Combined comparative genomics and molecular biology approaches provide insights into the terpene synthases inventory in Trichoderma

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Abstract

Trichoderma is a fungal genus comprising species used as biocontrol agents in crop plant protection and with high value for industry. The beneficial effects of these species are supported by the secondary metabolites they produced. Terpenoid compounds are key players in the interaction of Trichoderma spp. with the environment and with their fungal and plant hosts, however most of the terpene synthase (TS) genes involved in their biosynthesis have yet not been characterized. Here, we combined comparative genomics of TSs of 21 strains belonging to 17 Trichoderma spp., and gene expression studies on TSs using T. gamsii T6085 as a model. An overview of the diversity within the TS-gene family and the regulation of TS genes is provided. We identified 15 groups of TSs, and the presence of clade-specific enzymes revealed a variety of terpenoid chemotypes evolved to cover different ecological demands. We propose that functional differentiation of gene family members is the driver for the high number of TS genes found in the genomes of Trichoderma. Expression studies provide a picture in which different TS genes are regulated in many ways, a strong indication of different biological functions.

Trichoderma

INTRODUCTION

Trichoderma is a genus of ubiquitous fungi, comprising beneficious species used as biocontrol agents (BCAs) in crop plant protection due to their ability to antagonize and mycoparasitize a wide range of phytopathogens (Harman et al., 2004). Some strains promote plant growth (Fiorini et al., 2016), and are also able to protect plants against pathogens indirectly by inducing the plant defence responses (Shoresh, Harman, & Mastouri, 2010). The beneficial effects of *Trichoderma* spp. are supported and often rely on the secondary metabolites (SMs) they produce (Lorito et al., 1996; Vinale et al., 2008; Viterbo et al., 2007), and the biological roles associated to these metabolites has been extensively reviewed (Contreras-Cornejo, Macías-Rodríguez, del Val, & Larsen, 2016; Contreras-Cornejo et al., 2018; Hermosa et al., 2014; Patil, Patil, & Paikrao, 2016; Rai, Solanki, Solanki, & Surapathrudu, 2019; Salwan, Rialch, & Sharma, 2019). These fungi produce a wide variety of SMs in a strain-dependent manner (Yu & Keller, 2005), with peptaibols, polyketides and terpenes as the most relevant (Reino et al., 2008).

Trichoderma spp. are reported to produce a broad diversity of terpenoids, including volatile compounds (Pachauri, Sherkane, & Mukherjee, 2019). Terpenoids play important roles in the physiology of *Trichoderma* and in the interactions with other organisms, acting as toxins, chemical messengers, structural components

of membranes, regulators of genes related to stress and inducers of plant defence responses (Pachauri et al., 2019; Zeilinger, Gruber, Bansal, & Mukherjee, 2016). Despite their huge variety, all fungal terpenes are synthesized from few precursors by terpene synthase enzymes (TSs). Isopentenyl-pyrophosphate and its isomer dimethyl-allyl pyrophosphate, both synthetized from acetyl-coA, are the five carbons (C) isoprene building blocks for the biosynthesis of linear polyprenyl pyrophosphates: 10C geranyl pyrophosphate (GPP), 15C farnesyl pyrophosphate (FPP) and 20C geranylgeranyl pyrophosphate (GGPP) (Quin, Flynn, & Schmidt-Dannert, 2014). These molecules are synthesized by the isoprenyl pyrophosphate synthases (IPSs), and constitute the precursors that undergo further modifications by terpene cyclases (TCs) and prenyl transferases (PTs), the core enzymes mediating the committed steps in terpenoid biosynthesis (Guzmán-Chávez et al., 2018). According to the origin of their scaffolds, terpenes can be distinguished in those exclusively formed by isoprenyl units (C10 monoterpenes, C15 sesquiterpenes, C20 diterpenes, C25 sesterterpenes and C30 triterpenes), and those of mixed origin (meroterpenoids, indole terpenoids and indole alkaloids).

Although many terpenes have been isolated from *Trichoderma*species, there is no extensive information about TS genes involved in their biosynthesis, and only few members of the TS family have been experimentally characterized (Bansal & Mukherjee, 2016). Functional characterization of TS genes in *Trichoderma* has been mainly focused on the trichodiene synthase (TRI5)-encoding gene, which catalyses the first committed step in the biosynthesis of trichothecenes harzianum A and trichodermin in *T. arundinaceum* and *T. brevicompactum* , respectively (Cardoza et al., 2011; Malmierca et al., 2013, 2014, 2015; Tijerino et al., 2011a,b). Other *Trichoderma* TS genes experimentally characterized are *erg-20* of *T. reesei* , encoding a farnesyl pyrophosphate synthase (Pilsyk et al., 2013), and *vir4* , required for the biosynthesis of mono- and sesquiterpenes in *T. virens* (Crutcher et al., 2013). Furthermore, genome mining studies have assessed the complete TS-gene family in*Trichoderma* , however in those cases, the diversity of the genus has been mainly limited to three species – *T. virens* , *T. atroviride* and *T. reesei* – (Bansal & Mukherjee 2016), or the study has been merely quantitative (Mukherjee et al., 2013; Kubicek et al., 2011; Kubicek et al., 2019).

Given the importance of terpenoids in the ecology of *Trichoderma* , and the scarce information available about the diversity within the TS-gene family, we focused on the genomic characterization of the complete set of TS genes of 21 strains belonging to 17*Trichoderma* spp., providing an overview of the terpenoid biosynthetic potential of the genus. In addition, aimed to decipher the environmental signals regulating the TS genes in *Trichoderma* , we assessed the expression patterns of some TSs in different conditions associated to the ecology of these fungi, using *T. gamsii* T6085 as a model. Strain T6085 presents a versatile lifestyle. During the past ten years, it has been evaluated as BCA against *Fusarium graminearum* , the most aggressive causal agent of Fusarium Head Blight (FHB) on wheat. T6085 is able to reduce the growth of the pathogen as well as the production of deoxynivalenol (DON) (Sarrocco, Mauro, & Battilani, 2019, Sarrocco et al., 2013a), growing in presence of high DON concentrations (50 ppm) and reducing FHB symptoms and the development of *F. graminearum* perithecia on wheat straw (Matarese et al., 2010, 2012; Sarrocco et al., 2013b, Sarrocco et al., 2020 - unpublished). In addition, the fungus establishes a beneficial interaction with wheat roots, behaving as an endophyte and inducing the plant defence responses (Sarrocco et al., 2020 - unpublished).

