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Antitumor astins originate from the fungal endophyte *Cyanodermella asteris* living within the medicinal plant *Aster tataricus*

Thomas Schafhausen a,b,c,1, Linda Jahn b,1, Norbert Kirchner d, Andreas Kulik e, Liane Flor f, Alexander Lang g, Thibault Caradec h, David P. Fewer i, Kaarina Sivonen j, Willem J. H. van Berkel j, Philippe Jacques k,h, Tilmann Weber a,i, Harald Gross d, Karl-Heinz van Pée c, Wolfgang Wohlleben k,e,f, and Jutta Ludwig-Müller b,2,3

*Microbiology and Biotechnology, Interfaculty Institute of Microbiology and Infection Medicine, Eberhard Karls University Tübingen, 72076 Tübingen, Germany; aInstitute of Botany, Technische Universität Dresden, 01217 Dresden, Germany; bGeneral Biochemistry, Technische Universität Dresden, 01062 Dresden, Germany; cPharmaceutical Institute, Department of Pharmaceutical Biology, Eberhard Karls University Tübingen, 72076 Tübingen, Germany; dInstitut Charles Viollette, Equipe d’accueil 7394, University of Lille, 59000 Lille, France; eDepartment of Microbiology, University of Helsinki, 00014 Helsinki, Finland; fLaboratory of Biochemistry, Wageningen University & Research, 6708 WE Wageningen, The Netherlands; gMicrobial Processes and Interactions, Terra Teaching and Research Centre, Gembloux Agro-Bio Tech, University of Liège, 5030 Gembloux, Belgium; and hThe Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

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Medicinal plants are a prolific source of natural products with remarkable chemical and biological properties, many of which have considerable remedial benefits. Numerous medicinal plants are suffering from wildcrafting, and thus biotechnological production processes of their natural products are urgently needed. The plant *Aster tataricus* is widely used in traditional Chinese medicine and contains unique active ingredients named astins. These are macrocyclic peptides showing promising antitumor activities and usually containing the highly unusual moiety 3,4-dichloroprolinol. The biosynthetic origins of astins are unknown despite being studied for decades. Here we show that astins are produced by the recently discovered fungal endophyte *Cyanodermella asteris*. We were able to produce astins in reasonable and reproducible amounts using axenic cultures of the endophyte. We identified the biosynthetic gene cluster responsible for astin biosynthesis in the genome of *C. asteris* and propose a production pathway that is based on a nonribosomal peptide synthetase. Striking differences in the production profiles of endophyte and host plant imply a symbiotic cross-species biosynthesis pathway for astin C derivatives, in which plant enzymes or plant signals are required to trigger the synthesis of plant-exclusive variants such as astin A. Our findings lay the foundation for the sustainable biotechnological production of astins independent from aster plants.

Astins are natural products that bind to a crucial human regulatory protein, the stimulator of interferon genes (STING), which is a promising new therapeutic target for cancer and immune disorders. Astins have long been regarded as phytochemicals of a Chinese medicinal plant. Here, we show that they are produced by the newly identified fungal endophyte *Cyanodermella asteris* via a nonribosomal biosynthetic pathway. Moreover, we provide evidence that key astin variants are produced during symbiosis with the aster plant. The production of specific phytochemical variants during symbiotic interactions is poorly studied and might be more widespread than previously expected. These findings pave the way for a cost-effective biotechnological astin production.

**Significance**

Astins are natural products that bind to a crucial human regulatory protein, the stimulator of interferon genes (STING), which is a promising new therapeutic target for cancer and immune disorders. Astins have long been regarded as phytochemicals of a Chinese medicinal plant. Here, we show that they are produced by the newly identified fungal endophyte *Cyanodermella asteris* via a nonribosomal biosynthetic pathway. Moreover, we provide evidence that key astin variants are produced during symbiosis with the aster plant. The production of specific phytochemical variants during symbiotic interactions is poorly studied and might be more widespread than previously expected. These findings pave the way for a cost-effective biotechnological astin production.


The authors declare no competing interest.

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1 These authors contributed equally to this work.
2 W.W. and J.L.-M. contributed equally to this work.
3 To whom correspondence may be addressed. Email: wolfgang.wohlleben@biotech.uni-tuebingen.de or Jutta.Ludwig-Mueller@tu-dresden.de.

