Heterologous Gene Expression and Transcriptional Regulation of Manganese Peroxidase Gene Expression in the Basidiomycete *Phanerochaete chrysosporium*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract

Heterologous Gene Expression and Transcriptional Regulation of Manganese Peroxidase Gene Expression in the Basidiomycete *Phanerochaete chrysosporium*

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Lignin peroxidase (LiP) and manganese peroxidase (MnP) are the major components of the lignin degradation system in the white-rot fungus *Phanerochaete chrysosporium*. Expression of *P. chrysosporium* LiP-encoding genes and MnP-encoding genes (*mnp*) is activated by nutrient nitrogen depletion. Moreover, expression of *mnp* requires the presence of Mn^{2+}. Both nitrogen and Mn^{2+} levels regulate *mnp* expression at the transcriptional level.

The enhanced green fluorescent protein (GFP) gene (*egfp*) was used as a reporter in *P. chrysosporium* for gene expression driven by the glyceraldehyde-*p*-dehydrogenase gene (*gpd*) promoter and the MnP isozyme 1 gene (*mnp1*) promoter. The 1.1-kb *gpd* promoter or the 1.5-kb *mnp1* promoter was joined with the 5′ end of the *egfp* coding region, while the 250-bp *mnp1* 3′ untranslated region (UTR) was joined with the 3′ end of the *egfp* coding region. Efficient expression of *egfp* was only observed when an additional intron-containing sequence was inserted in the 5′ end of the *egfp* transcription unit. The GFP fluorescence levels faithfully reported the

activity of both the *gpd* and *mnp1* promoters. The 1.5-kb *mnp1* promoter was sufficient for promoting *egfp* expression only under nitrogen-limited and Mn^{2+}-sufficient conditions, similar to that required for endogenous MnP expression. A series of deletion, replacement and translocation mutations were introduced in this promoter, and their effects on *egfp* expression in response to Mn^{2+} status were examined. A 33-bp *cis*-element residing 521 bp upstream of the translation start codon in the *mnp1* promoter was identified that participates in the Mn^{2+}-dependent regulation of *mnp1* promoter activity. Negative control is exerted on this promoter element under Mn^{2+}-deficient conditions. All mutations constructed in the 1.5-kb *mnp1* promoter did not affect nitrogen-dependent gene regulation.

An efficient transformation system for *P. chrysosporium* was developed using the bacterial Bialaphos resistance gene (*bar*) as a selectable marker. The plasmid containing the *bar* coding region joined with the *gpd* promoter and the *mnp1* 3′ UTR onfers resistance to phosphinothricin (the active component of Bialaphos) when introduced into the *P. chrysosporium* wild-type strain OGC101. An efficient homologous expression system for *P. chrysosporium* MnP isozyme 1 was constructed using *bar* as a selectable marker.

CHAPTER 1
INTRODUCTION

1.1 WHITE-ROT FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

1.1.1 The Biology of *Phanerochaete chrysosporium*

Phanerochaete chrysosporium is classified as a Homobasidiomycete, Aphyllophorale, Polyporaceae [Burdsall and Eslyn, 1974]. It is a white-rot fungus that can digest both the lignin and cellulose in wood [Crawford, 1981].

Lignin, cellulose and hemicellulose are the primary polymeric components of the plant cell wall. They occur in wood and in other true vascular plants, ferns, and club mosses. After cellulose, lignin is the second most abundant renewable carbon source on earth and constitutes ~15-36% of the dry weight of wood [Sarkanen and Ludwig, 1971]. Lignin is an extremely complex, recalcitrant, insoluble and optically inactive polymer composed mostly of phenylpropanoid units, and it is resistant to hydrolytic attack. A structural scheme of spruce lignin is shown in Fig. 1.1. Lignin lends stability and rigidity to the plant cell wall and protects cellulose from degradation. It also forms a natural barrier in plants against pathogens. However, the decomposition of lignin is an essential step in the earth's carbon cycle as well as in the utilization of cellulose as a source for fuel and fiber by humans [Crawford, 1981; Eriksson, 1990]. White-rot fungi are the only known organisms that can degrade lignin to CO_2 and H_2O completely [Kirk and Farrell, 1987]. This fungal group includes many edible mushrooms as well as forest pathogens. Understanding the lignin-degrading pathways in white-rot fungi is a prerequisite for us to comprehend the earth's carbon cycle and to introduce biotechnology to the forest product industries.

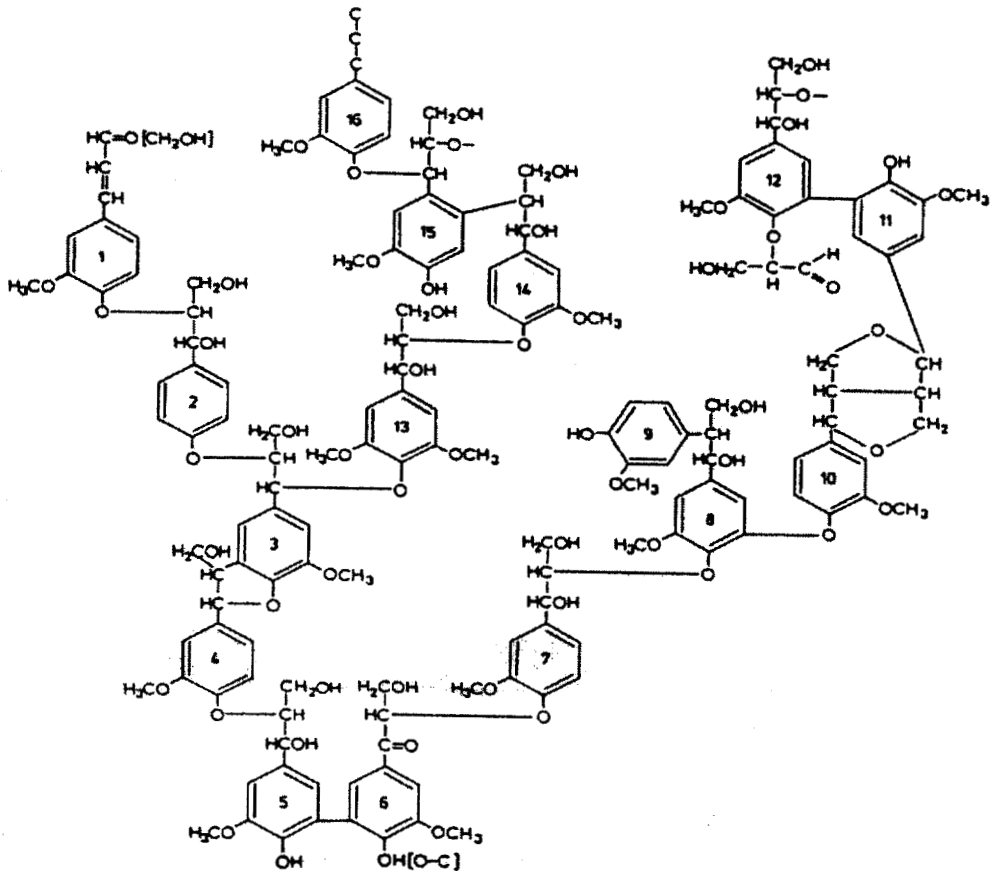

Fig. 1.1 Schematic structure of spruce lignin [Adler, 1977].

For the past 25 years, research in the Gold lab has been centered on the biochemistry, molecular biology and genetics of the lignin-degrading fungus *P. chrysosporium*. Several features make *P. chrysosporium* a very useful research subject among white-rot fungi. First, it grows relatively fast and makes copious asexual spores (conidia spores). Second, conditions for fruiting and crossing for this fungus have been developed in our lab. Third, under certain conditions, *P. chrysosporium* degrades lignin selectively and leaves the cellulose of wood behind. Fourth, it has a high optimal growth temperature (about 40°C), which allows it to grow on wood chips in compost piles that attain high temperature [Kirk, 1980]. Fifth, in addition to lignin, *P. chrysosporium* is capable of degrading a variety of aromatic pollutant compounds [Bumpus and Aust, 1987; Hammel, 1989; Joshi and Gold, 1993; Reddy et al., 1998]. These traits suggest possible roles for *P. chrysosporium* in biotechnological applications.

P. chrysosporium is one of the resupinate (crust) fungi [Burdsall and Eslyn, 1974]. In nature, it forms effused, very flat fruiting bodies that appear as a crust on the underside of a log. The diffuse and unrestricted growth is also observed on ordinary agar medium in petri dishes. However, the colonial growth of *P. chrysosporium* has been achieved by plating a dilute suspension of *P. chrysosporium* conidia on agar plates containing 4% sorbose [Gold and Cheng, 1978].

1.1.2 The Lignin-Degrading System of *P. chrysosporium*

Lignin degradation in *P. chrysosporium* only occurs during secondary metabolic growth phase. At this stage, the cell mass stops increasing and the essential nutrients are depleted. The limitation of nutrients including nitrogen, carbon or sulfur triggers secondary metabolism and induces lignin degradation [Kirk, 1980; Fenn and Kirk, 1981].

Nitrogen limitation is the most commonly used culturing condition in the study of lignin degradation in *P. chrysosporium*. A variety of nitrogen containing compounds, such as $(NH_4)_2SO_4$, asparagine, ammonium tartrate and $NaNO_3$ can support *P. chrysosporium* growth. However, regardless of nitrogen source, lignin

metabolism ensues only when nitrogen is depleted [Kirk, 1980]. The effect of nitrogen limitation is not surprising given the low levels of nitrogen found in wood [Buswell and Odier, 1987]. Nitrogen availability appears to control a general switch in the transition from primary to secondary metabolism in *P. chrysosporium*. The addition of excess nitrogen, such as NH_4^+ or glutamate, to ligninolytic cultures restores primary growth and consequently represses lignin degradation [Kirk, 1980; Fenn and Kirk, 1981]. In addition to lignin degradation, nitrogen depletion also derepresses several other secondary metabolic events including the production of extracellular polysaccharide [Leisola et al., 1982] and the synthesis of veratryl alcohol [Shimada et al., 1981].

As a secondary metabolic process, lignin degradation requires supplementation with a readily utilizable carbon source. A variety of carbon compounds, such as glucose, glycerol and cellulose can support both the growth and ^{14}C-lignin-degrading metabolism of *P. chrysosporium* in chemically defined media [Kirk, 1980]. *P. chrysosporium* grown on either a fermentable (glucose) or a non-fermentable (glycerol) carbon source produces ligninolytic activity within the same time course, suggesting there is no carbon catabolite repression (resulting from glucose metabolism) [Fenn and Kirk, 1981]. In fact, ligninolytic activity is higher in *P. chrysosporium* grown in media containing glucose than in media containing other carbon sources. The addition of glucose to cultures with glycerol as the sole carbon source enhances ligninolytic activity [Fenn and Kirk, 1981].

Other physiological factors that affect the lignin degradation in *P. chrysosporium* include the levels of oxygen and manganese. Increasing O_2 levels in *P. chrysosporium* cultures has a strong activating effect on the rate of lignin degradation [Kirk, 1980]. The presence of Mn is also very important and the accumulation of MnO_2 precipitates has been observed in wood decayed by some white-rot fungi [Blanchette, 1984].

The enzymes involved in lignin degradation in *P. chrysosporium* were discovered in the 1980s. Two families of unique heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), as well as hydrogen peroxide-

generating enzymes such as glyoxal oxidase (GLOX) and glucose oxidase, are the major components of *P. chrysosporium*'s lignin-degradation system [Kirk and Farrell, 1987; Gold et al., 1989; Gold and Alic, 1993]. MnP [Kuwahara et al., 1984; Paszczynski et al., 1985, 1986], LiP [Glenn et al., 1983; Tien and Kirk, 1983] and GLOX [Kersten and Kirk, 1987] are all extracellular enzymes, and are produced during the secondary metabolic phase, concomitant with the lignin-degrading activity of *P. chrysosporium*. Both MnP and LiP exist as a series of glycosylated isozymes containing one mol of iron heme per mol of protein. LiP isozymes have pIs ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa; MnP isozymes have pIs ranging from 4.2 to 4.9 and molecular masses ranging from 45 to 49 kDa [Glenn and Gold, 1985; Kirk et al., 1986; Paszczynski et al., 1986; Leisola et al., 1987b; Gold et al., 1989]. The catalytic cycles of both LiP and MnP involve three reactions. In the first reaction, the enzyme is oxidized by H_2O_2 to a two-electron oxidized state compound I [(Fe-heme)$^{5+}$]. During the second and third reactions, compound I is reduced back to the native enzyme [(Fe-heme)$^{3+}$] in two single-electron steps (Paszczynski et al., 1985; Renganathan and Gold, 1986; Wariishi et al., 1988; Gold et al., 1989]. The difference between MnP and LiP is the nature of their reducing substrates. Free Mn^{2+} is the substrate for MnP. Mn^{3+} is the product, and it is stabilized by chelation with organic acids such as oxalate and malonate. The chelated Mn^{3+} diffuses away from the surface of the enzyme and subsequently oxidizes the insoluble terminal substrate, lignin [Glenn and Gold, 1985; Paszczynski et al., 1985, 1986; Glenn et al., 1986; Gold et al., 1989; Wariishi et al., 1989, 1992). Unlike MnP, LiP directly oxidizes aromatic reducing substrates. Non-phenolic aromatic compounds such as veratryl alcohol are the preferred substrates for LiP and aromatic cation radicals are generated during the reactions. These radicals can act as mediators in the oxidation of lignin model compounds and aromatic pollutants [Kersten et al., 1985; Tien et al., 1986; Marquez et al., 1988; Harvey and Palmer, 1990; Schoemaker et al., 1994; Joshi and Gold, 1996; Teunissen and Field, 1998]. Both LiP and MnP are able to oxidize a variety of terminal aromatic substrates that have complex chemical structures. This relative non-specificity makes them ideal for degrading lignin as well as aromatic pollutants.

The *P. chrysosporium* LiP and MnP isozymes are encoded by a set of structurally related genes (designated *lip*s and *mnp*s, respectively) [Gaskell et al., 1991; Gold and Alic, 1993; Gold et al., 2000]. The expression of both *lip*s and *mnp*s is controlled at the transcriptional level by nutrient N. Moreover, the transcription of *mnp*s is regulated by Mn^{2+}, heat shock and oxidative stress. Details related to the structure of *lip*s and *mnp*s as well as the regulation of their expression are reviewed in Section 1.2.

1.1.3 The Genetics of *P. chrysosporium*

Basidiomycetous fungus produces sexual spores, basidiospores, from a distinctive structure, basidium. The basidiospores are haploid, and germinating basidiospores give rise to the primary mycelia, which contain cells that are haploid. Typically, when the primary mycelia derived from two basidiospores that possess compatible mating types encounter, the mycelia cells undergo plasmogamy to form diploid cells. At this point, the nucleic fusion does not occur and each diploid cell contains two different and separate haploid nuclei. Diploid cells divide and give rise to secondary mycelia. A specific mitotic mechanism, the formation of clamp connections, ensures that each daughter cell in the secondary mycelia receives the same two different types of haploid nuclei from their mother cell. Under the right conditions, basidia will be formed at the tips of secondary mycelia. In the basidia, nuclear fusion and meiosis will follow. The final products, basidiospores, are generated and then emitted from the sterigmata located at the tip of each basidium. However, for many basidiomycetes, the sexual reproduction mechanisms are not understood and they may be very different from the generalized and simplified model described above. In some species, neither obvious mating types nor clamp connections have been observed.

There are three common laboratory wild-type strains of *P. chrysosporium,* i.e., ME-446 [Burdsall and Eslyn, 1974], BKM-F-1767 [Burdsall, 1981], and OGC101 [Alic et al., 1987]. All of these strains have multinucleate septate mycelia with as many as 15 nuclei randomly dispersed throughout each cell [Alic et al., 1987].

Clamp connections have not been found in these *P. chrysosporium* strains [Burdsall and Eslyn, 1974; Alic et al., 1987]. These strains also possess heterokaryotic mycelia, which contain two or more types of genetically distinct nuclei [Alic et al., 1987].

Under certain conditions, *P. chrysosporium* fruits and produces basidiospores. The number of sterigmata per basidium varies between 2 and 8 in the strains mentioned above, with the majority of basidia having 4 sterigmata [Alic et al., 1987]. In the laboratory, the fruiting can be induced by growing *P. chrysosporium* in nutrient-poor medium, such as the medium containing a low level of nitrogen and only cellulose as the carbon source [Gold and Cheng, 1979]. The basidiospores all contain two identical nuclei, arising from a post-meiotic mitotic division [Alic and Gold, 1985; Raeder and Broda, 1986; Alic et al., 1987]. Therefore, single basidiospore isolates give rise to homokaryons, which may differ from each other and from parental wild-type heterokaryon in a variety of characteristics [Raeder and Broda, 1986; Alic et al., 1987].

P. chrysosporium exhibits a functionally homothallic or self-fertile mating system (Alic et al., 1987; Akileswaran et al., 1993]. This is strongly supported by the following data. First, basidiospores of any two auxotrophic strains that belong to different complementation groups can cross and form heterokaryons, and genetic recombination has been demonstrated in most of these heterokaryons (Alic et al., 1987; Akileswaran et al., 1993; Gold and Alic, 1993). Second, the single basidiospore isolates from various wild type strains, which germinate and fruit in the first generation, continue to yield fruiting progeny in succeeding generations [Alic et al., 1987]. These results indicate the absence of mating-type incompatibility in *P. chrysosporium*.

Many auxotrophic mutants of *P. chrysosporium*, which are deficient in the synthesis of a certain amino acid, nucleoside, or vitamin, were isolated by UV or X-ray mutagenic treatment of the conidia from strain ME-446 and OGC101, followed by colony isolation and complementation assays (Gold et al., 1982a; Alic and Gold, 1985; Molskness et al., 1986; Akileswaran et al., 1993]. Some of these auxotrophic

mutants have been used as recipients for foreign DNA in DNA transformation studies (see below).

Mutant strains with altered phenotypes in lignin degradation were also isolated. These include the strains that do not degrade lignin [Ander and Eriksson, 1976; Gold et al, 1982b] and the strains with nitrogen-deregulated lignin degradation. The latter were isolated through an assay that utilized the decolorization of a polymeric dye as the indicator of lignin-degradation activity [Glenn and Gold, 1983; Boominathan et al., 1990b]. Other nitrogen-deregulated mutants were isolated by mutagenizing a lysine auxotroph and selecting mutants that can grow on nitrogen-rich medium supplemented with an adduct of lysine and a lignin model compound [Tien and Myer, 1990]. Under nitrogen-sufficient growth conditions, high LiP and MnP activities were observed in nitrogen-deregulated mutants, but not in wild type strains. *P. chrysosporium* mutant strains with normal MnP activity but very low LiP activity were isolated using a poly-R decolorization assay specific for LiP [Glenn and Gold, 1983; Boominathan et al., 1990a]. This indicates the production of the two enzymes can be uncoupled. So far, no characterization of these mutant strains at the molecular level has been reported.

The haploid genome size of *P. chrysosporium* strain ME-446 was estimated to be 4.4×10^7 bp [Raeder and Broda, 1984, 1988]. Strains ME-446 and BKM-F-1767 have chromosomal (G+C) contents of 59% [Raeder and Broda, 1984], similar to other basidiomycetes [Kurtzman, 1985]. Using contour-clamped homogeneous electric field gel analysis, Gaskell et al. showed that the haploid *P. chrysosporium* genome contains at least seven chromosomes [Gaskell et al., 1991].

In 2001, the draft genomic sequence of *P. chrysosporium* strain BKM-F-1767 was completed using a shotgun method for whole genome sequencing (http://www.jgi.doe.gov/programs/whiterot.html). The information obtained from this accomplishment should lead to other breakthroughs in research on lignin degradation in white-rot fungi.

1.1.4 DNA Transformation of *P. chrysosporium*

The procedure for DNA transformation in *P. chrysosporium* is similar to that used in the basidiomycete *Schizophillum commune* [Munoz-Rivas et al., 1986]. It involves three steps: protoplast preparation, DNA uptake and selection for transformants. The protoplasts are obtained by digesting fungal cell wall with cellulase and Novozyme in osmotic stabilizing buffer [Gold et al., 1983; Alic et al., 1989]. The protoplasts can be generated from either basidiospores [Alic et al., 1989] or germinated conidia [Gold et al., 1983; Randall et al., 1989; Gessner and Raeder, 1994; Zapanta et al., 1998]. After an initial exposure of protoplasts to DNA, high concentration of Ca^{2+} and polyethylene glycol (PEG) is added to the protoplast-DNA mixture [Alic et al., 1989]. PEG may cause the protoplasts to clump and facilitate the trapping of DNA [Fincham, 1989]. The protoplast-DNA mixture is then diluted and plated on a selection medium.

Two methods have been employed for selection of transformed *P. chrysosporium* cells. The first method, pioneered in our lab, involves the selection of prototrophic transformants after complementation of auxotrophic *P. chrysosporium* strains with wild-type genes. Two adenine-requiring auxotrophs, ade2 and ade1, which are derivatives of *P. chrysosporium* strain OGC101, were transformed to prototrophy with plasmids containing the adenine biosynthetic genes *ade2* and *ade5* from *S. commune*, respectively [Froeliger et al., 1987; Alic et al., 1989, 1990]. A plasmid containing the *ade1* gene from *P. chrysosporium*, a homologue of *S. commune ade5* gene, also transformed auxotrophic strain ade1 to prototrophy [Alic et al., 1991]. The transformation frequency of these experiments is about 100 to 400 prototrophic transformants per μg of DNA, and approximately 0.05 to 0.15% viable protoplasts were transformed.

Two uracil auxotrophs, ura3 deficient in orotidylate decarboxylase (ODase) and ura5 deficient in orotate phosphoribosyl transferase (OPase) [Akileswaran et al., 1993], were isolated from *P. chrysosporium* strain OGC101. ura3 was transformed to prototrophy by plasmid containing the *ura3* gene from *S. commune* [Froeliger et al., 1989] or *P. chrysosporium* [Akileswaran et al., 1993]; likewise, ura5 was transformed

to prototrophy with the *ura5* gene from the ascomycete *Podospora anserina* [Begueret et al., 1984]. The plasmids containing *S. commune* and *P. chrysosporium* genes transformed a ura3 auxotroph with the frequency about 100 to 600 transformants per μg of DNA and approximately 0.3% viable protoplasts were transformed. In contrast, the ura5 auxotroph was transformed with the *P. anserina* gene at the frequency of only 1 to 25 transformants per μg of DNA and about 0.008% viable protoplasts were transformed [Akileswaran et al., 1993], suggesting the inefficient expression of this ascomycete gene in *P. chrysosporium*.

The systems discussed above take advantage of the variety of metabolic genes already cloned in other organisms, as well as the method of cloning a homologous gene from a *P. chrysosporium* genomic library based on known sequences. Zapanta et al. reported a different strategy to clone *P. chrysosporium* genes that function in biosynthesis [Zapanta et al., 1998]. This method employs the screening of a *P. chrysosporium* cDNA library constructed in an expression vector that allows expression in *Escherichia coli* for complementation of certain amino acid auxotrophs of *E. coli*. The cDNA molecule from the positive clone was isolated and subsequently used to probe a genomic library of *P. chrysosporium* to obtain the corresponding biosynthesis gene. Using such a method, the *P. chrysosporium leu2* gene encoding β-isopropylmalate dehydrogenase, which catalyzes a step in the pathway of leucine synthesis, was cloned and used to transform a leucine auxotroph [Molskness et al., 1986] to prototrophy [Zapanta et al., 1998]. The transformation frequency of this system was 2 to 10 transformants per μg of DNA.

All the prototrophic transformants discussed above were mitotically and meiotically stable on both selective and non-selective media. Southern blots showed that the transforming DNA was integrated into the host chromosome ectopically in single or multiple copies [Alic et al., 1989, 1991; Akileswaran et al., 1993]. In the case of multiple copy integration, the plasmid DNA may exist at one location in a tandem array or at various locations. A genetic cross between a single transformant resulting from transforming auxotrophic strain ade2 with pADE2 (containing *S. conmmune ade2* gene) and other auxotrophic strains all yielded some adenine

auxotrophs [Alic et al., 1989], which was suggestive of the ectopic integration of the transforming DNA. Ectopic and multiple copy integration is commonly observed in the transformation of filamentous fungi [Fincham, 1989]. Available evidence from transformation in other basidiomycetes, such as *Coprinus lagopus* [Binninger et al., 1987] and *Trametes versicolor* [Bartholomew et al., 2001], also indicates that ectopic integration is the predominant mode of stable transformation.

The second method for selecting *P. chrysosporium* transformants uses a dominant selectable marker, which involves introducing a gene conferring drug resistance to wild type strains. Low frequency (7 to 20 transformants per μg of DNA) transformation of strain ME-446 and BKM-F-1767 to G418 resistance has been reported [Randall et al., 1989; Randall and Reddy, 1992] using a plasmid containing the bacterial kanamycin resistance determinant of Tn903 [Grindley and Joyce, 1980] and an endogenous extrachromosomal *P. chrysosporium* sequence that apparently supports autonomous replication in *Saccharomyces cerevisiae* as well as in *P. chrysosporium* [Rao and Reddy, 1984]. This plasmid is maintained in *P. chrysosporium* transformants as an extrachromosomal circular plasmid at a very low copy number under both non-selective and selective conditions. It is recoverable from the total DNA via high-efficiency *E. coli* transformations [Randall et al., 1991]. Likewise, a plasmid containing the kanamycin resistance determinant inserted into the coding region of *lipD* gene (encoding LiP isozyme H2) was also found to be extrachromosomally maintained in *P. chrysosporium* transformants [Randall and Reddy, 1991]. Gessner and Raeder [1994] reported transforming *P. chrysosporium* strain ME-446 to phleomycin resistance (Ph^R) using a plasmid containing the *ble* gene [Drocourt et al., 1990] (a Ph^R gene from bacteria *Streptoalloteichus hindustanus*) joined with the promoter region of a *P. chrysosporium* gene encoding histone H4. The transformation frequency was 6–10 transformants per μg of DNA. The plasmid could either exist as an unstable extra-chromosomal form or be stably integrated. Another plasmid construct containing *ble* joined with the promoter region of *P. chrysosporium* cellobiohydrolase (CBH) gene *cbhI.1*, was reported to transform strain ME-446 to Ph^R [Birch et al., 1998]. Southern blot analysis revealed the

extrachromosomal maintenance of this plasmid. However, the transformation frequency was very low and it was assumed that less than 0.005% of regenerated protoplasts were transformed to Ph^R. The extrachromosomal maintenance of a recombinant plasmid containing the bacterial hygromycin resistance gene and fungal chromosomal sequences has been reported in another basidiomycete, *Pleurotus ostreatus* [Peng et al., 1992].

In Chapter 4, a new transformation system for *P. chrysosporium* strain OGC101 utilizing a bacterial bialophos-resistance gene (*bar*) as a selectable marker is reported. Unlike other positive selection systems discussed above, the plasmid that contains *bar* integrates into the host chromosome to form stable transformants. Furthermore, the transformation frequency is in the order of 100 transformants per μg of DNA.

A few factors have been reported that affect the efficiency of *P. chrysosporium* transformation. First, in the case of transforming auxotrophic strains with complementing wild-type genes, transformants generally appear earlier with the cloned *P. chrysosporium* genes than with genes from other fungi (3 to 5 days after plating, compared with 4 to 7 days). Second, the linearized transforming plasmids usually result in a higher transformation frequency when compared with the circular plasmid [Alic et al., 1989]. Third, there seems to be a correlation between the origin of the protoplast and the transformation frequency. All of the transformations carried out in strain OGC101, including transforming adenine and uracil auxotrophs to prototrophs as well as transforming the wild type strain to bialophos resistance, used the protoplasts derived from basidiospores. High transformation frequency ($=100$ transformants per μg of DNA) was observed in all these cases. In contrast, all the transformations done in strain ME-446, including transforming leucine auxotroph with the wild type *leu2* gene as well as transforming wild type strain to G418 and phleomycin resistance, used the protoplasts derived from germinated conidia spores. The transformation frequency in these cases was around 5 to 20 transformants per μg of DNA. These data suggest that protoplasts derived from basidiospores are the more efficient transformation hosts.

It is often necessary to purify the transformants since the protoplasts can undergo fusion in the presence of PEG, thus giving rise to heterokaryons. The homokaryotic derivatives of each transformant can be obtained through the fruiting process followed by isolating single basidiospores [Alic et al., 1989; Gold and Alic, 1993].

The transformation system is a basic tool for molecular manipulation in *P. chrysosporium*. The production of recombinant homologous and heterologous proteins, the study of gene expression regulation and the construction of gene disruption mutations are a few examples of how the transformation systems can be utilized.

