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RESEARCH PAPER

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Geosmin synthase *ges1* knock-down by siRNA in the dikaryotic fungus *Tricholoma vaccinum*

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Abstract

Genetic manipulation for generating knock-out experiments is essential in deciphering the precise function of a gene. However, dikaryotic fungi pose the inherent challenge of having two allelic versions of each gene, one in each nucleus. In addition, they often are slow-growing and do not withstand protoplasting, which is why *Agrobacterium tumefaciens*-mediated transformation has been adapted. To obtain knock-out strains, however, is not feasible with a mere deletion construct transformation and screening for deletions in both nuclear copies. Hence, a convenient method using chemically synthesized dicer substrate interfering RNA (DsiRNA) for posttranscriptional interference of targeted mRNA was developed, based on the fungal dicer/argonaute system inherent in fungi for sequence recognition and degradation. A proof-of-principle using this newly established method for knock-down of the volatile geosmin is presented in the dikaryotic fungus *Tricholoma vaccinum* that is forming ectomycorrhizal symbiosis with spruce trees. The gene *ges1*, a terpene synthase, was transcribed with a 50-fold reduction in transcript levels in the knockdown strain. The volatile geosmin was slightly reduced, but not absent in the fungus carrying the knockdown construct pointing at low specificity in other terpene synthases known for that class of enzymes.

KEYWORDS
dikarya, dsiRNA, terpene synthase, *Tricholoma vaccinum*

1 | INTRODUCTION

Understanding the function of genes for biological processes has been depending on genetic manipulation including single gene deletions [1–3]. One of the most intriguing aspects of fungal cell biology is the multinucleate nature of hyphal cells. Nuclear numbers range from close to one hundred in some ascomycetes to one nucleus per cell compartment [4]. Although many filamentous fungi, and prominently the ascomycete molds, can produce single-nuclei conidiospores or vegetative spores, this is not the case for many dikaryotic fungi carrying two nuclei within any single cell, a status mainly found in Basidiomycota. Transformation with extra copies thus is possible, but deletion needing independent knock-out of the two copies in either nucleus is usually not possible. In addition, transformation methods may need adaptation. Although protoplasting is an established method in some dikarya species, in others resuming growth after protoplasting and even after

maceration has been challenging. Fungi have a suite of cell wall types that differ between species and that change at different stages during growth and development. These differences likely explain why the ease and success of protoplasting vary between species [5]. A method to overcome difficulties in the transformation was established using *Agrobacterium tumefaciens*-mediated transformation (*At*MT, compare also [6]), but that still does not allow for a true knock-out in these dikaryotic fungi.

Newer approaches like CRISPR-Cas9, using Cas9-induced gene inactivation via double-strand break repair causing nonhomologous end joining (NHEJ) events also have some inherent challenges, as it has been suggested that the robust DNA damage machinery in basidiomycetes might be able to result in full repair [7]. In addition, the expression of bacterial genes like *cas9* may be limited, because for basidiomycetes it has been shown that they require introns for stable expression [8]. For the genetically well-studied basidiomycete *Schizophyllum commune*, transformation with a protein-RNA complex including Cas9 has been shown [7]. However, this again requires protoplasting, which is not feasible for *T. vaccinum* and many more slow-growing basidiomycetes. Also, for *S. commune*, homologous integration has been enhanced using deletion mutants for the NHEJ pathway genes *ku70* or *ku80* [9]. Again, owing to the lack of gene deletion strategies, this is not feasible for non-model basidiomycetes including *T. vaccinum*.

RNA interference (RNAi) has been introduced for understanding gene function [10]. This relies on argonaute and dicer proteins, which are present in almost all Basidiomycota [11]. Double-stranded RNA was first introduced as a potent and specific tool for genetic interference in *Caenorhabditis elegans* [12] and has developed into a very useful tool in plant-pathogen treatment [13]. The method became more popular when it was discovered that the dsRNA could be exogenously introduced into eukaryotic cells to induce specific gene knock-down [14,15].

RNAi is a sequence-specific posttranscriptional gene silencing mechanism mediated by small interfering RNAs (siRNAs). These short products of a double-stranded RNA are released by the action of the RNase III type enzyme dicer. The siRNAs are then incorporated into the RNA-induced silencing complex (RISC) reducing cognate mRNA levels [16].

