

Genetic transformation tools for an *Armillaria* species, a resource for studying the interaction between *Armillaria* fungus and the fully mycoheterotrophic plant *Gastrodia elata*

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Abstract

The genus *Armillaria* (Basidiomycota, Agaricales, Physalacriaceae) comprises pathogenic fungi that cause root-rot disease in plants, as well as species with low pathogenicity, some of which are hosts of the fully mycoheterotrophic orchid plant *Gastrodia elata* (Orchidaceae). To investigate the mechanisms underlying such special interactions between *Armillaria* fungi and *G. elata*, it is crucial to establish genetic transformation platforms for the *Armillaria* fungi and *G. elata*. In this study, an *Armillaria* strain Arm37 was isolated from *G. elata*, which can form symbiosis with *G. elata* in axenic culture under laboratory conditions. A vector pYT-EV containing a cassette for hygromycin-resistance selection and a cassette for expressing or silencing target genes was constructed. An *Agrobacterium tumefaciens* (Agrobacterium)-mediated transformation (AMT) system for Arm37 was successfully developed and optimized to achieve a transformation efficiency 32%. The AMT system was successfully used to express the reporter genes *enhanced green fluorescent protein* (eGFP) and β -glucuronidase (*GUS*) and to effectively silence the endogenous gene *orotidine 5'-monophosphate decarboxylase* (*URA3*) in Arm37. This AMT system established for Arm37 provides an efficient genetic tool for exploring the Arm37 genes that are involved in the unique interaction between the *Armillaria* fungi and fully mycoheterotrophic plant *G. elata*.

Introduction

In nature, almost all plants interact with certain fungi. Many fungi are pathogenic to plants (Doehlemann et al., 2017; Moller and Stukenbrock, 2017), while some are beneficial, e.g., mycorrhizal fungi (Genre et al., 2020; Shi et al., 2023). During evolution, some plants became parasites of specific fungal species (Merckx, 2013), and these plants are named mycoheterotrophic plants, which partially or completely rely on specific fungi to gain carbon and often other nutrients (Leake, 1994; Merckx and Gomes, 2023; Selosse and Roy, 2009). Due to their unique physiology, ecology, and evolutionary histories, mycoheterotrophic plants have been drawing much attention from plant biologists, especially in terms of how they interact with their symbiotic fungi. Fully mycoheterotrophic plants depend solely on fungal carbon throughout their entire life cycles, representing a way for plants to survive without sunlight or photosynthesis, a lifestyle that is similar to that of holoparasitic plants (Merckx and Gomes, 2023).

Gastrodia elata (Orchidaceae), a fully mycoheterotrophic orchid plant, has been used for medicinal purposes in eastern Asian countries (Wu et al., 2023; Zhan et al., 2016). *G. elata* is achlorophyllous and completes its life cycle by relying entirely on certain fungi, including some species of *Armillaria* (Basidiomycota, Agaricales, Physalacriaceae) (Hua et al., 2024). Some *Armillaria* species, such as *A. mellea*, *A. ostoyae*, and *A. borealis*, cause root rot disease in more than 500 host plants (Sipos et al., 2018). In contrast, several *Armillaria* species with low pathogenicity, such as *A. gallica*, *A. cepistipes*, *A. gallica*, and *A. nipponica*, can be associated with *G. elata* (Guo et al., 2016) or *Cyrtosia septentrionalis* (Umata et al., 2013) as their primary nutrient provider. In addition, *A. gallica* has been successfully isolated, identified, and used for cultivation of *G. elata* for more than 50 years (Xu et al., 1989; Zhou, 1981). Given that these *Armillaria* fungi can be artificially cultured and the symbiosis between *G. elata* and *Armillaria* can form under lab and field conditions, such *G. elata*-*Armillaria* interaction systems

provide exceptional opportunities to investigate the molecular mechanisms underlying mycoheterotrophic plant-fungus interactions. It is desirable to establish highly efficient genetic transformation systems for *G. elata* and the associated *Armillaria* fungi to facilitate further molecular and cellular research on their interactions.

Recently, multiple genomes of *Armillaria* spp. were sequenced (Sahu et al., 2023; Sipos et al., 2017). *Agrobacterium tumefaciens* (Agrobacterium)-mediated transformation (AMT) systems using the spores from *A. mellea* has also been established (Ford et al., 2015, 2016; Idnurm et al., 2017). However, induction of fruiting bodies and acquisition of spores in many *Armillaria* species are still challenging, including the *Armillaria* species that form symbiosis with *G. elata*. Furthermore, genetic manipulation of *G. elata* is still not available.

Here, we isolated and identified an *Armillaria* fungal strain Arm37, which can be a host fungus for the mycoheterotrophic *G. elata*. Importantly, we constructed a vector containing the hygromycin resistance gene *hygromycin phosphotransferase* (*HPT*) driven by the native *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*) promoter and a target sequence, which can be a coding sequence or an inverted-repeat RNAi (RNA interference) sequence, by the native *translation elongation factor 1 α* (*TEF1*). The vector was subsequently transformed into the Agrobacterium strain AGL-1 and used for transformation of Arm37. The relevant parameters were optimized to increase the transformation efficiency, including the concentration of acetosyringone, the ratio of fungi to bacteria, and the duration of coculturing. Using this AMT system, the *enhanced green fluorescent protein* (*eGFP*) and *β -glucuronidase* (*GUS*) were transformed into Arm37 for expression, and the endogenous *orotidine 5'-monophosphate decarboxylase* (*URA3*) gene was silenced by RNAi in Arm37. The development of an efficient AMT system for genetic manipulation of *Armillaria* provides a genetic tool for studying the interaction between *Armillaria* and *G. elata*.

Materials and methods

2.1. Isolation and identification of *Armillaria* strain

The fungal rhizomorphs from a tuber of *G. elata*, which was collected from Xiaocaoba, Zhaotong, Yunnan Province, China, were surface-sterilized by being immersed in 75% ethanol for 5 min and then 5% sodium hypochlorite for 5 min. The rhizomorphs were then cultured on a modified potato dextrose agar (mPDA) medium [200 g/L potato water (filtrate obtained after boiling peeled and chopped potatoes in clear water), 200 g/L sawdust water (filtrate obtained after boiling sawdust of *Cyclobalanopsis glauca* in water), 20 g/L dextrose, and 10 g/L agar] at 23°C. The medium was supplemented with 50 mg/L kanamycin, 50 mg/L ampicillin, and 200 mg/L timentin. After two weeks, the new rhizomorphs exhibited vigorous growth and were subcultured another three times to obtain the fungal strain, which was named Arm37. An established method (Guo et al., 2016) was used for molecular identification of its *Armillaria* genus.

2.2. Axenic culture of *Armillaria* strain Arm37 and *G. elata*

The isolated strain Arm37 was cultured on mPDA medium for two weeks at 23 °C in dark. Afterwards, it was inoculated on a RSTO medium (Sipos et al., 2017) for two weeks. Once the rhizomorphs showed vigorous growth, they were covered with sterilized sawdust of Fagaceae tree *Cyclobalanopsis glauca* and inoculated with *G. elata* protocorms. The axenic culture was kept in dark at 23 °C for 3 months to monitor the growth of *G. elata*.

2.3. Cultivation of bacterial strains

The *Escherichia coli* strain DH5α and *Agrobacterium tumefaciens* strain AGL-1 were cultured on the Luria-Bertani (LB) at 37 °C and yeast extract peptone (YEP) medium at 28 °C, respectively. Bacteria transformed with plasmids were cultured under selection of appropriate antibiotics.

