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Molecular methods unravel the biosynthetic potential of *Trichoderma* species

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Members of the genus *Trichoderma* are a well-established and studied group of fungi, mainly due to their efficient protein production capabilities and their biocontrol activities. Despite the immense interest in the use of different members of this species as biopesticides and biofertilizers, the study of their active metabolites and their biosynthetic gene clusters has not gained significant attention until recently. Here we review the challenges and opportunities in exploiting the full potential of *Trichoderma* spp. for the production of natural products and new metabolic engineering strategies used to overcome some of these challenges.

1.0 Introduction

Members of the fungal genus *Trichoderma* are ubiquitous inhabitants of soils, decaying wood and plant debris.[1] Their ability to survive in different geographical habitats can be attributed to their metabolic diversity, high reproductive capacity and competitive capabilities.[2] The genus *Trichoderma* is very widely researched mainly due to the well-established use of its members either in the production of bioenergy-related[3] and cell wall degrading enzymes[4,5] or as biocontrol agents against plant pathogens.[6–8] Members of this genus are highly ranked in the list of fungal biocontrol agents (BCA).[9] The proposed mechanism for their biocontrol activity includes: mycoparasitism (by secretion of cell wall degrading enzymes to facilitate pathogenic infection of the pest); antibiosis (by secretion of different antimicrobial secondary metabolites); and by competition with the phytopathogen for nutrients and space.[9–11] *Trichoderma* spp. have been used not only as biopesticides, but also as biofertilizers due to their ability to enhance plant growth, impart stress tolerance and induce systemic resistance of the plant to other fungi.[11,12] Although members of this genus have been used as biocontrol agents for decades and even sold as commercial biocontrol agents, for example Binab-TF-WP,[13] the main active constituents behind this activity are still under investigation.[9,11] *Trichoderma* spp. are known to produce important antibacterial, antifungal and antinematode natural products[14–16] and are also reported to be used in the mycoremediation of contaminated soils and water.[17,18]

Trichoderma species find use as BCA mainly by direct application of the fungi to the plant or the soil, which has some limitations. One of them is the variation in the environmental conditions that can affect the chemical profile of the fungi and that often leads to either a decline in the production of beneficial natural products or unintended production of mycotoxins.[19,20] Therefore, a more systematic and reliable approach would be the application of isolated secondary metabolites directly to the plant or soil after its isolation from the producing strain and biological testing of its activity and mode of action.[20] Linking this secondary metabolite to its biosynthetic gene cluster (BGC) would allow development of industrial production of useful *Trichoderma* natural products by facilitating process optimization. This process optimization can be done either by metabolic engineering of the BGC to enhance the production of this secondary metabolite or even produce new and more potent compounds using combinatorial biosynthesis or by heterologous expression of this BGC.[21–23]

Despite the great number of secondary metabolites isolated from different *Trichoderma* spp. (more than 390 non-volatile secondary metabolites and 480 volatile organic compounds have been reported so far),[24,25] few of them have been linked to their responsible biosynthetic gene clusters, and little biosynthetic engineering has yet been reported in this genus. This may be attributed to the fact that many fungal biosynthetic gene clusters are silent under normal laboratory conditions, making it challenging to exploit their full chemical and enzymatic potential.[20,24,26] In this review we will shed some light on challenges in investigating secondary metabolites and their biosynthetic gene clusters in *Trichoderma* species with a focus on some of the well-investigated PKS and hybrid PKS/NRPS biosynthetic gene clusters from *Trichoderma*, which until recently were the least studied class of biosynthetic gene clusters in *Trichoderma*.[27] Furthermore we highlight some of the opportunities which could be obtained if these problems were solved.

2.0 Fungal biosynthesis

Trichoderma spp. are rich sources of bioactive polyketides, nonribosomal peptides and terpenes. Many bioactive secondary

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metabolites have been isolated from different *Trichoderma* species and they have been thoroughly reviewed.[24,28,29] A wide range of bioactivities have been reported for different *Trichoderma* secondary metabolites, including antibacterial, antifungal, antineoplastic, plant growth promotion and several other activities related to the biocontrol potential of *Trichoderma*.[28,30–35] The most studied and well known secondary metabolites of *Trichoderma* are 6-pentyl-2*H*-pyran-2-one **1**, gliotoxin **2** and the peptaibols (Fig. 1). Peptaibols are a group of fungal peptides biosynthesized by nonribosomal peptide synthetases (NRPS), such as the antibiotic polypeptide alamethicin **3**, the first peptaibol isolated in 1967 from *T. viride*, a strain that was later reidentified as *T. arundinaceum*.[27,36–40]

Fungal polyketides and nonribosomal peptides are produced by highly programmed megasynth(et)ases named polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), respectively.[41–43] *Trichoderma* spp. also produce ribosomally synthesized and post translationally modified peptides (RiPP).[44] Fungal polyketide synthases are classified based on their catalytic domain structure into three different types; highly reducing polyketide synthases (hr-PKS), partially reducing polyketide synthases (pr-PKS) and non-reducing polyketide synthases (nr-PKS). Highly reducing polyketide synthases usually synthesize highly reduced, often linear, products and the others synthetize aromatic compounds with phenol groups.[42,45]

Non-ribosomal peptide synthetases are multimodular megasynthases, in which each module recognizes, activates and modifies a single amino acid residue of the final peptide.[46,47] Each single module of the NRPS consists of at least three core domains; adenylation (A), thiolation (T) and condensation (C) domains. The A-domain selects and activates the amino acid as amino acyl adenylate, the T-domain carries the activated amino acids and intermediates between the different catalytic domains, and the C-domain catalyzes the formation of the peptide bond. NRPS modules can also contain other catalytic domains such as thiolesterase (TE), reductase (R), epimerization (E), cyclization (Cy) and *N*-methylation (NM) domains.[41,46–49] The most studied nonribosomal peptide synthetases in *Trichoderma* species are the ones responsible for the biosynthesis of peptaibols (*e.g.* **3**) and the epithiodioxopiperazine (ETP) gliotoxin **2**.[47,50,51] Peptaibols such as **3** are linear peptides that are characterized by the occurrence of non-proteinogenic amino acids such as α-aminoisobutyrate (Aib) and a C-terminal amino alcohol, from which the name is derived.[47,52]

Terpenes are considered the most abundant and chemically diverse class of natural products, with an estimated number of more than 80 000 different compounds.[53–55] These highly diverse