The siRNA silencing requires transformation into the recipient cell [16–18]. More recently, however, chemically synthesized dsRNA has been used for interference targeting the RISC complex genes in many fungal species [19,20]. The direct uptake of small RNAs by fungal cells for gene knock-down has been reported in monokaryons, for example, in germinating spores [21,22]. Bioinformatic tools have been developed in RNAi design to predict the efficiency of the silencing and to estimate the probability of off-targets [23–25]. The specificity of the siRNA construct and the possibility for screening for off-targets allow for a precise and targeted silencing of genes in the target organism.

Here, we investigated the genetic machinery for the dicer substrate interfering RNA (DsiRNA)-mediated gene knock-down in *T. vaccinum* to see, whether the application of DsiRNA was feasible. We then went on to use chemically synthesized dsiRNA to target a gene involved in the production of the volatile, geosmin, in the ectomycorrhizal basidiomycete *T. vaccinum*. The biosynthetic gene for a secondary metabolite was chosen to avoid lethality that may interfere with the experiments. Hence, secondary metabolism was addressed, and here, geosmin synthesis is of special interest. It may be expected that a volatile is involved with the host and/or the ectomycorrhizosphere microbiome even at a distance before the symbiosis is fully established. Hence, the role of such volatiles was observed. In addition, terpene synthases are known to be promiscuous, allowing to substitute at least in part for other members of the enzyme family. Hence, negative effects of reduced volatile production may be avoided. Furthermore, *T. vaccinum* being a slow-growing basidiomycete fungus, the reduced growth effect while silencing genes involved in primary development in fungi (e.g., reduced growth in *Aspergillus nidulans* [16]) might be difficult to observe [26]. Therefore, a well-studied biosynthetic gene with a known product in *T. vaccinum*, *ges1* [26], was selected as a candidate gene to test for the efficiency of exogenous DsiRNA in the fungus. The knock-down was confirmed via reverse transcription-polymerase chain reaction (RT-PCR) and the volatile was measured. This enabled transcript level manipulation in spite of the fact that neither protoplasting nor gene deletion methods are available for this fungus that does not grow for extended periods of time as a monospore, monokaryotic strain. Hence, this fungus with interesting ecological interactions was a perfect candidate to show in a proof-of-principle that gene knock-down is possible in multinucleate (here dikaryotic) filamentous fungi.

This study, therefore, describes a very convenient, timely molecular tool for analysis of gene function in non-model dikaryotic fungi like the mushroom-forming ectomycorrhizal fungus, *T. vaccinum*.

2 | MATERIALS AND METHODS

2.1 | Growth and DsiRNA treatment of *T. vaccinum*

T. vaccinum GK6514 (FSU4731, Jena Microbial Resource Collection) grown in 30 ml liquid Modified Melin Norkrans b (MMNb) medium [27] at room temperature was

used after one week to target *ges1*, involved in the production of the volatile geosmin [26].

Chemically synthesized custom-designed DsiRNA primers (Table 1) were commercially obtained (Integrated DNA Technologies) to establish knockdown treatments, and a randomly generated scrambled DsiRNA sequence was used as a negative control. DsiRNA sequences were used with 10 μl of 2 μM solutions added to the cultures daily for 15 days and a short mixing gently by swirling the culture. Treatments were run in triplicates. The application for 15 days was performed to compensate for extracellular RNAse degradation, and the period of 15 days was chosen such as to allow the very slow-growing fungus that needs 4 weeks to form a visible mycelial growth in liquid culture to react with knockdown of the targeted gene expression and to form sufficient geosmin in the culture headspace for detection [26].

2.2 | Dicer and argonaute analyses

The protein sequences of *Neurospora crassa* dicer, Dcl-1(NCU08270) and Dcl-2 (NCU06766) and argonaute, Qde-2 (NCU04730), and Sms-2 (NCU09434) were obtained from NCBI. These protein sequences were used to BLAST the *T. vaccinum* genome (JGI ID 59348) with default BLASTp searching and filtered proteins database. The conserved domains of all sequences were examined with the online program SMART [28]; proteins containing both PAZ and PIWI domains were identified as argonaute and proteins containing at least two entire RNAse III domains were identified as dicer.