2.4. Identification of candidate genes and their regulatory sequences

To identify *GPDs* (*glyceraldehyde-3-phosphate dehydrogenases*), *TEF1s* (*translation elongation factor 1α*), and *URA3s* (*orotidine-5'-phosphate decarboxylases*) in Arm37, protein sequences of these genes' homologs in yeast were used as the queries to search the genomes of Arm37 (accession PRJCA013068 on <https://ngdc.cncb.ac.cn/>), *A. gallica*, *A. ostoyae*, *A. mellea*, *Aspergillus niger*, *Neurospora crassa*, and *Agricus bisporus* using the BLASTP program. Multiple sequence alignment and phylogenetic analysis were respectively performed using Clustal X (Larkin et al., 2007) and MEGA (Tamura et al., 2021). Moreover, the promoters and terminators of these genes were amplified by PCR using the Arm37 genomic DNA, based on the upstream and downstream sequences of *GPD* and *TEF1* in Arm37. The accession numbers of all sequences can be found in Table S1.

2.5. Nucleic acid extraction and PCR

Genomic DNA was extracted from fungal hyphae or rhizomorphs using the CTAB method (Allen et al., 2006). Total RNA was extracted from fungal hyphae or rhizomorphs using the TRIzol reagent (Thermo Fisher Scientific, USA), and cDNA synthesis was performed using a cDNA synthesis kit (Thermo Fisher Scientific, USA). PCR was done using 2 × Taq Master Mix kit (Vazyme, China). All primer sequences are listed in Table S2.

2.6. Plasmid construction

The *hygromycin phosphotransferase* (*HPT*) gene was artificially synthesized (Sangon Biotech, China), whose sequence was codon-optimized according to the codon usage bias in the genus *Armillaria* (Table S3). The expression vector pCAMBIA1301 was linearized using the restriction enzymes XhoI and EcoRI, and three fragments, the *GPD* promoter (Pgpd, primers: Pgpd-F/Pgpd-R), the *HPT* (primers: HPT-F/HPT-R), and the *GPD* terminator (Tgpd, primers: Tgpd-F/Tgpd-R), were recombined into the linearized pCAMBIA1301 plasmid using the ClonExpress II One Step Cloning Kit (Vazyme, China) to generate the intermediate plasmid pCAMBIA1301-Pgpd-HPT. Similarly, three fragments, the *TEF1* promoter (Ptef1, primers: Ptef1-F/Ptef1-R), the first intron of *GPD* (iGPD, primers: iGPD-F/iGPD-R), and the *TEF1* terminator (Ttef1, primers: Ttef1-F/ Ttef1-R), were integrated into the plasmid pCAMBIA1301-Pgpd-HPT, and the

resulting plasmid was designated pYT-EV (Fig. S1, GenBank accession number: PP437273) as the empty vector. The plasmid pYT-EV contains two expression modules: (i) the codon-optimized *HPT* with a 56-bp intron (iGPD) regulated by the *GPD* promoter and terminator, and this module is used for antibiotic hygromycin selection; (ii) the iGPD sequence with an upstream *TEF1* promoter and downstream *TEF1* terminator, and this module can be further modified to express or silence target genes.

The plasmids pYT-ieGFP and pYT-iGUS were respectively constructed by recombining the two fragments *enhanced green fluorescent protein* (*eGFP*, primers: eGFP-F/eGFP-R) and iGPD (primers: eiGPD-F/eiGPD-R) and by recombining the two fragments *β -glucuronidase* (*GUS*, primers: GUS-F/GUS-R) and iGPD (primers: giGPD-F/giGPD-R) into pYT-EV, which was pre-linearized by the restriction enzyme NcoI.

For construction of the plasmid to silence the *URA3* gene, 303-bp fragments of the sense *URA3* (*seURA3*) and the antisense *URA3* (*anURA3*) sequence were amplified from Arm37 cDNA using the primer pairs seURA3-F/seURA3-R and anURA3-F/anURA3-R, respectively. In addition, the first intron of *pyruvate orthophosphate dikinase* (iPDK) in *Flaveria trinervia* was also amplified from the plasmid pHELLSGATE 8 using the primers iPDK-F and iPDK-R. The fragment *seURA3* and the intron iPDK were recombined into the linearized plasmid pYT-EV (by NcoI digestion), resulting in the intermediate plasmid pYT-seURA3. pYT-seURA3 was further digested by SpeI and SbfI. The *anURA3* fragment was also digested by SpeI and SbfI and thereafter inserted into pYT-seURA3 to finally form the RNA interference plasmid pYT-iURA3. All restriction enzymes used in this study were purchased from New England Biolabs (Ipswich, USA). All primer pairs are listed in Table S2.

2.7. Test of fungal sensitivity toward antifungal substances

The sensitivity of the strain Arm37 to hygromycin and 5-fluoroorotic acid (5-FOA) was evaluated on mPDA medium supplemented with varying concentrations of hygromycin (0, 10, 20, 30, 40, 50 mg/L) or 5-FOA (0, 1, 2.5, 5, 7.5, 10 mg/L). After two weeks of incubation in dark at 23°C, the growth of the fungal hyphae and rhizomorphs was recorded.

2.8. Fungal transformation

A single colony of *Agrobacterium* strain AGL-1 carrying pYT-EV was inoculated in YEP liquid medium supplemented with kanamycin (50 mg/L) and cultured overnight at 28°C. Subsequently, 1 mL of the liquid culture was inoculated into 10 mL of YEP medium without antibiotics at 28°C to achieve an OD_{600 nm} value about 0.5. After the cells were centrifuged at 2000 *g* for 10 min, the supernatant was removed, and the pellet was resuspended in the liquid induction medium (IM) supplemented with acetosyringone (200 μ M) to achieve an OD_{600 nm} value of 0.6. Fresh Arm37 rhizomorph fragments (about 5 mm long) were transferred into resuspended bacteria to coculture at 23°C for 48 h. Rhizomorphs were subsequently transferred to mPDA medium supplemented with hygromycin (20 mg/L) and timentin (100 mg/L) and incubated at 23°C in dark for two weeks. Vigorously growing hyphae were picked and transferred to fresh medium (mPDA medium supplemented with 20 mg/L hygromycin and 100 mg/L timentin). After two weeks, the transformants were used for PCR confirmation of transformants.

2.9. PCR confirmation of transformants

Putative transformants were subcultured serially on the mPDA medium supplemented with hygromycin (20 mg/L) and timentin (200 mg/L). The presence of the transgene in the transformants was confirmed by PCR analysis of *HPT*, *eGFP*, and *GUS* gene using the primer pairs piHPT-F/piHPT-R, pieGFP-F/pieGFP-R, and piGUS-F/piGUS-R (Table S2), respectively. Furthermore, PCR was performed with the primers SLB-F and SHPT-R targeting the vector DNA of outside and inside the T-DNA borders to eliminate interference from bacterial contamination.

2.10. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was done on a CFX Connect Real-Time PCR Detection system (Bio-Rad, USA) using an *iTaq* Universal (SYBR Green) Kit (Bio-Rad, USA). The *GPD* gene was used as the internal reference gene. For each gene, a linear standard curve was constructed using a series of dilutions of cDNA and the threshold cycle numbers. Based on the standard curves, the extrapolated concentrations of transcripts were calculated. All the reactions were conducted with biological triplicate. Primers used are listed in Table S2.

