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Short Communication

Highly efficient *Agrobacterium*-mediated transformation of *Volvariella volvacea*

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ABSTRACT

Agrobacterium tumefaciens-mediated transformation (ATMT) was successfully applied to the edible straw mushroom, *Volvariella volvacea*. Mycelium pellets were transformed to cold stress resistance using the *afp* gene as both a selective marker and a reporter gene, under the control of a heterologous *Lentinula edodes gpd* promoter. The efficiency of transformation is over 100 times higher than that previously reported in *V. volvacea*. Stable integration of the *afp* gene with 1–4 copy numbers was confirmed in all 10 randomly selected transgenic events by Southern blot analysis. The mitotic stability of the transformants was demonstrated after five successive transfers on PDA medium without selection pressure and the PCR analysis of basidiospores harvested from transformants.

1. Introduction

The paddy straw mushroom, *Volvariella volvacea*, is the fifth most important edible mushroom in the world according to yield. Due to its cellulolytic characteristics, *V. volvacea* has been extensively cultivated on an array of agro-industrial residues under artificial conditions (Chang, 1993). However, *V. volvacea* has a number of deficiencies with respect to cultivation, particularly low biological efficiency, which limited production of *V. volvacea* and bioconversion of agricultural lignocellulosic wastes (Ding et al., 2006). Traditional breeding of *V. volvacea* has been hindered because of a lack of basic genetic information. Thus, genetic transformation could be a powerful means for introducing agriculturally valuable traits such as high efficiency of cellulose and lignin utilisation.

To date, only two transformation methods have been reported for *V. volvacea*. Jia et al. (1998) described a conventional polyethylene glycol (PEG)-mediated transformation using the *trp3*^{iar} gene as the selectable marker. In another report, particle bombardment transformation was applied in *V. volvacea* and the *hph* gene was used as the selective trait (Guo et al., 2005). According to these studies, low transformation frequency was a major drawback for both procedures employed. Recently, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been receiving great attention as a new technique in fungal transformation. Compared with other transformation technique, the ATMT method has been proved to increase transformation rate and show a greater degree of stability for the transgene (de Groot et al., 1998; Covert et al., 2001; Maria et al., 2004). But so far no transformation of *V. volvacea* has been reported using this method.

In this study, we report for the first time, establishment of a high efficient system for the genetic transformation of *V. volvacea* by applying the ATMT method. Antifreeze protein (*afp*) gene from budworm was chosen as a dominant selectable marker as well as a reporter gene by using the deficiency to cold stress of *V. volvacea* and the protection of *afp* for organism from cold (Chang, 1993; Huang et al., 2002). In addition, mitotic stability of transformants was explored.

2. Methods

2.1. Strains and plasmids

Volvariella volvacea strain V23 was purchased from the Institute of Edible Fungus (Shanghai, China). Strains EHA105 and LBA4404 of *A. tumefaciens* was used to transform *V. volvacea*. Plasmid pBlugfp, containing the *gfp* gene with the *gpd* promoter from *Agaricus bisporus* (*abgpd*), was kindly provided by Professor Lorna A. Casselton at the University of Oxford and Dr. Michael P. Challen at the Warwick HRI. The binary vector pLin235 with the *bar* gene expression cassette and *hph* gene expression cassette is conserved in our laboratory. Plasmid pTHP$_4$ containing *afp* gene and pTLgpd with *gpd* promoter gene cloned from *Lentinula edodes* (*legpd*) were constructed by us.

2.2. Construction of expression plasmid

A binary plasmid pLg-afp235 containing the *afp* gene driven by the *legpd* promoter was constructed. Intermediate plasmid pAgafp

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was generated by excising the *gfp* gene from pBlu-gfp with NcoI and BsrGI and inserting the *afp* gene sequence containing NcoI and SalI sites obtained from $pTHP_4$ by adding a adapter with SalI and BsrGI. A SacII/NcoI fragment of pTLgpd, containing the *legpd* gene, was isolated and cloned between the SacII and NcoI sites of pAgafp to produce pLgafp. The SacII/KpnI fragment from pLgafp containing the *afp* gene with the *legpd* promoter and the *trpC* terminator was inserted into the HindIII/KpnI sites of the binary vector pLin235 by adding an adapter with HindIII and SacII restriction sites, resulting in expression plasmid pLg-afp235 (Fig. 1).

2.3. Preparation of explant for transformation

Spores, fruiting body gill tissue pieces, mycelium pellets, blended mycelium from V23 were chosen as explants for transformation by assessing the regeneration frequency of these explants. Germinated spores were collected after inoculation in Potato Dextrose Broth (PDB) medium (1% maltose, 0.4% glucose, 0.4% yeast extract) for 2 days at 35 °C. Fruiting body gill tissue pieces were harvested and sterilized. Mycelium pellets were cultured in shaken flasks at 34 °C for 3 days. Blended mycelium was prepared by blending fresh mycelium in PDB for 30 s. All of the explants were inoculated with bacteria for 30 min and then transferred onto co-cultivation medium to test the optimal regeneration frequency.

