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Overexpression and repression of the tyrosinase gene in *Lentinula edodes* using the pChG vector

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Tyrosinase is an industrially useful enzyme, however, it causes gill browning of *Lentinula edodes* fruiting bodies during preservation. In this study, we constructed two vectors, pChG-gTs and pChG-gTa, expressing sense and antisense tyrosinase gene of *L. edodes*, respectively, using promoters derived from the glyceraldehyde-3-phosphate dehydrogenase gene. The host strain SR-1 of *L. edodes* was selected because of its fast growth, high protoplast yield, and high regeneration rate. Upon transformation of the host strain SR-1 with the pChG-gTs vector, a clone with 3.6-fold and 14.5-fold higher tyrosinase activity in vegetative mycelia and in fresh gills, respectively, than that of the host strain was obtained from nine transformants. Similarly, two clones containing the pChG-gTa vector with effectively repressed tyrosinase gene expression in vegetative mycelia and gills during the late stage of post-harvest preservation of fruiting bodies were obtained from 10 transformants. However, it remained unclear whether repression of the tyrosinase gene prevented gill browning, as the host strain also showed less browning than a commercial strain. Thus, this study highlights the usefulness of the pChG vector in expressing homologous enzyme coding genes in the vegetative mycelia and fruiting bodies of *L. edodes*.

[**Key words:** *Lentinula edodes*; Tyrosinase; pChG vector; Gene expression; Fruiting body; Gill browning]

Lentinula edodes (shiitake mushroom) is one of the most important edible mushrooms in Japan, and its fruiting body is used as a food as well as in medicine (1). Gill browning of the *L. edodes* fruiting body during post-harvest preservation results in poor quality and consequent loss of value. Kanda et al. (2,3) reported that the gill browning and tyrosinase activity of the *L. edodes* fruiting body during post-harvest preservation are closely correlated. Subsequently, tyrosinase was purified and characterized from fully browned *L. edodes* gills. Cloning and analysis of the *L. edodes* tyrosinase-encoding gene *Letyr* revealed that *Letyr* mRNA and its encoding protein are highly expressed in browned gills and only one copy of *Letyr* is present in the genome of *L. edodes*, suggesting that tyrosinase gene expression contributes to gill browning (4). To confirm the relationship between gill browning and tyrosinase expression, an efficient and reliable transformation system and heterologous expression vectors are required; additionally, analysis of *Letyr* gene-silencing is indispensable.

Sato et al. (5) developed an effective genetic transformation system for *L. edodes* by restriction enzyme-mediated integration (REMI) and constructed the vectors pLG-hph (6), pL-Cbx (7), and pChG-bar (8) for *L. edodes* transformation. The vector pChG consists of a selectable marker cassette that contains the hygromycin B phosphotransferase gene (*hph*) (9) driven by the chitin synthase (*chs*) gene promoter (10) and an exogenous gene expression cassette containing the *L. edodes* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene promoter (11) to express a heterologous gene. This vector has been used to generate double antibiotic-resistant *L. edodes* transformants, indicating its feasibility for exogenous gene expression in *L. edodes* (8).

Exploitation of fungal tyrosinase for food, pharmaceutical, and environmental applications, such as production of antioxidants and β-(3,4-dihydroxyphenyl) alanine (L-DOPA), polymerization of proteins, and bioremediation, has been reported by Halaouli et al. (12). This enzyme is highly attractive for industrial applications. Therefore, establishing an overexpression system for *Letyr* in *L. edodes* would be valuable. In this study, we constructed vectors to express the sense and antisense sequences of *Letyr* genomic DNA using the pChG vector and transformed *L. edodes*. The transformants were characterized to confirm expression of *Letyr* in *L. edodes*, and assess gill browning associated with *Letyr* repression during post-harvest preservation of fruiting bodies.

MATERIALS AND METHODS

Chemicals Unless otherwise stated, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) and were certified as reagent-grade.

Organisms and culture conditions A wild-type dikaryotic strain of *L. edodes*, strain SR-1 (13) and a commercial strain Hokken No. 600 (H600) (Hokken, Tochigi, Japan), were used in this study. Strain SR-1 was registered in the National Center

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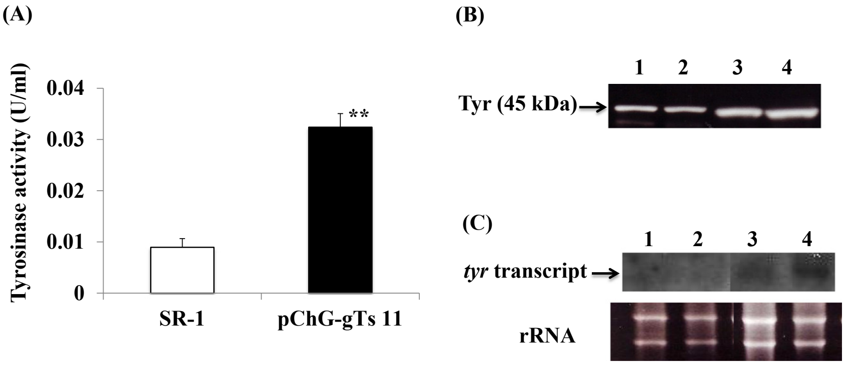

