Self-fusion and fusion cell isolation of transformants derived from white rot fungus Phanerochaete sordida YK-624 by simple visual method

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Self-fusion and fusion cell isolation of transformants derived from white rot fungus *Phanerochaete sordida* YK-624 by simple visual method

Short Title:

Self-fusion and fusion cell isolation of *P. sordida*

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Abstract

In order to develop a simple method for crossing two transformants, we first attempted to elucidate the fusion type (self-compatibility or -incompatible) of *Phanerochaete sordida* YK-624. Two transformants expressing green or red fluorescent protein derived from an auxotrophic mutant were constructed. Each recombinant protein fluoresced by expression as a fused protein with glyceraldehyde-3-phosphate dehydrogenase. On co-culture of both transformants, a number of sequential hyphal cells emitting dual fluorescence were formed at the contact areas of both hyphae. Some of the single cells isolated as protoplasts and chlamydospore from the co-cultures also expressed these fluorescent proteins. These results suggest that *P. sordida* YK-624 possesses a self-compatible fusion system. In addition, transformant strains with different fluorescence derived from this fungus can readily undergo self-fusion and nuclear interchange events by confrontational and mixed cultivation, and we developed a simple method that allows fused cells to be isolated as chlamydospores.

Introduction

White-rot basidiomycetes fungi are known to be unique microorganisms with the ability to degrade the natural recalcitrant aromatic polymer "lignin", one of the main components in plant biomass, to carbon dioxide and water. As the momentum to establish a sustainable society is rising, the expectation for biorefineries is also increasing. Many investigations relating to the application of white-rot fungi (WRF) that can extract fermentable carbohydrates from wood at low cost have been performed (1,2). Therefore, it is conceivable that WRF possessing superior ligninolytic characteristics (higher degradability and selectivity for lignin, less for polysaccharides) are preferable for wood biorefinery processes. The unique ligninolytic mechanism of WRF consists of ligninolytic oxidoreductases that mainly catalyze direct lignin oxidation and several accessory enzymes involved in the main oxidoreductase activities (3). To utilize this function, WRF have been employed in several studies for the pretreatment of lignocellulosic materials to obtain fermentable polysaccharides or monosaccharides (2).

However, details of the ligninolytic mechanisms, including lignin-degrading enzymes (e.g., those involved in degrading lignin fragments) have not yet been elucidated. We have attempted to elucidate the functions of several ligninolytic and related enzymes in wood decay by homologous expression of these genes in the white-rot fungus *Phanerochaete sordida* YK-624, which has good ligninolytic characteristics (4-6). However, the impact of the ligninolytic reaction of individual enzymes was relatively small, and it has been reported that a variety of enzyme genes are expressed during the onset of the ligninolytic reaction by *Phanerochaete chrysosporium* (7); therefore, it is expected that ligninolytic and related enzymes interact with one another in a complex manner for efficient lignin metabolism. Based on these facts, it was suggested that evaluation of the combination effects of ligninolytic enzymes is necessary to elucidate the details of the ligninolytic reaction.

In the case of genetic transformation, sequential recombination to introduce new phenotypes is possible by using the marker recycling method with the Cre/*LoxP* system (e.g. (8)). In contrast,

sequential transformation of the transformants generated using constructs without the *loxP* sequence is limited, and is caused by the type of selection marker. In this case, cell fusion or mating is a better choice for providing multiple beneficial characteristics in a single variant. In edible mushrooms containing WRF, mating breeding to create new strains exhibiting beneficial characteristics has traditionally been carried out. In the mating breeding method for basidiomycetes fungi, the mating type should be generally considered (9). Fungi including basidiomycetes are roughly divided into two main mating types; homothallic and heterothallic. Heterothallism is the common mating type in Basidiomycota, and heterothallic type needs another compatible individual for sexual reproduction. Homothallic fungi do not need another individual having mating compatibility for sexual reproduction. The model WRF *Phanerochaete chrysosporium* has been well investigated with regard to its mating system. For example, Alic et al. have reported on the genetic and cytological evidence indicating the primary homothallic mating system of a strain of *P. chrysosporium* (10). James et al. described the homothallic behavior of *P. chrysosporium*, and it has been predicted that the behavior was derived from hyper-polymorphism of a mating-type gene homologue, the homeodomain transcription factor genes (11).

Based on these facts, we expected that *P. sordida*, which is closely related to *P. chrysosporium*, has the potential for fruiting body and basidiospore formation under laboratory conditions. Therefore, in an attempt to evaluate self-compatibility (essential for homothallism) of *P. sordida* transformants, two transformants maintaining fluorescent protein (EGFP and mCherry) genes were generated to facilitate observation of hyphal fusion behavior on fluorescent microscopy. In addition, we attempted to establish a method to efficiently isolate the self-fusion cells that hold both recombinant genes.

