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Effect of Artificial Reconstitution of the Interaction between the Plant *Camptotheca acuminata* and the Fungal Endophyte *Fusarium solani* on Camptothecin Biosynthesis

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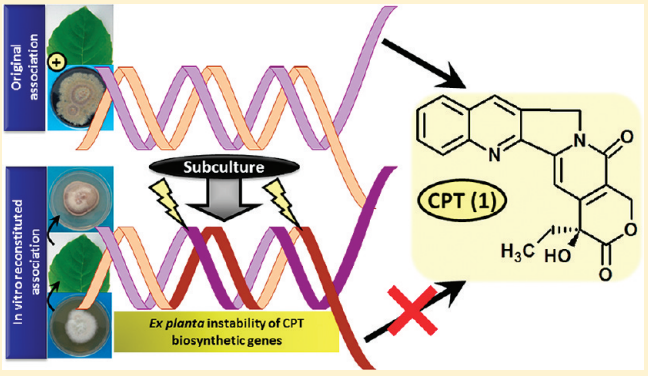
Supporting Information

ABSTRACT: Fungal endophytes inhabit healthy tissues of all terrestrial plant taxa studied and occasionally produce host-specific compounds. We recently isolated an endophytic fungus, *Fusarium solani*, from *Camptotheca acuminata*, capable of biosynthesizing camptothecin (CPT, **1**), but this capability substantially decreased on repeated subculturing. The endophyte with an impaired **1** biosynthetic capability was artificially inoculated into the living host plants and then recovered after colonization. Although the host−endophyte interaction could be reconstituted, biosynthesis of **1** could not be restored. Using a homology-based approach and high-precision isotope-ratio mass spectrometry (HP-IRMS), a cross-species biosynthetic pathway is proposed where the endophyte utilizes indigenous *G10H* (geraniol 10-hydroxylase), *SLS* (secologanin synthase), and *TDC* (tryptophan decarboxylase) to biosynthesize precursors of **1**. However, the endophyte requires host *STR* (strictosidine synthase) in order to condense the nitrogen-containing moiety (tryptamine, **2**) with the carbon-containing moiety (secologanin, **3**) to form strictosidine (**4**) and complete the biosynthesis of **1**. Biosynthetic genes of **1** in the seventh subculture generation of the endophyte revealed random and unpredictable nonsynonymous mutations. These random base substitutions led to dysfunction at the amino acid level. The controls, *Top1* gene and rDNA, remained intact over subculturing, revealing that instability of biosynthetic genes of **1** was not reflected in the primary metabolic processes and functioning of the housekeeping genes. The present results reveal the causes of decreased production of **1** on subculturing, which could not be reversed by host−endophyte reassociation.



Plants produce a diverse range of secondary metabolites to interact with dynamic environmental conditions, including defending themselves from natural enemies. Endophytic microorganisms are a diverse group of organisms associated with various tissues and organs of plants.[1] In recent years, there has been a drastic increase in discovering endophytes that produce novel metabolites with diverse biological activities (Figure S1, Supporting Information). Occasionally, novel endophytic microorganisms capable of producing associated plant secondary metabolites with established therapeutic value or potential have been discovered, for example, paclitaxel (Taxol),[2] podophyllotoxin,[3] deoxypodophyllotoxin,[4] hypericin and emodin,[5,6] and camptothecin (CPT, **1**).[7] However, there is no known published breakthrough in the commercial exploitation of endophytic fungi as a source of these drugs and drug lead compounds.

Recently, we isolated an endophytic fungus, *Fusarium solani* (strain INFU/Ca/KF/3), from the inner bark of *Camptotheca acuminata* Decne. (Nyssaceae), obtained from the Southwest Forestry University (SWFU) campus, Kunming (Yunnan Province), People's Republic of China. This endophyte is capable of indigenously producing CPT (**1**) in submerged in vitro axenic culture.[7] CPT (**1**), a monoterpenoid indole alkaloid that was originally isolated from the wood of *C. acuminata*,[8] is a potent antineoplastic agent by virtue of its ability to target the enzyme DNA topoisomerase I.[9] The discovery of an endophytic fungus that is capable of producing **1** led us to envisage the possibility of using this organism to produce **1** under controlled fermentation conditions in an economical, environment-friendly, and reproducible manner amenable to industrial scale-up. Unfortunately, it was observed that a substantial decrease occurred in the production of **1** by this in vitro-cultured endophyte following repeated subculturing (i.e., in successive subculture generations; hereafter called "generations").[7] Optimized fermentation conditions and the addition of precursors as well as various host plant tissue extracts did not restore the production of **1** (Table S2, Supporting Information).

Here we have attempted to restore the impaired CPT (**1**) biosynthetic capability of the endophytic fungus *F. solani* by artificially inoculating it in *C. acuminata* plants followed by

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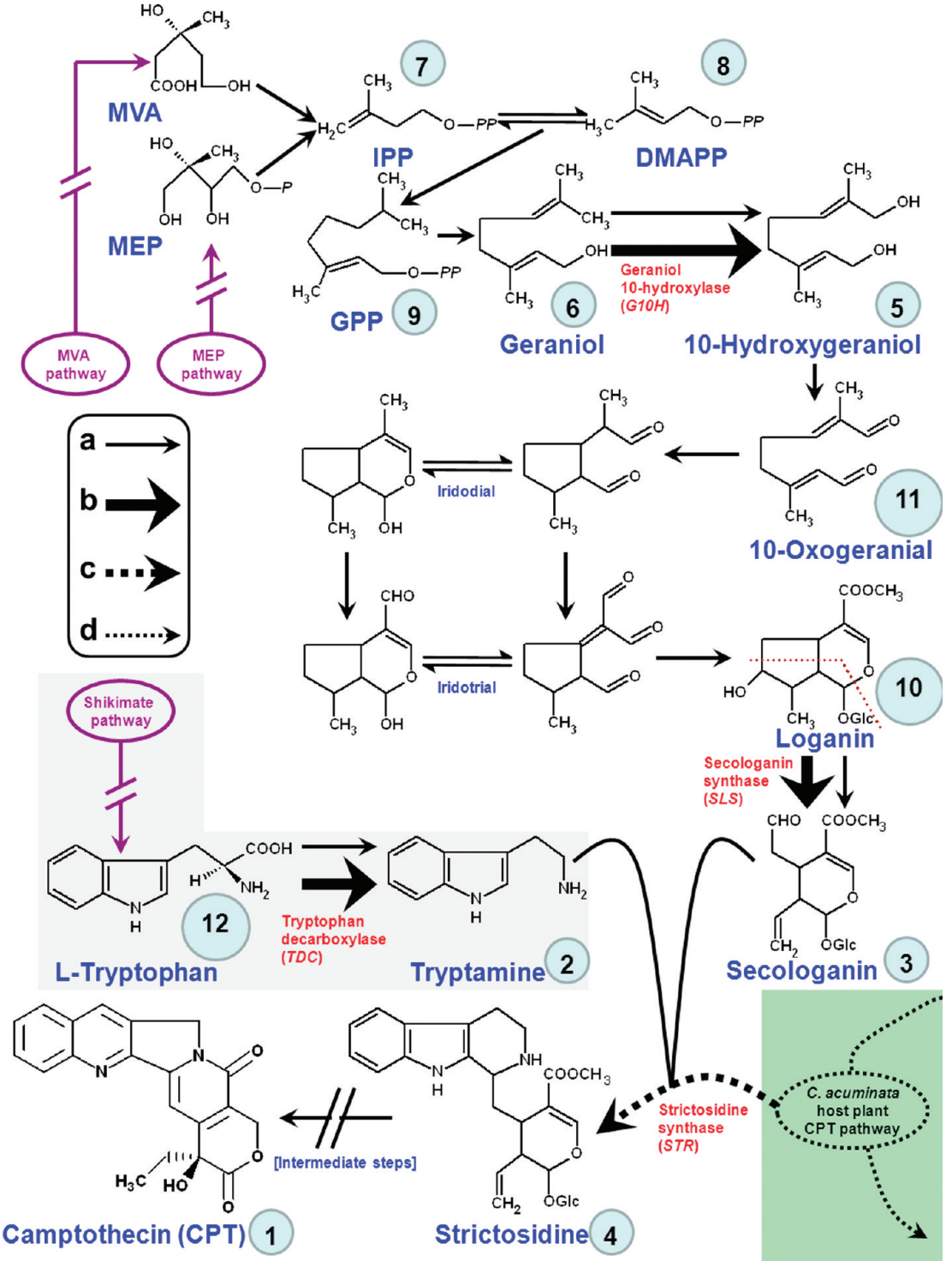