FIG. 1. Tyrosinase gene expression in mycelia cultured in MYPG liquid media. (A) Tyrosinase activity in the mycelia of wild-type host strain SR-1 and pChG-gTs transformant. Values are the mean ± SE of three samples. Asterisk (**) indicates the *p* value determined by Student's *t*-test as significantly different from that of the host strain ($p < 0.01$). (B) Western blot analysis. Lanes 1 and 2, wild-type strain SR-1; lanes 3 and 4, pChG-gTs transformant. (C) Northern blot analysis. Numbers represent the same as in panel B. rRNA represents ribosomal RNA stained with ethidium bromide after electrophoresis.

for Seeds and Seedlings (Japan) with registration number 18247. Mycelia were maintained on 2.0% agar plates (90 mm diameter) with 0.25 × MYPG medium (5). For tyrosinase expression, five mycelial blocks (7 mm diameter) were cut from a 0.25 × MYPG agar culture, inoculated into a 200 mL Erlenmeyer flask containing 100 mL of 0.25 × MYPG liquid medium, incubated at 25°C with shaking (7 cm stroke, 75 rpm) for 4 weeks in order to obtain mycelia necessary for analysis, and mycelia were cryopreserved at −80°C until analysis. To produce fruiting bodies, mycelia were cultivated on 200 g of sawdust medium for 3 months as described by Matsumoto (14) in a P2 culture chamber LPH-0.3BH-NC (Nippon Medical & Chemical Instruments Co., Ltd, Osaka, Japan). Fruiting bodies were harvested immediately after breakage of the veil and preserved in a desiccator at 20°C with 80% humidity. After preservation, the pileus, gill, and stipe were separated from the fruiting bodies and cryopreserved at −80°C until analysis (4).

Plasmid construction The vector pChG (8) was digested with *Mlu*I and dephosphorylated. The *Letyr* genomic DNA was amplified by PCR using the primers gT2605U (5′-TCTAAATCTTCTAACCCCACACA-3′) and gT5001L (5′-GACATTATTTATTTATCGCGATAGT-3′) and previously cloned the *Letyr* (DDBJ accession number AB033993) (4) as a template. The amplified PCR fragments were converted to blunt-ended forms using a Perfectly Blunt Cloning Kit (Merck Millipore, Darmstadt, Germany). Next, pChG-gTs (sense tyrosinase gene expression vector) and pChG-gTa (antisense tyrosinase gene expression vector) were constructed by ligation of the blunted pChG vector and the *Letyr* genomic DNA (Fig. S1). The direction of the gene inserted into the vector was confirmed by PCR.

Transformation procedure Protoplasts of *L. edodes* were prepared as described by Sato et al. (5). Transformation of *L. edodes* was carried out according to the REMI method with 2.5 U of *Sph*I and 2.5 μg of vector DNA and transformants were selected on 0.25 × MYPG plates containing 20 μg/mL hygromycin B as described by Sato et al. (5).

Preparation of crude extracts Crude extracts were prepared from mycelia using a FastProtein Kit (MP Biomedicals, Santa Ana, CA, USA) and FastPrep Instrument as described by Sato et al. (4), except that the samples were homogenized three times for 20 s at a speed setting of 6 on the instrument. The homogenate was centrifuged at 12,000 × *g* for 30 min. Thereafter, the supernatant was removed, centrifuged at 12,000 × *g* for 10 min, and collected for use as crude extract in enzymatic activity assays.

Fruiting bodies were harvested immediately after breakage of the veil and preserved in a desiccator at 20°C with 80% humidity. Fruiting bodies were sampled on day 0 (fresh stage), days 4–7 (middle stage), and days 7–11 (late stage). Following preservation, all samples were separated into the pileus, gill, and stipe and frozen immediately in liquid nitrogen. Crude enzyme from the frozen tissues was extracted by the same procedure as described above for mycelia.

Enzyme and protein assays Tyrosinase activity was determined according to the method described by Rodriguez et al. (15), using 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Sigma–Aldrich, St. Louis, MO, USA) as a trap for *O*-quinone generated by oxidation of the substrate L-DOPA by the enzyme. The reaction mixture for the standard assay contained 2 mM L-DOPA, 2 mM MBTH in McIlvaine buffer (pH 6.0), in a total volume of 100 μL. After incubation at 30°C for 10 min, the reaction was terminated by adding 100 μL 5% trichloroacetic acid. One unit of tyrosinase activity was defined as the amount of enzyme that produced the reaction product of 1 μmol of L-DOPAquinone and MBTH per minute.

Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

Western blot analysis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (16), using an NPU-10L PAGEL 10% polyacrylamide gel (ATTO Corporation, Tokyo, Japan). Samples were boiled in a buffer (Daiichi Pure Chemicals, Tokyo, Japan) for 3 min and loaded in the gel. Western blot analysis of proteins from mycelia or fruiting bodies was carried out as described by Sato et al. (4), using anti-tyrosinase polyclonal antibody (α-Tyr) as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Little Chalfont, UK) as the secondary antibody. Immunoreactive proteins were visualized using an ECL Detection Kit (GE Healthcare).

Northern blot analysis Total RNA was isolated using a FastRNA Pro Red Kit (MP Biomedicals) according to the manufacturer's protocol. The RNA concentration was measured using RiboGreen (Life Technologies, Carlsbad, CA, USA), and 10 μg of each RNA sample was used for northern blot analysis. Electrophoresis and blotting were performed as described by Hirano et al. (11). The probe used was an approximately 1.9 kb cDNA fragment of the tyrosinase gene amplified by PCR with primers 96U 5′-ATGTCTCATTATCTTGTCACTGGCG-3′) and 1985L (5′-CAACGAGGACATTATTTATTTATTATCGC-3′), as described by Sato et al. (4). Labeling of the DNA probes, hybridization, and signal detection were performed using an Alkphos Direct Labeling and Detection System (GE Healthcare) following the manufacturer's instructions. Ribosomal RNA (rRNA) as internal control was detected by ethidium bromide staining after electrophoresis.

Southern blot analysis For Southern blot analysis, genomic DNA was isolated from mycelia using Isoplant (Nippon Gene Co., Ltd., Tokyo, Japan), as described previously (5). Genomic DNA (10 μg) was digested with *Sph*I, separated by 0.7% agarose gel electrophoresis, and blotted on a Hybond N+ nylon membrane (GE Healthcare), following the method reported by Sato et al. (5). The probe, a 1.1-kb *hph* fragment, was prepared and used as described by Sato et al (5). Labeling of the probe, hybridization, and signal detection were performed using the ECL Direct Labeling and Detection System (GE Healthcare) according to the manufacturer's protocol.

Statistical analysis Data presented are average of three or four samples and standard errors. Statistical analysis of the 2 groups or more than 3 groups was carried out using student's *t*-test or by Dunnet's test, respectively.

RESULTS

Transformation of *L. edodes* by pChG-gTs and pChG-gTa vectors The vectors pChG-gTs and pChG-gTa, expressing the sense and antisense sequences, respectively, of the *L. edodes* tyrosinase gene, were constructed using the *L. edodes gpd* promoter and *hph* (9) expression cassette as a selectable marker, as described in Materials and methods (Fig. S1). These plasmids contained only one *Sph*I site that could be used for transformation by the REMI method. Integration of the vectors into the *L. edodes* genome was confirmed by PCR (data not shown), and the transformation efficiencies per microgram of pChG-gTs DNA and pChG-gTa DNA were calculated 2.12 and 2.20, respectively. The total number of transformants obtained was 21 for pChG-gTs and 22 for pChG-gTa.

Analyses of transformants with integrated sense vector pChG-gTs To compare the tyrosinase activity of nine randomly selected pChG-gTs-integrated transformants, crude extracts were

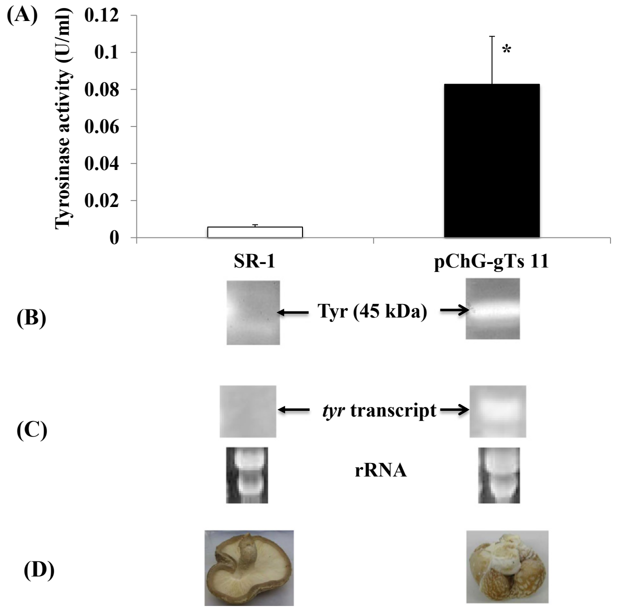

FIG. 2. Tyrosinase gene expression in gills during preservation. (A) Tyrosinase activity in the fresh gills of the wild-type host strain SR-1 and pChG-gTs transformant. Values are the mean ± SE of four samples. Asterisk indicates the *p* value determined by Student's *t*-test as significantly different from that of the host strain SR-1 F (*$p < 0.05$). (B) Western blot analysis. (C) Northern blot analysis. rRNA represents ribosomal RNA stained with ethidium bromide after electrophoresis. (D) Photographs of fruiting bodies.

