

Identification and applicability of *azg1* as counter-selectable marker in *Trichoderma* species

Clara Baldin

`clara.baldin@uibk.ac.at`

University of Innsbruck

Anna Oberdanner

University of Innsbruck

Laurenz Schark

University of Innsbruck

Julia Nagler

University of Innsbruck

Ulrike Schreiner

University of Innsbruck

Lesley Huymann

University of Innsbruck

Fabio Gsaller

Innsbruck Medical University

Susanne Zeilinger

University of Innsbruck

Research Article

Keywords: Counter-selectable marker, *Trichoderma* spp., 8-azaguanine, inducible system, fluorescent proteins, nucleobase analogs

Posted Date: October 28th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11522001/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: The authors declare no competing interests.

Abstract

Background

The widespread use of pesticides and antibiotics in agriculture and clinical settings has accelerated the emergence of antimicrobial resistance across diverse organisms. Although this phenomenon is widely recognized in pathogenic microorganisms, much less is known about the effect on beneficial fungi that are playing a role in sustainable agriculture, such as *Trichoderma*. Many *Trichoderma* isolates, especially those originating from intensively treated soils, exhibit low sensitivity to commonly used laboratory antifungals, complicating their genetic manipulation.

Results

In this study, we developed new genetic tools to facilitate the characterization and engineering of *Trichoderma* species, exploiting endogenous counter-selectable markers. Using *Trichoderma atroviride* as a model species, we first assessed fungal susceptibility to the nucleobase analogs 8-azaguanine, 5-fluorouracil and 5-fluorocytosine. While the fungus was sensitive to the first two compounds, 5-fluorocytosine showed no inhibitory effect. Using CRISPR/Cas9-mediated protoplast transformation, we generated mutants lacking a putative nucleobase analog transporter and identified the gene responsible for 8-azaguanine uptake. Deletion of this gene conferred resistance to 8-azaguanine without affecting the overall phenotype or growth behavior of *T. atroviride* under a range of tested conditions. This locus was subsequently employed as a counter-selectable marker for targeted integration events in *T. atroviride* and additional *Trichoderma* species. Furthermore, heterologous expression of the *Penicillium chrysogenum* cytidine deaminase gene, which catalyzes the intracellular metabolism of 5-fluorocytosine to 5-fluorouracil, rendered *T. atroviride* sensitive to this compound.

Conclusion

Together, our findings establish *azg1* as a multifunctional genomic platform and expand the genetic toolkit available for *Trichoderma* research, providing the foundation for advanced strain engineering in agricultural and biotechnological applications.

INTRODUCTION

The genus *Trichoderma* comprises fast-growing filamentous fungi within the phylum Ascomycota that are widely distributed across terrestrial and aquatic ecosystems [1, 2]. Species of this genus are well known for their metabolic versatility, ecological adaptability, and antagonistic interactions with other microorganisms [1, 3]. Many *Trichoderma* species exhibit a mycoparasitic lifestyle and produce a diverse array of hydrolytic enzymes and secondary metabolites, properties that underpin their extensive use as biological control agents (BCAs) in agriculture [4–6]. In addition, selected species have become indispensable in industrial

biotechnology, most notably *Trichoderma reesei*, which is widely exploited for the large-scale production of cellulolytic enzymes [7, 8].

Other *Trichoderma* species, including *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma virens* or *Trichoderma atroviride*, are commonly used both as BCAs and as model organisms to study fungal–fungal and fungal–plant interactions, with particular emphasis on mycoparasitism, secondary metabolism, and photoconidiation [3, 9–17]. However, despite the increasing interest in *Trichoderma* spp. for the above-mentioned reasons, the development of efficient genetic tools for these fungi has lagged behind that of other filamentous ascomycetes [18–22]. *T. reesei* represents an exception, as, due to its industrial relevance, it can count on a comparatively mature engineering stack built around auxotrophic and dominant markers, non-homologous end-joining (NHEJ)-deficient backgrounds, targeted integration, reusable/bidirectional selection, and Clustered Regularly Interspaced short palindromic repeats (CRISPR)-Cas9 assisted genome engineering [23–31].

Genetic manipulation in *Trichoderma* is particularly constrained by the limited repertoire of selectable markers [20, 21, 28, 32–35]. In contrast to genera such as *Aspergillus*, where numerous dominant, auxotrophic, and counter-selectable markers are available [36–45], gene editing in *Trichoderma* spp. relies predominantly on a small number of dominant resistance markers, most commonly the hygromycin B phosphotransferase-encoding gene (*hph*) [8, 20, 21, 30, 35, 46]. This limitation is strongly linked to the increased tolerance that some *Trichoderma* species exhibit to commonly used markers, complicating transformation-based selection. Such tolerance is frequently associated with their environmental origin, as many isolates used in research or industrial applications derive from soils extensively treated with agrochemicals, including fungicides and antibiotics [47–49]. This limitation, together with the unsuitability to use Ku70/Ku80 deletion mutants as wild type (WT) backgrounds [25], complicates the genetic engineering of these species, hampers marker recycling, and restricts the implementation of advanced genome-editing strategies, including iterative gene deletions and targeted integrations.

Counter-selection, also referred to as negative-dominant selection, represents a robust strategy to overcome these constraints by facilitating the isolation of mutants that lack genes involved in the uptake and/or metabolic activation of otherwise innocuous compounds, such as prodrugs [50, 51]. In filamentous fungi, recently-established examples include the use of nucleobase analogs such as 5-fluorocytosine (5-FC) and 8-azaguanine (8-AG) [41, 52–55]. *Aspergillus fumigatus* represents the perfect example for the development of counter-selectable markers. Starting with the optimization of 5-FC selection [40, 56], it was recently shown how disruption of genes responsible for uptake and key metabolic activities in the pyrimidine salvage pathway conferred resistance to different pyrimidine analogs [57–59]. Further implementation of this strategy, by including 8-AG [54, 60], allowed on top of consecutive selection even the possibility to achieve three simultaneous recombination events in one single transformation [45].

The use of endogenously encoded counter-selectable markers has emerged as an appealing and innovative approach to facilitate genetic manipulations. Nucleobase analogs are primarily known in cancer therapy, due to their cytotoxic activity, and their antimicrobial application was mainly restricted to a narrow spectrum of pathogens and often in combination with other drugs [61, 62]. However, recent discoveries

reopened the door for repurposing this class of compounds as promising antifungal [56]. Although the efficacy and versatility of selection systems based on nucleobase analogs have now been established in *Aspergillus*, their applicability to *Trichoderma* spp. has not been systematically explored.

In parallel, the toolbox for *Trichoderma* spp. is not only deprived of selectable markers, but also of other molecular instruments that are often used in the characterization of genes or protein functions, like fluorophores or other reporters [35, 63–66]. Similarly, most studies related to inducible promoters are conducted only in *T. reesei* [8, 67–69], due to the industrial pressure to accelerate large-scale heterologous protein production, but leave the possible use of such systems in other *Trichoderma* species largely underexplored. Moreover, the majority of established inducible expression options is triggered by sugars, amino acids, vitamins, metals and light [8, 68, 70], and similar strategies, even if adequately fitting in the context of industrial production, appear not well suited for studies in the mycoparasitic species. In a mycoparasitic confrontation setup, the tight control of factors such as amino acids or sugar content in the co-incubation medium would not be possible. The Tet-On/ Tet-Off system, with its regulation depending on tetracycline or doxycycline [71–74], could be a valuable option, but once again the few available studies have been focused only on *T. reesei* [8, 68] and never adapted to other species of the genus.

In this study, we addressed the lack of counter-selectable markers for *Trichoderma* species by investigating the sensitivity of *T. atroviride* to various nucleobase analogs. We demonstrated that the WT strain P1 is sensitive to 8-AG and identified a putative cellular importer based on protein sequence similarity to *Aspergillus nidulans* AzgA [55]. Deletion of the respective gene, hereafter referred to as *azg1*, resulted in stable resistance of *T. atroviride* to 8-AG. We further characterized the resulting deletion strain under different growth conditions and validated the *azg1* locus as a counter-selectable target for the integration of genetic constructs. We extended the applicability of this selection system to additional *Trichoderma* species, including *T. asperellum*, *T. virens*, and *T. reesei*, by targeting the respective *azg1* loci with reporter constructs. Finally, we combined the validation of *azg1* as counter-selectable marker with a first study on the Tet-On system applicability as well as with the validation of three new fluorescent reporters in *T. atroviride*. Together, our results establish *azg1* as a versatile counter-selectable marker and expand the genetic toolbox available for *Trichoderma* spp.

MATERIALS AND METHODS

Strains, media and growth conditions

All fungal and bacterial strains used in this study, including *Trichoderma* strains and prey fungi, are listed in Supplementary Table S1.

For plasmid amplification and plasmid assembly combining one insert and one vector, *Escherichia coli* Stellar cells (Takara, Saint-Germain-en-Laye, France) were employed, while assembly reactions combining three or more fragments were transformed and amplified in NEB 5-alpha Competent *E. coli* (New England Biolabs Inc., Ipswich, MA, USA).

Standard culture conditions for *T. atroviride* on solid media consisted of incubation at 25° C under a 12 h light/12 h dark photoperiod on either potato dextrose agar (PDA, BD Difco, Franklin Lakes, NJ, USA) or TMM [21]. For fungal protoplast transformation, TMM was supplemented with 1M sorbitol.

RNA handling and real-time quantitative PCR

For RNA extraction, *T. atroviride* WT spores at a final concentration of 1×10^6 spores/mL were inoculated in 50 mL of liquid TMM containing either 10 mM ammonium sulphate or 10 mM adenine as nitrogen source. Cultures were grown in biological triplicates for three days at 25°C shaking at 200 rpm. Mycelia were harvested using Miracloth (Merck KGaA, Darmstadt, Germany) and freeze-dried. Total RNA was extracted from 1 mg of each sample using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) as previously described [75]. Isolated RNA was treated with DNase I (ThermoFisher Scientific Baltic UAB, Vilnius, Lithuania) and RNA integrity as well as the absence of gDNA contamination were verified by electrophoresis using 1.5% agarose gels. For cDNA synthesis, 1 µg of DNase-treated RNA was reverse transcribed using the Revert Aid H- First Strand cDNA Synthesis Kit (ThermoFisher Scientific Baltic UAB, Vilnius, Lithuania) with a 1:1 combination of oligo(dT) and random hexamer primers supplied by the manufacturer. The resulting cDNA was diluted 1:10 with double distilled H₂O and stored at -70° C until use.