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human innate immunity (26). A total of 24 astin chemical variants (20, 27–32) are known, including the structurally related branched tataricins (33) as well as linear asterins (34, 35). Quantitatively, the astin variants A to C dominate in *A. tataricus* preparations (18, 31, 36). They consist of 5 amino acids including l-2-aminobutyric acid (2Abu), β-(R)-phenylalanine (jPhe), and the unique 3,4-dichlorinated l-proline (ProCl2) (Fig. 1A). Low natural availability (27, 31, 37) and unsuccessful chemical synthesis approaches (38, 39) of astins prevent a thorough exploration of their biological activity.

The biosynthetic origins of astins remained unclear, but it is widely assumed that they are made by the plant *A. tataricus* (40). It is hypothesized that astins are the product of a nonribosomal peptide synthetase (NRPS) biosynthetic pathway (33). NRPSs are large multidomain enzymes predominantly found in bacteria and fungi that catalyze a stepwise peptide synthesis by a thio-template mechanism (41). NRPSs are able to utilize a large number of nonproteinogenic amino acid substrates to generate nonribosomal peptides (NRPs) (41). The mycotoxin cyclochlorotine from the fungus *Talaromyces islandicus* (42) is an NRP showing remarkable structural similarities to astins, suggesting that astins likewise might be produced by a fungus.

Here, we show that astins do indeed have a fungal origin. The endophyte *Cyanodermella asteris* that has been recently isolated from inflorescences of *A. tataricus* (43) produces astin variants in pure culture, including astin C, one of the 3 main variants isolated from *A. tataricus* (25, 27, 31, 37). Plants free of *C. asteris* did not contain any astins, and reinfection with this fungus restored astin production, including plant-exclusive variants such as astin A. We identified the astin biosynthetic gene cluster in the recently sequenced fungal genome (44). *C. asteris* is characterized by stable and sound production of astins, which is unlike other endophytes that often only poorly and unreliably produce NPs originally known from their host plants (45–48). Our findings pioneer prospective biotechnological production of this valuable NP.

**Results**

*A. tataricus* Harbors an Endophytic Community Including the Astin-Producing Fungus *C. asteris*. Astins are primarily reported from dried roots and rhizomes of *A. tataricus* (20, 28, 29, 31). Extracts from fresh plant roots clearly also contained astins, according to high-performance liquid chromatography–mass spectrometry (HPLC-MS) analyses (Fig. 1B). Interestingly, some of the investigated *A. tataricus* plants did not contain any traces of astins (Fig. 1C), despite cultivation under identical conditions. This information together with structural similarity to the mycotoxin cyclochlorotine of *T. islandicus* (42) suggested that astins might have a fungal origin. Astins were abundant in inflorescences and leaves in addition to roots and rhizomes (Fig. 2), suggesting that the astin-producing fungus must live in close contact with the

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**Fig. 1.** The production of astins by *A. tataricus* and *C. asteris*. (A) Chemical structures of the main astin variants known from *A. tataricus*. (B–E) HPLC-MS chromatograms of extracts prepared from fresh plant roots and fungal cultures (each one is a representative chromatogram out of at least 3 biological replicates). The chromatograms show astin peaks at characteristic retention times (indicated in capital letters). Extracted ion chromatograms (EIC) depict the respective [M+H]+ ions of astins. TIC+ denotes total ion chromatogram, positive mode. Chromatograms are shown for a representative *A. tataricus* plant (initially being devoid of astins or C being in C), 3 mo after reinfection with the endophyte *C. asteris*. Intens., ion intensity in arbitrary units.

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The measurements were performed for 8 plants, and the most frequent symbol is displayed. (Scale bar, 30 mm.)

To investigate fungal endophytes of A. tataricus, roots were surface-sterilized, dissected, and incubated on agar plates until fungal colonies developed. Several endophytic fungi were isolated and assigned to morphotypes according to macroscopic and microscopic resemblances. A representative isolate of each morphotype was identified by ribosomal internal transcribed spacer (ITS) sequencing (SI Appendix, Tables S1 and S2). Fungal genera isolated and identified included Paraphoma, Colletotrichum, Epicocum, Cladosporium, and Sacrocladium, all of which have been reported as endophytes or plant-associated fungi from a variety of hosts. Besides these endophytes from roots, we recently reported the isolation of C. asteris (43), a hitherto unknown endophytic fungus, from the inflorescence of A. tataricus. C. asteris is a member of the heterogeneous lichen-forming family Lecanoromycetes (43), and the only member within the small genus Cyanodermella (43, 49) with an endophytic lifestyle.