prepared. The strains were cultivated for 4 weeks and the tyrosinase activity of the transformants was measured in the crude extracts of the cultured mycelia. Among nine pChG-gTs-integrated transformants (Fig. S2A), clone 11 showed approximately 3.6-fold higher (0.0325 U/mL) tyrosinase activity than that of host strain SR-1 (0.00895 U/mL, Fig. 1A). The other transformants demonstrated approximately same tyrosinase activity as that of SR-1 (Fig. S2A).

To confirm tyrosinase expression in the mycelia of clone 11, we performed western blotting using a rabbit polyclonal peptide antibody directed against Tyr (α-Tyr) (4). A single major immunoreactive signal of the active form tyrosinase with a molecular mass of 45 kDa, processed from the approximately 70 kDa of protyrosinase, reported by Sato et al. (4), was detected in the host strain SR-1. In clone 11, the same immunoreactive signal was detected at higher intensity than that of SR-1 (Fig. 1B). We also investigated *tyr* transcription in the mycelia by northern blot analysis. Although rRNA was clearly detected in all samples (Fig. 1C, rRNA), *tyr* transcripts were not detected in the SR-1 strain (Fig. 1C), and were observed in small amounts in clone 11 (Fig. 1C), correlating with tyrosinase activity.

Next, we measured tyrosinase gene expression in the fresh fruiting bodies of transformed clone 11. In the gills of SR-1 fresh fruiting bodies, low tyrosinase activity was detected (0.00573 U/mL, Fig. 2A, SR-1). In contrast, significant tyrosinase activity was observed in the gills of the fresh fruiting body in clone 11 (0.0828 U/mL, Fig. 2A, pChG-gTs 11). Tyrosinase activity in the fresh gills of clone 11 was approximately 14.5-fold higher than that in SR-1 (Fig. 2A, SR-1). We investigated tyrosinase activity in other parts of the fresh fruiting body, and found that activities in the fresh pileus and stipe of the transformant were approximately 1.6- and 8-fold higher, respectively, than that of SR-1 (data not shown). In western blot analysis, immunoreactive signal of the 45-kDa tyrosinase active form was detected in the gill of fresh fruiting body of the transformant, correlating with their respective tyrosinase activities (Fig. 2B, pChG-gTs 11), whereas the same immunoreactive signals were not detected in the gills of the fresh fruiting body of the host strain SR-1. The signal detected in Fig. 2B (and also in Fig. 4B) with slightly lesser molecular weight than the tyrosinase in both lanes was a non-specific one. We also investigated *tyr* transcription in the fresh gills of mature fruiting bodies. Although *tyr* expression was not observed in fresh fruiting bodies of SR-1 (Fig. 2C, SR-1), in clone 11, it was clearly detected in the gill of fresh fruiting bodies (Fig. 2C, pChG-gTs 11). These data indicate that tyrosinase activity in the whole fruiting body of clone 11 transformed with pChG-gTs, was higher than that in the host strain SR-1 fruiting bodies. On the other hand, the fruiting bodies in transformants showed abnormal morphology with unopened gills (Fig. 2D).

Analyses of transformants with integrated antisense vector pChG-gTa We also analyzed the transformants with integrated antisense vector pChG-gTa. Among 10 randomly selected transformants, 3 strains exhibited very low tyrosinase activity (Fig. S2B). In particular, tyrosinase activity in clone 2 (0.00133 U/mL) was less than one-sixth of that of host strain SR-1 (0.00896 U/mL), and tyrosinase activity was not detected in clone 3 (Fig. 3A). The activities of the other strains were similar or less than that of host strain SR-1 (Fig. S2B).

We also performed western blot analysis on crude extracts from vegetative mycelia of the transformants. The immunoreaction of tyrosinase with α-Tyr was barely detected in clones 2 and 3 (Fig. 3B, lanes 3–6). Among all lots of clones 2 and 3, only one lot of clone 2 showed a weak signal (Fig. 3B, lane 3), with tyrosinase activity of 0.004 U/mL, whereas the others showed no signals (Fig. 3B, lanes 4–6). Furthermore, northern blot analysis of *tyr* transcripts indicated that *tyr* expression was undetectable in clones 2 and 3 (Fig. 3C, lanes 3–6) same as in the host strain (Fig. 3C, lanes 1 and 2).

