Brazilian Journal of Microbiology (2020) 51:1631–1643
https://doi.org/10.1007/s42770-020-00329-7

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

An efficient transformation system for *Trichoderma atroviride* using the *pyr4* gene as a selectable marker

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Received: 19 December 2019 / Accepted: 25 June 2020 / Published online: 6 July 2020

Abstract

The development of an efficient transformation system is essential to enrich the genetic understanding of *Trichoderma atroviride*. To acquire an additional homologous selectable marker, uracil auxotrophic mutants were generated. First, the *pyr4* gene encoding OMP decarboxylase was replaced by the *hph* marker gene, encoding a hygromycin phosphotransferase. Then, uracil auxotrophs were employed to determine that 5 mM uracil restores their growth and conidia production, and 1 mg ml^{-1} is the lethal dose of 5-fluoroorotic acid in *T. atroviride*. Subsequently, uracil auxotrophic strains, free of a drug-selectable marker, were selected by 5-fluoroorotic acid resistance. Two different deletions in *pyr4* were mapped in four auxotrophs, encoding a protein with frameshifts at the 310 and 335 amino acids in their COOH-terminal. Six auxotrophs did not have changes in the *pyr4* ORF even though a specific cassette to delete the *pyr4* was used, suggesting that 5-FOA could have mutagenic activity. The $Ura^{-}1$ strain was selected as a genetic background to knock out the MAPKK Pbs2, MAPK Tmk3, and the blue light receptors Blr1/Blr2, using a short version of *pyr4* as a homologous marker. The Δ*tmk3* and Δ*pbs2* mutants selected with *pyr4* or *hph* marker were phenotypically identical, highly sensitive to different stressors, and affected in photoconidiation. The Δ*blr1* and Δ*blr2* mutants were not responsive to light, and complementation of uracil biosynthesis did not interfere in the expression of *blu1*, *grg2*, *phr1*, and *env1* genes upregulated by blue light. Overall, uracil metabolism can be used as a tool for genetic manipulation in *T. atroviride*.

Keywords Auxotrophy · Selectable marker · Sporulation · Cellular stress · Light response

Introduction

Ascomycota and Basidiomycota fungi are primarily the causative agents for diseases in an extensive variety of crops, but modern crop management can control them; however, a high risk of developing resistance to the fungicides exist [1, 2]. As an alternative, *Trichoderma* species are widely used as biofungicides and biofertilizers in agriculture; they improve crop quality and production, and the use of agrochemicals can be decreased [3, 4]. Nevertheless, the effects of fungi as biocontrol can be slow as compared with chemical control. For this reason, a deep understanding of *Trichoderma* species' biology would improve their biotechnological application.

Trichoderma atroviride is a filamentous fungus antagonist of phytopathogenic fungi. Its mycoparasitic capacity is attributed to competition for nutrients, the production of cell wall degrading enzymes, and antibiosis [4, 5]. In addition, it is considered an excellent model to understand asexual reproduction, as well as light and injury responses [6–8]. Thus, it is necessary to develop effective strategies for gene studies in *T. atroviride* in order to expand its industrial and agricultural applications.

The use of antibiotics, including fungicides, for selection is high priced and may interfere with the regular function of the targeted gene [9, 10]. One way to avoid the use of antibiotics is to generate auxotrophic mutants as a genetic tool, in which the same affected gene is utilized as a selectable marker, and its manipulation is less expensive [9]. Several auxotrophic strains have been reported in *Trichoderma* species. The *arg2* gene, which encodes the small subunit of carbamoyl phosphate synthetase, was

Responsible Editor: Derlene Attili Agellis.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42770-020-00329-7) contains supplementary material, which is available to authorized users.

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isolated and used to complement an arginine auxotroph strain in *Trichoderma virens* [11]. In *Trichoderma reesei*, the *argB* gene of *Aspergillus nidulans* can restore the arginine synthesis [12]. Adenine auxotrophs of *T. reesei* lacking the *ade2* gene, encoding a phosphoribosylaminoimidazole carboxylase required for production of purines, can be easily detected by selection of red colonies [13]. In *T. reesei* and *Trichoderma hypoxylon*, uracil auxotrophs selected by 5-fluoro-orotic acid (5-FOA) were used successfully as a genetic tool for the disruption of a single gene [14–16].

The pyrimidine analogue 5-FOA is generally used for positive selection of uracil auxotrophic mutants and is catalyzed by orotate phosphoribosyl transferase (OPRTase) and orotidine-5′-monophosphate decarboxylase (OMP decarboxylase) to obtain 5-fluorouridine 5′-monophosphate (5-FUMP) and 5-fluorouracil (5-FU) [17]. The 5-FU inhibits thymidylate synthase activity, consequently leading to thymine nucleotide depletion and affecting DNA synthesis [17–19]. Therefore, the toxic effect of 5-FOA on wild-type strains facilitates its selection, whereas mutants deficient in OPRTase or OMP decarboxylase, essential for uracil synthesis, can grow on 5-FOA medium supplemented with uridine/uracil [20, 21].

In order to obtain an alternative transformation system in *Trichoderma atroviride*, we generated uracil auxotrophic strains that are unable to grow in uracil-deficient media. Physiological and molecular results indicate that the *pyr4* gene can be used as a selectable marker in a uracil auxotrophic background. Four different genes related to stress and light responses in *T. atroviride* were effectively replaced in a uracil auxotroph strain, and the mutants showed identical phenotypes in comparison with knockouts generated by resistance to hygromycin B.

Methods

Strains and culture conditions

In this study, *T. atroviride* IMI206040 was used as the wild-type strain (WT) and as the parent strain for gene replacement experiments. The Δ*pbs2*–7 and Δ*tmk3*–13 strains were previously reported [8]. All strains were propagated on potato dextrose agar (PDA; DIFCO) at 27 °C. Transformants were selected on minimal medium (MM) containing per liter: 20 g glucose, 0.2 g $MgSO4.7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCl, 1 g NH_4NO_3, 2 mg $FeSO_4.7H_2O$, 2 mg $ZnSO_4.7H_2O$, 2 mg $MnCl_2.7H_2O$, and 15 g agar, and pH was adjusted to 5.5 with KOH. MM was supplemented with uracil 5 mM according to Gruber et al. [14]. When necessary, 100 μg ml^{-1} hygromycin B (Invitrogen) or 5-FOA was added to the medium. *Escherichia coli* DH5α strain was propagated in a Luria-Bertani (LB) medium with appropriate antibiotics for cloning and plasmid DNA isolation purposes.

Replacement of *pyr4* by the *hph* marker

To generate *pyr4* mutants, its sequence was identified in the *T. atroviride* genome database (v2.0) of the Department of Energy Joint Genome Institute through a BLASTp search against the amino acid sequence of *Trichoderma harzianum* Pyr4 (AAA51865.1), which has been previously reported [22]. The *pyr4* open-reading frame (ORF) was replaced by a hygromycin B resistance cassette based on the double-joint PCR method [23] with modified conditions as previously described [24]. Primers designed to replace the *pyr4* ORF by the selectable marker are listed in Table 1. The 5′ and 3′ flanking regions of the *pyr4* gene were amplified using the primers Ppyr4-F–PQpyr4-R and TQpyr4-F–Tpyr4-R, respectively. The hygromycin B resistance marker, *hph*, was amplified using the primers Hyg-F – Hyg-R. In a second PCR round, the three fragments were joined. The complementary sequences to *hph* were added to the chimeric primers (PQpyr4-R – TQpyr4-F). In a third PCR, the cassette was amplified using nested primers (N5pyr4-F – N3pyr4-R). The Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen) was used to construct the cassette, and the PCR conditions were as follows: first step at 94 °C for 2 min, 35 cycles at 94 °C/15 s, 60 °C/15 s, 68 °C/1 min per kb, and a final extension at 68 °C for 5 min. The final amplicon was used to transform *T. atroviride* protoplasts.