In total, 22 isolated fungal endophytes of A. tataricus were tested for production of astins. Fermentations in liquid potato dextrose broth (PDB) followed by culture extract screening via HPLC-MS clearly demonstrated the presence of astin C, F, and G in the culture medium of C. asteris (Fig. 1D). Astins were not detected from any of the other endophytes tested.

Growth rate and astin production of C. asteris were highest in malt extract autolysate (MEA) medium (SI Appendix, Fig. S1 and Table S3). Around 63 mg of astin C and 1.5 mg of astin F were isolated to purity from 3 L of culture (SI Appendix, Fig. S2). This allowed confirmation of the chemical structure, including the stereochemistry of both molecules. Their planar structures were established by high-resolution MS analysis and, in addition, either by direct comparison of \(^{1}\text{H}\) and \(^{13}\text{C}\) nuclear magnetic resonance (NMR) data with literature data or by de novo NMR-based structure elucidation. The absolute configuration was inferred from chiral amino acid analysis, NMR experiments, and comparison of the measured [\(\delta\)] values with literature data (SI Appendix, Figs. S3–S15 and Tables S4–S9). The dichlorinated astin C, for which antineoplastic and antiinflammatory activity has been reported (22–25, 50), was the main variant of the 3 astins produced by the endophytic fungus C. asteris. Production of astins in axenic culture was stable over time, even after more than a dozen rounds of cultivations.

**The Biosynthetic Origins of Astins.** The chemical structures of astins suggest that they are produced on an NRPS enzyme complex. We recently sequenced the genome of C. asteris de novo on an Illumina MiSeq system resulting in an 85× coverage, 37 scaffolds, a genome size of 28.42 Mb, and 10,309 predicted genes (44). The genome encodes 5 putative NPRS enzymes, each being modular type I NRPS systems. Since the fungus is not genetically tractable, we investigated each of these candidate NRPSs in silico in order to identify the one responsible for astin biosynthesis. The large enzyme AstN (614 kDa) is the only NRPS enzyme with the predicted 5-modular composition required for the assembly of the astin pentapeptide. AstN shows remarkably high global sequence similarity (65%, identity 47%) and an analog domain architecture to the NRPS CctN of T. islandicus. CctN is the key enzyme in the cyclochlorotine pathway, as has been experimentally demonstrated by RNA interference-based silencing experiments (51). It can therefore be assumed that AstN is the astin synthetase.

AstN has the deduced domain architecture A−T−(C−A−T)\(_{3}\)−C\(_{7}\), where A stands for adenylation domain, T for thiolation domain, C for condensation domain, and C\(_{7}\) for terminal condensation-like domain (52). The domain organization implies a stepwise assembly of the 5 amino acid building blocks to a linear precursor pentapeptide. A domains are responsible for selection and activation of the building blocks. Comparison of the active center amino acids of the A domains with the active centers of substrate-assigned A domains of other fungal NRPSs revealed considerable similarities that allow the prediction of the substrate specificity for AstN (SI Appendix, Table S10). Accordingly, 2Abu is incorporated by the first (A1) and the third (A3) A domains. The second building block is proposed to be Pro, due to high similarities of the respective domain (A2) with Pro-activating domains of other fungal NRPSs. The dichlorinated astin C, for which antineoplastic and antiinflammatory activity has been reported (22–25, 50), was the main variant of the 3 astins produced by the endophytic fungus C. asteris. Production of astins in axenic culture was stable over time, even after more than a dozen rounds of cultivations.

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assembly order of AstN thus is 2Abu–Pro–2Abu–Ser–βPhe (Fig. 3).

The terminal domain in AstN, C2, shows typical features of fungal cyclization domains. The domain has a characteristic variation of the first histidine residue (52) of the highly conserved H1xxxDCxxS motif of C domains (motif sequence in AstN: 53HIHSSQYDGS WS). Moreover, a characteristic signature within the downstream region of fungal C2 domains, the so-called WL1–WL2 signature (56), is present in this terminal domain. Thus, the C2 domain is expected to cyclize and simultaneously release the macrocyclic pentapeptide from the AstN enzyme.