2.11. Fluorescence observation and GUS activity analysis

To observe the expression of eGFP in hyphae, fungus was cultivated on mPDA medium without antibiotics for 10 days. The fluorescence signals of the reporter protein eGFP were detected under a fluorescence microscope (Leica, DM55008). To detect GUS activity, the fungus was stained with a GUS staining solution (1 mg/ml X-Gluc, 100 mM phosphate buffer [pH 7.0], 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA) at 37°C overnight in dark. The fungal tissues were washed three times with water to remove the GUS working solution, and images were captured under a light microscope (Leica, DM55008).

Results

Isolation of *Armillaria* strain Arm37 from *G. elata*

Several fungi of the genus *Armillaria* have been previously isolated, identified, and even utilized in the cultivation of the fully mycoheterotrophic plant *G. elata* (Xu et al., 1989; Zhou, 1981). The *Armillaria* strain Arm37 (hereafter Arm37) was isolated from a juvenile tuber of *G. elata* (Fig. 1A) and identified as an *Armillaria* fungal species (Fig. S2) using a previously established method (Guo et al., 2016). In an axenic culture system, we confirmed that Arm37 can form symbiosis with *G. elata*, supporting *G. elata* growth (Fig. S3).

The growth of basidiomycetes is influenced by environmental factors, such as temperature (Negrao et al., 2021) and light (Yu and Fischer, 2019). We found that the growth of Arm37 was the best at 23 °C on mPDA in dark (Fig. 1B and Fig. S4). These growth conditions are consistent with the findings in other fungi of the genus *Armillaria* (Doty and Cheo, 1974; Rishbeth, 1978). Like some *Armillaria* species that are composed of uninucleate cells with diploid characteristics during their vegetative stage (Ota et al., 1998; Peabody et al., 2005), microscope imaging analysis indicated that Arm37 is unicellular (Fig. 1C and D), a property that is preferred in genetic transformation.

Arm37 is sensitive to hygromycin and 5-fluoroorotic acid

Several antifungal agents, such as hygromycin, geneticin, and phleomycin, are commonly used in the genetic transformation of fungi (Li et al., 2017). For example, hygromycin has been shown to inhibit *Armillaria mellea* fungal growth (Ota et al., 1998; Peabody et al., 2005). In the present study, Arm37 was cultivated on mPDA medium supplemented with various concentrations of hygromycin. It was found that the growth of Arm37 hypha was completely inhibited by the treatment of 20 mg/L hygromycin (Fig. 2A), a concentration that is lower than the concentration (30 mg/L) required for the inhibition of three fungal forms (spores, hyphae, and rhizomorphs) of *A. mellea* (Baumgartner et al., 2010; Ford et al., 2015, 2016). Therefore, the hygromycin resistance gene *hygromycin phosphotransferase* (*HPT*) was used as the selection marker for genetic transformation of Arm37.

When a synthetic compound 5-fluoroorotic acid (5-FOA) is supplied to fungi, the fungal *URA3* (orotidine-5'-monophosphate decarboxylase) converts 5-FOA to a toxic compound 5-fluorouracil, which inhibits the growth of fungi (Staab and Sundstrom, 2003). We found that supplementation of 5 mg/L 5-FOA to the medium completely inhibited the growth of Arm37 (Fig. 2B). Thus, Arm37 is very sensitive to 5-FOA, and 5-FOA can be used as a selection agent for generating *URA3*-knockdown transformants of Arm37.

Agrobacterium-mediated transformation of Arm37

In fungi, the *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*) and *translation elongation factor 1 α* protein (*TEF1*) genes are often highly expressed genes and their promoters have been selected for driving the expression of target genes (Liu et al., 2023; Schuster and Kahmann, 2019; Wang et al., 2017). We sequenced the genome of Arm37 (Beijing Institute of Genomics, accession number: PRJCA013068). In the Arm37 genome, 25,100 genes were identified. By homolog searching, the *GPD* (GenBank accession number: PP437272) and *TEF1* (GenBank accession number: PP437274) coding sequences were identified in the Arm37 genome, which showed respectively 96.76% and 99.13% identity to the *A. mellea* *GPD* and *TEF1* on the amino acid level and high similarities to other fungal *GPD* and *TEF1* proteins (Fig. S5A and S6A). Phylogeny analysis also confirmed that the orthology of Arm37 *GPD* (Fig. S5B and S6B). Thereafter, the promoter of Arm37 *GPD* gene was amplified and cloned into the binary vector pCAMBIA1301 to drive the expression of a codon-optimized *HPT* gene (hygromycin resistance gene), and terminator of *GPD* was also cloned into this vector as terminator of *HPT*. Next, the *TEF1* promoter-first intron of *GDP* gene (iGDP)-*TEF1* terminator was inserted to the vector, forming the empty vector pYT-EV. In this manner, pYT-EV contains two expression cassettes: 1) cassette of *HPT* selection marker; 2) target gene expression cassette, in which the *TEF1* promoter and terminator are used to drive and terminate the expression of gene-of-interest, after the iGDP is replaced by the gene-of-interest (Fig. 3A).

Agrobacterium-mediated and protoplast-mediated transformation are frequently employed for transformation of filamentous fungi (Li et al., 2017; Wang et al., 2017). Previously, a genetic transformation system mediated by Agrobacterium was established for the pathogenic fungus *A. mellea* using spores (Ford et al., 2015, 2016; Idnurm et al., 2017). Although protoplast transformation is preferred, due to the fact that the regenerated cells after transformation are from single cells and thus

have uniform genetic backgrounds, protoplast transformation is not feasible in the *G. elata*-interacting *Armillaria*, as it is very difficult to induce spore formation in *Armillaria*, especially under in vitro culture condition.

Therefore, we next sought to establish an Agrobacterium-mediated transformation (AMT) system in Arm37. Agrobacteria strain AGL-1 carrying the plasmid pYT-EV ($OD_{600\text{ nm}} = 0.3$) were treated with 200 μM AS for 3 h. Ten days old Arm37 rhizomorphs were cut into 5 mm long fragments, and about 50 fragments were then cocultured with the Agrobacteria for 24 h. Subsequently, the rhizomorph fragments were placed onto a mPDA medium supplemented with hygromycin (20 mg/L, for selection of hygromycin-resistant Arm37) and timentin (200 mg/L, for elimination of Agrobacteria). After ten days, a few fungal colonies were obtained on each Petri dish of screening medium, with white hyphae evenly expanding out from the rhizomorph fragments (Fig. 3B). Finally, among these 46 rhizomorph fragments transformed, six generated fresh hyphae under hygromycin selection (13%) (Fig. 3B). All these six vigorously grown colonies were individually subcultured and DNA samples were isolated. PCR amplification of the *HPT* gene from these DNA samples was performed to verify the integration of T-DNA into the fungal genome (Fig. S7), and additionally, to eliminate the possibility that the samples of fungal DNA contained Agrobacterium DNA contamination, PCR analysis was performed to detect a region of vector DNA outside of the T-DNA borders (Fig. S8). Among all 200 rhizomorph fragments transformed, 11 were found to be genuinely transformed. Thus, the transformation efficiency was 5.5% (Fig. 3C). In another two independent experiments, 6% (15/250) and 8% (14/175) of rhizomorph fragments were found to grow fresh hyphae after transformation and selection, and respectively, and ultimately, 4% and 5% of all samples were found to be vigorously grown and genuinely transformed (Fig. 3C). Thus, Arm37 can be transformed by Agrobacterium, and in this experimental trial of AMT, the efficiency was in average about 5% (Fig. 3C).