Figure 1. Schematic representation of the biosynthetic pathway of CPT (**1**) (a–c represented by arrows). (a) Proposed/putative/discovered biosynthetic steps from the literature (as detailed in the text). (b) Biosynthetic steps in endophytic *F. solani* verified/discovered in the present study. (c) Biosynthetic step in endophytic *F. solani* aided in situ by the host plant (*C. acuminata*) enzyme (strictosidine synthase, product of *STR*) verified/discovered in the present study. (d) Biosynthetic events occurring indigenously inside the host plant (*C. acuminata*) for the production of plant CPT (**1**).

recovery after in planta tissue colonization. Furthermore, the key steps of the biosynthetic pathway of **1** have been investigated, as studied earlier in plants (Figure 1a, and Scheme S3, Supporting Information), in the first (coded INFU/Ca/KF/3/I) and seventh (coded INFU/Ca/KF/3/VII) generation of the endophytic fungus employing a homology-based molecular approach and high-precision isotope-ratio mass spectrometry (HP-IRMS). It is shown that the endophyte utilizes indigenous *G10H* (geraniol 10-hydroxylase), *SLS* (secologanin synthase), and *TDC* (tryptophan decarboxylase) to biosynthesize the precursors of **1**. However, the endophyte requires the host *STR* (strictosidine synthase) in order to condense the nitrogen-containing moiety (tryptamine, **2**)

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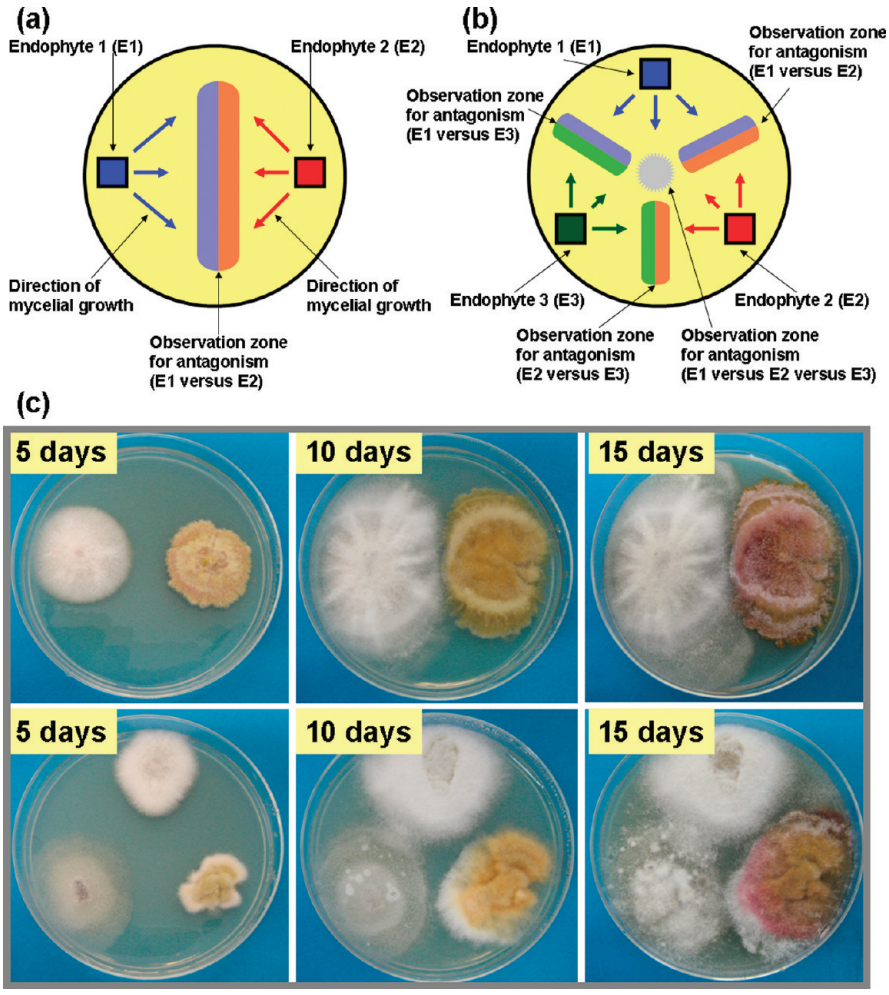

Figure 2. In vitro agar plate-based antagonism study between the various endophytic fungi. (a) Schematic representation of the inoculation zones, directions of growth, and zone(s) for observation of the in vitro antagonism between two endophytes. (b) Schematic representation of the inoculation zones, directions of growth, and zone(s) for observation of the in vitro antagonism between three endophytes simultaneously. (c) Representative plates showing how the agar plates were observed when considering the antagonism study between two endophytes and three endophytes in parallel. Growth after 5, 10, and 15 days is shown.

with the carbon-containing moiety (secologanin, **3**) to form strictosidine (**4**) and complete the biosynthesis of **1**. The present results reveal the causes of the impaired biosynthesis of **1** in the endophytic fungus over successive subculture generations. This investigation further emphasizes both short-term and long-term implications for endophyte biology, underlining not only the basic understanding of the plant−fungus cost−benefit mutualism but also the possibility of future industrial production of bioactive compounds from fungal endophytes.

RESULTS AND DISCUSSION

In Vitro Inoculation of Endophytic *Fusarium solani* for in Planta Colonization to Restore CPT (1) Biosynthesis. The metabolic regulation of endophytic fungi, cultured in vitro, differs substantially from that of the in planta association.[6,10] Endophytic *F. solani* starts to lose its capability for biosynthesizing **1** under axenic conditions. Drawing an analogy to the golden standards laid down by Koch's postulates for classical microbiology,[11,12] we attempted to restore the impaired fungal **1** biosynthesis by artificially reinfecting the *C. acuminata* host with the endophytic *F. solani* at its seventh-generation subculture (INFU/Ca/KF/3/VII). The different target host plants were selected based on the criteria for setting up the experimental model for the artificial inoculation of endophytic *F. solani* with the aim of recovering and characterizing it after in planta colonization. For this initial prescreening, endophytic fungi from the aerial parts of *C. acuminata* were isolated noting whether they contained the same endophyte under study and whether any of the endophytes isolated were capable of biosynthesizing **1** or related metabolites. First, none of the endophytic fungi isolated from the target host plants were capable of producing **1** or structural analogues. Second, none of the isolated endophytic fungi were *F. solani* (strain INFU/Ca/KF/3). Finally, using the rationale that the associated coexisting endophytes might pose additional antagonistic selection pressure to dictate the existence and in planta metabolomics of INFU/Ca/KF/3 after its artificial colonization postinoculation in the target hosts, in vitro antagonism of each of the isolated endophytes with INFU/Ca/KF/3 and with each other, and between that of INFU/Ca/KF/3 and INFU/Ca/KF/2 (Figure 2c), was evaluated. No apparent stress was observed in the plants after successfully inoculating the fungal endophyte, and there was no visible damage to the leaves and stems apart from the puncture wounds themselves (Figures 3a−c and 4a−g),