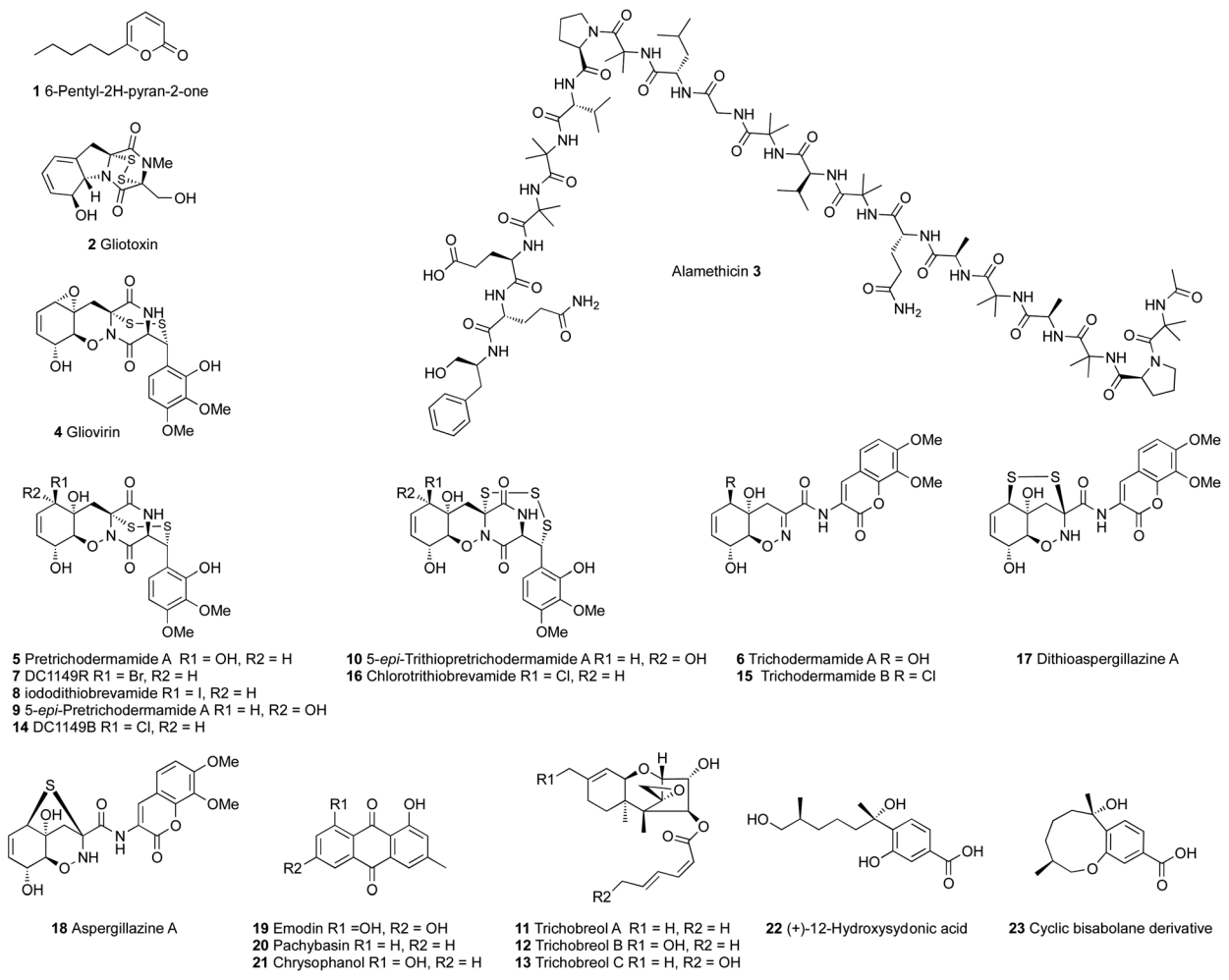


Fig. 1 Natural products isolated from *Trichoderma* spp.

natural products are derived from the cyclization of polyprenyl diphosphates such as geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP) and geranylfarnesyl diphosphate (GFPP) through the action of terpene cyclases (TC).[53–56] Several terpenoids have been characterized and reported from different *Trichoderma* spp., however only few of their responsible biosynthetic gene clusters have been experimentally characterized.[53,57] Nevertheless the interest in the terpene synthases inventory of different *Trichoderma* spp. has been clearly growing.[50,57]

3.0 Challenges in *Trichoderma* species

Despite the genomes of many *Trichoderma* species having been sequenced and published, research on the secondary metabolites of *Trichoderma* and their biosynthetic gene clusters is currently under-developed, compared to other genera such as *Aspergillus*.[58] However, the area is expected to grow rapidly. Genomic analyses of *Trichoderma* species have been performed to facilitate the prediction of biosynthetic gene clusters in these fungi and link them to already isolated secondary metabolites, as well as to identify the potential for producing new secondary metabolites. However the numbers of biosynthetic gene clusters are often large, and identified biosynthetic gene clusters are low. For example, analysis of the *T. reesei*, *T. atroviride* and *T. virens* genomes revealed a total of 47 PKS biosynthetic gene clusters, of which 7 are common to all species. However, very few of these have been investigated and linked to the biosynthesis of known compounds.[10] In a wider study of 12 *Trichoderma* genomes individual species were found to contain: 10–25 PKS biosynthetic gene clusters; 12–34 NRPS biosynthetic gene clusters; and 6–14 terpene synthases.[50] Likewise, *T. atrobrunneum* encodes 18 polyketide synthases, 8 non-ribosomal peptide synthetases, 5 PKS-NRPS genes and 5 terpene cyclases.[59] Sequencing of the *T. lixii* MUT3171 genome resulted in the identification of 23 polyketide synthases, 19 non-ribosomal peptide synthetases, and 8 NRPS-PKS hybrids.[17]

Nevertheless, the research on secondary metabolite biosynthetic gene clusters in *Trichoderma* spp. has lagged behind progress in other genera such as *Penicillium* and *Aspergillus*, which may be, at least partially, ascribed to the complex and tight control over secondary metabolism in *Trichoderma* species. Secondary metabolite production by *Trichoderma* spp., is often strongly influenced by factors including the presence of other microorganisms, environmental conditions and the activity of global and local transcription factors.[60] The energy expenditure to synthesize secondary metabolites by the fungi is thought to be stimulated by the presence of other threats to the organism or other strains competing for nutrients. Therefore isolation of many *Trichoderma* secondary metabolites was done by challenging *Trichoderma* with other fungal strains in the soil or by dual or multiculture of the fungal strains in the lab or by using the "one strain many compounds" (OSMAC) approach.[11,52,61,62]

Even minor changes in the cultivation conditions can lead to changes in the chemical profile of *Trichoderma* spp. Yamazaki *et al.*[63–66] investigated the effects of culture conditions on *T. brevicompactum* (*Trichoderma* sp. TPU199). They found that cultivating the fungus in freshwater media led to the production of gliovirin **4**, pretrichodermamide A **5** and trichodermamide A **6**, while supplementing the freshwater media with sodium halides, NaBr and NaI, led to the production of 5-bromo **7** and 5-iodo **8** derivatives of pretrichodermamide A, respectively. Cultivation of *T. brevicompactum* in freshwater media supplemented with NaI also resulted in the production of two new gliovirin-type epidiketopiperazines **9–10** and three new trichothecene derivatives **11–13**.[63] On the other hand, cultivation of the same fungus on seawater media led to the production of **14** and **15**, the 5-chloro derivatives of pretrichodermamide A and trichodermamide A, respectively.[64]

Supplementing the seawater medium with dimethylsulfoxide (DMSO) resulted in the production of the unprecedented epitrithiodiketopiperazine, chlorotrithio-brevamide **16**.[65] Also long-term static fermentation of the fungus resulted in the production of dithioaspergillazine A **17**, aspergillazine A **18** and three anthraquinones **19–21**, while long-term agitating fermentation resulted in the production of (+)-12-hydroxysedonic acid **22** and a new bisabolane sesquiterpene **23** as a cyclic artifact.[66]

Many so-called global transcription regulators have been reported to control secondary metabolite biosynthesis in different *Trichoderma* spp. Examples include CRE1,[67,68] the velvet complex (LAE1, Vel1, and Vel2)[52,69] and the pH regulator PacC.[52] The stress sensing mitogen-activated protein kinase (MAPK)-dependent signalling pathway has also been reported to influence the regulation and biosynthesis of *Trichoderma* secondary metabolites.[31,60,68,70,71] Furthermore, Derntl *et al.*[26] reported that the xylanase repressor transcription factor, xylanase promoter binding protein 1 (Xpp1), has a dual role in the regulation of primary and secondary metabolism in *Trichoderma reesei*. This was confirmed by the deletion of the Xpp1 transcription factor (TF) that led to a decline in primary metabolism, up-regulation of secondary metabolism genes, and impaired growth of the Xpp1 deletion strain in comparison with the parent strain. Therefore it was proposed that Xpp1 acts mainly as a switch between primary and secondary fungal metabolism.[26] Even some transcription factors in *Trichoderma* that were thought to be pathway-specific were later found to have a more generalized function. For example, the yellow pigment regulator-2 (YPR2) TF located in the sorbicillin (SOR) gene cluster in *T. reesei*,[72] was found later to have a more general regulatory function on balancing secondary metabolism with carbon metabolism.[73]