Protoplasts isolation

Protoplasts of *T. atroviride* were prepared by modifying the method of Baek and Kenerley [11]. A conidial suspension of the WT strain (1×10^6 conidia ml^{-1}) was cultivated in 100 ml of GYEC liquid medium (1.5% glucose, 0.3% yeast extract, 0.5% casein, and pH adjusted to 5.5 with KOH) and incubated in constant orbital agitation (160 rpm) at 27 °C for 18 h. Then, 0.2 g of mycelia were recovered by filtration and placed in a 50-ml centrifuge tube with a 7 ml of osmotic solution (50 mM $CaCl_2$, 0.5 M mannitol, 50 mM MES, pH 5.5) and 0.1 g of lyophilized lysing enzymes from *T. harzianum* (Sigma-Aldrich). Mycelia were incubated in orbital agitation (120 rpm) at room temperature for 2 h for the formation of protoplasts. Protoplasts were harvested through sterile Miracloth and carefully transferred into 1.5-ml microcentrifuge tubes. After centrifuging at 8000 rpm for 10 min at 4 °C, the supernatant was discarded, and the protoplast pellet was resuspended in osmotic solution at a concentration of $1 \times 10^{7-8}$ ml^{-1}.

Protoplast transformation by the polyethylene glycol (PEG)–$CaCl_2$ method

Transformation was conducted with 250 μl protoplasts and 100 μl DNA (50 μl PCR product plus 50 μl osmotic solution), and mixture remained on ice for 20 min. Afterwards, 350 μl of

Table 1 Primers used in this work

Gene	Primer	Sequence (5′ to 3′)	Target region
Orotidine 5′-phosphate decarboxylase (*pyr4*; EHK48963.1)	Ppyr4-F	CAG GGC TTC AAC TGC CAC GG	5′ flanking region of the *pyr4* gene
	PQpyr4-R	TGC TCC TTC AAT ATC AGT TAA CGT CGA TCA TGC CGT CTA ATG TCG GGT TG	
	TQpyr4-F	CCC AGC ACT CGT CCG AGG GCA AAG GAA TAG AGG TGG AGG CGA ACT GCA C	3′ flanking region of the *pyr4* gene
	Tpyr4-R	GTG TCT TGG CGC TCG GTC G	
	N5pyr4-F	GGG GGA GCT ACT GAG ACT C	Nested *pyr4* primers
	N3pyr4-R	GGA TGG GGG CGG TCC AAG	
	Q5pyr4-R	TCG CCT CCA CCT ATG CCG TCT AAT GTC GGG TTG	Chimeric *pyr4* primers with complementary sequences to the *pyr4* 5′ and 3′ regions
	Q3pyr4-F	TAG ACG GCA TAG GTG GAG GCG AAC TGC AC	
	smPyr4-F	CTC CCT ACG CTC TCC GCC ACC ACC AAT CGA TCT TTA TCC G	*pyr4* ORF
	smPyr4-R	CTC CAG CCT CTT CCA GCT CCG CAA CTT CTT TTT TTC TTA TCG CAG C	
	1Tpyr4-R	GAC GCA CTC AAC ATC TCG TAG C	
Hygromycin-B-phosphotransferase (*hph*; AEJ60084.1)	Hyg-F	GAT CGA CGT TAA CTG ATA TTG AAG GAG CA	*hph* marker
	Hyg-R	CTA TTC CTT TGC CCT CGG ACG AGT GCT GGG	
Mitogen activated protein kinase Tmk3 (*tmk3*; EHK43400.1)	Ptmk3-F	GCT GGT GCC CTC TTG TTC TC	5′ flanking region of the *tmk3* gene
	PQtmk3-R	GGT GGT GGC GGA GAG CGT AGG GAG ATT CGC GGG GAA ATA ACG ACG	
	TQtmk3-F	GCG GAG CTG GAA GAG GCT GGA GAG AGA GTA GAG GTG CAG AAT TCG	3′ flanking region of the *tmk3* gene
	Ttmk3-R	CTA CAG GTC GTC TCC ACA GC	
	N5tmk3-F	CTC TTG TGC TCC GAC GAA ACC	Nested *tmk3* primers
	N3tmk3-R	CGT CCA CCT CCA AAT CAT GGC	
	tmk3-F	GTT TGG TCT TGT CTG CTC TGC G	*tmk3* ORF
	tmk3-R	GCA GGT CGG TTC CGA GAA GC	
MAP kinase kinase Pbs2 (*pbs2*; EHK47459.1)	Ppbs2-F	CAG TGC GCT GGC TCC AGC	5′ flanking region of the *pbs2* gene
	PQpbs2-R	GGT GGT GGC GGA GAG CGT AGG GAG AGG TGG GGT TTT CGG ATG CTT C	
	TQpbs2-F	GCG GAG CTG GAA GAG GCT GGA GCA GAA GAA AAG CAT AGA GCG G	3′ flanking region of the *pbs2* gene
	Tpbs2-R	CGC GAA CCT ACC GGA AGC C	
	N5pbs2-F	TCA CGA CAG ACG AGT CTT GCC	Nested *pbs2* primers
	N3pbs2-R	TGA AAG AGG TAA TGG CAG TAT TCG G	
	pbs2-F	GTC ACG ATG AGC GGC ACC G	*pbs2* ORF
	pbs2-R	AAG CTT GGT GCC CCT CAC TGG CTG	
Blue-light receptor Blr1 (*blr1*; EHK39879.1)	Pblr1-F	GTT AGT TAG AGG ACG AAA GGC AG	5′ flanking region of the *blr1* gene
	PQblr1-R	GGT GGT GGC GGA GAG CGT AGG GAG AGT CGG AGA GGG AGA GGG C	
	TQblr1-F	GCG GAG CTG GAA GAG GCT GGA GAC GAG AAG AGC GAG AAA GTA GC	3′ flanking region of the *blr1* gene
	Tblr1-R	CCG AGA CGC TGC GAT GGC	
	N5blr1-F	GTA GGC CTT CAA GAC CGT GC	Nested *blr1* primers
	N3blr1-R	TCG GTC CAT TCA CAA AAG CTC C	
Blue-light receptor Blr2 (*blr2*; EHK40294.1)	Pblr2-F	GAA ACG CCG GTC AGC AGG C	5′ flanking region of the *blr2* gene
	PQblr2-R	GGT GGT GGC GGA GAG CGT AGG GAG AGC AGA CGA TGG CAC TGG AG	
	TQblr2-F	GCG GAG CTG GAA GAG GCT GGA GAC AAG GAA AGA GAG GAA GAA CGG	3′ flanking region of the *blr2* gene
	Tblr2-R	GGA TTA TGC AGC TGA GGT TAT GG	
	N5blr2-F	GTA CCT GTT TGG CGA TGC TAC G	Nested *blr2* primers
	N3blr2-R	GCA TCC GAG GCA AAT TGA GCG	
	blr2-F	ACC GCA GCC CTA CCC TCA TC	*blr2* ORF
	blr2-R	CAC CGC AAC CCG CAG GC	
Blu1 (*blu1*; EHK44319.1)	qblu1-F	CGT TGG CTC TCG CCT GAC C	*blu1* ORF
	blu1-R	GAA CGC CAT TGA AGG CCT CG	

Table 1 (continued)

Gene	Primer	Sequence (5′ to 3′)	Target region
Grg2 (*grg2*; EHK50625.1)	grg2-F	GAT TCC ATC AAG CAG GGT GCC	*grg2* ORF
	grg2-R	GTT TAG ATA GCC TGC TTG TGG G	
DNA photolyase Phr1 (*phr1*; EHK39920.1)	rtPhr1-F	TCG GAC AGC CAG GGA CAG G	*phr1* ORF
	rtPhr1-R	GTG ATT GAG CTG ACG CAT CGC	
Envoy (*env1*; EHK44161.1)	env1-F	GCC AAA ATG GTT CCT TCA GGG TC	*env1* ORF
	env1-R	GTT TGG TCG AGA CAC AAG TCG G	
Glyceraldehyde 3-phosphate dehydrogenase (*gpd*; EHK49005.1)	gpd-F	GCC GAT GGT GAG CTC AAG GG	*gpd* ORF
	gpd-R	GGT CGA GGA CAC GGC GGG A	

PEG (40% PEG 6000 dissolved in osmotic solution) at 42 °C was added and incubated at room temperature for 30 min. To select transformants using a *hph* marker, transformation was mixed with 7–10-ml soft PDA (PDB, 0.7% bacteriological agar, 1 M sorbitol and 100 μg.ml^{-1} hygromycin B) and poured on selective PDA plates (PDA, 1 M sorbitol and 100 μg.ml^{-1} hygromycin B). To select transformants using the *pyr4* marker, the transformation reaction was mixed with 7–10-ml soft MM (MM, 0.7% bacterial agar and 1 M sorbitol) and poured on MM plates (MM and 1 M sorbitol). Cultures were incubated at 27 °C for 4–5 days.