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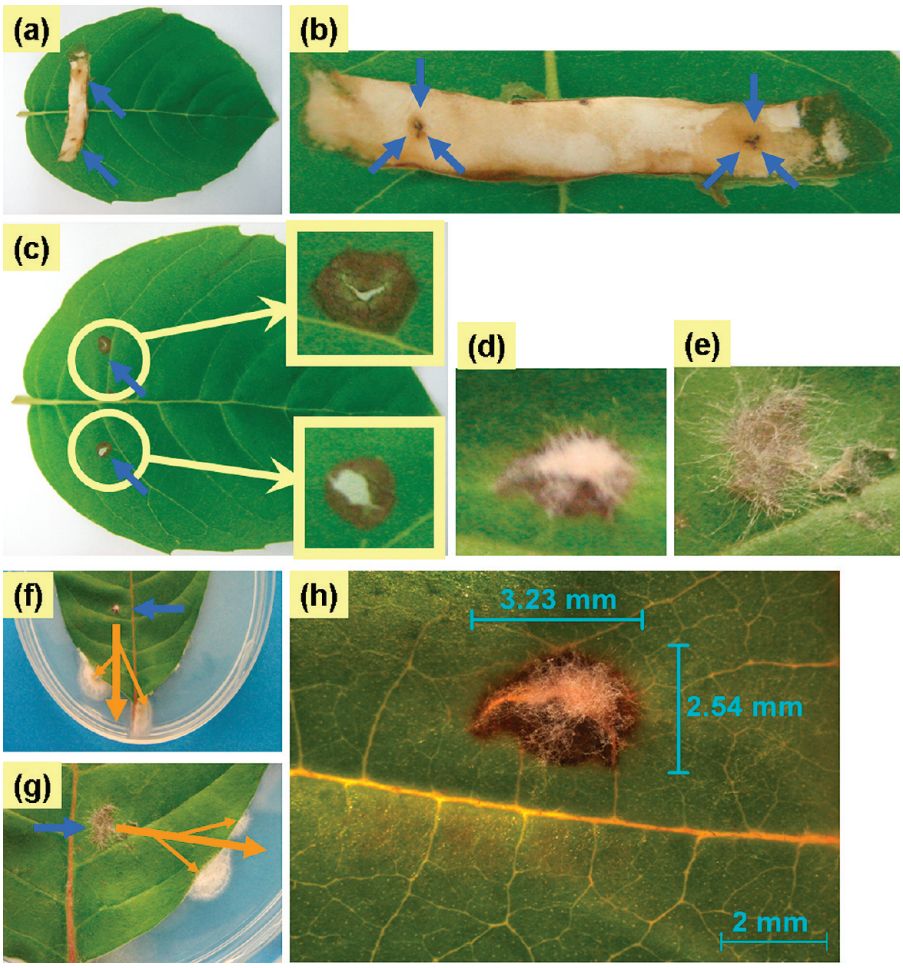

Figure 3. Representative pictures of artificial in vitro inoculation and in planta colonization of the seventh generation of endophytic *F. solani* (INFU/Ca/KF/3/VII) in the leaves of the target *C. acuminata* host plants followed by recovery, in an attempt to reverse the observed impairment of CPT (**1**) biosynthesis by the endophyte. (a, b) No apparent stress or visible manifestation could be observed except for the puncture wounds after removal of Parafilm (blue arrows) or after removal of endophytic mycelial mat (see c). (d, e) Endophytic *F. solani* could be recovered first at the site of wound. (f, g) Endophytic *F. solani* could be recovered later at the leaf-blade emerging a few cm away (orange arrows) from the original wound site (blue arrow), demonstrating the tissue colonization away from site of inoculation. (h) Close-up view of recovered endophytic *F. solani* INFU/Ca/KF/3 at the wound site, clearly showing no visible manifestation at other parts of the intact leaf.

similar to the control. From the leaf inoculations, fungi with white or off-white mycelia emerged on the agar plates both from the site of wound and in many cases from the edge of the leaf blades (Figures 3d−h) after 48 to 72 h. The fungi always emerged first at the wound site followed by emergence from the blade edge. The position on the plant where the leaf was situated did not affect the success of inoculation and recovery of the endophytic fungus. In those cases where the fungus emerged from the leaf blades (a few cm from the inoculation wound), tissue colonization had occurred well away from the inoculation site, demonstrating the affinity and specificity of the endophyte for the host. Additionally, there was no visible manifestation of the artificially established endophyte in the live host tissues even at the wound sites, corroborating the host affinity and the true endophytic nature of *F. solani* (INFU/Ca/KF/3). In the case of stem inoculations, recovery was higher for the inoculations in the upper part of the plants. The fungi emerging from the wound-inoculated stem pieces were visible only after 120 h of incubation, indicating that the colonization by the established endophyte was much slower in the stems than in the leaves. It might be either that the compactness of stem tissues was the main reason for slower colonization or that the fungus was better established within stem tissues, lessening the need to emerge. Unlike the leaves, no fungi emerged from the wound sites, maybe due to formation of callose at the wounded tissues; they emerged on all sides of the excised stem pieces starting close to the wound and then later further away (Figure 4h,i). The endophyte inoculated emerged from the leaf and stem tissues within 10−12 days (confirmed by ITS-5.8S rDNA sequencing), whereas no other resident endophyte emerged as fast, as expected.[4−6]

The CPT (1) Pathway Was Not Restored in Recovered Endophytic *Fusarium solani*. The axenic morphology of the infecting *F. solani* (INFU/Ca/KF/3/VII)[7] was compared with that of the recovered fungi (Figure 5a,b). It was noteworthy that the morphology of the recovered *F. solani* was similar (sporodochia could be seen by the naked eye, mostly creamy to pink but not blue or bluish-green) to that of the original *F. solani* INFU/Ca/KF/3 culture in its first generation.[7] The emergence pattern of the established endophyte from the plant tissues was evaluated (Figure 5c,d). The straight and pointed hyphae of the emerging fungi demonstrated the plausible inertia of growth gained during their existence under in vitro conditions.

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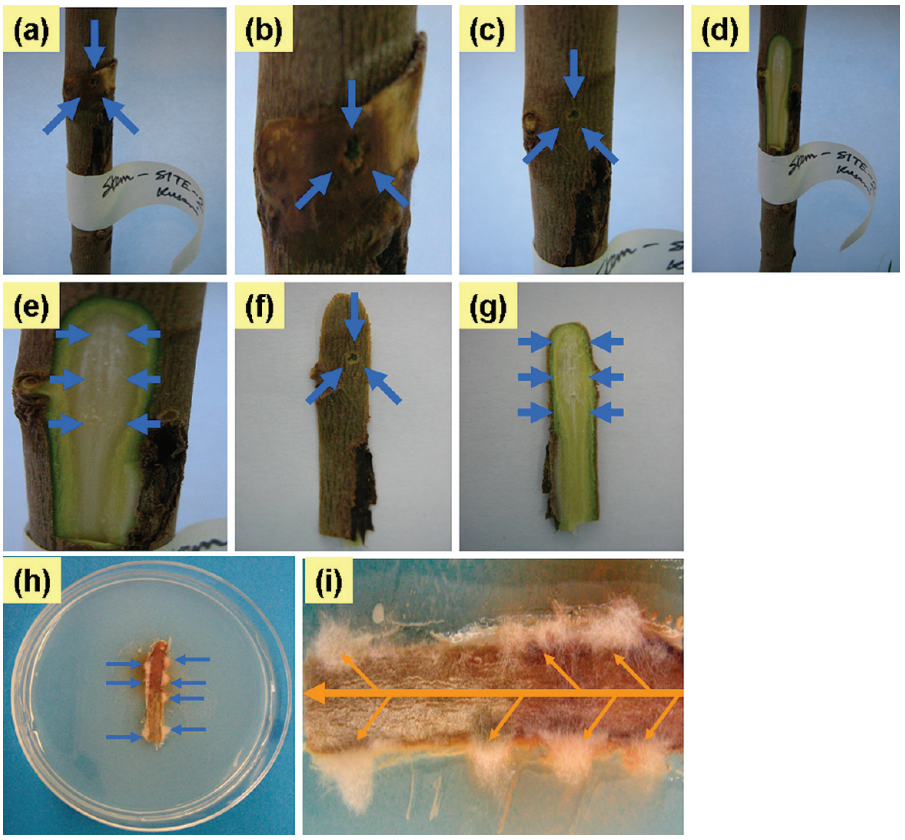

Figure 4. Representative pictures of artificial in vitro inoculation and in planta colonization of the seventh generation of endophytic *F. solani* (INFU/Ca/KF/3/VII) in the stems of the target host plants followed by recovery, in an attempt to reverse the observed impairment of CPT (**1**) biosynthesis by the endophyte. (a, b) Stem surface view with intact mycelia after one week of incubation. (c−g) Stem outer and inner surface view after removal of mycelial mat. (h, i) Endophytic *F. solani* could not be recovered at the site of wound. However, it could be recovered later at the stem edges emerging a few cm away, demonstrating the tissue colonization away from site of inoculation. The orange arrows show the direction of tissue colonization based on the gradual delay in recovery (emergence).

Furthermore, scanning electron microscopy (SEM) was performed on both the endophyte used for inoculating the plants and the recovered endophytes for comparative evaluation (Figure 5e−i). SEM revealed that the infecting *F. solani* was composed primarily of straight, overlapping, compact, and pointed hyphae in a particular direction before the host infection. However, the recovered *F. solani* contained several intertwined, criss-crossed, and meshed hyphae, resembling a net. It would seem that the endophytic fungus started in planta exploration for colonization after artificial inoculation. Further, after reconstitution of the endophyte−plant association, there might have been induction of certain genes responsible for morphological changes, even though the expected genes (pathway of **1**) were not activated. All the recovered fungi were established as axenic cultures and confirmed as being identical to the original *F. solani*, cultured under shake-flask conditions, and extracted and analyzed using the same procedures as for the original endophytic *F. solani*.[7] All recovered *F. solani* had completely stopped producing **1**, demonstrating the biosynthetic dysfunction. The reactive intermediates of the elucidated **1** pathway could also not be isolated and identified with LC-ESI-HRMS^n. Thus, even though the endophyte could be successfully colonized in its host (*C. acuminata*), the biosynthetic pathway of **1** was not restored.