The gene *ypr2* was found to affect the levels of alamethicin **3** and orsellinic acid **24** in *T. reesei*, where it was found to exert its function mainly in darkness and also depending on the carbon source.[37,73] Furthermore, Beier *et al.*[74] investigated a kinase present in the vicinity of the *sor* BGC (Section 4.1) with similarity to the YPK1 (serine/threonine protein kinase) type kinases. Phylogenetic analysis of this kinase revealed that it is unique in *Trichoderma* spp., therefore it was named unique *sor* cluster kinase 1 (USK1). Analysis of the effect of USK1 knockout on secondary metabolites showed that this gene does not only impact the production of sorbicillinoids, such as bisorbibutenolide **25**, but also affects other secondary metabolites such as alamethicin **3**, orsellinic acid **24** and paracelsin **26** (Fig. 2).[74] Further experiments by Hinterdobler *et al.*[75] revealed another

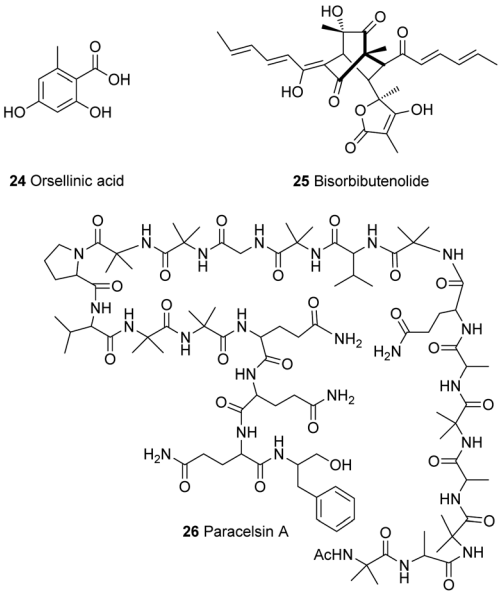
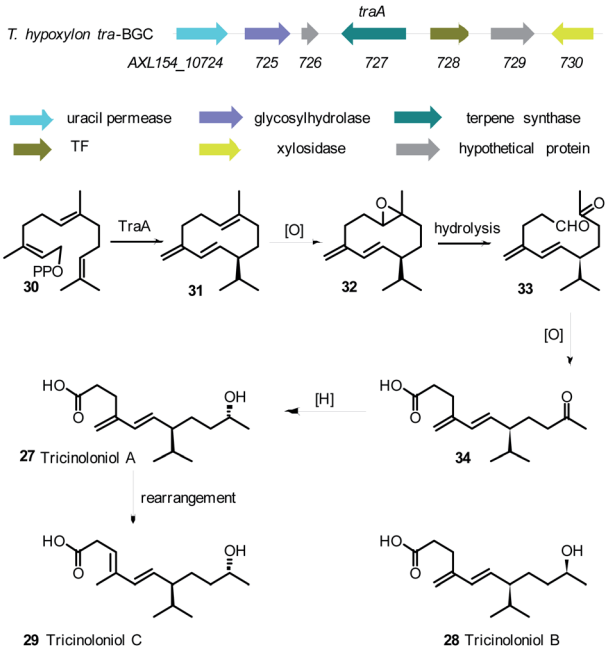


Fig. 2 Compounds from *Trichoderma* spp.



Scheme 1 Tricinoloniol acids BGC, structures of tricinoloniol acids A–C and proposed biosynthetic pathway.[76]

layer of complexity of the tight control of *T. reesei* on secondary metabolism. Another gene in the vicinity of the *sor* cluster (*gpr*8), which encodes a class VII G-protein coupled receptor, was shown to have a considerable influence on the regulation of secondary metabolism in *T. reesei* in darkness. This light dependent regulation of secondary metabolism by GPR8 was found to be mediated in part by the TF YPR2 and the function of the FAD/FMN containing dehydrogenase gene (*TrsorD*).[75]

Another challenge with linking *Trichoderma* secondary metabolites to their biosynthetic gene clusters is the intertwined and coordinated expression of different gene clusters. For example the disruption of *T. hypoxylon tri*5 gene which encodes the first step in trichothecene biosynthesis, disrupted trichothecene biosynthesis as expected, but also resulted in the suppression of another biosynthetic pathway responsible for the production of tricinoloniol acids A-C **27–29** (Scheme 1). Comparative genomics coupled with knockout experiments led to the identification of the terpene cyclase responsible for tricinoloniol acid **29** in *T. hypoxylon* and the biosynthetic pathway was also proposed (Scheme 1).[76] In the proposed biosynthetic pathway, the terpene cyclase (TraA) uses farnesylpyrophosphate (FPP) **30** as a building block to yield compound **31.** Subsequent oxidation and reduction steps of **30** result in the production of compound **27** *via* compounds **32–34.** Rearrangement of **27** leads to the final production of compound **29.** However it is noteworthy that the proposed BGC does not appear to encode proteins able to catalyse the required oxidative steps of the biosynthesis. The structure of the terpene **31** formed by TraA has not yet been determined, but it is presumably a germacrene-D type compound, for which specific cyclases are known from plants.[77]

Other challenges with the identification of biosynthetic gene clusters in *Trichoderma* spp. are the intraspecies diversity in secondary metabolite production and the difficulties in taxonomic classification of members of this genus that has led to a certain amount of confusion. This is clearly exemplified in the presence of two different strains of *Trichoderma virens*, Q- and P-strains, where the Q-strain can produce gliotoxin **2** and the P-strain can produce gliovirin **4.**[60,78–81] The name gliotoxin is originally derived from the fungus *Gliocladium fimbriatum*, from which gliotoxin was originally identified, however this fungus was reidentified later as *T. virens*.[27,79,82] A recent study on *T. virens* showed the ability of the Q-strain to produce heptelidic acid **35**, which was previously only reported from the P-strain. Heptelidic acid **35** (Fig. 3), the anticancer antibiotic also known as koningic acid, accumulated in *T. virens* Gv29-8 after knocking out an NRPS gene (*tex*7) and hence threw some doubt on the current classification of the *T. virens* strains.[83]

Another challenge with the assignment of the function of genes in *Trichoderma* spp. is the high programming of mega-synth(et)ases and the potential of the modules of some non-ribosomal peptide synthetases to bind multiple substrates,[47] where one multifunctional protein such as a PKS or NRPS can be responsible for the production of several metabolites. For instance, knocking out of the 14-module NRPS gene *tex*2 resulted in abolition of both the 14-residue and the 11-residue peptaibols, indicating a remarkable programming and module skipping of the NRPS. One protein Tex2 is reported to produce 88 different peptaibols; 53 different 14-residue and 35 different 11-residue compounds.[43,84]

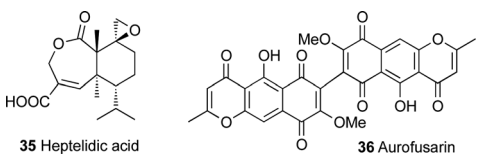
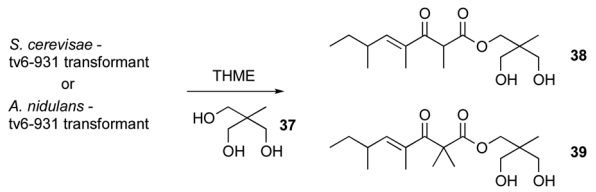


Fig. 3 Terpene and polyketide natural products.



Scheme 2 Heterologous expression of Tv6-931 in *S. cerevisae* and *A. nidulans* in the presence of THME **37**.

