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Highly Efficient Electroporation-mediated Transformation into Edible Mushroom *Flammulina velutipes*

Jong Kun Kim[1,2], Young Jin Park[3], Won Sik Kong[4] and Hee Wan Kang[1,2]*

[1]*Graduate School of Biotechnology & Information Technology, Hankyong National University, Ansung 456-749, Korea*
[2]*Institute of Genetic Engineering, Hankyong National University, Ansung 456-749, Korea*
[3]*National Academy of Agricultural Science, Rural Development Administration, NAAS, Suwon 441-707, Korea*
[4]*National Institute of Horticultural and Herbal Science, Development of Herbal Crop Research, RDA, Suwon 441-707, Korea*

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In this study, we developed an efficient electroporation-mediated transformation system featuring *Flammulina velutipes*. The flammutoxin (*ftx*) gene of *F. velutipes* was isolated by reverse transcription-PCR. pFTXHg plasmid was constructed using the partial *ftx* gene (410 bp) along with the hygromycin B phosphotransferase gene (*hygB*) downstream of the glyceraldehydes-3-phosphate dehydrogenase (*gpd*) promoter. The plasmid was transformed into protoplasts of monokaryotic strain 4019-20 of *F. velutipes* by electroporation. High transformation efficiency was obtained with an electric-pulse of 1.25 kV/cm by using 177 transformants/μg of DNA in 1×10^7 protoplasts. PCR and Southern blot hybridization indicated that a single copy of the plasmid DNA was inserted at different locations in the *F. velutipes* genome by non-homologous recombination. Therefore, this transformation system could be used as a useful tool for gene function analysis of *F. velutipes*.

KEYWORDS : Electroporation, *Flammulina velutipes*, Flammutoxin gene, Hygromycin B gene, Protoplast

Flammulina velutipes (Curt.: Fr.) Sing. is a well-known edible mushroom cultivated all over the world, especially in Asia. However, relatively few information is available regarding its genetic characteristics. The genome size of *F. velutipes* is approximately 33 Mb and is organized into 8 chromosomes [1, 2]. Moreover, the whole-genome draft sequence for the monokaryotic strain 4019-20 of *F. velutipes* in Korea is currently being determined. In addition, whole-genome sequencing projects for mushrooms, *Agaricus bisporus*, *Lentinula edode*, and *Pleurotus ostreatus*, are also being performed. Comparative genome analysis based on the sequence data provides a large amount of information that enables the exploration of gene function on a genome-wide scale. Insertional mutagenesis and targeted gene disruption studies are necessary to identify useful genes and analyze their functions after sequencing the whole-genome of the mushroom species.

Transformation is a basic technology for identifying the functions of other genes of interest involved in a variety of biological processes. Most protocols used in fungal transformation involve electroporation [3, 4], treatment with polyethylene glycol [5, 6], and restriction enzyme-mediated integration using protoplasts [7]. In addition, *Agrobacterium*-mediated transformation has been successfully applied to filamentous fungi containing mushrooms [8]. In principle, this method can generally be used for random genomic insertion of a target DNA fragment between both (left and right) borders derived from the transfer-DNA (T-DNA) of tumor-inducing (Ti) plasmids of *Agrobacterium*, and thus this method is useful in random mutagenesis. The most important limiting factor in the functional analysis of individual genes is low efficiency of transformation and targeted integration. Recently, the transposon-arrayed gene knockout system was demonstrated as a highly efficient method for gene discovery and gene function identification in filamentous fungi [9]. A high-throughput targeted gene disruption method using linear minimal elements (LMEs) has been developed for *Alternaria brassicicola* [10].

Flammutoxin (FTX) has previously been isolated from fruiting bodies of the edible mushroom *F. velutipes* as a cardiotoxic and cytolytic polypeptide 22 or 32 kDa in length. FTX, a 31-kDa pore-forming cytolysin from *F. velutipes*, is specifically expressed during fruiting body formation, and it causes efflux of potassium ions from human erythrocytes along with swelling of the cells before hemolysis [11, 12].