Perspectives on the Host−Endophyte Affinity. Colonization of the in vitro-cultivated endophytic *F. solani* could be successfully established in *C. acuminata*. This was independent of the origin or mode of propagation and growth of the host plants, revealing that the host affinity and specificity of this endophyte might be species-specific. The ability of the endophyte to infect was also completely unrelated to its ability to biosynthesize **1**. The pathway could not be recreated even by restoring the endophyte−host association, although the in planta strictosidine synthase was available to the endophytic fungus. In addition, none of the intermediates were found to be accumulated in the recovered endophytes. Thus, once the biosynthetic potential of the endophyte to produce **1** was impaired, it could not be restored even by providing the original host environment where the endophyte would have evolved the biosynthesis of **1**. It is possible that the ability of the endophytic fungus to infect the host and its regulation (i.e., its host affinity and specificity) might be partially or completely lost on preservation outside the host and on repeated subculturing in axenic conditions.

CPT (1) Biosynthetic Steps in the Endophytic Fungus. In order to understand the mutualistic association of the endophyte with the host plant and the reason for the reduction of **1** production on subculturing that could not be reversed even by recreating the host−endophyte interaction, the key steps of the biosynthesis of **1** were evaluated in the endophytic fungus based on the knowledge of these steps in plants. We screened for the presence of the *G10H*, *SLS*, *TDC*, and *STR* genes in the endophytic fungal genome (first generation, coded INFU/Ca/KF/3/I) using gene-specific and/or degenerate primers (Figure 6 and

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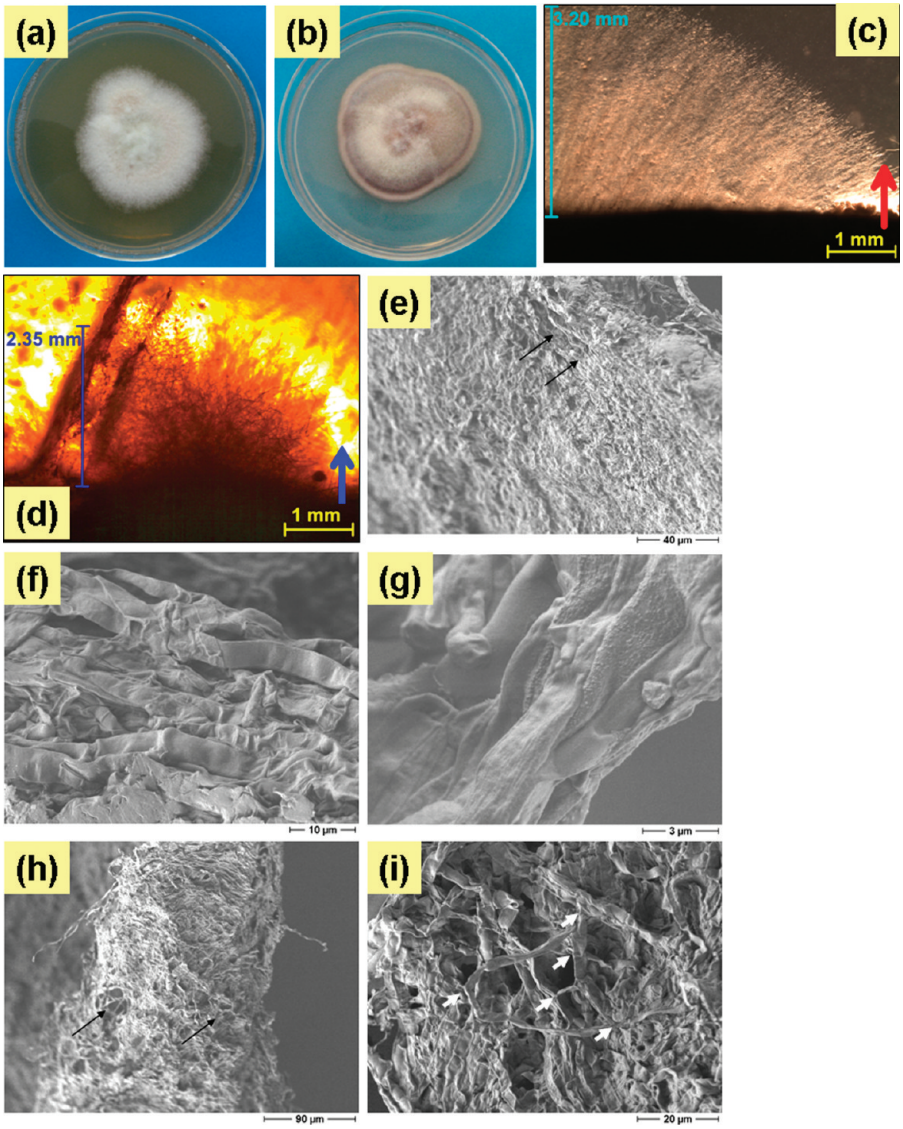

Figure 5. Macroscopic and microscopic evaluation of endophytic *F. solani* artificially inoculated in the target host *C. acuminata* plants as compared to the recovered *F. solani* postinfection. (a) Seventh generation of *F. solani* on SA medium before infection. (b) Representative morphology of the recovered *F. solani* after colonization in the target hosts on SA medium. (c) Representative bright field picture of endophytic hyphae emerging out of the *C. acuminata* leaf during recovery of *F. solani*. (d) Representative bright field picture of endophytic hyphae emerging out of the *C. acuminata* stem during recovery of *F. solani*. (e−g) SEM micrographs of the original *F. solani* before establishment in the host plants. (h, i) SEM micrographs of the recovered *F. solani* after colonization in the host plants (scales: e = 40 μm, f = 10 μm, g = 3 μm, h = 90 μm, i = 20 μm).

Table S4, Supporting Information). Amplification products of the expected sizes were obtained using the primers designed for *G10H*, *SLS*, and *TDC* (Figure 6a, and Tables S5 and S6, Supporting Information). The deduced amino acid sequence of the fungal *G10H* revealed 100% homology to the geraniol-10-hydroxylase enzyme (EC 1.14.14.1; UNIPROT: Q8VWZ7). The translated product of fungal *SLS* exhibited 100% homology to the cytochrome P450 (UNIPROT: Q42700) and secologanin synthase enzyme (EC 1.3.3.9; UNIPROT: Q05047). The translated *TDC* protein sequence from the endophyte *F. solani* showed homology (100%) to tryptophan decarboxylase enzyme (EC 4.1.1.28; UNIPROT: P93082). Interestingly, no product was obtained with any *STR* gene-specific primer (located in highly conserved regions) under any PCR condition (Figure 6b,d). Since no gene coding for strictosidine synthase, which is responsible for the condensation of **2** and **3**, was found in the genome of the endophytic fungus, the contribution of the host plant could be attributed to the completion of the biosynthesis of **1**.

