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Note

Protoplast-Mediated Transformation of the Filamentous Fungus *Cladosporium phlei*: Evidence of Tandem Repeats of the Integrative Transforming Vector

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To facilitate the genetic manipulation of *Cladosporium phlei*, a causal agent of leaf spot disease in timothy (*Phleum pretense*), protoplast-mediated transformation of *C. phlei* has been developed and the resulting transformants were characterized in this study. Hygromycin B resistance was applied as a dominant selection marker due to the sensitivity of *C. phlei* to this antibiotic. The transformation efficiency ranged from approximately 20-100 transformants per experiment. Southern blot analysis of stable transformants revealed that transformation occurred by way of stable integration of the vector DNA into the fungal chromosome. PCR analysis and plasmid rescuing of randomly selected transformants suggested that integration of tandem repeat copies of vector DNA was common. In addition, multiple integrations of the transforming vector at different chromosomal sites were also observed. The establishment of a transformation method for *C. phlei* facilitates strain improvement of this fungus and can be applied as an initial step in the molecular analysis of pigment production in this fungus.

Keywords : *Cladosporium phlei*, fungal transformation

The hypomycetous fungus *Cladosporium phlei* (C. T. Gregory) de Vries is known to cause leaf spot disease in timothy (*Phleum pratense*) (known as a purple eyespot), which is the most common foliar disease of timothy. Leaf-spot disease is recognized by a characteristic circular purple spot that is followed later by brown spots with white to grayish-fawn centers on the leaves (Shimanuki, 1987). During cultivation, this fungus showed characteristic deep red pigmented mycelia due to the presence of the secondary metabolite phleichrome (Yoshihara et al., 1975). Phleichrome, identified as a pigment of *C. phlei*, belongs to a group of fungal perylenequinones and it is a derivative of 4,9-dihydroxyperylene-3,10-quinone.

Recently, fungal perylenequinones, which include phleichrome, have been investigated with great interest because of their photodynamic properties (Olivo and Chin, 2006). Among those related compounds derived from 4,9-dihydroxy-3,10-perylenequinone, hypocrellin has gained considerable attention as a potential photosentitizer for photodynamic therapy (PDT) (Diwu, 1995) because of its light-induced antitumor properties (Lee et al., 2006; Wu et al., 2000a, 2000b; Zhang et al., 1998) and antiviral activity, particularly against the human immunodeficiency virus (HIV) (Fehr et al., 1995; Kraus et al., 1996). The recent progression of hypocrellins has further increased in value with the commercialization of hypocrellins A (HA), B (HB), and hypericin. The hypocrellins have several advantages over the currently used photodynamic therapeutic agent, Photofrin II (Reynolds, 1997), including high quantum yields of singlet oxygen ($^1O_2$), low toxicity, easy preparation and purification, low aggregation tendency and rapid metabolism *in vivo* (Hudson et al., 1997). However, hypocrellins also have several limitations; they do not have enough hydrophilicity in physiological conditions and do not show sufficiently strong absorption at the phototherapeutic window region (600-900 nm). Therefore, great efforts have been made to synthesize hypocrellin derivatives with improved hydrophilicity and polarity. One of major factors limiting the development and production of improved derivatives appears to be a lack of a sufficiently large source of hypocrellin and its sustainable production because of the inaccessibility of its natural source, *Hypocrella bambusae*, which is harvested in limited regions and seasons in China. In addition, although chemical synthesis of the key intermediate 4,9-dihydroxyperylene-3,10-quinone has been proposed, the synthesis of 4,9-dihydroxyperyene-3,10-quinone, the dimerization product of 1,2-naphthoquinone, which is in turn prepared from commercially available 3,5-dimethoxybenzaldehyden using a nine-step-reaction (Hauser et al., 1994), is itself a rate-limiting step and, consequently hampers the wide use of chemical

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synthesis. Therefore, as an alternative, use of other fungi that can provide fungal perylenequinone was suggested. In a previous study, we report the culture characteristics of *C. phlei* with respect to the sustainable production of phleichrome, a derivative of 4,9-dihydroxyperylene-3,10-quinone, and discuss putative chemical modifications for an improved PDT agent (Lee et al., 2007).