1.1.5 Homologous Gene Expression in *P. chrysosporium*

Homologous expression of MnP [Mayfield et al., 1994b], LiP [Sollewijn Gelpke et al., 1999] and cellobiose dehydrogenase (CDH) [Li et al., 2000] in *P. chrysosporium* were achieved during the past ten years in our lab. In all cases, the 1.1-kb promoter of the *P. chrysosporium* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was used to drive the expression of *mnp1* (encoding MnP isozyme 1 in strain OGC101), *LG2* (with synonym *lipE*, encoding LiP isozyme H8 in strain OGC101) and *cdh* (encoding CDH). The *gpd* promoter enables the production of recombinant MnP1 and LiPH8 in the primary metabolic cultures, where nutrient nitrogen is abundant and endogenous MnPs and LiPs are not produced. It also allows the production of recombinant CDH in cultures where glucose is the sole carbon source and endogenous CDH is not expressed. Since ectopic and multiple integration of plasmid DNA is the predominant mode of transformation in *P. chrysosporium*, the expression levels of the recombinant enzyme vary among individual transformants carrying the expression plasmids. Hence, a large number of transformants need to be screened for recombinant enzyme production and the most productive transformant is typically chosen for recombinant enzyme purification. Approximately 2 mg recombinant MnP1 (rMnP1), 2–3 mg recombinant LiPH8 (rLiPH8) and ~60 mg recombinant CDH (rCDH) per liter of extracellular culture media were obtained from

the transformants carrying *mnp1*, *LG2* and *cdh* expression plasmids, respectively. The production of rMnP1 and rLiPH8 is about one-third of the endogenous MnP and LiP produced by the wild type strain OGC101 under ligninolytic conditions, which are mixtures of different isozymes. Thus, the expression levels of these recombinant enzymes are comparable to the levels of their endogenous counterparts. The production of rCDH is also comparable to the wild type CDH and its high yield may result from its intrinsic stability [Li et al., 2000]. All the recombinant enzymes exhibit similar physical, spectral and kinetic characteristics as their endogenous counterparts. These homologous expression systems enable one to undertake detailed structure–function study for MnP, LiP and CDH.

The expression of rMnP and rLiP was also accomplished in other host organisms. In *E. coli*, the rMnP [Whitwam et al., 1995; Whitwam and Tien, 1996] and rLiP [Doyle and Smith, 1996; Nie et al., 1998] were expressed in the form of inclusion bodies. Through the process of reconstitution, low yield of active enzyme was obtained with incorporated heme. However, these recombinant enzymes were not glycosylated. No success has been reported using the yeasts *S. cerevisiae* and *Pichia radiata* as hosts. Recently the expression of active rMnP1 in the *Aspergillus niger* has been reported [Conesa et al., 2000]. In this system, rMnP was secreted into the medium and the production could reach 100 mg/liter upon supplementation of hemoglobin or hemin to the cultures. Overexpression of a chaperon, calnexin, which functions in *A. niger* secretion pathway, further increased rMnP production [Conesa et al., 2002]. The *A. niger* system is a very promising system for industrial production of the fungal peroxidases. However, the rMnP produced by *A. niger* exhibits a slightly lower mobility on SDS-PAGE than the native MnP, suggesting different posttranslational modification in *A. niger* compared to that in *P. chrysosporium*. Expression of active rLiP has not been reported in *A. niger*.

In conclusion, the homologous expression systems of rMnP and rLiP are still the primary choice for studying these enzymes in the laboratory. More research on how to improve the yield of the recombinant peroxidases in *P. chrysosporium* needs to be done in the future.

1.1.6 Heterologous Gene Expression in *P. chrysosporium*

Several biosynthetic genes from the basidiomycete *S. commune* and the ascomycete *P. anserina*, which complement *P. chrysosporium* auxotrophic mutants, are used as selectable markers for transformation (see Section 1.1.4). The bacterial derived genes conferring resistance to G418, phleomycin and bialaphos are also expressed in *P. chrysosporium* as dominant selectable markers. These are examples of heterologous gene expression in *P. chrysosporium*.

Recently, the Gold lab has developed a system using *P. chrysosporium* to express recombinant peroxidase from another basidiomycete. A recombinant MnP isozyme of *Dichomitus squalens* (rDsMnP) was successfully produced and purified in *P. chrysosporium* [Li et al., 2001]. In this study, the expression of *D. squalens mnp2* gene was driven by the *P. chrysosporium gpd* promoter, enabling the production of rDsMnP in nutrient nitrogen-rich cultures without contamination from *P. chrysosporium* MnPs. rDsMnP was secreted by *P. chrysosporium* and the yield was approximately 1.5 mg per liter of extracellular medium, sufficient to pursue further structure/function studies. For many basidiomycetes in which a DNA-mediated transformation system is not available, the heterologous expression system in *P. chrysosporium* may be a useful tool for protein structure/function studies.

Heterologous gene expression has been intensively studied in *S. commune*. It was reported that the cDNA coding sequence of the *Agaricus bisporus* hydrophobin gene (ABH1) inserted between the 5′ and 3′ untranslated regions (UTR) of the *S. commune* SC3 hydrophobin gene gave no expression in *S. commune* [Lugones et al., 1999]. In contrast, the genomic coding sequence of ABH1 (containing three introns) in the same configuration gave rise to high levels of ABH1 mRNA, suggesting introns are required for accumulation of ABH1 mRNA. The effect of an intron was also observed in the homologous expression of the *S. commune* SC3 and SC6 hydrophobin genes from three different types of constructs: containing only the cDNA, containing the genomic DNA, and containing the cDNA with an inserted intron at various locations. The result showed that only genomic DNA and cDNA with an intron that has the correct orientation as well as splice and branching sites resulted in mRNA

accumulation. Run-on analysis demonstrated that the enhancement of mRNA accumulation by the intron did not occur at the level of transcription initiation: genomic and cDNA sequences were equally active in this respect. The precise mechanism of the intron's effect remains unknown. Besides hydrophobin genes, it has been reported that insertion of an intron into the transcriptional unit of green fluorescent protein gene (*gfp*) from *Aequorea victoria* [Lugones et al., 1999] as well as hygromycin B resistance gene from *E. coli* [Scholtmeijer et al., 2001] (both contain no intron) yielded high expression of these genes in *S. commune*. We have examined the effect of intron insertion on the expression of a *gfp* variant (enhanced green fluorescent protein gene, *egfp*) in *P. chrysosporium*. As discussed in Chapter 2, insertion of an intron at the 5′ of *egfp* transcriptional unit resulted in the high expression of green fluorescent protein (GFP) in *P. chrysosporium*.

Another factor that interferes with heterologous gene expression in basidiomycetes is the (G+C) content of the foreign gene. High (G+C) content is frequently seen in many basidiomycete genomes; for example, the (G+C) content of *P. chrysosporium* is approximately 59%. However, many bacterial derived reporter genes (such as β-glucuronidase and β-galactosidase genes) and drug-resistance genes (such as hygromycin B phosphotransferase and aminoglycoside phosphotransferase genes) contain (A+T)-rich stretches [Scholtmeijer et al., 2001]. In *S. commune*, expressing these bacterial genes resulted in no full-length transcripts. The only transcripts produced were truncated in the 5′ part of the coding sequence at the position of the (A+T)-rich stretches [Schuren and Wessels, 1998]. Modifying the (A+T)-rich stretch in hygromycin B phosphotransferase gene from *E. coli* to increase its (G+C) content resulted in a low level accumulation of its full-length transcripts in *S. commune* [Scholtmeijer et al., 2001]. It appears that the mRNA accumulation caused by sequence modification was independent of the accumulation caused by intron insertion. In *P. chrysosporium*, the effect of (A+T)-rich stretches on heterologous gene transcription has not been investigated.

1.1.7 Gene Targeting and Disruption in *P. chrysosporium*

Gene disruption through homologous recombination enables the study of the physiological roles of specific genes. In the transformation experiment of *P. chrysosporium*, the positions on host chromosomes where the transforming DNA integrates may affect the expression of genes carried on the transforming DNA. The ability to target transforming DNA to a specific genomic location would diminish the position effects among different transformants. The only reported gene targeting study in *P. chrysosporium* involved a plasmid containing the *S. commune ade2* gene inserted into the coding region of the *P. chrysosporium ura3* gene [Alic et al., 1993]. This plasmid was used to transform *P. chrysosporium* adenine auxotroph to prototrophy (Ade^+). Inactivation of *ura3* enables *P. chrysosporium* to grow on medium supplemented with 5′-fluoroorotic acid (5′-FOA) and thus facilitates the selection of uracil auxotrophs (Ura^-) among the Ade^+ transformants. Stable Ade^+Ura^- transformants were obtained at a frequency of approximately one per μg of DNA. Southern blot analysis suggested that the plasmid insert (*ura3-ade2-ura3*) had replaced the chromosomal *ura3* in the Ade^+Ura^- transformants. Even with advantage of a two-way selection, the extremely low transformation frequency in this study supports the notion that homologous recombination is a relatively rare event in *P. chrysosporium*. A gene targeting experiment was also designed and performed in another white-rot fungus, *T. versicolor* [Dumonceaux et al., 2001]. A bacterial Ph^R gene, *shble*, was inserted in the coding region of the *T. versicolor* cellobiose dehydrogenase gene (*cdh*). The plasmids containing this construct were used to disrupt chromosomal *cdh* and confer Ph^R to the transformants. Less than 1% of *T. versicolor* Ph^R transformants were CDH defective mutants. Of the three CDH defective mutants, none had the chromosomal *cdh* replaced by the plasmid insert through a double recombination. Southern blot analysis suggested that the plasmid DNA might have integrated in tandem with the chromosomal *cdh*. In conclusion, the lack of gene targeting and gene disruption strategies remains an obstacle to the genetic study of white-rot fungi.

1.2 THE STRUCTURE OF *lips*, *mnps*, AND THE REGULATION OF THEIR EXPRESSION IN WHITE-ROT FUNGI

1.2.1 The Structure of *lips* in *P. chrysosporium*

P. chrysosporium LiPs are synthesized and secreted as a series of isozymes [Renganathan et al., 1985; Kirk et al., 1986; Farrell et al., 1989]. The principle criteria for identifying these LiP isozymes are their pIs and their order of elution from a Mono Q anion exchange column. Ten species of LiP have been identified and designated H1 through H10. Growth condition, purification methods and storage can affect relative isozyme levels [Kirk et al., 1986; Kirk and Farrell, 1987; Leisola et al., 1987a].

More than twenty cDNA and genomic sequences encoding LiP have been isolated from *P. chrysosporium* strains ME446, BKM-1767 and OGC101 [for reviews, see Gold and Alic, 1993, and Gaskell et al., 1994]. Many of these cDNA and genomic clones are alleles of a single *lip* gene. The finding that a single basidiospore isolate of *P. chrysosporium* contains two identical haploid nuclei, arising from a post-meiotic mitotic event [Alic et al., 1987], facilitates genetic analysis that is designed to differentiate alleles and closely related genes. Gaskell et al. described a genetic linkage analysis method, which employs the amplification of the allele sequences from several basidiospore isolates of strain BKM-1767 by polymerase chain reaction (allele-specific PCR) using the allele-specific oligos designed according to the published sequences, followed by probing the PCR product with radio-labeled allele-specific oligos [Gaskell et al., 1992, 1994]. Through this analysis, 10 closely related *lips* were identified and designated *lipA* through *lipJ* [Gaskell et al., 1994]. Among these *lips*, it is known that *lipA* and *lipD* encode LiP isozymes H8 and H2 in strain BKM-F-1767, respectively. However, the precise relationship between other *lips* and the specific isozymes remains unknown.

Genetic linkage analysis also revealed that eight *lips*, namely *lipA, lipB, lipC, lipE, lipG, lipH, lipI* and *lipJ* are linked and the *lipD, lipF* are separated from each other and the eight linked genes [Gaskell et al., 1994]. Mapping of cosmid clones

containing *P. chrysosporium* genomic DNA has established a physical map of the eight linked *lip*s, in which *lipA, lipB, lipC and lipE* reside within a 35-kb region and the rest lie in a 15-kb region [Stewart and Cullen, 1999]. This conclusion is confirmed by sequence information obtained from the recently finished *P. chrysosporium* genome project (http://www.jgi.doe.gov/programs/whiterot.html). The deduced amino acid sequence of LiP isozyme H8 was used to search for its homologues in translated *P. chrysosporium* complete genomic sequence by the BLAST program [Altschul et al., 1997]. Ten closely related sequences (TBLASTN, P = 2.7^{-112}) were found, in which eight sequences are clustered on scaffold 85 within a 110-kb region and the remaining two sequences are located on scaffold 21 and scaffold 19, respectively. The eight clustered sequences are assigned to the eight linked *lip*s based on the existing physical map. The diagram of the precise distance among the eight *lip*s and their individual transcriptional orientation are shown in Fig. 1.2. As shown, six genes are paired and transcriptionally convergent (*lipA* and *lipB*, *lipG* and *lipI* and *lipH* and *lipJ*). The regions that separate paired genes have different sizes and lack nucleotide similarity. *lipC*, *lipE* are unpaired and flank the *lipB, lipA* pair by 10 kb.

The *lipI* gene harbors a 1747-bp insertion immediately adjacent to the fourth intron, which transcriptionally inactivates this gene [Gaskell et al., 1995]. The inserted sequence, named Pce1, contains several transposon-like features including the inverted terminal repeats and a dinucleotide (TA) target duplication. Pce1 lacks homology to any known transposons and its function remains unknown.

All ten *lip*s contain either eight or nine introns ranging in size from 49 to 78 bp. The intron/exon junction sequences conform to the conserved 5′ -GT---AG- 3′ rule. The internal lariat formation sequences do not strictly conform to the conserved CTPuAPy [Ballance, 1986]. According to their intron/exon structures the ten *lip*s can be classified into five subfamilies [Gold and Alic, 1993; Stewart and Cullen, 1999]. As shown in Fig. 1.3A, six of the eight linked *lip*s (*lipA, lipB, lipE, lipG, lipH* and *lipI*) have the same intron/exon structures, which are grouped in one subfamily. The

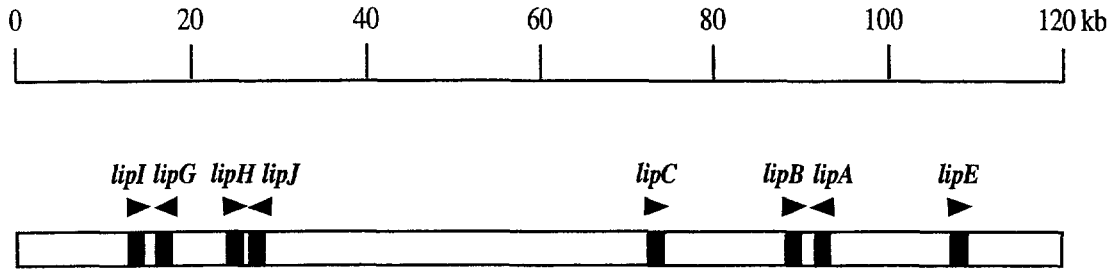

Fig. 1.2 Genomic organization of the eight linked *lip* genes. The arrowheads indicate the transcriptional orientations. Physical distances are in kilobases. The intergenic distances in paired *lipI–lipG*, *lipH–lipJ*, and *lipB–lipA* genes are approximately 1.3, 0.5, and 1.3 kb, respectively.

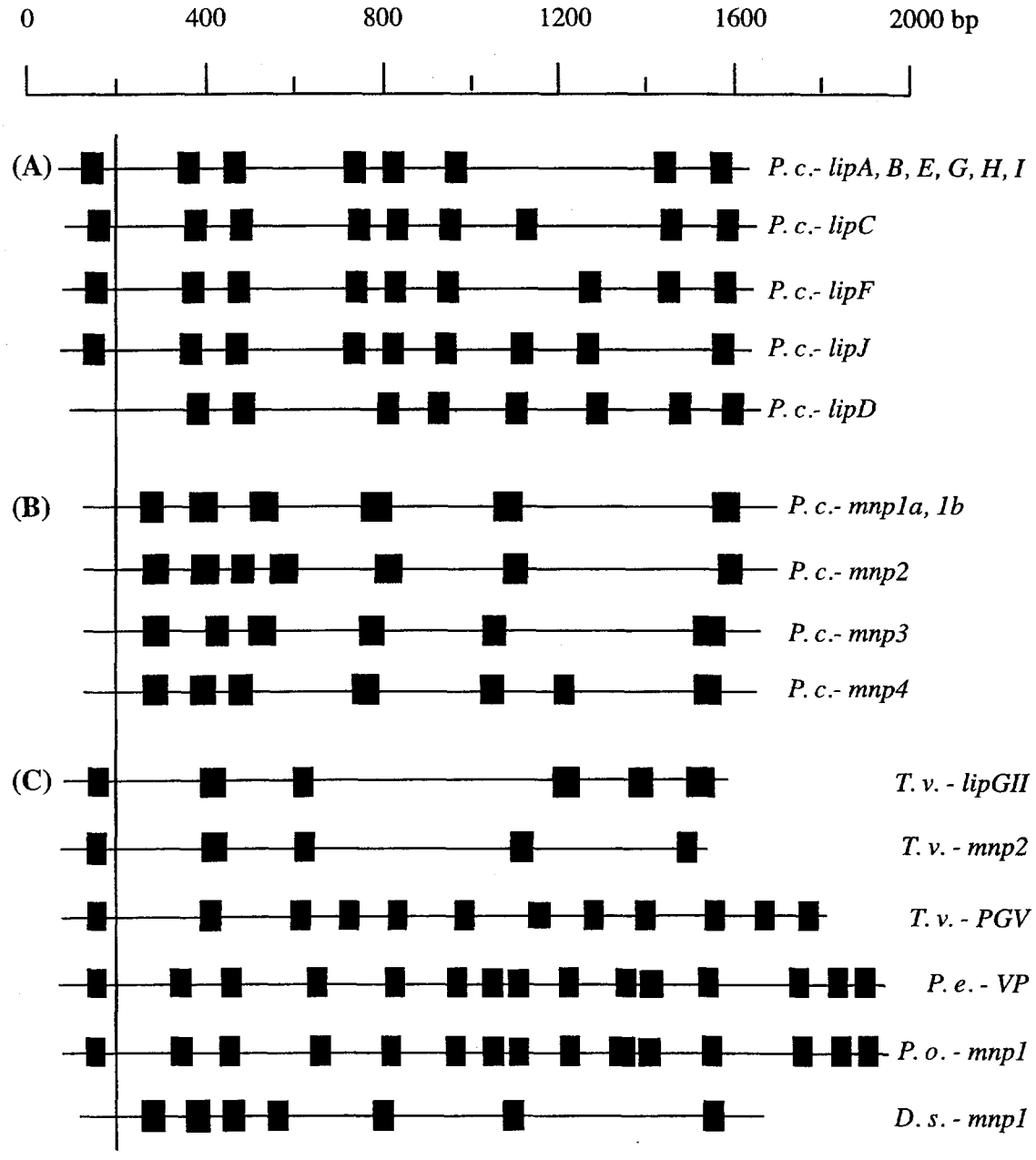

Fig. 1.3 Scheme of intron locations in *lip*- and *mnp*-related genes in various white-rot fungi. Introns are represented by black boxes. The fungal species shown are *P.c.* (*P. chrysosporium*), *T.v.* (*T. versicolor*), *P.e.* (*P. eryngii*), *P.o.* (*P. ostreatus*), and *D.s.* (*D. squalens*). The vertical line corresponds to the beginning of the nucleotide sequences that encode the mature proteins.

remaining two of the eight linked *lips* (*lipC* and *lipJ*) and the two unlinked *lips* (*lipD* and *lipF*) are each tentatively assigned to different subfamilies.

The deduced amino acid sequences of the ten *lips* share over 70% homology [Cullen, 1997]. Cladistic analysis based on the amino acid sequences establishes two main branches within the LiP family [Stewart and Cullen, 1999]. One branch comprises LiPD and LiPJ, the other branch comprises LiPA, LiPB, LiPE, LiPG, LiPH, LiPI, LiPF, and LiPC. Of the latter branch, LiPF and LiPC are only distantly related with other members. This observation is consistent with the classification based on the comparison of intron/exon structures. The emergence of *lipA, lipB, lipE, lipG, lipH* and *lipI* appears to be a relatively recent event, possibly facilitated by gene duplication or translocation. The evolutionary origin of the *lip* family and the reasons for the existence of multi-copy, clustered *lips* remain unclear. There have been reports showing the kinetic differences among various LiP isozymes [Farrell et al., 1989; Glumoff et al., 1990], suggesting the specific role for individual LiPs during lignin degradation. This might be one driving force for the emergence of multi-copy *lip* genes.

In the 5′ non-coding regions of many *P. chrysosporium lips*, sequence elements similar or identical to cAMP response element (CRE), activating protein 2 (AP2) binding site and mammalian xenobiotic response element (XRE) [Faist and Meyer, 1992] have been found. In Fig. 1.4, the positions of these putative elements in sequenced *LG2* (with synonym *lipE*) [Ritch and Gold, 1992] from strain OGC101 are shown. The involvement of these promoter sequences in the regulation of *lip* expression has not been analyzed.

1.2.2 The Regulation of *lip* Expression in *P. chrysosporium*

In *P. chrysosporium* strain OGC101, immunoblot analysis showed that LiP proteins were detectable in the extracellular media of nitrogen-limited, but not nitrogen-sufficient cultures [Li et al., 1994]. In accordance with this observation, Northern (RNA) blot analysis demonstrated that the *lip* transcripts were also detectable in nitrogen-limited, but not nitrogen-sufficient cultures [Li et al., 1994],

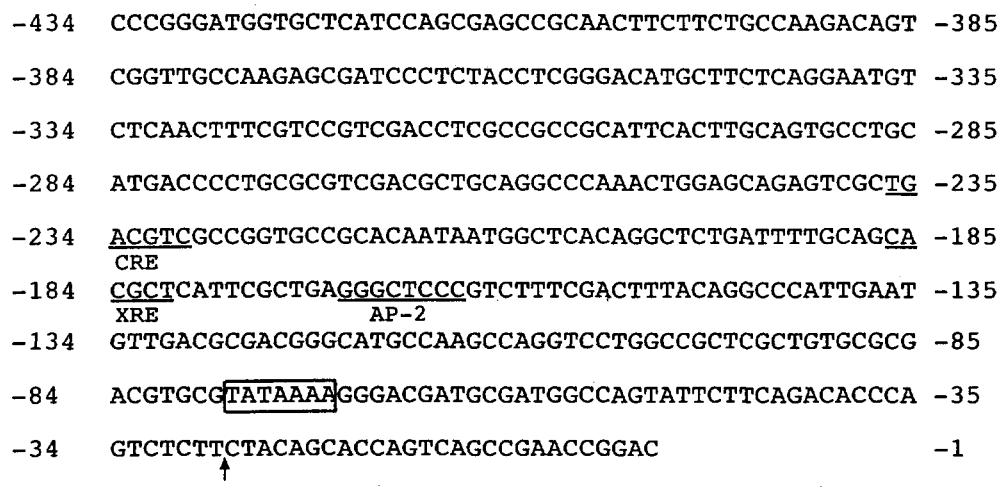

Fig. 1.4 The 5′ non-coding region of *LG2* (synonym *lipE*, encoding LiP isozyme H8) in *P. chrysosporium* strain OGC101. The TATAAA sequence is boxed. Three putative elements, namely a cAMP response element (CRE), a xenobiotic response element (XRE), and an AP-2 binding site, are underlined. The arrow denotes the 5′ end of the corresponding cDNA.

suggesting that the availability of nutrient nitrogen affects the regulation of *lip* expression at the transcription level.

Because of the high homology among *lips*, it is difficult for Northern blot to differentiate various *lip* transcripts. Two methods, nuclease protection assay [Reiser et al., 1993] and quantitative reverse-transcription PCR (RT-PCR) [Stewart et al., 1992; Stewart and Cullen, 1999], have been developed to detect all known *lip* transcripts in *P. chrysosporium* strain BKM-F-1767. Various studies have shown that in this strain, different *lips* may be differentially regulated by nutrient carbon (C) and nitrogen (N) at the transcriptional level [Stewart et al., 1992; Cullen, 1997; Stewart and Cullen, 1999]. It was shown that the transcript levels of *lipA, lipB* and *lipG* were similar in both C- and N-limited cultures. However, the transcript levels of *lipC, lipF, lipH* and *lipJ* were substantially increased under N-limitation, while increased transcript levels of *lipD*, *lipE* and *lipI* were observed under C-limitation. The *lipC* and *lipD* genes have highest transcript levels in N- and C-limited cultures, respectively. It is not yet known how the changes in *lip* transcripts reflect the changes in LiP isozyme profiles. Furthermore, no relationship has been found between the genomic organization and *lip* transcript levels. As reviewed in Section 1.2.1, eight *lips* in *P. chrysosporium* strain BKM-F-1767 cluster in a defined region on one chromosome. However, the eight genes, including those that are paired and closely linked to each other, were shown to be differentially transcribed under different culture conditions [Stewart and Cullen, 1999].

The results obtained from nuclease protection assays and RT-PCR experiments designed to examine the regulation of *lip* expression in response to nutrient nitrogen appear to be contradictory to those obtained from Northern blot analysis. This might be due to the difference in methodology. Moreover, factors, such as strain differences, different culture conditions including stationary versus agitating cultures and buffer composition, are shown to affect LiP isozyme profiles [Gold and Alic, 1993; Cullen, 1997]. Conceivably they may also affect the transcription of *lips*. For instance, it has been shown that in strain ME-446, *lipD* (synonym LIG5) transcripts were the most abundant when the fungus is grown in N-limited cultures [James et al.,

1992] or cultures containing more complex substrates such as ball-milled straw [Broda et al., 1995]. In strain OGC101, *LG2* (synonym *lipE*) was shown to be expressed at high levels [Ritch and Gold, 1992]. These reports complicate the study of *lip* expression in *P. chrysosporium*.

Quantitative RT-PCR was also used to examine different *lip* transcripts in *P. chrysosporium*-colonized wood chips [Janse et al., 1998] and organopollutant-contaminated soil [Lamar et al., 1995; Bogan et al., 1996a]. The utilization of magnetic oligo(dT) beads facilitates the isolation of mRNA from these complex substrates. In brief, the overall production pattern of various *lip* transcripts differed in *P. chrysosporium* grown in chemically defined medium, aspen wood chips or contaminated soil.

Other factors were also shown to regulate the level of *lip* transcripts or LiP activity. It was reported that the activity of LiP was increased by adding aromatic compounds such as benzyl alcohol [Gold and Alic, 1993] and is reduced by adding an inhibitor of cAMP production [Boominathan and Reddy, 1992] to *P. chrysosporium* cultures. Furthermore, high oxygen level and manganese deficiency appeared to increase LiP activity in N-limited cultures [Rothschild et al., 1999]. Nevertheless, very little is known about the mechanism of the complex regulation of *lip* expression. The large gene family and lack of molecular research tools continue to pose significant problems.

1.2.3 The Structure of *mnps* in *P. chrysosporium*

Three genes encoding various MnP isozymes, namely *mnp1* [Pribnow et al., 1989; Godfrey et al., 1990], *mnp2* [Mayfield et al., 1994a], and *mnp3* [Alic et al., 1997] in *P. chrysosporium* strain OGC101 have been reported. In strain BKM-F-1767, two cDNA sequences closely related to those derived from *mnp2* and *mnp3* were also reported [Pease et al., 1989; Orth et al., 1994]. When *mnp1* cDNA was used to search for homologues in the finished genome sequence of *P. chrysosporium* strain BKM-F-1767 through the BLAST search engine, five *mnp*s were found. Based on the identity of the coding regions of these five genes to those of *mnp1*, *mnp2* and

mnp3, they were identified as two copies of *mnp1*, which are tentatively named *mnp1a* and *mnp1b*, and single copies of *mnp2*, *mnp3* and *mnp4* (which was not previously detected). The messenger RNAs derived from all *mnp*s share over 75% homology and the encoded proteins share over 80% homology.

The two copies of *mnp1* in strain BKM-F-1767, *mnp1a* and *mnp1b*, reside on the same chromosome with an intervening 7-kb sequence and possess opposite transcriptional orientations, reminiscent of the gene-clustering phenomenon of *lip*s. The nucleotide identity is about 95% within the coding regions of *mnp1a*, *mnp1b* (BKM-F-1767) and *mnp1* (OGC101). The polypeptides encoded by these three genes are identical. However, the non-coding regions of *mnp1* (OGC101) is a "hybrid" of *mnp1a* and *mnp1b* (BKM-F-1767): the 5′ non-coding region of *mnp1b* shares over 95% homology with that of reported *mnp1*, whereas the 3′ non-coding region of *mnp1a* shares over 95% homology with that of *mnp1* (Fig. 1.5). The 3′ non-coding regions of *mnp1a* and *mnp1b* have very little homology, whereas the 5′ non-coding region of the two genes share 66% homology. The 375-bp regions immediately upstream of the start codon in *mnp1a*, *mnp1b* (BKM-F-1767) and *mnp1* (OGC101) are 92% identical (Fig. 1.5). We have performed PCR using genomic DNA from various homokaryon derivatives of strain OGC101 as templates and primers specifically hybridizing with the 5′ and 3′ non-coding regions of *mnp1a*, *mnp1b* (BKM-F-1767) and *mnp1* (OGC101). In all the homokaryons examined, only the PCR with the oligo pair designed according to *mnp1* (OGC101) yielded product,
confirming that only one copy of *mnp1* exists in strain OGC101 [Ma and Gold, unpublished data]. Approximately 95% identity can be found in both the non-coding region and coding region of *mnp2* and *mnp3* from either strain BKM-F-1767 or OGC101. *mnp4* in strain BKM-F-1767 contains non-coding regions different from those of the other *mnp*s. *mnp2*, *mnp3* and *mnp4* are not linked to each other. Moreover, they are not linked to *mnp1a* and *mnp1b* in the genome of strain BKM-F-1767, consistent with a previous report [Orth et al., 1994]. None of the *mnp*s are closely linked to the *lip*s.