Assay to identify uracil auxotrophy

Transformants resistant to hygromycin were subjected to three rounds of single-spore isolation in MM plus 5 mM uracil and 100 μg ml^{-1} hygromycin B. To identify auxotrophic mutants, drops of 500 conidia (5 μl of a 1×10^5 conidia per ml suspension) of the WT and transformants were inoculated on MM plates with 0.5% Triton X-100, with or without 5 mM uracil, and incubated for 4 days at 27 °C. Transformants unable to grow in uracil-deficient medium were selected, and the *pyr4* ORF replacement was confirmed by PCR using specific primers flanking the recombined sequences. Genomic DNA of the WT and mutant strains were prepared according to the procedure described by Raeder & Broda [25]. The DreamTaq DNA Polymerase (Thermo Scientific) was used for PCR reactions, and the conditions were as follows: first step at 95 °C for 3 min, 35 cycles at 95 °C/30 s, 60 °C/30 s, 72 °C/1 min per kb, and a final extension at 72 °C for 5 min.

Uracil assays to restore auxotrophy

The minimal amount of uracil required for the auxotrophic mutants to restore their growth was determined using MM with different concentrations of uracil. Drops of 500 conidia of the WT and mutant strains were inoculated on MM plates plus 0.5% Triton X-100 and with different uracil concentrations (0, 1, 2.5, 5, and 10 mM) for 4 days at 27 °C. To restore growth, mycelial plugs were taken from colonies grown on MM plates with 5 mM uracil (0.5 mm of diameter of the colony front) and inoculated on MM plates supplemented with uracil (0, 1, 2.5, 5, and 10 mM). Cultures were incubated at 27 °C during 72 h, and pictures were taken for radial growth measurements with ImageJ software (https://imagej.nih.gov/ij/). The experiment was conducted in triplicate.

5-FOA sensitivity assays

To establish the lethal dose of 5-FOA in *T. atroviride*, drops of 500 conidia of the WT and auxotrophic mutants were inoculated on MM plates with various concentrations of 5-FOA (0.5, 1, 2, 3, and 4 mg ml^{-1}), plus 5 mM uracil and 0.5%

Triton X-100, and incubated during 4 days at 27 °C. The experiment was conducted in triplicate.

Transformation based on a positive selection by use of 5-FOA

To obtain uracil auxotrophic strains free of an antibiotic-resistance marker, a co-transformation was carried out using a free-marker cassette created by joining the 5′ and 3′ flanking regions of the *pyr4* gene of *T. atroviride*, 5-FOA for positive selection [15, 26], and the plasmid pCB1004 [24], which is autoreplicative in *T. atroviride* (Esquivel-Naranjo and Herrera-Estrella, under review). Primers listed in Table 1 were designed to amplify the 5′ (Ppyr4-F – Q5pyr4-R) and 3′ (Q3pyr4-F – Tpyr4-R) flanking regions of *pyr4*, excluding the complete ORF. In a second PCR, 5′ and 3′ regions were joined through 22 complementary nucleotides added on the 5′-end of the chimeric primers (Q5pyr4-R - Q3pyr4-F). In a third PCR, the cassette was amplified using nested primers N5pyr4-F – N3pyr4-R and purified by QIAGEN columns. The Platinum™ Taq DNA Polymerase High Fidelity was used to construct the free-marker cassette, and the PCR conditions were as mentioned above. *T. atroviride* protoplasts were co-transformed with the free-marker cassette and pCB1004 in a 2:1 M ratio. Transformants were selected on MM supplemented with 5 mM uracil, 0.5 mg ml^{-1} 5-FOA (sublethal dose), and 100 μg ml^{-1} hygromycin B. Then, one round of single-spore isolation was performed in MM plus 5 mM uracil, 2 mg ml^{-1} 5-FOA, and 100 μg ml^{-1} hygromycin B and the next two rounds in MM plus 5 mM uracil, 0.5 mg ml^{-1} 5-FOA, and without hygromycin B. Auxotrophy tests were carried out as previously described. The *pyr4* gene deletion was confirmed by PCR using primers flanking the coding region and sequencing. The DreamTaq DNA Polymerase was used for PCR reactions, and the conditions were as detailed above.

The *pyr4* gene as a selectable marker for gene replacement

In order to explore of uracil auxotrophic mutants as a genetic tool, deletion cassettes to replace the *blr1*, *blr2*, *tmk3*, and *pbs2* genes by the *pyr4* gene were generated. The Ura−1 strain of *T. atroviride* was used as a parental strain. In a first PCR, the 5′ and 3′ flanking regions were amplified using primers for *tmk3* (Ptmk3-F – pPQtmk3-R and pTQtmk3-F – Ttmk3-R), *pbs2* (Ppbs2-F – pPQpbs2-R and pTQpbs2-F – Tpbs2-R), *blr1* (Pblr1-F – pPQblr1-R and pTQblr1-F – Tblr1-R), and *blr2* (Pblr2-F – pPQblr2-R and pTQblr2-F – Tblr2-R) genes, and the smPyr4-F - smPyr4-R primers were used to amplify 1247 bp of *pyr4* gene. In a second PCR, 5′ and 3′ flanking regions of each gene were joined to *pyr4* gene. In a third PCR, the cassettes were amplified using nested primers for *tmk3* (N5tmk3-F – N3tmk3-R), *pbs2* (N5pbs2-F – N3pbs2-R), *blr1* (N5blr1-F – N3blr1-R), and *blr2* (N5blr2-F – N3blr2-R) genes, and the transformation proceeded as described above. MM plates plus sorbitol 1 M were used as the selection medium. The gene replacement was determined by PCR with primer flanking the recombined 5′- and 3′-end sequences of *tmk3*, *pbs2*, *blr1*, and *blr2* genes. The DreamTaq DNA Polymerase was used for PCR reactions, and the conditions were as detailed above.

Cellular stress assays

Drops of 500 conidia of the WT, Δ*pbs2*, and Δ*tmk3* mutant strains were placed on PDA plates with 0.5% Triton X-100 plus KCl (0, 50 and 100 mM) for osmotic stress response; for heavy metal tolerance, cadmium (0, 400, and 800 mM $CdCl_2$); for oxidative stress tolerance, menadione (0, 200, and 400 mM); or Congo red (0, 50, and 100 mM) to challenge cell wall integrity. To analyze osmotic stress tolerance in mycelia, precultures were generated on PDA plates with 2 μl of conidia of the WT, Δ*pbs2*, and Δ*tmk3* strains and allowed to grow for 40 h at 27 °C in darkness. Mycelial plugs from precultures were inoculated on PDA with NaCl (0 and 0.2 M) for osmotic stress. The cultures were incubated at 27 °C during 4 days, and pictures were taken. The experiments were carried out in triplicate.

Analysis of conidiation induced by light and injure

Light response assays were performed as previously described [8]. Briefly, mycelial plugs from WT, Δ*blr1*, and Δ*blr2* precultures strains were inoculated on PDA plates and incubated for 40 h at 27 °C in darkness. To analyze conidiation stimulated by light, plates were exposed to blue light for 5 min (152.4 μmol m^{-2}) and incubated for 48 h at 27 °C in darkness. To analyze conidiation stimulated by injury, colonies grown on darkness for 40 h were cut with a sterile scalpel to induce conidiation and incubated 48 h at 27 °C in darkness. The experiments were carried out in triplicate.

