日本生物工学会誌　英文誌
Journal of Bioscience and Biotechnology

論文タイトル

Construction of Novel Vectors for Transformation of *Lentinula edodes* Using a Chitin Synthase Gene Promoter.

キチン合成酵素遺伝子プロモーターを利用した新規シイタケ形質転換ベクターの構築

要旨

シイタケ・キチン合成酵素遺伝子プロモーターによってハイグロマイシン耐性遺伝子 *hph* を発現させる発現ユニットを有する、シイタケ用の新規ベクター（pLCHS-hph と pChG-bar）を構築した。ランダムに選択した形質転換体における *hph* の発現を RT-PCR 法によって確認した。２種類のベクターはシイタケの形質転換に有用であることが明らかとなった。

Note

Construction of Novel Vectors for Transformation of *Lentinula edodes* Using a Chitin Synthase Gene Promoter.

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Key words

Lentinula edodes, novel vector, transformation, chitin synthase, gene expression

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Abstract

Novel vectors (pLCHS-hph and pChG-bar) containing expression unit driving *hph* as a selectable marker gene by chitin synthase gene promoter were constructed for *Lentinula edodes* transformation. Expression of the *hph* gene in random selected transformants was confirmed by RT-PCR method. Thus, both vectors are useful for *L. edodes* transformation.

Lentinula edodes is one of the most important edible basidiomycete in Japan. *L. edodes'* gills turn brown during post-harvest preservation, which is commercially undesirable because it causes an unpleasant appearance. We reported previously that the causative browning factors were tyrosinase (1, 2) and laccase (3). We then cloned the genes from browned fruit-bodies of *L. edodes*, and confirmed the increased expression of both genes during post-harvest preservation (4, 5). And also, tyrosinases (6) and laccases (7) are recognized for their potential in biotechnological applications in food and nonfood industries. At this point, the establishment of genetic engineering techniques for *L. edodes* was essential for confirmation of the genes function by a reverse genetic strategy and for overproduction system of useful enzymes. However, there were few reports except our studies (8-10). Although we developed an effective transformation method for *L. edodes* by restriction enzymes-mediated integration (REMI) (8) and constructed useful vectors, pLG (9) and pL-Cbx (10), it was necessary to construct a vector that could express simultaneously two exogenous genes, such as dominant selectable marker genes and another gene, to analyze the functions of genes useful in *L. edodes* breeding.

The cell wall of filamentous fungi comprises a variety of macromolecules, such as chitin and β-glucan. Chitin, the β-1, 4-linked polymer of N-acetylglucosamine

(GlcNAc), provides strength to the fungal cell wall and is therefore essential for the morphogenesis and survival of fungi (11, 12). Synthesis of chitin is mediated by chitin synthases (CHSs, EC 2.4.1.16) (11, 13). Thus, it is likely that the *chs* gene is constitutively expressed during all growth stages, and therefore, the *chs* promoter would be useful for exogenous gene expression, such as dominant selectable marker genes, for transformation of *L. edodes*. In previous study, we cloned a *LeChs2* gene from *L. edodes* and demonstrated the expression of the gene at various growth stages (14). In present study, we constructed the novel vectors, pLCHS and pChG, using *LeChs2* gene promoter, and confirmed its utility in the transformation of *L. edodes*.

To confirm the usefulness of the *LeChs2* promoter for heterologous gene expression, we constructed the novel vector, pLCHS-hph in which the *LeChs2* promoter drives the hygromycin B phosphotransferase (*hph*) gene (Fig. 1). pLCHS-hph (5.95kb) was constructed by ligation of the pLG vector 9) lacking the glyceraldehydes-3-phosphate dehydrogenase (*gpd*) gene promoter region and the *LeChs2* gene promoter region. The former region was amplified by PCR using primers pLC-rasU (5'-GGA TAT GAA AAA GCC TGA ACT CAC CGC G-3') and pLC-rasL (5'-CCC GGG CAC CTT GCG CCT CCA TAC TTT CAT-3') (9) and the latter region (1,154 bp) was amplified by PCR using primers CproU2 (5'-CTG ACT TAG CCG ACG ACA CAC GTA ATG ACA-3') and gCLA-L (5'-CCT TAG TTG TAG ATG GAA ATG GTG GGG TGG-3'), respectively (Fig. 1). pLG-bar was constructed by ligation of the pLG vector region amplified by pLC/hU-X (5’- GCT CTA GAT TGT GGC TCT CCA TCA TAT CGA CCC G-3’ containing an *Xba*I site at 5' end) and pG-L (5'-ATT CAA GCA GTC AAT GGA TTG GAG GG-3') using pLG-hph as a template, and a 0.55 kb fragment of *bar* gene, which encodes a phosphinothricin acetyltransferase confering bialaphos resistance, derived from the pLC-bar (15) by digestion with *Bam*HI (Fig. 1).