MATERIALS AND METHODS

1. Genomic platform

Genomes including gene annotation, available in public databases (National Center for Biotechnology Information,*www.ncbi.nlm.nih.gov*; Joint Genome Institute,*www.mycocosm.jgi.doe.gov/mycocosm/home*), comprising 21 strains belonging to 17 *Trichoderma* species, were used for computational analyses (Supporting Information Table**S1**). Model organism *Beauveria bassiana* was used as outgroup due its close phylogenetic relation with *Trichoderma*spp.

2. Prediction of TS proteins

InterProScan v5.44-79.0 (Jones et al., 2014) was used to identify TS proteins in *Trichoderma* spp. and *B. bassiana* proteomes, based on the terms associated to their conserved domains (Isoprenoid synthase domain:

IPR008949, Terpene cyclase/prenyl transferase domain: IPR008930). Pfam, PIRSF, Prosite and Panther algorithms included in the interface, were used to identify motifs relatives to sites, superfamily membership, variants of prenyl transferase domains, and trans-membrane (TM) regions associated to TS proteins.

3. Genomic characterization of TS proteins

In order to characterize TS enzymes in *Trichoderma* spp. and*B. bassiana* we used a combination of different approaches: i) PTs proteins were identified based on Pfam and Panther motifs, which in turn enabled differentially identification of TCs proteins; ii) Conserved aspartate-rich metal-binding motifs associated to Class I (D[D/E]xx[D/E]) and Class II (DxDD) TS-folds (Gao, Honzatko, & Peters, 2012) of mono- and bifunctional enzymes were identified searching for profile Hidden Markov Models (pHMM) in the amino-acidic sequences; iii) Substrate-specificity and putative functions were assigned by phylogenetic analysis: *Trichoderma* spp. and *B. bassiana* TS proteins were aligned by MAFFT v7.450 (Katoh et al., 2013) along with TS proteins of known functions and described in literature (Supporting Information Table **S2**). Phylogenetic tree was built with: MrBayes (Ronquist & Huelsenbeck, 2003), FasTree v2.1.11 (Price, Dehal, & Arkin, 2010) and PhyML v3.3.2 (Guindon et al., 2010). The best substitution model was obtained using ProtTest (Abascal, Zardoya, & Posada, 2005). Phylogenetic tree was reconstructed using the WAG + I evolutionary model (Whelan & Goldman 2001). The probabilities and bootstrap values threshold were 50%. Phylogenetic trees were visually checked and topology conservation evaluated. Sequences used for alignments and corresponding to each phylogenetic cluster identified were individually screened for conserved domains using InterProScan as described above.

4. Fungal and plant material

Trichoderma gamsii T6085 (*Tgam*) (Baroncelli et al., 2016; Matarese et al., 2012) and *Fusarium graminearum* ITEM 124 (*Fgra*) (Zapparata et al., 2017) were grown on PDA (Sigma) plates at 25, 12 h/12 h light/darkness. Seeds from *Triticum aestivum*cv. *Apogee* (wheat) were sown in pots in a potting mix and incubated in a growth chamber with photoperiod 16 h light/8h dark, at 20/22. Before all experiments, wheat seeds were surface sterilized with a NaClO solution (0.6% active chlorine) for 3 min on shaking, followed by three washing steps of 10 min each with sterile distilled water. Seeds were stored in sterile distilled water at 4 for 3 days for vernalization.

5. Liquid cultures of *Tgam*

Mycelium of *Tgam* was obtained following a two-step liquid culture procedure. Spores of *Tgam* were collected from 1-week-old PDA plates and inoculated in 50 mL flasks containing 25 mL of minimal medium (MM) 0.9% sucrose (9 g L^{-1} sucrose, 1 g/L NH_4NO_3, 5 g $L^{-1}C_4H_{12}N_2O_6$, 1 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4$, 0.13 g L^{-1} $CaCl_2$, 0.1 g L^{-1}NaCl, 0.0183 g L^{-1} $FeSO_4$, 0.0035 g L^{-1} $ZnSO_4$, 0.002 g L^{-1} $MnCl_2$) at a final concentration of 10^6 spores ml^{-1}. Flasks were incubated at 28 on a rotatory shaker at 180 rpm for 60 h. Mycelium was collected by centrifugation at 10000 rpm for 10 min and supernatants were discarded. Mycelial pellet was resuspended in sterile distilled water and centrifugated at 10000 rpm for 10 min to wash it. Mycelium was then inoculated in 50 mL flasks containing 25 mL of MM, MM 0.9% sucrose, and MM modified by adding different stressors, such as 0.5 mM H_2O_2, only 0.01% of NH_4NO_3 and $C_4H_{12}N_2O_6$(N starvation), and 200 mM NaCl. Flasks were incubated at 28 on a rotatory shaker at 180 rpm for 4 days. Mycelium was collected by filtration using Miracloth (475855-1R, Millipore), frozen in liquid N_2 and stored at -80 until RNA extraction. Three independent biological replicates were included for each condition.

6. *Tgam* interactions in FHB scenario

Wheat seeds were sown in pots in a potting mix and incubated in a growth chamber with photoperiod 16 h light/8 h dark, at 20/22 respectively, until plants reached the anthesis stage (5 weeks). Three biological replicates of three plants each were inoculated per each condition, i.e.*Tgam* alone, *Fgra* alone and *Tgam* + *Fgra*theses. For *Tgam* inoculation, spores were collected by washing 1-week-old PDA plates with 20 mL of sterile 0.01% Tween-80 solution, and a 10^7 spores mL^{-1} suspension was sprayed on spikes of *Tgam* alone and *Tgam* + *Fgra*plants. Plants were covered with a white bag, previously moistened inside with water

to maintain humidity, and with a black bag to facilitate penetration by the fungus (Dufresne M., personal communication). Plants were incubated in a growth chamber for 48 h in the same conditions described above. For inoculation of the pathogen, conidia of *Fgra* were collected by washing 2-week-old PDA plates with 20 mL of sterile 0.01% Tween-80 solution, and a 10^5 spores mL^{-1} suspension was sprayed on spikes of *Fgra* alone and *Tgam* + *Fgra*plants. Plants were covered with a white bag, previously moistened inside with water, and a black bag was placed above to facilitate penetration by the pathogen. Plants were incubated in a growth chamber in the same conditions described above for additional 24 h. Bags were then removed for plant aeration and after 1h, white bags were placed back for additional 24 h. Six days after inoculation of *Fgra* , ten spikes colonized by fungi were collected from each condition, frozen in liquid N_2 and stored at -80 until RNA extraction. Reduction of FHB symptoms was evaluated by calculating the percentage of healthy and symptomatic spikelets (Disease severity – DS), in*Fgra* alone and *Fgra* + *Tgam* plants. Differences on DS values were determined statistically by ANOVA after angular transformation, (P value (P) < 0.05).