Since the first transformation of filamentous fungi (Ballance et al., 1983; Yelton et al., 1984), several transformation techniques have become available. The vast majority of transformation protocols require the preparation of protoplasts, followed by permeabilizing the cell membranes with electroporation or a combination of $CaCl_2$ and polyethylene glycol (PEG), and then screening putative transformants showing the characteristics of the selectable marker in the integrating vector. In this study, we describe an efficient protoplast-mediated transformation system based on expression of a dominant selectable marker gene (*hph*) encoding hygromycin B phosphotransferase activity, which confers resistance to the aminoglycoside antibiotic hygromycin B. Developing a well-defined, efficient system for introduction of a DNA sequence into *C. phlei* will facilitate strain improvement of this fungus by cloning of *C. phlei* genes that govern biosynthesis of phleichrome and its regulation. This paper describes experiments representing a first step toward the genetic manipulation of the relatively uncharacterized fungus *C. phlei*.

To obtain a dominant selectable marker, sensitivity of *C. phlei* to hygromycin B was tested on V8 juice-media complemented with different concentrations of hygromycin B. When media was supplemented with 10 μg of hygromycin B per ml, marginal or superficial growth of mycelia was observed. However, no growth of *C. phlei* was observed when more than 50 μg/ml of hygromycin B was applied (Fig. 1). Therefore, 50 μg of hygromycin B per ml of media was determined to be selective for the transformed strain.

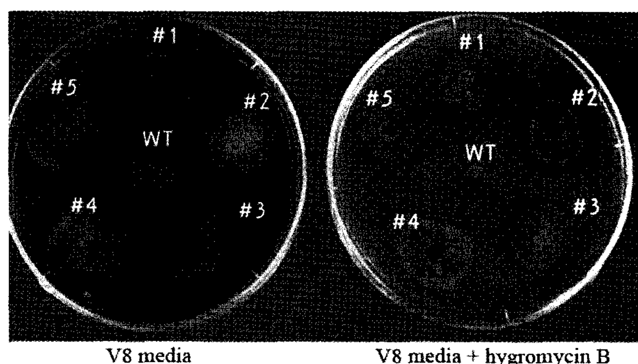

Fig. 1. Colony morphology of *C. phlei* on hygromycin B containing selective V8-agar medium. WT and numbers indicate the wild-type recipient strain and strain numbers of transformants, respectively.

C. phlei protoplasts were prepared using the procedures of Churchill et al. (1990). 5×10^8 freshly harvested conidia were inoculated into 200 ml of V8-juice media supplemented with 5% glucose. The cultures were incubated with shaking at 20°C under continuous darkness for 24 h, harvested by centrifugation at 2,500 g for 5 min at 4°C, and washed three times with 0.6 M $MgSO_4$. The washed mycelium was resuspended in 0.6 M $MgSO_4$ solution, harvested by filtration through a Miracloth, and blotted with sterile paper towels to remove excess liquid. The cells were resuspended in a filter-sterilized osmotic medium, then filter-sterilized glucuronidase and Novozym234 were added as described previously (Churchill et al., 1990). The protoplast concentration was determined with a hemacytometer and stored at −70°C in 100 μl of 4 parts STC buffer (1 M D-sorbitol, 100 mM Tris-HCl pH 8.0, 100 mM $CaCl_2$) and 1 part PTC buffer (40% PEG 4000, 100 mM Tris-HCl pH 8.0, 100 mM $CaCl_2$) at a final concentration of 1×10^8 cells/ml until use. Each 200 ml culture yielded 1-5 $\times 10^8$ protoplasts (0.4-1.0 $\times 10^7$ protoplasts/g mycelium) with 40-60% viability after transfer to nonselective regeneration medium. When the protoplasts were subjected to transformation, 10 mg of DNA of pSHG26, which cloned a 2.4 kb *Sal*I fragment of pDH25 hygromycin B resistant gene into the *Sal*I site of pBluescript II SK (Stratagene, La Jolla, CA, U.S.A.), was added to a 100 μl aliquot of protoplasts (1 $\times 10^7$ cells) and then PEG and $CaCl_2$ treatments were performed as described previously (Churchill et al., 1990). The transformation procedure itself caused 50%-70% mortality among viable cells. Protoplasts subjected to treatment with no DNA did not show any colonies on the selective media and the rate of spontaneous mutation to resistance was estimated to be less than one out of 5×10^8. Hygromycin B-resistant colonies were visible on the selective top agar 7-10 days after plating the DNA-treated protoplasts although slower-growing colonies appeared up to 3 weeks later. All colonies, except a few due to the sparse background growth on hygromycin B-containing regeneration media, were stably resistant upon transfer from the selective regenerating media to fresh selective media. The transformation efficiency was low compared to that of *Cryphonectria parasitica*, which exceeded 10^5 transformants per mg vector DNA. Approximately 2-10 transformants per mg vector DNA were obtained.