Next, we evaluated the expression of tyrosinase in the fruiting bodies of clones 2 and 3. We also examined the changes in

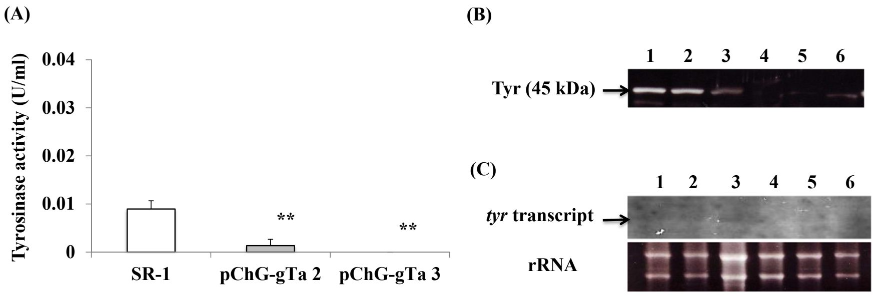

FIG. 3. Tyrosinase gene expression in mycelia cultured in MYPG liquid media. (A) Tyrosinase activity in the mycelia of the wild-type host strain SR-1 and pChG-gTa transformants. Values are the means ±SE of three samples. Asterisks (**) indicate the *p* values determined by Dunnett's test as significantly different from that of the host strain ($p < 0.01$). (B) Western blot analysis. Lanes 1 and 2, wild-type strain SR-1; lanes 3 and 4, pChG-gTa 2; and lanes 5 and 6, pChG-gTa 3. (C) Northern blot analysis. Numbers represent the same as in panel B. rRNA represents ribosomal RNA stained with ethidium bromide after electrophoresis.

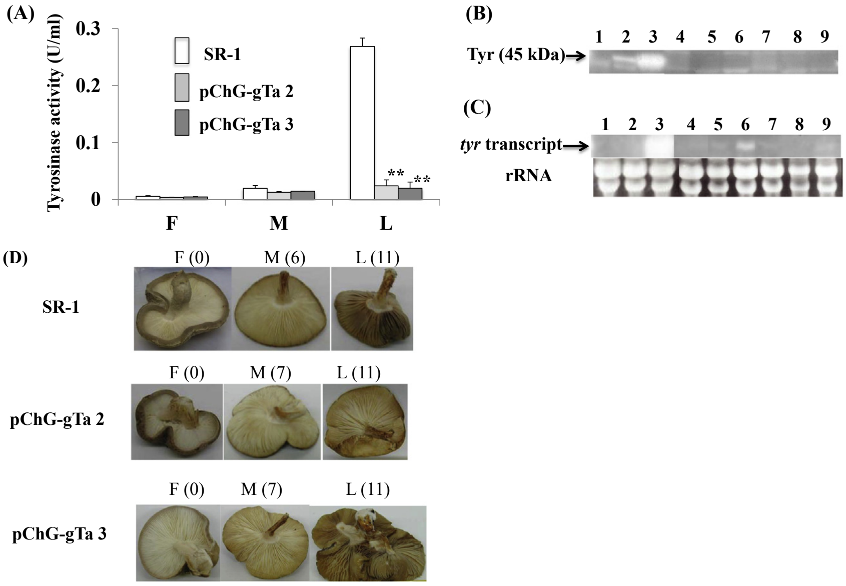

FIG. 4. Tyrosinase gene expression in gills during preservation. (A) Tyrosinase activity in gills of the wild-type host strain SR-1 and pChG-gTa transformants during preservation. F, M, and L indicate the fresh, middle, and late stages of preservation, respectively. Values are the mean ± SE of four samples (F and L stages), three samples (M stage), or one sample (pChG-gTa 3 M). Asterisks (**) indicate the p values determined by Dunnett's test as significantly different from that of the host strain ($p < 0.01$). (B) Western blot analysis. Lanes 1–3, F, M, and L of wild-type strain SR-1: lanes 4–6: F, M, and L of pChG-gTa 2; and lanes 7–9: F, M, and L of pChG-gTa 3. (C) Northern blot analysis. Numbers represent the same as in panel B. rRNA represents ribosomal RNA stained with ethidium bromide after electrophoresis. (D) Photographs of fruiting bodies.