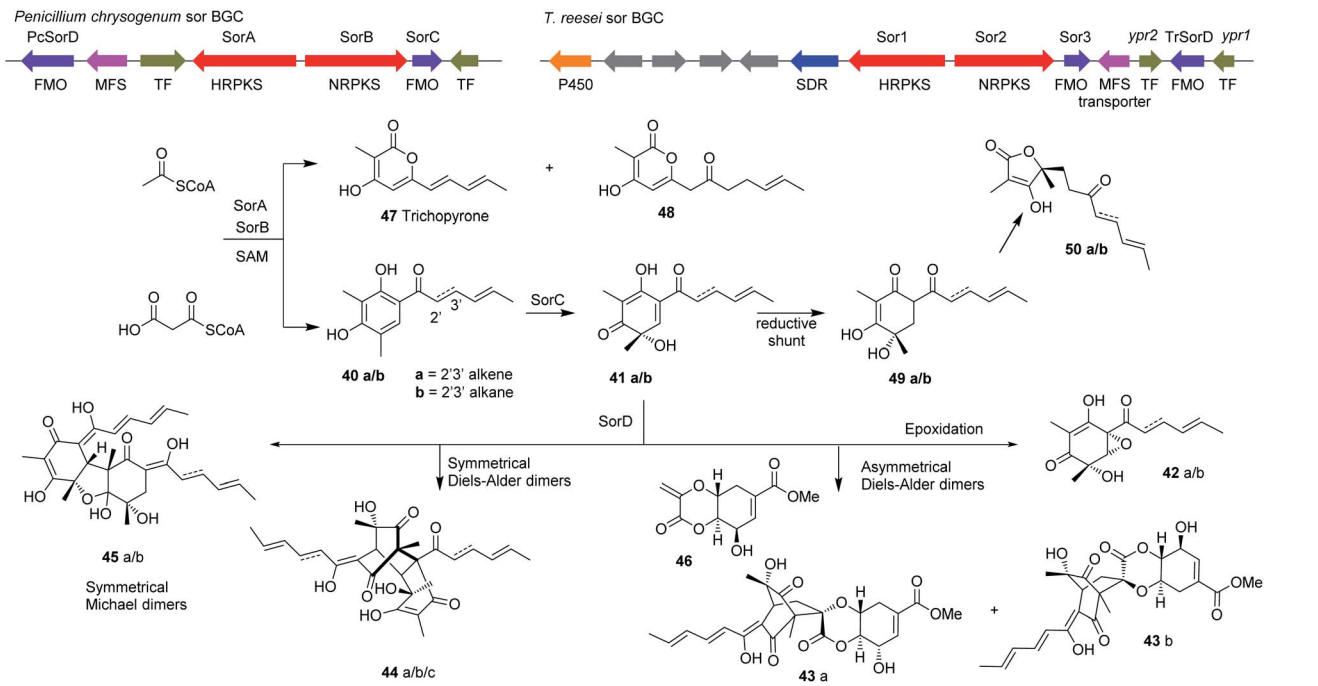
This sophisticated control over secondary metabolism in *Trichoderma* spp. further complicates the identification of new metabolites and efforts to link them to their biosynthetic gene clusters. As a result, knockout experiments alone have proved insufficient in linking some *Trichoderma* secondary metabolites to their biosynthetic gene clusters. For example, a phylogenetic analysis of *Trichoderma* type I polyketide synthases resulted in the prediction of a PKS, namely *pks*4, that was orthologous to the pigment-forming PKS associated with the synthesis of aurofusarin **36** (Fig. 3) in *Fusarium graminearum*. The *pks*4 gene was proposed to be responsible for the yellow-green pigmentation of *T. reesei*, *T. atroviride* and *T. virens*.[85,86] An attempt to knockout *pks*4 in *T. reesei* did lead to abolition of the green colour of conidia, but the structure of the compound responsible for the green conidial pigmentation was not elucidated.[86] Another attempt to knockout *pks*2 in *T. harzianum* also resulted in no clear link between this PKS and any chemical compound, although the results showed a link between this PKS and conidial pigmentation.[87]

Even heterologous expression methods have proven challenging. For example, heterologous expression was used to link PKS *Tv*6-931 from *T. virens* to its product. Studies on this PKS by Hang *et al.*[88] showed that this gene is well conserved across several *Trichoderma* species. However several attempts to heterologously express this gene in yeast and *Aspergillus nidulans* did not yield any new product. Even after the addition of the genes surrounding this PKS into the yeast, no new metabolites were detected.[88] Enzymatic assay of Tv6-931 combined with serendipity and keen eyes revealed that the problem was the absence of the proper releasing substrate.

Repeating the heterologous expression with the addition of 1% releasing substrate, for example 1,1,1-tris(hydroxymethyl) ethane (THME) **37**, led to the production of new tetraketide products **38–39** (Scheme 2). But the question still remains, what could be the natural offloading substrate of Tv6-931 in *T. virens*? The fact that this substrate is absent in both yeast and *A. nidulans* indicates that it might be a unique natural substrate to *T. virens*, which adds yet another layer of complexity to efforts to understand the full potential of *Trichoderma* natural products.[88]

Knockout experiments have also proven sometimes insufficient to predict the function of the genes in *Trichoderma*. For example, prediction of the function of a new gene in the sorbicillin BGC *sor*4/*D* in *T. reesei* based merely on a knockout experiment in the native host was later proved inaccurate after heterologous expression of this gene in *A. oryzae* (Scheme 3, Section 4.1).[89,90]

Therefore the application of more than one metabolic engineering strategy is often preferable when trying to link *Trichoderma* metabolites to their biosynthetic gene clusters. In the following sections, we will discuss some selected examples of PKS, terpene and PKS-NRPS hybrid biosynthetic gene clusters that have been successfully linked to their secondary



Scheme 3 The organization of the sorbicillin BGC in *T. reesei* and the proposed biosynthetic pathway. Enzyme abbreviations: MFS, Major facilitator superfamily transporter; TF, Transcription factor; SDR, Short-chain dehydrogenase; P450, cytochrome P450 monooxygenase; FMO, Flavin-dependent monooxygenase.[90]

metabolites in different *Trichderma* spp. and the metabolic engineering strategies used.

4.0 Genome mining and activation of local transcription factors

4.1 Sorbicillinoids

Sorbicillinoids (also called vertinoids)[91] are complex cyclic polyketides that were reported for the first time as pigments produced by the penicillin-producing fungus *Penicillium notatum* (syn. *P. chrysogenum*) by Cram and Tishler.[92,93] Over the years, the family has grown in size to include over 90 sorbicillinoids isolated from different fungal strains from both terrestrial and marine environments. They are also reported to have a wide range of biological activities that includes cytotoxic, antimicrobial, antiviral and antioxidant activities.[91,94,95]

The term "sorbicillinoid" was proposed in 2002 by Abe *et al.*[96] to describe hexaketide compounds having a sorbyl chain and isolated from bisorbicillinoid-producing strains.

The first report on isolation of sorbicillin **40a** and related compounds from *Trichoderma* was made by Andrade *et al.*[78] in 1992, yet the biosynthesis of these compounds in *Trichoderma* was not established until 2007 by a feeding experiment.[78,97] Since its first isolation from *Trichoderma*, several sorbicillin-related compounds have been isolated from different *Trichoderma* species.[91,94,98,99] However, the BGC of sorbicillin in *Trichoderma* was not identified untill 2013 when Jørgensen *et al.*[100] reported a BGC in *T. reesei* closely related to the sorbicillin BGC in *P. chrysogenum* (Scheme 3).[100,101]

In an attempt to unravel the secondary metabolism of *T. reesei*, Jørgensen *et al.*[100] overexpressed several transcription factors located in the vicinity of PKS and NRPS genes. Overexpression of one of these transcription factors, later termed *ypr*1, resulted in significant increase in the production of 78 different compounds, many of which were sorbicillin-related.