Real-time quantitative PCR (qPCR) was performed as previously described [76] using the LUNA Universal qPCR Master Mix (New England Biolabs Inc., Ipswich, MA, USA) in an qTOWER3 G cycler (Analytik Jena AG, Jena, Germany). Reactions were carried out in a final volume of 20 µL, containing 10 µL of Master Mix, 4 µL of cDNA template, 0.8 µL of each primer and 4.4 µL of sterile water. Relative expression of *azg1* was assessed using primers RT-azg1_FW3 and RT-azg1_RV. The reference gene *gapdh* [76] was used for normalization. The $2^{-\Delta\Delta C_t}$ method was used to calculate relative fold change gene expression, setting the condition with ammonium as nitrogen source as control. All primer sequences and other oligos used in this study are listed in Supplementary Table S2.

Generation of transformation constructs

For PCR amplification of transformation constructs, Q5 high-fidelity DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA) was employed according to the manufacturer's instructions. Further information regarding primers and oligos are listed in Supplementary Table S2.

Deletion of *azg1* in *T. atroviride* was initially achieved using the *hph* selection cassette flanked by 1 kb upstream and downstream homologous regions for targeted recombination. To this end, the plasmid pΔazg1-HPH was generated via the assembly of four fragments. The backbone was amplified from pGFP-XYR1 [77] using primers pRITA-bb-F1 and pRITA-bb-R1. The *hph* cassette was amplified from the same plasmid using primers *hph_F* and *hph_R*. Homology arms corresponding to approximately 1 kb upstream and downstream of the *azg1* locus were amplified from *T. atroviride* P1 gDNA using primer pairs *azg1_5F_bb/azg1_5R_hph* (5' flank) and *azg1_3F_hph/azg1_3R_bb* (3' flank), respectively.

The plasmids used for transformation of various *Trichoderma* species with a CRIB-mBasicGFP reporter targeted into the *azg1* locus, named with the species abbreviation in front (e.g. *T. atro* or *T. rees*) followed by pΔ*azg1*-CRIB-mVisGreen, were produced in a similar way. For *T. atroviride*, the above described pΔ*azg1*-HPH was used as template to PCR amplify the backbone using primers *azg1_3F_hph* and *azg1_5R_hph*, while the reporter expression construct was amplified from pLH2-Tasp [64] with primers *Ppki_FW_azg1* and *Tcbh2_RV_azg1*. For *T. reesei*, *T. virens* and *T. asperellum*, species-specific primers were designed to amplify the corresponding upstream and downstream flanking regions of the respective *azg1* loci, with primer overhangs for assembly with the backbone. Reporter constructs were generated using the same strategy described above, with species-specific primer overhangs. The backbone was amplified from pGFP-XYR1 [77] using primers pRITA-bb-F1 and pRITA-bb-R1 [64].

To assess whether introduction of a cytosine deaminase-encoding gene would induce 5-FC sensitivity in *T. atroviride*, four plasmids were generated: pΔ*azg1_Pcfcy1*, pΔ*azg1_Anigfcy1*, pΔ*azg1_Anidfcy1* and pΔ*azg1_Ncfcy1*. The common backbone was amplified from pΔ*azg1*-HPH using primers *Tgpdh_FW* and *Pgpdh_RV*, and contained the necessary elements for plasmid amplification in *E. coli*, *azg1* flanking regions for homologous recombination and the 5' and 3' regulatory regions of *T. reesei gpdh*. The sequence of *fcy1* from the selected species were amplified from the respective gDNA using species-specific primers (Supplementary Table S2) provided with tails for assembly to the backbone.

The two plasmids generated to test Tet-On inducibility in *T. atroviride*, pΔ*azg1_Tet-On(PoliC)_mVisGreen* and pΔ*azg1_Tet-On(Ppkia)_mVisGreen*, were assembled similarly. Both constructs had the same backbone, amplified from the template pΔ*azg1*-HPH using the primers *azg1_3F_hph* and *azg1_5R_hph*, as well as the same reporter construct (mBasicGFP followed by the *cbh2* terminator from *T. reesei*), obtained via PCR from the template Tatro_pΔ*azg1*-CRIB-mVisGreen using the primers *mVisGreen_FW* and *Tcbh2_RV_azg1*. For the inducible promoters, the Tet-On(*PoliC*) system was amplified from the template plasmid pΔ*fcyB_sGFP*^{Tet-On(*PoliC*)} [78] using *PoliC_azg1_5F* and *PoliC_mVisGreen_RV*, while the Tet-On(*Ppkia*) sequence was amplified from the template pΔ*fcyB_sGFP*^{Tet-On(*Ppkia*)} [78] using primers *Ppkia_azg1_5F* and *Ppkia_mVisGreen_RV*.

To evaluate FPs expression, each of the selected fluorophores was placed under the control of the constitutive *Pgpdh* promoter from *T. reesei* in the three respective plasmids pΔ*azg1_PgpdH_mko2opt*, pΔ*azg1_PgpdH_mtagbfp2opt*, and pΔ*azg1_PgpdH_k2s*. A common backbone for all FPs expression plasmids, containing an origin of replication and ampicillin resistance-encoding gene for amplification in *E. coli*, and the 3' and 5' flanking regions of the *azg1* gene, was amplified using pΔ*azg1*-HPH as template DNA with primers *TgpdH_FW* and *PgpdH_RV*. The specific FP coding sequences were amplified from plasmids pBM12 (mKO2opt), pBM123 (mTagBFP2opt), and pFG39 (Katushka2S) [59] respectively, using primers *FluoLinker_FW* and *FluoLinker_RV*.

PCR fragments were purified using the Monarch PCR & DNA Cleanup Kit (New England Biolabs Inc., Ipswich, MA, USA) and assembled using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's instructions. All generated plasmids were initially verified by colony PCR using OneTaq Quick-Load (New England Biolabs Inc., Ipswich, MA, USA)

according to the manufacturer's instructions. Positive clones were inoculated, plasmid extraction was performed using the Monarch Plasmid Miniprep kit (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's instruction, and further verification of the assembly was performed by restriction digestion and Sanger sequencing (Microsynth AG, Balgach, Switzerland). Positively verified constructs were used as template in a PCR reaction to generate the repair template for fungal transformation using species-specific primers *azg1*_5F and *azg1*_3R.

CRISPR/Cas9-assisted transformation

Trichoderma transformation was performed as previously described [21], with slight modifications. Briefly, two CRISPR-associated RNAs (crRNAs) were designed to target sequences near the 5' and 3' ends of the *azg1* coding sequence (Supplementary Table S2 and Supplementary Figure S1A). Before transformation, each crRNA was used to form the respective gRNA duplex, by incubating a 1:1 mix of crRNA and tracrRNA together with Duplex buffer (IDT, Coralville, IA, USA) at 95°C for 5 minutes, before letting the mixture cool down slowly. crRNAs, tracrRNA and Cas9 endonuclease were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Protoplasts were generated by incubating conidial germlings at 30°C with shaking at 70 rpm in a buffering solution (0.6 M KCl, 50 mM CaCl₂, 5 mM Tris-HCl) containing 0.1 g/mL VinoTaste Pro (Novozymes, Bagsværd, Denmark) for 1.5 to 2 h. The RNP complexes were prepared combining both gRNAs with 1 µg of Cas9 and 10 µL Cas9 working solution (20 mM HEPES, 150 mM KCl, pH 7.5) in a single tube and incubating the reaction for 5 minutes at room temperature. Each transformation reaction contained about 5×10^8 protoplasts, 1–2 µg of repair DNA and 3 µL of the prepared RNP mix. After polyethylene glycol (PEG) treatment, protoplasts were plated on selective medium containing 10 µg/mL 8-AG and incubated at 25°C under 12 h light/12 h dark conditions for up to 10 days.

Emerging transformants were purified to mitotic stability by two rounds of single spore isolation. Thereafter, spores were collected and used for gDNA extraction, to allow verification of correct in-locus integration by PCR genotyping. gDNA extraction was achieved using a combination of mechanical and chemical rupture with glass beads and lysis buffer (0.4 M Tris, 60 mM EDTA, 150 mM NaCl, 10 g/L SDS, pH 8.0). Homogenization was performed using a FastPrep-24 (MP Biomedicals, Eschwege, Germany) device followed by incubation at 65°C for 30 minutes. Potassium acetate precipitation was used to separate the nucleic acid from proteins and other debris, and finally gDNA was precipitated with isopropanol. For the genotyping PCR, a primer internal to the repair template (generally the reverse primer used to generate the first fragment downstream of the *azg1* 5' flank) was used in combination with Mx_*azg1*_5'_FW, which binds upstream the designated *azg1* 5' flank (Supplementary Table S2).

Phenotypic characterization

To test sensitivity of *T. atroviride* to various nucleobase analogs, 1×10^4 spores of the WT strain in a total volume of 5 µL were spotted in the center of plates filled with 20 mL of TMM (control) and TMM containing various compounds under investigation, which were added in concentrations previously reported to induce inhibition in *A. fumigatus* [40, 45].

To verify induced resistance to 8-AG upon deletion of *azg1*, 1×10^4 spores of *T. atroviride* WT and mutant strains in a total volume of 5 μ L were spotted in the center of plates filled with 20 mL of TMM in absence (control) or with increasing concentrations of the selective agent (1 μ g/mL and 30 μ g/mL 8-AG). To assess putative effects of *azg1* deletion on radial growth, *T. atroviride* WT and Δ *azg1* strains were further tested under a variety of conditions. TMM containing 1% glucose as carbon and 10 mM glutamine as nitrogen source was used as control. Tested conditions included: different nutrient sources (1% fructose and, separately, 10 mM sodium nitrate), complete darkness, osmotic stressors (0.5 M sodium chloride or 1.5 M sorbitol), oxidative stressors (20 mM hydrogen peroxide or 0.2 mM menadione), cell-wall stressor (50 μ g/mL calcofluor white), different temperatures (18°C and 37°C), different agar concentrations (2.5% and 3.5%), and different pH values (pH 3 and pH 7.5). To reach pH 3, 10% phosphate-citrate buffer was added after autoclaving.

Plates were incubated at 25°C (except those testing different temperatures) in light/dark condition (12 h cycles). Plates testing growth under complete darkness were kept in a dark box, and pictures and measurements were taken in a dark room. Incubation was carried on for up to 10 days, taking daily measurements of the colony diameter and pictures at days 3, 5 and 7. All assays were performed in technical duplicates for three independent clones. Significant variability of colony growth was evaluated from diameter measurements with an intermediate samples t-test assuming different variances ($p < 0.05$)

For evaluating putative effects of *azg1* deletion on the mycoparasitic activity of *T. atroviride*, WT and mutant strains were confronted on PDA plates with either *B. cinerea* or *F. graminearum* as prey fungi in a dual interaction assay. 1×10^4 spores (in a total volume of 5 μ L) of each interaction partner were spotted at a distance of about 7 cm on opposite sides of agar plates. Confrontation assays were conducted as well in technical duplicates for three independent clones. Plates were incubated at 25°C under light/dark conditions for up to 10 days, and pictures were taken daily for documentation.