Analysis of gene expression

Expression of *blu*1, *grg*2, *env*1, and *phr*1 genes was determined in the WT strain, Δ*pbs2*, Δ*tmk3*, Δ*blr1*, Δ*blr2* mutants generated with the *pyr4* marker, and Δ*pbs2*–7; Δ*tmk3*–13 mutants were selected with the *hph* marker. Total RNA from mycelia exposed to blue light for 30 min (914.4 μmol m^{-2}) or in darkness was extracted using TRIzol. DNase I and RNase-free (Thermo Scientific) were used to eliminate genomic DNA. Then, complementary DNA was synthesized following the manufacturer's recommendations (RevertAid Reverse Transcriptase; Thermo Scientific). To estimate transcript levels of genes regulated by light, 1 μl cDNA was used as template, and the DreamTaq

DNA Polymerase was used for PCR reactions, following the conditions detailed above.

Statistical analysis

Statistical analysis and graphs were made with GraphPad Prism (version 5). The presented graph shows the average of three different experiments, and standard deviation is indicated. The data were analyzed through a one-way analysis of variance (ANOVA) with a Tukey-Kramer post-test.

Results

Functional analysis of the *T. atroviride pyr4* gene encoding an OMP decarboxylase

The *pyr4* gene identified in the *T. atroviride* genome encodes an OMP decarboxylase of 379 amino acids, with 91.6%, 67.3%, 56.0%, and 46.0% amino acids identities with OMP decarboxylases from *T. reesei* (ETS06629.1), *Neurospora crassa* (CAD21085.1), *A. nidulans* (AAB66359.1), and *Saccharomyces cerevisiae* (AAB64498.1), respectively. *Trichoderma atroviride pyr4* does not contain introns similar to those of *T. reesei* and *N. crassa pyr4* genes unlike *pyrG* of *A. nidulans* and *Aspergillus niger* which contains two introns [27], suggesting that also *T. atroviride* is more closely related to *N. crassa* than to the two *Aspergillus* species.

In order to generate uracil auxotrophic mutants of *T. atroviride*, a knockout cassette to replace the *pyr4* gene by the *hph* selectable marker was constructed and used to transform *T. atroviride* protoplasts. Transformants were selected in MM plus 5 mM uracil and 100 μg ml^{-1} hygromycin B. Eight uracil auxotrophic mutants were identified and were able to grow only on MM supplemented with uracil (Fig. 1a). The gene replacement in auxotrophic strains was confirmed by PCR (Fig. S1). To evaluate the minimal amount of uracil required to supply the uracil starvation, the WT and mutant strains were cultured on MM with different uracil concentrations, and the growth rate was determined. The auxotrophic strains restored their growth with 5 mM uracil added to the MM (Fig. 1b), and conidiation was induced by light and injury at a similar level as occurred in the WT strain (Fig. S2).

5-FOA has commonly been used in fungi to select uracil auxotrophic strains without drug-resistance markers [20, 21, 27]. In order to establish a positive selection, a conidial suspension of uracil auxotrophs and parental strain was inoculated on MM plates with 0.5% Triton X-100, 5 mM uracil, and different concentrations of 5-FOA (0.5, 1, 2, 3, and 4 mg ml^{-1}). All auxotrophic strains were resistant to 4 mg ml^{-1} 5-FOA, whereas the WT strain showed a strong reduction of growth in 0.5 mg ml^{-1} of 5-FOA and was unable to grow at 1 mg ml^{-1} or even higher concentrations of 5-FOA (Fig. 2a). Interestingly, all auxotrophic strains were still able to produce conidia in 0.5 mg ml^{-1} 5-FOA, but at higher concentrations, conidiation was severally inhibited, thus suggesting a relationship between conidiation and 5-FOA.

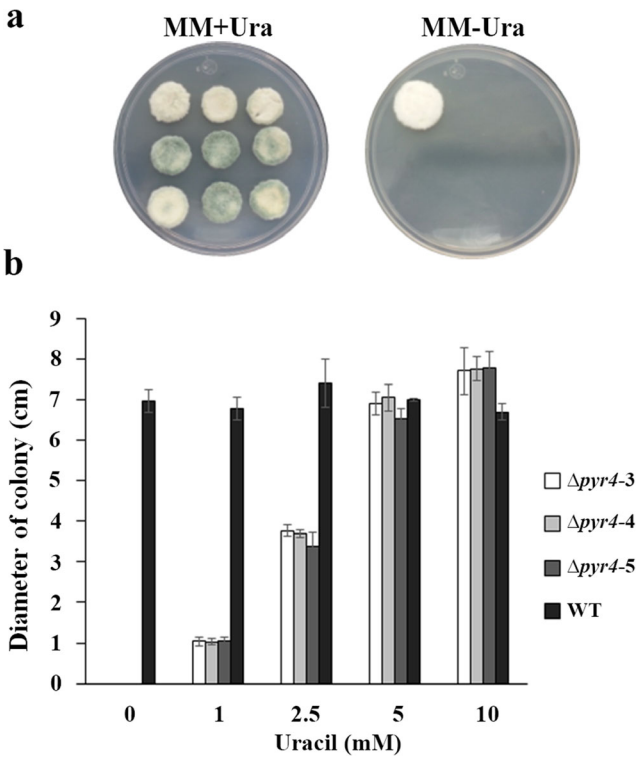

Fig. 1 Uracil auxotrophic strains lacking *pyr4* gene and uracil concentrations to restore auxotrophy. **a** Auxotrophy test for mutant identification. Drops of 500 conidia of WT, Δ*pyr4*–1, − 2, − 3, − 4, − 5, − 6, − 7, and − 8 strains (from top-left to bottom-right) were inoculated on MM plates with or without 5 mM uracil and incubated at 27 °C for 4 days. **b** Recover of uracil auxotrophy. Comparison among the mycelial growth rates of WT, Δ*pyr4*–3, − 4, and − 5 strains. Mycelial plugs were inoculated on MM plates plus different uracil concentrations. After 72 h, colony diameter was measured. Lines on bars represent standard errors. All experiments were carried out in triplicate

Metabolic pathways can be subject to negative feedback inhibition, in which the final product decreases enzymatic activity or gene expression. For the above-mentioned reason, we wanted to examine if the selected uracil concentration has an effect on sensitivity to 5-FOA. A conidial suspension was inoculated on MM plus 2 mg ml^{-1} 5-FOA and different uracil concentrations. Parental and auxotrophic strains showed the same phenotype, without changes in 5-FOA toxicity (Fig. 2b). Altogether, 5 mM uracil is the optimal amount to restore uracil auxotrophy of *T. atroviride* properly, and 2 mg ml^{-1} 5-FOA can be used for a positive selection approach to select uracil auxotrophic strains in this fungus.

Generation of uracil auxotrophy by positive selection

In fungi, uracil auxotrophy has been widely explored to use *ura5*/*pyr4*/*pyrG* or *ura3*/*pyr2* genes as selectable marker [15, 26, 28–30]. In order to create uracil auxotrophic strains using