tyrosinase activity in the gills of fruiting bodies of both clones throughout preservation. Although tyrosinase activity in the host strain SR-1 was low in the gills of fresh fruiting bodies (F: 0.00573 U/mL), it increased gradually during preservation (M: 0.0198 U/mL, L: 0.269 U/mL, Fig. 4A). In contrast, tyrosinase activity in the gills of fresh fruiting bodies of the two clones was slightly lower (clone 2: 0.00388 U/mL, clone 3: 0.00464 U/mL) than in the host strain SR-1. Tyrosinase activity of the transformants in the middle and late stages of preservation was lower than in SR-1 in the respective stages, although it gradually increased (Fig. 4A, M and L). Specifically, tyrosinase activity of the two clones in the late stage of preservation was less than 10% (clone 2: 0.0241 U/mL, clone 3: 0.0200U/mL) and tyrosinase activity in the host strain SR-1 was 0.269 U/mL in the same stage (Fig. 4A, L). Finally, we confirmed tyrosinase expression and *tyr* transcription by western blot and northern blot analyses. Tyrosinase protein and its transcript in SR-1 fruiting body were highly expressed in the late stage (Fig. 4B and C, lane 3), and low expression of only the tyrosinase protein was detected in the middle stage (Fig. 4B, lane 2). On the contrary, tyrosinase protein in the two clones was not detected during preservation (Fig. 4B, lanes 4–9), although the expression of the *tyr* transcript was low in the late stage (Fig. 4B, lanes 6 and 9). This data indicated that the levels of tyrosinase protein and its transcripts in the two clones were lower at each stage of preservation than that of the host strain, correlating with the respective tyrosinase activities. Gill browning was not observed in the fruiting body preservation during fresh stage to middle stage in all strains. However, slight gill browning was observed in the host strain fruiting bodies compared to the transformant fruiting bodies in the late stage (Fig. 4D).

Southern blot analysis of transformants To analyze the patterns of plasmid integration into the host genome, total DNA was prepared from the vegetative mycelia of transformants. Initially, 10 µg of all DNA samples were digested with *Sph*I, which was used in REMI transformation, and then Southern blot analysis was performed using a 1.1 kb *hph* fragment as hybridization probe (5). If plasmid integration into the genomic DNA occurred through a REMI, a signal at the position corresponding to 10.06 kb plasmid DNA (Fig. 5, lane P) was expected in the analysis of *Sph*I-digested DNA from these transformants. Following *Sph*I digestion, the 10.06 kb band was detected as a very weak signal and three other bands were detected in pChG-gTs 11 (Fig. 5A, lane 1), whereas the 10.06 kb band was not detected in pChG-gTa 2 (Fig. 5A, lane 2), although approximately four other bands were detected (Fig. 5A, lane 2). In clone pChG-gTa 3 (Fig. 5A, lane 3), it was not clear whether a 10.06 kb band was present because of multiple integrations of the vector into the genome. Therefore, in these transformations, plasmid DNA was reliably integrated as an REMI event only in clone pChG-gTs 11. In contrast, no band was detected in host strain SR-1 (Fig. 5A, lane S). To estimate the copy number of integrated plasmid DNA, a second Southern blot analysis was performed using DNA from the same *Sph*I-derived transformants digested with *Sph*I and *Spe*I (Fig. S1). As a result, a 3.4 kb band derived from plasmid DNA was detected in all transformants, except for the host strain SR-1 (Fig. 5B). This confirmed that *hph* was present in at least 3 copies in pChG-gTs 11 (Fig. 5B, lane 1), 5–10 copies in pChG-gTa 2 (Fig. 5B, lane 2), and more than 15 copies in pChG-gTa 3 (Fig. 5B, lane 3).

Comparison of fruiting body browning of host strain SR-1 and commercial strain H600 during preservation As one of the objectives of this study was to assess the potential to regulate *L. edodes* gill browning by introducing an antisense tyrosinase gene expression vector into the host strain SR-1, we compared the browning properties of the transformants and host strain fruiting bodies during preservation. No significant difference in browning at late stage between host strain and the transformants was observed (Fig. 4D). Therefore, we investigated tyrosinase activity and gill browning in the host and commercial strain fruiting bodies. The gills of the fruiting body of the host strain SR-1 in the late stage of preservation were slightly more browned than that in fresh stage (Fig. 6A). In contrast, the commercial strain H600 in the middle and late stages of preservation (Fig. 6A) showed much more browning, particularly in the late stage, compared to the host strain SR-1 (Fig. 6A). The gills of the host strain SR-1 were hardly browned after 11 days of preservation compared with

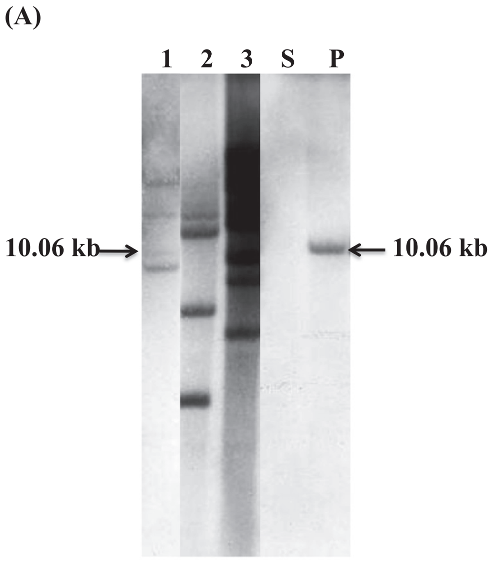

FIG. 5. Southern blot analysis of pChG-gTs and pChG-gTa transformants. (A) Samples of genomic DNA from *L. edodes* transformants were digested with *Sph*I. (B) Samples were digested with *Sph*I and *Spe*I. A DNA fragment containing *hph* was used as a probe. Lane 1, pChG-gTs 11; lane 2, pChG-gTa 2; lane 3, pChG-gTa 3; lane S, SR-1; lane P, plasmid.

those of the commercial strain H600, which browned within 7 days of preservation (Fig. 6A) (17).