Optimization of transformation efficiency

Next, we further optimized the transformation of Arm37. The efficiency of AMT in filamentous fungi is often influenced by various factors, such as the concentration of AS (a phenolic compound that induces pathogenicity of Agrobacterium), the ratio of fungus to Agrobacterium, and the time of infection (Li et al., 2017). For Arm37, it was found that the transformation efficiency increased as the density of the Agrobacterium cells increased from 0.2 to 0.5 ($OD_{600\text{ nm}}$), but when the $OD_{600\text{ nm}}$ of Agrobacterium cell were greater than 0.5, the number of transformants decreased due to bacterial overgrowth, resulting in inhibited fungal growth (Fig. 4A). For transformation of *A. mellea*, hyphae was used and the optimal density of Agrobacteria was $OD_{600\text{ nm}} 0.6$ (Teng et al., 2022), while in another study the best density of Agrobacteria was found to be 0.28 (Baumgartner et al., 2010). With respect to Arm37, the transformation efficiency was less than 5% when AS was not used (Fig. 4B). When we increased the AS concentration, the transformation efficiency increased to 22% at 200 μM but decreased at 250 μM (Fig. 4B). These results are consistent with the previous studies on filamentous fungi: supplementation of AS to Agrobacteria greatly enhances transformation efficiency (Hooykaas et al., 2018; Li et al., 2017; Wang et al., 2017). Next, the time of coculture between rhizomorphs and Agrobacteria was also optimized. It was

found that the transformation efficiency gradually increased to the highest until 48 h of coculture but declined when the time was extended to 60 h (Fig. 4C).

In summary, the following conditions appeared to be the best for Arm37 transformation: *Agrobacteria* with a density of OD_{600 nm} 0.6 should be treated with 200 μ M AS before coculturing with Arm37 rhizomorph fragments, and the coculture should take 48 h. In this manner, the transformation efficiency of Arm37 reached 32% (Fig. 4).

Transformation of Arm37 for expression of eGFP and GUS gene

Next, we examined whether such an AMT can be used for expression of target genes/proteins in Arm37. To this end, we respectively cloned the *eGFP* and *GUS* gene from pEGFP and pCambia1301 vector, and these two fragments were further cloned into the pYT-EV, respectively. The capacity of introns to increase gene expression has been well documented in diverse eukaryotic organisms (Chorev and Carmel, 2012; Shaul, 2017). Thus, iGPD was also inserted into both *eGFP* and *GUS* to form the plasmids pYT-eGFP and pYT-GUS (Fig. 5A). All these constructs were used to transform Arm37. To identify genuine transformants, PCR analysis was first used to amplify *HPT* gene from hygromycin-resistant colonies, and from those positive ones, we further employed PCR to identify those harboring the reporter genes *eGFP* (Fig. S9A) or *GUS* (Fig. S9B).

Thereafter, for each construct, we selected six different strains of Arm37 transformed with pYT-eGFP for RNA extraction. The RNA samples were reverse transcribed and the cDNA samples were used for PCR amplification of a partial *eGFP* sequence (Fig. 5B): expression of *eGFP* was indeed detected in all these six strains, while it was not detected in the wild-type (WT) Arm37. To examine the activity of eGFP, transgenic Arm37 rhizomorphs carrying pYT-eGFP were first squashed to release the hyphae, and we observed the green fluorescence of these hyphae under a fluorescence microscope. Indeed, the fungal hyphae emitted green fluorescent light along the hyphal bodies (Fig. 5C). Furthermore, the transformants carrying the *GUS* gene were confirmed to have GUS activity, as the rhizomorphs and hyphae turned blue (Fig. 5D). In contrast, Arm37 transformed with pYT-EV did not show any fluorescent signals (Fig. 5C) or GUS activity (Fig. 5D). Thus, *Agrobacteria* carrying constructs derived from the pYT-EV vector, in which *TEF1* promoter and terminator are used to drive and terminate the expression of target genes, can be used for Arm37 transformation, and the target genes/proteins can be expressed in the transformants.

Establishment of RNA interference in Arm37

RNA interference (RNAi) is a conserved regulatory mechanism in eukaryotes, in which small noncoding RNAs mediate posttranscriptional and transcriptional gene silencing; this approach has also been commonly used to study gene functions in fungi (Chang et al., 2012). Although the RNA-silencing pathway is highly conserved throughout the fungal kingdom, some budding yeasts, such as *Saccharomyces cerevisiae*, lack homologs of *Argonaute* (*AGO*), *Dicer-like* (*DCL*), or *RNA-dependent RNA polymerase* (*RdRP*) (Chang et al., 2012). In Arm37, five *AGO* genes, whose encoding proteins contain an ArgoN domain at the N-terminus followed by MID, PAZ, and PIWI domains at the C-terminus (Fig. S10,

Table S1), were identified. Three *DCL* genes, which encode proteins possessing a DExD domain at the N-terminus, a helicase-C domain, a Dicer dimerization domain, and two ribonuclease III domains (Fig. S11, Table S1), were found in the Arm37 genome. Furthermore, twelve *RdRP* genes with the conserved RdRP domain were found (Fig. S12, Table S1). Thus, it is very likely that the RNAi system can be used in Arm37.

Fungal *URA3* can convert 5-FOA to 5-fluorouracil, resulting in toxicity to fungal cells (Staab and Sundstrom, 2003). Silencing or knocking out *URA3* enables fungi to tolerate 5-FOA in high concentrations, and thus *URA3* gene has been widely used as auxotrophic selection marker (Staab and Sundstrom, 2003). In Arm37, we first identified the *URA3* gene (Fig. S13, Table S1), and the RNAi plasmid pYT-irURA3 was constructed by inserting a partial *URA3* sequence (303 bp) in the sense and anti-sense orientation, which were separated by a 767-bp PDK intron, into pYT-EV (Fig. 6A). *Agrobacteria* carrying pYT-irURA3 was used to transform Arm37. Six randomly selected transformants with hygromycin resistance were transferred to a medium supplemented with 5 mg/L 5-FOA but without hygromycin. These transformants were found to be able to grow, while the control strains, which were transformed with the empty vector pYT-EV, were unable to grow on the 5-FOA-containing medium (Fig. 6B). These results suggest that the *URA3* was likely silenced in these RNAi transformants. Indeed, qRT-PCR results showed that all these six strains presented clear decreases of *URA3* expression levels, compared to those of the control strains transformed with empty vector (Fig. 6C), especially the ir5 strain (79% downregulated). Thus, we concluded that candidate genes in Arm37 can be stably silenced using the RNAi approach.

Discussion

Armillaria-G. elata symbiosis systems are probably thus far the best system for studying fungus-mycoheterotrophic plant interactions, as this system can be easily established in laboratories and even has been used for agricultural production of *G. elata* for medicinal purposes. Genetic manipulation is needed for understanding the unique physiology and interaction between the fungal host *Armillaria* and the mycoheterotrophic plant *G. elata*. In this study, we isolated a *Armillaria* strain Arm37, which can form symbiosis with *G. elata*, and an effective AMT platform was established for transformation of Arm37.