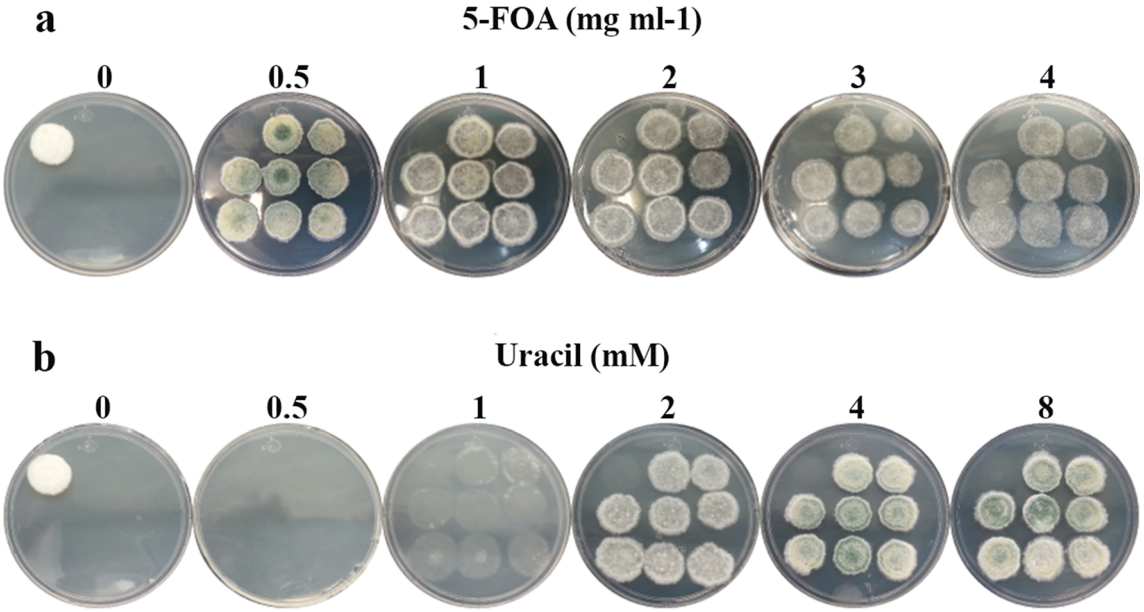

Fig. 2 Lethal dose of 5-FOA. **a** Drops of 500 conidia of WT, Δ*pyr4*–1, −2, −3, −4, −5, −6, −7, and −8 strains (from top-left to bottom-right) were inoculated on MM plates plus 5 mM uracil and the indicated 5-FOA concentrations. **b** Effect of uracil concentrations on the sensitivity to 5-FOA. Drops of 500 conidia of strains indicated in Fig. 2a were inoculated on MM plates plus 2 mg ml^{-1} 5-FOA and different uracil concentrations. Plates were incubated at 27 °C for 4 days. All experiments were performed in triplicate

positive selection with 5-FOA, a free-marker cassette was constructed to eliminate the *T. atroviride pyr4* gene. Given the fact that the plasmid pCB1004 acts as auto-replicative in *T. atroviride* (Esquivel-Naranjo and Herrera-Estrella, under review), we co-transformed protoplasts with the free-marker cassette and pCB1004 (2:1). To assure the auxotrophic mutants selection, transformants were recovered on MM plus 0.5 mg ml^{-1} 5-FOA, 5 mM uracil, and 100 μg ml^{-1} hygromycin B. *Trichoderma atroviride* protoplasts are multinucleated cells harboring transformed and non-transformed heterokaryotic nuclei. For this reason, we chose a 5-FOA sublethal concentration to select transformants. To obtain homokaryons, transformants were subjected to three rounds of single-spore isolation with modifications. Firstly, the selective medium comprised 5 mM uracil, 2 mg ml^{-1} 5-FOA, and 100 μg ml^{-1} hygromycin B. Secondly, the colonies were grown on MM supplemented with 5 mM uracil and 0.5 mg ml^{-1} 5-FOA to produce conidia. Ten stable mutants were identified by an auxotrophy test and designated Ura^{-}1-10 (Fig. 3a). All were unable to grow on uracil-deficient MM and sensitive to hygromycin B, indicating that the pCB1004 plasmid was lost in all auxotrophic strains when this antibiotic was not added to the MM. The auxotrophic strains were then analyzed in their mycelium growth, production of conidia stimulated by light, and injury on PDA plates plus 5 mM uracil. All uracil auxotrophic mutants were stable and showed a similar behavior to the parental strain, suggesting that these strains can be used as a reliable receiver strain for genetic manipulation (data not presented). Unexpectedly, when we amplified the coding region of the *pyr4* gene, the amplicons of six auxotrophic strains showed an electrophoretic mobility similar to that of the WT strain, whereas the amplicon of four auxotrophic strains (Ura^{-}1, Ura^{-}4, Ura^{-}9, and Ura^{-}10) was slightly shorter (Fig. S3). In order to identify mutations in the coding region of the *pyr4* gene, it was amplified from WT and Ura^{-}1, −2, −3, −4, −5, −6, −7, −9, and −10 strains and sequenced. As expected, the Ura^{-}2, −3, −5, −6, and −7 strains did not have changes in the *pyr4* nucleotide sequences and were identical to WT strain, whereas Ura^{-}1, −4, and −10 had a deletion of 139 bp and Ura^{-}9 deletion of 23 bp at the positions 928 bp and 997 bp, respectively (Fig. S4), considering the start codon. The deletion caused a frameshift, where the Ura^{-}1, −4, and −10 strains encoded a polypeptide of 351 amino acids of which 309 were identical, and Ura^{-}9 encoded 345 amino acids of which 334 were identical. Both of these were shorter than the Pyr4 protein (379 amino acids) of the WT strain (Fig. 3b–c). Given that, all produced auxotrophic strains showing at least two different mutations, and our results suggest that 5-FOA could be a mutagenic chemical or it could promote genomic instability, provoking mutagenesis. Furthermore, these data shows that the last 45 amino acids in the COOH-terminal of Pyr4 are essential for uracil biosynthesis.

The Ura^{-}1 and Ura^{-}9 strains were selected for complementation tests due to their truncated *pyr4* gene. The coding region of the WT *pyr4* gene, including 1372-bp upstream and 1548-bp downstream, was amplified using the primers Ppyr4-F and Tpyr4-R (4060 bp), cloned into a pCR2.1-TOPO vector (Invitrogen) and used to complement uracil auxotrophic strains. In both strains, the *pyr4* gene transformed the uracil

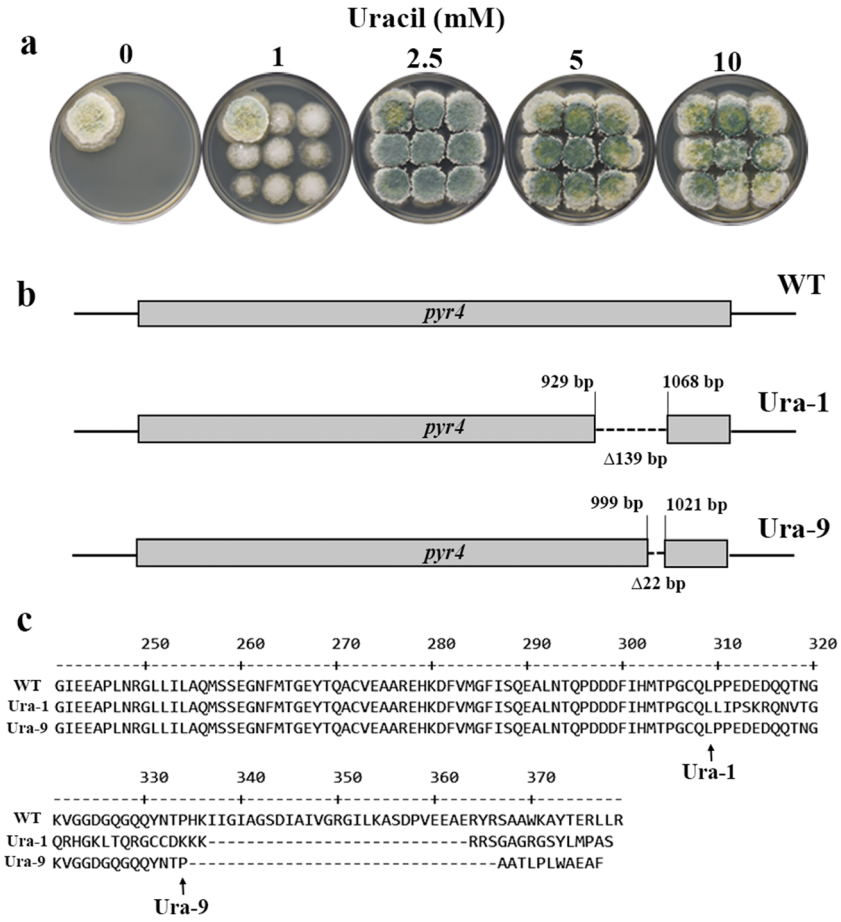

Fig. 3 Analysis of auxotrophic mutants selected by resistance to 5-FOA. **a** Drops of 500 conidia of WT, Ura^{-}1, − 2, − 3, − 4, − 5, − 7, − 8, and − 9 strains (from top-left to bottom-right) were inoculated on MM plates plus different uracil concentrations and incubated at 27 °C for 4 days. This assay was carried out in triplicate. **b** Schematic representation of *pyr4* deletions. Deletions and positions were determined by alignment of the nucleotide sequence using the Clustal V method. **c** Protein alignment conducted among Pyr4 proteins from WT, Ura^{-}1, and Ura^{-}9 strains using the Clustal V method. Arrows indicate the end of the conserved amino acid sequence for the indicated strain

auxotrophy to prototrophy (Fig. S5), indicating that the uracil auxotrophy was due to a truncation in the *pyr4* gene, as it was demonstrated by *pyr4* sequencing analysis.

