacon CW, Hywel-Jones NL, Spatafora JW (2003) *Clavicipitacean Fungi, Evolutionary Biology, Chemistry, Biocontrol, and Cultural Impacts*. Marcel Dekker, New York