In this study, we describe a highly efficient transformation method by using protoplasts released from *F. velutipes*. The monokaryotic strain 4019-20 of *F. velutipes* was used in this study. We were interested in targeted gene disruption of the *ftx* gene and thus constructed the plasmid pFTXHg to flanki parts of the *ftx* gene and the *hygB* gene expression cassette. Thus, we constructed a circular plasmid vector and an LME vector that were composed of the hygromycin B phosphotransferase gene (*hph*) and flammutoxin (*ftx*) cDNA gene and then transformed them into protoplasts by electroporation and polyethylenegly-

*Corresponding author <E-mail : kanghw2@hknu.ac.kr>

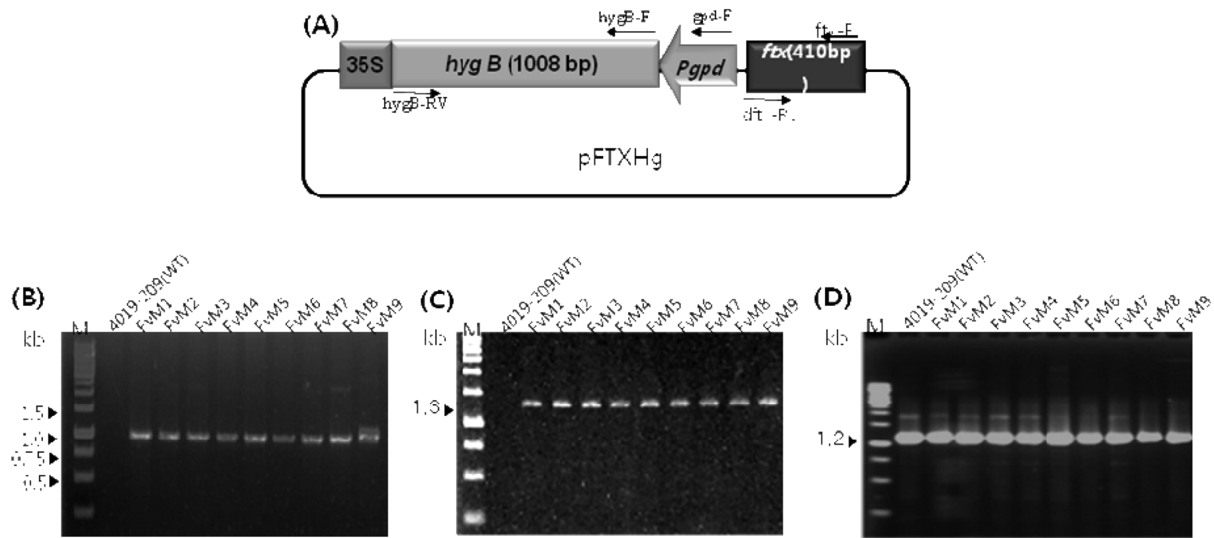

Fig. 1. PCR analysis of DNA isolated from putative pFTXHg transformants of *Flammulina velutipes* (4019-20). A, PCR amplification was carried out on genomic DNA using various primers (arrows) on plasmid pFTXHg; B, Amplicons by primers hygB-F and hygB-RV; C, Amplicons of primers gpd-F and hygB-RV; D, Primers ftx-F and ftx-RV covering full-length *ftx* gene. Lane M, 1 kb marker; Lane 1, *F. velutipes* wild type 4019-20; Lanes 2-10, transformants.

col (PEG)-mediated methods under different conditions to develop a transformation protocol with high efficiency.