Phylogenetic analysis

Protein sequences of putative *azg1* orthologs in *Trichoderma* species were retrieved using *azgA* from *A. nidulans* as query in a protein-protein blast (blastp, NCBI, Bethesda, MD, USA) and the obtained dataset was aligned with the online tool MAFFT version 7 [79, 80] using the default settings. The alignment was checked and manually curated (trimmed and corrected for unaligned positions) in Bioedit [81, 82]. The resulting curated file was used to create a maximum likelihood phylogenetic tree using IQTREE [83, 84]. To assess the robustness of the tree, 1000 fast Bootstrap replicates were conducted. Final editing and visualization of the tree were performed in ITOL [85].

Plate reader assay

Assessment of acquired sensitivity to 5-FC was performed in 96-well plates with 1:2 serial dilutions (ranging between 500 μ g/mL and 7.8 μ g/mL). Depending on the layout, wells were pre-inoculated with 2X TMM (containing 10 mM glutamine as nitrogen source), which was then diluted 1:1 with either sterile water or a 2×10^5 spores/mL suspension of the desired strain. The *T. atroviride* WT strain was included as control,

and two transformants per constructs were analyzed. Plates were incubated at 25° C in dark condition for up to 3 days and checked daily.

The mBasicGFP reporter signal expressed under the control of various Tet-On systems was measured using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Wavelengths for excitation and emission were set to 488 nm and 507 nm, respectively. Fungal growth development was evaluated at 600 nm (OD₆₀₀). Doxycycline stock solution was prepared by dissolving doxycycline monohydrate in 70% ethanol followed by filter sterilization. Depending on the layout, wells were pre-inoculated with 2X TMM (containing 10 mM glutamine as nitrogen source), which was then diluted 1:1 with either sterile water or a 2x10⁵ spores/mL suspension of the tested strain. 10 µL of doxycycline, diluted to the appropriate concentration (1:2 serial dilution, starting from 100 µg/mL), was added at the time point of inoculation (T0) or after 20 h of incubation at 25° C (T1). After an initial incubation for 20 h at 25° C in dark conditions, fluorescence and OD₆₀₀ measurements were taken at regular intervals (2 h) until reaching a maximum of 64 h.

Microscopy

Strains used for microscopic analyses were grown for 24 h at 25°C in dark conditions on plates with TMM and 1% phytigel (instead of agar), placed into inncelly experimentation chambers ib02 (inncellys GmbH, Mils, AT), as previously described [65], and incubated for 24 h at 25°C in dark conditions.

Visualization of CRIB_mBasicGFP reporter fluorescence was performed using a Nikon Eclipse Ti2 (Nikon Europe B.V., Amstelveen, Netherlands) inverted fluorescence microscope equipped with Nikon CFI Plan-Fluor 40x/0.75 NA and 60X/0.85 NA objectives and an Andor Zyla 5.5sCMOS monochrome camera. 490 nm LED light from a CoolLED p4000 LED unit was used at 5% of the full intensity to excite CRIB-GFP reporter molecules, and emission light between 515–545 nm was collected through a Nikon F66-413 quadband filter cube. Images were acquired using NIS-Elements software and saved in .ND2 format. For image inspection and analysis, ImageJ (Fiji distribution) was used with the Bio-Formats importer plugin.

Visualization of mKO2opt, mTagBFP2opt and K2S was performed using a Leica SP8x laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar Germany). Images were captured with a 40x objective and a resolution of 1,024 x 1,024 and 400 Hz. The pinhole was set to 429.95 µm. The laser intensity was 20%. Excitation wavelengths (Ex) and emission ranges (Em) were adapted to the corresponding FP: Ex 551 nm and Em 561–610 nm for mKO2opt, Ex 405 nm and Em 430–500 nm for mTagBFP2opt, and Ex 561 nm and Em 600–700 nm for K2S. The fluorescence gain was set around 800 V.

RESULTS

Identification of counter-selectable markers in *T. atroviride*

A schematic representation of putative metabolic pathways for nucleobase analogs, based on the recent characterization in *Aspergillus* species, is presented in Fig. 1A. A blastp analysis run against the *T. atroviride*

P1 genome database revealed the presence of multiple putative orthologs of the *A. fumigatus* cytosine permease FcyB and uracil phosphorybosiltransferase Uprt, as well as orthologs of the *A. nidulans* adenine-guanine-hypoxanthine transporter AzgA and for the major transporter of 5-fluorouracile (5-FU) FurD (Table 1). In contrast, no putative ortholog of the cytosine deaminase FcyA (AFUB_005410), crucial for the metabolic activation of 5-FC, could be identified.

To complement the bioinformatic analysis and evaluate potential counter-selectable systems experimentally, we tested the sensitivity of *T. atroviride* to 5-FC, 5-FU, 5-fluorouridine (5-FUR) and 8-AG (Fig. 1B). The fungus displayed strong resistance to high concentrations of both 5-FC and 5-FUR, in line with the absence of a cytosine deaminase and with its reported general broad resistance to various antifungal compounds. High concentrations of 5-FU (100 µg/mL), however, considerably inhibited *T. atroviride*'s growth, whereas 8-AG completely prevented growth, even at a concentrations as low as 10 µg/mL. Notably, these results were observed only upon cultivation in minimal medium (TMM); at least threefold higher concentrations were required to achieve comparable inhibition on rich media such as potato dextrose agar (PDA) (data not shown).

Table 1. BlastP search results						
Selection	Query	Results				
	A. fumigatus	T. atroviride P1	Score	E value	%identity	%coverage
5-FC	AFUB_025700/FcyB	Triatrov1_414389	675	3.55E-23	37.9	70.3
		Triatrov1_422238	446	1.89E-25	34.4	52.9
		Triatrov1_415578	437	5.03E-25	37.2	43.3
		Triatrov1_422021	388	1.71E-16	30.9	44.0
5-FC	AFUB_005410/FcyA	-				
5-FC/5-FU	AFUB_053020/Uprt	Triatrov1_439215	990	9.84E-131	77.8	97.2
		Triatrov1_173768	233	4.39E0.25	35.2	29.9
5-FUR	AFUB_001570/CntA	Triatrov1_232098	1726	6.38E0-156	60.0	83.7
	A. nidulans	T. atroviride P1	Score	E value	%identity	%coverage
8-AG	XP_681803.1/AzgA	Triatrov1_60818	2181	0.00E + 00	73.1	94.6
		Triatrov1_358927	1306	1.25E-72	57.2	80.0
5-FU	ABR22526.1/FurD	Triatrov1_203172	771	7.81E-36	43.1	59.7
		Triatrov1_468840	577	3.33E-27	31.4	29.8

Deletion and functional characterization of azg1 in T. atroviride

Given the low concentration of 8-AG required to inhibit *T. atroviride* growth, we selected this compound for further investigation as a potential counter-selective agent. Among the two putative orthologs identified, we initially focused on the gene encoding TRIATROV1_60818, which showed the highest sequence similarity to *A. nidulans* AzgA [54, 55]. We first analyzed the expression of this gene, hereafter designated *azg1*, upon fungal cultivation in TMM containing adenine as sole nitrogen source. Relative expression analysis revealed a considerable fold change in transcript levels in the presence of adenine compared to ammonium sulphate, supporting a role for *azg1* in adenine transport (Fig. 2A). For functional characterization of *azg1*, a respective *T. atroviride* deletion mutant was generated. To enhance transformation efficiency, a split-marker strategy combined with CRISPR/Cas9 RNPs was employed. Several transformants (> 10) were obtained, eight of which were randomly selected for single-spore isolation, genomic DNA (gDNA) extraction and genotyping. Seven of the eight transformants produced the expected PCR band corresponding to correct integration of the selection cassette at the target locus. Cloning strategy and genotyping results are available in Supplementary Figure S1A-B. Three independent deletion strains were subsequently selected for further characterization.

All tested $\Delta azg1$ mutants displayed growth comparable to that of the WT on TMM containing the preferred nitrogen source glutamine. In the presence of 10 $\mu\text{g/mL}$ 8-AG, the mutants remained unaffected while the drug completely inhibited growth of the WT. At concentrations higher than 30 $\mu\text{g/mL}$, the $\Delta azg1$ mutants exhibited reduced growth but were still able to sporulate (Fig. 2B).

Similar to the WT, $\Delta azg1$ mutants exhibited slightly faster growth on fructose-containing TMM compared to medium containing glucose as the sole carbon source, although sporulation was reduced (Fig. 2C and Supplementary Figure S2). Growth on sodium nitrate as a nitrogen source resulted in colony diameters comparable to those observed with glutamine; however, colonies displayed a thinner hyphal mat and delayed sporulation. Cultivation in complete darkness did not substantially affect vegetative growth but strongly impaired sporulation. Regardless of the differences observed between conditions, no significant variation (p value > 0,05) was detected between the WT and the $\Delta azg1$ mutants (Fig. 2C and Supplementary Figure S2).

To determine whether deletion of *azg1* affects the response of *T. atroviride* to stress, the mutants were exposed to osmotic stress (sodium chloride and sorbitol), oxidative stress (hydrogen peroxide and menadione), as well as cell-wall stress (calcofluor white). In addition, strains were tested at different temperatures (18°C and 37°C), under increased agar concentrations (2.5% and 3.5%) and at different pH values (pH 3 and pH 7.5). No significant growth difference between the WT and the $\Delta azg1$ mutants (p value > 0,05) was detected under any of the tested conditions (Fig. 2C and Supplementary Figure S2).

The mycoparasitic ability of the $\Delta azg1$ mutants was tested in dual confrontation assays using the phytopathogens *Botrytis cinerea* and *Fusarium graminearum* as prey fungi. After five days, the prey fungi were partially overgrown by *T. atroviride* with no differences between WT and $\Delta azg1$ strains (Fig. 2D).

Collectively, these results indicate that deletion of *azg1* confers resistance to 8-AG without affecting the overall phenotype of *T. atroviride*. The $\Delta azg1$ mutants exhibited growth, stress responses, sporulation, and mycoparasitic activity comparable to those of the WT under all tested conditions.

Validation of *azg1* as counter-selectable marker in *Trichoderma* species

In order to test the possible application as counter-selectable marker, the *azg1* locus of the *T. atroviride* WT strain was targeted to introduce a construct of interest, applying 8-AG as selective agent for transformation. To facilitate screening of transformants, a previously established fluorescent reporter construct consisting of the improved green fluorescent protein mBasicGFP fused to the Cdc42/Rac1-interactive binding (CRIB) motif [64] was used. Transformation was assisted by CRISPR/Cas9 to enhance homologous recombination efficiency (Supplementary Figure S3A). Eight transformants were screened by genotyping PCR to verify correct integration of the reporter construct at the target locus (Supplementary Figure S3B). Six transformants displayed the expected integration PCR band, and three independent strains were selected for single-spore isolation and fluorescence microscopy analysis. In all six tested strains that had integrated the construct at the *azg1* locus, a strong fluorescent signal at the hyphal tips was detected, whereas the WT displayed only weak autofluorescence (Fig. 3).