Deletion of genes encoding the MAPK Tmk3 and MAPKK Pbs2 using *pyr4* as selectable marker

To evaluate if the uracil auxotrophic mutants can be used as a genetic tool for gene replacement and to validate if *pyr4* restores the uracil starvation, we designed cassettes to replace the *tmk3* and *pbs2* genes with the *pyr4* gene. The *tmk3* and *pbs2* genes encode for MAPK Tmk3 and MAPKK Pbs2, respectively, related to light and stress responses [8]. These genes were selected to compare our results with those that have been previously reported in mutants selected by *hph* gene (Δ*tmk3*–13:*hph* and Δ*pbs2*–7:*hph* mutants). To avoid gene replacement at the *pyr4* locus and favor the targeted knockouts, the Ura^{-}1 strain was chosen because the truncation is bigger than in Ura^{-}9. To construct the deletion cassettes, the *pyr4* ORF was amplified with smPyr4-F and smPyr4-R primers to generate a selectable marker of 1247 bp (71-bp upstream—coding region—36-bp downstream). In this sense, the *pyr4* marker was positioned under the control of the promoter and terminator of the deleted genes.

Transformation was carried out, and eight out of 24 transformants (33.3%) were mutants lacking the *tmk3* gene (Δ*tmk3*:*pyr4* mutants), and three out of 20 (15%) were Δ*pbs2*:-*pyr4* mutants (Fig. S6–S7), all able to grow on MM without uracil. Conidiation stimulated by light in Δ*tmk3*:*pyr4* and Δ*pbs2*:-*pyr4* strains was analyzed, and in all mutants, strains conidiation was reduced to 80%, similar to data obtained with Δ*tmk3*:*hph* and Δ*pbs2*:*hph* strains (Fig. 4a–b). As reported before [8], all mutants were sensitive to osmotic stress, Congo red, $CdCl_2$, and menadione (Fig. 4c–d). There was no difference between mutants selected by *hph* or *pyr4* markers, indicating that the Ura^{-}1 strain was not affected neither in conidia production stimulated by light nor cellular stress responses.

Deletion of genes encoding the blue-light receptor Blr1/2 using the *pyr4* marker

To acquire more evidence on the application of Ura^{-}1 strain as a genetic background, cassettes were designed to replace the *blr1* and *blr2* coding regions by the *pyr4* gene marker. Eight out of 20 transformants (40%) were *blr1* mutants, and ten out of 18 transformants (55.5%) were *blr2* mutants (Fig. S8–S9). The Δ*blr1*:*pyr4* and Δ*blr2*:*pyr4* mutants were exposed to a blue-light pulse (152.4 μmol m^{-2}). As expected, the

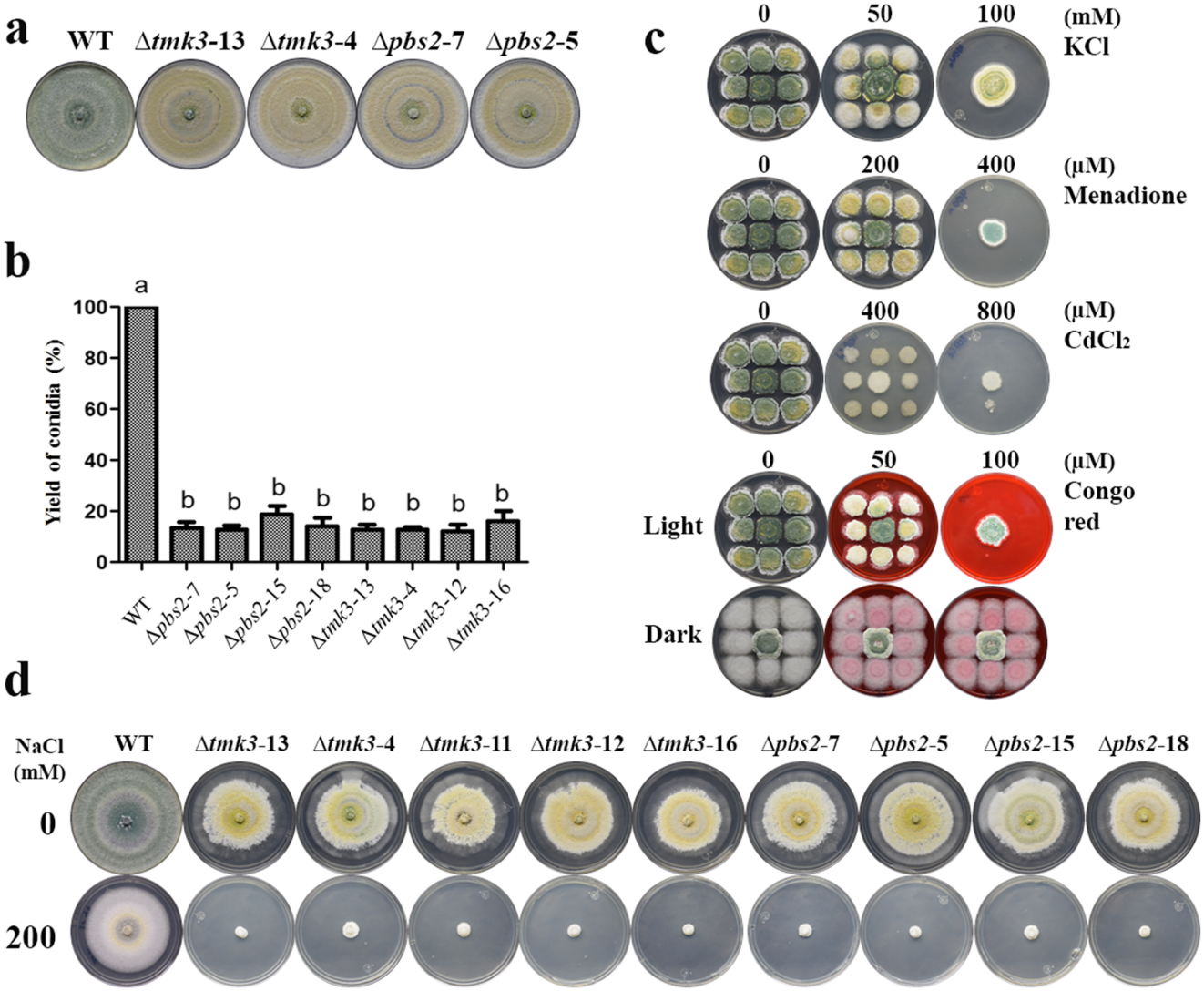

Fig. 4 Validation of *pyr4* gene as a selectable marker in *T. atroviride* for gene deletion. **a** Comparison between WT and mutant strains selected with the *hph* marker (Δ*tmk3*–13 and Δ*pbs2*–7) and *pyr4* marker (Δ*tmk3*–4 and Δ*pbs2*–5) grown on PDA plates in constant white light (0.586 μmol m^{-2} s^{-1}) for 7 days at 27 °C. **b** Conidial production of the WT strain and mutants selected with the *hph* marker (Δ*tmk3*–13 and Δ*pbs2*–7) and the *pyr4* marker (Δ*pbs2*–5, − 15, − 18, Δ*tmk3*–4, − 12, and − 16) grown on PDA plates in constant white light for 7 days at 27 °C. **c** Stress sensitivity in conidia of Δ*tmk3* and Δ*pbs2* strains. Drops of 500 conidia of Δ*tmk3*–4, − 12, − 16, − 13, WT, Δ*pbs2*–7, − 5, − 15, and − 18 strains (from top-left to bottom-right) were inoculated on PDA plates plus different concentrations of the indicated stressors. They were then, incubated in constant white light or darkness for 4 days at 27 °C. **d** Osmotic stress sensitivity in mycelia of Δ*tmk3* and Δ*pbs2* strains. Mycelial plugs of the indicated strains were inoculated on PDA plates with or without 200 mM NaCl and incubated for 4 days at 27 °C. All experiments were carried out in triplicate

Δ*blr1* and Δ*blr2* strains were not photoresponsive, and the phenotype in light was the same as the control strain kept in darkness. Thus, mutant strains could not form a conidia ring in response to light on PDA medium (Fig. 5a–b), but they conidiated in response to injury as described before [31].