Use of the Host Strictosidine Synthase by the Endophytic Fungus. In order to evaluate whether the host plant (*C. acuminata*) actually contributes to the completion of the in planta fungal biosynthesis of **1**, the *STR* gene was isolated and characterized from the *C. acuminata* genome using *STR* gene-specific primers (Table S4, Supporting Information). An amplification product of the desired size was obtained using a suitable *STR* primer set, under optimized PCR conditions and multiple purification of the genomic DNA (gDNA) from interfering agents (Figure 6c,d, and Tables S5 and S6, Supporting Information). The deduced amino acid sequence revealed 100% (UNIPROT: P18417) and 86% (UNIPROT: P68175) homology to the strictosidine synthase enzyme (EC 4.3.3.2). This suggested that the endophytic fungus utilized host strictosidine synthase to

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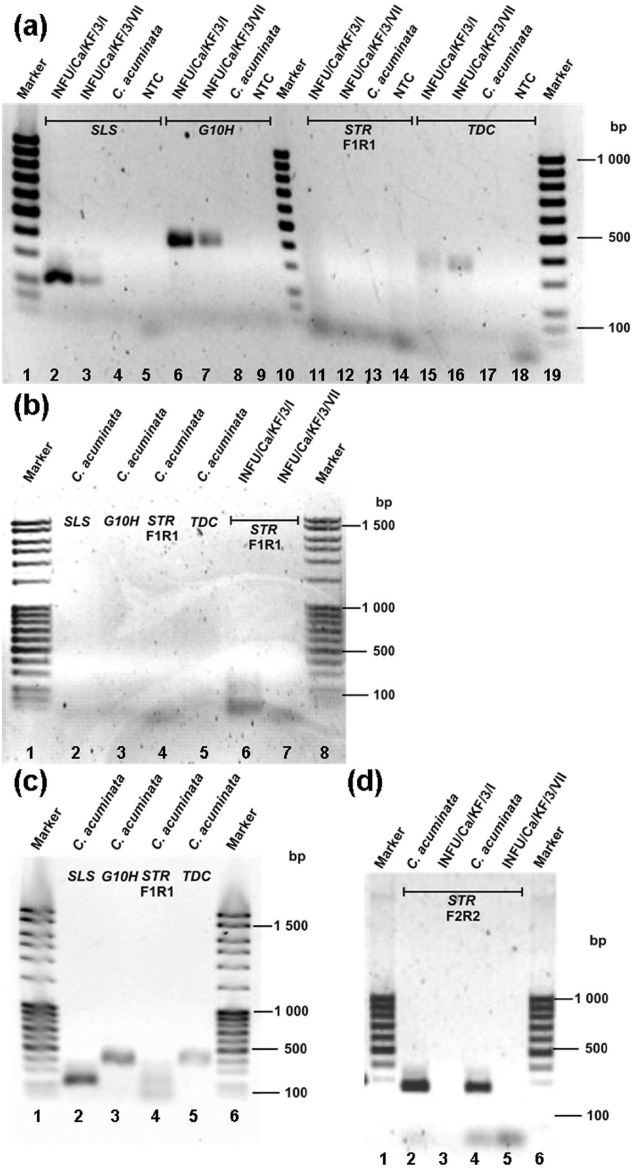

Figure 6. Stained agarose gels of PCR-amplified DNA from endophytic *F. solani* in its first (INFU/Ca/KF/3/I) and seventh (INFU/Ca/KF/3/VII) generation subcultures and from the *C. acuminata* host plant, encoding geraniol 10-hydroxylase (*G10H*), secologanin synthase (*SLS*), tryptophan decarboxylase (*TDC*), and strictosidine synthase (*STR*). (a−d) Desired products were obtained in each case using gene-specific and/or degenerate primers under optimized PCR conditions with specific templates, as detailed in the text. NTC, no template control.

condense **2** with **3** to form **4**, which it might have carried over into its biomass during the isolation procedure. The absence of *STR* in the endophytic fungus (Figure 6d) and the analyses of the plant *G10H*, *SLS*, and *TDC* sequences (Figure 6c) further confirmed that none of the genes identified in the endophyte were the remnants of host plant DNA in the fungal biomass. Thus, a cross-species biosynthetic pathway of **1** may be proposed. However, this biosynthetic mechanism is not the sole cause of the observed reduction of in vitro fungal **1** production (vide infra), as all attempts in reversing it (optimized fermentation conditions and recreating host−endophyte interaction) have been unsuccessful.

Confirmation of the Contribution of the Host Plant by HP-IRMS. It was confirmed that the endophytic fungus actually utilizes host strictosidine synthase using high-precision isotope-ratio mass spectrometry by compound-specific carbon isotope (CSCI) and compound-specific nitrogen isotope (CSNI) modules. HP-IRMS relies on the fact that the stable isotope composition of a secondary metabolite is determined by the precursor isotope composition and the isotopic fractionation processes occurring during enzyme-mediated biosynthetic incorporation, namely, the enzyme-kinetic isotope effect (E-KIE).[13−17] Compound **1** produced by the cultured endophyte (first generation, INFU/Ca/KF/3/I) outside the host plant in a nitrogen-free medium was compared to that from the tissue (not containing *F. solani* INFU/Ca/KF/3, INFU/Ca/KF/2, or other **1**-producing endophytes) of the original *C. acuminata* host (from SWFU), to check both the $\delta^{13}C/^{12}C$ (by CSCI) and the $\delta^{15}N/^{14}N$ (by CSNI). It was possible to trace the exact pattern of the accumulation of both "carbons" and "nitrogens" with the source of the enzyme(s) (fungal or plant) concerned up to and including the formation of

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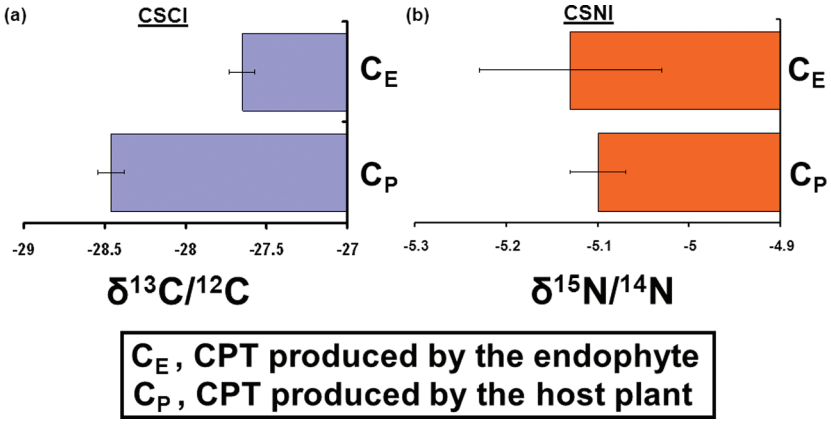

Figure 7. HP-IRMS plots by CSCI and CSNI showing the $\delta^{13}C/^{12}C$ and the $\delta^{15}N/^{14}N$ ratios between the CPT (**1**) biosynthesized by the endophytic fungus (*F. solani* INFU/Ca/KF/3) and the original host plant (*C. acuminata*). (a) CSCI. (b) CSNI.

1 in the endophytic fungus and in the host plant. The $\delta^{13}C/^{12}C$ of the endophytic and plant **1** were found to be 27.65 ± 0.1‰ and 28.46 ± 0.08‰, respectively (Figure 7a). This significant difference between the stable carbon isotope ratios of the **1** biosynthesized by the endophyte and the plant thus corroborates the hypothesis from the homology-based approach that the building of the carbon skeleton in the endophyte has been achieved independently by the fungal enzymes. The carbon signature of the culture media contributed partly toward the observed variation in $\delta^{13}C/^{12}C$ values. On the other hand, the $\delta^{15}N/^{14}N$ ratios of the endophytic and plant **1** were found to be 5.13 ± 0.1‰ and 5.10 ± 0.03‰, respectively (Figure 7b). Thus, no significant difference between the stable nitrogen isotope ratios of **1** produced by the endophyte and the plant was observed. This established without a doubt that the endophytic fungus utilizes the plant enzyme strictosidine synthase, which had been carried over into the biomass of the isolated endophyte.

Perspectives on the Proposed Cross-Species CPT (1) Biosynthesis. Combining the homology approach and HP-IRMS, a plant−fungal cross-species **1** biosynthesis may be proposed in which the endophytic fungus utilizes indigenous geraniol 10-hydroxylase, secologanin synthase, and tryptophan decarboxylase to biosynthesize **1** precursors (Figure 1b), but then requires the host strictosidine synthase to complete the biosynthesis (Figure 1c). The endophyte accumulates 10-hydroxygeraniol (**5**) from geraniol (**6**), using the geraniol 10-hydroxylase enzyme, which in turn is made from IPP (**7**) and DMAPP (**8**) via the formation of GPP (**9**) (Scheme S3, Supporting Information). This suggests that either the mevalonate (MVA) or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which have been studied in many plants and fungi, might be responsible for initially forming **7** and **8**.[18−20] Further, the fungal secologanin synthase converts loganin (**10**) to **3**. Loganin (**10**) is synthesized from **5** via 10-oxogeranial (**11**) and further iridotrial intermediates, by cyclization and randomization of methyl groups.[21] The biosynthetic pathway in the fungal endophyte up to **3** demonstrates the incorporation of carbon alone; the assimilation of nitrogen occurs via an indole moiety (**2**). Fungal tryptophan decarboxylase is responsible for the enzymatic conversion of L-tryptophan (**12**) to **2**, channeled through the shikimate pathway.[22] Since *F. solani* strains from other sources are incapable of the biosynthesis of **1**, endophytic *F. solani* has de facto been subjected to some kind of in planta selection pressure to enable the activation of the development of biogenesis of **1**. It is compelling that during the coexistence of this endophyte with *C. acuminata* these two interacting organisms coevolved (host−microbe coevolution) to develop the production pathway of similar compounds (in this case, **1**) that exert similar biotic effects on other organisms.