7. *Tgam* – wheat roots interaction

Four wheat seeds were placed on PDA at 1.5 cm of distance from the centre of the Petri dish. Plates were sealed with tape and incubated for 24 h at 22. After 24 h, 1 cm^2 agar plug cut out at the border of 1-week-old colony of *Tgam* was placed in the centre of the plates containing wheat seedlings, as well as in PDA plates without plants as controls. Plates were sealed with tape and incubated for 3 days in the same conditions described above. Wheat roots colonized by *Tgam* were collected, frozen in liquid N_2 and stored at -80 until RNA extraction. Mycelium from *Tgam* PDA control plates was collected and stored until use. Three biological replicates were included for each condition.

8. Nucleic acid manipulation

Fungal biomass from liquid cultures, wheat roots colonized by*Tgam* and fungal mycelium from the *Tgam* - root interaction control plates were ground in liquid N_2 using pre-chilled mortar and pestle. Samples containing 100 mg of powder were used for total RNA extraction using the RNeasy*(r)* Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Wheat spikes colonized by the fungus were ground in liquid N_2 using pre-chilled mortar and pestle. Samples containing 300 mg of powder were used for total RNA extraction according to the method described by Logemann, Schell and Willmitzer (1987). RNA samples were treated with DNase I (DNase I Amplification Grade, AMPD1 Sigma-Aldrich) for gDNA removal, according to the manufacturer's instructions. A total of 400 ng of RNA were used for cDNA synthesis using Maxima First Strand cDNA synthesis kit (K1642 Applied biosystems) according to the manufacturer's instructions.

9. Gene expression analyses

Gene expression was analyzed by quantitative real-time PCR performed in Rotor-Gene Q cycler (Qiagen) with QuantiNova SYBER(r) Green PCR Master Mix 2X (Qiagen). All PCR reactions were performed in triplicate in a total volume of 20 µL for 40 cycles under the following conditions: initial activation, 95°C, 2 min; 40 cycles of denaturation, 95°C for 5 sec and combined annealing/extension, 60°C, 10 sec. Threshold cycles (Ct) were calculated using the β-*tubulin* gene as endogenous control, which was selected due to its expression stability among other housekeeping genes, such as *actin* and *tef-1* . Data were expressed as $2^{-\Delta\Delta^{\gamma}\tau}$ (Livak & Schmittgen, 2001). Values from three biological replicates were consistent and used for ANOVA statistical analysis, using SYSTAT©v.13.2 software. Data from liquid cultures of*Tgam* was analysed by ANOVA and Tukey test. Primers used for assessing expression patterns of *ts1* , *ts3* , *ts4* ,*ts5* , *ts6* , *ts7* , *ts9* , *ts11* , *tri5*genes and those of β-*tubulin* (Supporting Information Table**S3**) were checked for efficiency and dimmer formation.

RESULTS

1. Characterization of TSs provides an overview of the terpenoid biosynthetic potential in *Trichoderma*

We used an *in-silico* approach in order to assign putative functions to 387 TS-encoded proteins currently found in the genomes of*Trichoderma* spp. Initially, we identified PT and TC proteins according to their

conserved domains. Subsequent detection of the metal-binding motifs enabled protein classification as Class I, Class II or Bifunctional enzymes. Clustering-based phylogenetic analysis using biologically characterized fungal TSs also enabled determining substrate-specificity, as well as assigning putative functions to 15 groups of proteins. TSs sharing conserved domains and metal-binding motifs clustered in the same phylogenetic group, each one highlighted in different colour (Fig. **1**). TSs accession numbers, TS-content per each species showing specific portions of the TS inventory, and phylogenetic tree including protein accession numbers are available in Supporting Information Table **S4** and Fig. **S1** .

Analysis revealed specific TSs sharing N-terminal HAD-like (Pfam 13419; PTHR43611:SF3) and C-terminal TS domains (IPR008930) (light-blue colour in Fig. 1). Although they did not cluster with known TSs, the presence of both Class I DDxxE and Class II DxDTT motifs indicates they are bifunctional enzymes. Interestingly, these seems to be exclusive of species belonging to clade Viride (Supporting Information Table **S4**).

We found a vast group of sesquiterpene synthases (sesquiTSs) belonging to the TRI5 superfamily (Pfam 06330) (dark-green colour in Fig. 1), which was particularly represented in species of Viride clade (Supporting Information Table **S4**). It contained 7 trichodiene synthases (TRI5) (PIRSF001388), 15 longiborneol synthases, and two groups of proteins that did not cluster with known TSs, and were therefore named as "uncharacterized group 1" and "2", respectively. TRI5 was found in species of the Brevicompactum clade, *T. gamsii* ,*T. asperellum* and *T. guizhouense* . This phylogenetic distribution indicates that TRI5 is not a monophyletic trait in*Trichoderma* , opening questions about its evolutionary origin. Species of Viride clade were the only lacking longiborneol synthases, which were found highly conserved among species from the other clades. Instead, proteins of "uncharacterized group 1" were only present in Viride and some species of the Longibrachiatum clade. In species of Viride, we found two TSs of "uncharacterized group 2", while *T. arundinaceum* and *T. virens* had only one.