In comparison with the cyclochlororotine synthetase CctN, the astin synthetase AstN differs primarily in the selection of the first building block during peptide assembly. While the first module of AstN incorporates 2Abu (Table 1), it is Ser in the case of CctN (51), which accounts for the small structural differences between the 2 groups of macrocyclic pentapeptides (SI Appendix, Fig. S16).

The Astin Biosynthetic Gene Cluster Encodes Further Biosynthetic Enzymes, Although There Is No Evident Halogenase. The genetic region around astN contains the astin biosynthetic gene cluster (ast BGC) (Fig. 4). The cluster boundaries were estimated manually based on predicted gene functions and by identifying genetic elements that likely belong to the primary but not to the astin metabolism (SI Appendix, Fig. S17). According to that, the ast BGC consists of 13 distinct genes encoded in an area of about 50 kilo base pairs (kb) in size. Most of the cluster genes show striking sequence similarity to genes of the cyclochlorotine biosynthetic gene cluster (Fig. 4), which strongly supports the conclusion that the proposed genetic region constitutes the ast BGC. For several of the gene products, putative functions in astin biosynthesis are suggested (Table 1). Besides the already described NRPS AstN, these functions are transcriptional regulation (AstM, X), transportation (AstO, T, W), and biosynthesis of astin building blocks (AstR, P).

A halogenase is assumed to catalyze the chlorination of the Pro residue in the course of pentapeptide synthesis to yield the dichlorinated astin C variant and the monochlorinated astin F variant. Unexpectedly, no apparent halogenase is encoded in the ast BGC. To our best knowledge, no enzymes are described so far that convert Pro into ProCl2. The regiospecific and stereoselective chlorination of the pyrrolidine ring constitutes a challenging chemical reaction, which is principally only attributed to flavin-dependent and α-ketoglutarate/iron-dependent halogenases (57–59). Remarkably, according to protein function predictions and amino acid sequence comparisons, not a single gene in the whole genome codes for any of these halogenases (SI Appendix, Tables S11 and S12). In view of this, it is likely that a member of a novel type of halogenase, which has yet to be identified, achieves chlorination of the astins.

The Astins in A. tataricus Are Produced by the Endophyte C. asteris and Are Further Metabolized inside of the Host Plant. A. tataricus plants were investigated for the distribution of the C. asteris endophytes and astins. Plants that contained astins always contained C. asteris. The distribution of astins in the different plant parts of A. tataricus is consistent with the incidence of the endophyte C. asteris (Fig. 2). C. asteris was not detected from plants lacking astins. A. tataricus plants devoid of C. asteris were infected with the endophyte, and HPLC-MS analysis of root extracts prepared 3 mo after infection showed a range of astins comparable to that of other astin-containing plants (Fig. 1E), whereas the noninfected control plant remained totally devoid of astins. These findings clearly demonstrate that the endophyte C. asteris is responsible for astin production in A. tataricus.

Interestingly, a higher number of astin variants could be detected in the host plant than are produced in axenic fungal cultures. Especially, astin A, which is one of the main variants in planta, was not detected in C. asteris culture extracts. It is known that fungal biosynthetic pathways and their produced metabolites are influenced by growth conditions and media compositions (60). Therefore, a large variety of cultivation conditions were tested for C. asteris to induce production of more astin variants (e.g., different cultivation media, temperatures, durations, and supplements; see SI Appendix, Table S13). In addition, the fungus was also cultivated in the presence of phytohormones, plant extracts, and plant pieces (SI Appendix, Table S13) in order to test for plant stimuli. Moreover, since it is possible that astin A arises from incorporation of 1-alko-Thr (instead of 2Abu) at module 1 of the NRPS, the fungal culture broth was supplemented with high amounts (500 mg/L) of that building block. However, in all tested approaches, neither astin A nor other hydroxylated variants were produced in the fungal cultures. If, on the contrary, C. asteris was reintroduced into astin-devoid A. tataricus plants, most astins, including the plant-exclusive variant A, reemerged (Fig. 1E). This indicates that some astins are the result of a symbiosis in planta. Apparently, their biosynthesis needs more than one biological partner and requires an inducer or a biosynthetic enzyme from a further party like the host plant itself or endophytes other than C. asteris residing therein.