pLC-bar was kindly donated by Dr. Yanai, Meiji Seika Kaisha, Ltd. We then prepared a pGPD-*bar*-tGPD fragment by PCR using pG-U/S (5'- GGA CTA GTC GAA GTT TGA GGT GGT TGC GAA TAC G-3' containing an *Spe*I site at 5' end) and tG-L2/N (5'- CTA GCT AGC TAT CAG GGA GAC GGC GGA CAC AGG TA -3' containing an *Nhe*I site at 5' end) with pLG-bar as a template (Fig. 1). The resulting fragment was blunting and ligated to the pLCHS-hph *Ehe*I site resulting in pChG-bar (8.12kb, Fig. 1).

Preparation of *L. edodes* protoplasts for the transformation was by the method of Irie *et al*. (10) About 1×10^8 protoplasts were obtained from 1 g of *L. edodes* strain SR-1 mycelia. Protoplasts of *L. edodes* were transformed with 2.5 μg of vectors according to the restriction enzyme-mediated integration (REMI) method as described by Sato *et al*. (8). In the case of pLG-hph and pLCHS-hph, *L. edodes* protoplasts were transformed twice with 5 units of *Bgl*II and selected with MYPG agar medium containing 20μg/ml hygromysin B by the method of Sato *et al*. (8). The transformation efficiencies of the vectors, pLG-hph and pLCHS-hph, were both about 20 transformants per 2.5 μg vector DNA (Table 1). These results suggested that *LeChs2* gene promoter was useful for *L. edodes* transformation, similarly to the *L. edodes gpd* gene promoter. In the case of pChG-bar, protoplasts of *L. edodes* were transformed three times with the vector and 25 units of *Hin*dIII instead of *Bgl*II because of the existence of *Bgl*II site in the *bar* gene. First selection was done with MYPG agar medium containing 20μg/ml hygromysin B, and then hygromycin B resistant transformants were selected with MYPG agar medium containing 20 μg/ml hygromycin B and 2 μg/ml bialaphos. The final transformation efficiency achieved was three transformants per 2.5 μg vector DNA (Table 1). The result indicates that the bar gene can be used as a selection marker gene in *L. edodes* transformation besides *hph* (8, 9) and carboxin resistant gene (10). The transformation efficiencies of pChG-bar was lower than that of pLG-hph and pLCHS-hph. One

possible reason for the low efficiency of pChG-bar transformation is that the restriction enzymes used for REMI transformation; *Hin*dIII was used to transform *L. edodes* for pChG-bar, and *Bgl*II for pCHS-hph and pLG-hph. Hirano *et al*. reported that transformation efficiency of *L. edodes* using *Bgl*II with pLG-hph by REMI method was higher than using *Hin*dIII (9). Another possible reason is the vector size; pCHS-hph and pLG-hph are 5.95 kb and 5.85 kb respectively, whereas pChG-bar is 8.12 kb. To confirm the integration of each vector in the transformants, Southern blot analysis was performed for five randomly selected clones of each transformants using *hph* gene as a probe, with or without restriction enzymes digestion. The vectors were shown to be integrated into the chromosome of all five *L. edodes* clones at single or multiple sites (data not shown).