After optimization, 32% efficiency was achieved for AMT of Arm37 based on rhizomorphs, and this AMT system for Arm37 can be used to obtain independently transformed strains in one month. For basidiomycetes, including the genus *Armillaria*, the most effective method for constructing a genetic transformation system is through the use of spores and protoplasts (Li et al., 2017). Previously, for *A. mellea*, 20 ~ 70% efficiency of AMT was achieved using spores (Ford et al., 2016). However, obtaining spores from fruiting bodies can be challenging in most species of the genus *Armillaria*, including the Arm37 strain. Protoplast-based transformation systems are yet to be developed for Arm37.

Recent evidence has indicated that mRNAs, small RNAs, and proteins are transferred between host plants and parasitic plants, such as dodder (*Cuscuta*) (Shen et al., 2023). During the interactions between plants and pathogenic fungi, proteins and small RNAs are also delivered between plants and fungi (Vincent et al., 2019; Weiberg et al., 2015). For example, cross-kingdom transfer of small RNAs play important role in

the interactions between the pathogenic fungus *Botrytis cinerea* and plant *Arabidopsis thaliana* (Cai et al., 2018; Wang et al., 2016; Weiberg et al., 2013). Moreover, a small RNA from the ectomycorrhizal fungus *Pisolithus microcarpus* is transported to the host plant *Eucalyptus grandis* and is involved in the formation of stable symbiosis (Wong-Bajracharya et al., 2022). Taking advantage of the genomes of Arm37 and *G. elata* (Xu et al., 2021), it would be interesting to explore whether proteins and small RNAs are transported between Arm37 and *G. elata*, and using our AMT system, the target genes encoding important mobile proteins and small RNA biogenesis- or function-related proteins can be overexpressed or silenced to study the roles of these mobile molecules from Arm37 to *G. elata*. Candidate genes from Arm37, which are potentially involved in nutrient transfer are also worth to study using this AMT system for Arm37.

As a special orchid plant, transformation of *G. elata* is still not feasible. Host-induced gene silencing (HIGS) has been successfully used in studying plant interactions with pathogenic fungi, oomycetes, and insect pests: by expressing homologous double-stranded RNAs (dsRNAs) or artificial microRNAs (amiRNAs) in the host plants, the interacting organisms, such as pathogenic fungi, take up dsRNAs, small RNAs derived from dsRNAs, or amiRNAs, resulting in silencing of the target genes in the interacting organisms (Zand Karimi and Innes, 2022). Recently, HIGS has been successfully used to study the functions of genes in mycorrhizal fungi, including genes encoding the HOG1-MAPK protein (Wang et al., 2023), the secreted LysM protein RiSLM (Zeng et al., 2020), the high-affinity phosphate transporter GigmPT (Xie et al., 2016), the high-affinity monosaccharide transporter MST2 (Helber et al., 2011), and the strigolactones-induced putative secreted protein SIS1 (Tsuzuki et al., 2016). In the Arm37 fungus, we show that the RNAi system can effectively silence the transcription of target genes (Fig. 6), and this system may be used as a HIGS tool to target *G. elata* genes for silencing by expressing RNAi constructs targeting *G. elata* genes in Arm37.

Conclusions

In this study, we isolated a fungal strain Arm37 (*Armillaria* sp.), which can form symbiosis with *G. elata*, supporting the growth and development of such a special mycoheterotrophic orchid. A vector carrying hygromycin resistance gene *HPT*, which can be used for selection of transformed Arm37, was constructed. Based on this vector, we developed an AMT system, which achieved a relatively high transformation efficiency in Arm37 (32%). This AMT system was used to successfully express the reporter genes *eGFP* and *GUS*. Importantly, this AMT system allow us to silence target genes in Arm37 and possibly target genes in *G. elata* by HIGS.

Taken together, our established AMT system provides a new platform for genetic manipulation of *Armillaria* Arm37, which likely can be easily used for other closely related *Armillaria* species and strains. Importantly, this platform also allows future studies on the molecular mechanisms underlying the interplay between *Armillaria* and *G. elata*.

Declarations

Acknowledgments

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Author contributions

JW designed and supervised the entire project and revised the manuscript; YX, ZS, and CZ, and HL performed bioinformatics analysis, YL, ZS, HL, and CZ performed the experiments, analyzed data, and wrote the manuscript. All authors read, edited, and approved the manuscript.

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Data availability

All data used have been included in the manuscript.

Conflicts of interest

The authors declare there is not conflict of interest. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

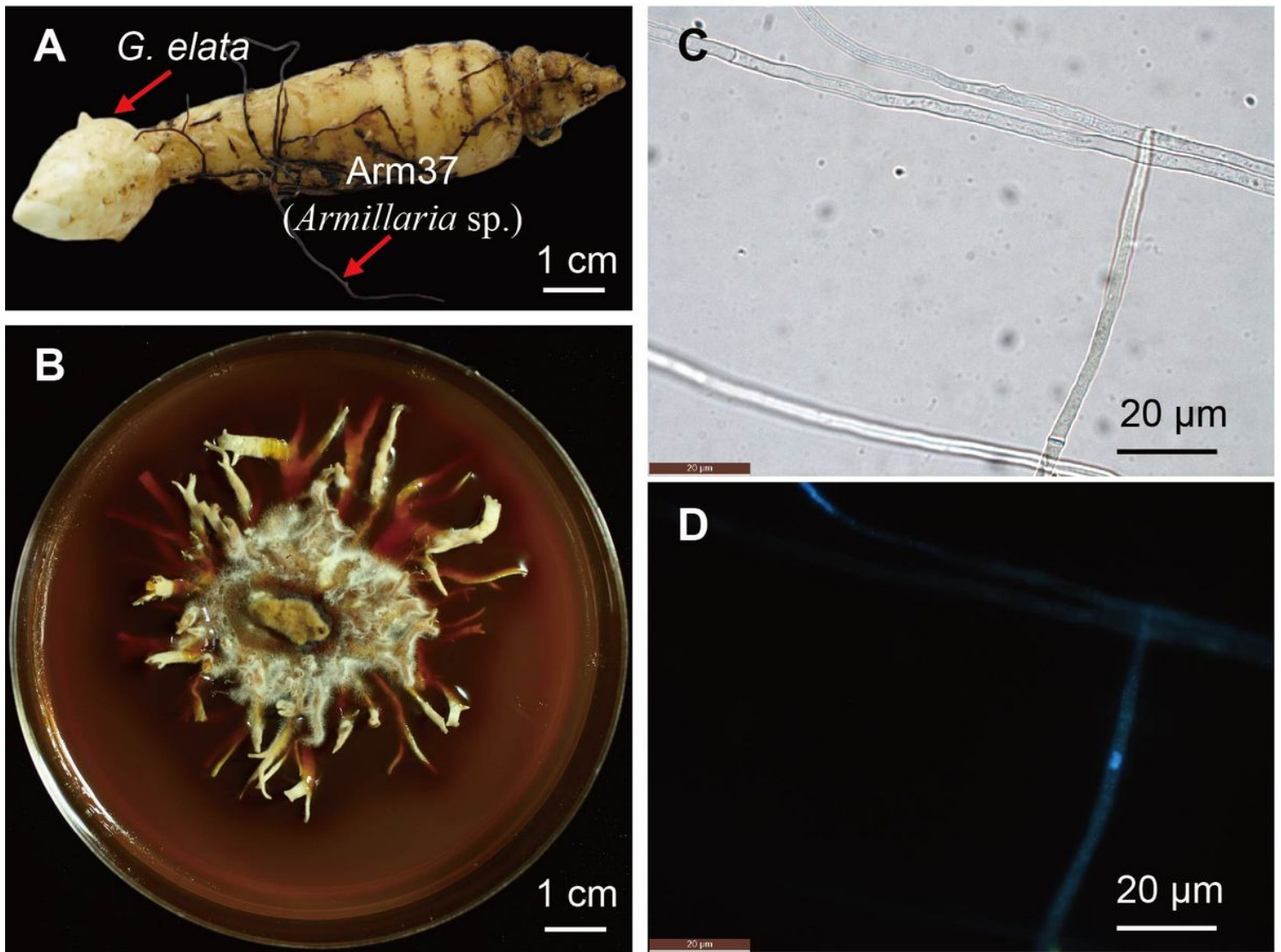


Figure 1

Morphological phenotypes of the *Armillaria* strain Arm37 isolated from *Gastrodia elata*.