Mitotic stability of transformants was examined by successive transferring on alternating selective and nonselective media as described previously (Kim et al., 1995). Of the 50 transformants tested, 49 showed normal growth on selective media even after 3 weeks of serial growth on nonselective media. However, one transformant showed restricted growth around the transferring agar plug on the

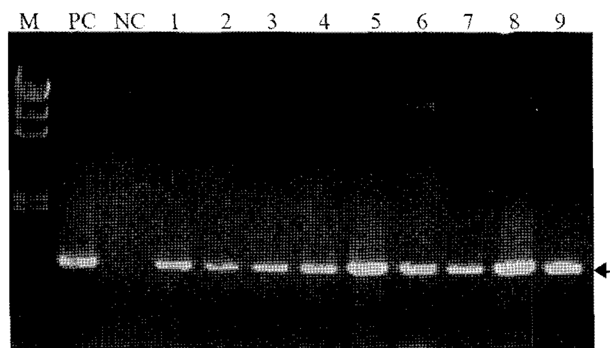

Fig. 2. PCR analysis of transformed *C. phlei* using primers corresponding to the coding region of the hygromycin B resistance gene (*hph*). Lane M indicates the *Hin*dIII-digested λ DNA as a size marker. PC and NC represent results using DNA of pSHG26 and the wild-type strain as positive and negative controls, respectively. Lanes 1-9 show the PCR amplicons of transformants. Strain identifications are equal to lane numbers. The arrow on the right indicates the PCR-amplified *hph* fragment with a size of 1.0 kb.

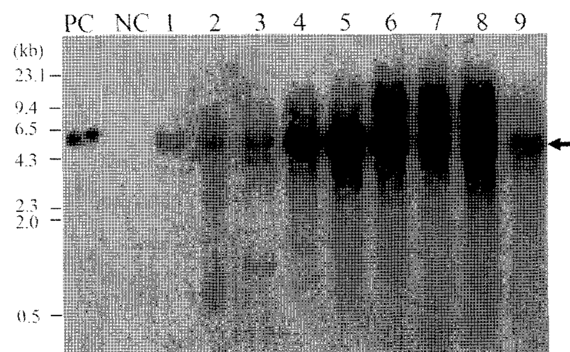

Fig. 3. Southern blot analysis of transformants. Genomic DNA from randomly selected transformants was digested with *Eco*RV, which restricts the plasmid at one site outside the region of the probe. NC and PC are DNA from the wild-type strain and pSGH26 as negative and positive controls, respectively. Lanes 1-9, DNA from transformants 1-9 (TNF-1 to TNF-9). Numbers on the left indicate the DNA sizes in kb. An arrow indicates the unit-size of the transforming vector when digested with the corresponding restriction enzyme.

selective media and reverted to hygromycin B sensitivity. These results indicated that the selective marker gene, *hph*, for hygromycin B phosphotransferase appeared to be mitotically stable regardless of the selection conditions, possibly due to the stable integration of transforming vector DNA. However, the one example of an abortive transformant suggests possible loss or rearrangement of portions of the incoming vector DNA.