Next, we measured the tyrosinase activities of the fruiting body gills of host strain SR-1 and a commercial strain H600 at each preservation stage: fresh, middle, and late stages. The tyrosinase activity of the H600 strain at all preservation stages was much higher than in the SR-1 strain (Fig. 6B). The tyrosinase activity of the commercial strain H600 fruiting bodies was 3-fold higher in the fresh stage (H600: 0.0182 U/mL), 45-fold higher in the middle stage (H600: 0.844 U/mL), and 6-fold higher in the late stage (H600: 1.96 U/mL) than in the host strain SR-1 at each preservation stage (SR-1, fresh stage: 0.00573 U/mL, middle stage: 0.0198 U/mL, late stage: 0.269 U/mL). Particularly, the tyrosinase activity even in the middle stage in the H600 strain was more than 3-fold higher than that in the host strain SR-1 in the late stage during preservation (Fig. 6B, M and L).

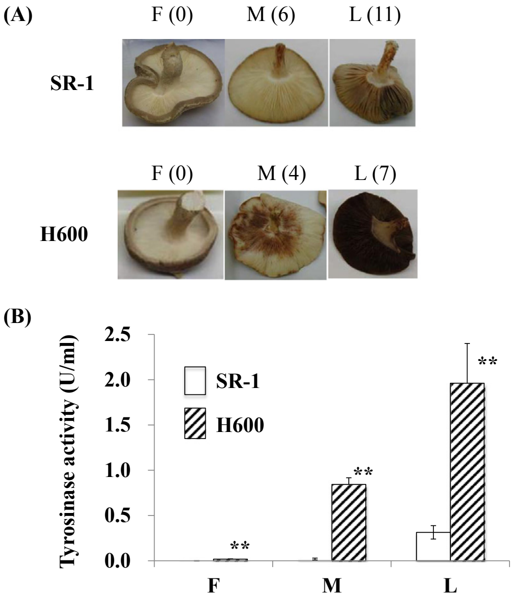

FIG. 6. Photographs of fruiting bodies and tyrosinase activity change in gills of SR-1 strain and commercial strain H600 during preservation. (A) Photographs of fruiting bodies. Fruiting bodies were preserved at 20°C and 80% humidity up to the 11th day. F, M, and L indicate the fresh, middle, and late stages of preservation, respectively. Numbers in parentheses indicate preservation days. (B) Tyrosinase activity in gills of the wild-type host strain SR-1 and commercial strain H600 during preservation. Values are the means ±SE of four samples (SR-1) or three samples (H600). Asterisks (**) indicate the *p* values determined by Student's *t*-test as significantly different from that of the host strain ($p < 0.01$).

There were three reasons for selecting the SR-1 strain as a transformation host strain: its fast growth rate, high protoplast yield, and high regeneration rate from the protoplast. The SR-1 strain grew 2-fold faster than H600 strain on an agar plate (data not shown). The protoplast yield of SR-1 strain was 10^7–10^8 cells/g wet mycelia, whereas H600 strain yielded 10^6–10^7 cells/g wet mycelia. The regeneration rate of SR-1 protoplast was approximately 1–3%, while H600 showed only around 1/10 regeneration rate of SR-1. These results were obtained by comparing more than 20 strains of *L. edodes* (data not shown), therefore, we used SR-1 as the host for transformation.

DISCUSSION

We constructed the *Letyr* expression vector pChG-gTs and repression vector pChG-gTa using the pChG vector (8) and used them to transform *L. edodes*. A clone showing elevated tyrosinase activity was selected from among the pChG-gTs-integrated transformants, and two clones with repressed tyrosinase activity were selected from the pChG-gTa-integrated transformants. The transformation efficiency of these vectors using the REMI method and 2.5 U of *Sph*I was approximately 2.2 transformants/μg of vector DNA.

We evaluated the tyrosinase activity of nine clones transformed with the sense tyrosinase expression vector pChG-gTs. Clone 11 was selected due to its high level of tyrosinase activity (Fig. S2A). The clone showed 3.6-fold higher activity in the vegetative growth stage (Fig. 1A) and 14-fold higher activity in the fresh fruiting body stage (Fig. 2A) compared to the host strain SR-1. These results indicate that the pChG vector is useful for expressing exogenous genes in *L. edodes*. Differences in tyrosinase gene expression between the host strain and transformant were confirmed at the transcriptional and translational levels by northern and western blot analyses, respectively (Fig. 2). These data suggest that tyrosinase was synthesized *de novo* in the fresh fruiting bodies of the transformant.