The sister clade of the TRI5-superfamily group (light-green colour in Fig. 1) contains Class I proteins sharing a conserved terpene synthase C domain (Pfam 03936), which can be found in sesquiTS and monoterpene synthases (monoTS). It contains two groups of sesquiTS including 16 presilphiperfolan-8β-ol synthases and 22 pentalenene synthases, along with two groups of proteins that did not cluster with known proteins ("uncharacterized 3" and "4", respectively) (Supporting Information Table **S4**). Presilphiperfolan-8β-ol synthases are absent in Viride species, *T. arundinaceum* and *T. atrobrunneum* , whereas pentalenene synthases were found in all the genomes analysed. Although most of these lasts share highly conserved metal-binding motifs, some proteins lack on the NSD/DTE triad but contain an additional DDxxD motif. This suggests they could actually synthesize sesquiterpenes others than pentalenene. TSs of "uncharacterized group 4" are widely distributed across the species, but are particularly represented in *T. virens* and *T. pleuroticola* . Differently, proteins of "uncharacterized group 3" seems to be exclusive of species belonging to the Harzianum clade, and their phylogenetic proximity to both groups of sesquiTS suggests this group is also composed by this type of TSs.

We found a large group of PTs, identified as squalene synthases (SQSs) (Pfam 00494; PTHR11626:SF2; PS01044) (orange colour in Fig. 1) and enzymes involved in protein prenylation (Jeong et al., 2018) (red colour in Fig. 1), such as type I geranylgeranyl transferases (GGTases 1) (PTHR11774:SF4), type II geranylgeranyl transferases (GGTases 2) (PTHR11774:SF11) and farnesyl transferases (FTases) (PTHR11774:SF6). In SQSs, we also identified a C-terminal TM helix region of 23 residues, which is universally conserved in all eukaryotic SQSs and it is responsible of binding the protein to the endoplasmic reticulum (Linscott et al., 2016). Although each genome contains one of these TSs, an additional SQS is present in *T. pleuroti* , indicating that at least one of them is probably pathway-specific (Supporting Information Table **S4**).

All genomes of *Trichoderma* here analysed contain one copy of oxidosqualene cyclase (OSCs) (Pfam 13249; Pfam 13243; PTHR11764; PS01074) (light-brown colour in Fig. 1) showing DCTSE or DCISE aspartate-rich motifs, both variants of the classical DCTAE reported in these enzymes (Abe, Naito, Takagi, & Noguchi, 2001). Furthermore, these OSCs contain 5 conserved QW motifs, which are thought to be responsible of strengthening the structure of the enzyme and stabilize the carbocation intermediates (Kushiro et al., 2000).

Interestingly, TSs from the sister clade of OSCs contain a conserved squalene synthase-phytoene synthase domain (Pfam 00494; PTHR21181:SF13) (dark-brown colour in Fig. 1), but they did not cluster with SQSs neither with reference lycopene-phytoene synthases. They were therefore named as "uncharacterized group 5", of which one is present in each genome.

Copalyl-pyrophosphate/Ent-kaurene synthases (CPS/KS) (PTHR31739:SF4; PIRSF 026498) were found in *T. asperellum* , known for its ability for gibberellin biosynthesis (Zhao & Zhang 2015). In addition, we found bifunctional enzymes clustering with CPS/KS in *T. citrinoviride* ,*T. parareesei* , *T. reesei* and species of Brevicompactum clade (PTHR31739:SF4) (grey colour in Fig. 1), but their low sequence similarity with CPS/KS indicates these are diterpene synthases (diTS) not involved in ent-kaurene biosynthesis.

The last cluster (dark-blue colour in Fig. 1) contain proteins sharing a conserved polyprenyl synthase domain (Pfam 00348). Within this group, GGPP synthases (PTHR12001:SF47; PS00723; PS00444) and FPP synthases (PTHR11525:SF0; PS00723; PS00444) were identified, showing the two characteristic DDxxD motifs usually found on these enzymes (Wendt, & Schulz, 1998; Gao et al., 2012). Some species belonging to Harzianum and Brevicompactum clades have 2-3 copies of these PTs class, suggesting that at least some of them could be actually pathway-specific (Supporting Information Table **S4**). Analysis also revealed a set of highly conserved indole diTS, of which some species have more than one. Considering that production of indole diterpenes has not been reported in *Trichoderma* , our results reveal that these species have at least the potential to produce these compounds. The last group contains Class I TSs clustering with known chimeric TSs from fungi, and were therefore named as chimeric-like, which were absent in species of Viride clade. Most of these proteins contain only polyprenyl synthase or terpene synthase C domains. Nevertheless, we found one protein in *T. asperellum* TR456 containing both domains which is highly similar to ophiobolin F synthase from *Aspergillus clavatus* , suggesting this specie is able to produce sesterterpenes.

2. Assessment of the genomic context of *tri5* genes reveals its potential involvement in unknown biosynthetic pathways in *Trichoderma*

The presence of *tri5* orthologs in *Trichoderma* spp. that have not been described as trichothecene-producers, such as *T. gamsii* , *T. asperellum* and *T. guizhouense* , suggests this gene could be involved in the biosynthesis of different trichodiene-derivatives. Multi-sequence alignment of TRI5 proteins showed the active centre is highly conserved, sharing DDSRE/DDSIE aspartate-rich motif and NDLFSFYKE triad (Fig. **2a**). Pairwise alignments of each TRI5 protein with that of *T. arundinaceum* and *T. brevicompactum* showed 77% of amino-acid identity in *T. guizhouense* , 80-82% in *T. asperellum* , respectively, and 87% in *T. gamsii* .

Assessment of the genomic context of *tri5* genes by antiSMASH 5.0 (Blin et al., 2019) revealed this gene is included in a 21.2 kb cluster in *T. gamsii* , enclosing another 6 genes that were named as *A* , *B* , *C* , *D* , *E* and *F* (Fig.**2b**). Manual characterization based on conserved domains and similarity with characterized proteins in other systems enabled the identification of four tailoring enzymes, one efflux transporter and one regulatory protein. The three genes located upstream of *tri5* encode a Zn2-C6 transcription factor (TF) (*A*), oxygenase (*B*), and alpha-beta hydrolase (*C*), while the three located downstream were identified as oxygenase (*D*), Major Facilitator Superfamily (MFS) transporter (*E*) and carbonic anhydrase (*F*).