(A) Photograph of the *G. elata* tuber covered with *Armillaria* rhizomorphs. The rhizomorphs were used to isolate the Arm37 strain. (B) Arm37 cultured and maintained on a medium of modified potato dextrose agar (mPDA) at 23 °C for ten days in dark. (C and D) Bright field (C) and fluorescence (D) microscopic images of Arm37 hyphae stained with DAPI (4',6-diamidino 2-phenylindole).

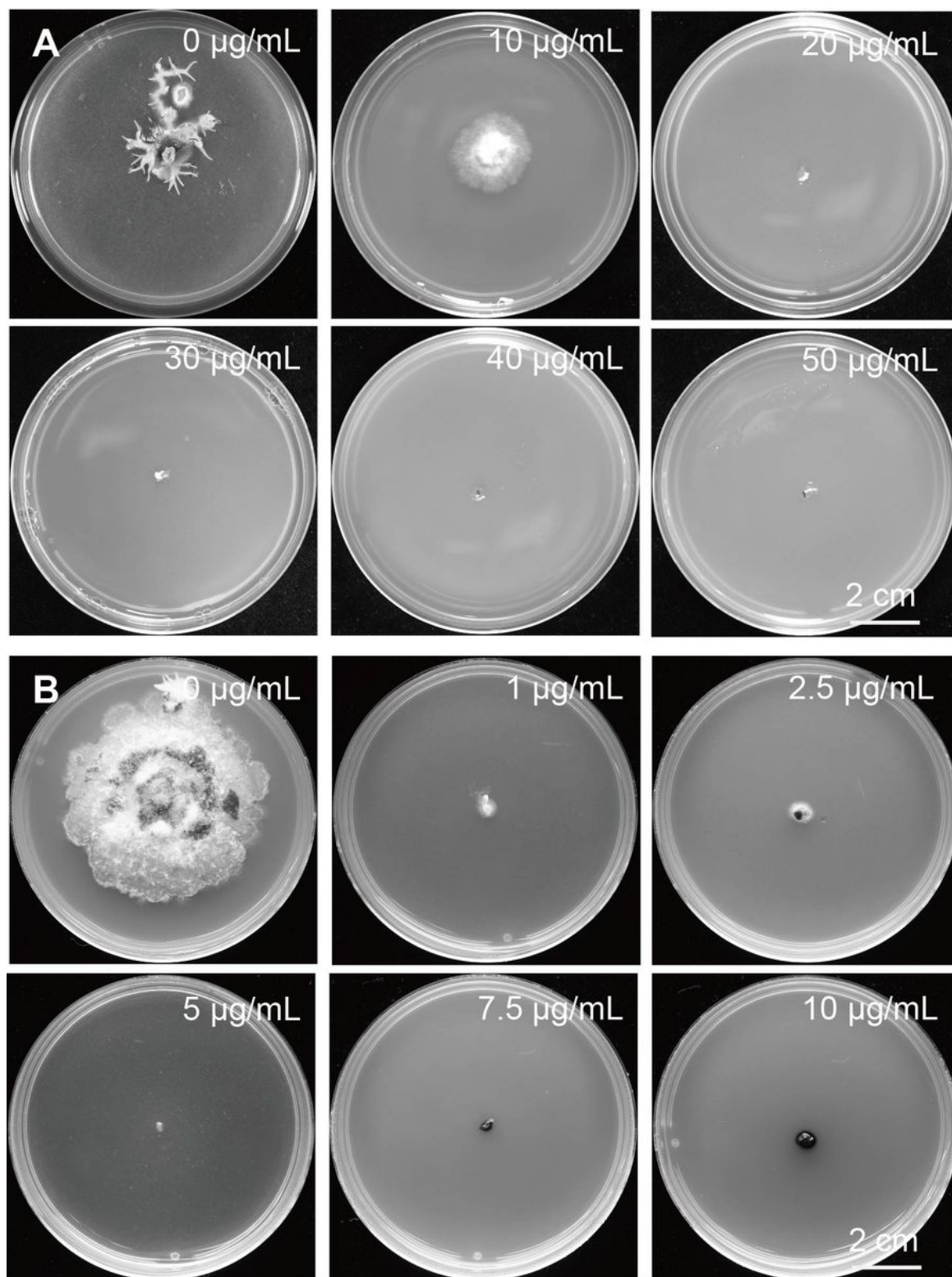


Figure 2

Growth of *Armillaria* strain Arm37 under the treatment of hygromycin B and 5-fluoroorotic acid (5-FOA).

The Arm37 strain was cultured on a mPDA medium containing different concentrations (0, 10, 20, 30, 40, 50 mg/L) of hygromycin B (A) or different concentrations (0, 1, 2.5, 5, 7.5, 10 mg/L) of 5-FOA (B). The plates were incubated at 23 °C for ten days (A) and two weeks (B), respectively.

