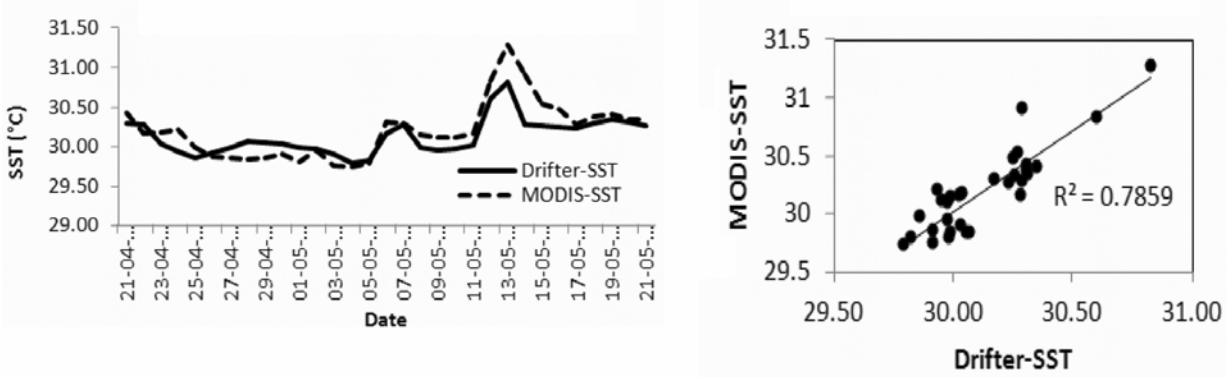

Figure 3. Comparison of daily averaged absolute SST between MODIS-Aqua and Pradyu.

captured every 15 min and it was found that for a period of one month (21 April 2012 to 25 May 2012) the drifter whirled in a steady location (7°N, 83°E) may be due to reversal of wind speed and surface current which cause the stratification. To know its scientific evidence, the SST was compared with MODIS-Aqua 3-days composite SST with a spatial resolution of 4 km, as shown in Figures 2 and 3.

A V-shaped thermal front was observed from MODIS-Aqua images (Figure 2 *a*). Later due to strong stratification, the average temperature in this area has been increased within an anomaly of 3°C (Figure 2 *b*). During this one month observation, two peaks were observed in Pradyu SST which compares well with MODIS-Aqua SST. The absolute value of satellite-derived SST (as appeared in the areas of thermal front in Figure 2) is the skin temperature of water at sea surface overestimates about 0.5°C with Pradyu SST which is spatially distributed due to the action of ocean current and surface wind speed. The first peak indicates the stratification caused by the surface wind and ocean current, which leads to an eddy which persists for one week, i.e. 6–8 May 2012. Whereas the secondary peak indicates that the mean temperature is about 31.5°C due to strong stratification induced by strong current and wind speed. The straight comparison between Pradyu SST and MODIS SST is significant and R^2 is 0.78. However, the vertical profile of SST is unknown.

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Do endophytic fungi possess pathway genes for plant secondary metabolites?

Endophytic fungi which live inside plant tissues as asymptomatic mutualists have been recognized as an important and novel source of bioactive compounds[1]. They produce a number of important secondary metabolites, including anti-cancer, anti-fungal, anti-diabetic and immunosuppressant compounds[2]. Some of these compounds are those produced by their respective host plants as well. For example, Stierle *et al.*[3] showed that an endophytic fungus, *Taxomyces andreanae* isolated from the yew plant, *Taxus brevifolia* produced paclitaxol, the multi-billion dollar anti-cancer compound, just as it is produced by the yew plant. Following this report, a number of endophytic fungal sources of important plant secondary metabolites including camptothecin (CPT), podophyllotoxin, vinblastine, hypericin, diosgenin, azadirachtin and rohitukine have been reported (Table 1). These findings have triggered the expectation that endophytic fungi can be a source of important plant metabolites, thus relieving our dependence on plants for these metabolites.

However, this expectation remains unfulfilled. One of the main reasons for this is the attenuation (loss of) of metabolite production by the endophytes upon sub-culturing[4,5]. Although the reasons for such attenuation are not studied, it could be due to lack of host stimulus in the culture media and/or silencing of genes in axenic cultures[6]. Attempts to reverse the attenuation by amending axenic cultures with host tissue extracts have not been successful[5,7]. This behoves a critical evaluation of the possible mechanisms of the synthesis of plant metabolites by endophytic fungi.

