MUTATIONAL ANALYSIS OF A *GPD* PROMOTER SEQUENCE USING TRANSIENT GENE EXPRESSION – A NEW PROMOTER ASSAY SYSTEM FOR BASIDOMYCETOUS FUNGI

YOICHI HONDA, EIJI TANIGAWA, TAKAHITO WATANABE, TAKASHI WATANABE
Research Institute for Sustainable Humanosphere (RISH), Kyoto University,
Gokasho, Uji, Kyoto 611-0011,
Japan
yhonda@rish.kyoto-u.ac.jp

ABSTRACT

During the development of a transformation system in the selective lignin-degrading white rot fungus, *Ceriporiopsis subvermispora*, we observed many unstable transformants showing transient resistance to the selection drug, hygromycin B, in addition to a few stable and integrative transformants. The number of drug resistant transformants changed when distinct promoter sequences were used to drive the gene for hygromycin phosphotransferase (*hph*). We used this phenomenon to estimate promoter activity by using the number of colonies on the first screening plate as a *merkmal*. A series of deletion mutants of *C. subvermispora gpd* (glyceraldehyde-3-phosphate dehydrogenase) promoter fused to *hph* were constructed and transformation experiments showed that a 141-bp sequence was required for the basic promoter function. Subsequent point- and insertion-mutation analyses were conducted to test the functional significance of putative *cis* elements predicted from conserved sequences in ascomycetes. The assay system presented in this report may serve as a new tool to understand the regulation mechanism for gene expression in basidiomycetes, minimizing undesirable effects from multiple and random integration of the reporter construct in the chromosomes of the stable transformants.

Keywords: Promoter assay; Transformation; Transient gene expression

INTRODUCTION

In mushroom-forming fungi, transformations are brought about by the integration of the introduced DNA sequence into the host chromosome. Generally, integration occurs randomly at multiple sites and the copy number of the insert varies among the transformants [1, 2]. The resultant phenotype is stably maintained in mitotic and meiotic cell divisions. This situation has made it very difficult to do promoter assays in these stable transformants since the expression of a gene is affected by its dosage and position in the chromosome. In 2004, in order to overcome this problem, Bertossa et al.[3] used a large number of transformants for each reporter construct containing deletion or insertion mutant derivatives of the *Coprinopsis cinerea cgl2* promoter sequence. They demonstrated that a 627-bp sequence is a minimal promoter and that a 120-bp internal region was required for induction of the gene in darkness by measuring the transcript level in 100-120 different strains gathered from plate cultures [3]. However, it is not always so easy to obtain such a large number of transformants in other mushroom-forming fungi. It is also

very difficult to collect RNA samples from so many transformants in liquid culture. In this context, a precise, simple and rapid promoter assay method is required.

In addition to stable and integrative transformation, unstable transformants have occasionally been reported in some mushroom-forming fungi [4-7]. They were sometimes described as "false" or "abortive" transformants, although it was often difficult to say if they were all background unless similar phenomenon was also observed equally in the control experiment with no DNA introduction. These unstable transformants might be useless for isolating desirable strains by molecular breeding, but it is possible that some could be used as strains in a temporal stage with transient expression of the introduced gene.

Transcription is often controlled in its initial efficiency, and *cis*-acting regulation elements have been found near to or within the promoter region of a gene in various organisms. In eukaryotes, they are recognized by specific *trans*-acting factors including general transcription factors and activators. General transcription factors are thought to recognize the fundamental structure of the promoter to recruit the pre-initiation complex, which releases RNA polymerase to produce mRNA. Activators interact with general transcription factors and influence the efficiency of several transcriptional steps including initiation. Many kinds of transcriptional factors and their binding sequences have been reported and registered in the database that allow us to find putative regulation elements in a DNA sequence with computer software such as MOTIFS [8]. Also, many reports have described the existence of such consensus sequences in the promoter regions of basidiomycetous genes. However, most of the data relating to the fungal transcription factors and their target sites have come from experimental results in ascomycetes. Confirmation of the physiological function of those reported in basidiomycetes is largely lacking. Previously, it was thought that transcriptional regulation was very similar among fungi, and ascomycetous promoters were used to express recombinant genes in basidiomycetes. However, with a few exceptions, most of these trials were unsuccessful. Moreover, gene expression sequences were often incompatible between different basidiomycete species. However, it is now thought that, in basidiomycetes, there might be special features associated with the fundamental sequence, or conformational requirements for promoter function and its regulation. In other words, the molecular mechanisms underlying transcriptional recognition and regulation in basidiomycetes are mostly unclear.

MATERIALS AND METHODS

Plasmids and strains. Recombinant plasmids, pCsGi-*hph* and pCsbtubi-*hph* contain the gene for hygromycin phosphotransferase (*hph*) driven by *gpd* (glycelaldehyde-3-phosphate dehydrogenase) and *β-tublin* promoters from *Ceriporiopsis subvermispora*, respectively (unpublished). *C. subvermispora* Fp-90031 ATCC 90467 was used as the host strain for all transformation experiments. Plamids for the promoter assay were constructed from pCsGi-*hph* by introducing deletion, base-substitution and insertion of synthesized oligo-DNA.