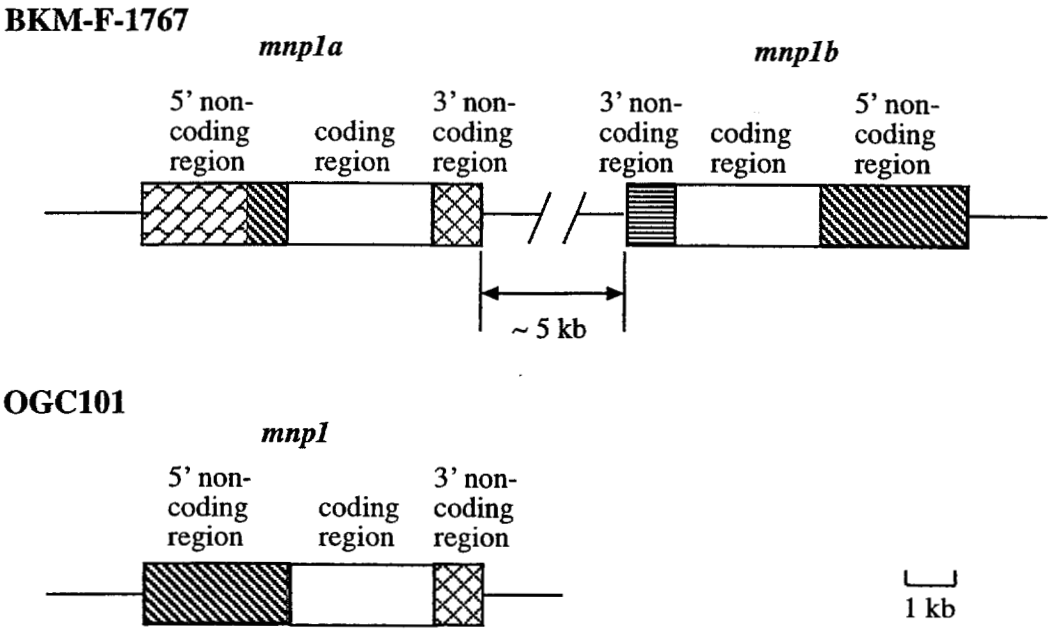

Fig. 1.5 Gene structures of *mnp1* from *P. chrysosporium* strain BKM-F-1767 and OGC101. In BKM-F-1767, two copies of *mnp1* (*mnp1a* and *mnp1b*) are closely linked. OGC101 has only a single copy of *mnp1*. The coding regions of all three genes share over 95% homology. The sequences in their 5′ and 3′ non-coding regions that share more than 90% homology are represented by the same filling pattern.

Among the *mnp*s discussed above, *mnp2* and *mnp4* contain seven introns, while *mnp1a*, *mnp1b* and *mnp3* contain six. The intron/exon structures of these genes are shown in Fig. 1.3B. The size of introns ranges from 50 to 72 bp. All introns contain the consensus intron/exon junction sequence as well as the putative internal lariat formation site [Ballance, 1986].

The 5′ non-coding regions of all the *mnp*s contain the TATAAA element at positions -120 to -80 relative to the translation start codon. Sequence analysis has also revealed the existence of many putative *cis*-elements in the 5′ non-coding regions of the *mnp*s. These include the inverted CCAAT element (ATTGG), recognition site for AP-2 (consensus sequence CCCMNSSS, M=A+C, N=A+G+C+T, S=G+C) [Faist and Meyer, 1992], metal response element (MRE, TGCRCNC, R=A+G) [Thiele, 1992] and heat shock element (HSE, CNNGAANNTTCNNG) [Fernandes et al., 1994]. These elements, characterized in many eukaryotic genes, are believed to be responsible for the regulation of gene transcription under various conditions. For instance, MRE and HSE are recognized by metal response element-binding transcription factor and heat shock transcription factor, respectively. MRE is conserved in fruit fly, mouse and human and is responsible for transcriptional regulation by transition metals ions [Thiele, 1992]. HSE is conserved among baker's yeast, fruit fly and human, and is responsible for transcriptional activation by heat shock stress [Morimoto, 1993]. Fig. 1.6 shows the locations of selected putative *cis*-elements relative to the associated translation start codon in the 1.5 kb of the 5′ non-coding regions of *mnp1a*, *mnp1b*, *mnp2*, *mnp3* and *mnp4* from strain BKM-F-1767. The pattern of the *cis*-elements is identical in the 5′ non-coding regions of *mnp1* (OGC101) and *mnp1b* (BKM-F-1767). The nucleotide sequence of the 1,528-bp *mnp1* 5′ non-coding region (OGC101) is shown in Fig. 1.7, in which the various putative *cis*-elements are indicated.

One noteworthy observation is that some consensus MREs are arranged in pairs with overlaps and form 4-bp palindromes in the 5′ non-coding regions of *mnp1a*, *mnp1b* and *mnp2*. This arrangement is not observed in MREs found in other genes. *mnp1a* contains one MRE pair located at 320 bp upstream of the translation

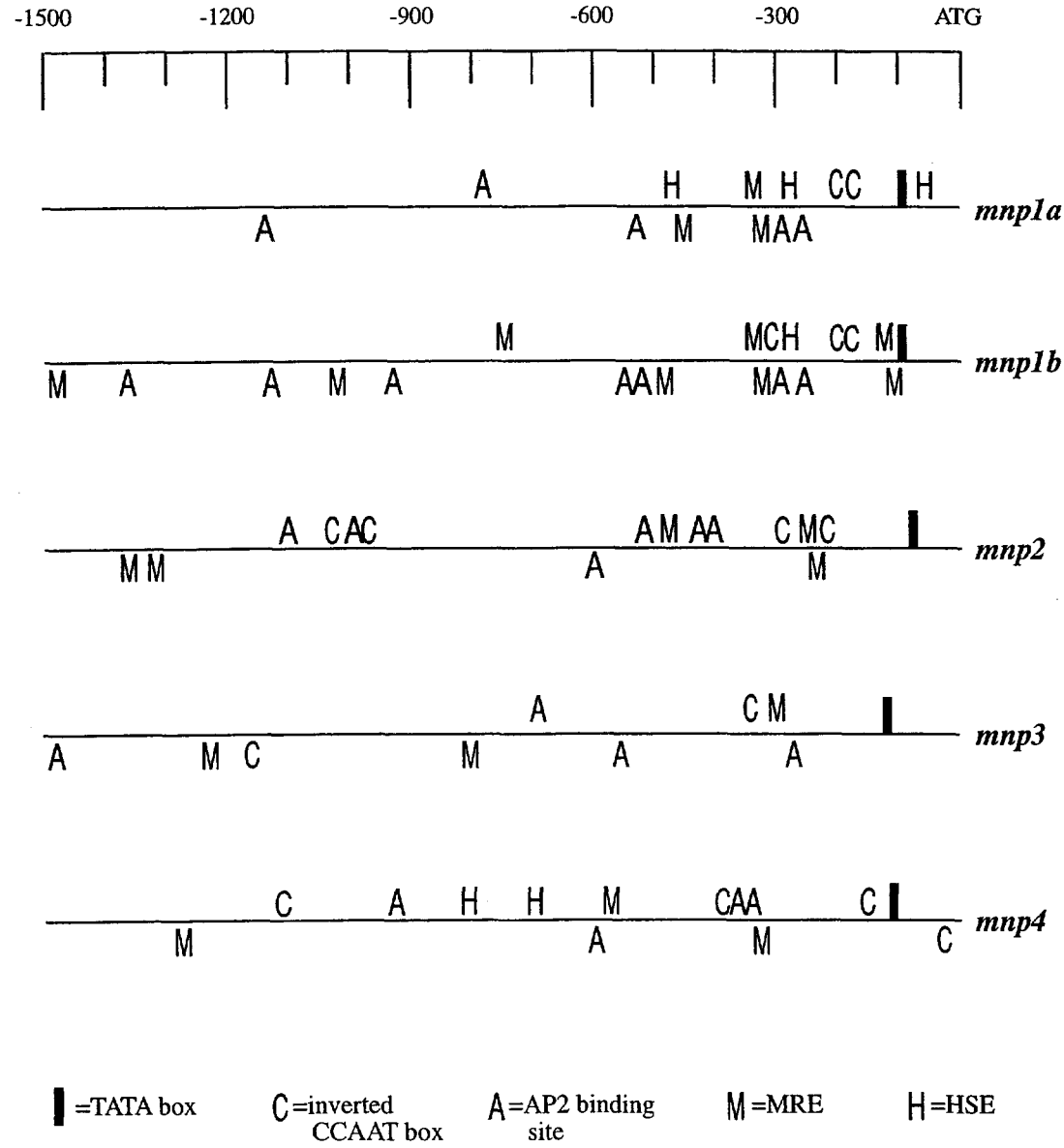

Fig. 1.6 Comparison of the 5′ non-coding regions of *mnp1*, *mnp2*, *mnp3*, and *mnp4* in *P. chrysosporium*. The sequence information is obtained from http://www.jgi.doe.gov/programs/whiterot.html.

Fig. 1.7 The 5′ non-coding region of *mnp1* from *P. chrysosporium* strain OGC101. The TATAAA sequence is boxed. The putative MREs are in bold and underlined. The overlapping MREs are labeled as MRE D (MRE distal pair) and MRE P (MRE proximal pair). The inverted CCAAT boxes are both overlined and underlined. The putative AP-2 binding sites are overlined with arrows. The 48-bp sequence that contains a putative Mn^{2+}-responsive *cis*-element is in bold and boxed (described in Chapter 3).

start codon (Fig. 1.6). *mnp1b* contains two MRE pairs located at 100 and 320 bp upstream of translation start codon (Figs. 1.6 and 1.7). There is one MRE pair in *mnp2* located at 250 bp upstream of the translation start codon (Fig. 1.6). No direct evidence has demonstrated the connection between the putative *cis*-elements in the 5′ non-coding regions of *mnp*s and the regulation of *mnp* expression.

1.2.4 The Regulation of *mnp* Expression in *P. chrysosporium*

The expression of *mnp*s is regulated in response to nutrient nitrogen at the transcription level. In *P. chrysosporium* strain OGC101, Northern blot analysis showed that *mnp* transcripts were only detectable in N-limited, but not N-sufficient cultures [Pribnow et al., 1989]. Furthermore, quantitative RT-PCR analysis demonstrated that transcripts from *mnp1*, *mnp2* and *mnp3* were all present at a very low basal level in stationary, N-sufficient cultures, whereas in N-limited cultures, the transcripts of *mnp*1, *mnp*2 and *mnp*3 increased approximately 400-, 1700-, and 100-fold, respectively [Gettemy et al., 1998]. At the protein level, N- and C-limitation affect the production of each MnP isozyme differently in strain BKM-F-1767 [Pease and Tien, 1992]. In general, it was shown that MnP activity appeared and peaked much earlier in N-limited than C-limited cultures. Other factors including strain differences, culture conditions (such as agitating versus stationary incubation) and buffer composition also affect MnP isozyme profiles [Gold and Alic, 1993]. Quantitative RT-PCR analysis has shown that, in strain OGC101, the *mnp2* transcript was predominant in stationary cultures, while the *mnp1* transcript was predominant in agitated cultures [Gettemy et al., 1998].

In addition to N-limitation, the presence of Mn^{2+} is required for MnP expression in *P. chrysosporium*. Immunoblots of both intracellular and extracellular *P. chrysosporium* proteins have demonstrated that MnPs were only present in N-limited and Mn^{2+}-supplemented cultures [Brown et al., 1990]. The extent of induction of MnP activity correlated with Mn^{2+} concentration up to 180 μM [Bonnarme and Jeffries, 1990; Brown et al., 1990]. Moreover, Mn^{2+} cannot be replaced by other metal ions including Fe^{3+}, Mo^{3+}, Ni^{2+}, Zn^{2+}, Co^{2+}, and Mg^{2+} as

the inducer of MnP [Brown et al., 1990, 1991]. Northern blot analysis demonstrated that *mnp* transcripts were only detectable in N-limited cultures supplemented with Mn^{2+} [Brown et al., 1990, 1991; Pease and Tien, 1992]. In the presence of 180 μM Mn^{2+}, *mnp* transcripts were first detected on day 4 and reached a maximum level on day 5. Addition of Mn^{2+} to 5-day-old N-limited, Mn^{2+}-deficient cultures resulted in a detectable level of *mnp* transcripts within 40 min, and the amount of *mnp* transcripts was a function of the final concentration of Mn^{2+} in the medium, up to a maximum of 180 μM. Only poly(A) RNAs from the Mn^{2+}-supplemented cultures, but not Mn-deficient cultures, yielded MnP proteins in an *in vitro* translation experiment [Brown et al., 1991]. All these data suggest that Mn^{2+} status regulates the expression of *mnp*s at the transcriptional level. Quantitative RT-PCR analysis demonstrated that Mn^{2+} might differentially regulate various *mnp*s [Gettemy et al., 1998]. In 5-day-old, N-limited stationary cultures, the levels of *mnp1* and *mnp2* transcripts were dramatically increased in the presence of 180 μM Mn^{2+}; however, *mnp3* transcript concentration remained at a low basal level regardless of Mn^{2+} supplementation. In contrast to its effect on transcription of *mnp*s, Mn^{2+} at a concentration of 180 μM has no significant effect on the growth, consumption rate of C- and N-source, and synthesis of secondary metabolites in *P. chrysosporium* [Bonnarme and Jeffries, 1990; Brown et al., 1990].

The transcription of *mnp*s in *P. chrysosporium* is also induced by heat shock [Brown et al., 1993], hydrogen peroxide and chemical stress [Li et al., 1995]. In N-limited, Mn-deficient cultures, *mnp* transcripts were detectable within 15 min and reached the highest level in 1–2 hrs after increasing the incubation temperature from 37°C to 45°C or supplementing with 1.0 mM H_2O_2. However, in the absence of Mn^{2+}, MnP protein and activity were not observed after these treatments. In contrast, supplementation with both Mn^{2+} and H_2O_2 or heat shock in the presence of Mn^{2+} resulted in a synergistic induction of *mnp* expression. Higher levels of *mnp* transcripts and MnP activity were seen after the combined treatment than with Mn^{2+} alone. The induction effect of either H_2O_2 or heat shock was not observed under N-sufficient conditions. Furthermore, a whole range of chemicals, such as ethanol,

sodium arsenite, and 2,4-dichlorophenol that are known to induce heat shock-like responses in other organisms [Nover, 1991], could also induce the accumulation of *mnp* transcripts in the same manner as H_2O_2 and heat shock in *P. chrysosporium*. The various environmental conditions described above that affect *mnp* expression are summarized in Fig. 1.8.

In addition to heat shock, H_2O_2 and chemical stress, it has been demonstrated that purging 5-day-old N-limited, Mn^{2+}-supplemented *P. chrysosporium* cultures with 100% O_2 resulted in an increase in *mnp* transcript levels and in MnP activity [Li et al., 1995]. However, in contrast to the effect of heat shock and H_2O_2, purging with O_2 in the absence of Mn^{2+} has no effect on the accumulation of *mnp* transcripts.

Although sequences identical to the consensus MRE and HSE are found in the 5′ non-coding regions of various *mnp*s, no direct evidence has demonstrated the involvement of these sequences in the transcriptional regulation of *mnp*s by Mn^{2+} status, heat shock, hydrogen peroxide and chemical stress. At present, the precise mechanisms of the regulation in response to these agents remain unknown.

The levels of *mnp* transcripts were also measured by quantitative RT-PCR in *P. chrysosporium* strain BKM-F-1767 grown on polycyclic aromatic hydrocarbon (PAH)-contaminated soil [Bogan et al., 1996b] and aspen wood chips [Janse et al., 1998]. *mnp1*, *mnp2* and *mnp3* transcripts were all expressed under both culture conditions. The variations in the levels of the three transcripts were not substantial. In addition, PAH disappearance in the soil was concurrent with the expression of MnP in *P. chrysosporium*.

1.2.5 Reporter Systems to Study the Regulation of *mnp* Expression in *P. chrysosporium*

In the majority of cases, the regulation of a certain gene (designated X gene) expression at the transcription level is accomplished by *trans*-acting transcription factors interacting with specific *cis*-acting elements residing in X gene's promoter (promoter X). Such interactions modulate the transcription activity of promoter X in response to changes in environmental conditions. In order to identify *cis*-acting

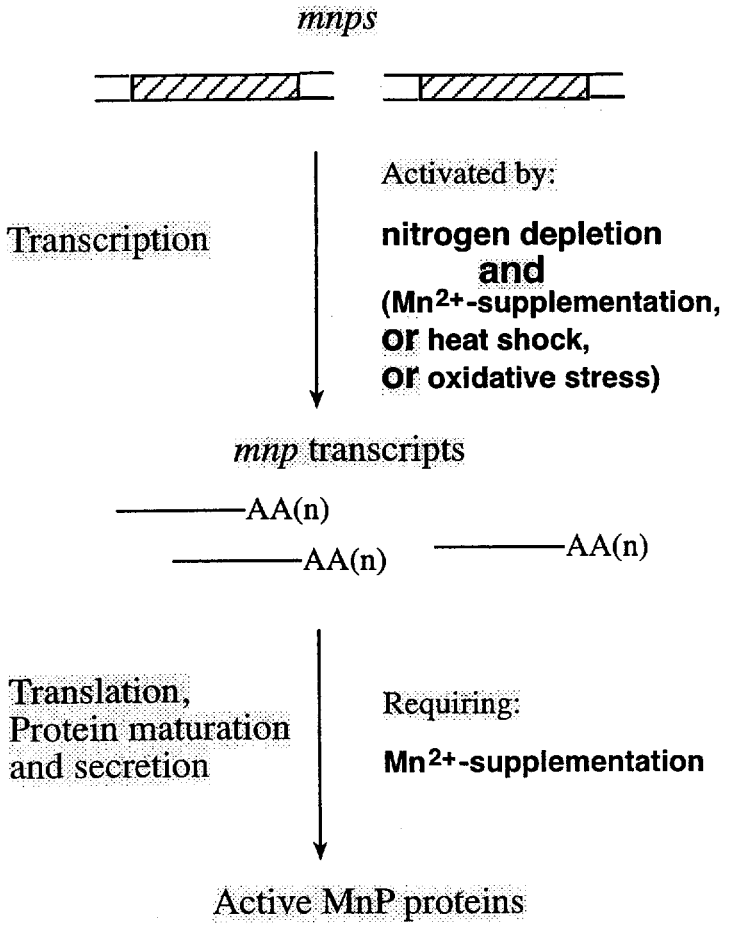

Fig. 1.8 Environmental conditions that affect the expression of *mnps* in *P. chrysosporium*.

elements in promoter X, it is necessary to generate mutated derivatives of this promoter and place a sensitive reporter gene under the control of each mutated promoter. The effects of each mutation on the promoter X's transcription activity are assessed by measuring the reporter expression (usually through measuring the activity of the reporter product) under the conditions that modulate X gene expression. Significant change in reporter expression should be observed when a functional regulatory element is mutated. Characterization of the *cis*-acting sequences comprising such a regulatory element is often the first step in elucidating the molecular mechanism of the gene expression regulation.

Previously, two reporter systems were developed in our lab for studying the transcriptional regulation of *mnp* expression in *P. chrysosporium*. The first system used *S. commune* orotidylate decarboxylase (ODase) gene (*ura1*) as a reporter [Godfrey et al., 1994]. The 1.5-kb *P. chrysosporium mnp1* 5′ non-coding region was joined immediately upstream of the *S. commune ura1* coding region in a plasmid (pAMO) containing *S. commune ade5* as a selectable marker. pAMO was used to transform a *P. chrysosporium* ade$^-$ ura$^-$ strain that lacks endogenous ODase activity. The resulting transformants expressed ODase only in N-limited, Mn^{2+}-sufficient cultures in the same manner as the endogenous MnP. When Mn^{2+} was added to N-limited and Mn^{2+}-deficient cultures, both ODase and MnP activity were induced synchronously. These data indicate that this reporter system can be used to study the regulation of *mnp1* promoter activity. However, there are three disadvantages of this system. First, it utilizes a double-auxotrophic strain that exhibited lower transformation frequency compared to the wild type or to the single-auxotrophic strain of *P. chrysosporium*. Second, the ODase activity assay, which measures the evolution of $^{14}CO_2$ from ^{14}COOH-orotidyl monophosphate, is tedious. Third, among the transformants carrying pAMO, the induction of ODase activity in response to exogenous Mn^{2+} varied between 0 to 25-fold, with an average of 8-fold [Godfrey et al., 1994]. This large variability in apparent induction ratios suggests that ODase is an inefficient reporter of the minor changes in *mnp1* promoter activity. The second system used truncated *mnp* genes as the reporter [Gettemy et al., 1997]. This was

accomplished by making internal deletions within the coding regions of the *mnp1* or *mnp2*, which were then individually subcloned into a plasmid carrying the *S. commune ade5* as a selectable marker. When the plasmid containing the deletion was introduced into *P. chrysosporium*, the truncated version of *mnp1* or *mnp2* transcript was expressed and could be differentiated from the wild type *mnp1* or *mnp2* transcript by Northern blot or RT-PCR analysis. The advantage of this system is that it directly measures the transcript level of the reporter gene. The disadvantage of this system is the tedium of RNA preparation and the following Northern blot or RT-PCR analysis, rendering it unsuitable for processing a large number of *P. chrysosporium* transformants.

The best reporter systems lend themselves to rapid and highly sensitive reporter assays, often based on colorimetry and photometry. Examples of reporters for which colorimetric assays are available, include β-galactosidase (*LacZ*) [Casdaban et al., 1983] and 3-glucoronidase (GUS) [Nacken et al., 1996] from bacterial origins; those possessing assayable photometric activities include luciferase (*luc*) from firefly *Photinus pyralis* [de Wet et al., 1987] and GFP (*gfp*) from jellyfish *Aequorea victoria* [Chalfie et al., 1994]. These reporters have been introduced into various cell types from a variety of organisms, including bacteria, fungi, plants, animals and human. However, as discussed in Section 1.1.6, many factors affect the expression of heterologous genes in basidiomycetes. These factors may exert different effects on individual heterologous genes. Therefore, the expression of the reporter genes mentioned above remains an obstacle in basidiomycetes. In some cases, modifying the reporter gene according to the codon preference and (G+C) content of the basidiomycete host may result in its expression [Scholtmeijer et al., 2001]. In *P. chrysosporium*, the expression of reporters such as *LacZ*, GUS and *luc* has not been successful [Alic and Gold, unpublished data]. However, when an intron is inserted at the 5′ end of a *gfp* variant (*egfp*), it appears to be a faithful reporter for both *P. chrysosporium gpd* and *mnp1* promoter functions [Ma et al., 2001]. The construction and characterization of this reporter system is discussed in Chapter 2.

GFP from *A. victoria* contains 238 amino acids in a single polypeptide chain. It absorbs blue light, with maxim at 395 nm and 470 nm, and emits green light with a maximum at 509 nm [Cormack, 1998]. Formation of the fluorophore in GFP requires molecular oxygen, but unlike many reporters that are enzymes, the GFP fluorescence requires neither substrates nor cofactors. Several variants of the wild type GFP have been created to improve the fluorescence intensity [Cormack et al., 1996; Heim and Tsien, 1996]. One of them is enhanced green fluorescent protein, EGFP, which contains a change of two amino acids in the fluorophore [Cormack et al., 1996]. Its maximal excitation peak shifts to approximately 490 nm and its fluorescence is 35-fold brighter than wild-type GFP. The gene encoding EGFP, *egfp*, contains a (G+C) content of 59%, similar to that of the *P. chrysosporium* genome. The codon preference is also similar among *egfp* and various *P. chrysosporium mnp*s. Furthermore, GFP has been scrutinized for its efficacy as a reporter in the functional characterization of promoters in baker's yeast, *S. cerevisiae* [Niedenthal et al., 1996]. When three constructs each containing *gfp* joined downstream of *S. cerevisiae GAL1*, *URA3* or *GAL4* promoters (strong, weak or very weak promoter respectively) were introduced into yeast cells, the GFP fluorescence were readily distinguishable in accordance with the promoter strengths. In addition, the fluorescence levels faithfully reported the induction of *GAL1* transcription by galactose and repression of *GAL4* transcription by glucose. All these data suggest that the use of GFP as a reporter for gene expression in *P. chrysosporium* would be feasible. GFP and its variants have also been used as markers for living cells and for the subcellular localization of proteins and organelles. This is often achieved by fusing GFP with a particular host protein or with a targeting signal for specific organelles. Since GFP fluorescence requires no cofactors, it can be detected by fluorescence activated cell sorting (FACS) and fluorescence microscopy without disrupting living cells.

Since plasmid DNA typically integrates ectopically in multiple copies in the *P. chrysosporium* genome, caution must be taken in utilizing any gene expression reporter system in this organism. The copy number of the integrated DNA and the sites of integration can affect the expression of reporter gene in individual

transformants. Thus, it is often necessary to examine the reporter activity from a large number of transformants in order to assess the transcription activity of a particular promoter or its mutant derivative. When concluding that an observed change in reporter activity is caused by a particular mutation constructed in the promoter concerned, such a change must be statistically significant. Utilizing *egfp* as the reporter gene, we have used this approach to identify a 33-bp *cis*-acting sequence in the *mnp*1 promoter, which may be responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity. This study will be described in Chapter 3.

1.2.6 The *lip*- and *mnp*-Related Genes in Other White-Rot Fungi

The best-characterized extracellular lignin-modifying enzymes in white-rot fungi are LiP, MnP and laccase. Laccase is a copper-containing oxidase, which utilizes molecular oxygen as an oxidant and oxidizes phenolic compounds to the corresponding radicals [Eriksson et al., 1990]. The activities of MnP and laccase have been found in almost all white-rot fungi, including most species in the Order Aphyllophorales [Hatakka, 1994; Pelaez et al., 1995]. However, LiP activity was observed only in a small number of white rot fungi, including species in the Genus *Phanerochaete*, *Phlebia*, *Trametes* and *Bjerkandera* [Hatakka, 1994]. Laccase activity has not been found in *P. chrysosporium* [Gold and Alic, 1993]. Based on their enzyme production patterns, white-rot fungi that are efficient lignin degraders (estimated by $^{14}CO_2$ evolution from ^{14}C-[Ring]-labeled synthetic lignin) could be categorized into two groups: LiP–MnP group (e.g., *P. chrysosporium* and *Phlebia radiata*) and MnP–laccase group (e.g., *Dichomitus squalens* and *Ceriporiopsis subvermispora*) [Hatakka, 1994]. The physiological roles of LiP, MnP and laccase in each white-rot fungus are still not known.

Genes encoding LiP have been characterized in several white-rot fungi. These include four genes in *T. versicolor* [Black and Reddy, 1991; Jonsson and Nyman, 1992; Jonsson and Nyman, 1994; Johansson and Nyman, 1996], one in *Bjerkandera adusta* [Asada et al., 1992] and one in *P. radiata* [Saloheimo et al., 1989]. Several genomic and cDNA sequences encoding MnP have also been isolated from white-rot

fungi such as *T. versicolor* [Johansson and Nyman, 1996], *P. ostreatus* [Irie et al., 2000], *C. subvermispora* [Lobos et al., 1998; Tello et al., 2000], *D. squalens* [Li et al., 1999] and *Elfvingia applanata* [Maeda et al., 2001]. Pairwise amino acid sequence comparisons of LiPs from the strains mentioned above and from *P. chrysosporium* showed similarities ranging from 53% to 98%. A similar level of homology is also found among the amino acid sequences of MnPs from various white-rot fungi. Furthermore, approximately 38% to 50% similarity is shared between any known LiP and MnP. All these LiPs and MnPs are evolutionarily related to other heme peroxidases from plants, fungi and bacteria [Welinder, 1992]. Together they comprise a plant peroxidase superfamily. The residues believed essential to peroxidase activity, including those as ligands for the heme prosthetic group, are conserved among these proteins. Moreover, the residues involved in the binding of Mn^{2+} are conserved among all MnPs [Gold et al., 2000].