Tan and Zou[8] suggested that genetic recombination of the endophyte with its host in evolutionary time could have lead to the incorporation of the pathway genes of the host into the endophyte[8]. However, as yet there is no proof for the horizontal transfer of genes coding for secondary metabolites between a plant host and its endophytic fungal associate[9]. In effect, the mechanism underlying the production of plant secondary metabolites by an endophytic fungus remains enigmatic.

Against this background, we briefly review here the biosynthetic pathway of plant secondary metabolites with specific reference to terpenoid indole alkaloid (TIA) and ask if this indeed occurs in endophytic fungi. We further discuss possible scenarios that might explain: (a) the production of plant secondary metabolites by endophytic fungi and (b) their attenuation on sub-culturing the endophytes. Finally, we discuss the implications of these scenarios in reversing the attenuation of production of plant secondary metabolites by the endophytic fungus.

In plants, TIAs are one of the largest groups of plant secondary metabolites comprising more than 1800 different alkaloids[10]. They are predominantly distributed in plant families such as Apocynaceae, Loganiaceae and Rubiaceae and have significantly contributed to the development of modern-day drugs[11]. The TIAs are characterized by three key structures, including a tryptamine part, a C9 or C10 residue with a terpenoid origin and a secoiridoid part. Consequently, the biosynthetic pathway of all TIAs involves both the shikimate and the mevalonate (MVA)/deoxyxylulose 5-phosphate (DXP) pathways (Figure 1).

The shikimate pathway converts simple carbohydrate precursors obtained from

Table 1. Illustrative list of endophytic fungi producing plant secondary metabolites

Endophytic fungi	Host	Metabolite	Reference
Taxomyces andreanae	*Taxus brevifolia*	Taxol	3
Entrophospora infrequens	*Nothapodytes foetida*	Camptothecin	43
Fusarium solani	*Apodytes dimidiata*	Camptothecin	44
Alternaria sp.	*Sinopodophyllum hexandrum*	Podophyllotoxin	45
Fusarium oxysporum	*Sabina recurva*	Podophyllotoxin	46
Alternaria sp.	*Catharanthus roseus*	Vinblastine	47
Fusarium oxysporum	*Catharanthus roseus*	Vincristine	48
Chaetomium globosum	*Hypericum perforatum*	Hypericin	49
Cephalosporium sp.	*Paris polyphylla* var. *yunnanensis*	Diosgenin	50
Acremonium sp.	*Huperzia serrata*	Huperzine A	51
Fusarium proliferatum	*Dysoxylum binectariferum*	Rohitukine	52
Eupenicillium parvum	*Azadirachta indica*	Azadirachtin	53