Materials and Methods

Strains and Plasmids

The auxotrophic mutant strain UV-64 derived from *P. sordida* YK-624 was used for transformation (12). These were maintained on potato dextrose agar (PDA) medium, and the p*PsGPD* and p*PsGPD-EGFP,* and p*PsURA5* plasmids were as described in a previous report (12). The mCherry expression plasmid (p*PsGPD-mCherry*) that expresses the PsGPD-mCherry fusion protein under regulation of the *PsGPD* promoter was prepared by the same method as p*PsGPD-EGFP*, as reported previously (12). Synthetic *mCherry* (accession number: LC466107) redesigned based on *P. chrysosporium* codon usage was used in this experiment (GenScript Japan Inc.). *P. sordida* actin gene promoter (*Psact0* promoter, accession number: LC377778) sequence was obtained from the genome using inverse and thermal asymmetric interlaced PCR. Subsequently, restriction sites were attached to the 3'- (*Sbf*I) and 5'- (*Kpn*I, *Bmf*I, *Mfe*I, and *Xba*I) ends of the *Psact0* promoter using a primer pair (5'-cctgcaggcatgggttcgccgccgtc-3' and 5'-tctagacaattggctagcggtacctgtgagggcgagggctgctttg-3'). The restriction site attached-*Psact0* promoter and p*PsGPD* plasmid were digested with *Sbf* I and *Xba* I, respectively. The *GPD* promoter region was removed from the plasmid by this operation. The digested products were ligated to the *Psact0* promoter following purification, then the p*Psact0* pro vector was obtained. Primers (5'-ggtaccatgccggtgagcacacag-3' and 5'-tctagattacttgtacagctcgtccatg-3') were utilized to amplify the *PsGPD* linked *EGFP* sequence (*Kpn* I (5') and *Xba* I (3') restriction sites attaching) from the p*PsGPD-EGFP* plasmid. The *PsGPD-EGFP* sequence was joined to the p*Psact0* pro vector at these restriction sites. Thus, plasmid p*Psact0-EGFP* for GPD fused EGFP protein expression under control of the actin gene promoter was obtained.

Transformation

Co-transformation to the auxotrophic strain UV-64 with EGFP or mCherry expression plasmid and p*PsURA* was performed as reported previously (12). Regenerated prototrophic transformants were confirmed for the recombination of fluorescent protein genes by genomic PCR using the respective primer pairs (for *EGFP*: 5'-gacaaccactacctgagcac-3' and 5'-gggtacttgtacaatcctcc-3'; for *mCherry*: 5'-gggcgcgccttacttgtacagctcgtcca-3' and 5'-ggtaccatggtgagcaaggg-3'). Positive clones

were grown on PDA at 30°C for 10 days, and green (EGFP) or red (mCherry) fluorescence was assessed on a UV-transilluminator.

Preparation for fluorescence microscopic samples

Wild-type and individual transformants were grown on PDA medium. Before incubation, a wet cover glass was placed on the PDA side of inoculum. After 3-7 days of incubation, cover glass with thin mycelium on the upper surface was recovered for fluorescence microscopy. A confrontational culture was prepared by inoculation with both fluorescent transformants at a distance 4-6 cm on PDA medium in a 9-cm petri dish, and a wet cover glass was placed on the midpoint of inocula.

The mycelia-attached cover glasses were used for fluorescence microscopy and nuclei staining. Staining of nuclei was performed by HCl-Giemsa staining (13). An Olympus BX51 fluorescence microscope was used to obtain EGFP and mCherry images. Nuclei of protoplast was stained by Hoechst 33342.

Isolation of fusion cells

In order to obtain single cells, protoplasts were prepared from the mycelium of single or co-culture. Mycelium pellets (on co-culture, around the zone line) were punched and collected, then cultivation and protoplast preparation were performed as described previously (12). Small portions of protoplast suspension were diluted and put onto slide glass for microscopic analysis.

Homogenates obtained from PDA cultures of green and red fluorescent transformants were well mixed and spread on PDA. After 20 days of incubation at 30°C, 2.0 ml of 0.1% Tween 20 was added to the culture, the surface of grown mycelium was washed with a spreader. The wash was collected and passed through Miracloth (Merck Millipore), and then chlamydospore fluorescence was observed. Obtained spore suspensions were spread onto PDA and were incubated for several days at 30°C. The generated hyphal colonies were isolated, sub-cultured onto another PDA, and hyphal fluorescence was observed, as described above.