Expressions of the *gpd* gene, *hph* gene, and *bar* gene in five randomly selected clones of *L. edodes* pChG-bar transformants were confirmed by RT-PCR (Fig. 2). Total RNAs were prepared from mycelia grown in MYPG liquid culture of the transformants for two weeks at 25ºC and cDNA synthesis was by the method of Hirano *et al*. (16). The expression of the *gpd* gene, as a control gene, was detected as a 792 bp band by PCR using cG298U (5'-TCG TCG ACG GAA AGG AAA TCT C-3') and cG444L (5'-GGC AGC AAA CAC CAA GAG AT-3') as primers (Fig. 2A), and *hph* gene expression was detected as a 626 bp band by PCR using 122U (5'-TTC GAT GTA GGA GGG CGT GGA T-3') and 726U (5'-CGC GTC TGC TGC TCC ATA CAA G-3') as primers (Fig. 2B). These bands were clearly detectable in all five clones of pChG-bar transformants. However, *bar* gene expression was only detected in one clone as a 436 bp band by PCR using 117U (5'-TAC CGA GCC GCA GGA ACC-3') and 535L (5'-CTC GGT GAC GGG CAG GAC-3') as primers (first PCR, Fig. 2 C). We then performed nested PCR with the first PCR product as a template using 242U (5'-CAC GCA ACG CCT ACG

ACT-3') and 492L (5'-AAG TCC AGC TGC CAG AAA-3') as primers. As a result, a 268 bp band was detected in all five transformants (Fig. 2 D). This result indicate that *bar* gene expression was weaker than *hph* gene expression, suggesting that *bar* gene expression could be unstable at a transcriptional level and could be resulted in low transformation efficiency. Although *bar* gene expression by the *gpd* gene promoter in the pChG-bar transformants was weak than *hph* gene expression by the *LeChs2* gene promoter, it has been confirmed that *hph* gene expression by the *gpd* gene promoter was effective in *L. edodes* (9) and *Pleurotus oseteatus* (17). Therefore, the low expression of *bar* gene in this study is probably not due to the activity of *gpd* gene promoter.

Collectively, these data described here suggest that the *LeChs2* gene promoter is effective in *L. edodes* exogenous gene expression, and that the pChG is very useful vector expressing two heterologous genes simultaneously in *L. edodes* for molecular breeding. Future studies will include overproduction and repression of interesting homologous genes or exogenous genes using this vector. In particular, overproduction and repression by antisense method of the *L. edodes* tyrosinase gene are currently underway.

Acknowledgements

We thank Meiji Seika Kaisha, Ltd., for providing the bialaphos resistance gene.

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Figure Legends

Fig. 1. Construction of pLCHS and pChG-bar.

GPD and *CHS* are *L. edodes* glyceraldehyde-3-phosphate dehydrogenase gene and chitin synthase gene (*LeChs2*), respectively. *pGPD, pCHS and pRAS* are promoter sequences of the *GPD, CHS* and *ras* genes of *L, edodes*, and *tGPD* and *tPriA* are the terminator sequences of *L. edodes GPD* and *priA* gene. *hph*, *amp*, and *bar* are the hygromycin B phosphotransferase gene, ampicillin resistance gene, and bialaphos resistance gene, respectively. The thin arrows represent primers.

Fig. 2. Marker genes expressions in the pChG-bar transformants by RT-PCR.

RT-PCR products were separated on a 1% agarose gel. Expression of *gpd* gene was used as a control gene (A). The *hph* gene expression (B), and *bar* gene expressions (C) (first PCR) and D (nested PCR), respectively, are shown. The numbers indicate the clone number of the transformants, and S and N indicate host strain SR-1 and no template DNA, respectively. M and M' indicate 500 bp ladder and 100 bp ladder marker DNAs, respectively.

Table 1. Number of *Lentinula eodes* transformants generated with the vectors by REMI method.

Protoplasts of *L. edodes* were transformed with *Bgl*II or *Hin*dIII. REMI transformation was done with 2.5 μg of circular plasmid DNA. The values represent the average of two or three replications.