2.3 | Measurements of geosmin via volatile collection

Volatiles in the headspace of the treated *T. vaccinum* cultures (DsiRNA-ges1 and DsiRNA-scrambled mock-treatment) were sampled over 48 h via solid-phase microextraction (SPME; Supelco DVB/CAR/PDMS) [26], followed by gas chromatography-mass spectrometry (GC-MS) analyses (Trace 1310 coupled to Q-Exactive mass spectrometer, Thermo). MS-parameters were: resolution, 120,000; AGC target: 1×106; maximum ion time, 200 ms; scan range: 40–500 m/z. Transfer Lines 1, 2, and 3 as well as the ion source temperature were set to 300°C. The GC parameters were: after an initial 1 min at 40°C, the GC oven temperature was raised to 320°C with 10°C/min and held for 3 min. The split/splitless injector was operated in split mode at 250°C with a flow rate of 10 ml/min, the split flow was 10, and the column flow was 1 ml/min. The SPME fiber was kept for 10 min for sampling and 1 min inside the injector. Spectra of commercially available geosmin (Sigma-Aldrich) was used for standard.

2.4 | Confirmation of gene knock-down by DsiRNA treatment

Total RNA of DsiRNA treatments was isolated [29] and expression changes for *ges1* were observed by RT-qPCR using designed primers (see Table 1) spanning an intron (primer synthesis: Eurofins Genomics). For reference, genes *act1, cis1*, and *tef1* coding for actin, citrate synthetase, and translation elongation factor EF1α (primer sequences, see Table 1), respectively, were used with three technical replicates for each cDNA sample. The qPCR (initial denaturation 95°C, 10 min; denaturation 95°C, 20 s; annealing 55°C, 20 s; elongation 72°C, 20 s with 34 cycles was followed by a melting curve 65–95°C with 0.1°C/s) was performed using qTOWER 3 (Analytik Jena) with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Primer efficiencies were calculated using a dilution series of the cDNA and expression ratios were normalized [30].

2.5 | Statistical analyses

To compare two different treatments, a two-tailed unpaired Student's *t* test was used. Significance levels are indicated, $p < 0.05$ with small letters. Groups of data are given as average values ± standard deviation.

3 | RESULTS

3.1 | *T. vaccinum* dicer-like protein and argonaute analysis

Five copies of argonaute, similar to the argonaute protein in *N. crassa*, were detected in the genome of *T. vaccinum* (gene identifiers: *arg1* g5566.t1, *arg2* g28.t1, *arg3* g210.t1, *arg4* g4909.t1, *arg5* g9239.t1), and two copies were detected for dicer-like proteins (*dcl1* g2416.t1, *dcl2* g4389.t1). All *T. vaccinum* argonaute proteins had the conserved structure containing a PAZ domain, a PIWI domain, and additional domains found in argonaute proteins such as DUF1785, ArgoN, and ArgoL2 (Figure 1a). *T. vaccinum* dicer orthologs were composed of a DExH box, an RNA helicase domain (HELICc), an RNA binding dicer dimer domain, and two RNase III domains (Figure 1b).

TABLE 1 Duplex sequences used for DsiRNA treatment of *Tricholoma vaccinum* in gene knockdown experiments, and primers used in this study

Duplex sequences				
Gene	**Sequence**			
ges1	5′-rArUrCrUrGrGrArUrArUrUrCrCrUrGrArUrGrArArGrUrCAT-3′			
	5′-rArUrGrArCrUrUrCrArUrCrArGrGrArArUrArUrCrCrArGrArUrUrG-3′			
Scrambled	5′-rArUrCrGrCrArArUrUrArArCrUrArArUrGrCrArUrUrGrArGrC-3′			
	5′-rUrUrUrCrGrUrUrUrGrGrCrCrCrArArArGrGrGrGrCrCrCrC-3′			
Primer sequences used for RT-qPCR				
Gene		**Sequence**	**bp**	**Temperature (°C)**
act1	Forward	5′-ACAACCATGTTCCCCGGTATCT-3′	22	60.3
	Reverse	5′-TTCGCTCAGGAGGAGCAGGCAAT-3′	23	60.3
cis1	Forward	5′-CAAATTCGTGCCGAGCATGG-3′	20	59.4
	Reverse	5′-AACCGTCCCAGATGAGAGCA-3′	20	59.4
tef1	Forward	5′-GGCAACTTATTGTTGCTGTGAACAA-3′	25	59.7
	Reverse	5′-GACCTTCTTGATAAAGTTGGAGGTT-3′	25	59.7
ges1	Forward	5′-CACTTCCCAAATACAGACCGTTCCC-3′	25	64.9
	Reverse	5′-AAATCTTCGCTGGGTGCCCTCT-3′	22	64.9

Abbreviation: RT-qPCR, quantitative reverse transcription-polymerase chain reaction.