2.4. A. tumefaciens-mediated transformation

A. tumefaciens strains EHA105 and LBA4404 carrying the binary vector pLg-afp235 were cultivated at 28 °C for 2 days in 5 ml liquid YEB (Yeast extract 10 g L^{-1}, Tryptone 10 g L^{-1}, NaCl 5 g L^{-1}) medium with spectinomycin (80 mg L^{-1}) and streptomycin (150 mg L^{-1}). One milliliter fresh culture was transferred to 100 ml of YEB medium containing spectinomycin and streptomycin and grown overnight at 28 °C to an optical density at 600 nm of 0.3–0.4. Bacteria were collected by centrifugation and resuspended in YEB medium containing 200 mM acetosyringone (AS) to an optical density at 600 nm of 0.2. Mycelium pellets obtained as described above were immersed in the suspension of induced bacteria for 20–30 min with gentle shaking at 80 rpm. Inoculated mycelium pellets were transferred to sterile filter papers overlaid on co-cultivation medium with 200 mM AS. Following co-cultivation for 3 days at 28 °C, mycelium pellets were covered with 0.8% PDA containing 200 mg L^{-1} cefotaxime. Each experiment included a non-transformed control and was repeated thrice.

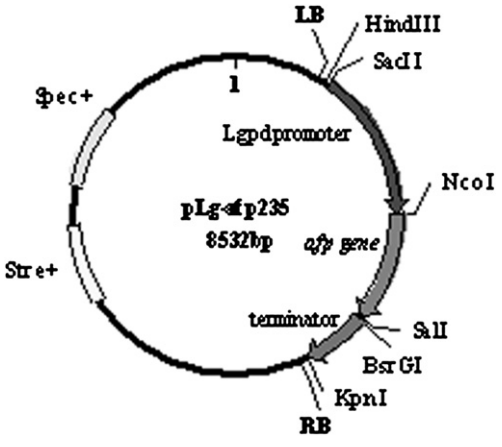

Fig. 1. Construction of binary vector pLg-afp235. pLg-afp235 is 8.532 kb in size and consists of a pLin235 backbone containing the spectinomycin and streptomycin resistance (R) genes and the right border (RB) and left border (LB) sequences of *Agrobacterium* T-DNA. The *afp* gene are located between the border sequences, and each is joined to the *L. edodes* glyceraldehyde-3-phosphate dehydrogenase promoter (*legpd*) and the *trpC* terminator. Shown are unique restriction sites.

2.5. Selection of cold-resistant transformants

An novel method for selection of transformants was established in this study. After regeneration from layer medium, mycelium, together with medium, was sectioned into 7–8 mm^2 of pieces, then transferred to PDA plates. PDA plates with mycelium pieces were exposed to 0 °C for 30 h (The lethal time of non-transformed mycelium was 10 h under 0 °C), then cultured at 34 °C. Mycelia which can re-grow at 34 °C was considered as putative transformants.

2.6. PCR analysis

Hypha from the margin of the cold-resistant colonies were cultivated in PDB. Genomic DNA was extracted from the mycelium. PCR analysis for detection of the *afp* gene in putative transformants was performed using the primer pair *afpF* (5′-TATACCATGGACTGCAGGAATCGGCACGA-3′) and *afpR* (5′-CGAGGTCGACATGGCTTAATTAGCACTG-3′). The PCR amplification protocol consisted of an initial denaturing cycle of 5 min at 95 °C, followed by 35 cycles with 30 s denaturation (94 °C), 30 s annealing (56 °C) and 1.5 min elongation (72 °C).

2.7. Southern blot analysis

Ten microgram of DNA samples from transformed and non-transformed (negative control) strains were digested with HindIII and separated on 0.8% agarose gel. The 442 bp fragment containing the *afp* gene amplified from pLg-afp235 was used as the probe. DNA probe labelling and hybridisation were performed under conditions recommended for the digoxigenin (DIG) hybridisation system by Roche (Mannheim, Germany).

2.8. Examination for mitotic stability of transformants

Following 10 rounds of culture on PDA medium without selection pressure, mycelium of the transformants were exposed to 0 °C for different time (20, 30, 40, 50, 60, 70, 80, 90, and 100 h), and then cultured at 34 °C for days. Cold resistant abilities of the transformants were observed. Basidiospores were harvested from fruiting bodies of the transformants and incubated at 36 °C on agar plates. Genomic DNA was extracted from the mycelium germinated from the single spore. The mitotic stability of transformants randomly selected was detected by PCR.

3. Results

3.1. Explant preparation

To select the most appropriate explants, four explants of *V. volvacea* were chosen to test their hypha regeneration frequency after co-cultivation with bacteria. The results showed blended mycelium could barely regenerate on co-cultivation medium. Spores and fruiting body gill tissue pieces displayed low regeneration frequency. While mycelium pellets exhibited a highest regeneration rate of 100%. Thus, mycelium pellets was considered to be an appropriate explant for this transformation.

3.2. Transformation

In an attempt to develop a highly efficient transformation system for *V. volvacea*, we tested in this study the applicability of the ATMT method. Two strains EHA105 and LBA4404 harboring