Alignment of these proteins with the TRI (trichothecene) proteins functionally associated to *tri5* in the trichothecene-producer species of the Brevicompactum clade showed no sequence similarity. Furthermore, the genome of *T. gamsii* lacks on the entire set of genes encoding the TRI proteins, with the exception of a distant related homolog of the gene *tri101* , which has been already reported in other *Trichoderma* species (Proctor et al., 2018). We used the protein sequences encoded in the cluster found in *T. gamsii* as queries in BLASTp analyses to search for homologous proteins in the *Trichoderma* genomes used here. Genes *A* , *B* and *C* were also found in all the other *Trichoderma* spp. belonging to the Viride clade, with conserved synteny (Fig.**2c**), and preliminary BLAST analyses suggest that these genes may be originated by horizontal gene transfer (HGT) from a donor belonging to the Eurotiomycetes. In any case, further analyses are needed in order to better understand the evolutionary origins of these genes. Instead, genes *D* and *F* are present in some of the genomes analysed in closely related species; while gene *E* seems to be specific to *T. gamsii* .

These findings suggest the origin of a novel *tri5* -associated cluster in *T. gamsii* , which is likely involved in the biosynthesis of trichodiene-derivates with unknown functions. According to this, *tri5* could participate in two different sesquiterpene biosynthetic pathways in *Trichoderma* .

3. Functional differentiation of TS family members as the driver for the high genomic potential for terpenoid biosynthesis in *Trichoderma*

We identified 16 TS-encoding genes in the genome of *Tgam* (Table**1**). For gene expression studies, we focused on 9 genes encoding TSs belonging to Class I – TS1, TS3, TS4, TS5, TS6, TS7, TS9, TS11 and TRI5 – which represented a high diverse functional group, well distributed across *Trichoderma* spp., according to our analyses. Thus, we excluded genes encoding Class II proteins and those involved in the biosynthesis of terpene precursors and protein prenylation. In order to assess the environmental signals regulating the expression of TS genes selected in *Tgam* , the fungus was grown i) under different simulated stress conditions and in presence/absence of C source, ii) in a FHB scenario, and iii) in interaction with wheat roots.

We firstly investigated changes on TSs expression in 4-day-old liquid cultures of the fungus grown in MM, MM 0.9 % sucrose, MM 0.5 mM H_20_2, MM with low N concentration, and MM 200 mM NaCl, using MM as reference control (Fig. **3a**). The availability of C source had contrasting effects on TS gene expression. Addition of 0.9% sucrose to the culture medium did not change the transcript levels of *ts6* and *ts11* . Nevertheless,*ts3* was significantly down-regulated (0.1-fold), whereas an up-regulation was observed in *ts1* , *ts9* and particularly, in *ts4* (18.7-fold). In the same way, addition of 0.5 mM H_2O_2 to the culture medium induced opposite changes on gene expression, indicating that regulation of TS genes occurs in different manners in response to oxidative stress. While expression of *ts1* and *ts11* did not change, *ts3* and*ts6* were down-regulated (0.3-, 0.6-fold, respectively). In contrast, *ts9* , encoding a putative indole diTS, was up-regulated (2.7-fold), suggesting that biosynthesis of indole diterpenes might occur in response to oxidative stress in *Tgam* . We observed that N starvation tended to negatively regulate TS genes, although significant differences were only found in *ts3* and *ts11* (0.4-fold). Similarly, addition of 200 mM NaCl to the medium down-regulated the expression of TS genes (0.02 to 0.33-fold), with the exception of*ts1* , which expression was not affected. We did not detect transcripts of *tri5* , *ts5* or *ts7* in any of the conditions tested.

Since *Tgam* is able to suppress *Fgra* on wheat spikes, we investigated whether a differential expression of TS genes of*Tgam* takes place in this scenario. To this end, we assessed relative changes in TS expression when the fungus was interacting with*Fgra* on wheat spikes, where a reduction of FHB symptoms was observed (57.9 ± 4.7 % DS) compared to *Fgra* alone plants (88.3 ± 1.2 % DS), using spikes inoculated with *Tgam* alone as control (Fig. **3b**). Analysis revealed that *ts1* , *ts6* ,*ts9* and *ts11* were active when *Tgam* was on wheat spikes, regardless the presence/absence of the pathogen. Expression of*ts1* , *ts6* and *ts9* did not change significantly between the two conditions, indicating that these genes are not particularly involved in the triple interaction in the conditions tested. Instead, *ts11* was slightly up-regulated (1.45-fold) when *Tgam* was on spikes with *Fgra* , suggesting the presence of the pathogen could directly induce changes in its expression or could mediate physiological changes in spikes that promoted changes on*ts11* expression. These results indicate that *Tgam* did not induce prominent changes in terpene biosynthesis when interacted with*Fgra* on wheat spikes, under the conditions tested.

In order to determine whether a differential regulation on terpene biosynthesis occurs when *Tgam* colonizes wheat roots, *Tgam*was incubated for 3 days on PDA in presence of 1-day-old wheat seedlings, using *Tgam* grown alone on PDA under the same conditions as control. Root colonization affected TSs expression in *Tgam* , which differentially regulated most of its TS genes, with the exception of *ts7* , whose transcripts were not detected in any condition, and *ts1* , which expression did not change (Fig.**3c**). This clearly indicates a reprogramming in terpene biosynthesis in *Tgam* when colonizing wheat roots. In particular, a modulation in sesquiterpene biosynthesis occurred in *Tgam*during root colonization. Whereas *ts5* was slightly up-regulated (1.58-fold), the contact with the roots strongly repressed the expression of *ts4* (0.03-fold) and in a lesser extent, that of*ts3* (0.59-fold). Nevertheless, the most remarkably difference was found in the expression of *tri5* , which was 137-fold up-regulated when the fungus was on the roots, suggesting that signals from the roots are responsible of triggering the expression of this gene in *Tgam* . In addition, the interaction with the roots

up-regulated*ts6* (1.52-fold) and *ts11* (2.58-fold). Since *ts6* was predicted to encode an SQS, this suggests that triterpene biosynthesis was slightly enhanced in *Tgam* when colonizing the wheat roots. Interestingly, *ts9* was found highly down-regulated (0.28-fold), thus suggesting a repression of indole diterpene biosynthesis in the fungus during the interaction with wheat roots.