To isolate the *ftx* gene, the monokaryotic strain 4019-20 of *F. velutipes* was grown in 100 mL of potato dextrose (PD) broth (Duchefa, Haarlem, The Netherlands) by shaking at 28°C for 7 days. Total RNA was extracted from the mycelium by using a RNeasy mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. RNA was treated with DNase (Promega, Madison, WI, USA) for 20 min at 37°C. Reverse transcription (RT)-PCR was performed using a standard kit (ReverTra Ace, TRT-101; Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. For synthesis of *ftx* cDNA, the following primers FTX-F 5'-ATGCCTCAAGTCAA-GACAAG-3' and FTX-R 5'-TCACTCAGGACCAGGA-ACCATG-3' were generated from the 3'-ending sequences of the known nucleotide sequence of *ftx* gene. We added 400 ng of total RNA sample to a 20 μL reaction mixture, including 20 pmol of the downstream primer, 4 μL of 5 × RT buffer, 2 μL of dNTPs (10 mM), 10 units of RNase inhibitor, and 10 units of reverse transcriptase, followed by incubation in a PTC-225™ thermocycler (MJ Research, Inc., Waltham, MA, USA) at 42°C for 30 min. After the cDNA was synthesized, 10 μL of cDNAs were used for the PCR using KOD DASH (Toyobo Co., Ltd.) in a 50 μL reaction mixture according to the manufacturer's instructions under the following conditions: 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1.5 min. Full-length cDNA (819 bp) of *ftx* was obtained from the RT-PCR and then cloned into the pGEM-T Easy vector (Promega) to yield the plasmid pFTX. On the basis of the cDNA sequence, pFTX was digested using the restriction enzymes *sal* I (restriction site at position 410 downstream of the initial codon) and *spe* I (restriction site in the multi-cloning site of the vector) and then ligated into the *sal* I/ *spe* I sites of the pHg plasmid that contained the *hph* gene under the control of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter, generating pFTXHg (Fig. 1A).

Mycelia of the monokaryotic strain 4019-20 of *F. velutipes* were grown in 200 mL of PDB by gentle shaking at 25°C for 7 days. The mycelia were ground using a homogenizer, incubated for an additional day under the above conditions, harvested by centrifugation at 2,000 ×g for 10 min, and then resuspended in 40 mL of 0.5 M $MgSO_4$ containing cellulase onozuka R-10 (Sigma, St. Louis, MO, USA) and Glucanex® 200G (Novozyme, Bagsvaerd, Denmark) to a final concentration of 500 μg/mL. After incubation for 6 hr, these mycelia were suspended in 10 mL of 0.5 M $MgCl_2 \cdot 7H_2O$ and then filtered through a sintered glass filter P-1 (Duran, Mainz, Germany). The protoplasts were collected by centrifugation at 6,000 ×g for 10 min at 4°C, washed twice with 10 mL of 0.6 M sorbitol, and then washed with 10 mL of electroporation buffer (0.6 M mannitol, 10 mM sodium phosphate [pH 7.0]). The protoplasts were resuspended at a concentration of 1×10^7 in electroporation buffer (200 μL), after which 10 μg of plasmid pFTXHg was added. Transformation was conducted by electroporation using BTX ECM 3000 and 0.2-cm cuvettes (BTX, San Diego, CA, USA). The electric pulse delivery test required several settings: capacitor, 25 μF; resistance, 200 Ω; and field strength, 0.8 kV/cm to 2.5 kV/cm of the electroporation apparatus. The protoplasts were plated directly onto sorbitol agar plates (1.2 M sorbitol, 10 mM Tris/HCl pH 8.0, 1.3% agar).

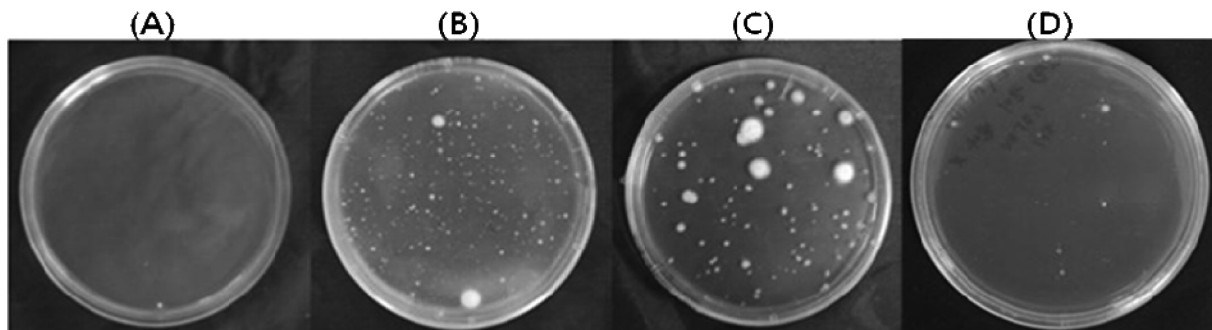

Fig. 2. Transformants formed on selective mushroom complete medium plate containing 30 μg/L of hygromycin after incubation of 14 days. A, Negative control (without plasmid vector); B, Electroporation with electric-pulse 1.25 kV; C, Electroporation with electric-pulse 2.5 kV; D, Polyethyleneglycol method.