Knocking out the two polyketide synthases in the *sor* cluster, both individually and simultaneously, abolished the production of sorbicillinoids in *T. reesei*.[100] Further knockout experiments of the two transcription factors located in the sorbicillin BGC (*ypr*1 and *ypr*2) were performed and complemented with reinsertion of the genes into the respective deletion strains to understand the regulatory functions of these genes on the sorbicillin BGC. The results suggested that the identified BGC responsible for the yellow pigment formation is indeed the sorbicillin BGC.[72]

Comparison of the sorbicillin BGC in *T. reesei* and *P. chrysogenum* shows high homology in the three core genes in the sorbicillin pathway, namely *sorA*, *sorB* and *sorC*. Subsequent knockout experiments of *sor*1 and *sor*3 (homologous to *sorA* and *sorC* in *P. chrysogenum*, respectively) confirmed the functions of the two genes.[89] Interestingly, the *sor* BGC in *T. reesei* includes another gene in the cluster encoding a flavin-containing dehydrogenase with unknown function that shares very low homology to the *P. chrysogenum*, flavin-dehydrogenase termed *sor*4/*D*. Knockout of *sor*4/*D* in *T. reesei* (*TrsorD*) resulted in accumulation of dihydrosorbicillinol **41b** and reduction in sorbicillinol **41a** production. These results led the authors to suggest that TrSorD might catalyse the reduction of the 2′,3′-olefin in the linear side-chain of dihydrosorbicillinol **41b** to yield sorbicillinol **41a**. The authors also reported that no late sorbicillinoids, *i.e.* dimeric sorbicillinoids, were produced in the Δ*TrsorD* strain, however at that point they did not link that to the function of TrSorD.[89]

Further studies on *TrsorD* using heterologous expression in *A. oryzae* and feeding experiments revealed that TrSorD actually catalyzes the epoxidation of sorbicillinol **41a** to epoxysorbicillinol **42a** in addition to catalyzing intermolecular Diels–Alder and Michael reactions to form dimeric sorbicillinoids **43–45** (Scheme 3). Hence, TrSorD was the first flavin-dependent enzyme reported to catalyze epoxidation, Diels–Alder and Michael addition reactions, which emphasize the immense chemical, genetic and enzymatic potential of *Trichoderma* spp.[90]

Another knockout of *sorA* in *T. reesei* resulted not only in abolition of sorbicillinoid production, but also in the accumulation of a compound that was identified as scytolide **46**, which was not previously reported from *Trichoderma*.[90] Isolation of spirosorbicillinol **43a**/**b** from *Trichoderma* species was reported previously and its biosynthesis was proposed to be a result of Diels–Alder reaction between sorbicillinol **41a** and scytolide **46**.[24,102] This Diels–Alder reaction was later confirmed to be catalyzed by TrSorD by feeding *bona fide* scytolide **46** to *A. oryzae* strains expressing *sorABC* and *sorABCD*. Spirosorbicillinols **43a**/**b** were only produced in the latter strain.[90]

Kahlert *et al.*[90] then proposed the sorbicillinoid biosynthetic pathway (Scheme 3) based on the reconstitution of the *sor* BGC from *T. reesei* Qm6a (*TrsorA–D*) in the heterologous host *A. oryzae* NSAR1. Heterologous expression of *TrsorA* or *TrsorB* alone in *A. oryzae* led to the production of no new compounds. However, heterologous expression of the two polyketide synthases *TrsorA* and *TrsorB* together led to the production of compounds **40a** and **40b** and the pyrones **47** and **48**. Addition of *TrsorC* then unexpectedly led to the production of the reduced sorbicillinol **49** and the vertinolides **50a** and **50b**, which were proposed to be reduced shunt products of sorbicillinol **41a** due to the action of an unknown enzyme in the host *A. oryzae*. *In vitro* assays of SorC using **40a** and **40b** led to the production of **41a** and **41b** respectively, which confirmed the function of TrsorC. Expression of all four genes (*TrsorA–D*) finally led to the production of compounds **42a**/**b**, **44a**/**b**/**c** and **45a**/**b**.[90]

Another interesting finding reported by Derntl *et al.*[89] is that sorbicillinoid production results in reduction of cellular biomass, a finding that was related then to the possible growth limiting effect of sorbicillinoids against *T. reesei* itself, metabolic burden or due to the intracellular accumulation of sorbicillin and dihydrosorbicillin.[89] However, subsequent investigations by the same group reported the presence of an Xpp1 TF that acts as a switch between primary and secondary metabolism in *T. reesei* and leads to either accumulation of secondary metabolites or production of cell biomass in *T. reesei*. The same TF was also reported to be present in other fungal species including *Trichoderma atroviride*.[26]

4.2 Tricholignans

Bioinformatic analysis of a *T. harzianum* strain to identify all the iterative polyketide synthases led to the identification of 25

biosynthetic gene clusters, one of which contained two polyketide synthases, an nr-PKS and an hr-PKS.[20] The main natural product produced by this strain is pachybasin **51**,[20] which is unrelated to this cluster. RT-PCR revealed that this cryptic BGC is silent under normal laboratory conditions.

Activation of the BGC, by cloning the cluster-specific TF *tlnI* under the *gpdA* promoter (P_{gpdA}) and intergration of this cassette in *T. harzianum* t-22, led to the production of many new metabolites, including two major compounds named tricholignan A **52** and B **53** (*ca* ≈ 2 mg·L^{-1}), their precursors **54–55** and dimers **56–57** (Fig. 4).[20] Biological testing of **52** showed that it can reduce Fe(III) to Fe(II) and hence may promote plant growth under iron-deficient conditions. This redox activity was attributed to the *O*-hydroquinone moiety of the compound, which is a rare feature among fungal metabolites.

To fully elucidate the biosynthetic pathway of the identified compounds, especially the logic behind the production of *O*-hydroquinone through a polyketide pathway, the individual steps of the biosynthesis were reconstituted in *Saccharomyces cerevisiae*.[20] Unlike the sorbicillin pathway, heterologous expression of the two polyketide synthases, *tlnA* and *tlnB*, alone or together in *S. cerevisiae* resulted in no detectable products. Only after the coexpression of thiolesterase (TE), *tlnD*, was the truncated pentaketide pyrone **58** produced as a major product and hexaketide β-resorcylic acid **54** as a minor product (Scheme 4). These results imply that TlnD is the TE that releases the product from the PKS by hydrolysis. Coexpresseion of *tlnC*, which is a gene that encodes an unusual di-domain protein with an N-terminal acyl carrier protein (ACP) and a C-terminal C-methyl transferase (C-MeT), with the other three genes in yeast resulted in the production of compound **55**. Further coexpression of the *tlnE* (FMO) and *tlnF* (*O*-MeT) genes led to the production of **53** *via* compounds **62–64** (Scheme 4). In addition, the catalytic roles of TlnA-TlnF were further verified by performing purified enzyme assays *in vitro*.[20]

An interesting finding was that TlnC is a *trans*-acting C-MeT, since methylation can only take place during nr-PKS TlnB-catalyzed chain elongation and not post-PKS. This methylation by the C-MeT domain of TlnC serves as a checkpoint in the nr-PKS (TlnB) programmed steps to produce compound **55** rather than **54**, to ensure the final production of tricholignans by the subsequent enzymes (Scheme 4). This might also explain the production of trichopyrone **47** and related pyrone **48** as side products upon heterologous expression of *sorA* and *sorB* in *A. oryzae* (Scheme 4).[20,90]

As in the sorbicillinoid pathway, the hr-PKS TlnA synthesize the triketide **66**. The nr-PKS TlnB accepts the triketide **66** and extend it to the tetraketide **67**, which is then methylated by the action of TlnC to the tetraketide **68**. After another TlnB-catalyzed two rounds of chain extension to yield the hexaketide **69**, the product template domain of TlnB catalyzes