Discussion

Astin Are Nonribosomal Peptides from a Lecanoromycetes Fungus. The true origin of plant-derived NPs is often a matter of debate (61). Similar to the recent findings in marine invertebrates, where bacteria and fungi are increasingly made responsible for the production of various NPs (61, 62), there has been a growing number of reports of microbial endophytes able to produce NPs initially known from their host plants (12–16), including the pharmacologically interesting compounds paclitaxel (16), camptothecin (15), and FR900359 (14). Apparently, several plant-derived NPs are not true phytochemicals but are rather made by endophytic microorganisms. This clearly applies to the anti-tumor pentapeptides astins, which have long been regarded as phytochemicals of the traditional Chinese medicinal plant A. tataricus (40). We could show that they actually originate from C. asteris, a Lecanoromycete fungus living inside the plant as an endophyte. At present, no other microorganisms are reported to produce astins.

The prominent structural analogy between astins and cyclochlorotines from the mold T. islandicus (Eurotiomycetes) is reflected in a related biosynthetic pathway that involves key enzymes with high degrees of similarity. For example, the astN gene is more similar to cctN than to any other gene of a Lecanoromycetes strain from which genome data are available. In light of this and the distant relation between C. asteris and T. islandicus, horizontal gene transfer might be involved in the dissemination of the BGCs encoding the biosynthesis pathways. Notably, however, both BGCs show only a weak gene synteny (Fig. 4), presumably caused by events of gene rearrangements, which is not an unusual observation in fungal BGCs (63).

Astin A and Further Plant-Exclusive Variants Are Only Produced In Planta by a Symbiotic Biosynthesis. The endophytic fungus C. asteris produces the compounds astin C, F, and G under submerged axenic cultivation conditions. Further variants such as the hydroxylated astin A were only found inside the endophyte-containing plant A. tataricus. Thus, neither the plant nor the fungus alone are able to produce these plant-exclusive astins, but they are a symbiotic product of both organisms.

Since no incorporation of the hydroxylated building block allo-Thr was observed in C. asteris cultures, we assume that the hydroxylated plant-exclusive variants like astin A and astin H result from a hydroxylation subsequent to the macrocyclic peptide assembly. Similarly, the variants D and H show an alternative chlorination pattern likely originating from dehydrogenation and monochlorination of astin G (Fig. 3). Although it cannot be
Fig. 3. Biosynthesis model for the main astin variants. In conjunction with in silico analysis of the NRPS adenylation (A) domains and gene function prediction of the ast biosynthetic gene cluster, a biosynthesis model has been deduced. AstR and AstP are proposed here to supply the nonproteinogenic building blocks 2Abu and βPhe, respectively. The NRPS AstN assembles 5 building blocks to a macrocyclic pentapeptide. Dotted line arrows indicate incorporation by the A domains. The products released from AstN are proposed to be the fungal astins G, F, and C, which differ in the degree of chlorination. The plant-exclusive variants A, D, and H likely derive from the fungal astins as a result of a symbiosis between host and endophyte. Asterisk (*) denotes that the enzymes involved in tailoring of the astins are unknown.
ruled out that these reactions are catalyzed by \textit{C. asteris} enzymes that are only expressed in the native environment after receiving a certain trigger from the host plant, all attempts to induce the proposed tailoring reactions by adding plant components to the fungal culture failed. This makes an enzymatic contribution by a species other than \textit{C. asteris} more likely. It is well known that plants can hydroxylate aliphatic molecules in the course of biotransformation of toxic xenobiotics (64–66). Therefore, the occurrence of modified astin variants is probably best explained by a subsequent modification of the fungal NRP via plant enzymes. We assume that all of the other variants described from the aster (20, 27–35) arise in a similar way.

Such cross-species metabolic pathways, which require symbiosis between 2 or more biological partners, are quite unexplored. A symbiotic biosynthesis has only been shown for rhizoxin [joint bacterial and fungal biosynthesis (67)]. Furthermore, joint plant and fungal biosynthesis was proposed for one case of camptothecin (68) and for maytansine (69). Taking these examples and our findings on the plant-exclusive astins into consideration, it cannot be ruled out that many further NPs whose biosyntheses are obscure actually are the result of a symbiotic achievement of more than one species.