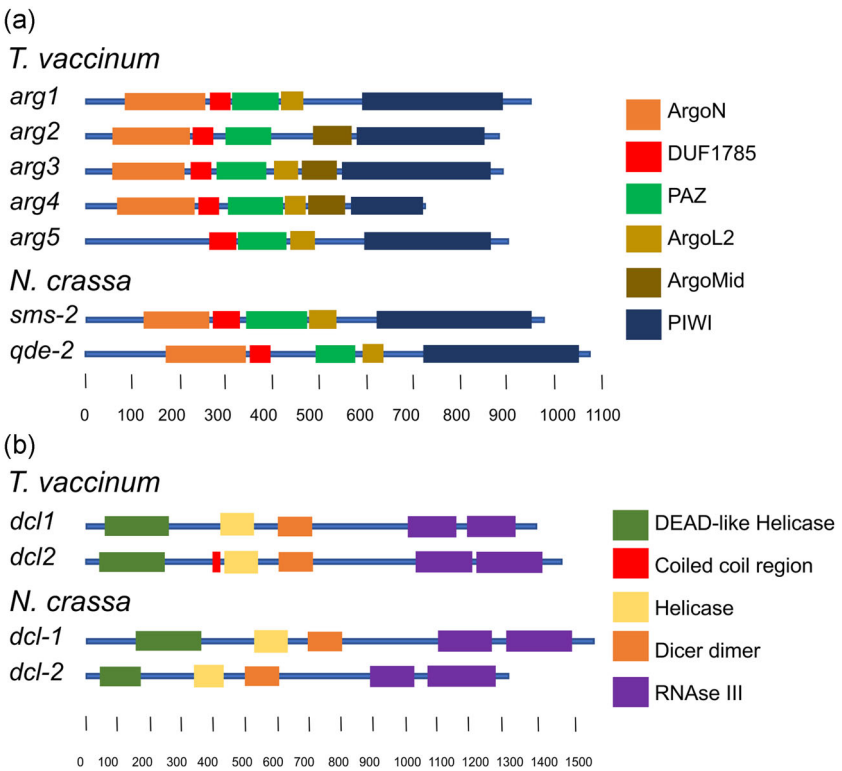

FIGURE 1 Architecture of argonaute and dicer proteins predicted from the *Tricholoma vaccinum* genome sequence. (a) The five *T. vaccinum* argonaute proteins were obtained as best hits of a BLAST search using *Neurospora crassa* proteins Qde-2 and Sms-2 as queries. All contained the conserved PAZ and PIWI domains known for argonaute proteins. (b) For dicer-like proteins predicted from genes of the *T. vaccinum* genome, two RNase III domains were detected, similar to dicer-like proteins characterized in *N. crassa*

The presence of the relevant proteins for DsiRNA knock-down indicated a fully functional RNA degradation pathway through the RISC complex. This allowed us to pursue the idea of using dsRNAs for minimizing transcript levels of target genes.

3.2 | DsiRNA-mediated gene knock-down

The application of oligonucleotides for gene knock-down allows for an easy gene characterization in organisms that are less well amenable to full knock-out recombination like the ectomycorrhizal *T. vaccinum*. As a proof-of-principle, geosmin production was targeted with the terpene synthase gene *ges1* [26]. As a negative control for RNA interference, a scrambled sequence was used that did not yield a hit in BLAST search within the genome of *T. vaccinum*. After 15 days of DsiRNA application, transcript levels were checked for the targeted gene *ges1*. Indeed, the knock-down did show a dramatic effect of DsiRNA in the transcript levels of *ges1* compared to the control (Figure 2). A 50-fold reduction indicates successful RNA interference.

3.3 | RNA interference effect on volatile production

The successful RNA interference allowed us to screen for changes in geosmin production. Mass spectrometric analyses of both treatments (*ges1* DsiRNA and scrambled DsiRNA) showed the presence of geosmin in the headspace of the respective cultures. However, the amount of geosmin was somewhat lower in the DsiRNA-ges1 treatment (Figure 3). We, therefore, can conclude that either a feedback loop regulates geosmin production even with 50-fold lower transcript and hence enzyme amounts, or that the other terpene synthases by promiscuous substrate acceptance carried out the reaction.