Promoter assay using transient gene expression. The basic protocol for transformation of *C. subvermispora* was the same as the PEG/$CaCl_2$ method used for *Pleurotus ostreatus* [2]. Hygromycin B (Hyg), 100 μg/ml, was used to screen for drug resistant transformants. The activity of promoter sequences was estimated by counting the number of Hyg-resistant colonies on the first screening plate.

RESULTS AND DISCUSSION

To develop a transformation system in *C. subvermispora*, we observed isolates with drug resistance on the first screening plate containing 100 µg of hygromycin B (HygB). When pCsGi-*hph* and pCsbtubi-*hph* were used as vector plasmids, about 4% of the isolates showed stable drug resistance after four successive subcultures on screening plates containing the same concentration of HygB. Remaining isolates failed to grow after the second or third subcultures. Using Southern hybridization, the introduced marker sequence was shown to exist in stable transformants but not in unstable transformants (data not shown). No HygB-resistant colonies were recorded in the control experiment in which no DNA was introduced into the protoplasts. These results indicated that the unstable transformants were not background colonies but transient transformants showing a part-time drug resistance caused by expression of the introduced constructs, most likely in an extra-chromosomal manner. Furthermore, when plasmid pLG-*hph*, containing *hph* under the control of *Lentinus edodes ras* promoter, was used as a vector plasmid, less drug-resistant isolates were observed and no stable transformants were obtained. This suggested that differences in the promoter sequence affected the number of drug resistant isolates and also the possibility of stable transformation. The emergence of drug resistant colonies on the screening plate depends on the balance between growth inhibition by the drug and expression of the relevant drug marker gene, irrespective of its intra- or extra-chromosomal existence. In the case of the extrachromosomal state especially, this balance can be changed during mycelial growth or transfer to a new culture plate, which leads to unstable maintenance of drug resistance.

Table 1: Deletion analysis of the *gpd* promoter

Promoter length (bp)	Number of HygB-resistant colonies	%
1233	139	100
836	85	61
637	92	66
503	81	58
317	67	48
201	47	34
141	65	47
120	5	4
52	0	0
0	0	0

We used this phenomenon to estimate promoter activity by using the number of the colonies on the first screening plate as a *merkmal*. A series of deletion mutants of *C. subvermispora gpd* promoter fused to the *hph* coding sequence was constructed and introduced into wild-type *C. subvermispora* (Table 1). It was demonstrated that a 141-bp sequence is required for the basic promoter function. A point mutation analysis was then conducted using a 200-bp promoter sequence. A significant decrease in the colony numbers was observed when the mutation was introduced into one of the putative sequences for the GCN4-binding site, the AP-1 binding site and the TATAA box. In contrast, almost no effects were observed for mutants in the

consensus sequence for the GATA factor and Sp-1. Furthermore, introduction of a synthetic GCN4-bonding sequence suppressed the mutation in the original GCN4-binding site. These results strongly suggested that, in *C. subvermispora*, the GCN4-binding site, the AP-1 binding site and the TATAA box play an important role in the function of *gpd* promoter.

CONCLUSIONS

Unstable transient transformants were observed in the selective lignin-degrading fungus, *Ceriporiopsis subvermispora* and used for a new promoter assay system. The number of transformants on the first screening plate changed when distinct promoter sequences were used to drive the marker gene. A deletion mutant analysis was successfully used to show that a 141-bp sequence was required for the basic function of the *C. subvermispora gpd* promoter. The promoter assay system presented in this report may serve as a new tool for understanding the basic organization of promoter sequences and the regulation mechanism(s) of gene transcription in basidiomycetes.

ACKNOWLEDGEMENT

We thank Dr. Toshitsugu Sato, Kitami Institute of Technology, for the gift of plasmid pLG-*hph*. This work was supported by a grant-in-aid for the promotion of science (to Y.H).

REFERENCES

[1] Binninger D.M. et al. (1987). DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. *EMBO J*. 6: 835-840.
[2] Honda Y, et al. (2000). Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. *Curr*. *Genet*. 37: 209-212.
[3] Bertossa R.C. et al. (2004). Promoter analysis of *cgl2*, a galectin encoding gene transcribed during fruiting body formation in *Coprinopsis cinerea* (*Coprinus cinereus*). *Fungal Genet*. *Biol*. 41: 1120-1131.
[4] Peng M. et al. (1992). Recovery of recombinant plasmids from *Pleurotus ostreatus* transformation. *Curr*. *Genet*. 22: 53-59.
[5] Kim B.G. et al. (2003). Transformation of the edible basidiomycete *Pleurotus ostreatus* to phleomycin resistance. *Mycobiology*. 31: 42-45.
[6] Randall T.A. and Reddy C.A. (1992). The nature of extrachromosomal maintenance of transforming plasmids in the filamentous basidiomycete *Phanerochaete chrysosporium. Curr*. *Genet*. 21: 255-260.
[7] Birch P.R. et al. (1998). A reporter system for analysis of regulatable promoter functions in the basidiomycete fungus *Phanerochaete chrysosporium*. *J*. *Appl*. *Microbiol*. 85: 417-24.
[8] http://molbiol-tools.ca/Motifs.htm