Selection by *pyr4* did not affect the expression of genes regulated by light

All mutants selected by the *pyr4* marker showed the same phenotype in stress tolerance and photoconidiation, such as previously described using *hph* marker [8, 31]. However, gene expression epistasis has been associated with the use of auxotrophic strains in *S. cerevisiae* [32]. In order to discard changes in gene expression generated by the selectable marker, the expression of four light-regulated genes was examined in the Δ*blr1*:*pyr4*, Δ*blr2*:-*pyr4*, Δ*tmk3*:*hph*, Δ*tmk3*:*pyr4*, Δ*pbs2*:*hph*, and Δ*pbs2*:-*pyr*4 strains. As reported previously [8, 31, 33], *blu1*, *grg2*, *phr1* (CPD-photolyase), and *env1* (photoreceptor for blue-light tolerance) gene expression was not detected in darkness; however, after a blue-light pulse, their transcript levels were induced in the WT strain. In the Δ*tmk3*

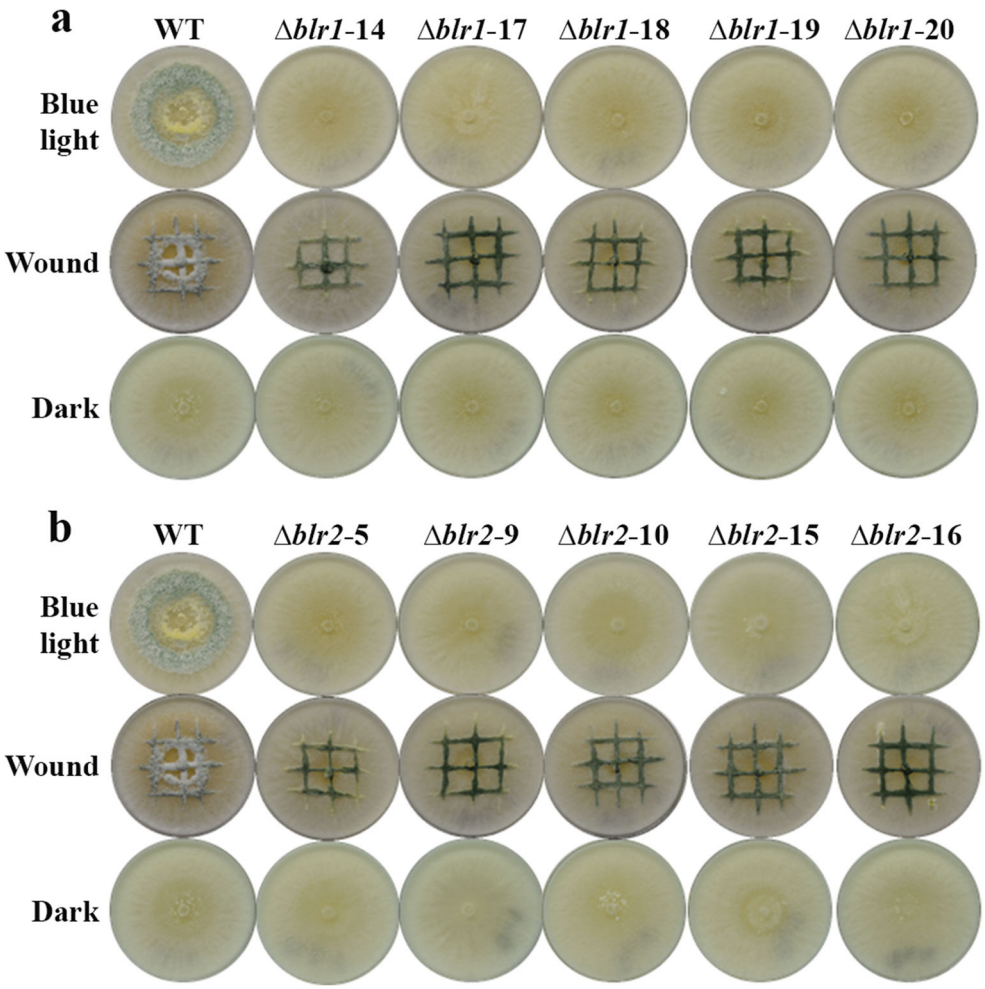

Fig. 5 Phenotypes of *T. atroviride* WT, Δ*blr1*, and Δ*blr2* strains. **a** Mycelial plugs of WT, Δ*blr1*– 14, − 17, − 18, − 19, and − 20 strains or **b** Δ*blr2*–5, − 9, − 10, − 15, and − 16 strains were inoculated on PDA plates and incubated for 40 h at 27 °C. Then, a blue-light pulse (152.4 μmol m^{-2}) was applied for light-response assays or the mycelia were injured with a sterile scalpel for wound response. Pictures were taken 48 h later. Strains kept in darkness are shown as control. All experiments were performed in triplicate

and Δ*pbs2* strains, expression of *blu1* and *grg2* genes was not induced, whereas *phr1* and *env1* genes were responsive to blue light at a similar level as the WT strain. Under the same conditions, the blue-light-regulated genes presented similar patterns in the Δ*tmk3* and Δ*pbs2* mutants obtained with *pyr4* and *hph* markers (Fig. 6). In the Δ*blr1* and Δ*blr2* strains, *blu1*, *grg2*, *phr1*, and *env1* genes were not induced by blue-light (Fig. 6), indicating a preponderant function of Blr1/2 blue-light receptors on light responses in *T. atroviride*. Altogether, these data provide evidence that the *T. atroviride* $Ura^{-}1$ strain can be used as a background for genomic functional studies.

Discussion

The use of auxotrophic mutants as a genetic tool is an option to improve our knowledge of gene function in a low-cost way. Here we describe our transformation system in *T. atroviride* to obtain uracil auxotrophs free of drug-selectable markers, based on the 5-FOA positive selection. The *pyrG*/*pyr4* gene in *T. reesei*, *T. harzianum,* and *T. hypoxylon* encodes orotidine-5′-monophosphate decarboxylase [15, 22, 27]. The absence of *pyr4* in *T. reesei* can be restored by the *pyrG* gene of *A. niger* and the *pyr4* gene of *N. crassa* [14], indicating that *pyr4* is highly conserved in fungi. Furthermore, it is possible to use uracil auxotrophs in *T. atroviride* to establish homologous and heterologous transformation system.

To determine growth conditions, uracil auxotrophs were generated using resistance to hygromycin B. The results demonstrate that the knockout of *pyr4* in *T. atroviride* leads to a uracil-deficient strain. Uracil in the medium restores growth of mutants, and the Δ*pyr4* strains were able to grow in media containing 5-FOA. This drug has already been used in *Trichoderma* species to select strains mutagenized through UV-light irradiation [14] and knockout [15, 26]. The 5-FOA lethal dose was determined to begin at 1 mg ml^{-1}, and high uracil concentrations have no effect on 5-FOA toxicity. Together, data obtained were used to establish conditions for a markerless transformation in *T. atroviride*.