DISCUSSION

The impressive number of TSs we found in the genomes analysed demonstrates that terpenoid biosynthesis has a great impact in the diversity and complexity of SMs in *Trichoderma* . Indeed, the TS-gene inventory of these species (15-23 genes per genome) clearly outnumbers those found in other fungi considered as rich producers of SMs, such as *Aspergillus* spp. (2-10 genes per genome) (de Vries et al., 2017; Kubicek et al., 2019). This likely reflects the importance of TSs and terpenoids in the ecology of these fungi. However, most of the *Trichoderma* TSs have not yet been characterized (Kubicek et al., 2019), and available information about the diversity of TSs in *Trichoderma* is scarce. Thus, we focused on characterising the TS-gene arsenal of this genus, providing a complete overview of the nature and diversity of the *Trichoderma* terpenoid biosynthetic inventory.

Although TS-family size is very homogeneous within *Trichoderma* , we were able to identify clade-specific TSs, which reflect particular portions of the terpenoid inventory shared by phylogenetically close species. Thus, despite their similar terpenoid biosynthetic potential, the species of *Trichoderma* have adapted their terpene production according to different environmental demands. Species of Viride clade constitute an example, as they are missing in some groups of TSs that are present in the other clades, but have evolved specific TSs which are absent in the other species.

According to our results, *Trichoderma* spp. have a huge potential for sesquiterpene biosynthesis. We identified eight groups of sesquiTSs, which constitute almost the third of the total number of TSs found in this work. Species of Viride are particularly rich in sesquiTS belonging to the TRI5-superfamily, and they also contain HAD-like TSs which are absent in species of other clades. These HAD-like proteins harbour a DxDTT motif, which is a variant of the DxDD found in Class II diTS (Nakano et al., 2005, 2009). Shinohara, Takahashi, Osada and Koyama (2016), reported that some sesquiTS can contain HAD-like domains and DxDTT motifs, leading to FPP cyclization through a protonation step, instead of by an ionization step. We hypothesized that HAD-like TSs might be particular bifunctional sesquiTS synthesizing specific metabolites of Viride clade.

Previous studies reported that some *Trichoderma* spp. have the potential to synthesize longiborneol (Bansal & Mukherjee 2016), an intermediate of the culmorin biosynthetic pathway (Bansal & Mukherjee 2016; McCormick, Alexander, & Harris, 2009). According to our results, longiborneol biosynthesis is very widespread in *Trichoderma* , but is absent in species of the Viride clade. Since culmorin production has not been reported in *Trichoderma* , longiborneol could be synthesized as a solely compound or as an intermediate of unknown biosynthetic pathways in these species. Most of these proteins show a conserved D(D/E)HFD motif, which is partially conserved (NDHFD) in proteins of *T. arundinaceum* and *T. brevicompactum* . Site-directed mutagenesis and crystallography studies on TSs have revealed that the first aspartate residue (D) of the metal-binding motif interacts directly with Mg^{2+} (Starks, 1997), and its replacement can lead to anomalous cyclization products or a product mixture (Cane, Xue, & Fitzsimons, 1996). According to this, longiborneol synthases of Brevicompactum species could be actually involved in terpenoid blend formation or in the biosynthesis of newly terpenoids.

We found that most *Trichoderma* spp. can potentially produce presilphiperfolan-8β-ol. This compound is thought to play a central role in the biosynthesis of a wide range of polycyclic sesquiterpenes in fungi (Pinedo et al., 2008). Thus, these TSs may contribute in generating a variety of structurally complex terpenoids in *Trichoderma* . Proteins sharing similarity with fungal pentalenene synthases were also identified, which are involved in the biosynthesis of the parent hydrocarbon of the pentalenolactone family of fungal antibiotics (Kim, Cheong, & Yoo, 1998). Nevertheless, the variability found on the structure of the active centre of these proteins suggests that some of them probably synthesize sesquiterpenoids others than pentalenene.

Our analyses revealed that single-copy SQS and OSC enzymes provide the linear and cyclic precursors required for triterpene biosynthesis in most *Trichoderma* species. However, the presence of an additional SQS in the genome of *T. pleuroti* indicates that pathway-specific SQSs could be present as well. In the same way, additional GGPP and FPP synthases may act as donors of terpenoid precursors in specific biosynthetic pathways in members of the clades Harzianum and Brevicompactum. This most likely reflects specific portions of the terpenoid inventory of these species, and guarantees an efficient distribution of terpenoid precursors between primary and secondary metabolism.

Harziandione was the first diterpene isolated from *Trichoderma*spp. (Ghisalberti et al., 1993), and a number of these compounds have been recently reported in these species (Adelin et al., 2014; Chen et al., 2019; Miao et al., 2012; Song et al., 2018; Zhao et al., 2019). Nevertheless, the low number of diTS found here indicates the ability of*Trichoderma* spp. for diterpene biosynthesis is scarce and not very widespread within the genus.

Interestingly, our analyses revealed that *Trichoderma* spp. have the potential to produce sesterterpenes and indole diterpenes. Sesterterpenes are rare among terpenoids, and their antimicrobial and nematocidal properties (Tian, Deng, & Hong, 2017) could confer competitive advantages to some *Trichoderma* spp. In the other hand, production of indole diterpenes have been reported in some Sordariomycetes being involved in protecting their reproductive structures from fungivores (Saika et al., 2008). Many of the indole diterpene-producer fungi stablish symbiotic relations with plants, thus, biosynthesis of these compounds may confer ecological advantages on*Trichoderma* -host associations as well (Parker & Scott 2004).

No monoTS were found among the genomes analysed in this work, although production of monoterpenes has been reported in *T. virens*(Crutcher et al., 2013; Inayati, Sulistyowati, Aini, & Yusnawan, 2019). No *bona fide* monoTS have been identified in fungi (Schmidt-Dannert, 2014), and the scarce availability of sequences of these enzymes probably leaded to miss-predict them. Biochemical studies have shown that fungal sesquiTS are able to cyclize GPP (Lopez-Gallego et al., 2010); thus, we hypothesize that enzymes involved in monoterpene biosynthesis in *Trichoderma* could be actually included within "uncharacterized group 4", since they are phylogenetically close to sesquiTS, and other uncharacterized proteins were found restricted to some clades not including *T. virens* or fell into the TRI5-superfamily.