After incubation at 25°C for 24 hr, plates were overlaid with 5 mL of sorbitol top agar containing 30 μg/mL of hygromycin B. Hygromycin-resistant transformants were observed after incubation for 20 days (Fig. 2).

Using the electroporation method, a low electric-pulse of less than 1 kV resulted in no mycelia colonies, whereas a high-voltage electric-pulse (2.5 kV) dramatically reduced the regeneration rate of the protoplasts (0.008%), indicating physical damage to the protoplasts. Consequently, the highest transformation efficiency was obtained at an electric-pulse of 1.25 kV/cm with 177 transformants/μg of DNA in 1×10^7 protoplasts (Table 1). On the other hand, the PEG-mediated transformation efficiency was extremely low; less than 2 mycelial colonies in the protoplasts density. The efficiency of transformation by electroporation was higher than that of PEG-mediated transformation. Transformants produced by this method were repeatedly transferred to PD agar (PDA) media without hygromycin B and then transferred onto selective media containing 30 μg/mL of hygromycin B. All transformants were vigorously grown on selective media, although they were cultivated continuously several times. These results show that the hygromycin resistance of our transformants was mitotically stable. As shown in Fig. 3, nine transformants

Table 1. Transformation efficiency into *Flammulina velutipes* protoplasts corresponding to electroporation conditions

Electric pulse (kV)	Regeneration frequency (%)/ 1×10^7 protoplasts	Transformants
0.8	9.3×10^4	0
1	7.2×10^4	0
1.25	2.4×10^4	177
2.5	8×10^2	77

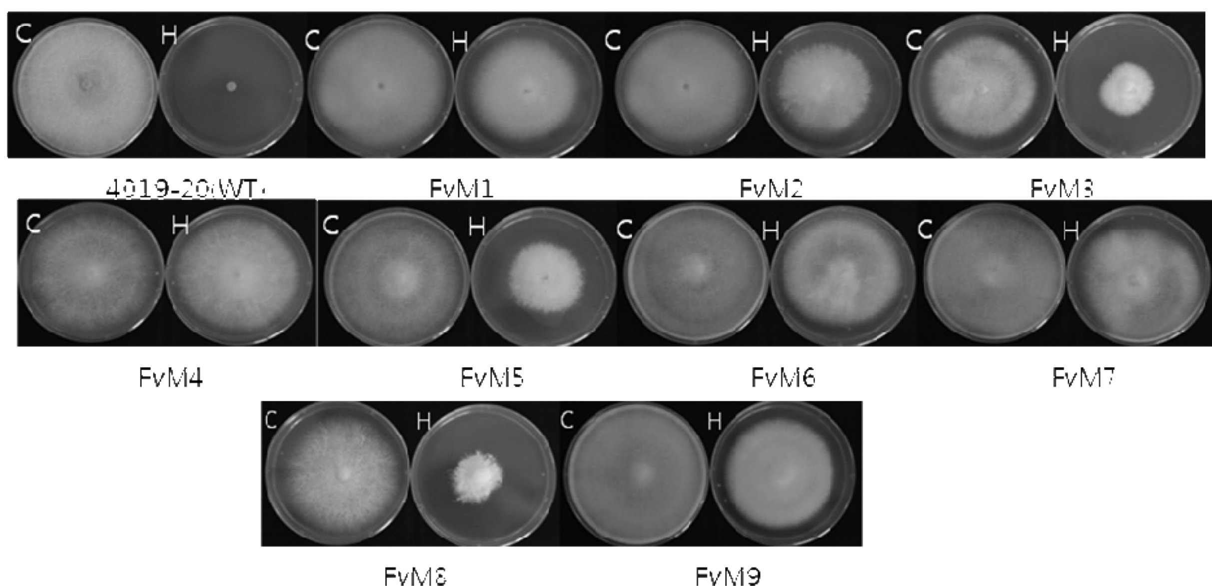

Fig. 3. Morphological features of transformants grown on potato dextrose agar without (C) and with (H) hygromycin 30 μg/L. The transformants were randomly selected from mycelial colonies formed in Fig. 1B.