Techniques have been developed to eliminate the *pyr4* gene without a drug-selectable marker in *T. reesei* and *T. hypoxylon* [15, 26]. In *T. reesei*, a genetic background lacking a non-homologous end-joining (NHEJ) repair system was

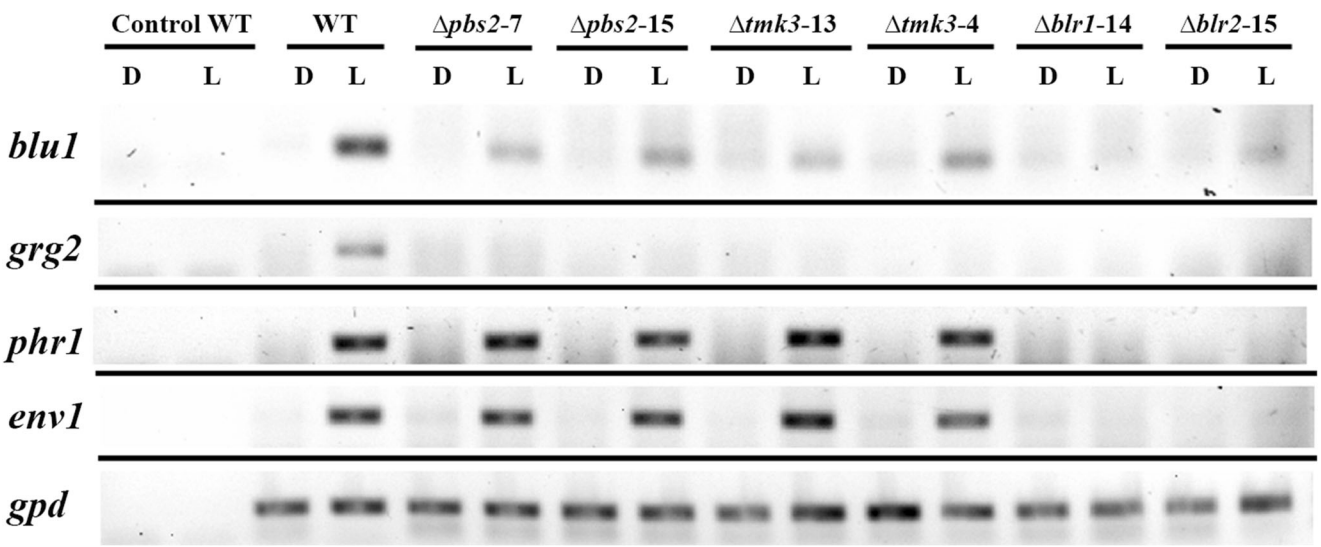

Fig. 6 Expression of light-responsive genes in Δ*pbs2*, Δ*tmk3*, Δ*blr1*, and Δ*blr2* strains. An RT-PCR was performed to compare the expression of *blu1*, *grg2*, *phr1*, and *env1* genes in WT and mutants selected with the *hph* marker (Δ*pbs2*–7 and Δ*tmk3*–13) and the *pyr4* marker (Δ*pbs2*–15, Δ*tmk3*–4, Δ*blr1*–14, and Δ*blr2*–15). The strains were kept in darkness for 48 h. Then, the strains were exposed to blue light for 30 min (914.4 μmol m^{-2}). After the blue-light induction, total RNA was extracted with TRIzol. The *gpd* gene was used as control, and this experiment were carried out in duplicate obtaining practically the same results. Control WT: control without RT. D: darkness. L: light.

used to efficiently eliminate the coding region of *pyr4*, but in *T. hypoxylon*, the WT strain was employed. Although a free-marker cassette was designed as previously described [15, 26], with the promoter and terminator fragments joined, excluding the ORF, our results showed that the mutant strains were generated by a different mechanism. Analysis of *pyr4* deletion strains by PCR and sequencing revealed that the *pyr4* ORF of four uracil auxotrophs (Ura^{-}1, Ura^{-}4, Ura^{-}9, and Ura^{-}10) was truncated by at least two independent events. Even though the *pyr4* gene was not completely deleted, the produced alleles encoded a non-functional Pyr4 protein that was unable to produce uracil, which was demonstrated by auxotrophy, and were successfully complemented with the WT *pyr4* gene. On the other hand, six auxotrophic strains showed bands with electrophoretic mobility comparable with WT *pyr4* locus, suggesting that the mutations occurred outside *pyr4* coding region as confirmed by sequencing in Ura^{-}2, −3, −5, −6, and −7 auxotrophic strains. The auxotrophs without changes in *pyr*4 ORF could be explained by a mutation in a cis or trans element necessary for *pyr4* expression. Another possibility that able to explain our results is that a mutation took place in another gene involved in pyrimidine biosynthesis, such as the *ura3*/*pyr2* gene encoding OPRTase. These results suggest that 5-FOA may be mutagenic or a stress condition caused by genomic instability during transformation process. In this sense, 5-FU and FdUMP, toxic molecules produced by 5-FOA metabolism, can be incorporated into RNA or DNA matching 5-FU:A or 5-FU:G and induce genomic instability [18, 19]. *Saccharomyces cerevisiae* mutants lacking Apn1, the major abasic site endonuclease, showed a pronounced sensitivity to 5-FU, suggesting that abasic sites formed during DNA repair are more toxic, which can provoke DNA strand breaks [34]. However, 5-FOA has not been documented to cause DNA deletions. More data supporting this hypothesis comes from experiments in *Candida albicans*. When *C. albicans* was exposed to 5-FOA, its chromosomes suffered alteration [35, 36], presumably as an adaptive mutation under the stress conditions by 5-FOA that caused genetic changes. The above can explain the mutation that occurred in *T. atroviride*, as well as the partial loss of the *pyr4* ORF by 5-FOA added in the culture medium.

Furthermore, we demonstrated that the Ura^{-}1 strain can be used as background to knockout genes in *T. atroviride*. The *blr1*, *blr2*, *pbs2*, and *tmk3* genes previously characterized [8, 31] were successfully replaced by *pyr4* in the Ura^{-}1 strain. The efficiency ranged from 15 to 55% among the genes included in our analysis. Noticeably, expression of *blr1* and *blr2* genes has been described to be undetectable by Northern-blot analysis, indicating that those genes are little expressed, whereas higher transcript levels were detected in *tmk3* and *pbs2* genes [31, 37]. Despite that the *pyr4* gene was under control of the promoter of these deleted genes, in all cases uracil auxotrophy was successfully complemented. The conidial production and gene expression in mutants obtained from the auxotroph strain were evaluated, and the results are comparable with those of mutants obtained with the *hph* marker. Although the average efficiency of gene replacement was 36%, it was similar using the *hph* marker [8], suggesting that the knockout cassettes were targeted mainly to the corresponding locus.

In *S. cerevisiae*, auxotrophies alter the genetic background which induces different transcriptional responses [32].

Therefore, we evaluated the level of expression of four light-regulated genes in mutants obtained with a uracil auxotrophic strain, and the analyzed genes did not exhibited alterations in transcript levels. This suggests that the behavior of *T. atroviride* is different from that described in *S. cerevisiae*, although we cannot discard epistasis in other genes not included in this work. Our results show that the Ura^{-}1 strain serves as a reliable genetic background, given that transcriptional perturbations were not detected under our experimental conditions.

In conclusion, the Ura^{-}1 strain is an alternative tool to disrupt or complement genes in *T. atroviride*. Despite the fact that the uracil auxotroph was not obtained by knockout as initially designed, we did not observe differences in phenotype in comparison with mutants obtained with the *hph* marker. It would be interesting to know if a heterologous *pyr4* gene from other fungi can improve the gene replacement efficiency in *T. atroviride*.

Funding information This work was supported by CONACYT (Consejo Nacional de Ciencia y Tecnología, CB-2011-169,045) and FOFI-UAQ-2012 (Fondo para el fortalecimiento de la investigación—Universidad Autónoma de Querétaro, FNB-2012-10) grants to E.U. E-N. G. C-H is indebted to CONACYT for a doctoral fellowship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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