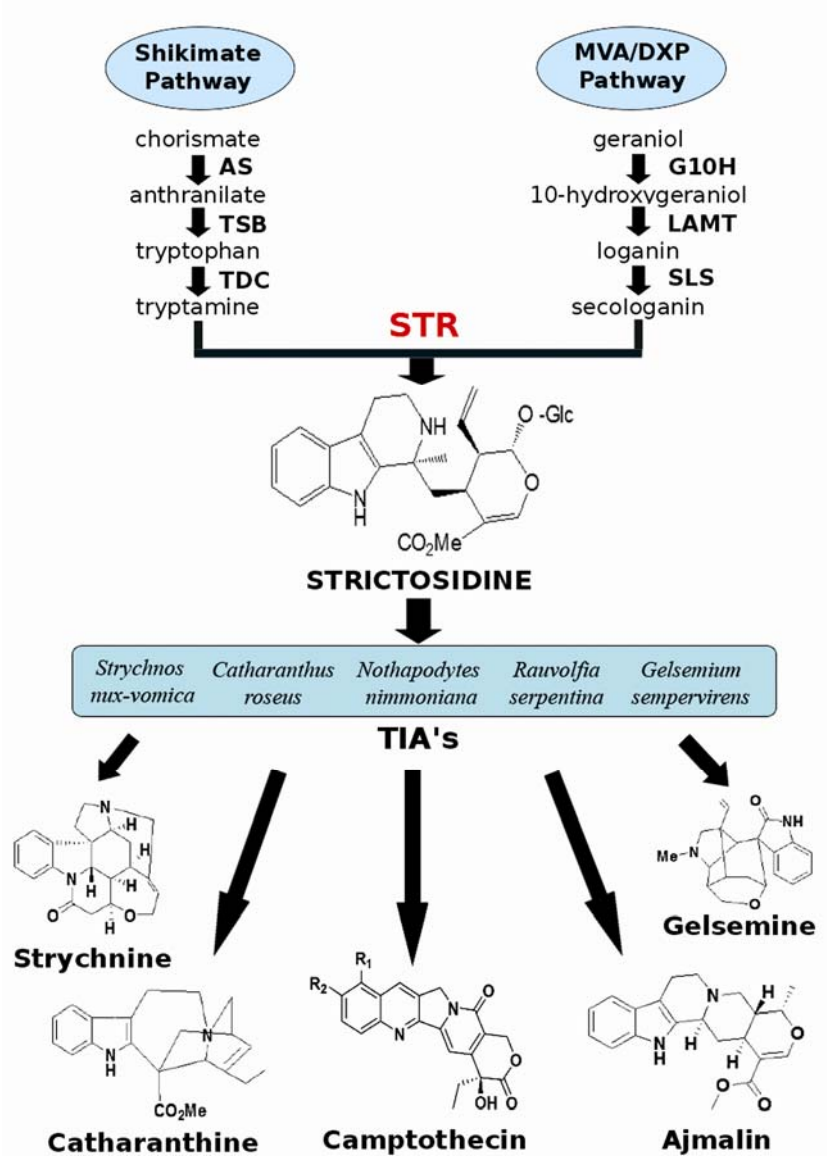

Figure 1. Terpenoid indole alkaloid pathway showing the shikimate and mevalonate (MVA)/deoxyxylulose 5-phosphate (DXP) pathway leading to the production of strictosidine and thereafter to a number of terpenoid indole alkaloids (TIA) by respective plant species. (Shikimate pathway: *AS*, Anthranalite synthase; *TSB*, Tryptophan synthase beta; *TDC*, Tryptophan decarboxylase. Non-mevalonate pathway: *G10H*, Geraniol 10-hydroxylase; *LAMT*, Loanic acid-*o*-methyltransferase and *SLS*, Secologanin synthase; *STR*, Strictosidine synthase.)

glycolysis and pentose phosphate pathway (PPP) into aromatic amino acids such as tryptophan, phenylalanine and tyrosine. The pathway is present in plants, fungi and bacteria, but not in animals. Tryptophan and its decarboxylated product tryptamine serve as the precursors for the biosynthesis of a large class of indole alkaloids. Isoprenoids produced from the MVA/DXP pathway serves as precursors for the synthesis of secologanin. Condensation of tryptophan/tryptamine with secologanin, a monoterpenoid glucoside, gives rise to strictosidine, a nitrogenous glucoside. The latter is the precursor for more than 1800 indole alkaloids, including quinine, strychnine, and the anticancer compounds vinblastine, vincristine and CPT[12]. The formation of strictosidine is catalysed by the enzyme strictosidine synthase (STR). This enzyme is located in the vacuole[13].

A number of genes upstream of strictosidine, both in the shikimate pathway [tryptophan synthase (*TSB*)[14], tryptophan decarboxylase (*TDC*)[15]] and MVA/DXP pathway [3-hydroxy-3-methylglutaryl-CoA synthase (*HMGR*)[16], deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*)[17], hydroxy geraniol oxidoreductase (*10HGO*)[18]] have been identified in several alkaloid-producing plants. However, the pathway downstream of strictosidine is largely unresolved.

With specific reference to CPT, a monoterpene alkaloid, the biosynthetic pathway is only partially characterized in plants[13,19]. The first step after strictosidine formation is the removal of the glucose moiety, a reaction that is catalysed by the enzyme strictosidine β-D-glucosidase. This reaction results in a highly reactive dialdehyde. Depending on the conditions (e.g. solvent, pH), various products can be formed from this dialdehyde. In the next step, the double bond in ring B is broken by oxidation, forming two carboxyl groups. An aldol-type condensation yields pumiloside, which undergoes allylic isomerization, reduction and oxidation to yield CPT.

Although some endophytic fungi elaborate their host metabolites in culture[1,20], few studies have attempted to look for plant secondary metabolite pathway genes in endophytic fungi. Zhang *et al.*[21] showed that the endophytic fungus, *Cladosporium cladosporiodes* MD213 isolated from *Taxus* × *media* (yew species) houses the gene 10-deacetylbaccatin-III-10-*O*-acetyl transferase responsible for taxol biosynthesis. This gene shares 99% and 97% identity respectively with *Taxus* × *media* and *T. wallichiana* var. *marirei* (host plants). Taxadiene synthase (*txs*), a gene unique to the formation of the primary taxane skeleton as well as baccatin amino phenylpropanoyl transferase (*bapt*) catalysing the final acylation of the core structure of taxol are present in the endophytic fungus *Taxomyces andreanae* isolated from *Taxus*[22]. Besides these, surprisingly there are no studies on the pathway genes of taxol biosynthesis in the endophytic fungi.