A new type of heme peroxidase, the so-called versatile peroxidase (VP) was first characterized in *Pleurotus eryngii* [Ruiz-Duenas et al., 1999b]. This enzyme may be a hybrid of typical MnP and LiP. The special feature is that VP is a Mn-oxidizing peroxidase exhibiting Mn-independent activity. In the presence of Mn^{2+}, VP oxidizes Mn^{2+} to Mn^{3+} as a typical MnP. In the absence of Mn^{2+}, VP is also able to oxidize a spectrum of non-phenolic compounds and synthetic dyes including veratryl alcohol, which is a typical substrate for LiP but not for MnP [Ruiz-Duenas et al., 1999b]. Besides *P. eryngii*, VP was also characterized in other *Pleurotus* species such as *P. ostreatus* [Sarkar et al., 1997], *P. pulmonarius* [Camarero et al., 1996]. Genes encoding VP have been cloned from *P. eryngii* [Ruiz-Duenas et al., 1999b]. The amino acid sequence comparisons among VP, MnP and LiP have shown that VP contains not only the conserved residues for Mn binding in MnP, but also the conserved residues involved in the interaction with aromatic substrates in LiP. Higher homology was observed among VP and *P. chrysosporium* LiPs than VP and *P. chrysosporium* MnPs (58–60% versus 50–55%) [Ruiz-Duenas et al., 1999b].

Other genes structurally related to *P. chrysosporium lip*s and *mnp*s include *PGV* [Jonsson et al., 1994] and *npr* [Collins et al., 1999] from *T. versicolor*. The

deduced amino acid sequences from both *PGV* and *npr* contain the conserved residues for Mn binding in MnPs. However, the former has higher homology with *P. chrysosporium* LiPs than with MnPs (54–60% versus 44–48%) [Cullen, 1997]. The latter shares less than 50% homology with both *P. chrysosporium* LiPs and MnPs. No peroxidases encoded by *PGV* and *npr* have been isolated; hence, the function of these genes remains unclear.

A gene cluster in the order of *LPGIII*, *LPGIV* (both encoding LiP) and *MPGI* (encoding MnP) was found in *T. versicolor* [Johansson and Nyman, 1996]. The three genes are arranged in the same transcriptional orientation within a 10-kb region and with intergenic distances of approximately 2.4 kb. The gene clusters of *lips* and *mnps* in *P. chrysosporium* are reviewed in Sections 1.2.1 and 1.2.3. However, the close linkage of MnP- and LiP-encoding genes seen in *T. versicolor* is not observed in *P. chrysosporium*.

All the *lip*- and *mnp*-related genes discussed above contain introns, but the number of introns differs in genes according to their origin [Camarero et al., 2000]. The genes encoding VP and MnP in some species of genus *Pleurotus* have the highest number of introns (a total of 15 with similar size and disposition). *PGV* from *T. versicolor* also has a high number of introns (12 introns). However, other genes encoding MnP and LiP in *T. versicolor* contain only 5–6 introns, comparable to their counterparts in *P. chrysosporium* (6–9 introns). The diagram of intron distributions in some peroxidase genes is shown in Fig. 1.2C.

The 5′ non-coding region of all *mnp*- and *lip*-related genes from white-rot fungi other than *P. chrysosporium* contain TATA boxes in the neighborhood of 60–100 bp upstream of the translation start codon. Other identified putative promoter elements include inverted CCAAT, MRE, HSE, and binding sites for transcription factors AP-1, AP-2. Among them, MRE(s) and HSE(s) have been found not only in MnP-encoding genes from *D. squalens* [Li et al., 1999], *T. versicolor* [Johansson and Nyman, 1996], *P. ostreatus* [Asada et al., 1995] and *C. subvermispora* [Tello et al., 2000], but also VP-encoding gene in *P. eryngii* [Ruiz-Duenas et al., 1999b] and LiP-

encoding gene in *T. versicolor* [Johansson and Nyman, 1996]. Again, the function of these putative promoter elements has not been examined.

1.2.7 The Regulation of *lip*- and *mnp*-Related Gene Expression in Other White-Rot Fungi

Physiological studies have shown that in white-rot fungi *B. adusta*, *Bjerkandera sp.* Strain BOS55, *P. ostreatus* and *Lentinula edodes*, high levels of ligninolytic enzyme activities, including the activities of LiP, MnP and VP, were observed in N-sufficient cultures, but not in N-limited cultures [Kaal et al., 1995]. This is opposite to what has been observed in *P. chrysosporium*, which only produces LiP and MnP under N-depleted conditions. The form of nitrogen source also affects the production of LiP and MnP in several white-rot fungi. For example, the highest MnP activity of *D. squalens* was found in N-limited cultures in which amino acids Asn and Phe, instead of ammonium salt, were used as the N-source [Périé and Gold, 1991]. Several species in the genus *Pleurotus*, such as *P. eryngii* and *P. ostreatus*, do not produce ligninolytic enzymes when grown in medium containing NH_4^+ salt as the N-source; however, when peptone was used as the N-source, these fungi produced a large amount of VP or both VP and MnP [Camarero et al., 1996; Martinez et al., 1996; Sarkar et al., 1997; Ruiz-Duenas et al., 1999a]. The enhancement of MnP production by peptone was also observed in *B. adusta, Bjerkandera* sp. Strain BOS55 and *L. edodus* [Kaal et al., 1995]. The molecular mechanism of how nitrogen availability and different nitrogen source regulate the expression of *lip*- and *mnp*-related genes awaits future experimentation.

The induction of *mnp*-related gene expression by Mn^{2+}, as shown in *P. chrysosporium*, was also observed in *D. squalens* [Périé and Gold, 1991], *T. versicolor* [Johansson et al., 2002], *C. subvermispora* [Tello et al., 2000] and *P. ostreatus* [Asada et al., 1995]. RT-PCR results showed a large increase of *mnp* transcript levels in *T. versicolor* and *C. subvermispora* in Mn^{2+}-supplemented cultures, in comparison with Mn-deficient cultures. Differential regulation of the expression of various *mnps* by Mn^{2+} was also observed in several white-rot fungi. In

C. subvermispora, the transcription of *Cs-mnp2*, but not that of *Cs-mnp3*, was activated by Mn^{2+} [Tello et al., 2000]. In *T. versicolor*, adding 200 μM Mn^{2+} to the Mn-deficient cultures resulted in an 8-fold and a 250-fold increase in *mnp1* and *mnp2* transcript concentrations, respectively [Johansson et al., 2002]. Interestingly, the putative MRE was only found in *Tv-mnp1*, but not in *Tv-mnp2*, suggesting that MRE might not be responsible for *mnp* regulation by Mn^{2+} in this fungus.

Besides acting as an inducer for *mnp* expression, Mn^{2+} also appears to be a repressor for the expression of VP-encoding genes in *P. eryngii* [Ruiz-Duenas et al., 1999a] and *npr* in *T. versicolor* [Collins et al., 1999]. Neither transcript from VP-encoding genes nor VP activity could be detected in glucose-peptone culture of *P. eryngii* when Mn^{2+} concentration of 25 μM or higher was added. In *T. versicolor*, the transcript of *npr* was most abundant in Mn-deficient culture and was greatly reduced when the Mn^{2+} concentration reached as low as 0.5 μM. Hence, it is possible that the emergence of VP and the enzyme encoded by *npr* may be temporally separated from that of MnP.

Finally, it should be noted that the research on the regulation of *lip*- and *mnp*-related gene expression in white-rot fungi is still at an early stage. Little is known about the physiological role of each ligninolytic enzyme in these organisms. However, more answers will be obtained when new molecular tools are developed for these organisms.

1.3 TRANSITION METAL ION REGULATION OF GENE EXPRESSION

For all living organisms, many transition metals are essential nutrients. Among these metals, Fe, Cu, Co, Mo, Mn, Zn and Ni play a central role in enzymatic catalysis. Zn is also essential for the structure integrity of many proteins. However, high levels of these metals are toxic to living cells. Therefore, the maintenance of transition metal ion homeostasis in living cells is critical, since both the depletion and over-feeding of these metals can cause adverse and even lethal effects. One key mechanism for controlling transition metal ion homeostasis is to

regulate the expression of metal-binding proteins and metal transporters according to the environmental availability of transition metals. In this section, several well-characterized models of this regulation are reviewed. As frequently observed, the basic regulatory mechanism is often conserved among many organisms to regulate biological related cellular events. Information acquired in other organisms about how metal ions regulate gene expression may shed some light on our study of how Mn^{2+} regulates *mnp* expression in *P. chrysosporium*.

1.3.1 Regulation of Metallothionein Gene Expression by Transition Metals

Metallothioneins (MTs) are small, cysteine-rich proteins that have been found in all eukaryotes and some prokaryotes. Based on structural similarity, MTs can be divided into three distinct classes. All vertebrates contain class I MTs, while fungi, plants and some cyanobacteria contain class II and class III MTs [Heuchel et al., 1995]. Under physiological conditions, MTs bind multiple Zn and Cu atoms, but differences exist among MTs from single-cell eukaryotes and mammals. It has been shown that MTs from single-cell eukaryotes bind Cu predominantly, whereas most mammalian MTs bind Zn predominantly [Kagi, 1991; Palmiter, 1998].

In higher eukaryotes, as exemplified by mouse and human, the main functions of MTs are the maintenance of heavy metal homeostasis and the detoxification of overloaded heavy metals. MTs also act as potent radical scavengers and play prominent roles in cellular antioxidant defense and redox balancing [Andrews, 2000]. In general, MTs are stress proteins, which respond to many environmental changes and control the distribution of heavy metal ions in the cell.

Human, mouse and other vertebrates contain four types of MTs, of which MT-I and MT-II are ubiquitously expressed, while MT-III and MT-IV are only expressed in certain tissues [Palmiter, 1998]. The expression of MT-I and MT-II is induced by elevated levels of heavy metals such as Zn as well as Cd, and also by oxidative stress, hypoxia, hormone treatment and viral infection [Andrews, 2000; Ghoshal et al., 2001]. It is believed that this regulation occurs at the transcriptional level. The promoter regions of MT-I and MT-II genes from human, mouse, rat, fish and many other vertebrates contain *cis*-regulatory sequences, named metal response elements

(MREs), which are essential for the regulation of MT genes in response to heavy metals and oxidative stress [Andrews, 2000; Samson et al., 2001]. The consensus sequence of MRE is also found in fruit fly *Drosophila melanogaster* and shown to be functional [Silar et al., 1990], but it is not found in yeast and plants. As mentioned before, MRE-like subsequences were found in the 5′ non-coding regions of many MnP-encoding genes in white-rot fungi. However, the function of these MRE-like subsequences in white-rot fungi is unknown.

Genes encoding MT-1 and MT-2 in mouse and human all contain multiple MREs in their promoters. The *trans*-acting factor that interacts with these MREs was first discovered in mouse and named MTF-1 (MRE-binding transcription factor-1) [Radtke et al., 1993]. Subsequently, MTF-1s from human and *D. melanogaster* were obtained [Brugnera et al., 1994; Zhang et al., 2001]. MTF-1 is a zinc-finger protein that belongs to the Cys_2His_2 transcriptional factors family. It contains six zinc finger motifs within its N-terminal half. This domain functions in zinc sensing and DNA binding. The transcription activation domain resides in the C-terminal half.

Both *in vivo* and *in vitro* experiments show that the DNA binding activity of MTF-1 (both mouse and human) is activated by elevated levels of Zn, but not those of other transition metals, such as Cd and Cu [Bittel et al., 1998; Daniels et al., 2002]. The six zinc finger motifs in MTF-1 may function differently in response to Zn. The zinc finger 1 (ZF-1) that is the closest to the N-terminus is essential for MTF-1 to sense the cellular Zn level, while ZF-2, -3 and -4 constitute the core DNA-binding domain. ZF-5 and -6 are required for stabilizing the DNA–protein interaction [Bittel et al., 2000]. The proposed mechanism of action is that Zn binds to ZF-1, causing a conformation change of MTF-1. This allows ZF-2, -3 and -4 to bind DNA and eventually to bring the transcription activation domain close to the MT gene promoter to exert its function.

Relocation of MTF-1 from cytoplasm to nucleus is another important event that occurs in response to elevated Zn levels. The accumulation of MTF-1 in the nucleus is concomitant with increased DNA binding activity of MTF-1 and the induction of reporter gene expression driven by the MT-1 gene promoter [Smirnova et

al., 2000; Saydam et al., 2001]. The Zn levels have no effect on the amount of MTF-1 in mammalian cells [Andrews, 2000].

Recent findings have revealed that the post-translational modification, especially phosphorylation is involved in the activation of MTF-1 [LaRochelle et al., 2001]. The Zn treatment caused a 2–4 fold increase in the overall phosphorylation status of MTF-1 *in vivo* and such an increase required the function of signal transduction cascades containing protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and c-Jun N-terminal kinase (JNK).

In conclusion, Zn induces the transcription of MT genes through a combination of three mechanisms: activating MTF-1 DNA-binding activity, increasing the translocation of MTF-1 from cytoplasm to nucleus and enhancing the phosphorylation of MTF-1.

It is not yet known how other heavy metals, especially Cd, and stress conditions activate the production of MTs. Cd is a highly toxic transition metal with no known physiological function. Chemical induced oxidative stress also threatens cell survival. Mammalian cells treated with Cd or oxidative stress inducers exhibit a global change in cellular metabolism, including the induction of MTs production. There is evidence that Cd and oxidative stress directly affect MTF-1. First, both conditions result in the accumulation of MTF-1 in the nucleus [Saydam et al., 2001]. Second, Cd-treatment resulted in increased phosphorylation of MTF-1 [LaRochelle et al., 2001]. However, unlike elevated Zn level, Cd-treatment and oxidative stress did not activate the DNA-binding activity of MTF-1 [Bittel et al., 1998; Daniels et al., 2002]. One possible explanation is that the response to Cd and oxidative stresses requires the function of other transcription factors. Many mammalian MT genes contain one or more copies of a *cis*-regulatory element, the antioxidant response element (ARE). In mouse MT-1 gene, the single copy of ARE overlaps with another *cis*-element, the binding site for upstream stimulatory factor (USF). There is evidence that in addition to MREs, ARE and USF-binding site are required for the induction of MTs production by Cd-treatment and oxidative stresses [Andrews, 2000]. Furthermore, it can't be excluded that Cd and oxidative stress may cause the temporary increase of intracellular Zn level through redistributing Zn from its

reservoir, which subsequently causes the activation of MTF-1. Thus, multiple cellular pathways may be responsible for inducing MT gene expression in response to Cd-treatment and oxidative stress. These mechanisms may be distinct but highly related with the mechanism of Zn-dependent regulation.

Other genes regulated by MTF-1 through the interaction with MRE include the zinc transporter-1 gene (ZnT-1) [Langmade et al., 2000], the glutamylcysteine synthetase heavy chain gene [Gunes et al., 1998] and the placental growth factor gene [Green et al., 2001]. Recently, it has been shown that the MREs in rat Cu/Zn superoxide dismutase (Cu/Zn SOD) gene are responsible for the induction of Cu/Zn SOD production by Zn, Cu and Cd [Yoo et al., 1999]. So far, the transcriptional regulation in response to transition metals is best characterized in the mammalian MT genes, yet it is still stunning to see its complexity. It exemplifies that living cells activate a network of inter-connected molecular pathways in response to environmental stresses.

1.3.2 Post-Transcriptional Regulation of Gene Expression by Iron

Iron is essential for many basic cellular functions. However, because of its high reactivity in oxygen atmosphere, iron is the source of reactive oxygen species (ROX) that damage living cells. Thus, the uptake, storage and utilization of iron are tightly regulated depending on its supply and demand. Many organisms, including vertebrates, invertebrates and bacteria, adopt an elegant post-transcriptional regulatory system to control the expression of iron uptake, storage and utilization related genes [Alen and Sonenshein, 1999; Thomson et al., 1999; Theil and Eisenstein, 2000]. This regulatory system operates in the cytoplasm and involves the interactions between structural elements, IREs (iron responsive elements) residing in mature, fully processed mRNAs, and cytoplasmic proteins, IRPs (iron regulatory proteins) [Theil and Eisenstein, 2000]. Under physiological conditions, IRE forms a stable stem-loop structure, which is recognized and bound by IRP [Theil and Eisenstein, 2000]. IREs may reside in either the 5′- or the 3′-end of the untranslated region (UTR) in a mRNA and the binding of IRPs to IREs may exert either repressing or enhancing effects on translation depending on the location of IREs. When IREs are located at

the 5′ UTR, the binding of IRPs to IREs can repress translation by sterically hindering the formation of translation initiation complex. The well-studied genes subjected to this regulation encode ferritin, erythroid 5-aminolaevulinate synthase (eALAs), mammalian mitochondrial aconitase (mt-acon) and *Drosophila* succinate dehydrogenase subunit b [Thomson et al., 1999]. Ferritin is the major iron-storage protein in mammalian cells. eALAs is a key enzyme in heme synthesis. Both mt-acon, succinate dehydrogenase are important enzymes in the tricarboxylic acid cycle. When IREs are located at the 3′ UTR, the binding of IRPs to IREs can block the degradation of mRNA and prolong its half-life, which in turn enhances translation efficiency. The best-studied mammalian genes subjected to this regulation encode transferrin receptor (TfR) and divalent metal transporter-1 (DMT-1) [Thomson et al., 1999; Gunshin et al., 2001]. Both TfR and DMT-1 are central players for cellular iron uptake. In mammals, the majority of iron in the circulation system is bound to a serum protein-transferrin. TfR captures the iron-loaded transferrin at the cell plasma membrane and internalizes it by receptor-mediated endocytosis into endosomes. DMT-1 belongs to an ancient metal ion transporter family, the NRAMP family (natural resistance–associated macrophage protein) containing members in bacteria, fungi, invertebrates, vertebrates, and plants [Cellier et al., 1995; Curie et al., 2000]. The dual functions of DMT-1 are: transporting iron across endosome membrane to the cytoplasm and transporting iron from the lumen of the gut into duodenal enterocytes. Hence, all the genes known to be regulated by IRP–IRE interaction are directly related to cellular iron and oxygen metabolism.

How is the IRP–IRE interaction responsive to environmental and cellular iron levels? There are two iso-IRPs, IRP-1 and IRP-2 in mammals and each of them is regulated by iron in a distinct way. IRP-1 is a bifunctional protein, acting either as a cytoplasmic aconitase (holoprotein) under iron-replete conditions or as an IRE-binding protein (apoprotein) under iron-depleted conditions [Haile et al., 1992]. This functional switch is dependent upon the assembly of a cubic iron-sulfur (4Fe-4S) cluster in IRP-1's activity center, which is located in a "cleft" formed between domains 1–3 and 4 in IRP's tertiary structure. The holoprotein contains the covalently bound 4Fe-4S cluster in the "cleft" and is an active aconitase, while the

"cleft" of the apoprotein is devoid of the 4Fe-4S cluster and open for IRE-binding. The removal of the 4Fe-4S cluster depends on the cytoplasmic iron level and the mechanism of this process is not understood.

IRP-2 has approximately 60% sequence identity with IRP-1. Although it has a similar structure as IRP-1, IRP-2 neither binds the iron-sulfur cluster nor possesses aconitase activity. Intracellular iron level regulates the quantity of IRP-2 through iron-dependent oxidation of this protein and subsequent ubiquitin-mediated degradation [Guo et al., 1994]. One major difference between IRP-1 and IRP-2 is the extra 73 residues that are unique to IRP-2 near the N-terminus. This sequence is responsible for the oxidation and degradation of IRP-2 under iron-replete conditions. Thus, when iron is abundant, both IRP-1 and IRP-2 interact poorly with IREs and the translation of ferritin, eALAS and mt-aconitase is derepressed to promote cellular iron storage and utilization. Additionally, the translation of TfR and DMT-1 is repressed to reduce iron uptake. The whole process reverses itself when iron is deficient.

Besides iron status, many cellular signal transduction pathways are also involved in controlling the IRP-IRE interaction. The phosphorylation of both IRP-1 and IRP-2 as a result of activating signal cascades containing PKCs and MAPKs (mitogen activated protein kinases) plays a pivotal role in regulating their functions. The known signals that regulate the RNA-binding activity of IRP-1 include nitric oxide (NO), oxidative stress (such as H_2O_2 treatment), various hormones (such as erythropoietin and thyroid hormone), cytokines (such as IL-1β) and hypoxia. The known signals that interfere with the turn-over of IRP-2 include NO, hormones and cytokines [Thomson et al., 1999].

Invertebrates such as the fruit fly and nematode *Caenorhabditis elegans* [Thomson et al., 1999], and bacterial species such as *Bacillus subtilis* [Alen and Sonenshein, 1999] contain only IRP-1. The bacterial and insect IRP-1s possess similar functions as their mammalian counterparts. IRE-containing mRNAs and IRP have not been found in plants [Thomson et al., 1999]. In plants, the expression of ferritin genes is controlled at the transcriptional level by iron [Petit et al., 2001]. This regulation is mediated by a *cis*-acting iron-dependent regulatory sequence, which is located in the promoters of maize and *Arabidopsis thaliana* ferritin genes.

1.3.3 Regulation of Gene Expression by Transition Metals in *S. cerevisiae*

As mentioned previously, yeast contains MTs that are different from their Zn-binding vertebrate counterparts. Yeast MTs predominantly bind Cu and play a central role in detoxifying this heavy metal [Heuchel et al., 1995]. Cu is an essential element to all living organisms. It is an indispensable cofactor for enzymes involved in basic cellular functions, exemplified by cytochrome oxidase, Cu/Zn SOD and lysyl oxidase. However, Cu also engages in the generation of hydroxyl radicals that are highly toxic to the cell. Thus, as with iron, the uptake, storage and distribution of copper is tightly controlled in the cell with respect to its supply and demand. In *S. cerevisiae*, there is a single MT gene, designated *CUP-1*. The MT encoded by this gene binds and stores Cu, contributing to the low intracellular free copper concentration required to protect the cell. Elevated intracellular copper levels induce the transcription of *CUP-1*. This regulation is mediated through the interaction of a *cis*-element, namely UAS (upstream activator sequences), in the *CUP-1* promoter and a *trans*-acting factor Ace-1 [Heuchel et al., 1995]. No sequence similarity is found between UAS and the consensus mammalian MRE. The transcriptional activator Ace-1 contains an N-terminal region that exhibits striking similarity to the MT encoded by *CUP-1*. The Cu-binding as well as the DNA-binding domain of Ace-1 are located in this region. The *trans*-activation domain of Ace1 is located in the C-terminal half [Hu et al., 1990]. Intracellular Cu levels control the Cu-bound status of Ace1. Under Cu-deficient conditions, Ace-1 does not bind Cu and its DNA binding domain lacks an ordered structure. In contrast, under Cu-excess conditions, Ace-1 binds copper and its DNA binding domain adopts a so-called "copper-fist" structure. This allows Ace1 to access DNA and activate *CUP-1* transcription [Furst et al., 1988]. High intracellular Cu level activates the transcription of the Cu/Zn SOD gene through a similar mechanism.

Cu levels regulate the transcription of genes encoding Cu-uptake proteins in *S. cerevisiae* through a different mechanism. Yeast cells import Cu only in the form of Cu^{+}. In this process, the extracellular free Cu^{2+} is first reduced to Cu^{+} by cell-surface metalloreductases and then is transported into the cell through both high- and

low-affinity Cu^+-transporters [Eide, 1998]. *S. cerevisiae* has seven homologous genes that appear to encode metalloreductases, designated *FRE1* to *7*. Among them, *FRE1* and *2* were found to encode functional enzymes that reduce both Cu^{2+} and Fe^{3+} to Cu^+ and Fe^{2+} [Labbe and Thiele, 1999]. The high-affinity Cu^+-transporters are Cu-specific and responsible for Cu-uptake under Cu-deficient conditions. Two yeast genes, *CTR1* and *CTR3*, encode functional high-affinity Cu-transporters [Labbe and Thiele, 1999]. Cu-starvation activates the transcription of *CTR1*, *CTR3*, *FRE1* and *FRE7*. In this regulation, the required promoter *cis*-elements, CuREs (Cu response element), are conserved in all four genes [Labbe et al., 1997]. A nuclear transcriptional activator, Mac1, was found to bind CuRE and activate gene transcription only under Cu-deficient conditions [Yamaguchi-Iwai et al., 1997]. Upon elevated Cu concentrations, Mac1 is released from the CuREs and may undergo a conformational change to inactivate its trans-activating function. In addition, at very high environmental Cu concentrations ($>10\ \mu M$), MAC1 is rapidly and specifically degraded [Zhu et al., 1998]. It is noteworthy that a concentration as low as 20 pM of extracellular Cu^{2+} can achieve half-maximal attenuation of *CTR1* and *FRE1* expression [Labbe et al., 1997]. However, the connection between the cellular Cu level and Mac1 function is not fully understood. The low-affinity Cu-transporters support cellular Cu metabolism under Cu-sufficient conditions. In *S. cerevisiae*, the known low-affinity Cu-transporter is Fet4 (encoded by *FET4*), which is not Cu-specific and is responsible for low-affinity uptake of Fe, Cu, Zn and several other metals [Hassett et al., 2000]. The regulation of *FET4* expression is discussed below.

Fe and Cu uptake are interconnected in *S. cerevisiae*. Both high- and low-affinity Fe-uptake systems require the reduction of extracellular Fe^{3+} to Fe^{2+}. This is accomplished by a set of metalloreductases (encoded by *FRE1* and *FRE2*) similar to those in the Cu-uptake systems [Eide, 1998]. However, the process of high-affinity Fe-uptake is complex. It functions through a plasma membrane-bound protein complex, consisting of a multicopper ferroxidase Fet3 (encoded by *FET3*) and a high-affinity iron transporter Ftr1 (encoded by *FTR1*). It is proposed that Fe^{2+} is re-oxidized to Fe^{3+} by Fet3, which is subsequently passed to Ftr1 and transported across

the plasma membrane [Eide, 1998]. Each Fet3 acquires four Cu atoms as cofactors from the lumen of the *trans*-Golgi network (TGN). Ccc2 (encoded by *CCC2*) is a Cu-transporter located on the membrane of the TGN. It obtains Cu from cytoplasmic Cu-chaperon molecules and transports Cu to the lumen of TGN [Labbe and Thiele, 1999]. Hence, Fet3 forms a direct tie between cellular Cu and Fe metabolism. Fe-starvation activates the transcription of *FRE1*, *FRE2*, *FET3*, *FTR1* and *CCC2* [Winge et al., 1998]. Notably, *FRE1* and *FRE2* are under dual-control from both Cu and Fe. In addition, Fe interferes with intracellular Cu-distribution by regulating the expression of *CCC2*. These controls result in an interdependent and harmonized cellular Cu and Fe metabolism in response to environmental inputs and cell demands. *S. cerevisiae* produces two homologous transcription activators, Aft1 and Aft2, which bind a conserved CACCC promoter element in each Fe-responsive gene under Fe-deficient conditions [Yamaguchi-iwai et al., 1996; Blaiseau et al., 2001]. Elevated environmental iron levels diminish the function of Aft1 and Aft2. The mechanism of how both Aft1 and Aft2 respond to Fe status is unknown. Recently it was shown the Fe status regulates the translocation of Aft1 from cytoplasm to nucleus [Yamaguchi-iwai et al., 2002]. Fe-starvation results in the accumulation of Aft1 in the nucleus, whereas under Fe-replete conditions, the majority of Aft1 localizes in the cytoplasm. The low-affinity metal ion transporter Fet4 is responsible for Fe-uptake under Fe-sufficient condition. Unlike the high-affinity Fe-uptake system, Fet4 directly transports Fe^{2+} into the cell [Eide, 1998]. The regulation of *FET4* expression by Fe status requires the function of Aft1. Furthermore, Smf3 (encoded by *SMF3*), a yeast NRAMP family member, functions as an Fe-transporter on the vacuole membrane [Portnoy et al., 2000]. Upon Fe-starvation, Smf3 transports the stored iron in the vacuole to the cytoplasm for further utilization. Aft1 and Aft2 may participate in the activation of the *SMF3* gene transcription by Fe-starvation [Portnoy et al., 2002].

S. cerevisiae absorbs Zn from the environment through two plasma membrane-bound transporters, Zrt1 and Zrt2 (encoded by *ZRT1* and *ZRT2*) [Eide, 1998]. Zrt1 and Zrt2 are high- and low-affinity Zn^{2+}-transporters, respectively. Both transporters are essential for maintaining cellular Zn-homeostasis and Zn-depletion enhances the

expression of both *ZRT1* and *ZRT2* [Winge et al., 1998]. This regulation is achieved at the transcriptional level through an activator, Zap1 (encoded by *ZAP1*) [Zhao and Eide, 1997]. Several features suggest that Zap1 itself might be the sensor for intracellular Zn levels. The DNA-binding domain of Zap1 is located in its C-terminus, which contains five classical Zn-finger motifs. Two candidate trans-activation domains are located in the N-terminal half of Zap1. The proposed mechanism is that Zn-binding to Zap1 may reduce its DNA-binding and/or transcription activating function. Moreover, it has been shown that Zap1 enhances the transcription of its own gene (*ZAP1*) under Zn-deficient conditions [Zhao and Eide, 1997]. Such auto-regulation endows the yeast cell with a rapid, amplified response to environmental changes in Zn status. This auto-regulation is not found in the control of other metal-responsive systems in *S. cerevisiae*.