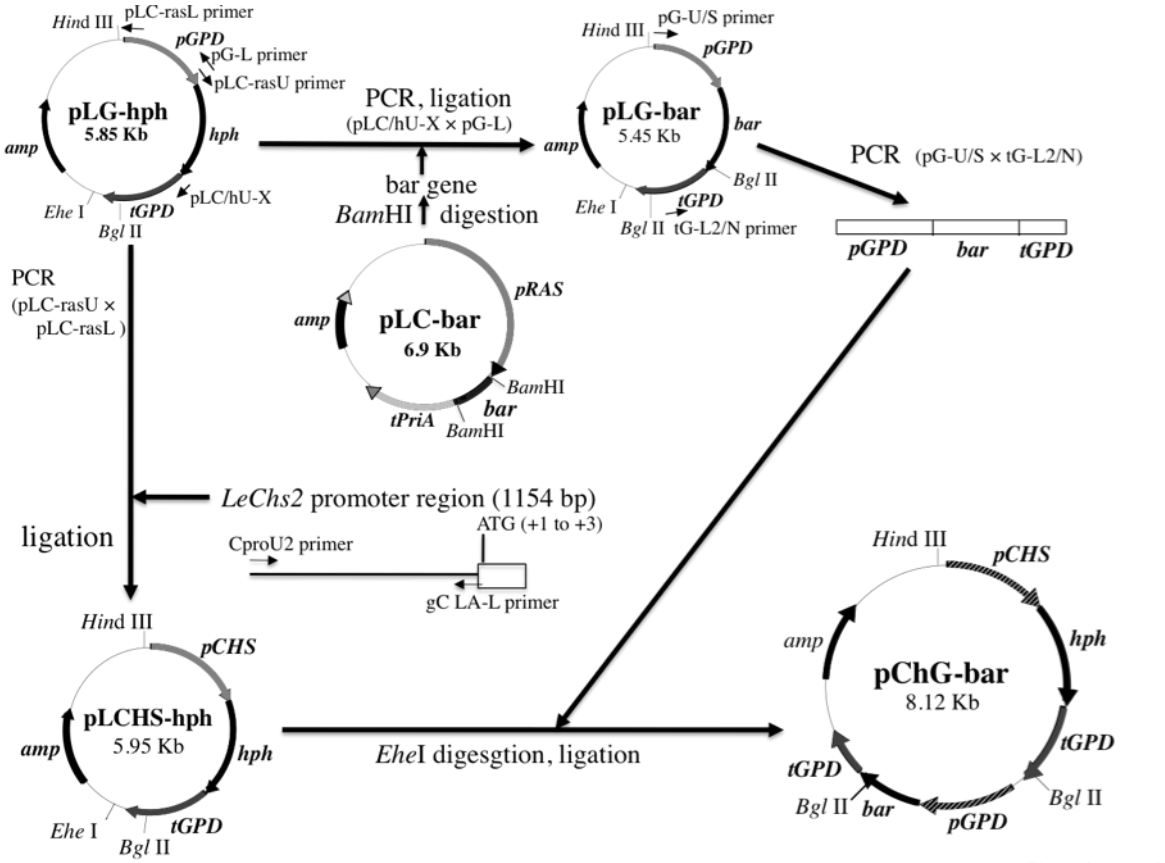

Fig. 1. Sato *et al.*

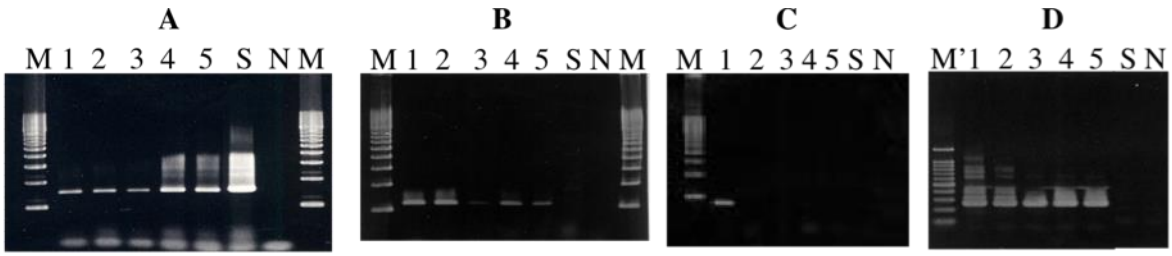

Fig. 2. Sato *et al*.

Table 1. Number of *Lentinula eodes* transformants generated with the vectors by REMI method.

Vector	Restriction enzyme (units)	No. of replication	No. of transformants/2.5μg vector DNA
pLG-hph	*Bgl*II (5)	2	19.0
pLCHS-hph	*Bgl*II (5)	2	20.5
pChG-bar	*Hin*dIII (25)	3	3.0

Protoplasts of *L. edodes* were transformed with *Bgl*II or *Hin*dIII. REMI transformation was done with 2.5 μg of circular plasmid DNA. The values represent the average of two or three replications.