Kusari *et al.*[7] attempted to study CPT biosynthetic genes upstream of strictosidine in the CPT-producing endophytic fungus *Fusarium solani* isolated from *Camptotheca acuminata*. They reported the presence of *TDC* (from shikimate pathway) and *G10H* and *SLS* (from MVA pathway) genes but not the presence of *STR* and argued that the endophyte might be using the host *STR* to catalyse the condensation of tryptamine with secologanin. However, this is inconceivable in fungi which produce CPT in axenic cultures where they clearly do not have access to host *STR*.

Attempts have been made to study the secondary metabolite genes and their clusters in fungi using whole genome sequences (Secondary Metabolite Unique Regions Finder (SMURF)[23], Kyoto Encyclopedia of Genes and Genomes (KEGG)[24] and FUNGIpath v3.0 (ref. 25)). Analysis of whole genome sequences of 53 fungi, representing both plant and animal fungi failed to detect the presence of *STR* as also other downstream genes of TIAs, including those known in the biosynthesis of vindoline, resperine and ajmalcine (http://www.genome.jp/kegg/pathway.html). These results were also confirmed by the FUNGI3 pathway database where orthologues of *STR* and other downstream genes could not be located in any of the fungal genomes analysed[25]. The databases also failed to detect any of the genes responsible for taxol biosynthesis.

Some of these findings are also supported by recent studies on the evolution of *STR* and strictosidine synthase-like proteins (*SSLs*). Besides being found in plants, the latter proteins have been recovered from algae, cyanobacteria and insects, but never significantly from fungi[26].

In summary, while fungi possess the major pathway genes of both the shikimate and MVA pathways, they seem to singularly lack the crucial *STR* gene as well as other downstream genes. This finding is intriguing in the context of previous studies that have clearly demonstrated the production of several TIAs by the endophytic fungi.

In the absence of *STR* and other downstream genes, how are the fungi able to condense the tryptamine and secoirdiod scaffolds into making terepenoid indole alkaloids? Here, we propose three alternative hypotheses to explain: (a) the observed production of plant secondary metabolites by endophytic fungi and (b) their attenuation on sub-culturing (Figure 2) with specific reference to *STR*.

First, it is likely that the function of *STR* (that is condensation) is actually rendered by another enzyme, a new *SSL* or an entirely different protein. If this were true, quite obviously attempts to identify orthologues of the genes based on either whole genome sequencing or by conventional use of heterologous probes may not have been successful. An alternative strategy and a more conclusive approach would be to evaluate the functional ability of the fungi to catalyse the conversion of tryptamine and secologanin to strictosidine, an experiment that surprisingly has not yet been reported. In the event that such a gene does exist, both the observed production of secondary metabolite and its attenuation could be explained easily. The latter could be explained by gene silencing or methylation process.

Second, we hypothesize that endophytic fungi may not possess the *STR* gene by themselves, but might harbour it on certain extra-chromosomal elements (ECEs) in the fungal cytoplasm. The *STR* in the ECEs might have been horizontally transferred from the host plant during the course of evolutionary interactions. This argument stems from the fact that fungi are known to carry ECEs, including plasmids[27,28].

The third alternative is an extension of the second. Here we propose that the *STR* gene is carried in a plasmid within endohyphal bacteria. Like in the former hypothesis, the plasmid could have acquired the genes through horizontal gene transfer from the host plant. There are many reports of the presence of endohyphal bacteria in fungi[29–33]. Hoffman and Arnold[34] showed that diverse bacteria inhabit hyphae of phylogenetically diverse fungal endophytes.