Random and Unpredictable Nonsynonymous Mutations in CPT (1) Biosynthetic Genes in the Seventh-Generation Subculture. Although the endophyte shares the biosynthesis of **1** with its host plant (using strictosidine synthase), the pathway could not be restored even after recreating the endophyte−host association. Hence, the availability of the host enzyme did not restore the biosynthesis of **1** in the endophytic fungus. Therefore, using knowledge of the key biosynthetic steps of **1** in the first generation of the endophytic fungus, it was checked whether or not there might be additional explanations for the impaired **1** biosynthesis over successive generations. Similar products were isolated from the genome of the endophytic fungus in its seventh subculture (INFU/Ca/KF/3/VII) as performed for the first generation. Using the same primers and PCR conditions as for the first generation, specific products with the desired sizes were also obtained for *G10H*, *SLS*, and *TDC* in the seventh generation (Figure 6a, and Tables S4 and S5, Supporting Information). Like the first generation, *STR* was not found in the seventh generation (Figure 6b,d). We sequenced the obtained PCR products to check whether there might be some alteration or instability in the CPT (**1**) biosynthetic genes under in vitro conditions over several subcultures. This approach consisted in comparing all the obtained deduced translation products with the databases, as was carried out with the first generation (Table S6, Supporting Information). *G10H*, *SLS*, and *TDC* revealed nonsynonymous mutations, which exhibited highly reduced similarities to the original products: at least 96% for *G10H*, at least 86% for *SLS*, and at least 48% for *TDC* at the amino acid level. The similarities always varied in different replicates, revealing that the mutations in CPT (**1**) biosynthetic genes were random and not always the same on repeated subculturing.

Instability of CPT (1) Biosynthetic Genes Led to Dysfunctional Proteins. The final enzyme identities (ORF-based translated) of the above biosynthetic genes were compared from the first- and the seventh-generation subculture to confirm the cause of impaired biosynthesis of **1**. The EMBOSS-WATER bioinformatics tool based on the Smith−Waterman local alignment algorithm[23] was utilized to align (local) and compare the predicted structure of the fungal enzymes in the seventh-generation

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subculture to that of the original enzymes in the first generation. Random alterations leading to irreversible dysfunction at the amino acid level were revealed; the seventh-generation-predicted proteins exhibited only 98.1−99.4% (*G10H*), 92.0−98.8% (*SLS*), and 47.6−81.7% (*TDC*) similarities to the original enzymes. The similarities always varied within this range in different replicates, revealing that the mutations in the biosynthetic genes of **1** were not always of the same type on repeated subculturing. The EMBOSS-NEEDLE bioinformatics tool based on the Needleman−Wunsch global alignment algorithm[24] was further used to evaluate the optimal globally aligned dynamic scores of the seventh-generation-predicted proteins with that of the general structures of the actual enzymes. The results agreed with the local alignment scores, revealing that degradation of the biosynthetic genes of **1** under ex planta axenic conditions led to dysfunctional proteins. The seventh-generation-predicted proteins exhibited only 86.6−99.1% (*G10H*), 84.6−98.2% (*SLS*), and 22.4−70.2% (*TDC*) similarities to the general structures of the original proteins, in different replicates. The primary structure of the control gene *Top1* was, however, not degraded or destabilized over repeated in vitro subculturing, revealing that instability of the CPT (**1**) biosynthetic genes was not reflected in the primary metabolic processes in the endophytic fungus. Furthermore, the rRNA gene in the endophytic isolate at its seventh generation was checked. It was revealed that the fungal rDNA was still intact as the first parent isolate even in the seventh subculture. Thus, it was proved that the primary metabolic processes and the functions of the housekeeping genes were not destabilized on repeated subculturing.

Perspectives on Impaired CPT (1) Biosynthesis on Subculturing. The pattern of decrease in production of **1** by the cultured endophyte[7] revealed a gradual decrease from the first to second generation followed suddenly by a drastic drop to practically negligible amounts from the third generation. The present results might clarify this observation in that the endophytic fungus probably carried over the plant strictosidine synthase enzyme during the isolation procedure, enough to last only up to the second-generation subculture, thereby causing the sudden drop in amounts of **1** from the third generation. This may be a practical possibility owing to the high stability of this enzyme, even on repeated freezing and thawing.[25] Evaluation of the biosynthetic genes in the seventh-generation subculture of the endophytic fungus revealed nonsynonymous alterations leading to irreversible dysfunction at the amino acid level, rendering the enzymes of the pathway dysfunctional. This was also evidenced by the fact that optimized fermentation conditions and the addition of precursors as well as of various host plant tissue extracts did not restore the production of **1**. Furthermore, artificial inoculation and colonization of the endophytic fungus within the host plant did not restore **1** biosynthesis. Finally, none of the subculture generations demonstrated accumulation of the reactive intermediates, revealing that the destabilization of the genes of the CPT (**1**) pathway would have commenced immediately under the in vitro axenic conditions due to the lack of in planta selection pressures. As expected, the primary structure of the control gene, *Top1*, was not degraded or destabilized over repeated in vitro subculturing. This revealed that instability of the CPT (**1**) biosynthetic genes (secondary metabolism) was not reflected in the primary metabolic processes in the endophytic fungus. Furthermore, the fungal rDNA was still intact in the seventh subculture as in the first parent isolate, showing that the functions of the housekeeping genes were not destabilized on repeated subculturing.

The results reported in this study emphasize the difficulties ahead if endophytic fungi are to be exploited for industrial production of bioactive secondary metabolites. The discovery of an endophytic microorganism might not be sufficient to clear the way for drug production. It is also necessary to understand the endophyte−host mutualism and biosynthetic pathway(s) employed for production of host-specific metabolites by endophytes in order establish or restore in vitro biosynthetic potential. Multistep processes are now needed to secure information about the exact endophyte−host mutualism, and only the holistic understanding of this relationship on a case-by-case basis can lead the way toward commercialization.

EXPERIMENTAL SECTION

Isolation, Identification, Culturing, Maintenance, and Storage of Endophytic Fungi. The endophytic *F. solani* used in the present study was initially isolated from asymptomatic inner bark explanted from a fully matured *C. acuminata* tree collected from the Southwest Forestry University (SWFU) campus, Kunming, Yunnan Province, People's Republic of China.[7] Following isolation, the fungus was fully characterized using classical morphology and physiology, macroscopic and microscopic evaluations, and ITS-5.8S rDNA sequencing, and its indigenous biosynthetic capacity was elucidated by shake-flask fermentations followed by multicomponent high-resolution tandem mass spectrometry (LC-ESI-HRMS^n), when the phenomenon of the reduction of **1** biosynthesis on subculturing was also observed.[7] The axenic culture of *F. solani* thus obtained was coded as INFU/Ca/KF/3 and deposited at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ), Braunschweig, Germany. Another endophytic fungus incapable of producing **1** was also obtained from the same bark explant as *F. solani*, which was isolated, identified, and characterized as *Albonectria rigidiuscula* using the same procedure[7] and coded INFU/Ca/KF/2 (for in vitro antagonism studies). Both the fungal endophytes have been routinely maintained on PDA (potato dextrose agar; DIFCO), SA (Sabouraud dextrose agar; DIFCO), and CDA (Czapek-Dox agar; Merck, Darmstadt, Germany) in active form. For long-term storage, the colonies were preserved in the vegetative form in 15% glycerol at −70 °C. Agar blocks impregnated with mycelia were used directly for storage of the vegetative forms.