We found highly conserved *tri5* orthologs in non-trichothecene-producer *Trichoderma* species missing on the entire TRI cluster, some of which have been previously reported (Gallo, Mulè, Favilla, & Altomare, 2004). This leaves open some questions about the role of *tri5* in beneficious *Trichoderma* spp. non producer of trichothecenes. Unlike other *tri5* -containing species,*tri5* is embedded in a BGC in *T. gamsii* , enclosing tailoring enzymes, a TF and a transporter. The presence of a TF within the cluster suggests a pathway-specific regulation, while the presence of a transporter suggests the production of a sesquiterpenoid with extra-cellular functions. Some of these genes were likely transferred to*Trichoderma* spp. by HGT from Eurotiomycetes, but the entire cluster is only present in *T. gamsii* . suggesting the origin of a novel *tri5* -associated BGC in this species. This cluster could lead to an uncharted trichodiene-derived sesquiterpenic biosynthetic pathway producing novel metabolites with potential agronomic interest. Since *tri5* seems to be functionally associated to two different BGCs (TRI and that found in *T. gamsii*), we hypothesize this sesquiTS is involved in different metabolic pathways in*Trichoderma* .

The striking genomic potential for terpenoid production of*Trichoderma* spp. found in this work suggests that functional differentiation of gene family members is the driver for the high TS gene numbers of these species. Assessing changes in the relative expression of TS when the fungus grows under different environmental conditions, or when interacts with other organisms, enables individuating genes that could play a role in these frameworks and hypothesizing about processes they could be involved in. Here, we provide a picture showing that different TS genes are differentially regulated, a strong indication of different biological functions.

Availability of C source had contrasting effects on the expression of TS genes, as observed in SMs genes from other fungi (Calvo, Wilson, Bok, & Keller, 2002; Jiao, Kawakami, & Nakajima, 2008). Similarly, TS genes were regulated in opposite ways in response to oxidative stress. Association of oxidative stress with

SMs biosynthesis in fungi has been extensively demonstrated, and it has been suggested that it is induced to prevent fungi from ROS damage (Hong, Roze, & Linz, 2013). In particular, up-regulation of *ts9* suggests that biosynthesis of indole diterpenes might occur in *T. gamsii* T6085 under oxidative stress conditions, as it has been observed in *Aspergillus* spp. (Fountain et al., 2016). Interestingly, addition of 0.9% sucrose or 0.5 mM H_2O_2 to the medium did not induce*tri5* expression in *T. gamsii* T6085, differently from what observed in *T. brevicompactum* when grown in presence of 1% or 2% sucrose or in presence of 0.5 mM H_2O_2 (Tijerino et al., 2011a). This suggests different types of regulation of *tri5* in *T. gamsii* T6085 and *T. brevicompactum* .

Nitrogen availability has a considerable impact on secondary metabolism in fungi (Hautbergue et al., 2018); in *Fusarium fujikuroi* , it affected the expression of 3 out of 10 TS genes (Wiemann et al., 2013). Similarly, saline stress modulates changes in SMs production, as it has been shown in *T. harzianum* (Bualem, Mohamed, & Moulay, 2015). N starvation and saline stresses negatively regulated TS genes in*T. gamsii* T6085, suggesting terpenoid biosynthesis does not confer particular advantages to the fungus to overcome these stresses.

In *T. arundinaceum* , *tri* gene expression is affected when grown in dual cultures with *B. cinerea* , while polyketides and harzianum A (HA) produced by the first induce changes in some *B. cinerea* genes linked to its virulence (Malmierca et al., 2015). Since*T. gamsii* T6085 is able to suppress *F. graminearum* on wheat spikes and to reduce DON production by the pathogen (Matarese et al., 2012; Sarrocco et al., 2019, 2013), we investigated whether a differential expression of TS genes of *T. gamsii* T6085 occurs when the fungus interacts with *F. graminearum* on wheat spikes. However, our results show that the presence of the pathogen did not induce prominent changes in TS expression in *T. gamsii* T6085 when both fungi were on wheat spikes, although *ts11* was found slightly up-regulated. Gene expression patterns are highly dynamic, and more extensive time-course experiments are needed to provide more information about the role of TSs in this scenario.

Root colonization by *Trichoderma* is an intimate relationship involving a tightly regulated exchange of molecular signals including SMs (Hidangmayum & Dwivedi, 2018). When *Trichoderma* colonizes the roots, it releases a variety of SMs that promote substantial changes in plant biochemistry, which in turn, cause changes in the fungal physiology (Contreras-Cornejo et al., 2018). Our results indicate that a significative reprogramming of terpene biosynthesis occurs in *T. gamsii* T6085 when colonizes wheat roots. Down-regulation of *ts9* , suggests that root colonization induces a repression in indole diterpene biosynthesis in the fungus. In addition, the contrasting effects observed in the expression of sesquiTS and the up-regulation of the SQS, suggest that root colonization induces a modulation on sesquiterpene and triterpene biosynthesis in *T. gamsii* T6085 through FPP as central node. The absence of *tri5* expression in response to wheat spikes and the strong up-regulation observed during root colonization was remarkable, as it suggests that signals from the roots induce the expression of this gene in *T. gamsii* T6085. Although activation of *tri5* usually leads to the production of phytotoxic compounds, such as trichodermin in *T. brevicompactum* (Tijerino et al., 2011a), HA produced by *T. arundinaceum* lacks on phytotoxic activity and has a crucial role in plant protection against *B. cinerea* (Malmierca et al., 2013). This example illustrates how different ecological demands led to an adjustment in metabolic pathways governed by a single signature gene in species within the same fungal genus (Mukherjee et al., 2013). In this context, we can imagine the involvement of *tri5* in the biosynthesis of a sesquiterpenoid involved in promoting a beneficial relationship between *T. gamsii*T6085 and wheat plants, a question that must be further addressed by using *tri5* -deletion mutants of the fungus. Results strongly indicate the involvement of TS genes in the interaction of *T. gamsii* T6085 with plant roots, and further studies determining their impact on the plant physiology will provide more information about the roles of these genes in the *Trichoderma* -plant beneficial interaction.