were normally grown on PDA media with or without hygromycin B, as with the wild type strain. Exceptionally, transformants FvM3, FvM5, and FvM8 showed slow growth rates and different morphological characteristics in media containing hygromycin B, which implies high sensitivity to antibiotics. In a previous study, the *gpd* gene was transformed into the germinated basidiospores of *F. velutipes* by electroporation [13]. That study revealed that the transformation efficiency is dependent on a high-voltage electric pulse of 1.25 kV/cm, which is in good agreement with our result. Nevertheless, our protocols show three-time higher transformation efficiency compared to a previously reported method [13]. Although *Agrobacterium*-mediated transformation of *F. velutipes* was also performed using gill tissues of the fruiting body [8], the protoplast-mediated electroporation method of this study is the first to utilize *F. velutipes.* The *Agrobacterium*-mediated transformation system has been extensively used to study the genomic function of fungal species based on mutation of the fungal genome via T-DNA insertion.

Transformation technology can be used to transfer useful foreign genes. In addition, transformation can be used for targeted gene disruption based on homologous recombination to analyze the functions of genes of interest in the genomic sequences of fungal species. As a remarkable transformation technique, LME constructs were devised for high-throughput targeted gene disruption of the *A. brassicicola* genome [10]. Typically, 100% targeted gene disruption events occur in transformed mutants when using LME constructs. Therefore, we amplified a DNA fragment covering the 35S terminator from the *ftx* gene by PCR in order to generate LME from pFTXHg (Fig. 1A). The LME construct was then transformed into protoplasts of the *F. velutipes* strain 4019-20 on the basis the protocol established in this study. However, we could not obtain any transformants using the LME method (data not shown).

Molecular confirmation of the transformants was conducted by PCR and Southern hybridization analysis. For PCR, approximately 50 ng of genomic DNA was used in each 50 μL PCR reaction using a PTC-225™ thermocycler (MJ Research, Inc.). Sequence information from the *hygB* gene and *gpd* promoter was used to design specific primer sets to amplify the target genes. The primers gpd-F (5'-GAAGAAGCTTTAAGAGGTCCG-3') and hygB-R (5'-CTATTTCTTTGCCCTCGGACG-3') were designed to amplify a 1.3-kb region covering the *hygB* gene and *gpd* promoter in pFTXHg. PCR reactions were performed by denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, and elongation for 2 min at 72°C. A final elongation was performed at 72°C for 7 min. PCR products were size-fractionated on a 1% agarose gel by electrophoresis and then visualized by ethidium bromide staining. As shown in Fig. 1, the primer sets hygB-F/hygB-RV and gpd-F/hygB-RV amplified the expected PCR bands of 1.0 and 1.3 kb in genomic DNA samples from all transformants, but not in samples of the wild type strain 4019-20. Furthermore, PCR amplification using the primers ftx-F 5'-ATGCCTCAAGTCAAGACAAG-3' and ftx-RV 5'-