Mn is also an important transition metal for all living organisms. Enzymes such as Mn-superoxide dismutase (Mn-SOD) and arginase require Mn as cofactors [Eide, 1998]. Mn-SOD, which scavenges ROX in the cell, is an essential enzyme involved in oxidative stress response. Mn also plays an important role in controlling *S. cerevisiae* cell-cycle progression and filamentous growth; however, the molecular mechanisms for these controls are not understood [Loukin and Kung, 1995; Asleson et al., 2000].

In eukaryotes, very little is known about the regulation of gene expression in response to Mn. In *S. cerevisiae*, Mn exerts no regulation on the expression of Mn-uptake related genes at either the transcriptional or the post-transcriptional levels. Yeast cells acquire Mn from the environment in the form of Mn^{2+} through a plasma membrane-bound metal ion transporter, Smf1 [Eide, 1998], a member of the Nramp family. Another close homologue, Smf2, functions as a vesicle membrane-bound metal ion transporter [Portnoy et al., 2000]. Neither Smf1 nor Smf2 is Mn-specific, however, they both are essential for maintaining Mn-homeostasis in *S. cerevisiae*. Both Smf1 and Smf2 are only functional under Mn-deficient, but not Mn-sufficient conditions [Portnoy et al., 2002]. This regulation in response to Mn status occurs at the level of protein degradation and sorting through the secretory pathway. When cells have ample Mn, Smf1 and Smf2 are targeted to the vacuole for degradation and

this movement requires the function of an intracellular protein encoded by *BSD2* [Liu and Culotta, 1999]. Conversely, when cells are starved for Mn, Smf1 and Smf2 fail to reach the vacuole, instead, they are translocated to their functional sites-the plasma and the vesicle membrane, respectively. It is not yet known how cellular Mn-levels determine the destination of Smf1, Smf2 and what function the *BSD2*-encoding protein performs.

In summary, almost all metal-responsive genes in *S. cerevisiae* are involved in the maintenance of transition metal homeostasis. Regulation at the transcriptional level functioning through *cis*-elements and *trans*-factors appears to be the major regulatory mode in response to transition metals in yeast.

1.3.4 Metal-Dependent Regulation of Gene Expression in Other Fungi

Heavy metal-dependent regulation of gene expression has been studied in the opportunistic pathogenic yeast *Candida glabrata*. Cu-binding MTs in *C. glabrata* are similar to those in *S. cerevisiae*. However, in contrast to *S. cerevisiae*, which possesses only one MT-encoding gene, *C. glabrata* has two classes of MT-encoding genes, namely *MT-1* and *MT-2* [Heuchel et al., 1995]. *MT-1* occurs as a single copy, while there are multiple copies of *MT-2* in the *C. glabrata* genome. Elevated intracellular Cu and Ag levels activate the transcription of both classes of *MT*s in *C. glabrata*. In this type of regulation, both the required *cis*-element and *trans*-activator are homologous to their counterparts in *S. cerevisiae* [Heuchel et al., 1995]. The *trans*-activator in *C. glabrata* is Amt1 (encoded by *AMT-1*), a Cu-binding protein and possibly contains both Cu-sensing and transcription activation functions similar to its *S. cerevisiae* homologue, Ace1 [Zhou and Thiele, 1991]. However, unlike Ace1, which is constitutively expressed, Amt1 activates the expression of its own gene in response to elevated Cu levels [Zhou and Thiele, 1993]. Therefore, under Cu-excess conditions, the transcription of *MT*s in *C. glabrata* exhibits rapid activation kinetics. The rapid accumulation of MTs resulting from this activation protects the cells from damage by heavy metals such as Cu.

Structural analysis has revealed that the single Cu-responsive *cis*-element (tentatively named UAScg) in the *AMT-1* promoter is located in a stably positioned nucleosome. The access of Amt1 to the nucleosomal UAScg is facilitated by an adjacent 16-bp homopolymeric dA:dT DNA structural element (A16 element) [Koch and Thiele, 1999]. Additional evidence has shown that the Swi/Snf nucleosome remodeling complex and the histone acetyltransferase Gcn5 are both essential for *AMT1* autoregulation [Koch et al., 2001]. The requirement for these chromatin-remodeling factors is target gene (*AMT1*) specific.

Cu-modulated gene expression has also been studied in the ascomycete *Podospora anserina*. In this fungus, a putative transcription factor Grisea was identified as an ortholog of the *S. cerevisiae* Cu-responsive transcription activator Mac1 [Osiewacz and Nuber, 1996]. As mentioned previously, Mac1 activates the transcription of several Cu-uptake-related genes upon Cu-starvation. In *P. anserina*, the function of Grisea is dependent on Cu status and it appears to play important roles in many cellular events, including Cu-uptake, mitochondrial DNA rearrangement and the induction of an alternative respiratory pathway (Fe-dependent, instead of Cu-dependent) [Borghouts and Osiewacz, 1997; Borghouts et al., 2001]. However, very little is known about the precise mechanism through which Grisea functions. Interestingly, *P. anserina* cells with a mutated Grisea showed a prolonged life span, which suggests an important role of Cu-homeostasis in the control of ageing and senescence.

The acquisition of Fe in most fungi and bacteria is facilitated by the low molecular weight and high-affinity Fe-chelators called siderophores that are synthesized and excreted by these microorganisms. The siderophore-bound Fe is actively taken up by cells through specific receptors on cell membrane [Bagg and Neilands, 1987]. In this respect, *S. cerevisiae* is an exception since it lacks the ability to synthesize siderophores, although it can utilize siderophores produced by other microorganisms [Yun et al., 2000]. In ascomycetes and basidiomycetes, siderophore biosynthesis and siderophore-mediated Fe uptake are controlled by homologous, negative-acting GATA transcription factors, e.g., *Aspergillus nidulans* SREA [Haas et

al., 1999], *Neurospora crassa* SRE [Zhou et al., 1998], *Ustilago maydis* URBS1 [Voisard et al., 1993] and *Penicillium chrysogenum* SREP [Haas et al., 1999]. The GATA factors are defined as GATA-binding proteins that recognize the core nucleotide sequence motif GATA. In each case, disruption of the URBS1-encoding gene in *U. maydis* [Voisard et al., 1993], the SRE-encoding gene in *N. crassa* [Zhou et al., 1998] and the SREA-encoding gene (*sreA*) in *A. nidulans* [Haas et al., 1999] resulted in derepressed extracellular siderophore biosynthesis as well as increased accumulation of Fe in cells provided with sufficient Fe supply. This suggests that these GATA factors function as repressors for siderophore synthesis and siderophore-mediated iron uptake under Fe-replete conditions. The extracellular siderophore production in an *A. nidulans* strain containing the deletion of *sreA* is derepressed but still regulated negatively by iron availability in a reduced degree, suggesting the presence of other Fe-dependent regulatory mechanism(s) in this fungus [Oberegger et al., 2001]. The candidate genes that are regulated by SREA in *A. nidulans* include *mirA*, encoding a putative siderophore permease, and *amcA*, encoding a putative mitochondrial carrier for the siderophore precursor, ornithine. The mechanism of how Fe-responsive GATA factors control gene expression in fungi is not understood.

1.3.5 Gene Expression Regulation by Manganese in Bacteria

Gene expression modulated by transition metals has been extensively studied in bacteria. Here this topic will be reviewed in general, but with an emphasis on gene expression regulated in response to Mn, since Mn is also an agent that modulates *mnp* expression in *P. chrysosporium.*

In bacteria, transition metal ions regulate gene expression predominantly at the transcriptional level. The best characterized metal-responsive transcription regulators fall into four distinct families: Fur (ferric uptake repressor), DtxR (diphtheria toxin repressor), MerR (mercury-resistance related) and ArsR (arsenical resistance related) [Que and Helmann, 2000; Jakubovics and Jenkinson, 2001]. Representative members of all four families share the same structural features. In brief, they all function as dimers with each subunit containing an N-terminal helix-turn-helix DNA-binding

domain and an adjacent metal-binding domain. Some DtxR-family members also contain an extra C-terminal SH_3-like domain that might modulate their DNA- and metal-binding activity. In the target operon, the *cis*-elements that interact with these metal-responsive regulators all reside near the -35 or within the -35 to -10 region in the promoter and all feature inverted repeat nucleotide sequences.

Both Fur and DtxR are involved in cellular Fe-metabolism. Fur is found in Gram-negative and Gram-positive bacteria with low (G+C) content (such as *B. subtilis*), whereas DtxR exists in Gram-positive bacteria with high (G+C) content (such as Streptomycetes and Mycobacteria) [Hantke, 2001]. Both Fur and DtxR regulate a similar set of genes that are mostly related to Fe-uptake, virulence factor production and oxidative stress response [Hantke, 2001]. Indeed, DtxR was first characterized in *Corynebacterium diphtheriae* as a repressor for the production of a Fe-dependent toxin, diphtheria toxin, which is directly related to virulence [Jakubovics and Jenkinson, 2001]. The consensus nucleotide sequence that is recognized and bound by Fur is well defined and designated the Fur-box [Escolar et al., 1999]. Such a consensus sequence has also been identified for DtxR, but it shares no homology with the Fur box [Dussurget and Smith, 1998]. Both Fur and DtxR bind Fe^{2+} under Fe-sufficient, but not Fe-deficient conditions and Fe^{2+}-binding results in conformational changes, which enable the regulators to bind DNA and repress the transcription of target operons [Dussurget and Smith, 1998; Escolar et al., 1999]. Fur itself is under the control of transcriptional regulators that are involved in oxidative stress response [Hantke, 2001]. Moreover, several known Fur-family members regulate genes that are not involved in Fe-metabolism. These regulators include: Zur in *E. coli* and *B. subtilis*, which is a Zn-binding repressor that regulates Zn-uptake related genes, and PerR in *B. subtilis*, which appears to bind Mn^{2+} and regulates oxidative stress-related genes such as the catalase gene [Hantke, 2001].

Recently three DtxR-family members, MntR, ScaR and TroR, were identified as Mn-dependent transcription regulators for Mn-uptake genes in *B. subtilis*, *Streptococcus gordonii* and *Treponema pallidum*, respectively [Posey et al., 1999; Jakubovics et al., 2000; Que and Helmann, 2000]. Under sufficient Mn supply, both ScaR and TroR repress the transcription of operons containing Mn^{2+}-specific ABC

(ATP-binding cassette) metal transporter genes, namely *scaCBA* (*S. gordinii*) and *troABCD* (*T. pallidum*). However, MntR appears to be a dual-functional transcription regulator: it activates the transcription of an operon (*mntABCD*) encoding Mn^{2+}-specific ABC transporter under Mn-deficient condition, but represses the transcription of *mntH*, which encodes a Nramp-family proton-coupled Mn^{2+}-transporter under Mn-sufficient condition. All three regulators bind Mn^{2+} only when the intracellular Mn concentration is high. In the case of ScaR and TroR, Mn^{2+}-binding might activate their DNA-binding activity. As for MntR, it is proposed that Mn^{2+}-binding switches its DNA-binding specificity to different promoters. The promoter regions that are recognized and bound by all three regulators contain AT-rich palindrome sequences, which are different from the consensus sequence defined for other DtxR-family members. Many nucleotides are conserved in the promoter sequences bound by MntR and TroR, but not in that bound by ScaR [Jakubovics et al., 2000]. It is not yet known whether these Mn^{2+}-dependent regulators affect the expression of genes other than those related to Mn-uptake.

The physiological significance of Mn in bacteria has been thoroughly examined. Bacteria, especially those that are parasitic, face a constant limitation of essential transition metals, especially Fe. In higher eukaryotic organisms almost all Fe is protein-bound and stored in subcellular organelles. Parasitic bacteria must wrest Fe from their hosts. However, excess free Fe is deleterious because it results in the formation of ROX that damage bacterial cells. Thus, many bacteria have evolved metabolic strategies with decreased dependence on Fe, but increased dependence on Mn. A group of bacteria (such as the Lyme disease pathogen *Borrelia burgdorferi*) are known to have a definite requirement for Mn, but not for Fe [Posey and Gherardini, 2000; Jakubovics and Jenkinson, 2001]. It is not fully understood how these bacteria perform basic cellular functions with the substitution of Fe with Mn. However, the significant roles of Mn in oxidative stress response, nutrient metabolism and bacterial development have been well documented [Jakubovics and Jenkinson, 2001]. In oxidative stress response, two enzymes, MnSOD and Mn-catalase that require Mn^{2+} function as ROS scavengers [Jakubovics and Jenkinson, 2001]. Recently it has been shown that the accumulation of intracellular Mn^{2+} can protect

bacteria from oxidative damage through a non-enzymatic mechanism [Tseng et al., 2001]. In nutrient metabolism, it is known that a variety of bacterial enzymes involved in photosynthesis, glycolysis, sugar metabolism and amino acid metabolism require Mn^{2+} [Jakubovics and Jenkinson, 2001]. The role of Mn in development is best studied in *B. subtilis*. In this bacterium, both endospore formation and spore germination are dependent on Mn. SpoIIE, a serine phosphatase that is pivotal in the signal cascade controlling endospore formation, requires Mn^{2+}. SpoIIE belongs to the PPM phosphatase family and many members of this family require Mn^{2+} for catalytic activity [Barford, 1996]. All of these facts highlight the physiological and evolutionary importance of Mn. However, it is not yet known whether Mn exerts any control over the expression of the genes encoding various Mn-containing enzymes.

Finally, bacteria detoxify heavy metals through complex efflux machineries. In response to the intracellular status of heavy metals, members of the MerR and ArsR families modulate the expression of genes related to metal-efflux. When heavy metal is scarce, the representative regulators from these two families are in an apoprotein form that bind the promoters of target operons and thus repress transcription. Elevated heavy metal concentrations result in metal-binding of the regulators and the concomitant conformational changes, which derepress transcription [Wu and Rosen, 1993; Heuchel et al., 1995]. Studies on MerR have revealed that upon metal binding, the transcriptional regulator still binds the promoter DNA, but the whole regulator–DNA complex undergoes a conformational change that allows transcription initiation.

In conclusion, I have reviewed the transition metal-regulated gene expression in both eukaryotic and prokaryotic organisms. The majority of genes subjected to this regulation are involved in the maintenance of cellular homeostasis of transition metals. The predominant regulatory mechanism adopted by cells in response to transition metals is to control gene transcription through the interaction of *cis*-acting elements and *trans*-acting regulators. It is frequently observed that *trans*-acting regulator and *cis*-acting element pairs are structurally and functionally conserved over a broad range of species. Furthermore, transition metal-dependent regulation of gene

expression is connected to many cellular signal transduction pathways and, thus, is integrated into the regulatory network within the cell and the entire organism.

1.4 THESIS OVERVIEW

The goal of my dissertation research is to advance the molecular biology of *P. chrysosporium*. Specifically, we intend to explore the requirements for heterologous gene expression in this fungus, to develop tools for studying Mn^{2+}-dependent induction of *mnp* expression and to characterize the promoter *cis*-acting sequences required for this regulation. In the following chapters, I report the results from three studies: first, development of GFP as a gene expression reporter in *P. chrysosporium*; second, characterization of a *cis*-acting sequence responsible for Mn^{2+}-dependent regulation of *P. chrysosporium mnp1* expression; third, construction of a new transformation system for *P. chrysosporium* using bacterial bialaphos-resistance gene as a dominant selectable marker. In the last chapter, I propose future studies that could extend from my present results.

CHAPTER 2
THE GREEN FLUORESCENT PROTEIN GENE FUNCTIONS AS A REPORTER OF GENE EXPRESSION IN *PHANEROCHAETE CHRYSOSPORIUM*[1]

2.1 INTRODUCTION

The white-rot basidiomycete, *Phanerochaete chrysosporium*, has been the focus of numerous studies on the degradation of the plant cell wall polymer lignin [Gold and Alic, 1993; Cullen, 1997] and various aromatic pollutants [Bumpus and Aust, 1987; Hammel, 1989; Valli et al., 1992; Joshi and Gold, 1993]. Two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), along with an H_2O_2-generating system are the major components of its extracellular lignin-degrading system [Gold and Alic, 1993; Cullen, 1997].

The lignin degradation of *P. chrysosporium* is an event occurring only during secondary metabolic phase, whose onset is triggered by depletion of nutrient nitrogen, carbon or sulfur in the culture [Gold and Alic, 1993]. Previous data have shown that the expression of MnP- and LiP-encoding genes (*mnp*s and *lip*s, respectively) is regulated at the transcriptional level in response to nitrogen-depletion [Pribnow et al., 1989; Li et al., 1994]. Moreover, it has been found that the transcription of *mnp*s is activated by supplementation of Mn^{2+} in the culture medium [Brown et al., 1990, 1991; Gettemy et al., 1997, 1998]. The production of MnP protein also requires the presence of Mn^{2+}. In addition, the transcription of *mnp*s is activated by oxidative

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[1] Parts of this chapter have been modified from material published in *Applied and Environmental Microbiology* and is used here with permission of the American Society for Microbiology.

Ma, B., Mayfield, M. B., and Gold, M. H. (2001) The green fluorescent protein gene functions as a reporter of gene expression in *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **67**, 948–955.

stress and heat shock [Brown et al., 1993; Li et al., 1995]. Sequence analysis has revealed that the promoter regions of the *P. chrysosporium mnp*s contain several putative *cis*-acting elements; however, the function of these elements is unknown [Gold and Alic, 1993; Alic et al., 1997]. To study the regulation of genes encoding lignin-degrading enzymes, a suitable gene reporter system is required. Previously, we developed a reporter system based on the orotidylate decarboxylase gene from *Schizophyllum commune* [Godfrey et al., 1994]. While this reporter system is sufficient for reporting the regulation of *mnp* expression in response to Mn^{2+}- and nitrogen-status, the assay is complicated and uses a radioactive substrate.

Green fluorescent protein (GFP) from *Aequorea victoria* has several advantages as a reporter system for gene expression. The enhanced green fluorescent protein gene (*egfp*) contains a (G+C) content of 59%, which is the same as that of the *P. chrysosporium* genome [Raeder and Broda, 1984; Cormack et al., 1996; Cormack, 1998]. GFP presents strong intrinsic fluorescence that can be easily detected and quantified [Cormack et al., 1996]. Moreover, GFP has been successfully expressed in many fungal species and used as a reporter for gene expression [Spellig et al., 1996; Cormack, 1998; Henriksen et al., 1999; Lugones et al., 1999]. In this Chapter, I'll describe the establishment of a new reporter system in *P. chrysosporium* using *egfp* as a gene expression reporter. We have found that efficient expression of *egfp* in *P. chrysosporium* requires an intron inserted into its transcription unit.

2.2 MATERIALS AND METHODS

2.2.1 Organisms

P. chrysosporium wild-type strain OGC101 and the auxotrophic strain *ura11* were maintained as described previously [Akileswaran et al., 1993]. *Escherichia coli* strain DH5α was used for plasmid construction and preparation.

2.2.2 Construction of Transformation Vectors

The *P. chrysosporium* ura transformation vector (pUB) contained the full coding region of the *S. commune ura1* orotidylate decarboxylase gene (including 200 bp of the promoter region) and was constructed as described previously [Sollewijn Gelpke et al., 1999].

2.2.3 Construction of Expression Plasmids

2.2.3.1 pUGGM3'. This plasmid contained the *P. chrysosporium gpd* promoter [Mayfield et al., 1994b], the *egfp* gene coding sequence (Clontech) [Heim, 1996], and 250 bp of the *mnp1* 3′ untranslated region (UTR) [Godfrey et al., 1990] immediately following the stop codon. The *gpd* promoter was modified to contain a *Kpn*I site 5 bp upstream of the translation start codon. Using a forward primer 5′-upstream of a native *Bam*HI site and a reverse primer located 5 bp upstream of the translation start codon including the *Kpn*I site, a *Bam*HI–*Kpn*I polymerase chain reaction (PCR) fragment was prepared. This fragment replaced a *Bam*HI–*Kpn*I fragment in pGPDstu1.8 (containing the 1.14-kb *gpd* promoter), which had been constructed previously [Mayfield et al., 1994b]. The entire 1.14-kb *gpd* promoter was isolated as an *Xba*I–*Kpn*I fragment and subcloned into pUC18 for further use.

The coding region of the *egfp* gene and the 3′ UTR of the *mnp1* gene [Godfrey et al., 1990] were fused at the stop codon using the megaprimer method [Barik, 1997] in a two-step reaction. A 250-bp megaprimer was prepared in an initial PCR using the *mnp1* genomic DNA as a template. The forward primer contained 18 nucleotides (nt) encoding the C-terminal 6 amino acids of GFP and a stop codon (TAA) followed by 13 nt encoding the proximal end of the *mnp1* 3′ UTR. The 18-nt reverse primer (ncMR1) annealed 250 bp downstream of the *mnp1* stop codon and included a new *Eco*RI at its 5′ end. The megaprimer was purified by agarose gel electrophoresis and obtained using a Gel Extraction Kit (Qiagen). A second PCR mixture included pEGFPC-3 (Clontech Laboratories, Palo Alto, CA) that contains the entire *egfp* coding region as the template. The forward primer contained the 5′ end of the *egfp* coding region with a new 5′ *Kpn*I site 4 nt upstream of the translation

start codon. This second PCR mixture also contained the megaprimer described above and the reverse primer used in the first PCR step (ncMR1). The final PCR product was a 1.0-kb fragment containing the *gfp* coding sequence fused to the *mnp1* 3′ UTR. This PCR fragment was extracted with $CHCl_3$, precipitated with ethanol, double digested with *Kpn*I and *Eco*RI, and purified by gel electrophoresis. The *Kpn*I–*Eco*RI (*egfp-mnp*) fragment and the *Xba*I–*Kpn*I *gpd* promoter fragment described above were then ligated with *Xba*I and *Eco*RI digested pUB in a three-way ligation to yield pUGGM3′ (Fig. 2.1).

2.2.3.2 pUGiGM3′. This plasmid (with 5′ intron) contained the *gpd* promoter followed by the fragment iGM3′, which includes, from 5′ to 3′, the *gpd* exon 1 (6 bp), intron 1 (55 bp), the 5′ end of exon 2 (9 bp), the *egfp* coding sequence (717 bp), and the *mnp1* 3′ UTR ending at the *Eco*RI site (Fig. 2.2). The megaprimer method was used to generate the junction between *gpd* and *egfp* sequences. In the first PCR for constructing the megaprimer, the template was pGPD stu1.8 constructed from the *gpd* genomic DNA of *P. chrysosporium* as described previously [Mayfield et al., 1994b]. The 18-nt forward primer contained a native *Hin*dIII restriction site located 170 bp upstream of the translation start codon. The 36-nt reverse primer included an 18-nt nonhybridizing tail encoding the 6 N-terminal amino acids of GFP excluding the start codon, followed by 9 nt encoding 3 amino acids at the beginning of the second exon of *gpd*, followed by 9 nt encoding the 3′ end of intron 1 of *gpd* [Mayfield et al., 1994b]. The 260-bp megaprimer PCR product was amplified and gel purified. The second PCR used pGM3′ as a template. pGM3′ was constructed by subcloning the *Kpn*I–*Eco*RI fragment from pUGGM3′ into *Kpn*I and *Eco*RI double-digested pUC18. The reaction also contained the same forward primer used in the first PCR, the ncMR1 reverse primer, and the megaprimer. The 1.3-kb final PCR product was amplified and gel purified as a *Hin*dIII–*Eco*RI fragment. The pGPD stu1.8 was double digested with *Xba*I and *Hin*dIII to generate an *Xba*I–*Hin*dIII *gpd* promoter fragment. This *Xba*I–*Hin*dIII *gpd* promoter fragment and the *Hin*dIII–*Eco*RI PCR fragment were ligated to *Xba*I and *Eco*RI digested pUB in a three-way ligation to yield pUGiGM3′ (Fig. 2.2).

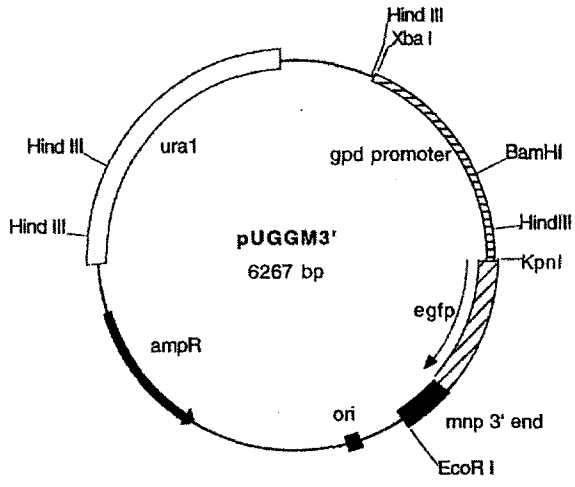
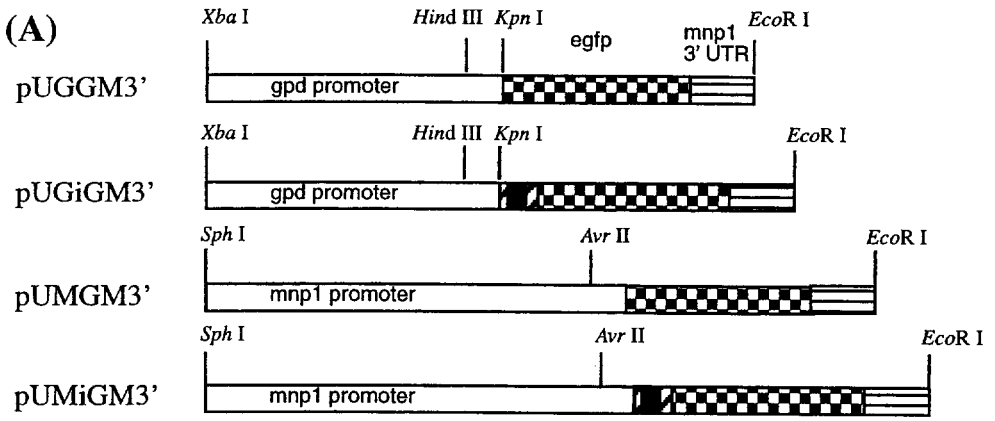


Fig. 2.1 Restriction map of pUGGM3′. The relative position of the *egfp* expression cassette (including the *gpd* promoter, the *egfp* coding region, and the *mnp1* 3′ untranslated region) and the two genes, amp^R and *ura1*, for selecting transformants in *E. coli* as well as in *P. chrysosporium*, respectively, are indicated.



(B)

pUMGM3' ...aacggcttgg tattccag ATG GTG AGC AAG...

pUMiGM3' ...aacggcttgg tattccag **ATG CCG** gtcagtacac cacacagccc gaccgcgacg accgcgtgct gacttcgctt tccag **GTC AAA GCA** GTG AGC AAG...

pUGiGM3' ...cccctacact acttgaac **ATG CCG** gtcagtacac cacacagccc gaccgcgacg accgcgtgct gacttcgctt tccag **GTC AAA GCA** GTG AGC AAG...

Fig. 2.2 *egfp*-expression cassettes in pUGGM3′, pUGiGM3′, pUMGM3′, and pUMiGM3′. **(A)** Restriction map of the *egfp*-expression cassette in each of the four plasmids. The black boxes represent the first intron of *gpd* (55 bp). The hatched boxes 3′ and 5′ to the intron represent the coding sequences of *gpd*. The positions of the *gpd* promoter (white), the *mnp1* promoter (white), the *egfp* coding region (checkered), and the *mnp1* 3′ UTR (horizontally striped) are indicated. **(B)** Sequences surrounding and including the 5′ inserted intron in pUGiGM3′ and pUMiGM3′. pUMGM3′ was used as a control. The 5′ non-coding sequences of *mnp1* and *gpd* are in lower case. The intron sequences are in lower case and underlined. The two short *gpd* coding sequences are in bold upper case, and the *egfp* coding sequences are in upper case.

2.2.3.3 pUMGM3'. This plasmid is similar to pUGGM3', except that the *gpd* promoter is replaced by the *mnp1* promoter. The *mnp1* promoter and *egfp* coding region were fused at the start codon using the megaprimer method in a two-step PCR. In the first PCR, a 158-bp megaprimer, containing 140 bp of the 3' end of *mnp1* promoter followed by 18 bp of the 5' end of *egfp* coding region, was amplified. This megaprimer was used in the second PCR with pUGGM3' as the template. A 1.1-kb fragment was generated, which contained 140 bp of the 3' end of the *mnp1* promoter joined with the entire *egfp* coding region and *mnp1* 3' UTR ending at an *Eco*RI site. This fragment was digested at a native *Avr*II site located at the 3' end of the *mnp1* promoter and the *Eco*RI site. The *Avr*II–*Eco*RI fragment was exchanged with the *Avr*II–*Eco*RI fragment in pMO, which was constructed before and contained 1.5-kb *mnp1* promoter sequence [Godfrey et al., 1994], yielding pMGM3'. Finally, the whole *egfp* expression construct, containing 1.5 kb of the *mnp1* promoter, the *egfp* coding region, and the *mnp1* 3' UTR, was isolated as a *Sph*I–*Eco*RI fragment and inserted into pUB to yield pUMGM3' (Fig. 2.2).