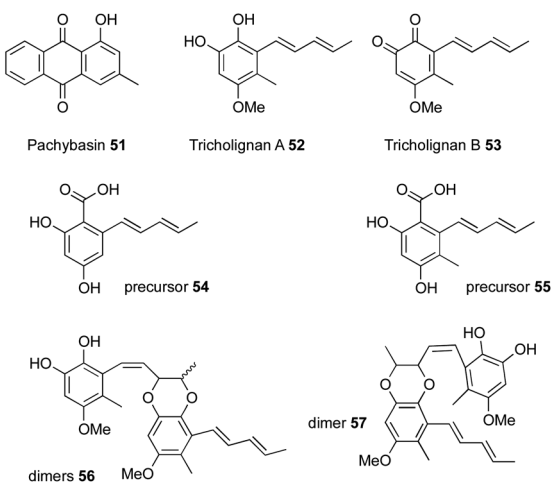
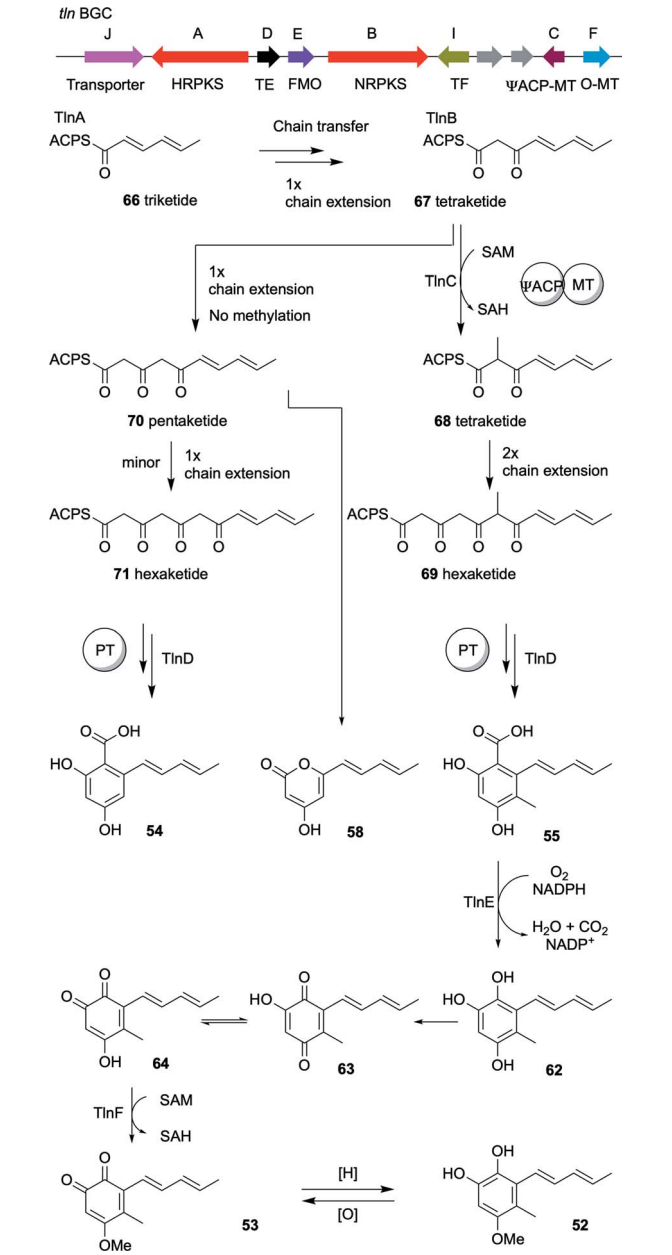


Fig. 4 Compounds from *T. harzianum*.



Scheme 4 The organization of the tricholignan BGC in *T. harzianum* t-22 and the 4.2 Tricholignans and the proposed mechanism of TlnC-assisted biosynthesis of **52**.[20]

a regioselective cyclization. Finally, in contrast to the sorbicillinoid pathway, TlnD catalyzes hydrolytic release to yield compound **55**. However, in the absence of TlnC-catalyzed α-methylation of compound **67**, TlnB programming is affected resulting in the production of the pentaketide **70**. Compound **70** can then either be cyclized to give the pyrone **58** as a major product or produce the unmethylated hexaketide **71**, which in turn undergo regioselective cyclization and hydrolytic release to yield compound **54** as a minor product.[20]

Analysis of the amino acid sequence of TlnC showed that the phosphopantetheine modification site of its ACP domain is mutated and hence this ACP domain is likely to be inactive. This was proved when the standalone ACP was not post-translationally modified when treated with CoA and the fungal phosphopantetheinyltransferase NpgA.[20] However, the efficiency of methylation was severely affected without this inactive ACP domain. These findings were attributed to the possible role of the *apo-ACP* in providing protein–protein interaction between TlnB and TlnC to ensure that the regioselective methylation by the C-MeT domain takes place.[20]

5.0 Genome mining and comparative genomics

5.1 Trichobrasilenol

Although *Trichoderma* spp. are reported to produce many terpenes, their responsible biosynthetic gene clusters in *Trichoderma* have been under-investigated.[53,82,103,104] *T. viride* J1-030 was mined for terpene cyclases, one of which, named *Tvi*09626, was expressed in an FPP-overproducing strain of *S. cerevisiae*. This produced a new 5/6 bicyclic brasilane-type sesquiterpene **72** (Scheme 5).[55]

In a parallel experiment by a different group, Murai *et al.*[54] identified a terpene cyclase gene in *T. atroviride* FKI-3849 (TaTS) that is not clustered with other biosynthetic genes. Heterologous expression of this gene in *A. oryzae* also resulted in the production of the brasilane-type sesquiterpene trichobrasilenol **72**. Using extensive isotopic labelling studies they proved the operation of unusual rearrangement during its production. Phylogenetic analysis revealed the presence of closely related enzymes in several other *Trichoderma* strains. The corresponding gene from *T. reesei* Qm6a also yielded the same compound upon its heterologous expression in *A. oryzae*.[54] Brasilanes are also made by other fungi such as *Annulohypoxylon truncatum* where the TC is encoded within a more complex BGC.[105]

5.2 Trichoxide[45]

Sordarial **73**, pyriculol **74** and aurocitrin **75** are a group of salicylaldehydes biosynthesized by hr-PKS-containing biosynthetic gene clusters, representing a clear deviation from the standard PKS classification (Fig. 5). Genome mining of sequenced fungal genomes using **73** (*srd*) BGC as a lead resulted in the identification of a BGC that is well conserved in many *Trichoderma* species.

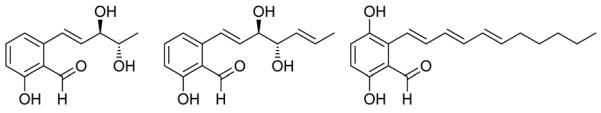


Fig. 5 Fungal secondary metabolites containing a salicylaldehyde core.

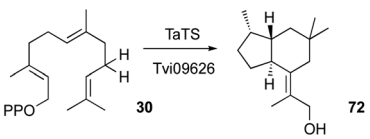
The identified cluster from *T. virens* (*vir*) encodes an hr-PKS megasynthase together with 11 tailoring genes, five of which belong to the SDR family of proteins (Scheme 6). Heterologous expression of all 12 *vir* genes from *T. virens* in *A. nidulans* resulted in the accumulation of four new metabolites: one epoxycyclohexenol, trichoxide **76**; two substituted salicylic alcohol derivatives, virensols A **77** and B **78**; and one known salicylaldehyde 5-deoxyaurocitrin **79**. Upon removal of the hr-PKS gene *virA* from the heterologous host, the production of the four compounds was abolished. Reconstitution of the individual steps of the biosynthetic pathway in the heterologous host led to complete elucidation of the biosynthetic pathway of salicylaldehydes and epoxycyclohexenol-containing natural products (Scheme 6). Both trichoxide **76** and 5-deoxyaurocitrin **79** showed antifungal activities against *S. cerevisiae* and *Candida albicans*.[45]

Interestingly, expression of *virA* alone in *A. nidulans* led to the production of a new compound virensol C **80**, which indicates an intriguing programming of this hr-PKS. In the polyketide chain of virensol C **80**, the first two ketides of the polyketide chain are fully reduced, followed by three ketides that undergo β-dehydration and the last three ketides are only reduced to β-hydroxys. Two of these three β-hydroxy groups are selectively oxidized back to the β-ketones later by SDR enzymes. This enables the aldol reaction to take place, which results in the production of 5-deoxyaurocitrin **79**, a polyketide with both reduced and aromatic portions.