4 | DISCUSSION

The number of copies of argonaute proteins is similar to the number of copies found in *Fomitopsis pinicola* and also in the range of copies of argonaute and dicers found in other agaricomycetes [11]. The dicer-like proteins in *T. vaccinum*, however, lack the PAZ domain that was reported [31]. The two RNase III domains and the interspersed structures are essential for snRNA synthesis, while the function of other domains may also be maintained by separate genes [6]. Hence the *T. vaccinum*

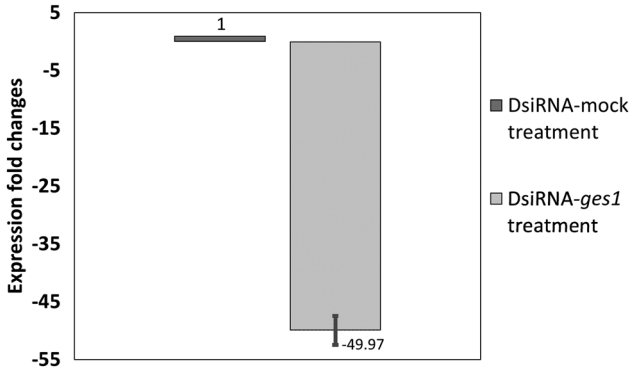

FIGURE 2 Expression of *ges1* during dicer substrate interfering RNA (DsiRNA) treatment (fold change) measured by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and compared to mock treatment for control

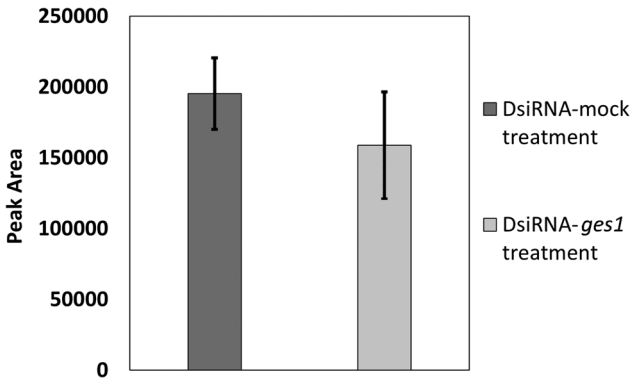

FIGURE 3 Production of geosmin in the headspace of *Tricholoma vaccinum* cultures during treatments with *ges1* DsiRNA and the mock control measured by gas chromatography-mass spectrometry ($p > 0.05$, $n = 3$)

genes are in good accordance with agaricomycete argonaute and dicer-like protein-encoding genes, allowing for dsRNA interference approaches.

Methods for knocking down translation of transcribed mRNAs usually involve transformation with suitable vectors that express short hairpin RNAs [10,32]. Previously, transformation strategies for fungi like *T. vaccinum* have been established using *A. tumefaciens*-mediated transformation [33,34] and require transformant selection. A new system using chemically synthesized, exogenously added double-stranded RNAs has been developed for plant protection in agriculture [35,36]. These dicer substrates form small interfering RNAs that associate with argonaute within the cell to form a RISC. This complex cleaves the targeted mRNA [37].

One of the major advantages of this method is the complete absence of a permanent genetic manipulation

that allows for acceptance by the general public. Also, in contrast to the use of CRISPR/Cas9, no introduction of endonuclease is necessary, when using the fungal system where it is present already.

Using the chemically synthesized DsiRNA for direct uptake into the growing fungus in RNA-mediated knock-down, we could show a markedly reduced abundance of the transcript. However, the volatile gene product, geosmin, was still produced. This could be due to the promiscuity of synthetases present in the eukaryotic cells. Indeed, another gene, g2958, had been reported earlier to potentially perform a similar function in *T. vaccinum* [26]. Therefore, complementation of *ges1* function by g2958 or other promiscuous synthetase is proposed.

Previously we had shown by deuterium labeling that geosmin is synthesized via the terpene synthesis pathway [26]. The genome sequence of *T. vaccinum* [38] yielded multiple terpene synthase genes. However, the conceptually translated Ges1, in addition to similarity to *Coprinus cinerea* sesquiterpene synthases Cop1 and Cop3, was the only one showing similarity to the known characterized germacradienol/germacrene D synthase in *Termitomyces* sp. [26]. Therefore, we sought a way to delete or knock down the expression of Ges1.

All in all, our proof-of-concept allows for wide application of DsiRNA knock-down in genetically not easily amenable fungi, specifically dikaryotic basidiomycetes that do not propagate by uninucleate spores.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All sequence information is available at the given accession numbers with GeneBank.

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