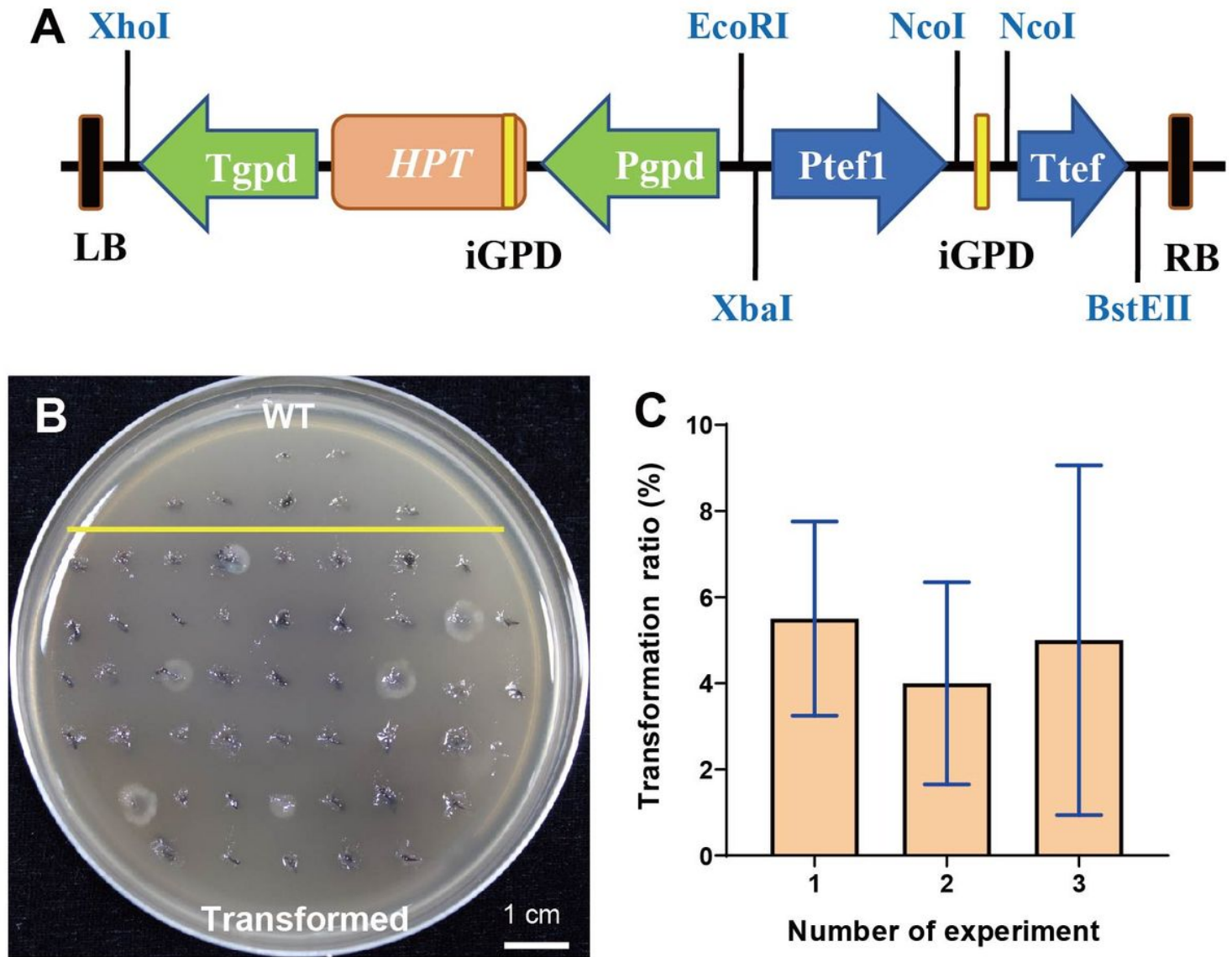


Figure 3

Agrobacterium-mediated transformation of the *Armillaria* strain Arm37.

(A) Schematic indicating the T-DNA region of the empty vector plasmid pYT-EV. The region contains the *hygromycin phosphotransferase* (*HPT*) gene with the intron (iGPD) from *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*), and *HPT* is driven by the *GPD* promoter (*Pgpd*) and terminator (*Tgpd*). iGPD is located between the *translation elongation factor 1 α* (*TEF1*) promoter (*Ptef1*) and terminator (*Ttef1*). (B) Photograph showing wild-type (WT) Arm37 and transformed Arm37, after the rhizomorph fragment grew for ten days on a mPDA medium supplemented with 20 mg/L hygromycin B. (C) Transformation efficiency of Arm37 from three independent experiments. In each experiment, rhizomorph fragments were plated on four Petri dishes and the vigorously grown colonizes were used for PCR confirmation of transformation. Data are means $\pm$ SD (n = four biological replicates).

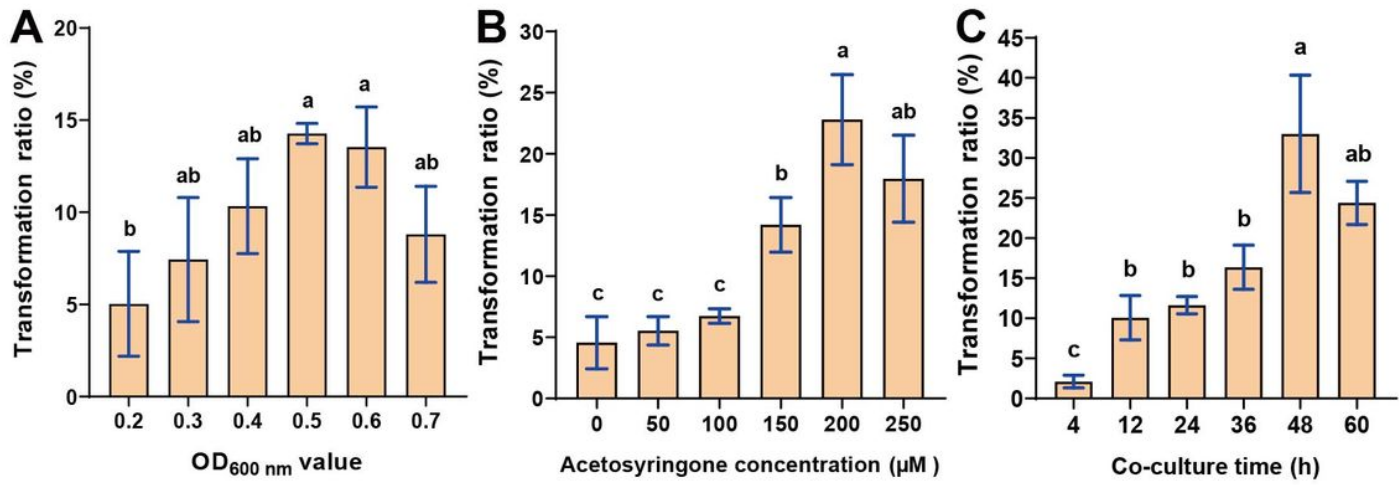


Figure 4

Optimization of Arm37 transformation.

Effect of the density of *Agrobacterium* cells (**A**), concentration of acetosyringone (**B**), and the co-culture time (**C**) on the transformation efficiency of Arm37. The data were from three independent experiments. Data are means ± SD. Statistic differences were analyzed by one-way ANOVA between different groups, and the different letters indicate significant differences ($p \leq 0.05$).