Integration of vectors into *L. edodes* was reported for a drug resistance gene as a selection marker (5–8), and the expressions of genes except drug resistance genes was reported only for the β-glucuronidase (GUS) gene (18) and the *Pleurotus ostreatus* manganese peroxidase (*mnp3*) gene (19). All these were expressed only in vegetative mycelia, not in the fruiting bodies. In the edible basidiomycetes, expression of the green fluorescent protein (GFP) gene in the fruiting body was confirmed in *Flammulina velutipes* (20). However, as far as we know, there is no report on the expression of commercially useful proteins in the fruiting bodies of edible basidiomycetes. In this study, we expressed *L. edodes tyr* gene by *gpd* promoter and confirmed for the first time its high expression in fruit bodies (Fig. 2). In the future, expression of commercially useful proteins using this vector is expected.

On the other hand, the morphology of the transformant fruiting bodies showed abnormal gills opening (Fig. 3D). It is unknown whether this result is due to the high expression of *tyr* upon introduction of the pChG-gTs vector or the position on the inserted genome. It is necessary to further study other pChG-gTs integrated strains to understand this mechanism.

To demonstrate repression, 2 pChG-gTa transformants, clones 2 and 3, were selected from among 10 transformants as they expressed lower tyrosinase activity than the host strain SR-1 (Fig. S2). Although the tyrosinase activity of the host strain and pChG-gTa clones in the fresh fruiting bodies was low, the activity of clones in the late-stage fruiting body increased by approximately 4–6-fold (Fig. 4A), while the tyrosinase activity of host strain SR-1 in the late-stage fruiting body increased by approximately 47-fold (Fig. 4A). The tyrosinase activity in the late-stage fruiting bodies of the host strain SR-1 was 11–13-fold higher than that of the pChG-gTa clones (Fig. 4A), indicating that the antisense method effectively repressed the tyrosinase gene in *L. edodes*. Successful repression of tyrosinase gene expression by the antisense method is feasible as the tyrosinase gene is synthesized *de novo* during preservation of the *L. edodes* fruiting body (4).

Stoop and Mooibroek succeeded in repressing *Agaricus bisporus* tyrosinase gene expression by the antisense method (21). However, the effect of tyrosinase expression on browning remained unclear. In *L. edodes*, transcription of *lcc4* and laccase activity increased in fruiting bodies during preservation (17,22), suggesting that *lcc4* is possibly involved in gill browning after harvest. Therefore, combined repression of *lcc4* and *tyr* in H600 as a host strain are necessary.

We compared browning of the fruiting bodies of the host strain SR-1 and commercial strain H600 during preservation and found that the SR-1 strain showed less browning than H600. This indicates that the SR-1 strain was not suitable for this study. The tyrosinase activity of the commercial strain H600 fruiting bodies during preservation were 3.2-fold, 42.6-fold, and 7.3-fold higher in the fresh, middle, and late stages, respectively, compared to that in the host strain SR-1. Moreover, the gill color of the host strain SR-1 at the late stage of preservation (Fig. 6A) was less brown than that of the commercial strain H600 (Fig. 6A). This suggests that tyrosinase contributes to browning of fruiting bodies during preservation. Further studies in which the vector pChG-gTa is transformed into the H600 strain should be carried out to determine the phenotype of fruiting body browning during preservation.

To confirm the copy numbers of the introduced vectors, we performed Southern blot analysis of the transformants (Fig. 5). More than 3 copies of the vector were confirmed in the pChG-gTs-transformed strain 11 (Fig. 5, lane 1) and 5–10 copies in the pChG-gTa-transformed clone 2 (Fig. 5, lane 2). In the pChG-gTa-transformed clone 3, multiple vector integrations (more than 15 copies) were observed (Fig. 5, lane 3), and tyrosinase gene repression was slightly stronger than that of clone 2 (Fig. 3A, B and Fig. 4C). In previous basidiomycetes transformation studies, Coconi-Linares et al. (23) and Yanai et al. (24) suggested that the copy number of exogenous gene integrations and enhanced expression of the gene were correlated. The results of this study suggest that tyrosinase gene repression was caused by a high integration of vector copy in the genome. However, more studies are needed to confirm this possibility. Additionally, exogenous gene expression by the vector may be affected by the position of integration into the genome as the vector is randomly integrated into the genome by the REMI method.

There have been previous reports of successful antibiotic-resistant or reporter gene expression in *L. edodes* (5,6,8,18); however, commercially useful applications of gene expression in *L. edodes* fruiting body has not been reported to date. To our knowledge, this is the first report of the successful expression of a useful gene in *L. edodes* fruiting body. In conclusion, we showed that the pChG vector was effective for homologous gene expression and could be used for both suppression and expression of a gene of interest in the mycelia and fruit bodies of *L. edodes*. We expect that the pChG vector will be useful for heterologous gene expression and functional analysis in this system.

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