We next investigated whether the *azg1*-based selection system can be applied to other *Trichoderma* species. *Azg1* orthologs were identified using *A. nidulans* *AzgA* as query and resulting protein sequences were used to generate a phylogenetic tree. Two highly similar orthologs clustering on opposite sides of the unrooted tree emerged from this analysis in all analyzed species (Fig. 4A). The upper branch contained the ortholog with the higher similarity to *A. nidulans* *AzgA*, including *T. atroviride* *Azg1*, whereas the lower branch grouped the second paralogues of each species. A closer examination of the upper branch (Fig. 4B) revealed four major clusters. Cluster I included *T. atroviride* IMI 206040, several *Trichoderma viride* isolates, *Trichoderma asperellum*, and *Trichoderma gamsii*. Cluster II, the smallest group, comprised *Trichoderma ceciliae*, *Trichoderma arundinaceum* and *Trichoderma cornu-damae*, while cluster III included, among others, *Trichoderma reesei* QM6a, *Trichoderma citrinoviride* and *Trichoderma longibrachiatum*. Finally, Cluster IV, the largest group, encompassed *Trichoderma virens* Gv29-8, several *Trichoderma harzianum* isolates as well as less-characterized species. One representative species from each cluster, except cluster II, was selected for CRISPR/Cas9 assisted transformations targeting the respective *azg1* locus for integration of the CRIB-mBasicGFP reporter construct.

Not many transformants were obtained for the selected species upon 8-AG selection, but the goal of these experiment was not to optimize the transformation protocol but to see the efficiency of the *azg1* as marker. Therefore, all transformant colonies, three for *T. asperellum* and *T. reesei* and two for *T. virens*, were screened via genotyping PCR (Supplementary Figure S4A). Despite the presence of some unspecific bands, all tested mutants showed the expected amplicon of about 2 kb, while the respective WT resulted in no amplification. All transformants displayed colony morphology comparable to that of the corresponding WT strains, and fluorescence microscopy confirmed mBasicGFP accumulation at hyphal tips in all analyzed colonies (Supplementary Figure S4B).

Possible application for the *azg1* locus as marker

To further explore the advantages of the newly established counter-selectable marker system in *Trichoderma* spp., we next investigated potential applications of the *azg1* locus as a platform for targeted integration of additional genetic tools. As a first proof of concept, the locus was used to investigate the

susceptibility of *T. atroviride* to other nucleobase analogs, focusing specifically on 5-FC. Because *T. atroviride* lacks an ortholog involved in the initial step of the pyrimidine salvage pathway, we hypothesized that its intrinsic resistance to 5-FC results from the absence of a functional cytosine deaminase. We therefore tested whether heterologous expression of *fcy1* orthologs from 5-FC-sensitive fungi could confer sensitivity to this compound. Orthologs of the *A. fumigatus fcyA* gene [40] were identified and amplified from four species phylogenetically related to *Trichoderma*: *Penicillium chrysogenum* (EN45_039270), *Aspergillus niger* (An01g04210), *A. nidulans* (Aspnid_9622), and *Neurospora crassa* (NCU07413). Each coding sequence was cloned into an integration vector targeting the *azg1* locus under *Trichoderma*-specific regulatory sequences (Supplementary Figure S5A). Resulting *T. atroviride* transformants were designated $\Delta azg1_Pcfcy1$ (*P. chrysogenum*), $\Delta azg1_Anigfcy1$ (*A. niger*), $\Delta azg1_Anidfcy1$ (*A. nidulans*), and $\Delta azg1_Ncfcy1$ (*N. crassa*). CRISPR/Cas9-assisted transformation with 8-AG selection yielded four transformants per construct, except for $\Delta azg1_Anigfcy1$ with only two. Genotyping confirmed variable integration patterns, with one $\Delta azg1_Pcfcy1$ clone, both $\Delta azg1_Anigfcy1$ transformants, one $\Delta azg1_Anidfcy1$ strain, and two $\Delta azg1_Ncfcy1$ clones displaying the expected integration pattern (Supplementary Figure S5B). Two representative transformants per construct were selected for further analysis. For the construct of *P. chrysogenum* and *A. nidulans* only one clone showed the band of the expected size; nevertheless, a second clone - $\Delta azg1_Pcfcy1$ #3 resulting in a product slightly bigger than expected and $\Delta azg1_Anidfcy1$ #2 exhibiting a random band pattern that could be due to degradation - was included in further tests. 5-FC sensitivity was assessed in 96-well plate assays to estimate the minimum inhibitory concentration (MIC) of 5-FC using *T. atroviride* WT as control. Because optical density measurements at OD₆₀₀ did not provide reliable quantification of filamentous fungal growth at late time points, inhibition was assessed visually. $\Delta azg1_Pcfcy1$ #2 and both $\Delta azg1_Anigfcy1$ selected clones displayed increased 5-FC sensitivity compared to the WT and other transformants (Fig. 5A), with a MIC equal to 125 µg/mL. These findings were confirmed by plating spores of $\Delta azg1_Pcfcy1$ #2 and $\Delta azg1_Anigfcy1$ #1 on TMM supplemented with either 100 µg/mL or 500 µg/mL 5-FC. Both selected transformants were able to grow on 5-FC but failed to sporulate at 100 µg/mL and produced only a thin hyphal layer at 500 µg/mL, while the WT showed unaltered growth and sporulation at both 5-FC concentrations (Fig. 5B). Overall, expression of functional *fcy1* orthologs rendered *T. atroviride* susceptible to 5-FC, suggesting that through further sequence optimization the cytosine deaminase could have a potential use as an additional counter-selectable marker.

As a second application, the *azg1* locus was used to evaluate inducible expression systems in *T. atroviride*. Two Tet-On variants optimized for *A. fumigatus*, Tet-On (*PpkiA*) and Tet-On (*PoliC*) [86, 87], were integrated at the *azg1* locus and used to drive the expression of a *mBasicGFP* reporter. Previous studies [39, 86, 87] reported that the Tet-On (*PoliC*) system achieved expression levels exceeding those of the constitutive *PgpdA* promoter at relatively low doxycycline concentrations (6 µg/mL), whereas the Tet-On (*PpkiA*) system displayed comparatively weak induction even at higher inducer concentrations (100 µg/mL). Consistent with these reports, the *T. atroviride* Tet-On (*PoliC*) strain ($\Delta azg1_TetOn(PoliC)mVisGreen$) displayed a clear fluorescence signal that increased proportionally with doxycycline concentration, whereas the Tet-On (*PpkiA*) strain ($\Delta azg1_TetOn(PpkiA)mVisGreen$), similar to the WT control, produced no detectable signal (Fig. 6A). Because high doxycycline concentrations are known to affect fungal growth [88], fluorescence measurements were normalized to OD₆₀₀ values, although the use of OD₆₀₀ as a proxy for growth in

filamentous fungi remains debated. Background-corrected FL/OD values, however, revealed patterns that differed only slightly from raw fluorescence measurements. In cultures induced immediately after inoculation (T0), the fluorescence signal increased during early growth before gradually plateauing and declining after approximately 42 h, with stronger fluorescence signals at higher doxycycline concentrations. In contrast, delayed induction (T1) triggered a steadier increase in fluorescence after doxycycline addition that subsequently remained constant for the rest of the experiment (Supplementary Figure S6).

Finally, integration into the *azg1* locus was used to investigate the expression and suitability of selected fluorescent proteins (FPs). Among the evaluated fluorophores were monomeric Kusabira Orange 2 (mKO2), monomeric TagBFP2 (mTagBFP2opt), and Katushka2S (K2S), all characterized by high in vivo brightness and minimal spectral overlap with each other and with mBasicGFP, making these fluorophores suitable for future multicolor imaging applications in *T. atroviride*. Expression cassettes encoding the individual fluorophores under control of the constitutive *gpdh* promoter and terminator were integrated into the *azg1* locus and 8-AG selected transformants were analyzed by confocal microscopy to assess fluorophore expression and intracellular localization. Imaging of the periphery of actively growing colonies revealed strong fluorophore-specific signals for mKO2 (yellow), mTagBFP2opt (blue), and K2S (red) (Fig. 6B), that enabled visualization of hyphal morphology. WT background fluorescence was negligible under imaging conditions used for mKO2 and K2S, whereas settings optimized for mTagBFP2opt produced a stronger background signal in the blue and green channels, indicating intrinsic autofluorescence in this spectral range.

By applying the *azg1* locus as counter-selectable marker and effective integration point, we managed to validate multiple innovative tools for *T. atroviride*, such as a new putative marker, an inducible promoter system and various FPs, providing additional options to better characterize this species.

DISCUSSION

Compared with established fungal model systems such as *Aspergillus* spp., the molecular toolbox available for *Trichoderma* remains limited despite the increasing agricultural and industrial relevance of this genus. The molecular resources available for strain engineering have been mainly developed for *T. reesei*, whereas biocontrol-relevant species still lack a broad set of validated selection markers, reporter systems, and controllable expression tools [8, 31]. Recent works in *T. atroviride* still point to limited marker availability as a major bottleneck for successive genetic manipulations [21, 89].

In this study, we developed a set of genetic tools aiming to overcome this longstanding methodological gap. Endogenous counter-selectable systems based on nucleobase uptake and salvage pathways have recently been developed in *A. fumigatus*, enabling site-directed integration without the need for exogenous resistance markers and supporting sequential, as well as multigene engineering workflows [40, 45, 56]. These approaches established endogenous genes required for toxic analog uptake as particularly attractive engineering targets, where successful construct integration is directly coupled to resistance while the underlying gene disruption is physiologically well tolerated. In the attempt to extend this strategy to *Trichoderma*, however, we observed intrinsic resistance of *T. atroviride* to several antifungal drugs, including

5-FC and other pyrimidine analogs. We therefore focused on the purine uptake pathway. Building on the AzgA transporter in *A. nidulans*, which mediates uptake of adenine, guanine, hypoxanthine and the toxic analogue 8-AG [54, 55, 60], we functionally characterized one of the two identified orthologs of *T. atroviride*. Although filamentous fungi typically encode multiple purine transporters, functional redundancy does not necessarily extend to toxic substrate uptake [90]. Deletion of the selected gene, *azg1*, conferred robust resistance to 8-AG, consistent with previous observations in other fungi, where loss of a single transporter is sufficient to confer drug resistance despite the presence of paralogs [55, 90].