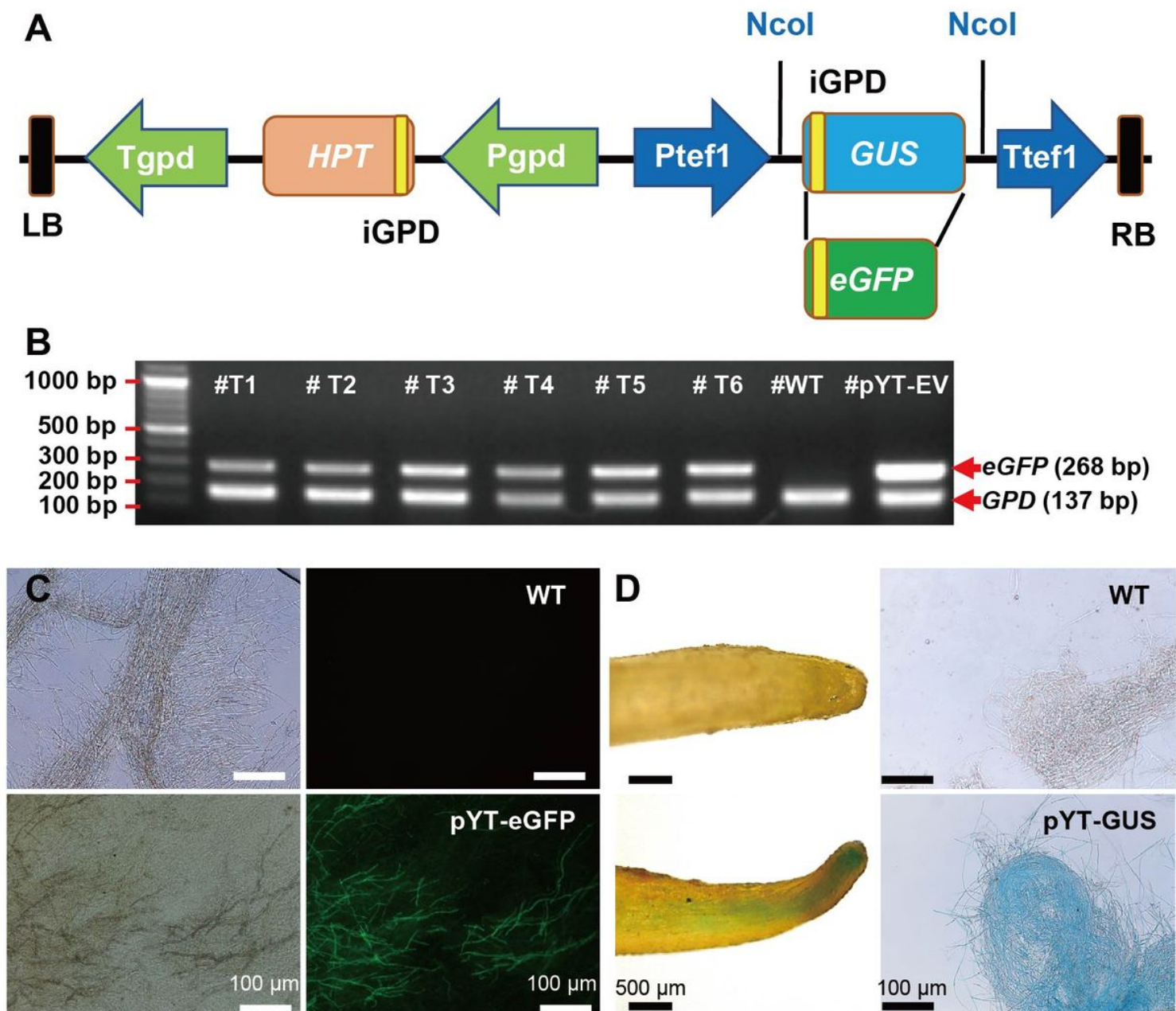


Figure 5

Transformation of *enhanced green fluorescent protein (eGFP)* and *β -glucuronidase (GUS)* into Arm37.

(A) Schematic indicating the T-DNA region of the constructed plasmids pYT-eGFP and pYT-GUS, including the *eGFP* and *GUS* coding region containing the intron iGPD. *TEF1* promoter (*Ptef1*) and terminator (*Ttef1*) were located up- and downstream of these two coding regions. (B) Semi-quantitative PCR analysis of the *eGFP* gene expression. Six randomly selected fungal transformants (T1-T6), the WT Arm37 (negative control), and the plasmid pYT-EV (positive control) were used for reverse-transcription PCR analysis. The house-keeping gene *GPD* was used as the internal reference. (C) Bright field (left) and fluorescence (right) microscopy images of pYT-EV mycelia and mycelia transformed with *eGFP*. (D) Microscopy image of the rhizomorph tip and the hyphae after GUS straining.

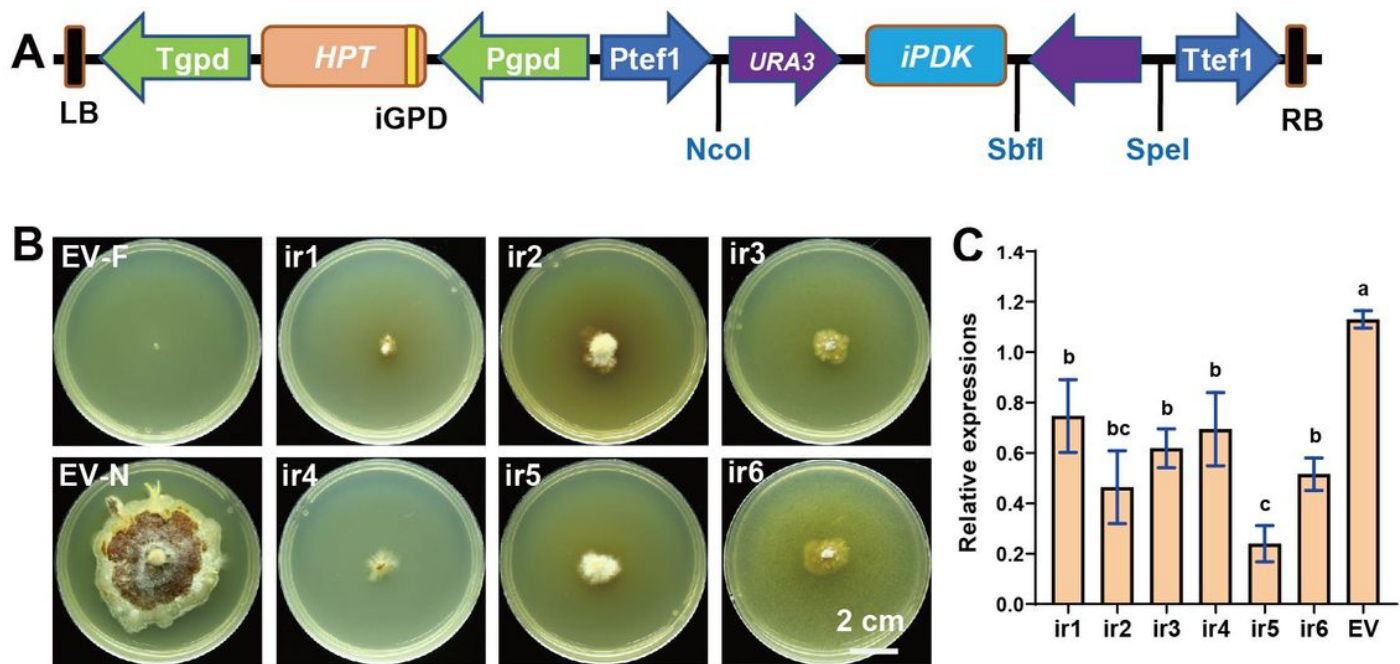


Figure 6

RNA interference (RNAi) of the endogenous gene *orotidine 5'-monophosphate decarboxylase* (*URA3*) in Arm37.

(A) Schematic indicating the T-DNA region of the constructed plasmid pYT-irURA3. The RNAi cassette contains an inverted repeat of partial *URA3* sequence flanking the intron *iPDK*. (B) Photograph indicating the growth of Arm37 transformed with pYT-EV or pYT-irURA3. ir1-ir6: Six randomly selected transformants transformed with pYT-irURA3. EV: a transformant transformed with pYT-EV (negative control). All were cultured on mPDA medium supplemented with 5 mg/L 5-FOA, except that EV-N (a transformant transformed with pYT-EV on mPDA without 5-FOA). (C) Relative transcript levels of the gene *URA3* in six randomly selected transformants (ir1-ir6) and EV strain. EV and ir1-ir6 were subcultured in triplicates. RNA was extracted from these samples and used for reverse transcription and PCR analysis. Data are means $\pm$ SD (n = three biological replicates). Statistical differences were analyzed by one-way ANOVA between different groups, and the different letters indicate significant differences ($p \leq 0.05$).

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