Results and Discussion

Number of nuclei in P. sordida cells

The number of nuclei in mycelium was investigated in *P. sordida* YK-624. The majority of mycelial cells seemed to have two to five nuclei (average 3.2 ± 1.1 nuclei per cell) on microscopy with HCl-Giemsa staining (Fig. 1). It was obvious that the presence of fusion was not clear based on nuclei number, because there was variation in nuclei number in *P. sordida* cells. Some *Phanerochaete* fungi are known to form multi-nuclei hyphae, and these fungi probably lost strict control of nuclei number in the hyphae (10,14). Therefore, we attempted to evaluate the self-compatibility of *P. sordida* YK-624 by using two recombinant genes encoding different fluorescent proteins, and investigated the expression of transformed genes in the fused hyphae. Transformed genes coding non-fluorescent protein can be assessed by PCR or enzymatic activities to obtain the fused strains having both characteristics. However, to omit these labors and to evaluate easily the self-compatibility of the fungus, fluorescent proteins were chosen as transformed genes in the present study.

Self-compatibility test by using of fluorescent transformants

EGFP and mCherry expression cassettes were co-transformed into a uracil auxotrophic strain. EGFP and mCherry fusion proteins were successfully expressed under the control of *actin* and *gpd* promoter, respectively. Nuclei number of protoplasts using for transformation was counted by Hoechst 33343 staining, around 29.2% of stained protoplast were single nucleus as a result (average nuclei number was 2.1 nuclei per cell). Photomicrographs of the mycelium around the confrontation line between the green and red fluorescent transformants in a confrontational culture are shown in Fig. 2. After 3 days of incubation, hyphae from the two transformants came into contact on a cover glass. At this time, cells showing both types of fluorescence (yellow to orange hyphae in overlay panel in Fig. 2) were observed between the green and red fluorescent hyphae. Cells showing both types of

fluorescence were present between the hyphae with single fluorescence and were connected to the respective fluorescent hyphae. This type of fluorescence pattern indicates that both EGFP and mCherry genes were retained in single cells; therefore, this suggests that nuclei from two transformants are able to migrate or be interchanged after fusion. In addition, it was frequently observed that the one to a few cells with dual fluorescence were sequentially connected. In *P. velutin*a and *Stereum* species, it has been reported that hyphal fusion occurs by binding between the hyphal apices and specific sites at the side cell wall of other hyphae, and then nuclei were interchanged before septum formation (14,15). If a rejection response occurs, cytoplasmic lysis and vacuolation are observed in fused cells. In the present study, the similar hyphal fusion forms were observed, and no cytoplasmic lysed hyphae were seen. These facts indicate that two transformants were fused by hyphal contact, and nuclei exchange events occurred around the contact region. On the other hand, the range of cells exhibiting both types of fluorescence scarcely changed, even when cultivation time was extended to several weeks. Because the protoplasts also had variation of nuclei number, how many nuclei number had been retained in the original protoplast transformed, and how many nuclei in the transformants have been modified with recombinant gene are uncertain. Nevertheless, cells showing both type of fluorescent were observed. Therefore, this result indicates that the nuclei migration or interchange events had been occurred between the two transformants.

Isolation of fusion cells

In order to confirm this fusion, protoplasts were prepared for separation to individual cells, and observed for fluorescence. Typical fluorescent protoplasts are shown in Fig. 3. Because protoplasts were prepared from sub-cultured mycelia grown from a mixture of yellow and other fluorescent hyphae, the yellow/green/red ratio probably does not reflect the original balance. Nevertheless, a protoplast showing both green and red fluorescence was observed. Therefore, this indicates that nuclei derived from each transformant coexist in single cells as a result of self-fusion, and fusion cells are able