In Vitro Inoculation of *Fusarium solani* in Living *Camptotheca acuminata* and Its Recovery after Colonization. *C. acuminata* plants were sampled from different botanical gardens and tissue culture laboratories across Germany (Table S7, Supporting Information). We isolated the endophytic fungi from the aerial parts (leaves and stems) of the above plants with special emphasis on whether they contained the same endophyte under study (*F. solani* INFU/Ca/KF/3) and whether any of the endophytes isolated were capable of biosynthesizing **1** or related metabolites. We employed established methods to do this.[7] Only those plants were selected as suitable target hosts where INFU/Ca/KF/3 was not one of the endophytes and that did not produce **1** or related metabolites. Finally, using the rationale that the associated coexisting endophytes might pose additional antagonistic selection pressure to dictate the existence and in planta metabolomics of INFU/Ca/KF/3 after its artificial colonization postinoculation in the target hosts, we evaluated the in vitro antagonism of each of the endophytes isolated with INFU/Ca/KF/3 and with each other and also between that of INFU/Ca/KF/3 and INFU/Ca/KF/2 (Figure 2a,b). The only plants considered as potential target hosts for the artificial fungal establishment were those that did not possess any in vitro antagonistic association with each other or with endophytic *F. solani* INFU/Ca/KF/3.

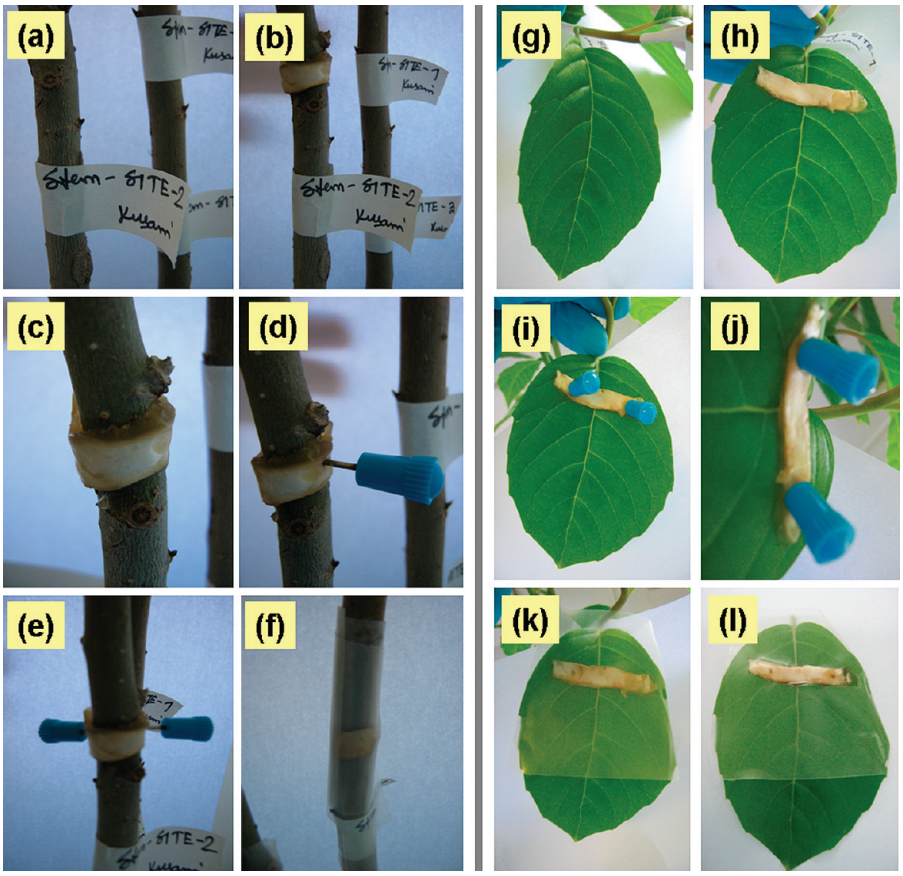

Figure 8. Representative pictures of artificial in vitro establishment of the seventh generation of endophytic *F. solani* in the stems and leaves of the target *C. acuminata* host plants. (a, g) Representative target stem and leaf for artificial inoculation of endophyte. (b, c, h) A 10-day-old mycelial mat placed over the surface-sterilized stem and leaf surface. (d, e, i, j) Pinpoint puncture wounds created through the mycelial mat into the stem and leaf surface below with sterilized needles mediating breakage of mycelia and plant tissue at the same point. (f, k, l) Undisturbed experimental setup for endophyte infection and colonization within the living host tissues.

The seventh generation of endophytic *F. solani* (INFU/Ca/KF/3/VII), with its CPT (**1**) biosynthetic potential impaired, was used to inoculate the selected target *C. acuminata* host plants using previously reported methodology,[26] suitably modified. The plants were first acclimatized under controlled laboratory conditions at the site of the infection studies for a few weeks, by planting them in medium-sized pots containing high-value fertile potting soil (Floragard Vertriebs GmbH, Germany) with very specific physicochemical properties (Table S8, Supporting Information) and maintaining them in a moderate-humidity chamber with natural light. The temperature was maintained at 25−28 °C, and the plants were watered moderately on alternate days. Using the rationale that the plants in their natural environment constantly interact with their dynamic surroundings, we maintained only a precisely controlled and not a totally sterile environment during the experiments. Each plant was inoculated at the leaves and stems in triplicates in three zones, namely, upper (toward the apex), central, and lower (toward the base/roots), in order to get an overall notion as to whether the age or physiology of host tissue dictates the endophyte affinity, infectivity, and metabolomics. The target sites of infection were briefly decontaminated from the unwanted surface microorganisms by swabbing the surfaces with sterile paper tissues dampened with 70% ethanol. Each plant site was inoculated by placing a 10-day-old mycelial mat of endophytic *F. solani* carefully teased from the SA surface, over the tissue surface (leaf and stem), then puncturing both mat and plant surface with sterilized needles (Figure 8a−e, g−j). The puncture wounds allowed the breakage of both the mycelia and the plant tissue at the same point. This provided the perfect opportunity for the new hyphae growing at the fungal wound site where the mycelial mat was damaged to enter the plant tissue through the plant wound site. The mycelial mats adhering to the plant surfaces were wrapped with Parafilm to prevent dislocation of the wound sites and monitored regularly without disturbance (Figure 8f,k,l). A control set of leaves and stems was also set up but without the fungal inoculum having been placed on the surface. A week after inoculation, the incubation was terminated by removing the fungal mycelial mats from the plant surfaces. The surfaces were again swabbed with sterile paper tissues dampened with 70% ethanol. The infected leaves and stems (including the wound sites) were then excised from the plants, and surface-sterilization and recovery of the in planta colonized endophyte were conducted using established procedures.[7] The recovered endophytes were pure-cultured in SA subsequent to emersion from the plant tissue explants into the water agar (supplemented with antibiotic) medium.

Microscopic Examination of Endophytic Fungus before Infection and after Recovery. The endophytic hyphae emerging from the plant tissues were observed directly and characterized under a bright field stereomicroscope to evaluate the emergence pattern of the colonized endophyte from the plant tissues. In addition, the hyphae from the agar plate were aseptically transferred to slides for microscopy. A Leica S8 APO Greenough stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Schott KL 1500 compact halogen cold light source (Schott AG, Mainz, Germany) was used to examine the microscopic features of the plant-associated and axenic endophytic fungi. The images were captured using a Leica EC3 digital camera (Leica Microsystems) and were processed using the Leica

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Application Suite LAS EZ version 1.6.0 (Leica Microsystems). The *F. solani* inoculum (INFU/Ca/KF/3/VII) and the recovered endophytes were further processed for SEM (scanning electron microscopy). The fungi growing on SA were processed as described elsewhere,[26] critical-point dried, and carbon coated, and micrographs were recorded with a Hitachi S4500 SEM in an accelerating voltage of 1.0 kV using an SE (secondary electron) detector.