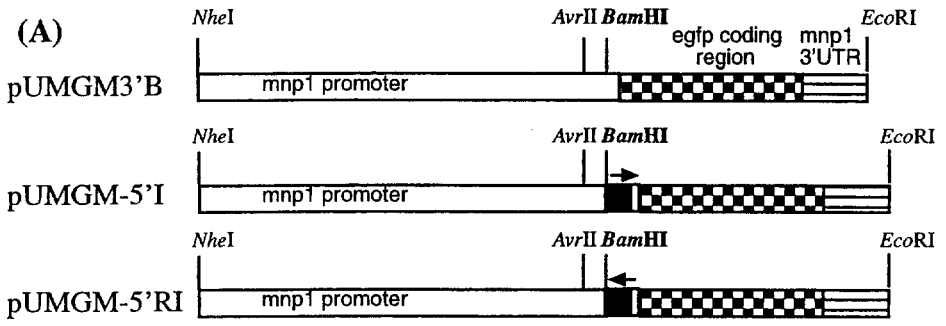
2.2.3.4 pUMiGM3'. Replacing the *gpd* promoter in pUGiGM3' with the *mnp1* promoter yielded pUMiGM3'. The megaprimer method was employed for the promoter replacement. In the first step, a 158-bp megaprimer was produced which includes 140 bp of the 3' end of the *mnp1* promoter followed by 18 bp of the 5' end of iGM3' fragment as described above. The megaprimer was used in the second PCR with pUGiGM3' as the template. The final product was a 1.15-kb fragment, containing 140 bp of the 3' end of the *mnp1* promoter joined with the entire iGM3' sequence (see above). An *Avr*II–*Eco*RI digest was performed on the PCR product, and the isolated *Avr*II–*Eco*RI fragment was exchanged with the *Avr*II–*Eco*RI fragment in pMO, yielding pMiGM3'. Finally, the whole *egfp* expression construct, containing 1.5 kb of the *mnp1* promoter followed by iGM3', was isolated as a *Sph*I–*Eco*RI fragment and inserted into pUB to yield pUMiGM3' (Fig. 2.2).

2.2.3.5 pUMGM-5'I and pUMGM-5'RI. These two plasmids are similar to pUMGM3', except both plasmids contain 76-bp DNA insertions positioned at 25 bp upstream of the translation start codon in the *mnp1* promoter. In pUMGM3', the 6-

bp sequence (TCCTCC) in the *mnp1* promoter located at 25 bp upstream of the translation start codon was mutated into a unique *Bam*HI restriction site (GGATCC) using the Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). Two oligonucleotide primers, a mutagenic primer and a selection primer, were synthesized. The mutagenic primer contained the desired nucleotide changes, and the selection primer was designed to mutate an unique *Sph*I (located at the 5′ end of the *mnp1* promoter) to a *Nhe*I site. Following the kit's instructions, the two primers were used in synthesizing a mutated DNA strand of pUMGM3′. The mutated plasmid was recovered after transformation to *E. coli* and named as pUMGM3′B (Fig. 2.3). The fourth intron of the *P. chrysosporium mnp1* gene [Godfrey et al., 1990] was amplified by PCR using a forward and a reverse primer that hybridized with the 5′ and 3′ end of the intron sequence. The forward and reverse primer also contained a new *Bam*HI and *Bgl*II restriction site, respectively. Finally, the PCR product was digested with *Bam*HI as well as *Bgl*II, and ligated to *Bam*HI-digested pUMGM3′B. The intron-containing fragment was inserted in two orientations. The plasmid that contains the inserted sequence with consensus intron splicing site (5′ GT---AG 3′) was named pUMGM-5′I, whereas the plasmid that contains an inverted insertion was named pUMGM-5′RI (Fig. 2.3).

2.2.4 Fungal Transformations

Protoplasts (2×10^6) of the *P. chrysosporium ura11* strain and ~ 2 μg of each expression plasmid linearized at the unique *Eco*RI site were used for each transformation as described previously [Akileswaran et al., 1993; Sollewijn Gelpke et al., 1999]. After the transformation, the prototrophic colonies that grew in the absence of uracil were selected on plates. Approximately 50 Ura^+ colonies obtained from each transformation were reexamined for their ability to grow on minimal medium in the absence of uracil and were subsequently purified by fruiting and basidiospore plating as described previously [Alic et al., 1991; Akileswaran et al., 1993].



(B)

```
                                  gga tcc
pUMGM3'B ...attccagttc tacttcctcc tcctccggtc aacggcttgg tattccag ATG...

pUMGM-5'I ...attccagttc tacttccg gatccGTAA GCAGTCTTTC AATGACTCGG
           TTTGTGCGCG ACAGTCGTTT CTGACCACTG ACCATGCCTG TGTGGCAGatcc
           tccggtcaac ggcttggtat tccag ATG...

pUMGM-5'RI...attccagttc tacttccg gatCTGCCAC ACAGGCATGG TCAGTGGTCA
           GAAACGACTG TCGCGCACAA ACCGAGTCAT TGAAAGACTG CTTACg gatcc
           tccggtcaac ggcttggtat tccag ATG...
```

Fig. 2.3 *egfp*-expression cassettes in pUMGM-5′I and pUMGM-5′RI. **(A)** Restriction maps of the *egfp*-expression cassettes in pUMGM-5′I and pUMGM-5′RI. In pUMGM3′B, a *Bam*HI site was created in the *mnp1* promoter 25 bp upstream of the translation start codon. Insertion of a DNA sequence containing an intact intron (black box) into the *Bam*HI site results in pUMGM-5′I or pUMGM-5′RI. The inserted sequence can adopt opposite orientations, which are indicated by the arrows. The positions of the *mnp1* promoter (white box), the *egfp* coding region (checkered box), and the *mnp1* 3′ UTR (horizontally striped box) are also indicated. **(B)** Inserted sequences in the *mnp1* promoter in pUMGM-5′I and pUMGM-5′RI. The *mnp1* promoter sequences are in lowercase. The six nucleotides changed into a *Bam*HI site in pUMGM3′B are underlined. The *Bam*HI site is shown in bold and italic lowercase. The arrows indicate the 5′ end of the corresponding cDNA. The inserted sequences are underlined. Those nucleotides corresponding to an intron are in uppercase. The 5′ and 3′ splice sites of the intron are in bold uppercase. The translation start codon is also in bold uppercase.

2.2.5 Culture Conditions

Transformants carrying pUGGM3′ and pUGiGM3′ were grown at 37°C from a conidial inoculum in liquid stationary cultures. Each culture contains 20 ml of HCHN (high-carbon and high-nitrogen) medium (Kirk's salts, 2% glucose, and 24 mM ammonium tartrate, 20 mM sodium succinate, pH 4.5) in a 250-ml flask [Kirk et al., 1978; Mayfield et al., 1994b]. The mycelia from 40-h-old cultures were filtered through Miracloth (Calbiochem), dried between layers of paper towel, frozen in liquid nitrogen, and stored at -80°C. The intracellular GFP fluorescence of the mycelia was assayed. Transformants carrying pUMGM3′, pUMiGM3′, pUMGM-5′I and pUMGM-5′RI were individually grown at 37°C from conidial inoculum in stationary cultures containing 20 ml HCLN (high-carbon and low-nitrogen) medium (Kirk's salt, 2% glucose, 1.2 mM ammonium tartrate, 20 mM sodium succinate, pH 4.5) in the presence or absence of 180 μM $MnSO_4$ in 250-ml Erlenmeyer flasks. Cultures were incubated under air for 4 days, then purged with 100% O_2 at 48-h intervals. Normally, 7-day-old cultures were harvested as described above, and the intracellular fluorescence was assayed. For the time course experiment, the cells were harvested on days 4, 5, 6, 7, and 8. For the Mn^{2+} induction experiment, cultures were grown for 4 days under air in HCLN or HCHN medium without Mn^{2+} at 37°C. On day 5, 180 μM $MnSO_4$ was added, cultures were purged with O_2, and the cells were harvested after additional 12-, 24-, 36-, or 48-h periods.

2.2.6 Intracellular GFP Determination

The frozen mycelia were homogenized with 1 g of acid-washed glass beads in a mini-bead beater (Biospec) in a buffer containing 10 mM Tris-Cl (pH 8.0), 10 mM EDTA, and 0.002% NaN_3. The mixture was centrifuged at 10,000 $\times$ g at 4°C. The supernatant was decanted and assayed for protein concentration by the BCA method (Sigma) [Smith et al., 1985]. The crude supernatant was diluted to 1 mg/ml of protein, and a fluorescence spectrum (500–600 nm) was determined using a 488-nm excitation wavelength with an SLM Aminco 8000C spectrofluorometer. Maximum fluorescence occurred at $\sim$509 nm.

2.2.7 RNA Extraction and Northern Blotting Analysis

Filtered and dried *P. chrysosporium* mycelia (100 mg) were homogenized in Tri-reagent (Sigma) with 1 g of glass beads as described previously [Brown et al., 1993]. RNA was extracted as described in the protocol supplied with the reagent. The RNA pellet was resuspended in 4 M LiCl and kept at 4°C for 4 h. After microcentrifugation at 10,000 × *g*, the pellet, containing high molecular weight RNA, was washed with ethanol, dissolved in 0.5% SDS, and stored at -80°C. After spectrophotometric quantitation at 260 nm, the RNA (20 μg per lane) was denatured in the presence of 2.2 M formaldehyde for 15 min at 68°C and electrophoresed in a denaturing (0.6 M formaldehyde, 1% agarose) gel. Northern blots were performed as described previously [Brown et al., 1993]. The [α-^{32}P]-dCTP labeled probes were synthesized using a Multiprime DNA labeling kit (Amersham). Probed RNA blots were washed and exposed to XAR-5 X-ray film.

2.2.8 RT-PCR Analysis

Total RNA from the cultures of transformants carrying various plasmids was extracted as described above. The reverse transcription (RT) reaction contained 1 μg of total RNA as the template, 150 nM oligo(dT)$_{15}$ (Promega) as the primer, 1 mM dNTP and 200 U of M-MLV reverse transcriptase (GIBCO BRL) in a total volume of 25 μl. The reaction was performed according to the protocol supplied with the reverse transcriptase. 1 μl of the RT-product was directly used as the template in the following PCR [Gettemy et al., 1998]. The forward primer used in the PCR hybridized to an 18-nt sequence in the *mnp1* promoter approximately 100 bp upstream of the translation start codon. The reverse primer hybridized to an 18-nt sequence in the *egfp* coding region 200 bp downstream of the translation start codon (Fig. 2.7). The PCR protocol was described previously [Gettemy et al., 1998]. Following the amplification, 5 μl of each PCR product was analyzed by electrophoresis on a 1% agarose gel.

2.2.9 Southern Blotting Analysis

DNA from two transformants of each *egfp* expression plasmid and two control transformants carrying only pUB was extracted as described previously [Alic et al., 1989], digested with *Bam*HI, gel electrophoresed, and transferred to nylon membranes. The *Nhe*I–*Hin*dIII fragment containing the *egfp* coding region was labeled with [α-^{32}P]-dCTP. Southern hybridization and auto radiography were performed as described [Alic et al., 1989].

2.2.10 Fluorescence Microscopy

P. chrysosporium mycelia were grown on microscope cover slips and observed in a Leila TCS SP microscopic system with appropriate fluorescein isothiocyanate filters (Leica Microsystems, Heidelberg). Normal phase contrast images of each sample were taken as controls. The digital image was further processed using Photoshop 5.0 (Adobe).

2.3 RESULTS

2.3.1 GFP Expression in Transformants Carrying pUGGM3′, pUGiGM3′, pUMGM3′, and pUMiGM3′

As shown in Fig. 2.2, four *egfp* expression plasmids were made. pUGGM3′ contains the *gpd* promoter, followed by the *egfp* coding region and then the *mnp1* 3′ UTR. pUGiGM3′ contains the *gpd* promoter, followed by the iGM3′ sequence that includes, from 5′ to 3′, the *gpd* exon 1 (6 bp), intron 1 (55 bp), the 5′ end of exon 2 (9 bp), the *egfp* coding region and the *mnp1* 3′ UTR. pUMGM3′ is similar to pUGGM3′ except that the *mnp1* promoter replaces the *gpd* promoter. pUMiGM3′ contains the *mnp1* gene promoter, followed by the iGM3′ sequence. All four plasmids contain the *S. commune ura1* gene as the selectable marker as illustrated in a restriction map for pUGGM3′ (Fig. 2.1). All four plasmids were also sequenced to confirm that no mutations were introduced during plasmid construction.

Transformation of the *P. chrysosporium ura11* auxotrophic strain with each of the plasmids described above, resulted in the isolation of ~50 prototrophic transformants per μg of DNA. The transformants carrying pUGGM3′ and pUGiGM3′ were grown in stationary culture at 37°C in 20 ml of HCHN medium [Mayfield et al., 1994b]. After 40 h, the cells were filtered, homogenized, and the fluorescence intensity of the cell-free extract was measured. The transformants containing pUMGM3′ and pUMiGM3′ were grown at 37°C in stationary cultures containing 20 ml of HCLN medium supplemented with 180 μM $MnSO_4$ for 7 days. Likewise, the fluorescence intensity of the cell-free extract from these cultures was measured. Under both culture conditions, the background fluorescence shown in transformants carrying only pUB was also determined. Only the transformants that carried *egfp* expression plasmids and exhibited more than three times the background fluorescence were considered to express GFP. None of the transformants carrying pUGGM3′ or pUMGM3′ (both contain no intron) exhibited significant fluorescence under the specified culture conditions. In contrast, approximately 50% of the transformants carrying plasmids that contain introns (pUGiGM3′ and pUMiGM3′) expressed significant amount of GFP. The cell-free extract from each transformant that expressed GFP exhibited a maximum emission at 509 nm and a weak shoulder at 545 nm when excited at 488 nm, identical to the emission spectrum exhibited by purified recombinant EGFP [data not shown]. Fig. 2.4 shows the fluorescence intensity at 509 nm of the two most fluorescent transformants carrying each expression plasmid. As shown, the transformants (T-19, T-23) carrying the pUGiGM3′ and those (T-5, T-11) carrying pUMiGM3′ exhibited high levels of fluorescence, whereas the transformants carrying pUGGM3′ (T-5, T-28) and those (T-3, T-27) carrying pUMGM3′ exhibited only negligible fluorescence. This suggests that the presence of an intron is important for efficient expression of GFP in *P. chrysosporium*.

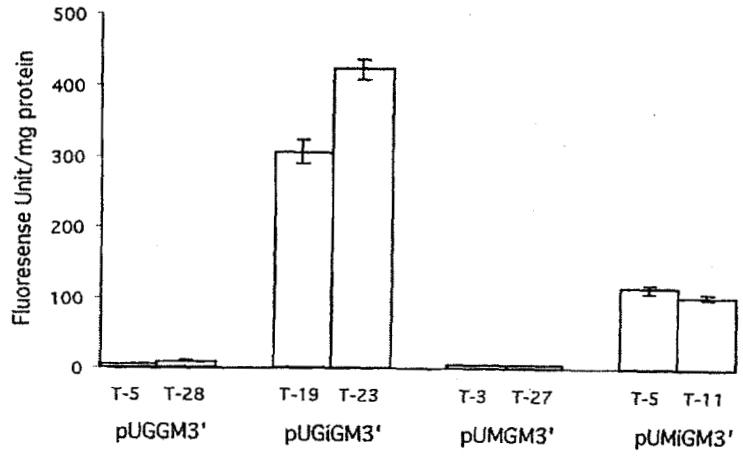


Fig. 2.4 GFP fluorescence intensity in transformants carrying pUGGM3′, pUGiGM3′, pUMGM3′, and pUMiGM3′. Only the data from two transformants of each plasmid exhibiting the highest fluorescence intensity are shown. The fluorescence was measured from crude extracts of triplicate cultures of each transformant. The means and deviations of triplicate values are shown.

2.3.2 Northern Blots and Southern Blots of Transformants Carrying pUGGM3′ and pUGiGM3′

Total RNA extracted from the cultures of the two most fluorescent transformants carrying pUGGM3′ (T-5, T-28) and pUGiGM3′ (T-19, T-23) was probed with ^{32}P-labeled *egfp* coding region DNA (Fig. 2.5A). The weak band from transformants carrying pUGGM3′ is suggestive of the low level of *egfp* transcription. In contrast, a high level of *egfp* transcript is observed in transformants carrying pUGiGM3′. The same RNA was also probed with ^{32}P-labeled *gpd* gene to show the quality of the total RNA samples (Fig. 2.5B).

Genomic DNA extracted from transformants T-5 and T-28 carrying pUGGM3′ as well as T19 and T-23 carrying pUGiGM3′ was analyzed by Southern blot analysis. When probed with ^{32}P-labeled *egfp* coding region, only high molecular weight DNA corresponding to the undigested genomic DNA, but no rapidly migrating DNA fragments were detected, indicating the transforming plasmids all integrated into the host chromosomes [data not shown]. Fig. 2.5C shows the same genomic DNA samples digested with *Bam*HI and hybridized with the same *egfp* probe. *Bam*HI linearizes both pUGGM3′ and pUGiGM3′ in the *gpd* promoter regions. As shown, no significant difference in the band intensity can be observed between transformants carrying pUGGM3′ and transformants carrying pUGiGM3′. The various bands whose sizes are different from that of linearized pUGiGM3′ (lane 6, Fig. 2.5C) indicate the plasmids have integrated into different locations on the host chromosomal DNA. In addition, bands with the similar size as linearized pUGiGM3′ were detected in transformant T-5 carrying pUGGM3′ and transformant T-23 carrying pUGiGM3′ (lanes 1 and 4, respectively). This suggests that multiple copies of the transformed plasmids were possibly integrated into the genome of these transformants.

2.3.3 Fluorescent Mycelia

Fig. 2.6 shows a fluorescent micrograph of transformed mycelia carrying pUGiGM3′ (from transformant T-23). It is apparent that the green fluorescence of GFP is located within the mycelium. A phase contrast image of the same mycelia is

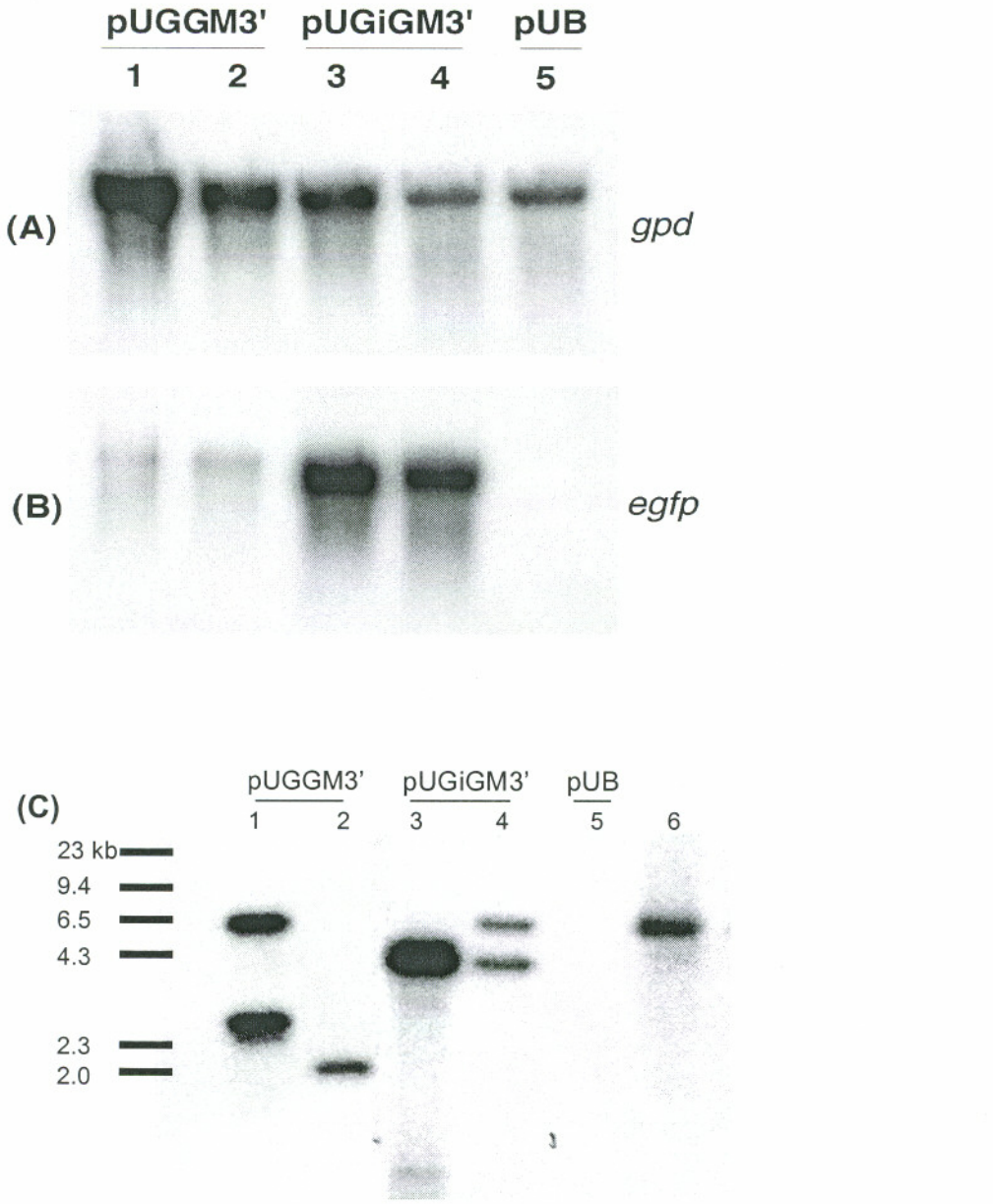


Fig. 2.5 Northern blot and Southern blot analysis of transformants carrying pUGGM3′ and pUGiGM3′. Only the results from the two most fluorescent transformants carrying these two plasmids are shown. (**A**) Gel-separated total RNA was probed with the ^{32}P-labeled *gpd* genomic DNA. (**B**) The same RNA blot was probed with the ^{32}P-labeled *egfp* coding sequence. (**C**) 1 mg of genomic DNA from various transformants was digested with *Bam*HI, separated on a 1% agarose gel, and probed with the ^{32}P-labeled *egfp* coding sequence. In all panels, lanes 1 and 2, T-5 and T-28 carrying pUGGM3'; lanes 3 and 4, T-19 and T-23 carrying pUGiGM3'; lane 5, T-1 carrying blank vector, pUB. In (C), lane 6, 100 pg of *Bam*HI-digested pUGiGM3′.

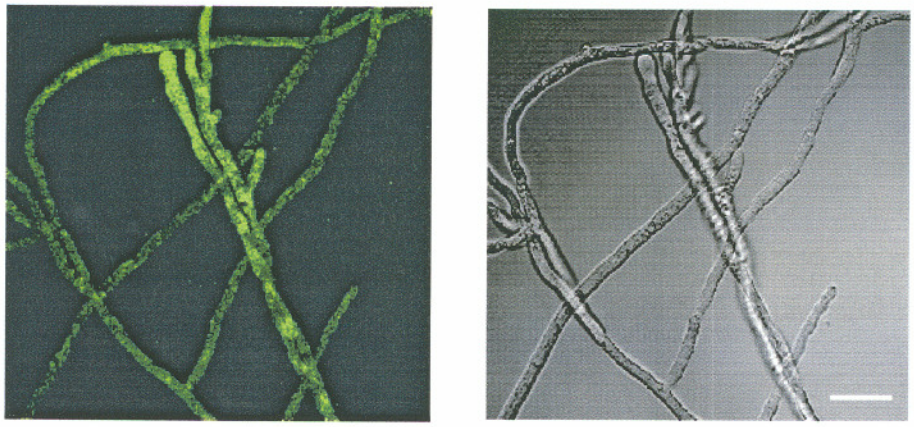


Fig. 2.6 Fluorescence micrograph of *P. chrysosporium* mycelia carrying pUGiGM3′ (left). The picture on the right is the phase-contrast image of the same mycelia. The green color in the mycelia is due to GFP. Mycelia carrying pUGGM3′ did not show significant fluorescence [data not shown].

also shown. Transformants containing pUGGM3′ did not exhibit green fluorescence under the experimental condition [data not shown].

2.3.4 Expression of *egfp* Requires a Functional Intron

RT-PCR was performed on total RNA extracted from transformant T-5 carrying pUMiGM3′. The oligonucleotide primers used in the PCR were designed to amplify a 320-bp fragment encompassing the intron region (Fig. 2.7). However, if the intron is correctly spliced out in mRNA produced from the *egfp* expression cassette in pUMiGM3′, RT-PCR should amplify a 250-bp fragment. This is clearly shown in Fig. 2.7 (compare lanes 1 and 2). Using the genomic DNA from T-5 carrying pUMiGM3′ as the template, only a 320-bp fragment was amplified. Instead, when the cDNA synthesized from the total RNA of T-5 using oligo$(dT)_{15}$ as the primer was utilized as the template, only a 250-bp fragment was amplified. Sequencing of the 250-bp RT-PCR fragment showed the precise junction between the *gpd* exon1 and partial *gpd* exon 2, indicating accurate splicing of the intron in transformants carrying pUMiGM3′.

To further investigate the requirement of an intron in expression of *egfp* in *P. chrysosporium*, two expression plasmids, pUMGM-5′I and pUMGM-5′RI, were constructed. Both plasmids are similar to pUMGM3′ (with no intron) except that both contain insertions of 76-bp DNA (encompassing an intact intron) at the position 25 bp upstream of the translation start codon (Fig. 2.8). However, the inserted DNA can adopt two opposite orientations. pUMGM-5′I contains inserted DNA with consensus intron splicing site (5′ GT---AG 3′), whereas the inserted DNA in pUMGM-5′RI is an inversion of that in pUMGM-5′I and contains no intron splicing site (Fig. 2.8). Transformants carrying pUMGM-5′I exhibited significant GFP fluorescence comparable to those carrying pUGiGM3′ and pUMiGM3′ (Fig. 2.8). Conversely, transformants carrying pUMGM-5′RI showed negligible GFP fluorescence similar to those carrying pUGGM3′ and pUMGM3′ (Fig. 2.8). This result suggests that the efficient expression of *egfp* in *P. chrysosporium* requires an intron with the functional splicing site.

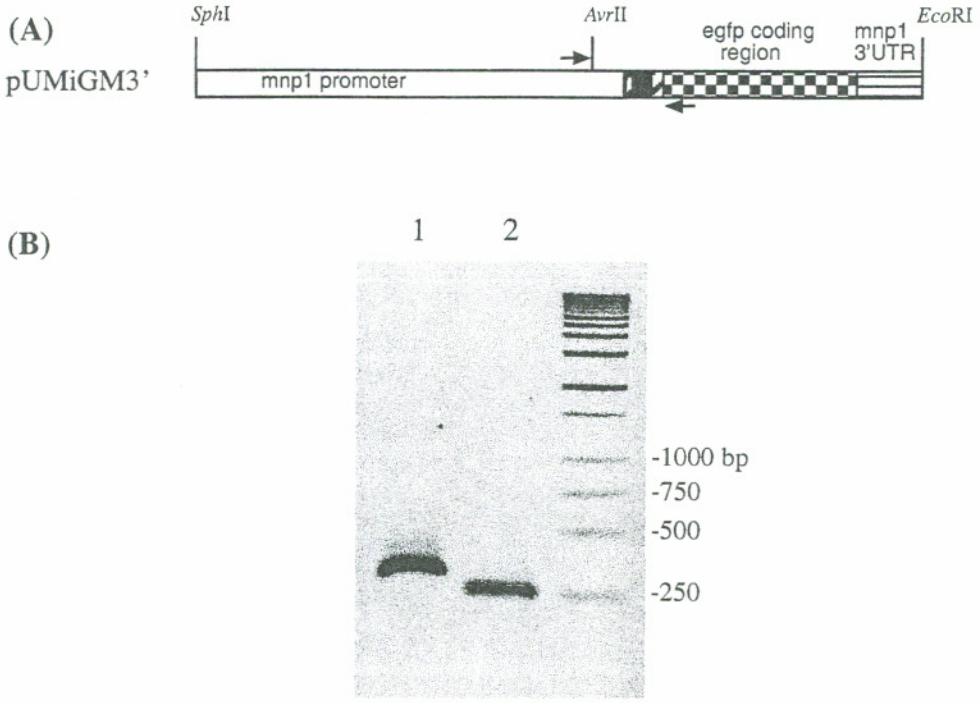


Fig. 2.7 RT-PCR analysis of transformant T-5 carrying pUMiGM3′. (**A**) Restriction map of the *egfp* expression cassette in pUMiGM3′, as described in Fig. 2.2. The arrows indicate the locations where the primers used in PCR hybridize. (**B**) Picture of PCR products separated on a 1% agarose gel and stained with ethidium bromide. Lane 1, control PCR product with genomic DNA of T-5 as the template; lane 2, RT-PCR product from the total RNA of T-5.