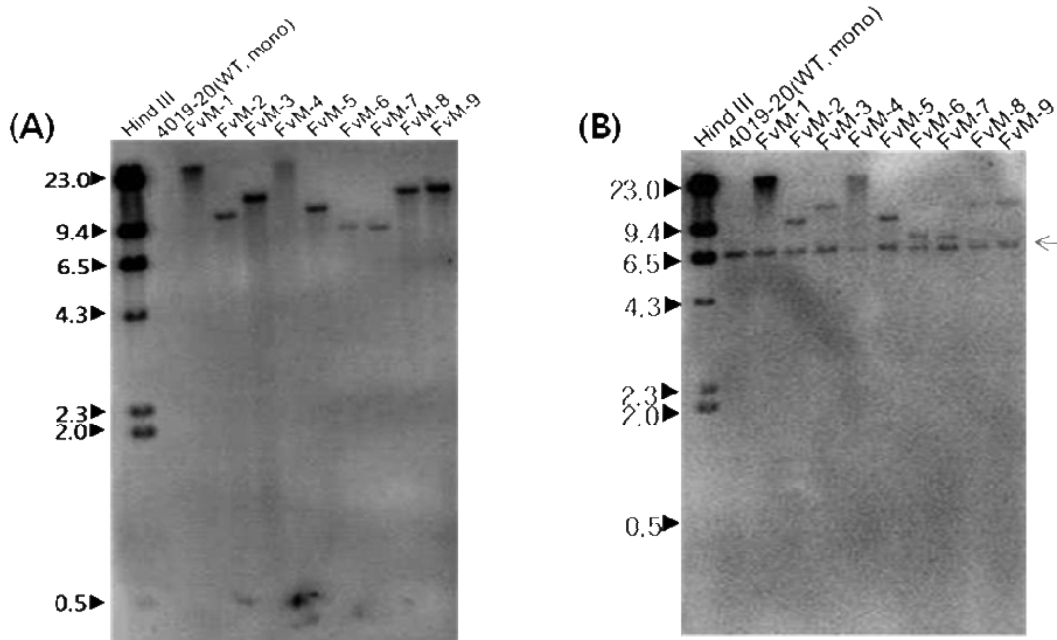

Fig. 4. Southern hybridization of genomic DNA isolated from pFTXHg transformants of *Flammulina velutipes* (4019-20) genomic DNA was digested with *EcoRV,* separated on a 0.9% agarose gel, and blotted onto a nylon membrane. The 32P-labeled *hyg* B (A) and *ftx* (B) genes were used as probes.

TCACTCAGGACCAGGAACCATG-3'), which were designed from both ends of the full-length *ftx* cDNA gene (819 bp), was performed on the nine transformants. An increased band size was expected due to insertion of pFTXHg by homologous recombination. However, the amplified PCR products had the same molecular size (1.2 kb) and contained some introns in the transformants and wild type strains (Fig. 1D).

Southern blot hybridization using the *hygB* gene as a probe was conducted to determine the copy number of pFTXHg integrated into the transformants of *F. velutipes.* The genomic DNA was extracted from the mycelia of *F. velutipes* strain 4019-20 by following a previously reported protocol [14]. The extracted genomic DNA (about 5 μg) was then digested with *Eco*RV, which does no cut the *hygB* and *ftx* genes, fractionated on a 0.9% agarose gel in NEB buffer (100 mM Tris-acetate, 1 mM EDTA pH 8.0), and transferred onto a Hybond™ N^+ nylon membrane (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions, followed by fixing in 0.4 M NaOH for 20 min. Hybridization probes were prepared by labeling the DNA fragments with [α^{32}P] dCTP (Amersham) using a random labeling kit (Takara, Osaka, Japan). Hybridization and washing of the membrane was performed according to the protocol provided by the manufacturer. The membrane was exposed to an X-ray film. The *hygB* probe hybridized with the genomic DNA of each transformant (Fig. 4A), as shown by different band patterns together with a single band, except for the wild type strain. Furthermore, Southern hybridization was performed using the *ftx* gene (819 bp) as a probe. Hybridization bands of the same size (6 kb) were detected from the nine transformants and wild type strain. Interestingly, additional hybridized bands of higher molecular weight were observed to have the same patterns (Fig. 4A), indicating that the plasmid pFTXHg was randomly integrated into different genomic locations of the *ftx* gene in the wild type genome without homologous recombination. In conclusion, although we did not achieve homologous recombination with the *ftx* target gene of *F. velutipes*, these results show that the electroporation method using protoplasts was highly efficient to stably transform *F. velutipes.* Furthermore, this protocol could be used as a basic tool for the random disruption of genes and as a major tool to study gene function.

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