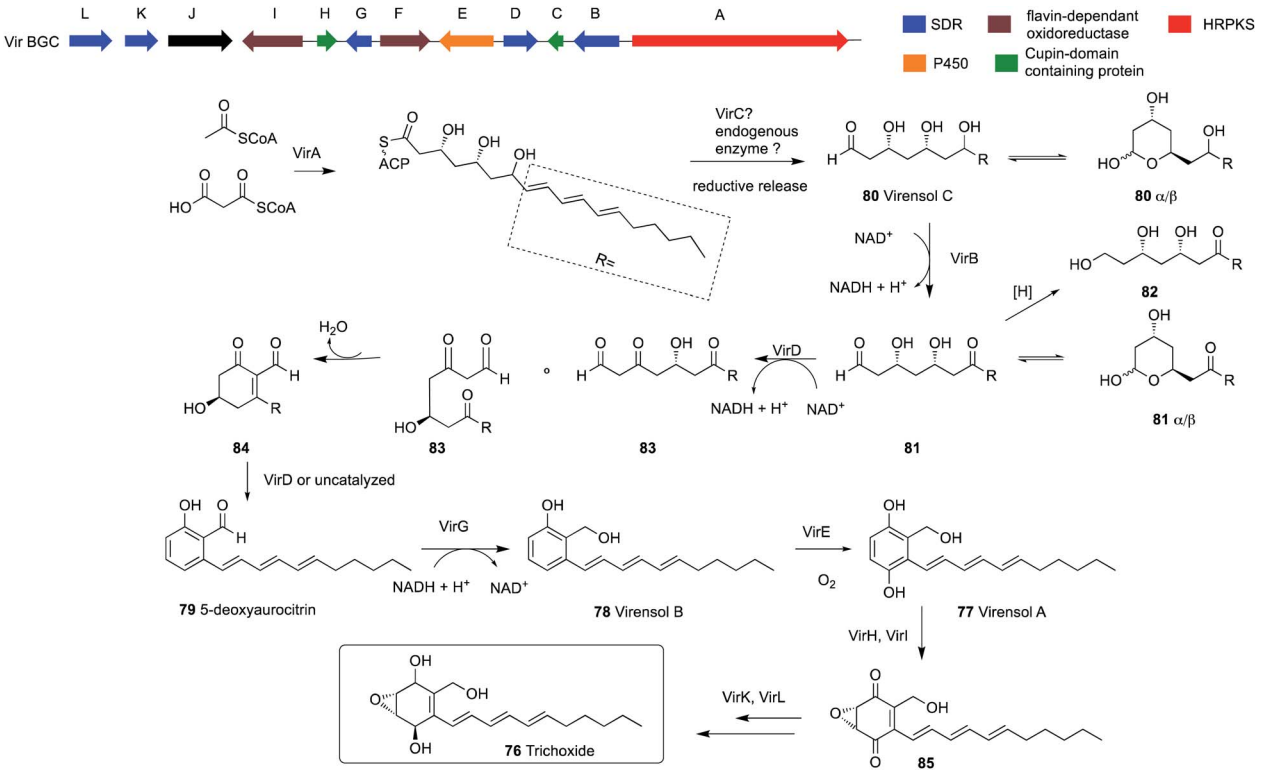
This is in contrast to the two previously mentioned examples of secondary metabolites from *Trichoderma*, sorbicillins and tricholignans, where two PKS genes are used to synthesize the final product (Scheme 7). This involves an hr-PKS to synthesize the reduced portion of the chain, which is then transferred to an nr-PKS to synthesize the aromatic portion. However, the release mechanism of VirA is still under investigation, since VirA lacks a reductase (R) domain and efforts to obtain the pure protein was not successful.[45]

Compound **80** exists in solution mostly as a pair of hemiacetals **80α** and **80β**. Oxidation of C-7 alcohol in **80** by the action of VirB results in the production of **81**, which also exists as hemiacetals **81α** and **81β** in solution.

Coexpression of *virD* together with *virA* and *virB* led to the production of the salicylaldehyde **79** *via* compounds **83–84** and trace amounts of **82**, which was proposed based on purified



Scheme 5 Proposed biosynthetic pathway of trichobrasilenol.



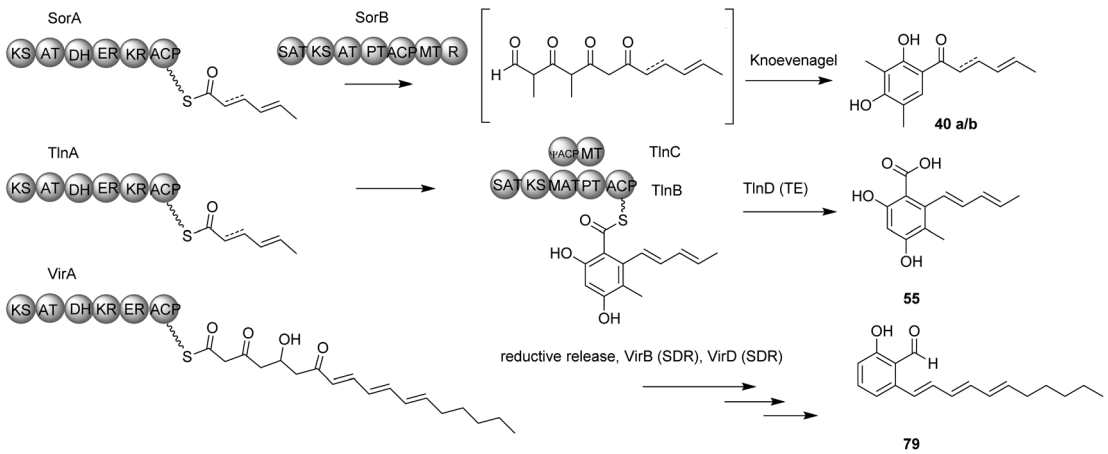
Scheme 6 The organization of the trichoxide BGC in *T. virens* Gv29-8 and the proposed biosynthetic pathway.[45]

enzyme assays. Compound **79** is then reduced by the action of VirG to produce **78**, which is a substrate for VirE to form **77**. Further expression of *virI* and *virH* led to the production of **85**. Finally, co-expressing all the genes in the BGC led to the production of **76**.

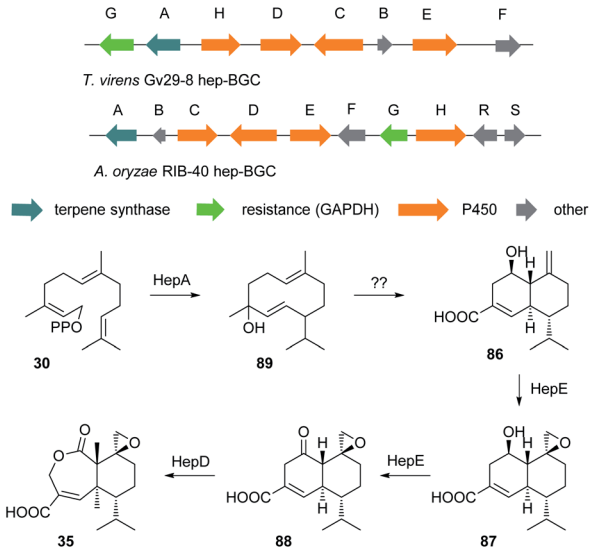
It is worth mentioning that the salicylaldehyde aurocitrin **75** and 5-deoxyaurocitrin **79** were previously reported as metabolites of *Hypocrea citrina* (currently valid name *Trichoderma citrinum*) along with other benzofuran and dihydroisocoumarin derivatives.[106,107] This indicates the outstanding chemical diversity of *Trichoderma* species that awaits to be explored, which was clouded by the complexity and difficulty of the genus taxonomy.

5.3 Heptelidic acid

The cultivation of *T. virens* Gv29-8 in potato dextrose broth (PDB) at 28 °C while shaking resulted in the production of the anticancer antibiotic heptelidic acid **35**. However, changing the



Scheme 7 The proposed functions of the main PKS genes in the biosynthesis of the three secondary metabolites; sorbicillin **40a**, tricholignan **52** and trichoxide **76**. Enzyme/domain abbreviations: KS, ketosynthase; MAT, malonyl-CoA: ACP transacylase; AT, Acyltransferase; DH, dehydratase; KR, ketoreductase; ER, enoylreductase; ACP, acyl-carrier protein; SAT, starter acyltransferase; MT, methyltransferase; R, reductive release; PT, product template; SDR, short chain dehydrogenase; TE, thiolesterase.