A critical requirement for the practical utility of any selectable or counter-selectable marker is that its disruption does not confer a substantial fitness cost. Extensive phenotypic characterization of $\Delta azg1$ strains across a wide range of environmental conditions, including osmotic, oxidative, and cell wall stress, as well as varying temperatures, pH, light exposure, and nutrient conditions, revealed consistently comparable phenotypes between WT and deletion mutants. Likewise, ecological fitness, assessed through confrontation assays against *B. cinerea* and *F. graminearum*, remained unchanged. The absence of an obvious defect in the present study supports the view that *azg1* is not only a useful counter-selectable target, but also a strong candidate for a neutral integration locus for routine construct insertion in *T. atroviride*. The broader utility of the locus is further supported by its conservation across the genus. Sequence-based identification of *azg1* homologs in additional *Trichoderma* species enabled extension of the strategy beyond *T. atroviride*, showing that the marker concept is not restricted to a single species. This is an important practical advantage, as transformation efficiency and marker performance can vary substantially among *Trichoderma* species [8, 33]. A conserved endogenous target therefore offers a path toward more comparable engineering workflows across taxa. In that sense, the *azg1* system may help reduce a persistent limitation in *Trichoderma* genetics, namely that tools often remain species- or laboratory- specific rather than broadly portable.

The successful use of the *azg1* locus for CRISPR/Cas9 mediated integration also highlights its value as a genomic landing site rather than merely a resistance determinant. This matters because one of the recurring limitations in *Trichoderma* genetic engineering has been the difficulty of combining precise editing with reusable selection strategies and predictable transgene behavior [8, 21, 31]. By coupling targeted editing to a selectable phenotype, the locus provides a simple framework for repeated construct delivery. In practical terms, this opens the way to workflows in which reporter cassettes, regulatory modules, or complementation constructs can be introduced at a defined chromosomal position instead of being dispersed across the genome.

This feature became especially clear when evaluating heterologous gene or construct integrations. As first practical application of *azg1* as counter-selectable marker, we attempted to dissect the intrinsic resistance of *T. atroviride* to 5-FC. This prodrug activity strictly depends on uptake and subsequent conversion through the pyrimidine salvage pathway [62]. Cytosine deaminase, encoded as *FCY1*, *FCA1*, or *fcyA* depending on the species, is a central step in this pathway, but drug susceptibility also depends on efficient uptake and downstream metabolic activation [40, 62]. The partial restoration of 5-FC sensitivity in *T. atroviride* after heterologous expression of *fcy1* orthologs strongly supports the idea that the intrinsic resistance of this species is predominantly linked to the absence of a functional cytosine deaminase. At the same time, the

incomplete phenotype is equally informative. It suggests that cytosine deaminase is not the only limiting factor and that additional barriers, such as suboptimal uptake or efflux, transport specificity, expression level, or downstream flux through the salvage pathway, still constrain full susceptibility. This interpretation is consistent with studies in *A. fumigatus*, where FcyB, FcyA, and Uppt each make distinct contributions to 5-FC activity and where loss of any one of these steps is sufficient to alter drug response [40, 56, 62]. In that sense, the *azg1* locus could serve as a standardized test bed for expanding a missing metabolic function and for evaluating how transferable antifungal selection strategies are across genera.

Importantly, the *azg1* locus also enabled the development of advanced genetic tools. Several studies have emphasized that inducible and synthetic expression systems in *Trichoderma* have been developed mainly for *T. reesei* and that tetracycline-controlled systems, although well established in *Aspergillus*, have not been broadly implemented across the genus [8, 68]. We therefore tested two systems that recently proved useful in *A. fumigatus* for different purposes [78, 86, 87]. The Tet-On (*PoliC*) construct showed clear, doxycycline-dependent induction of gene expression, confirming its functionality in *T. atroviride*. The differing performance of the tested Tet-On variants shows that successful transfer without extensive species-specific redesign cannot be assumed. Promoter architecture, codon usage, genomic context, and transactivator design are all likely to influence system behavior, as has also been seen during optimization of Tet circuits in other fungi [71, 72]. However, the successful employment of the *PoliC*-based system without prior optimization represents a strong starting point for further implementation of tetracycline-inducible expression systems in *Trichoderma*.

In parallel, we employed the *azg1* locus for integrating a set of FPs for live-cell imaging applications, and our results likewise have value beyond simple proof of integration. Reporter based imaging has long been useful in *Trichoderma*, including early host-inducible GFP systems in *T. atroviride* [63], and later codon optimized vector platforms using eGFP and mCherry derivatives [35]. Even so, the palette of fluorophores routinely used in the genus remains modest compared with more intensively engineered fungal models [8, 35]. The three selected and tested FPs exhibited strong cytoplasmic expression when integrated into the *T. atroviride azg1* locus, enabling clear visualization of hyphal structures. Minimal autofluorescence under most imaging conditions and adequate spectral separation between fluorophores support their suitability for multi-color imaging approaches. The observed signal strength and spectral separation indicate that these reporters should be suitable not only for basic localization studies, but also for future multicolor experiments aimed at following hyphal differentiation, organelle dynamics, or fungal interactions in living cells.

Taken together, this study establishes *azg1* as a multifunctional genomic platform in *Trichoderma*. First, it functions as a robust counter-selectable marker based on 8-AG resistance. Second, it serves as a candidate neutral landing site for targeted integration. Most importantly, these two features are combined in a way that is highly useful for iterative strain engineering. In the present study the suitability of the *azg1* locus was demonstrated across three distinct applications: restoration of 5-FC susceptibility via heterologous pathway complementation, installation of a doxycycline responsive expression system, and deployment of new FPs. This multifunctionality distinguishes the system from conventional dominant resistance markers, which are typically consumed after a single engineering step and can hinder

subsequent manipulations [8, 21]. In contrast, an endogenous counter-selectable locus is well matched to workflows that require repeated genome editing, metabolic pathway testing, reporter exchange, or regulated transgene insertion.

The broader implication is that *Trichoderma* genetics may benefit from moving toward endogenous, reusable, and locus defined engineering strategies similar to those already explored in *Aspergillus* [40, 45]. The present study provides an initial framework for that shift. Although more extensive validation will be required, these preliminary findings strongly support the use of *azg1* as a counter-selectable marker across multiple *Trichoderma* species. This system therefore represents a promising strategy to facilitate genetic manipulation in species for which efficient selection systems are currently unavailable, thereby expanding the molecular toolbox for the genus.

Future work should test how robustly the *azg1* platform performs across additional *Trichoderma* backgrounds, whether homologous integration efficiencies can be further improved in strains with strong NHEJ, and how far the Tet-based system can be optimized for tighter repression and a wider induction range. The 5-FC experiments also point to a clear next step, namely reconstructing additional elements of the pyrimidine salvage and uptake pathway to determine which combination is required for fully efficient counter selection in *Trichoderma*. It will likewise be important to determine whether the locus remains neutral when used for larger constructs, stronger promoters, or more demanding physiological assays. Even with these open questions, the data presented here mark a substantial advance toward a more versatile and standardized molecular toolbox for *Trichoderma* species.

CONCLUSION

In this study, we established *azg1* as a versatile counter-selectable marker and genomic landing site for genetic engineering in *Trichoderma*. Deletion of *azg1* confers stable 8-AG resistance without detectable fitness defects, enabling targeted and reusable integration of genetic constructs. The successful application of the locus for pathway complementation, inducible gene expression, and fluorescent reporter integration demonstrates its potential as a multifunctional platform for strain engineering. Moreover, the conservation of *azg1* across *Trichoderma* species supports the broader applicability of this strategy. Together, these findings provide a foundation for more flexible, standardized, and iterative genetic engineering approaches and contribute to expanding the molecular toolbox available for *Trichoderma*.

Declarations

ETHIC DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

FUNDING

This work was funded by the Austrian Science Fund (FWF; <https://www.fwf.ac.at/>; grant number PAT 1385625 to CB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHORS' CONTRIBUTION

Conceptualization: C.B., F.G. Data curation: C.B., L.H. Software, Validation, Formal analysis: C.B. Investigation: C.B., A.O., L.S., J.N., U.S. Methodology, Supervision: C.B., S.Z. Writing – Original Draft, Funding acquisition, Project administrator: C.B. Writing – Review & Editing: C.B., L.H., F.G., S.Z.

ACKNOWLEDGMENT

We would like to thank Ursula Peintner (University of Innsbruck, Department of Microbiology) for the assistance with phylogenetic analysis, as well as Selin Slavova, Mario Bertelsen-Schreiner and Diana Zerlauth for helping with the experiments in the context of their bachelor thesis.

DATA AVAILABILITY

No dataset was generated or analyzed in this study.

References

1. Chaverri P, Castlebury LA, Overton BE et al (2003) Hypocrea/Trichoderma: species with conidiophore elongations and green conidia. *Mycologia*
2. Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A et al (2011) Trichoderma: the genomics of opportunistic success. *Nat Rev Microbiol.* 10.1038/nrmicro2637
3. Woo SL, Hermosa R, Lorito M et al (2023) Trichoderma: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. *Nat Rev Microbiol.* 10.1038/s41579-022-00819-5
4. Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V et al (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. *Genome Biol.* 10.1186/gb-2011-12-4-r40
5. Mukherjee PK, Horwitz BA, Herrera-Estrella A et al (2013) Trichoderma research in the genome era. *Annu Rev Phytopathol.* 10.1146/annurev-phyto-082712-102353