Detection, Screening, Amplification, and Characterization of CPT (1) Biosynthetic Genes. The endophytic fungus (first and the seventh generation in parallel, namely, INFU/Ca/KF/3/I and INFU/Ca/KF/3/VII) was grown on SA for 5 days at 28 ± 2 °C. Then, the mycelia were scraped directly from the agar surface and weighed, and the total genomic DNA was then isolated and purified using the Macherey-Nagel NucleoSpin Plant II Maxi Genomic DNA extraction kit, following the manufacturer's guidelines.[27] Fresh *C. acuminata* bark tissue (5 g) that did not contain INFU/Ca/KF/3, INFU/Ca/KF/2, or other **1**-producing endophyte was used for isolating plant gDNA. Here, an additional purification step was performed for plant gDNA in order to completely eliminate any contaminants and interfering agents, by precipitation of 2 × 500 μL of DNA solution with 2% 2-propanol, then pooling, followed by Q-sepharose cation purification, and final cleanup and concentration with a Qiagen MinElute purification kit (Qiagen, Hilden, Germany) to a final volume of 12 μL. The purified DNA template qualities were evaluated using a NanoDrop Micro-Volume UV−vis spectrophotometer. Amplification of the *G10H*, *SLS*, *TDC*, and *STR* genes from the endophytic fungal (two clones in parallel each from the different generations) as well as from the plant gDNAs (duplicates) was attempted using the gene-specific and/or degenerate primers designed for each gene by aligning the nucleotide sequences (Clustal 2.0) of the respective genes reported so far and by choosing the maximum conserved regions of these sequences (Table S4, Supporting Information). The PCRs were performed in triplicate by optimizing the conditions in each case based on the template used and the target product as detailed in Table S5 (Supporting Information). The PCR products were purified and desalted using the Chargeswitch purification kit (Invitrogen, Carlsbad, CA) and sequenced (bidirectional, at least 2× coverage) on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The final base sequences were established by base calling (noting the data quality and confidence for each base) and trimming with the Phred 0.020425c software. The final sequences were subjected to six-frame translation and interpreted based on the open reading frames (ORFs) to obtain the putative amino acid sequences of the expected products (Table S6, Supporting Information). The final product alignments were performed by the EMBOSS-WATER bioinformatics tool based on the Smith−Waterman local alignment algorithm[23] and the EMBOSS-NEEDLE bioinformatics tool based on the Needleman−Wunsch global alignment algorithm,[24] using the Blosum62 matrix (EMBL). The sequences of all the products have been deposited at the EMBL-Bank.

Screening, Amplification, and Characterization of Control Genes. Since *F. solani* from other sources is incapable of producing **1**, suitable control genes were selected for this study to examine whether the instability of the CPT (**1**) biosynthetic genes (i.e., secondary metabolism) over successive subculture generations was reflected in the primary metabolic processes and functioning of the housekeeping genes in the endophytic fungus. The *Top1* gene, encoding topoisomerase I enzyme, and the rDNA of the endophyte in its first and seventh generation were used as controls. The *Top1* cDNA fragments from the first and seventh generation of the endophyte were amplified by RT-PCR (reverse transcriptase-polymerase chain reaction) with degenerate primers. The template for RT-PCR (cDNA) was prepared by reverse transcription of total RNA with 200 U M-MLV reverse transcriptase (Promega, Madison, WI) and 10 mM anchored oligoT primer at 42 °C. The RNA from the endophyte was isolated using an RNeasy-Plant Mini Kit (Qiagen, Valencia, CA). The PCR primers (Table S4, Supporting Information) were designed using GenTool Lite 1.0 software based on the nucleotide sequence alignment (ClustalX 2.0) of *Top1* coding sequences from *C. acuminata* (AB372511), *Fusarium culmorum* (FJ93-8238), and a hypothetical protein from *Giberrella zeae* (XM_387050). Several sets of PCR primers were constructed to cover the whole functional and direct/indirect **1**-binding domains of *Top1*. The desired products were amplified by touchdown-style RT-PCRs. PCRs were performed in 30 μL reaction volume [0.5 μM forward and reverse primer; 1x iQ Supermix (Biorad, Hercules, CA) containing 0.2 mM dNTPs; 3 mM $MgCl_2$, and 0.7 U hot start iTaq DNA polymerase; and 30−50 ng reverse transcribed RNA/cDNA]. The reaction conditions were as follows: 4 min at 95 °C, 30 cycles (30 s at 94 °C, 30 s at 69 °C decreased by 0.4 °C per cycle, 45 s at 72 °C), 20 cycles (30 s at 94 °C, 30 s at 54 °C, 50 s at 72 °C), and 4 min at 74 °C. The sizes of the amplified products were checked by electrophoresis in 2% agarose TAE gel stained with GoldView (0.005% v/v, SBS, Beijing, People's Republic of China). The amplified products were purified by Wizard, SV Gel, and PCR Clean-Up System (Promega, Madison, WI) and directly sequenced with the forward and reverse primer. Overlapping fragments of *Top1* were assembled and aligned with available *Top1*s. Based on the ORF alignments, the acquired sequences were interpreted and putative amino acid sequences were deduced (numbered according to human Topo 1) and compared with the coding amino acid sequences from different plants and fungi. The fungal rDNA analysis was performed following the previously established procedure[7] in its first and seventh generation; additionally, rDNA was analyzed after recovering the endophyte from the artificial plant inoculation experiments. The *Top1* and rDNA sequences obtained have been deposited at the EMBL-Bank.

High-Precision Isotope-Ratio Mass Spectrometry (HP-IRMS). CPT (**1**) biosynthesized by the cultured endophyte (first generation, INFU/Ca/KF/3/I) outside the host plant and that from the tissues (not containing *F. solani* INFU/Ca/KF/3, INFU/Ca/KF/2, or other **1**-producing endophytes) of the original host plant (*C. acuminata*, from SWFU), respectively, were the samples that were analyzed by HP-IRMS. The fungal **1** was obtained by shake-flask fermentation in nitrogen-free potato dextrose broth (PDB), followed by extraction and analysis, as detailed earlier.[7] The host plant **1** was extracted in a similar manner to Kusari et al.[28] from tissue that did not contain INFU/Ca/KF/3, INFU/Ca/KF/2, or other **1**-producing endophytes, and pure **1** was isolated by preparative HPLC and identified by LC-ESI-HRMS^n (vide infra). The samples were readied for HP-IRMS in each case by placing 0.5 mg of **1** in 3.5 × 5 mm tin capsules (HEKAtech GmbH, Germany), lyophilizing completely, and finally rolling the capsules into small spheres. The HP-IRMS measurements were performed by suitable modifications of established methods.[13−17] Briefly, the HP-IRMS measurements were performed in compound-specific carbon isotope and compound-specific nitrogen isotope modules, using a FlashEA 1112 elemental analyzer (Thermo Fisher, Italy) coupled to a DELTA V Plus isotope-ratio mass spectrometer (Thermo Fisher, Bremen, Germany) interfaced through a ConFlo IV universal continuous flow interface (Thermo Fisher, Bremen, Germany). The combustion furnace (oxidation reactor) was maintained at 1020 °C, and flash combustion initiated by injecting a pulse of O_2 at the time of sample drop. Helium was used as the carrier with a flow rate of 120 mL min^{-1}. NO_x species were reduced to N_2 in a reduction furnace at 680 °C. Water was removed by phosphorus pentoxide in a water trap, and CO_2 was separated from N_2 using a Porapak-packed N_2/CO_2-separation column (3 m × 6.5 mm, Thermo Electron S.p.A.) operated isothermally at 85 °C. Each sample was analyzed in quadruplet. Acetanilide (Fisons Instruments) was used as the reference standard.

Preparative HPLC and LC-ESI-HRMS^n for Isolation of Pure CPT (1) from *Camptotheca acuminata*. For the isolation of pure **1**, gradient separation was performed using a Gilson preparative HPLC

system (Middleton, WI) with a model 322 pump, a model 152 UV/vis detector (288 nm), a model 204 fraction collector (collection of **1** peak at t_R 18.5 min), and Gilson-Unipoint software. Compounds were separated at a flow rate of 4 mL min^{-1} on a C_{18} column (Alltima, 5 μm, 10 × 250 mm) using a Millipore water (solvent A)−distilled methanol (solvent B) gradient: 70% A isocratic for 2 min, linear gradient to 100% B within 25 min. After 100% B for 9 min, the system was returned to its initial conditions within 1 min and held for 6 min. Purity of the fraction collected was checked by multicomponent high-resolution tandem mass spectrometry (LC-ESI-HRMS^n) according to Kusari et al.[7]

Accession Numbers. The endophytic fungus *Fusarium solani* (strain INFU/Ca/KF/3) was deposited at DSMZ (accession number DSM 21921). All the sequences and products verified/discovered in the present study have been deposited at the EMBL-Bank under the accession numbers FN582355−FN582360 (*G10H, SLS, TDC*), FN667579−FN667581 (*A. rigidiuscula*, *STR*), FN669774 (*Top1*), and FM179605 (ITS-5.8S rDNA).

■ ASSOCIATED CONTENT

Supporting Information. Publications and patents on endophytes over the last 20 years; composition of different media, including various host plant tissue extracts, for optimizing fermentation conditions; general scheme of the biosynthetic pathway of CPT (**1**); various degenerate and/or gene-specific primers, information on the used templates, and the different optimized PCR conditions; botanical gardens and laboratory details from where plants were sampled; the physicochemical properties of soil used in the present study. This material is available free of charge via the Internet at http://pubs.acs.org.

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