PCR amplification of the transforming plasmid and Southern blot analysis were performed to determine whether the hygromycin B resistant colonies arising on selective regenerating media were indeed transformants. PCR amplification using internal primers for the coding region of *hph* showed the expected size of 1.0 kb for all selected transformants, while no amplicon was observed for the untransformed recipient strain (Fig. 2). In addition, genomic DNA from nine representative transformants as well as untransformed recipient strain were isolated and hybridized with an 0.8 kb *Eco*RI/*Bam*HI fragment of the *hph* gene. Undigested DNA from transformants yielded hybridizing signals only in the chromosomal-sized-DNA region, whereas no hybridization signal was observed in the recipient strain (data not shown). These results clearly indicate that the transformation of *C. phlei* occurred by integration of the vector DNA into the host chromosomal DNA and suggest that the integrated vectors persist stably in the fungal chromosome. No evidence of autonomous replication of the transforming plasmid was observed; attempts to transform *E. coli* to ampicillin resistance with a total DNA preparation from the transformants were not successful. The nature of integration of the vector was further inferred from Southern blot analysis using various enzyme and probe combinations. When the DNA was digested with the restriction enzyme *Eco*RV, which restricts the plasmid at one site outside the region of probe, and probed with the 0.8 kb *Eco*RI/*Bam*HI *hph* fragment, six of nine transformants showed more than two hybridizing bands and, more interestingly, all yielded a 5.4 kb band (Fig. 3). Because *Eco*RV digestion yields a hybridizing band at 5.4 kb which is the same size as that of the transforming vector and the intensity of the signal from the unit-sized band was stronger than that of the other band, it is likely that tandem repeats of the transforming vector integrated into the fungal chromosome. To confirm the tandem integration of the vector, we also examined hybridization patterns with the enzymes *Hin*dIII and *Eco*RI, which restrict the plasmid at one and two sites outside of the probe, respectively (Fig. 4). Again, all transformants showed the unit-sized bands of 5.4 kb and 4.8 kb, indicating integration as a tandem repeat of vectors. In addition, transformant TNF-3 showed two additional hybridizing bands besides the unit-sized band, suggesting multiple integrations at different chromosomal sites. We then examined whether the integration of tandem repeats of the transforming vector depended upon the physical status of transforming DNA (i.e., linear or circular form). Transformation using linear DNA was conducted and Southern blot analysis of eight randomly selected transformants resulted in five transformants with a hybridizing band at the unit size of the vector (data not shown). Although further studies using different transforming vectors are required, these results suggested that integration as a tandem repeat of the transforming vector is characteristic of this fungus.

Plasmid rescue from a representative transformant TNF-1 was conducted according to the method of Kim (1997).

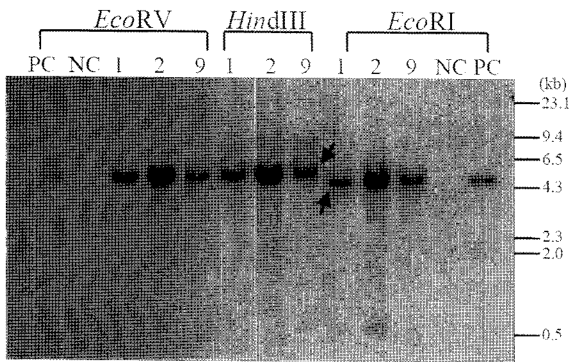

Fig. 4. Southern blot analysis of selected transformants using the restriction enzymes *Eco*RV, *Hin*dIII, and *Eco*RI. PC and NC are the results obtained using DNA of pSHG26 and the wild-type strain as positive and negative controls, respectively. Strain identifications are given at the top of each lane. Numbers on the right indicate the DNA sizes in kb. Arrows indicate the unit-size of the transforming vector when digested with the corresponding restriction enzyme.