6. Schuster A, Schmoll M (2010) Biology and biotechnology of *Trichoderma*. *Appl Microbiol Biotechnol*. 10.1007/s00253-010-2632-1
7. Bischof RH, Ramoni J, Seiboth B (2016) Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microb Cell Fact*. 10.1186/s12934-016-0507-6
8. Tomico-Cuenca I, Mach RL, Mach-Aigner AR et al (2021) An overview on current molecular tools for heterologous gene expression in *Trichoderma*. *Fungal Biol Biotechnol*. 10.1186/s40694-021-00119-2
9. Baazeem A, Almanea A, Manikandan P et al (2021) Vitro Antibacterial, Antifungal, Nematocidal and Growth Promoting Activities of *Trichoderma hamatum* FB10 and Its Secondary Metabolites. *J Fungi (Basel)*. 10.3390/jof7050331
10. Berini F, Caccia S, Franzetti E et al (2016) Effects of *Trichoderma viride* chitinases on the peritrophic matrix of Lepidoptera. *Pest Manag Sci*. 10.1002/ps.4078
11. Bonanomi G, Lorito M, Vinale F et al (2018) Organic Amendments, Beneficial Microbes, and Soil Microbiota: Toward a Unified Framework for Disease Suppression. *Annu Rev Phytopathol*. 10.1146/annurev-phyto-080615-100046
12. da Silveira AA, Andrade JSP, Guissoni ACP et al (2021) Larvicidal potential of cell wall degrading enzymes from *Trichoderma asperellum* against *Aedes aegypti* (Diptera: Culicidae). *Biotechnol Prog*. 10.1002/btpr.3182
13. Fiorentino N, Ventorino V, Woo SL et al (2018) *Trichoderma*-Based Biostimulants Modulate Rhizosphere Microbial Populations and Improve N Uptake Efficiency, Yield, and Nutritional Quality of Leafy Vegetables. *Front Plant Sci*. 10.3389/fpls.2018.00743
14. Harman GE, Uphoff N (2019) Symbiotic Root-Endophytic Soil Microbes Improve Crop Productivity and Provide Environmental Benefits. *Scientifica (Cairo)*. 10.1155/2019/9106395
15. Li N, Islam MT, Kang S (2019) Secreted metabolite-mediated interactions between rhizosphere bacteria and *Trichoderma* biocontrol agents. *PLoS ONE*. 10.1371/journal.pone.0227228
16. Ngo MT, Nguyen MV, Han JW et al (2021) Vitro and In Vivo Antifungal Activity of Sorbicillinoids Produced by *Trichoderma longibrachiatum*. *J Fungi (Basel)*. 10.3390/jof7060428
17. Xiao-Yan S, Qing-Tao S, Shu-Tao X et al (2006) Broad-spectrum antimicrobial activity and high stability of Trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. *FEMS Microbiol Lett*. 10.1111/j.1574-6968.2006.00316.x
18. Baek JM, Kenerley CM (1998) The *arg2* gene of *Trichoderma virens*: cloning and development of a homologous transformation system. *Fungal Genet Biol*. 10.1006/fgbi.1997.1025
19. Calcáneo-Hernández G, Rojas-Espinosa E, Landeros-Jaime F et al (2020) An efficient transformation system for *Trichoderma atroviride* using the *pyr4* gene as a selectable marker. *Brazilian J Microbiol*. 10.1007/s42770-020-00329-7
20. Gruber S, Omann M, Rodriguez CE et al (2012) Generation of *Trichoderma atroviride* mutants with constitutively activated G protein signaling by using geneticin resistance as selection marker. *BMC Res Notes*. 10.1186/1756-0500-5-641
21. Gründlinger ME, Drechsel C, Schreiner L, Pierson U, Baldin S, Zeilinger C (2025) DOI S. *Simply cut out – Combining CRISPR/Cas9 RNPs and transiently selected telomere vectors for marker free-gene deletion in*

Trichoderma atroviride. bioRxiv, : <https://doi.org/10.1101/2025.05.06.652419>

22. Zeilinger S (2004) Gene disruption in *Trichoderma atroviride* via *Agrobacterium*-mediated transformation. *Curr Genet*. 10.1007/s00294-003-0454-8
23. Derntl C, Kiesenhofer DP, Mach RL et al (2015) Novel Strategies for Genomic Manipulation of *Trichoderma reesei* with the Purpose of Strain Engineering. *Appl Environ Microbiol*. 10.1128/AEM.01545-15
24. Gruber F, Visser J, Kubicek CP et al (1990 DOI) Cloning of the *Trichoderma-Reesei-PyrG* Gene and Its Use as a Homologous Marker for a High-Frequency Transformation System. *Curr Genet*. 10.1007/Bf00309915
25. Guangtao Z, Hartl L, Schuster A et al (2009) Gene targeting in a nonhomologous end joining deficient *Hypocrea jecorina*. *J Biotechnol*. 10.1016/j.jbiotec.2008.10.007
26. Liu GG, Li S, Wei YD et al (2015) Efficient genome editing in human pluripotent stem cells through CRISPR/Cas9. *Hereditas(Beijing)*. 10.16288/j.ycz.15-240
27. Penttilä M, Nevalainen H, Ratto M et al (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene*. 10.1016/0378-1119(87)90110-7
28. Schuster A, Bruno KS, Collett JR et al (2012) A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. *Biotechnol Biofuels*. 10.1186/1754-6834-5-1
29. Smith JL, Bayliss FT, Ward M (1991) Sequence of the cloned *pyr4* gene of *Trichoderma reesei* and its use as a homologous selectable marker for transformation. *Curr Genet*. 10.1007/BF00362084
30. Steiger MG, Vitikainen M, Uskonen P et al (2011) Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers. *Appl Environ Microbiol*. 10.1128/AEM.02100-10
31. Wang YC, Ma H, Gong L, Wu M, Bao Y, Zou D (2022) Use of CRISPR-Cas tools to engineer *Trichoderma* species. *Microb Biotechnol*. 10.1111/1751-7915.14126
32. Bischof R, Seiboth B *Molecular Tools for Strains Improvement in Trichoderma spp.*, in *Biotechnology and Biology of Trichoderma*. 2014: Institute of Chemical Engineering, Vienna University of Technology and Austrian Centre of Industrial Biotechnology (ACIB), Vienna, Austria <https://doi.org/10.1016/B978-0-444-59576-8.00012-6>
33. Cai F, Kubicek CP, Druzhinina IS (2021) Genetic Transformation of *Trichoderma* spp. *Methods Mol Biol*. 10.1007/978-1-0716-1323-8_12
34. Kubodera T, Yamashita N, Nishimura A (2002) Transformation of *Aspergillus* sp. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. *Biosci Biotechnol Biochem*. 10.1271/bbb.66.404
35. Nogueira-Lopez G, Padilla-Arizmendi F, Inwood S et al (2019) TrichoGate: An Improved Vector System for a Large Scale of Functional Analysis of *Trichoderma* Genes. *Front Microbiol*. 10.3389/fmicb.2019.02794
36. Kubodera T, Yamashita N, Nishimura A (2000) Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. *Biosci Biotechnol Biochem*. 10.1271/bbb.64.1416

37. Punt PJ, Oliver RP, Dingemanse MA et al (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene*. 10.1016/0378-1119(87)90164-8
38. Son YE, Park HS (2021) Genetic Manipulation and Transformation Methods for *Aspergillus* spp. *Mycobiology*. 10.1080/12298093.2020.1838115
39. Baldin C, Kühbacher A, Merschak P et al (2021) Inducible Selectable Marker Genes to Improve *Aspergillus fumigatus* Genetic Manipulation. *J Fungi (Basel)*. 10.3390/jof7070506
40. Birstonas L, Dallemulle A, Lopez-Berges MS et al (2020) Multiplex Genetic Engineering Exploiting Pyrimidine Salvage Pathway-Based Endogenous Counterselectable Markers. *mBio*. 10.1128/mBio.00230-20
41. Krappmann S, Bayram O, Braus GH (2005) Deletion and allelic exchange of the *Aspergillus fumigatus* *veA* locus via a novel recyclable marker module. *Eukaryot Cell*. 10.1128/EC.4.7.1298-1307.2005
42. Krappmann S, Sasse C, Braus GH (2006) Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end-joining-deficient genetic background. *Eukaryot Cell*. 10.1128/EC.5.1.212-215.2006
43. Nayak T, Szewczyk E, Oakley CE et al (2006) A versatile and efficient gene-targeting system for *Aspergillus nidulans*. *Genetics*. 10.1534/genetics.105.052563
44. Nielsen ML, Albertsen L, Lettier G et al (2006) Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. *Fungal Genet Biol*. 10.1016/j.fgb.2005.09.005
45. Sastre-Velasquez LE, Mach N, Mertens B et al (2025) Simultaneous multigene integration in *Aspergillus fumigatus* using CRISPR/Cas9 and endogenous counter-selectable markers. *J Biol Eng*. 10.1186/s13036-025-00539-3
46. Schmoll M, Zeilinger S (2021) Resistance Marker- and Gene Gun-Mediated Transformation of *Trichoderma reesei*. *Methods Mol Biol*. 10.1007/978-1-0716-1048-0_4
47. Cheng P, Liu B, Su Y et al Genomics insights into different cellobiose hydrolysis activities in two *Trichoderma hamatum* strains. *Microb Cell Fact*, 2017 10.1186/s12934-017-0680-2
48. Escudero-Leyva E, Alfaro-Vargas P, Muñoz-Arrieta R et al (2021) Tolerance and Biological Removal of Fungicides by *Trichoderma* Species Isolated From the Endosphere of Wild Rubiaceae Plants. *Front Agron*. 10.3389/fagro.2021.772170
49. He X, Wubie AJ, Diao Q et al (2014) Biodegradation of neonicotinoid insecticide, imidacloprid by restriction enzyme mediated integration (REMI) generated *Trichoderma* mutants. *Chemosphere*. 10.1016/j.chemosphere.2014.01.023
50. d'Enfert C, Weidner G, Mol PC et al (1999) Transformation systems of *Aspergillus fumigatus*. New tools to investigate fungal virulence. *Contrib Microbiol*. 10.1159/000060292
51. Reyrat JM, Pelicic V, Gicquel B et al (1998) Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infect Immun*. 10.1128/IAI.66.9.4011-4017.1998
52. Gardiner DM, Howlett BJ (2004) Negative selection using thymidine kinase increases the efficiency of recovery of transformants with targeted genes in the filamentous fungus *Leptosphaeria maculans*. *Curr Genet*. 10.1007/s00294-004-0488-6