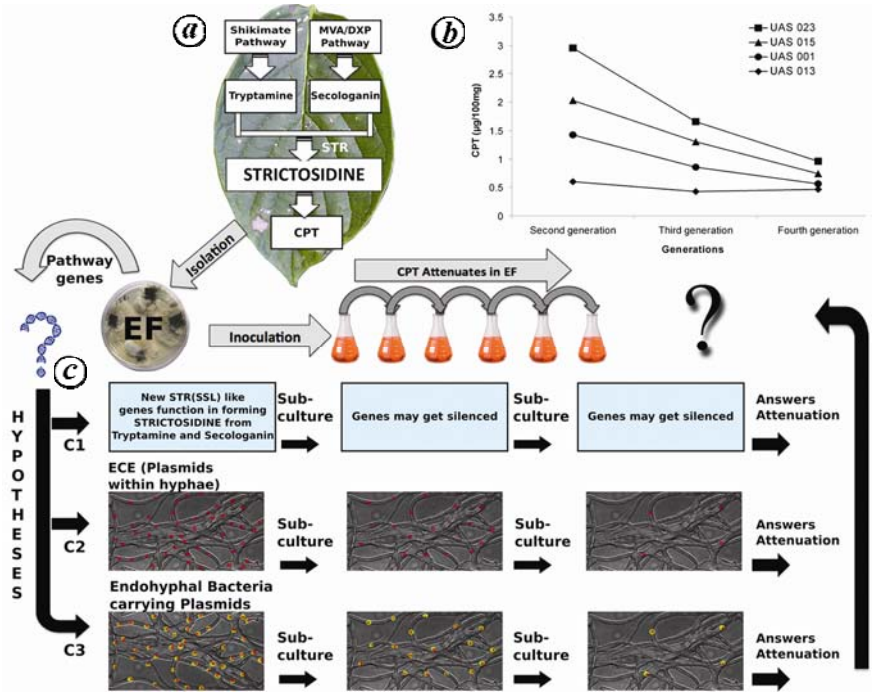

Figure 2. Schematic representation of the hypotheses describing the mechanism of production of plant secondary metabolites by endophytic fungi (EF). ***a***, Formation of strictosidine by the enzyme strictosidine synthase (*STR*) in the plant. ***b***, Typical attenuation of camptothecin (CPT) by CPT-producing endophytic fungi from *Nothapodytes nimmoniana*[5]. ***c***, Flow of events on isolation of EF from the plant. C1 – Hypothesis 1, C2 – Hypothesis 2 (red circles represent the extra-chromosomal elements (ECEs) carried within the fungal mycelia) and C3 – Hypothesis 3. (Yellow dots are endohyphal bacteria presumed to carry plasmits (red) bearing secondary metabolite genes.)

The above hypothesis gains credence from the recent discovery of giant linear plasmids isolated from many antibiotic-producing *Streptomyces* species. These plasmids have been shown to carry the secondary metabolite gene clusters. For example, the plasmid SCP1 of *Streptomyces coelicolor* was shown to carry the gene cluster for methylenomycin A (ref. 35); similarly, the plasmid pSLA-2 of *S. rochei* was shown to possess four secondary metabolite biosynthetic gene clusters, three polyketide synthase (*PKS*) clusters and a carotenoid biosynthetic cluster[36]. Recently, Medema *et al.*[37] reported the presence of a unique 1.8 Mb linear giant megaplasmid in *S. clavuligerus* carrying 25 putative secondary metabolite gene clusters, including *PKS* gene clusters and those coding for terpene synthases or cyclases. Although it is not immediately clear why these plasmids carry the secondary metabolite genes, it is believed that they contribute to horizontal transfer of antibiotic production[38]. In fact, the role of such giant plasmids or transposable elements may be overwhelming as evident from genome sequencing projects that have shown that nearly 45% of the human genome[39] and even more in some plants[40] may be encoded by transposable elements.

Both of the hypotheses (based on ECE and plasmids in endohyphal bacteria) are consistent with the observed production of secondary metabolites and their attenuation endophytic fungi in pure cultures. For example, attenuation could be due to the steady decay in the number or loss of the endohyphal bacteria or plasmids bearing the genes. Loss of endohyphal bacteria from filamentous fungus and plasmids from bacteria on subculture is well known[34,41,42].

In summary, we propose that the genes for plant secondary metabolites may be housed in the endophyte hypha either in plasmids or in endohyphal bacteria. A careful analysis of endophytic fungi elaborating plant metabolites for their endohyphal bacteria could resolve their role in producing such compounds. It would be worthwhile to undertake specific plasmid-cure experiments of both endophytic fungi and their bacterial constituents to prove this conclusively. Furthermore, whole genome sequencing of endophytic fungi should include the first few sub-culture generations as later generations/sub-cultures may not represent the full complement of genes due to their time-related decay.

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