Scheme 8 Heptelidic acid biosynthetic pathway and BGC.

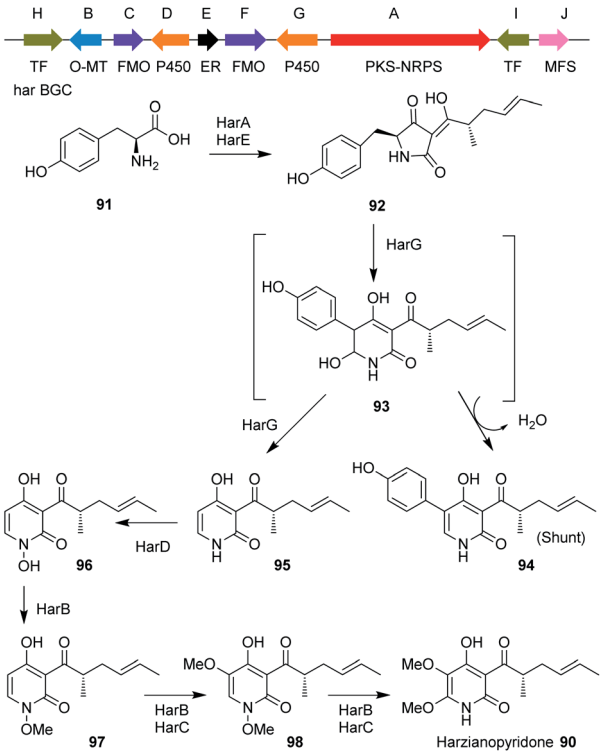
culturing conditions to stationary incubation resulted in the production of another three new metabolites **86–88.** The molecular weights of these new metabolites indicated that they might be biosynthetic intermediates of heptelidic acid **35.** Since the BGC of heptelidic acid (*hep-BGC*) in *Aspergillus oryzae* was already reported,[108] bioinformatic analysis led to the identification of the *hep-BGC* in *T. virens* Gv29-8. Further biotransformation and biochemical assays suggested a biosynthetic pathway for heptelidic acid **35** (Scheme 8) and hence the production of the new metabolites was suggested to be the result of lower aeration under stationary conditions.[109] As in the tricinoloniol acid **29** case (Section 3.0) the precise product of the terpene cyclase HepA has not yet been identified, but may again be a germacrene-D type system such as **89.**

6.0 Prediction of the biosynthetic gene clusters based on the proposed biosynthetic pathway and genome mining

6.1 Harzianopyridone

Harzianopyridone **90**, an antifungal metabolite produced by *T. harzianum*,[110,111] is a potent mitochondrial complex II inhibitor. It is active in the nanomolar range at inhibiting the mammalian succinate ubiquinone oxidoreductase, which is one of the five complexes in oxidative phosphorylation. Oxidative phosphorylation is considered an attractive target for antifungal and anticancer therapies.[112] It was the first pyridone isolated from *Trichoderma* species, when Dickinson *et al.*[110] isolated this compound in 1989. It is structurally similar to other 2-pyridone natural products such as tenellin and aspyridone, for which the biosynthetic gene clusters have been well-studied.[113–116]

Until recently the BGC for **90** was unknown but its biosynthetic pathway was proposed based on the biosynthesis of other 2-pyridone natural products and on isotope feeding studies.[112]



Scheme 9 Organization of the harzianopyridone BGC in *T. harzianum* and the proposed biosynthetic pathway.

This proposal led to the identification of a candidate BGC from the genome of *T. harzianum*, a *PKS-NRPS* gene in addition to a potential ring expansion cytochrome P450 monooxygenase ($P450_{RE}$) homologous to *tenA*.[112,113]

Other tailoring enzymes were also encoded by the cluster such as: an additional P450; an ER; two FMO; a potential *N*-hydroxylase; and an *O*-MeT (Scheme 9). The detailed biosynthetic pathway together with the function of each gene of the cluster were verified by heterologous expression of the genes in *A. nidulans A*1145 *ΔEM*, which revealed that four out of the six tailoring enzymes perform iterative catalysis.

Analysis of **90** biosynthesis also revealed an unexpected biosynthetic logic, in which the fungus introduces an *N*-OMe group during the biosynthesis that was removed later at the last step of the biosynthesis. This *N*-methoxy group was proposed to serve as a directing group to increase the nucleophilic character of the nitrogen to subsequently promote electrophilic aromatic substitution (EAS) and not to protect the pyridone nitrogen from being methylated by the iterative methyltransferase HarB. These findings highlight the highly programmed and remarkable functions of fungal biosynthetic enzymes.[112] The pathway involves use of tyrosine **91** which is condensed with a tetraketide by HarA to produce the first enzyme free intermediate **92.**

Ring expansion *via* intermediate **93** was proposed, to give either substituted pyridone **94** or the de-phenylated **95.** *N*-hydroxylation gives **96,** and then *N*–*O*-methylation gives **97.** This is the substrate for HarB and HarC which hydroxylate to give **98** and methylate again to give harzianopyridone **90.**

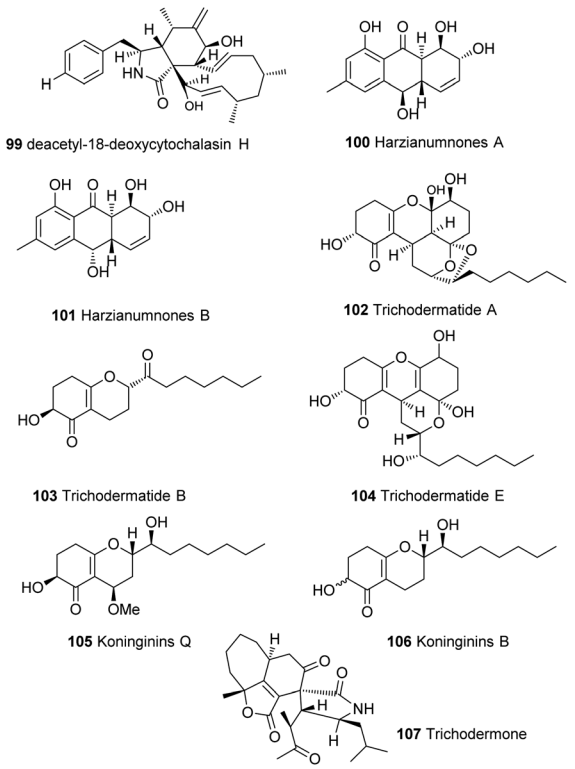


Fig. 6 Natural products of different classes isolated from *Trichoderma* spp.

7.0 Concluding remarks

Members of the genus *Trichoderma* show remarkable chemical and genetic diversity that has been barely tapped. Many secondary metabolites have been isolated from this genus, such as cytochalasins **99**,[117] hydroxyanthraquinones **100–101**,[118] trichodermatides **102–104**,[119,120] koninginins **105–106**[121] and many other secondary metabolites[24,28] that have not yet been linked to their biosynthetic gene clusters (Fig. 6). Some of these compounds showed unprecedented chemical diversity such as trichodermone **107**; the first spiro-cytochalasan with unprecedented tetracyclic nucleus (7/5/6/5).[122] With the urgent need for more reliable and greener alternative for chemical pesticides, the research on natural products from *Trichoderma* spp. is surely expected to grow rapidly. And while the biosynthetic gene clusters of *Trichoderma* have been shown to be highly programmed and quite challenging to manipulate, recent advances in metabolic engineering strategies, genome mining and comparative analysis tools[27,44,71,81,123–128] can help resolve this and help explore and exploit the full potential of this genus.

Author contributions

The topic of the review was originated by MLS. The review was written by MLS and RJC.

Conflicts of interest

There are no conflicts to declare.

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