The ecological benefits of the symbiotic production of plant-exclusive astins are unclear. However, regarding both the relative high concentration of astin A in plants and its increased bioactivity (20, 24, 70) compared to astin C, the modifications would constitute a selective advantage to the host plant by increasing the potency of the fungal peptides. The astins might serve as a defense system against predators or pathogenic opponents.

The Discovery of the Astin Pathway in \textit{C. asteris} Enables a Biotechnological Production of the Biologically Active Astin C. While NPs of medicinal plants remain a major source for lead compounds in anticancer therapy and other pharmaceutical applications (13), they are not suited to meet industrial demands, due to slow plant growth, low NP production rates, and time-consuming extraction processes. The increasing findings of endophytes to produce plant-derived NPs (12, 13, 15, 16, 61) raised high hopes regarding the usage of these microorganisms for sustainable NP production in bioreactors (17). However, there have been no major breakthroughs in terms of commercial exploitation for any NP produced by an endophyte (17, 71). Reasons for that are either extremely poor production levels or the substantial decrease in NP production upon repeated subcultivation in axenic conditions (13), which, for example, is commonly observed for endophytic producers of the high-value cancer therapeutics camptothecin and paclitaxel (45–48). Further challenges in achieving biotechnological production using microorganisms arise from missing information on biosynthetic pathways and the encoding genes, which applies even for high-value plant-derived NPs such as paclitaxel, podophyllotoxin, or artemisinin (8, 9, 12).

None of these obstacles is anticipated for microbial astin production. First, \textit{C. asteris} is characterized by a strong astin C

![Fig. 4. The ast biosynthetic gene cluster in comparison to the cyclochlorotine biosynthetic gene cluster. Percentages illustrate the global amino acid sequence similarity of astin cluster genes to cyclochlorotine cluster genes from \textit{T. islandicus} (only similarities of >30% are illustrated).](image-url)
production (20 mg/L). Second, no attenuation of astin production in *C. asteris* is observed, despite subcultivation for more than a dozen rounds. Third, and perhaps most importantly, the genome sequence of *C. asteris* is available, and the astin BGC has been elucidated. This opens the door for genetic engineering of astin production in *C. asteris* as well as heterologous production in more readily accessible microorganisms. Due to the findings reported here, a sustainable biotechnological astin production has now been made possible. Consequently, the prospective pharmaceutical exploitation of astins toward anticancer or immunosuppressive pharmaceuticals has moved a big step forward.

**Materials and Methods**

**General.** Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich, and H₂O used was deionized. *A. tataricus* plants were obtained from the company Sarastro Stauden (Ort im Innkreis, Austria). The plants were cultivated in the greenhouse under long-day conditions (16 h light at 23 °C and 8 h dark at 18 °C). *Zi Wan* dried roots (in coordinates 48.515987, 9.039934) were purchased from the Anguo Mayway Herb Company Ltd. (Hubei, China).

**Astin Identification in Plant and Fungal Material by HPLC-ESI-MS and HPLC-MS/MS.** To analyze astins from plant material, fresh plant tissue was ground with liquid nitrogen and extracted twice with 20 mL of methanol/acetone (1:1) per gram of tissue weight and centrifuged for 30 min (5,000 × g) at 4 °C. Astins were extracted 3 times with 30 mL of ethyl acetate. Alternatively, l-butanol was used as extracting solvent for fungal cultures. If applicable, extracts from the same sample were combined. The extracts were then evaporated in vacuo, resolved in 1 mL of H₂O/methanol (1:1) and analyzed by HPLC-MS and/or HPLC-MS/MS using a Zorbax 300SB-C18 column (5 μm, 250 × 2.1 mm; Dr. Maisch GmbH) was coupled to an ESI mass spectrometer (LC/MSD Ultra Trap system XCT 6330; Agilent Technologies). Detection of astin masses was conducted with Agilent DataAnalysis for 6300 series Ion Trap LC/MSD 6.1 version 3.4 software (Agilent Daltonics). Solvent A was 0.1% formic acid, and solvent B was 0.06% formic acid in methanol (gradient: 15% B to 50% B in 25 min; flow rate: 0.4 mL/min; column temperature: 40 °C). Electrospray ionization (alternating positive and negative ionization) in Ultra Scan mode with a Z-spray source was used to achieve high-resolution full-scan MS. A library of authentic astins was used to aid in astin identification using the**

**Astin Production in Plants by qPCR.** *A. tataricus* total DNA was isolated according to a protocol of Möller et al. (74) with the following modifications: Before DNA precipitation, 150 μL of 5% polyvinylpolypyrrolidone (PVPP) were added to the supernatant. After incubation at room temperature for 30 min with gentle shaking, the sample was centrifuged (16,000 rcf, 4 °C, 10 min) to remove the PVPP. The DNA was precipitated by incubation at −20 °C for up to 2 h.