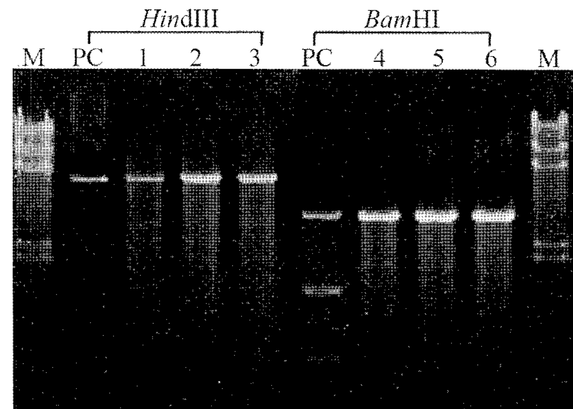

Fig. 5. Agarose gel electrophoresis of three rescued plasmids using *Hin*dIII- and *Bam*HI-digested DNA of TNF-1. Lane M indicates the *Hin*dIII-digested λ DNA size marker; PC, pSHG26 as a positive control, Lanes 1-6 are rescued plasmids from transformant TNF-1. Enzymes using for the rescuing and digestion of rescued plasmid are indicated at the top of panel. Note that the plasmid rescued using *Bam*HI showed a 3.3 kb band when digested with *Bam*HI, which is equal to the largest fragment size of *Bam*HI-digested plasmid pSHG26 and contains the cloning vector pBluescript II SK. No additional bands at 1.4 and 0.7 kb corresponding to pSHG26 were observed in the rescued plasmid.

Briefly, genomic DNA from TNF-1 was digested with *Hin*dIII, size-fractionated on a 0.6% agarose gel, and electroeluted for fragments larger than 3.0 kb from the gel. The electroeluted DNA fragments were self-ligated and used to transform *E. coli* strain TOP10, and the resulting ampicillin resistant colonies were screened. Marker rescue using 5 μg of recirculated DNA yielded 17 ampicillin resistant colonies from which plasmids were extracted for further analysis. Restriction map and size analysis of the rescue plasmids revealed that 16 plasmids were identical to the transforming vector pSHG26, which can be inferred as additional evidence of incorporation of the transforming vector as tandem repeats (Fig. 5). Similarly, marker rescue using *Bam*HI-digested DNA from TNF-1 was also conducted. *Bam*HI is the enzyme which restricts three sites within pSHG26, one from the multiple cloning site of the cloning vector and two in the hygromycin B resistance cassette. Again, rescued plasmids showed the 3.3 kb plasmid which is equal to the largest fragment of *Bam*HI-digested pSGH26 indicating integration of the vector as a tandem repeat (Fig. 5). No ampicillin resistant *E. coli* was obtained using undigested DNA from the transformant, which is in good agreement with the fact that no autonomously replicating transforming vector is present.

The results described here illustrate the ease with which foreign DNA can be introduced into *C. phlei*, a relatively uncharacterized fungus. We demonstrated that transformation of *C. phlei* occurred by chromosomal integration and multiple integrations of the vector at different chromosomal loci were also observed. More interestingly, integration in the form of tandem repeats of the transforming DNA vector was common (Fig. 6) and the integration site was likely to be random. An efficient method for transfer of foreign DNA into the *C. phlei* genome as well as its stable expression should greatly facilitate the genetic manipulation of this fungus to enable large-scale production of useful metabolites such as phleichrome. In addition, although expression levels may not be tightly correlated with gene copy number (van den Hondel et al., 1991), increasing transgene dosage is still a common strategy for improving heterologous expression. Therefore, *C. phlei* is a promising fungal expression host as transformation of this fungus

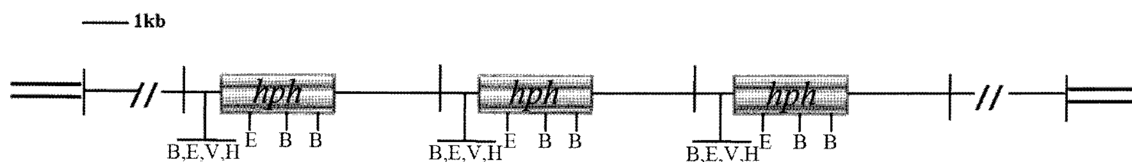

Fig. 6. Schematic diagram of the integration of tandem repeats of the transforming vector. Chromosomal DNA is indicated by the double line. The transforming vector pSHG26, consisting of the cloning vector pBluescript II SK and the 2.4 kb hygromycin B resistance cassette (*hph*^{R}), are represented by a single line and a closed bar, respectively. E. V, H, and B in pSHG26 indicate sites for restriction enzymes, *Eco*RI, *Eco*RV, *Hin*dSKI and *Bam*HI, respectively.

results in stable integration of tandem repeats of the transforming DNA.

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