to express both recombinant proteins. However, the appearance frequency of fusion cells was quite low with this method utilizing protoplast formation for single-cell formation (1 dual florescent cell per 300 or more protoplasts). Hence, in order to increase the frequency of contact between the green and red fluorescent hyphae, a mycelial homogenate mix of transformants was spread on PDA, followed by incubation. The fused hyphae were observed at several sites after incubation, and this suggests that fusions occurred everywhere on the mixed culture. In a previous report (16), although some strains of *P. sordida* produced chlamydospore, other types of vegetative spores were not observed in cultures of *P. sordida.* Therefore, we attempted to extract chlamydospores from the mixed culture. On fluorescence microscopy, several chlamydospores were observed and a part of spores showed dual fluorescence, and most of other spores have single fluorescence (Fig. 4 and Fig. S1). Fluorescence patterns of 45 chlamydospores were analyzed by fluorescence microscopy, and 14 dual, 22 single red, and 5 single green fluorescent spores were obtained. Four of these analyzed chlamydospores showed little or no fluorescence. In other words, the frequency of fusion cells was about 31.8%. As *P. sordida* is probably not able to form conidia under these culture conditions (16) and chlamydospores are developed from vegetative cells, we speculated that the ratio of fused and un-fused chlamydospores directly affected the appearance frequency of fusion cells. Therefore, it was expected that the increase in yield of chlamydospores that express both green and red fluorescent proteins resulted from the increase in hyphal fusion between transformants by the mixed cultivation method. Finally, hyphal colonies were regenerated from the chlamydospore suspension. Some of these colonies showed clear green and red fluorescence, and fluorescence photomicrographs of a regenerated hyphal colony is shown in Fig. 5. The fluorescence pattern had been kept after 5 time or more sub-culture, so it was suggested that both nuclei containing each fluorescent protein gene are stable in the fused cell. Thus, we demonstrated that it is possible to generate and isolate novel transformants inheriting both characteristics from two independent transformants of *P. sordida* YK-624 by a simple procedure.

In conclusion, we developed a simple method for the creation of a fusion strain expressing

two individual proteins derived from two independent *P. sordida* transformants retaining the respective recombinant genes. Firstly, in order to reveal the self-compatibility of *P. sordida* YK-624, and for observation of nuclear migration or interchange between self-fused cells, two types of recombinant fluorescent label (EGFP and mCherry) were constructed. We then aimed to establish an isolation method for fusion cells. On confrontational culture, green and red fluorescent hyphae were physically linked in the contact area, and some cells between both single fluorescent hyphae showed dual fluorescence. This indicates that nuclear exchange occurred following cell fusion between the two transformants, and that the cells producing recombinant proteins derived from parent transformants can be generated using a simple procedure. Finally, the efficient isolation and recovery of the dual fluorescence strains as hyphae regenerated from chlamydospores obtained from agar culture of homogenate mixtures of parent strains were accomplished using a simple experimental procedure. Therefore, the fusion strains derived from the *P. sordida* transformants that individually, homologously express ligninolytic or related enzymes constructed in previous studies may be able to be generated using this method (4-6). As a result, the investigation of interactions between various enzymes will become possible. The fusion strains can also be utilized for evaluation of interactions between the enzymes. In the future, it will be possible to observe the interaction effects between three or more enzymes after establishment of cultivation methods for basidiospore formation of *P. sordida* YK-624.

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Figure captions

Fig. 1. Microscopic analysis of number of nuclei in *P. sordida* YK-624 hyphal cells by Gimsae-HCl staining. Black and white arrow heads indicate nuclei and cell walls, respectively. Scale bar is 10 μm.

Fig. 2. Fluorescence at hyphal contact area in confrontational culture of EGFP and mCherry expression transformants. Merge panel is an overlay of photographs of bright field, EGFP, and mCherry fluorescence. BF, EGFP, and mCherry panels show microphotograph of bright field, green fluorescence, and red fluorescence, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.) Scale bar is 50 μm.

Fig. 3. Fluorescence patterns of protoplasts derived from EGFP, mCherry expression transformants, and fused cells of these transformants. Panel descriptions are the same as those in Fig. 2. Scale bar is 5 μm.

Fig. 4. Fluorescence microscopy images of a chlamydospore which showing both green and red fluorescence obtained from the 20-day agar cultures incubated with mixed hyphal homogenates of EGFP and mCherry expression transformants. Panel descriptions are the same as those in Fig. 2. Scale bar is 10 μm.

Fig. 5. Fluorescence pattern and bright field photomicrograph of regenerated hyphal colony from chlamydospore suspension. Panel descriptions are the same as those in Fig. 2. Scale bar is 50 μm.