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DATA ACCESIBILITY

Terpene synthases protein accession numbers shown in Supporting Information Fig. S1 are available in public databases of the Joint Genome Institute (*JGI*) and the National Center for Biotechnology Information (NCBI). Terpene synthases and β-tubulin gene accession numbers of *T. gamsii* T6085 used for gene expression analyses are available in NCBI as: XM_024549662 (*β-τυβυλιν*), XM_018805995 (*ts1*), XM_018801080 (*ts3*), XM_024550105.1 (*ts4*), XM_018800313 (*ts5*), XM_018800491 (*ts6*), XM_024549058 (*ts7*), XM_018806700 (*ts11*), XM_018800875 (*tri5*). Nucleotide sequence of *ts9* gene was extracted from the whole genome sequence of *T. gamsii* T6085 (NCBI WGS Project accession number JPDN00000000) using the *JGI* protein code Trigam1_9898.

AUTHORS CONTRIBUTION

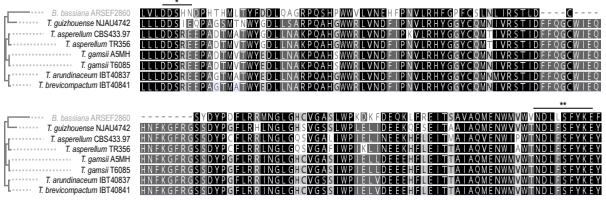
G.V., E.M., S.S., R.H. and R. Baroncelli. designed the research. I.V., R. Baroncelli, M.E.M-D., R. Bernardi and G.P. performed the experiments. I.V., S.S., R.B. and M.E.M-D analysed the data, and G.V., E.M. and R.H. contributed to data interpretation. I.V. wrote the manuscript, and all the authors contributed to its revision.

Conflict of Interests

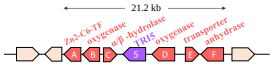
All authors declare no conflict of interest.

Phylogeny	Predicted function	Enzyme type	Aspartate-rich motif
	HAD-like	Bifunctional	DDXXE and DXDTT
	Uncharacterized group 1	Class I – TS	DDXXX
	Trichodiene synthases	Class I – TC	DDSRE / DDSIE
	Uncharacterized group 2	Class I – TS	[D/E]XX[D/E]
	Longiborneol synthases	Class I – TC	DDHFD / DEHFD / NDHFD
	Uncharacterized group 3	Class I – TS	DDIRE
	Presilphiperfolan-8ββ-ol synthases	Class I – TC	DDQFD / DDPAA
	Pentalenene synthases	Class I – TC	DD(M/V/L)FD
	Uncharacterized group 4	Class I – TS	DDXXD
	Squalene synthases	Class I – PT	DDMTI / DDMTH
	GGTases 1	Class I – PT	DEXXV / DAXXV
	GGTases 2	Class I – PT	DEWGE
	FTases	Class I – PT	D[D/E]XX[D/E]
	Uncharacterized group 5	Class I – TS	DXXXE
	Oxidosqualene cyclases	Class II – TC	DCTSE / DCISE
	Diterpene synthases	Bifunctional	DEXXE and DXDD
	GGPP synthases	Class I – PT (IPS)	DDVID and DDMLD
	FPP synthases	Class I – PT (IPS)	DDIMD and DDXXD
	Indole-diterpene synthases	Class I – PT	DDYLD and DDVQD
	Chimeric-like	Chimeric PT-TC PT/TC	D[D/E]XXD and/or D[D/E]XXD

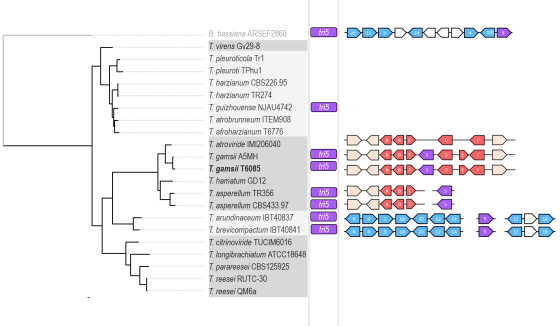
a)

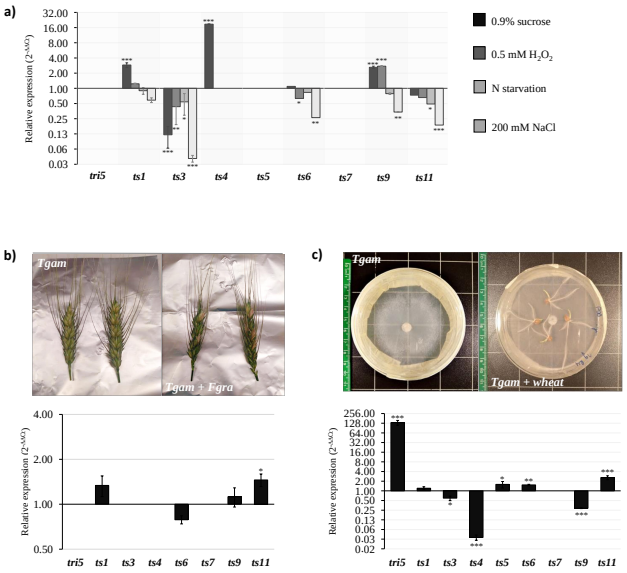


b)



c)





***JGI* Id.**	**Name**	**Putative function**
Trigam1|5596	TC4	bifunctional HAD-like
Trigam1|4742	**TRI5**	**trichodiene synthase**
Trigam1|162	**TS5**	**pentalenene synthase**
Trigam1|9843	**TS7**	**sesquiTS**
Trigam1|1824	**TS4**	**sesquiTS**
Trigam1|4947	**TS3**	**sesquiTS**
Trigam1|5367	**TS1**	**uncharacterized 4**
Trigam1|340	**TS6**	**squalene synthase**
Trigam1|3208	TC3	GGTase I
Trigam1|5139	TC5	GGTase II
Trigam1|3927	TC1	FPP transferase
Trigam1|4065	**TS8**	**GGPP synthase**
Trigam1|2917	**TS10**	**FPP synthase**
Trigam1|9898	**TS9**	**indole diTS**
Trigam1|8345	TC2	oxidosqualene cyclase
Trigam1|6072	**TS11**	**uncharacterized 5**