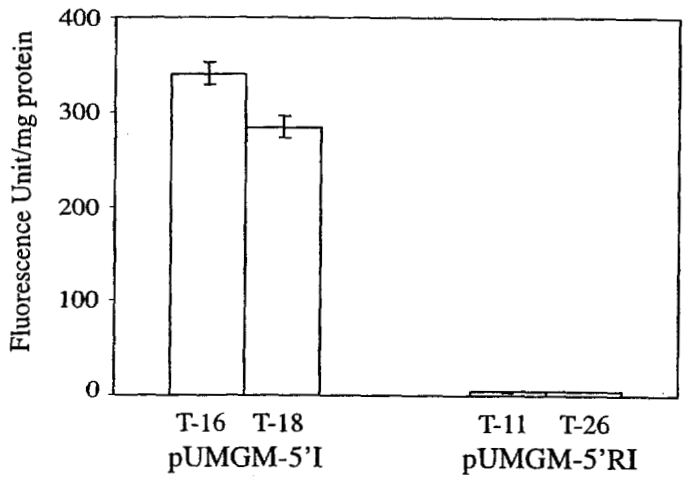


Fig. 2.8 GFP fluorescence intensity in transformants carrying pUMGM-5′I and pUMGM-5′RI. The data from the two transformants carrying each plasmid and exhibiting the highest fluorescence intensity are shown. The fluorescence was measured from crude extract of triplicate cultures of each transformant. The means and deviations of triplicate values are shown.

2.3.5 Effect of Mn^{2+} on the GFP and MnP Expression in Transformants Carrying pUMiGM3′

Time courses for the appearance of intracellular GFP and extracellular MnP activity in transformant (T-5) carrying pUMiGM3′ are shown in Fig 2.9. Cultures of this transformant in HCLN medium with no exogenous Mn^{2+} exhibited no detectable MnP activity through day 8 and had negligible expression of GFP during the same period. In contrast, the same cultures grown in the presence of 180 μM $MnSO_4$, exhibited both MnP activity and GFP fluorescence, which first appeared on day 4 and were present through day 8. MnP activity peaked on day 5 while GFP fluorescence continued to increase through day 8. Cultures of transformants carrying pUMGM3′ showed no GFP expression in a similar time course experiment [data not shown].

The Mn^{2+} induction of MnP activity and GFP fluorescence is shown in Fig. 2.10. In this experiment, the transformant (T-5) carrying pUMiGM3′ was grown for 5 days in HCLN medium in the absence of Mn^{2+}, after which $MnSO_4$ was added to a final concentration of 180 μM and the incubation continued. Neither significant MnP activity nor GFP fluorescence was detected in cultures without Mn^{2+}. After the supplementation of Mn^{2+} on day 5, MnP activity was observed and reached a maximum after $\sim$24 h, slowly declining thereafter. This is similar to our previous results with the wild-type *P. chrysosporium* cultures [Brown et al., 1991]. In parallel with the appearance of MnP activity, GFP fluorescence steadily increased for 36 h after the addition of Mn^{2+} and leveled off thereafter. The transformant (T-5) carrying pUMiGM3′ was also grown in HCHN medium; however, it exhibited neither MnP activity nor GFP fluorescence even after the supplementation of Mn^{2+}.

2.3.6 Effect of Mn^{2+} on the Transcription of *egfp* and *mnp* in a Transformant Carrying pUMiGM3′

Total RNA from 5-day-old stationary cultures of transformant (T-5) carrying pUMiGM3′ in HCLN media supplemented with 180 μM $MnSO_4$ or without Mn^{2+} was probed with ^{32}P-labeled *egfp* coding region or *mnp1* cDNA (Fig. 2.11). Both the *mnp1* and *egfp* transcripts were observed only in the cultures supplemented with Mn^{2+} (lane 4). The same blot was also probed with the constitutively expressed *gpd* gene to

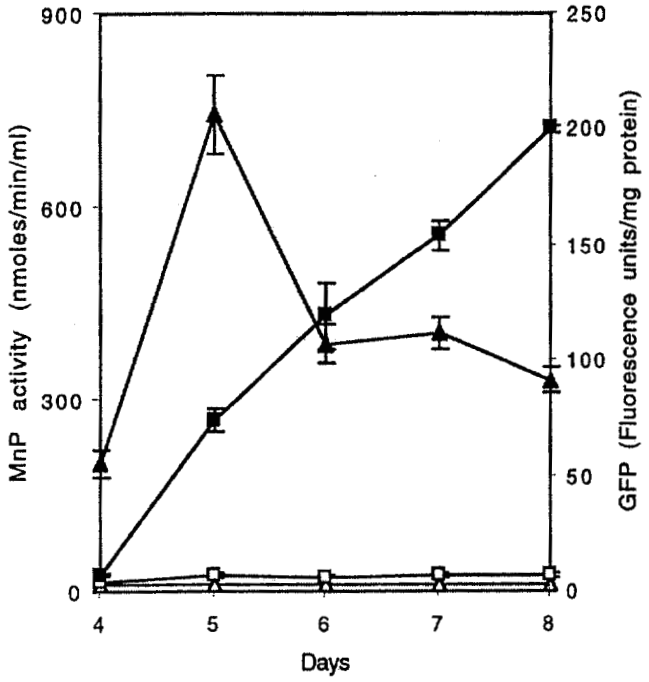


Fig. 2.9 Effect of Mn^{2+}-supplementation on the expression of extracellular MnP and intracellular GFP directed by the *mnp1* promoter. Transformant T-5 (carrying pUMiGM3′) was grown in HCLN media in the presence of 180 μM Mn^{2+} or no additional Mn from a conidial inoculum as described in the text. MnP activity in Mn^{2+}-supplemented (▲) or no additional Mn cultures (Δ) and GFP fluorescence in Mn^{2+}-supplemented (■) or no additional Mn (□) cultures were assayed. Experiments were run in triplicate and the deviation is shown.

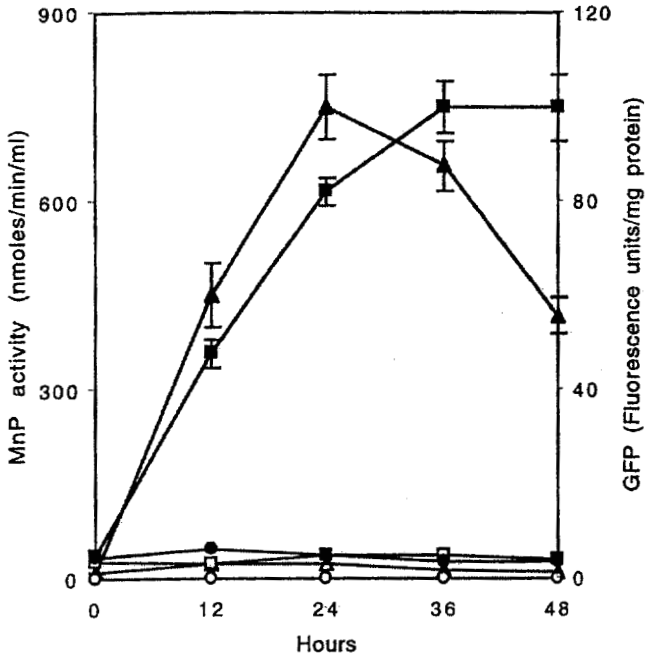


Fig. 2.10 Induction of extracellular MnP activity and the *mnp1* promoter-directed intracellular GFP activity by Mn^{2+}. HCLN or HCHN, Mn-deficient cultures of transformant T-5 (carrying pUMiGM3′) were grown for 5 days, after which Mn^{2+} was supplemented to a final concentration of 180 μM. GFP fluorescence in HCLN cultures induced with Mn^{2+} (■) or not induced (□) and in HCHN cultures induced with Mn^{2+} (•) was assayed. MnP activity in HCLN cultures induced with Mn^{2+} (▲) or not induced (△) and in HCHN cultures induced with Mn^{2+} (○) was also assayed. Experiments were run in triplicate and the deviation is shown.

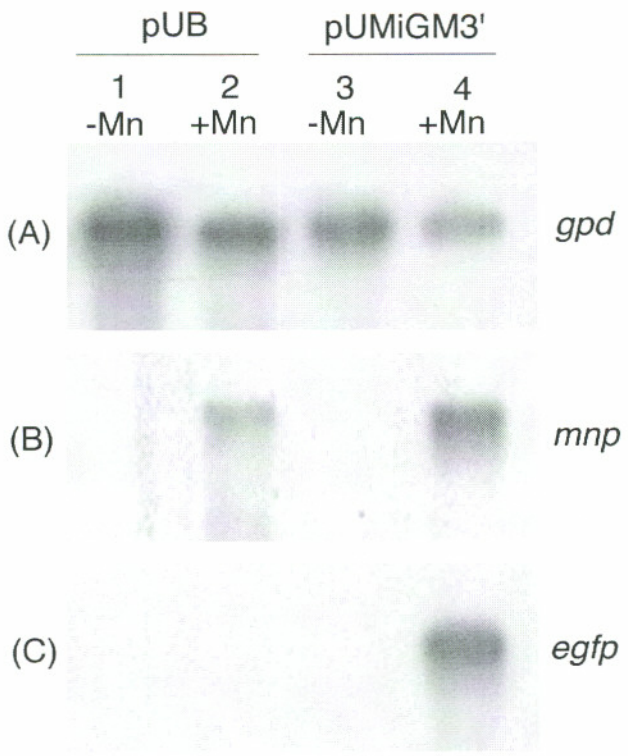


Fig. 2.11 Effect of Mn^{2+}-supplementation on the *mnp1* promoter-directed *egfp* expression at the RNA level. Transformants carrying pUB or pUMiGM3′ were grown in HCLN medium in the presence and absence of Mn^{2+}. Total RNA was extracted, and the Northern blot procedure is described in the text. (**A**) Northern blots probed with the ^{32}P-labeled *gpd* gene. (**B**) Northern blots probed with the ^{32}P-labeled *mnp1* cDNA, showing transcripts from the endogenous *mnp*s. (**C**) Northern blots probed with the ^{32}P-labeled *egfp* coding region. Lanes 1 and 2 contain RNA from the transformant T-1 carrying pUB; lanes 3 and 4 contain RNA from transformant T-5 carrying pUMiGM3′; lanes 2 and 4, from cells growing in the presence of 180 mM Mn^{2+}; lanes 1 and 3, from cells grown in the absence of exogenous Mn.

show the quality and quantity of the RNA sample. These results suggest that Mn^{2+} affects the transcript levels of both the endogenous *mnp* and exogenous *egfp* in a similar fashion in transformants carrying pUMiGM3′.

2.4 DISCUSSION

The activities of LiP and MnP, two major components of *P. chrysosporium*'s lignin degrading system, are only detectable in the extracellular culture media during the secondary metabolic growth phase [Gold and Alic, 1993; Cullen, 1997]. Our earlier studies indicated that MnP activity and *mnp* transcripts are observed exclusively in nitrogen-limited and Mn^{2+}-supplemented cultures [Pribnow et al., 1989; Brown et al., 1990, 1991].

To further investigate the regulation of *mnp* expression, a suitable gene reporter system is required. The *gfp* gene from *A. victoria* has been used in numerous studies as a reporter for gene expression and protein localization [Cubitt et al, 1995; Cormack, 1998]. In addition, it has been successfully expressed in several fungal systems [Spellig et al., 1996; Cormack, 1998; Henriksen et al., 1999; Lugones et al., 1999]. Lately, a *gfp* variant, *egfp*, was developed and it encodes a protein 35-fold more fluorescent than the original GFP from *A. victoria* [Cormack et al., 1996]. The utilization of *egfp* greatly improves the sensitivity of GFP as a reporter for gene expression. Our present study demonstrates that the efficient expression of *egfp* in *P. chrysosporium* requires an inserted intron. The commercial *egfp* gene contains no intron. In contrast, all sequenced genes from the basidiomycete *S. commune* and *P. chrysosporium* contain introns [Lugones et al., 1999] (Genbank), suggesting that introns may play an important role in gene expression of these fungi. Recently, it was shown that an intronless hydrophobin gene was very weakly expressed in *S. commune*; however, high expression was observed when a native or synthetic intron was introduced into this gene [Lugones et al., 1999].

Three types of *egfp* expression constructs were made in our present study. Type 1 constructs contain the *egfp* coding region placed between the *P. chrysosporium*

gpd or *mnp1* 5′ non-coding region and the *mnp1* 3′ UTR without an intron (pUGGM3′ and pUMGM3′). Type 2 constructs are similar to those of type 1, except that they contain fragments including an intron and short coding sequences from *P. chrysosporium gpd* gene joined in frame with the *egfp* coding region (pUGiGM3′, pUMiGM3′). The *P. chrysosporium gpd* gene has an important feature: its first exon is extremely short (6 bp including the start codon) and this is followed by a 55-bp intron. This intron contains the consensus intron/exon junction sequence and the putative internal lariat formation site. In type 2 *egfp* constructs, joining the *gpd* exon1, intron1, and 9 bp of exon2 in frame with the *egfp* coding region results in a GFP variant with four extra amino acids compared to the original protein encoded by *egfp*. Type 3 *egfp* constructs are also similar to those of type 1, except that they have insertions of intron-containing sequences at 25 bp upstream of the translation start codon. The inserted sequences in type 3 constructs contain either an intron with correct splicing site (pUMGM-5′I) or an inverted intron (pUMGM-5′RI). Our results showed that only the type 2 constructs and a type 3 construct that has an intron with correct splicing site resulted in efficient expression of *egfp*, whereas the type 1 constructs and a type 3 construct that has an inverted intron did not. RT-PCR clearly showed the precise splicing of the 5′ intron in transformants carrying a type 2 *egfp* construct (pUMiGM3′). Therefore, these data indicate that the expression of *egfp* in *P. chrysosporium* requires a functional intron and the intron can reside either within the coding region or in the untranslated region. Furthermore, northern blot data showed that the steady-state *egfp* transcript levels were clearly higher in transformants carrying a type 2 *egfp* construct (pUGiGM3′) than those carrying a type 1 construct (pUGGM3′). Previous results in *S. commune* indicated that the absence of an intron apparently did not affect transcriptional initiation of the hydrophobin genes [Huang and Gorman, 1990; Lugones et al., 1999]. Thus, the difference in RNA levels of the intron-containing versus the intronless *egfp* construct in *P. chrysosporium* is possibly due to some post-transcriptional events affecting the stability of nascent *egfp* transcripts.

Based on an earlier survey [Hawkins, 1988], introns located at 3′ downstream of the stop codon are rare, while roughly one-third of 328 vertebrate genes surveyed contained introns in the 5′ non-coding region. A study using mammalian cells suggested that introns can increase the efficiency of RNA 3′-processing and the accumulation of cytoplasmic RNA [Huang and Gorman, 1990]. More recent work using *Xenopus* oocytes suggests that the location of an inserted intron strongly affects the translation of the cytoplasmic RNA [Hawkins, 1988; Matsumoto et al., 1998]. Comparisons of the *mnp*, *lip*, *gpd*, and quinone reductase genes of *P. chrysosporium* found in the Genbank database indicate that more introns are found in the 5′ half of these genes than in the 3′ half. These observations suggest that the 5′ introns may have a role in gene transcription, RNA processing, or RNA translation.

Fluorescent micrograph of *P. chrysosporium* mycelia carrying pUGiGM3′ (with a 5′ intron) showed that the expressed GFP was intracellular and probably cytoplasmic. Significant green fluorescence was observed neither in the extracellular medium nor in the conidiospores [data not shown]. This suggests that GFP may be a good marker for viewing *P. chrysosporium* in wood or in a consortium of other fungi.

Transformation of *P. chrysosporium* with plasmid DNA often results in the ectopic integration of the DNA into the fungal genome [Alic et al., 1989, 1991], where the site of integration is not controlled. It is recognized that, in fungal transformations, the site of integration can affect the expression of transformed genes. This may explain why only 50% of the transformants carrying pUGiGM3′ or pUMiGM3′ showed significant expression of GFP and the expression levels among different transformants varied in a broad range. However, most importantly, we have shown that *egfp* faithfully reports the function of both *P. chrysosporium gpd* and *mnp1* promoters. Especially in the latter case, the efficient GFP expression under the control of approximately 1.5 kb of *P. chrysosporium mnp1* promoter (as seen in transformants carrying pUMiGM3′) is only observed in nitrogen-limited and Mn^{2+}-supplemented cultures the same as the endogenous MnP. The role of Mn^{2+} in regulating the expression of *egfp* in transformants carrying pUMiGM3′ was further examined by the Mn^{2+}-induction experiment and the Northern blot analysis. Our results show that supplementing Mn^{2+} to the 5-day-old nitrogen-limited and Mn^{2+}-

deficient cultures leads to the simultaneous appearance of both GFP and endogenous MnP. In addition, the *egfp* transcripts in transformants carrying pUMiGM3′ are abundant in cultures grown in the presence of Mn^{2+}, but below the detectable level in cultures grown in the absence of Mn^{2+}, similar as the endogenous transcripts of *mnp*s. This result further suggests that the effect of Mn^{2+} occurs at the gene transcription level as reported previously [Brown et al., 1990, 1991; Gettemy et al., 1998].

In conclusion, we have demonstrated that, when an intron is placed at the 5′ end of the *egfp* coding sequence (either in frame or in the untranslated region), *egfp* is an effective reporter of either *gpd* or *mnp1* promoter function in *P. chrysosporium*. We also conclude that approximately 1.5 kb of the *mnp1* promoter sequence can regulate *egfp* reporter expression in a manner similar to the regulation of endogenous *mnp* genes in response to exogenous Mn^{2+} and nutrient nitrogen levels. We plan to use the *egfp* construct with a 5′ intron to pinpoint the *cis*-acting sequences that may be responsible for the regulation of *mnp1* by Mn^{2+} and other factors.

CHAPTER 3

CHARACTERIZATION OF PROMOTER SEQUENCE REQUIRED FOR MANGANESE-DEPENDENT REGULATION OF MANGANESE PEROXIDASE ISOZYME 1 GENE EXPRESSION IN *PHANEROCHAETE CHRYSOSPORIUM*

3.1 INTRODUCTION

Manganese peroxidase (MnP) is a major ligninolytic enzyme produced by almost all white-rot fungi [Hatakka, 1994; Pelaez et al., 1995]. In *Phanerochaete chrysosporium*, MnP is secreted as a series of isozymes, which are encoded by structural related genes [Gold and Alic, 1993; Gold et al., 2000]. Over the past fifteen years, Gold lab has reported the cDNA and genomic sequences of three MnP-encoding genes (*mnp*s) in *P. chrysosporium*, namely *mnp1*, *mnp2* and *mnp3* [Pribnow et al., 1989; Godfrey et al., 1990; Mayfield et al., 1994a; Alic et al., 1997]. In this fungus, MnP isozymes (MnPs) are idiophasic proteins whose production is activated by the depletion of nutrient nitrogen in the culture medium [Tien and Tu, 1987; Pribnow et al., 1989; Gold and Alic, 1993; Li et al., 1994]. The production of MnPs is also dependent on the presence of Mn^{2+} in the cultures [Glenn and Gold, 1985; Pribnow et al., 1989; Brown et al., 1990, 1991]. Both Northern blot and RT-PCR analysis have shown that the nitrogen depletion and Mn^{2+}-supplementation activate the transcription of *mnp*s [Brown et al., 1990, 1991; Gettemy et al., 1997, 1998]. When *P. chrysosporium* cells were grown in nitrogen-limited cultures containing 180 μM Mn^{2+}, *mnp* transcripts were first detected on day 4 and reached a peak on day 5. In contrast, no *mnp* transcript was observed in nitrogen-sufficient cultures or in nitrogen-limited cultures in the absence of Mn [Brown et al., 1990; Gettemy et al., 1997]. Addition of Mn^{2+} to 5-day-old nitrogen-limited, Mn-deficient cultures resulted in a detectable level of *mnp* transcripts within 40 min, and the amount of *mnp* transcripts

was a function of the final concentration of Mn^{2+} in the media, up to a maximum of 180 μM [Brown et al., 1991]. Using quantitative RT-PCR analysis, it was shown that Mn^{2+} might regulate the transcription of *P. chrysosporium mnp1, mnp2 and mnp3* differently [Gettemy et al., 1998]. To date, the detailed mechanism of transcriptional regulation of *mnp*s expression in response to Mn^{2+}-supplementation and nitrogen depletion is unknown.

There are several short sequences found in the promoter regions of *P. chrysosporium mnp*s that conform to several *cis*-acting promoter elements, which include the inverted CCAAT box, metal response elements (*MRE*s), heat shock elements (*HSE*s) and the binding site of activator protein-2 (AP-2) [Gold and Alic, 1993]. However, experiments that prove the involvement of these sequences in the regulation of *mnp*s expression are lacking.

To study the transcriptional regulation of *mnp*s expression, a suitable gene reporter system is required. In Chapter 2, an efficient reporter system using the enhanced green fluorescent protein (GFP) gene (*egfp*) to monitor gene expression in *P. chrysosporium* is described. When the plasmid construct, pUMiGM3′ (Fig. 3.1), containing the 1,528-bp promoter region of *P. chrysosporium mnp1*, followed by *egfp* with an intron inserted at its 5′ and the 250 bp of *mnp1* 3′ untranslated region (UTR) was introduced into *P. chrysosporium*, high expression of GFP was observed only under nitrogen-limited and Mn^{2+}-supplemented conditions, which are similar to that required for endogenous MnPs production. In this Chapter, I report the characterization of a short sequence residing approximately 500 bp upstream of the translation start codon in the *mnp1* promoter that is at least partially responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity using the reporter system based on GFP.

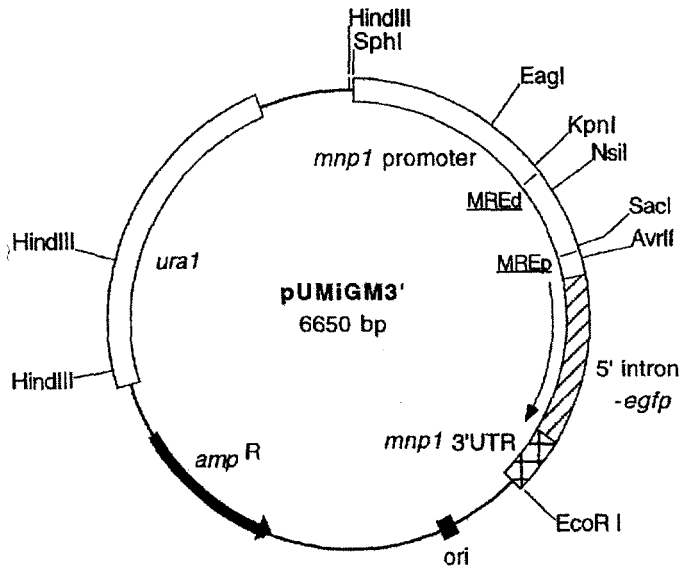


Fig. 3.1 Restriction map of pUMiGM3′. The relative locations of the *P. chrysosporium mnp1* promoter, the intron-containing *egfp* coding region, the *mnp1* 3′ UTR and the two genes, amp^R and *ura1*, for selecting transformants in *E. coli* and in *P. chrysosporium*, respectively, are indicated.

3.2 MATERIALS AND METHODS

3.2.1 Organisms

P. chrysosporium wild type strain OGC101 and the auxotrophic *ura11* strain were maintained as described previously [Alic et al., 1987; Akileswaran et al., 1993]. *Escherichia coli* strain DH5α was used for the preparation of plasmid constructs.

3.2.2 Construction of Plasmids

3.2.2.1 pUMiGM3′-A, pUMiGM3′-K, pUMiGM3′-E, and pUMiGM3′-ΔEK. In the *mnp1* promoter, unique *Avr*II, *Kpn*I and *Eag*I restriction sites reside 80 bp, 521 bp, and 774 bp upstream of the translation start codon, respectively (Fig. 3.1). Double digests were performed on pUMiGM3′ with *Sph*I/*Avr*II, *Sph*I/*Kpn*I, *Sph*I/*Eag*I or *Eag*I/*Kpn*I. In each case, DNA fragments of 1,448 bp, 1,007 bp, 750 bp, or 253 bp containing part of the *mnp1* promoter were released. The digests were fractionated on a 1% agarose gel, and the *egfp*-containing fragments were purified using a gel extraction kit (Qiagen). These fragments were blunt-ended with T4 DNA Polymerase (New England Biolabs) and re-ligated with T4 DNA ligase (New England Biolabs) to yield pUMiGM3′-A, pUMiGM3′-K, pUMiGM3′-E (Fig. 3.2) and pUMiGM3′ΔEK (Fig. 3.3A).

3.2.2.2 pUMiGM3′ΔE197 and pUMiGM3′ΔK(-48). In pUMiGM3′ΔE197, the 197-bp DNA downstream of the *Eag*I site in the *mnp1* promoter was deleted (Fig. 3.3A). In pUMiGM3′ΔK(-48), the 48-bp DNA upstream of the *Kpn*I site was deleted (Fig. 3.3A). These deletions were constructed by PCR. For constructing pUMiGM3′ΔE197, a forward primer was designed that hybridized with the *mnp1* promoter sequence 197 bp downstream of the *Eag*I site and contained an introduced *Eag*I site at its 5′ end. A reverse primer, which corresponded to the junction sequence between the *mnp1* promoter and the intron-containing *egfp*, was also synthesized. Using pUMiGM3′ as the template and the primers mentioned above, a 0.6-kb fragment was amplified. This fragment was digested at the introduced *Eag*I site and at the native *Avr*II site, and used to replace the *Eag*I–*Avr*II fragment in

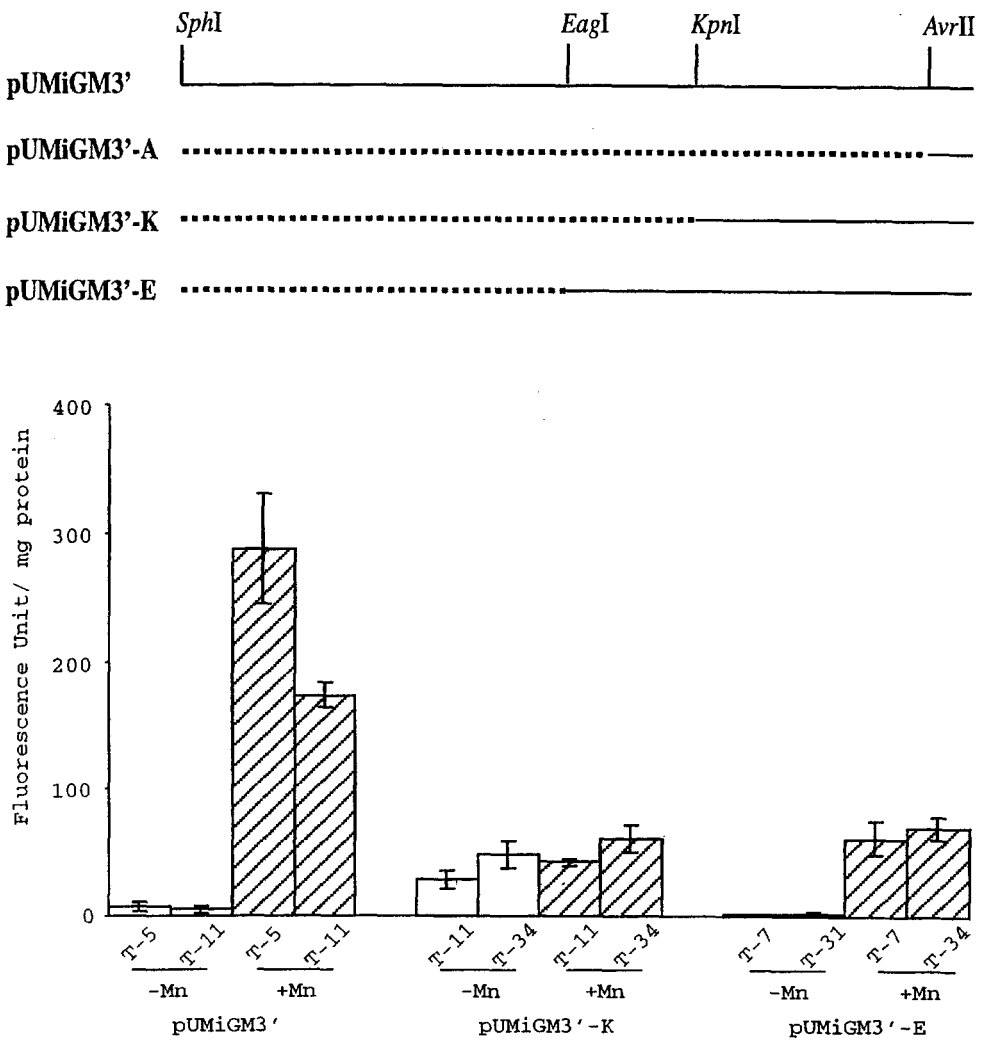


Fig. 3.2 GFP expression in transformants carrying pUMiGM3′, pUMiGM3′-K, and pUMiGM3′-E. The upper panel shows the deleted *mnp1* promoter regions (---) in indicated pUMiGM3′ derivatives. The lower panel shows the fluorescence intensity of the two most fluorescent transformants carrying each specified plasmid in 7-day-old HCLN cultures without exogenous Mn (–Mn) and in cultures supplemented with 180 mM Mn^{2+} (+Mn). The average fluorescence intensity per mg of total protein from triplicate –Mn and +Mn cultures of each specified transformant, after deduction of the background fluorescence intensity per mg total protein exhibited by transformant carrying only the blank vector, pUB, is shown.