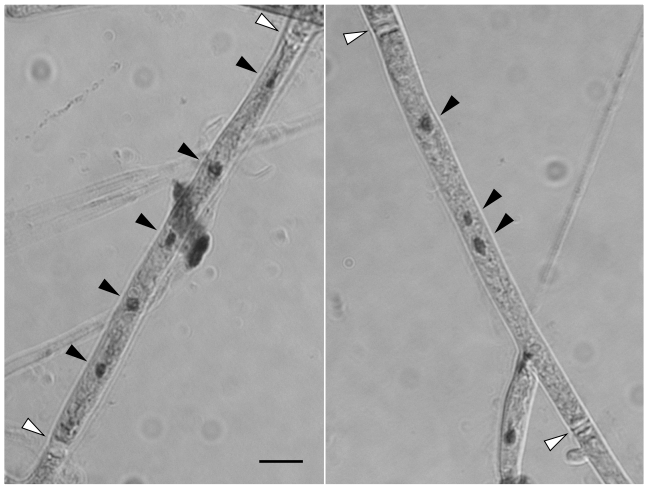

Fig. 1

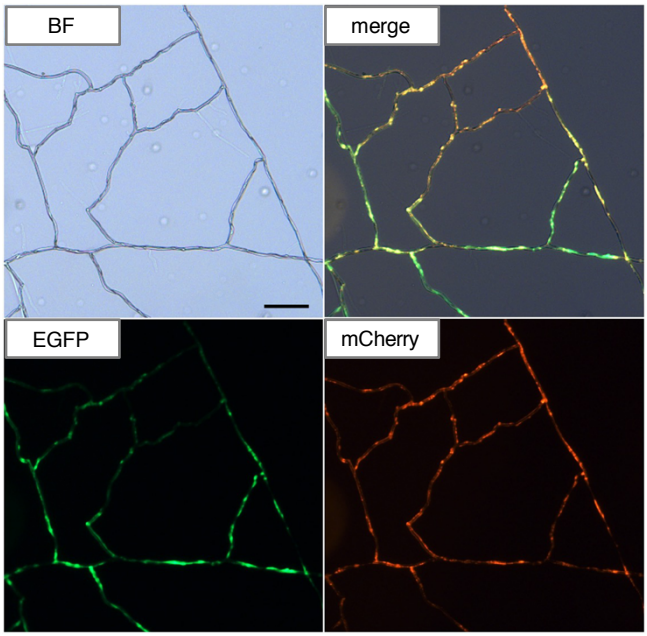

Fig. 2

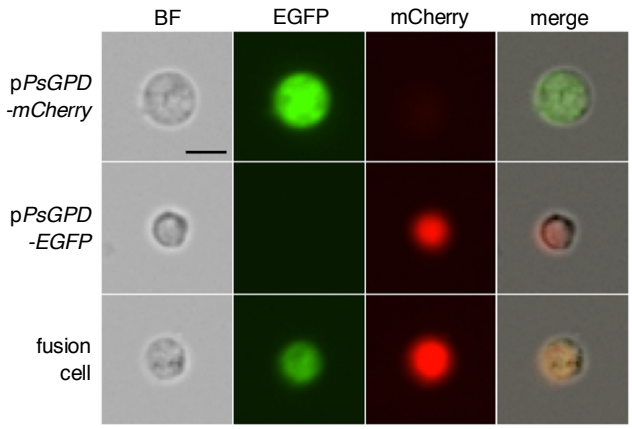
BF
EGFP
mCherry
merge
pPsGPD -mCherry
pPsGPD -EGFP
fusion cell

Fig. 3

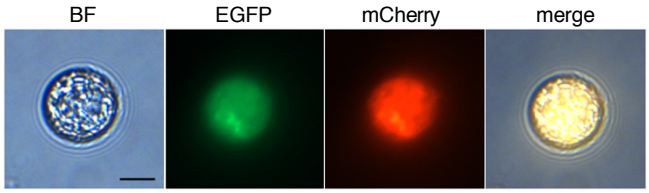

Fig. 4

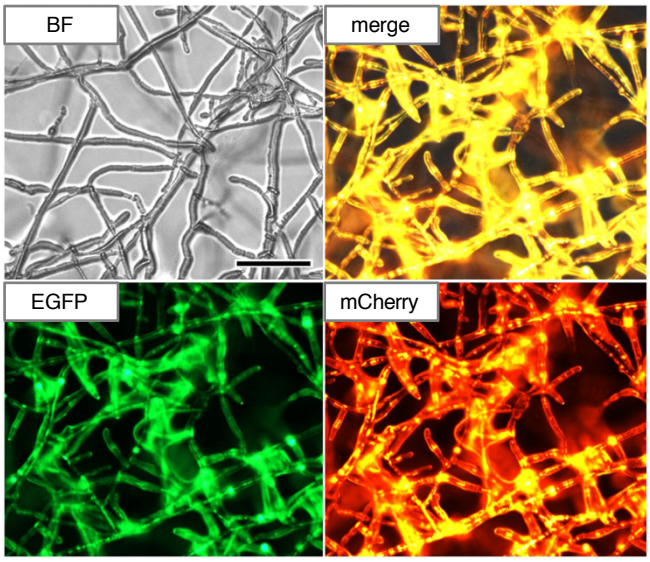

Fig. 5