53. Khang CH, Park SY, Lee YH et al (2005) A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. *Fungal Genet Biol*. 10.1016/j.fgb.2005.03.004
54. Diallinas G, Gorfinkiel L, Arst HN Jr. et al (1995) Genetic and molecular characterization of a gene encoding a wide specificity purine permease of *Aspergillus nidulans* reveals a novel family of transporters conserved in prokaryotes and eukaryotes. *J Biol Chem*. 10.1074/jbc.270.15.8610
55. Cecchetto G, Amillis S, Diallinas G et al (2004) The AzgA purine transporter of *Aspergillus nidulans*. Characterization of a protein belonging to a new phylogenetic cluster. *J Biol Chem*. 10.1074/jbc.M308826200
56. Gsaller F, Furukawa T, Carr PD et al (2018) Mechanistic Basis of pH-Dependent 5-Flucytosine Resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*. 10.1128/AAC.02593-17
57. Kuhbacher A, Birch M, Oliver JD et al (2024) Anti-*Aspergillus* activities of olorofim at sub-MIC levels during early-stage growth. *Microbiol Spectr*. 10.1128/spectrum.03304-23
58. Kuhbacher A, Merschak P, Haas H et al (2023) The cytochrome P450 reductase CprA is a rate-limiting factor for Cyp51A-mediated azole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*. 10.1128/aac.00918-23
59. Storer ISR, Sastre-Velasquez LE, Easter T et al (2024) Shining a light on the impact of antifungals on *Aspergillus fumigatus* subcellular dynamics through fluorescence imaging. *Antimicrob Agents Chemother*. 10.1128/aac.00803-24
60. Goudela S, Reichard U, Amillis S et al (2008) Characterization and kinetics of the major purine transporters in *Aspergillus fumigatus*. *Fungal Genet Biol*. 10.1016/j.fgb.2007.08.001
61. Parker WB (2009) Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer. *Chem Rev*. 10.1021/cr900028p
62. Delma FZ, Al-Hatmi AMS, Bruggemann RJM et al (2021) Molecular Mechanisms of 5-Fluorocytosine Resistance in Yeasts and Filamentous Fungi. *J Fungi (Basel)*. 10.3390/jof7110909
63. Lu Z, Tombolini R, Woo S et al (2004) In vivo study of trichoderma-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl Environ Microbiol*. 10.1128/AEM.70.5.3073-3081.2004
64. Moreno-Ruiz D, Salzmann L, Fricker MD et al (2021) Stress-Activated Protein Kinase Signalling Regulates Mycoparasitic Hyphal-Hyphal Interactions in *Trichoderma atroviride*. *J Fungi (Basel)*. 10.3390/jof7050365
65. Pierson S, Fricker M, Lichius A et al (2025) Revealing robust antioxidant defences of a mycoparasitic *Trichoderma* species. *Fungal Biol*. 10.1016/j.funbio.2025.101549
66. Zhong Y, Yu H, Wang X et al (2011) Towards a novel efficient T-DNA-based mutagenesis and screening system using green fluorescent protein as a vital reporter in the industrially important fungus *Trichoderma reesei*. *Mol Biol Rep*. 10.1007/s11033-010-0534-z
67. Bischof RH, Horejs J, Metz B et al (2015) L-Methionine repressible promoters for tuneable gene expression in *Trichoderma reesei*. *Microb Cell Fact*. 10.1186/s12934-015-0308-3
68. Fitz E, Wanka F, Seiboth B (2018) The Promoter Toolbox for Recombinant Gene Expression in *Trichoderma reesei*. *Front Bioeng Biotechnol*. 10.3389/fbioe.2018.00135

69. Rantasalo A, Vitikainen M, Paasikallio T et al (2019) Novel genetic tools that enable highly pure protein production in *Trichoderma reesei*. *Sci Rep*. 10.1038/s41598-019-41573-8
70. Adnan M, Ma X, Olsson S et al (2022) Promoter regulation and genetic engineering strategies for enhanced cellulase expression in *Trichoderma reesei*. *Microbiol Res*. 10.1016/j.micres.2022.127011
71. Wanka F, Cairns T, Boecker S et al (2016) Tet-on, or Tet-off, that is the question: Advanced conditional gene expression in *Aspergillus*. *Fungal Genet Biol*. 10.1016/j.fgb.2015.11.003
72. Meyer V, Wanka F, van Gent J et al (2011) Fungal gene expression on demand: an inducible, tunable, and metabolism-independent expression system for *Aspergillus niger*. *Appl Environ Microbiol*. 10.1128/AEM.02740-10
73. Das AT, Tenenbaum L, Berkhout B (2016) Tet-On Systems For Doxycycline-inducible Gene Expression. *Curr Gene Ther*. 10.2174/1566523216666160524144041
74. Fang Z, Yang F, Ma Y et al (2025) Harnessing the Tet-Off system for effective gene silencing in *Magnaporthe oryzae*. *Front Plant Sci*. 10.3389/fpls.2025.1641730
75. Gruber S, Zeilinger S (2014) The transcription factor Ste12 mediates the regulatory role of the Tmk1 MAP kinase in mycoparasitism and vegetative hyphal fusion in the filamentous fungus *Trichoderma atroviride*. *PLoS ONE*. 10.1371/journal.pone.0111636
76. Flatschacher D, Eschlbock A, Zeilinger S (2023) Identification and evaluation of suitable reference genes for RT-qPCR analyses in *Trichoderma atroviride* under varying light conditions. *Fungal Biol Biotechnol*. 10.1186/s40694-023-00167-w
77. Lichius A, Seidl-Seiboth V, Seiboth B et al (2014) Nucleo-cytoplasmic shuttling dynamics of the transcriptional regulators XYR1 and CRE1 under conditions of cellulase and xylanase gene expression in *Trichoderma reesei*. *Mol Microbiol*. 10.1111/mmi.12824
78. Baldin C, Kühbacher A, Merschak P et al (2022) Modular Inducible Multigene Expression System for Filamentous Fungi. *Microbiol Spectr*. 10.1128/spectrum.03670-22
79. Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform*. 10.1093/bib/bbx108
80. Kuraku S, Zmasek CM, Nishimura O et al (2013) aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. *Nucleic Acids Res*. 10.1093/nar/gkt389
81. Hall TA (1999) *BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. *Nucleic Acids Symposium Series*
82. Hall TA (2011) BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences*
83. Minh BQ, Schmidt HA, Chernomor O et al (2020) IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol Biol Evol*. 10.1093/molbev/msaa015
84. Nguyen LT, Schmidt HA, von Haeseler A et al (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 10.1093/molbev/msu300
85. Letunic I, Bork P (2024) Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. *Nucleic Acids Res*. 10.1093/nar/gkae268

- ## Figures



Uptake and metabolism of nucleobases and sensitivity to their analogs in *T. atroviride* P1. (A) Simplified diagram for the pyrimidine (top) and purine (bottom) metabolic pathways, based on available models for *Aspergillus* species and on the recent review of Ayoub et al. [91]. Nucleobase analogs are highlighted in red. Multiple steps in the pathway are indicated with a dashed arrow. The grey curved lines mark the cell membrane. (B) Sensitivity of *T. atroviride* to nucleobase analogs, shown as plate assay. Selected pictures were taken after 5 days of incubation at 25° C in light/dark conditions. Abbreviation: 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU), 5-fluorouridine (5-FUR), 8-azaguanine (8-AG), uridine 5-monophosphate (UMP), 5-fluorouridine 5-monophosphate (5FUMP), inosine 5-monophosphate (IMP), adenosine 5-monophosphate (AMP), guanine 5-monophosphate (GMP), 8-azaguanine 5-monophosphate (8AGMP).

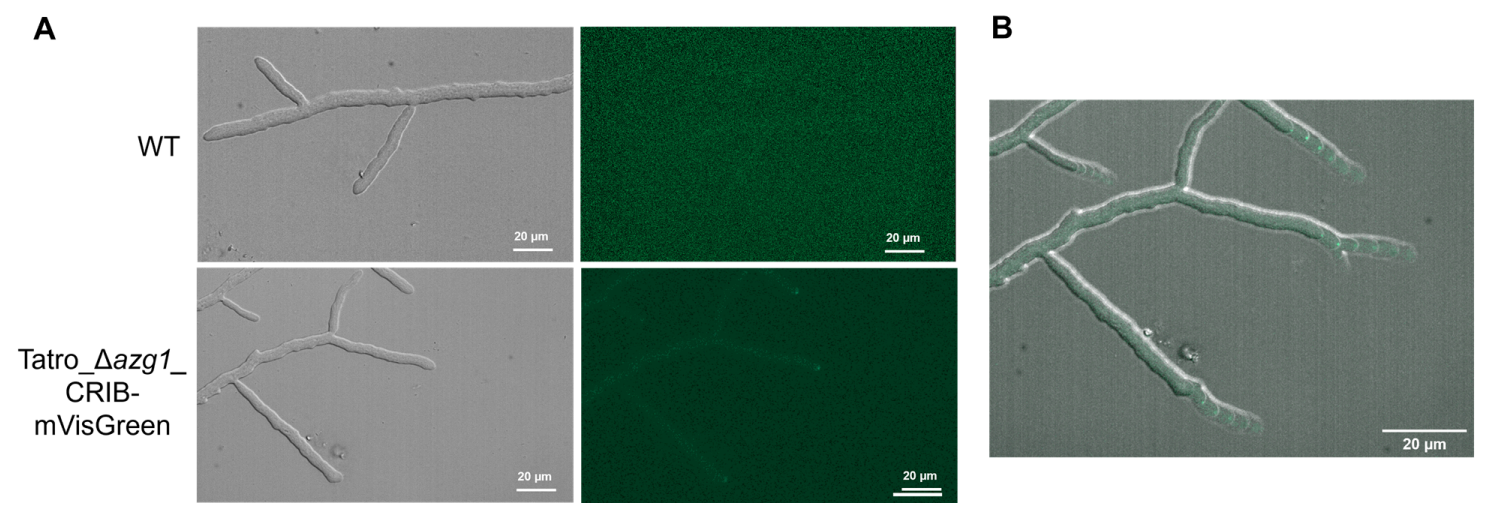


Figure 3

Validation of *azg1* as counter-selectable marker in *T. atroviride*. (A) Microscopy pictures confirming expression of the CRIB-mBasicGFP reporter [77]in the mutant strain. (B) Time-lapse overlay of Tatro_Δazg1_CRIB-mVisGreen pictures taken every minute for 5 min, showing real-time CRIB reporter localization at the actively growing hyphal tip.