The extracted plant DNA was then screened for *C. asteris* DNA by quantitative real-time PCR using the device qTOVER2.2 and the software qPCRsoft 3.2 (both Analytik Jena). Specific primers were used to amplify parts of the gene actin of *A. tataricus* and ribosomal DNA of *C. asteris* (Table S15). The qPCR protocol conducted as follows: 1 cycle with 95 °C for 2 min, then 40 cycles with 95 °C for 5 s, and 64 °C for 25 s, followed by a melting curve analysis for verification of the PCR products. Calibration curves were created for absolute quantification using pure DNA from *A. tataricus* (noninfected plant tissue) and pure DNA from an axenic *C. asteris* culture.

**Infection of Astin-Free Plants with *C. asteris*.** *Astin-free* *A. tataricus* plants were cultivated in a 4-compartment system (75) in autoclaved soil, where a gauge (pore size 20 μm) separated the plant compartment from the fungal one. The soil of the fungal compartment was infected with 150 mg of freshly homogenized *C. asteris* tissue. The roots are not able to grow through the gauge, whereas the fungal hyphae can. The 2-compartment system with the plant and infected soil was cultivated in a climate chamber under long-day conditions (16 h light with 23 °C, 8 h dark with 18 °C). Astin content was investigated in plant tissue after 3 mo of growth as described above. Three plants were infected with *C. asteris*; one noninfected plant served as control.

**Data Availability.** All data generated or analyzed during this study are included in this article and SI Appendix.

**ACKNOWLEDGMENTS.** The authors gratefully acknowledge the financial support by the European Union (European Regional Development Fund, Project Astinprod) and the Free State of Saxony (Grant 100271404 and Grant 22 °C, without lighting). The culture was refreshed every 2 to 4 wk by inoculating fresh medium with 1/100 volume of a gently homogenized preculture with preculture (using a glass pistil to force dispersed growth). To test the incorporation of allo-Thr into astins, a *C. asteris* culture grown in 100 mL of MEA medium was supplemented with 500 μg/l allo-Thr or l-Thr at day 7 and cultivated for a further 21 d before extracts were analyzed by HPLC-MS (see above). This experiment was performed twice. To test the influence of host plant extracts on astin production, *C. asteris* fungal cultures were supplemented with an aqueous extract of plant tissues. For this, different plant parts (about 5 g each) were ground with liquid nitrogen and extracted with 20 mL of H₂O per gram of tissue. The extracts were concentrated 10 times, sterile-filtered, and added to the fungal culture (up to 1% final concentration, experiment performed in triplicates). Fungal extracts were analyzed by HPLC-MS (see above) after 2 to 3 wk of growth. For all other variations performed to elicit astin A production in *C. asteris* cultures, the different conditions and details are given in **SI Appendix, Table S13**.

**Screening for *C. asteris* in Plants by qPCR.** *A. tataricus* DNA was isolated according to a protocol of Möller et al. (74) with the following modifications: Before DNA precipitation, 150 μL of 5% polyvinylpolypyrrolidone (PVPP) were added to the supernatant. After incubation at room temperature for 30 min with gentle shaking, the sample was centrifuged (16,000 rcf, 4 °C, 10 min) to remove the PVPP. The DNA was precipitated by incubation at −20 °C for up to 2 h. After purification, the extracted DNA was then screened for *C. asteris* DNA by quantitative real-time PCR using the device qTOVER2.2 and the software qPCRsoft 3.2 (both Analytik Jena). Specific primers were used to amplify parts of the gene actin of *A. tataricus* and ribosomal DNA of *C. asteris* (Table S15). The qPCR protocol conducted as follows: 1 cycle with 95 °C for 2 min, then 40 cycles with 95 °C for 5 s, and 64 °C for 25 s, followed by a melting curve analysis for verification of the PCR products. Calibration curves were created for absolute quantification using pure DNA from *A. tataricus* (noninfected plant tissue) and pure DNA from an axenic *C. asteris* culture.

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