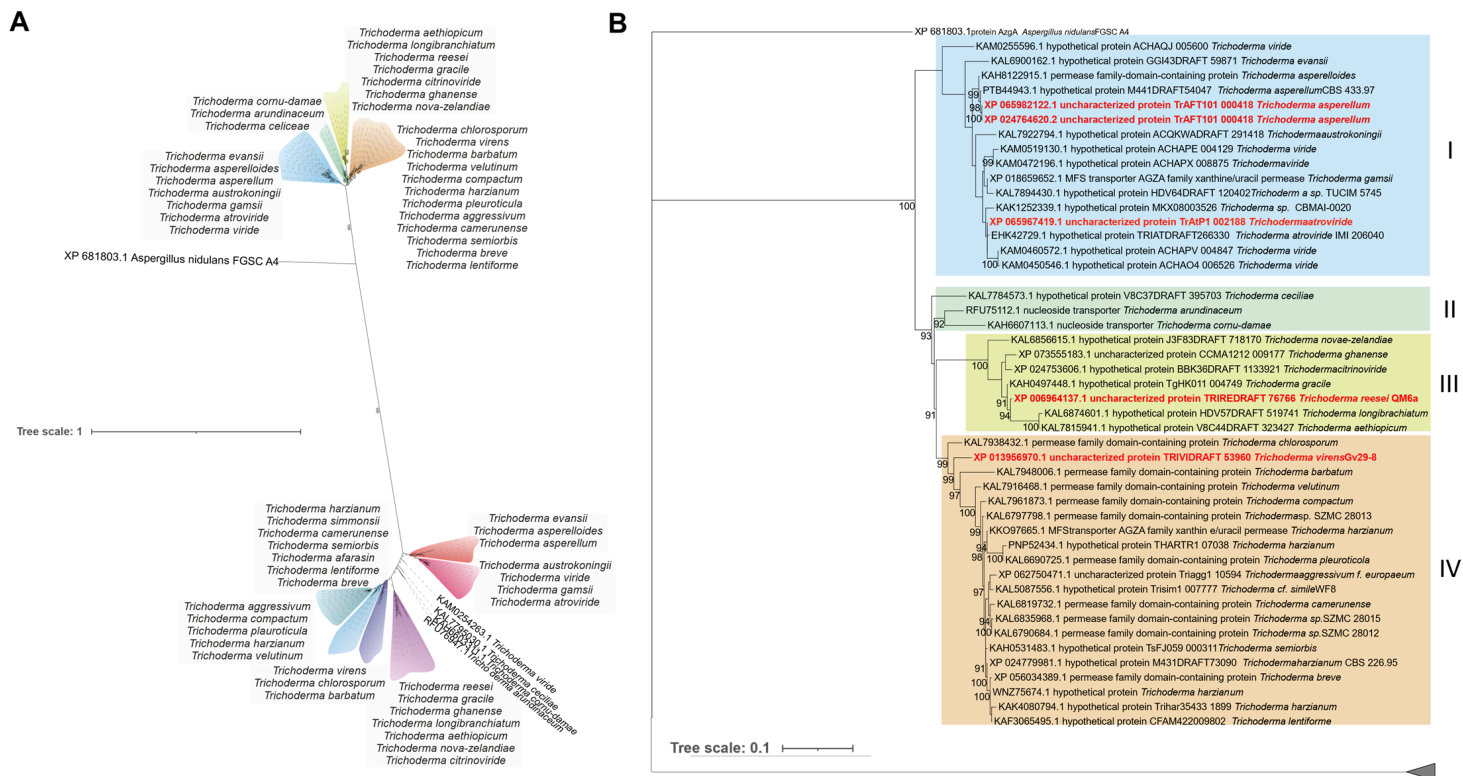


Figure 4

Maximum likelihood analysis of putative Azg1 orthologs of different *Trichoderma* species. Protein sequences of putative purine transporters of *Trichoderma* spp. were retrieved using *A. nidulans* AzgA as query [54, 55], in the tree indicated as XP_681803.1. (A) Unrooted tree, with colors indicating the different clusters. Tree scale indicating 1 change per site. (B) Expanded visualization of the phylogenetic tree upper branch in rooted form. Cluster identification is reported on the right side. Ultra-fast BS values (BS>90) are shown over the corresponding branches, tree scale indicating 0.1 change per site.

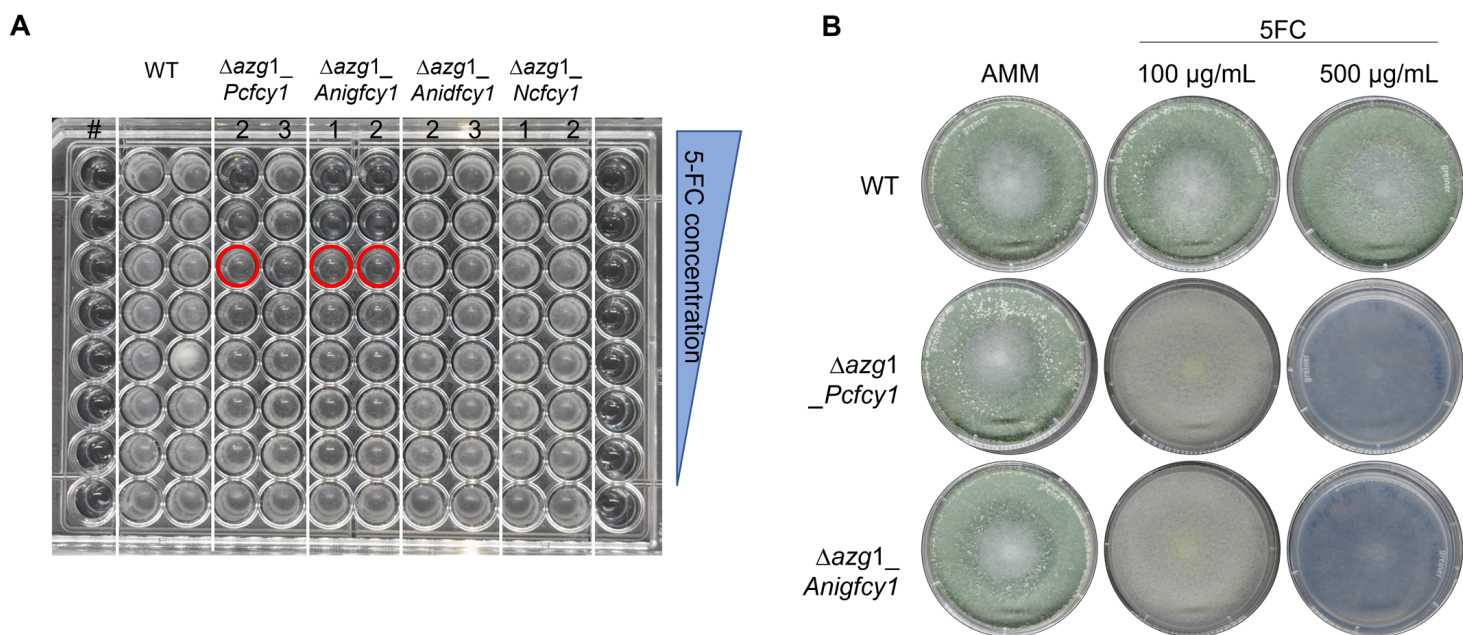


Figure 5

Induction of 5-FC susceptibility in *T. atroviride*. (A) Susceptibility assay verifying whether the presence of cytosine deaminase conferred sensitivity to the nucleobase analog 5-FC. Mutant strains were evaluated in comparison to the WT after growth on TMM containing decreasing concentrations of 5-FC. The bottom row did not contain any nucleobase analog and serves as control condition. In red are highlighted the first wells per column where no growth could be seen. The displayed picture was taken after 3 days of incubation at 25° C. (B) Strains that showed an increased sensitivity to 5-FC were tested on solid TMM containing 100 µg/mL or 500 µg/mL 5-FC. The displayed picture was taken after 7 days of incubation at 25° C in light/dark condition.

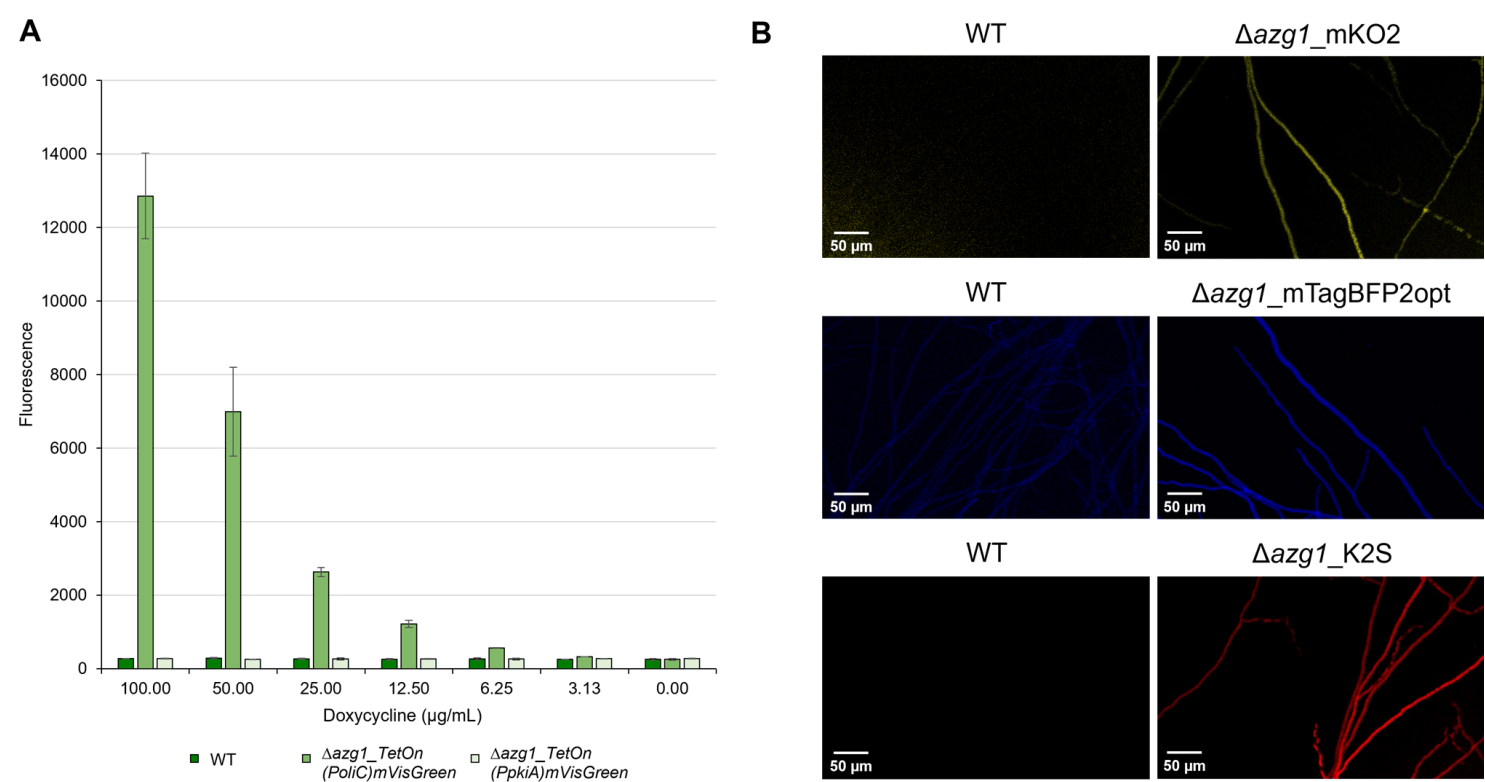


Figure 6

Implementation of new genetic tools in *T. atroviride*. (A) Fluorescence-based evaluation of two inducible Tet-On systems in *T. atroviride*. The reporter mBasicGFP was placed under the control of Tet-On(*PoliC*) [87] and Tet-On(*PpkiA*) [86], respectively, and integrated into the *azg1* locus of *T. atroviride*. WT is included as negative control to estimate autofluorescence. Plates were incubated for a total of 64 h at 25 °C in dark conditions. The bar plot shows values measured at the end point. (B). Assessment of FPs expression in *T. atroviride*. Pictures of the WT (left) and mutant strains carrying three FPs (right) were taken after 24 h of incubation at 25 °C in dark conditions. The FPs (mKO2, mTagBFP2opt and K2S) [59] were expressed under the control of the constitutive *gpdh* promoter and integrated into the *azg1* locus of *T. atroviride*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryfiles.pdf](#)
- [SupplementaryTableS2.xlsx](#)