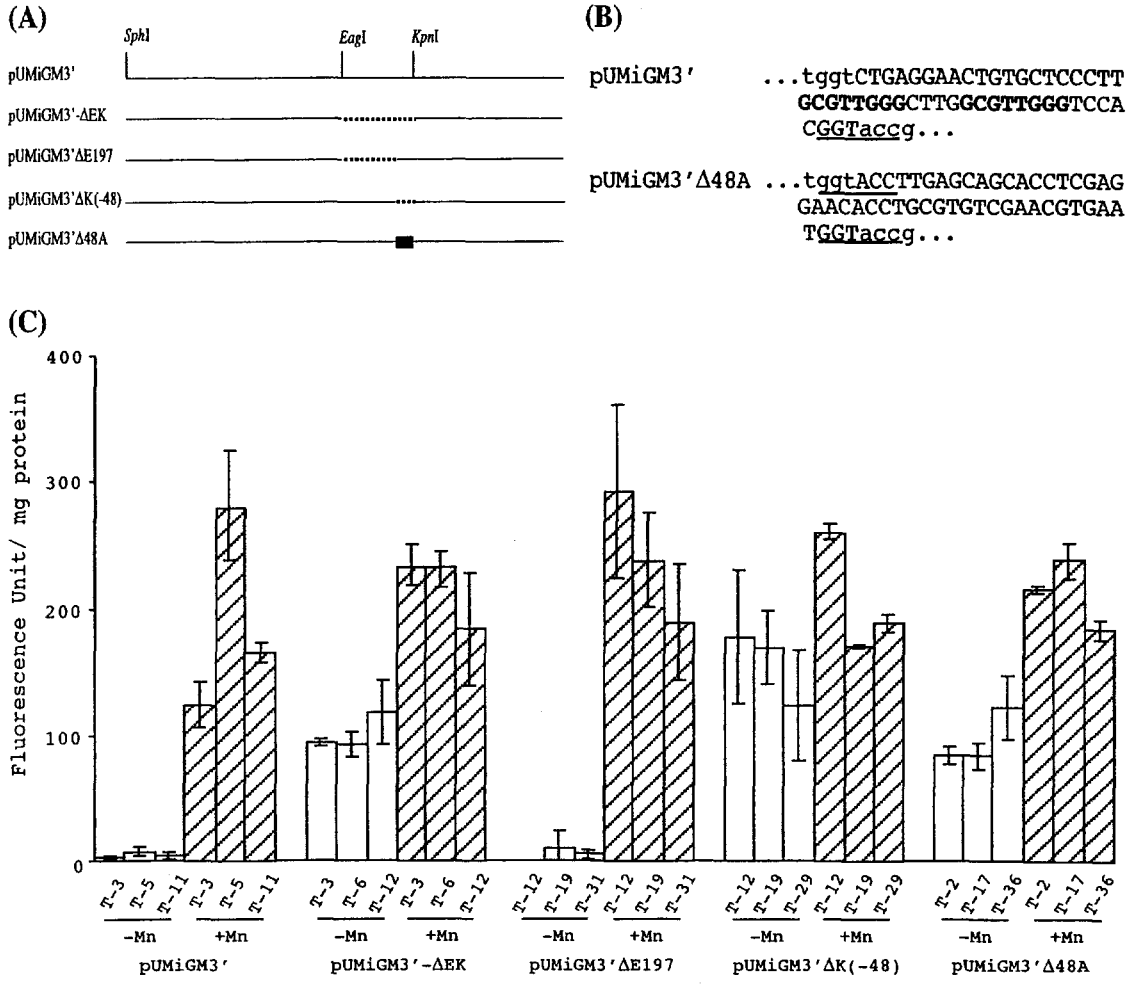


Fig. 3.3 GFP expression in transformants carrying pUMiGM3′ΔEK, pUMiGM3′ΔE197, pUMiGM3′ΔK(-48), and pUMiGM3′Δ48A. (**A**) The mutated *mnp1* promoter regions (deleted sequence, dashed line; replaced sequence, black box) in indicated pUMiGM3′ derivatives. (**B**) The nucleotide sequence of the 48-bp fragment immediately upstream of the *Kpn*I site in the *mnp1* promoter and the synthesized 48-bp fragment that replaced the original 48-bp *mnp1* promoter fragment in pUMiGM3′Δ48A. The two identical sequences that conform to consensus AP-2 binding site are in bold. The *Kpn*I site is underlined. (**C**) The fluorescence intensity of the three most fluorescent transformants carrying each specified plasmid. Data were calculated as described in Fig. 3.2.

pUMiGM3′, yielding pUMiGM3′ΔE197. For constructing pUMiGM3′ΔK(-48), a forward primer hybridizing with the *mnp1* promoter sequence 1.0 kb upstream of the translation start codon was synthesized. The reverse primer was designed that contained a new *Kpn*I site at its 5′ end and hybridized with the *mnp1* promoter sequence 48 bp upstream of the native *Kpn*I site. Using pUMiGM3′ as the template, a 0.5-kb fragment was amplified. This fragment was digested at the native *Eag*I site and at the introduced *Kpn*I site, and used to replace the *Eag*I–*Kpn*I fragment in pUMiGM3′, yielding pUMiGM3′ΔK(-48).

3.2.2.3 pUMiGM3′Δ48A. pUMiGM3′Δ48A was constructed from pUMiGM3′ΔK(-48) by inserting a 48-bp DNA with sequence of the 5′ end of the *mnp1* exon 6 [Godfrey et al., 1990] into the *Kpn*I site (Fig. 3.3A). A pair of complementary 48 mer oligonucleotides, Oligo 1 and Oligo 2, were designed that contained *Kpn*I ends. The sequences of Oligo 1 and Oligo 2 are 5′-CATTCACGTTCGACACGCAGGTGT-TCCTCGAGGTGCTGCTCAAGGTAC-3′ and 5′-CTTGAGCAGCACCTCGAGGAA-CACCTGCGTGTCGAACGTGAATGGTAC-3′, respectively. The oligonucleotides were phosphorylated with T4 polynucleotide kinase, denatured by heating to 85°C for 5 min, and annealed by cooling to room temperature. The resultant double-stranded DNA was ligated with *Kpn*I-digested pUMiGM3′ΔK(-48) to yield pUMiGM3′Δ48A.

3.2.2.4 pUMiGM3′-N48. This plasmid was constructed from pUMiGM3′ΔK(-48) by inserting the deleted 48-bp *mnp1* promoter sequence into the unique *Nsi*I site, located 398 bp upstream of the start codon (Fig. 3.7). Oligonucleotides corresponding to both strands of the 48-bp *mnp1* promoter sequence with *Nsi*I ends were synthesized, combined, annealed, and subsequently ligated with *Nsi*I-digested pUMiGM3′ΔK(-48), yielding pUMiGM3′-N48.

3.2.2.5 pUMiGM3′Δ24 and pUMiGM3′Δ33. In pUMiGM3′Δ24, a 24-bp fragment in the *mnp1* promoter residing between positions 48 bp upstream of the *Kpn*I site and 25 bp upstream of the *Kpn*I site was deleted (Fig. 3.8). This deletion was constructed using the Transformer site-directed mutagenesis kit (Clontech). Two oligonucleotide primers, a mutagenic primer and a selection primer, were synthesized.

The mutagenic primer contained the desired deletion, and the selection primer was designed to mutate a unique *Sph*I site (located at the 5′ end of *mnp1* promoter) into a *Nhe*I site. Following the kit's instruction, the two primers were used in synthesizing a mutated DNA strand of pUMiGM3′. The final plasmid, pUMiGM3′Δ24, was recovered after transformation of *E. coli*. In pUMiGM3′Δ33, a 33-bp fragment immediately upstream of the *Kpn*I site in the *mnp1* promoter was deleted (Fig. 3.8). This deletion was constructed by PCR. Two primers, a forward primer hybridizing with the *mnp1* promoter sequence 1.0 kb upstream of the translation start codon, as well as a reverse primer that contained a new *Kpn*I site at its 5′ end and hybridized with the *mnp1* promoter sequence 33 bp upstream of the native *Kpn*I site, were synthesized. Using pUMiGM3′ as the template, a 0.5-kb fragment was amplified. This fragment was digested at the native *Eag*I site and at the introduced *Kpn*I site, and used to replace the *Eag*I–*Kpn*I fragment in pUMiGM3′, yielding pUMiGM3′Δ33.

3.2.3 Fungal Transformation

Protoplasts (2×10^6) prepared from basidiospores of *P. chrysosporium ura11* strain [Akileswaran et al., 1993] and ~ 1 μg of each plasmid described above, which was linearized at the unique *Eco*RI site, were used for fungal transformation as described previously [Alic et al., 1989, 1990]. One to three hundred Ura^+ transformant colonies were obtained from each transformation. Among these colonies, 40 were isolated and examined for their ability to grow on minimal medium without uracil. They were subsequently purified by fruiting and basidiospore isolation as described previously [Alic et al., 1989, 1990].

3.2.4 Culture Conditions

All the transformants were grown initially from conidial inocula at 37°C in stationary cultures containing 20-ml of high-carbon, low-nitrogen (HCLN) (2% glucose, 1.2 mM ammonium tartrate, and Kirk's salt) [Kirk et al., 1978] media supplemented with 180 μM $MnSO_4$ in 250-ml Erlenmeyer flasks. Cultures were incubated under air for 4 days and then purged once with 100% O_2. The 7-day-old

cultures were harvested and the intracellular GFP fluorescence was determined [Ma et al., 2001]. The transformants that exhibited significant GFP fluorescence were subsequently grown in HCLN media without Mn or supplemented with 180 μM $MnSO_4$, and high-carbon, high-nitrogen (HCHN) (2% glucose, 12 mM ammonium tartrate, and Kirk's salt) media supplemented with 180 μM $MnSO_4$ under the same condition described above.

3.2.5 Intracellular GFP Determination

Approximately 100 mg dry mycelia were homogenized with 1 g glass beads in a minibead beater (Biospec) in 1.5 ml buffer [10 mM Tris-Cl (pH 8.0), 10 mM EDTA, and 0.002% NaN_3]. The mixture was centrifuged at 10,000 $\times$ *g* at 4°C for 30 min. 1 ml of the supernatant was used to determine the GFP fluorescence. The total protein concentration of the cell-free extract was determined by the bicinchoninic acid method (Pierce) [Smith et al., 1985]. The fluorescence assay was conducted on an SLM Aminco 8000C spectrofluorometer with an excitation wavelength at 488 nm and an emission wavelength at 510 nm [Ma et al., 2001]. 10 μg of purified recombinant GFP (Clontech) was used to calibrate the spectrofluorometer at an arbitrary reading of 1,000 fluorescence units.

3.2.6 RNA Extraction and Northern Blotting

Total RNA of *P. chrysosporium* transformants was extracted from 5-day-old HCLN cultures with or without 180 μM $MnSO_4$ as described previously [Brown et al., 1990]. After spectrophotometric quantitation at 260 nm, the total RNA (20 μg per lane) was denatured and electrophoresed in a denaturing (0.6 M formaldehyde, 1% agarose) gel. Northern blotting was performed as described previously [Brown et al., 1990; Li et al., 1994]. The coding region of *egfp*, cDNA of *P. chrysosporium mnp1* [Pribnow et al., 1989], and the *gpd* gene were used as templates for synthesizing [α-^{32}P] dCTP-labeled probes using a Multiprime DNA labeling kit (Amersham). Probed RNA blots were washed and exposed to XAR-5 X-ray film (Kodak).

3.2.7 Southern Blotting and Genomic PCR

Genomic DNA from various transformants was extracted as described previously [Alic et al., 1990]. For Southern blot analysis, 1 μg of genomic DNA from each transformant was digested with restriction enzyme *Sac*I, resolved by gel electrophoresis and transferred to nylon membranes. *egfp* coding region was used as the template for synthesizing the randomly primed and [α-^{32}P] dCTP-labeled probe. Southern hybridization and autoradiography were performed as described previously [Alic et al., 1990]. For genomic PCR, 100 ng of genomic DNA from each transformant was used as the template. The forward and the reverse primer corresponded to the *mnp1* promoter sequence 1.4 kb upstream of the translation start codon and to the *egfp* coding sequence 40 bp downstream of the start codon, respectively. The PCR products were gel-purified using the gel-extraction kit (Qiagen) and their sequences were determined by the Sanger dideoxy-mediated chain-termination method.

3.3 RESULTS

3.3.1 Construction of Expression Plasmids and Characterization of Transformants

Fig. 3.1 shows a restriction map of pUMiGM3′, which contains an *egfp* expression cassette, including the 1,528-bp *mnp1* promoter followed by the intron-containing *egfp* and the 250-bp *mnp*1 3′ UTR, subcloned into pUB [Ma et al., 2001]. The *Schizophyllum commune ura1* gene is carried by this plasmid and used as a selectable marker for fungal transformation. Various mutations were introduced in the *mnp1* promoter of pUMiGM3′. Each derivative of pUMiGM3′ was sequenced to confirm that only the desired mutation was present. Transformation of 1 μg of pUMiGM3′ or each of its derivatives into *P. chrysosporium ura11* strain resulted in over 100 prototrophic transformants, among which 40 transformants were isolated. GFP expression in the isolated transformants were initially screened by growing them at 37°C in nitrogen-limited (HCLN) stationary cultures supplemented with 180 μM

$MnSO_4$ and subsequently measuring the fluorescence intensity of the cell-free extract from 7-day-old cultures. The background fluorescence was determined from transformants carrying the blank vector (pUB). Only those transformants that carried pUMiGM3′ or its derivatives and exhibited over three times the background level of fluorescence were considered to express GFP. In addition, the transformation of each plasmid and the characterization of resulting transformants were accomplished at least twice. After transformation of each plasmid constructed in this study (except pUMiGM3′-A, see below), approximately 20% of the isolated transformants showed significant expression of GFP. The levels of GFP fluorescence varied among these transformants. Five to eight transformants, which carried each plasmid construct and showed the highest fluorescent intensity, were further purified by fruiting and isolating single basidiospores. Subsequently their GFP fluorescence levels were examined in HCLN cultures supplemented with 180 μM $MnSO_4$ or without Mn. No transformant in this study exhibited any GFP expression when grown in nitrogen-sufficient (HCHN) cultures.

3.3.2 GFP Expression in pUMiGM3′-A, pUMiGM3′-K, and pUMiGM3′-E

Long-range deletions were constructed from the 5′ end of the 1,528-bp *mnp1* promoter in pUMiGM3′ to yield pUMiGM3′-A, pUMiGM3′-K and pUMiGM3′-E, in which 80 bp, 521 bp, and 774 bp of the *mnp1* promoter (immediately downstream of a *Avr*II, *Kpn*I, and *Eag*I site, respectively) were used to direct *egfp* expression (Fig. 3.2). All transformants carrying pUMiGM3′-A exhibited only background fluorescence under all culture conditions, suggesting that the 80-bp *mnp1* promoter fragment in this plasmid is unable to drive *egfp* expression. In 7-day-old HCLN cultures containing 180 μM $MnSO_4$, the level of GFP fluorescence exhibited in transformants carrying pUMiGM3′-E or pUMiGM3′-K were lower than that in transformants carrying pUMiGM3′. As shown in Fig. 3.2, the GFP fluorescence intensity from the two most fluorescent transformants carrying pUMiGM3′ (T-5 and T-11) is 2.5 to 4 times stronger than that from the two most fluorescent transformants carrying pUMiGM3′-E (T-11 and T-34) or pUMiGM3′-K (T-7 and T-31) in HCLN cultures supplemented with 180 μM Mn^{2+}. This result suggests that the deletions in

mnp1 promoter constructed in pUMiGM3′-K and pUMiGM3′-E may affect the overall expression of GFP. Furthermore, transformants carrying pUMiGM3′ exhibited significant GFP fluorescence only in HCLN cultures supplemented with Mn^{2+}, but not in cultures without exogenous Mn [Ma et al., 2001]. This expression profile was observed in transformants carrying pUMiGM3′-E. However, transformants carrying pUMiGM3′-K exhibited GFP expression in HCLN cultures both in the absence and in the presence of Mn^{2+}. As shown in Fig. 3.2, transformants T-5, T-11 (pUMiGM3′) and transformants T-7, T-34 (pUMiGM3′-E) exhibited very low levels of fluorescence when grown in HCLN media without exogenous Mn^{2+}, but the levels of GFP fluorescence shown by transformants T-11 and T-34 (pUMiGM3′-K) in HCLN cultures without Mn is comparable to those in HCLN cultures supplemented with 180 μM Mn^{2+}. These data suggests that the deleted *mnp1* promoter in pUMiGM3′-K may contain the sequence responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity and this sequence may reside between the *Eag*I and the *Kpn*I site.

3.3.3 GFP Expression in pUMiGM3′-ΔEK, pUMiGM3′ΔE197, pUMiGM3′ΔK(-48), and pUMiGM3′Δ48A

The 253-bp *mnp1* promoter fragment in pUMiGM3′ between the *Eag*I and the *Kpn*I site was deleted, yielding pUMiGM3′-ΔEK (Fig. 3.3A). The levels of GFP fluorescence shown in transformants carrying pUMiGM3′-ΔEK were similar to those shown in transformants carrying pUMiGM3′ in HCLN cultures supplemented with 180 μM Mn^{2+}. However, unlike transformants carrying pUMiGM3′, transformants carrying pUMiGM3′-ΔEK also exhibited significant levels of GFP fluorescence in HCLN cultures without exogenous Mn. The ratio of GFP fluorescence intensity in cultures supplemented with 180 μM Mn^{2+} versus in cultures without Mn, [GFP(+Mn)/GFP(-Mn)], was over 40 in transformants carrying pUMiGM3′ (Table 3.1). In contrast, this ratio ranged from 1.6 to 2.5 in transformants carrying pUMiGM3′-ΔEK (Table 3.1). Fig. 3.3C shows the fluorescence intensity of three most fluorescent transformants carrying pUMiGM3′ (T-3, T-5 and T-11) and pUMiGM3′-ΔEK (T-3, T-6 and T-12) in 7-day-old HCLN cultures both without Mn

Table 3.1

[GFP(+Mn)/GFP(-Mn)] from Transformants Carrying pUMiGM3′ and Its Mutant Derivatives

Plasmid	No. Transformants	[GFP(+Mn)/ GFP(-Mn)]	Average [GFP(+Mn)/ GFP(-Mn)]
pUMiGM3′	6	≥ 40	ND
pUMiGM3′-ΔEK	5	1.6 - 2.5	2.1 ± 0.4
pUMiGM3′ΔE197	6	≥ 24	ND
pUMiGM3′ΔK(-48)	8	1.5 - 2.7	1.9 ± 0.5
pUMiGM3′Δ48A	8	1.5 - 3.0	2.1 ± 0.7
pUMiGM3′-N48	6	12 - 34	21 ± 8
pUMiGM3′Δ24	6	≥ 15	ND
pUMiGM3′Δ33	6	1.6 - 7.0	3.4 ± 2.5

[GFP(+Mn)/GFP(-Mn)] is the ratio of GFP fluorescence intensity exhibited by transformant carrying pUMiGM3′ or its mutant derivative in 7-day-old HCLN culture supplemented with 180 μM Mn^{2+} versus in culture without exogenous Mn^{2+}. Each transformant was grown in triplicate Mn^{2+}-supplemented (+Mn) and Mn^{2+}-absent (-Mn) cultures. The average fluorescence intensity per mg of total protein exhibited in each transformant under both (+Mn) and (-Mn) conditions was modified by subtracting the background fluorescence intensity per mg total protein exhibited in transformants carrying only the blank vector, pUB. The modified values were used to calculate [GFP(+Mn)/GFP(-Mn)] ratio. ND, not determined.

and supplemented with 180 μM $MnSO_4$. The fluorescence intensity of T-3, T-6, and T-12 (pUMiGM3′-ΔEK) in the absence of Mn^{2+} was approximately 50% of that in the presence of Mn^{2+}. This data suggests that the fragment between the *Eag*I and *Kpn*I site in *mnp1* promoter may contain an element that participate in Mn^{2+}-dependent regulation of *mnp1* promoter activity.

In order to further analyze the *Eag*I–*Kpn*I region, the following mutations were constructed and examined. pUMiGM3′ΔE197 contains the deletion of a 197-bp fragment immediately downstream of the *Eag*I site in the *mnp1* promoter (Fig. 3.3A). The GFP expression profile in transformants carrying pUMiGM3′ΔE197 was similar to that in transformants carrying pUMiGM3′. The [GFP(+Mn)/GFP(–Mn)] ratio was more than 24 in transformants carrying pUMiGM3′ΔE197 (Table 3.1). As shown in Fig. 3.3C, the three most fluorescent transformants carrying either pUMiGM3′ (T-3, T-5 and T-11) or pUMiGM3′ΔE197 (T-12, T-19 and T-31) exhibited similar levels of GFP fluorescence in HCLN cultures supplemented with 180 μM Mn^{2+}; however, they showed very little or no GFP fluorescence in HCLN cultures without Mn. Furthermore, deletions of a 150-bp and a 100-bp fragment immediately downstream of the *Eag*I site in the *mnp1* promoter were also constructed. Transformants carrying the plasmids that contain these deletions exhibited a similar GFP expression profile as those carrying pUMiGM3′ or pUMiGM3′ΔE197 [data not shown].

pUMiGM3′ΔK(-48) contains deletion of a 48-bp fragment immediately upstream of the *Kpn*I site in the *mnp*1 promoter (Fig. 3.3A). The deletions constructed in pUMiGM3′ΔE197 and in pUMiGM3′ΔK(-48) together encompass the entire *Eag*I–*Kpn*I region. Transformants carrying pUMiGM3′ΔK(-48) exhibited a similar GFP expression profile as those carrying pUMiGM3′-ΔEK. The [GFP(+Mn)/GFP(–Mn)] ratio ranged from 1.5 to 2.7 in transformants carrying pUMiGM3′ΔK(-48) (Table 3.1). Moreover, pUMiGM3′Δ48A was constructed, which contains a 48-bp DNA with sequence of the 5′ end of the *mnp1* exon 6 replacing the original 48-bp fragment that was deleted in pUMiGM3′ΔK(-48) (Fig. 3.3A and B). Transformants carrying pUMiGM3′Δ48A exhibited a similar GFP expression profile as those carrying pUMiGM3′-ΔEK or pUMiGM3′ΔK(-48). The

[GFP(+Mn)/GFP(–Mn)] ratio ranged from 1.5 to 3.0 in transformants carrying pUMiGM3′Δ48A (Table 3.1). As shown in Fig. 3.3C, in HCLN cultures without exogenous Mn, the three most fluorescent transformants carrying pUMiGM3′ΔK(-48) (T-12, T-19 and T-29) and the three carrying pUMiGM3′Δ48A (T-2, T-17 and T-36) exhibited approximately 40–60% of the GFP fluorescence intensity observed in HCLN cultures supplemented with 180 μM Mn^{2+}. These data suggest that the 48-bp DNA sequence immediately upstream of the *Kpn*I site in the *mnp1* promoter is responsible for the gene regulation that is responsive to Mn^{2+}-concentration.

3.3.4 Northern Blot Analysis of Transformants Carrying pUMiGM3′, pUMiGM3′-ΔEK, pUMiGM3′ΔE197, pUMiGM3′ΔK(-48), and pUMiGM3′Δ48A

Total RNA was extracted from 5-day-old HCLN cultures without exogenous Mn or supplemented with 180 μM Mn^{2+} of all the transformants specified in Fig. 3.4. The RNA was resolved on a denaturing agarose gel, transferred to a nylon membrane and analyzed separately using ^{32}P-labeled *egfp*, *mnp1* and *gpd* probes. As shown in Fig. 3.4A, when probed with the *egfp* coding region, significant amounts of *egfp* transcript were observed in transformants carrying pUMiGM3′-ΔEK, pUMiGM3′ΔK(-48) and pUMiGM3′Δ48A both in the absence and in the presence of Mn^{2+}. In contrast, they were observed only in HCLN cultures supplemented with 180 μM Mn^{2+}, but not without Mn, in transformants carrying pUMiGM3′ and pUMiGM3′ΔE197. The levels of *egfp* transcript in all the specified transformants correlated with their GFP fluorescence levels as shown in Fig. 3.3C. These data suggest that the deletion of the 253-bp *Eag*I–*Kpn*I region, the deletion of the 48-bp fragment upstream of the *Kpn*I site and the replacement of the 48-bp fragment in the *mnp1* promoter affect the transcription of *egfp* directed by this promoter in response to Mn^{2+}-status. These data again suggest that the 48-bp sequence immediately upstream of the *Kpn*I site in the *mnp1* promoter is responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity. The same RNA blot was probed with ^{32}P-labeled *mnp1* cDNA, which hybridized with the transcripts of *mnp1* and other *mnp*

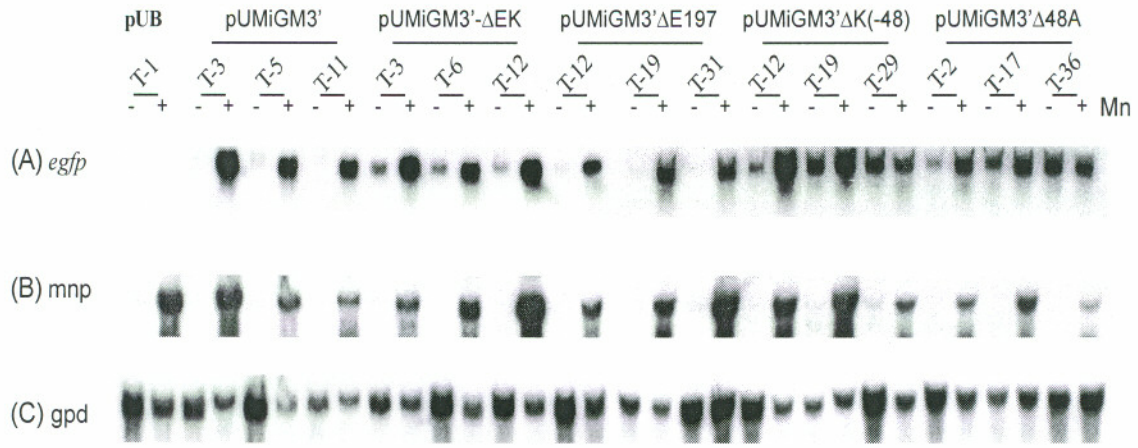


Fig. 3.4 Northern blot analysis of the *egfp* transcript levels in transformants carrying pUMiGM3′ΔEK, pUMiGM3′ΔE197, pUMiGM3′ΔK(-48), and pUMiGM3′Δ48A. Total RNA was extracted from 5-day-old HCLN culture without exogenous Mn (–Mn) and supplemented with 180 mM Mn^{2+} (+Mn) of single transformant carrying specified plasmid. The RNA blot was probed with the ^{32}P-labeled *egfp* coding region DNA (**A**), the *mnp1* cDNA (**B**), and the *gpd* gene (**C**).

genes. As shown in Fig. 3.4B, in all the specified transformants, significant amounts of *mnp* transcript were observed only in HCLN cultures supplemented with 180 μM Mn^{2+}. This result indicates that the transformation of *P. chrysosporium* with various *egfp* expression plasmids that contain mutations constructed in the *mnp1* promoter does not affect the activation of endogenous *mnp* gene transcription by Mn^{2+}-supplementation. Fig. 3.4C shows the presence of *gpd* transcripts in all specified transformants both in the presence and in the absence of Mn^{2+}, as a control to show the quality of the RNA samples.

Southern blot analysis was performed on the genomic DNA extracted from all the transformants shown in Fig. 3.3 and Fig. 3.4. All these transformants contained the *egfp* expressing plasmids that had integrated into the chromosomes [data not shown]. Fig. 3.5 shows the Southern blot result of the genomic DNA from various transformants shown in Fig. 3.3 and Fig. 3.4 digested with *Sac*I and hybridized with ^{32}P-labeled *egfp* coding region. *Sac*I linearizes pUMiGM3′ and all its derivatives constructed in this study in the *mnp1* promoter region. As shown, all transformants carry one or more copies of *egfp*-expressing plasmids and no significant difference in the *egfp* copy numbers is observed in the genomic DNA of these transformants.

Genomic PCR was performed on each transformant shown in Fig. 3.3 and Fig. 3.4 to amplify a fragment that spans from 1.4 kb upstream of the translation start codon in the *mnp1* promoter region to 40 bp downstream of the start codon in the *egfp* coding region. The PCR products were resolved on an agarose gel and visualized after ethidium bromide staining of the gel (Fig. 3.6). The specifically amplified fragments from each transformant DNA all migrated to expected positions (Fig. 3.6, as indicated by the arrow). Two non-specific fragments were also amplified, and they were present even when the genomic DNA from a transformant carrying only the blank vector (pUB) was used as the template (Fig. 3.6). Sequencing results of the gel-purified PCR fragments showed the accuracy of the constructed mutations in the *mnp1* promoter region of the integrated plasmids in these transformants [data not shown].

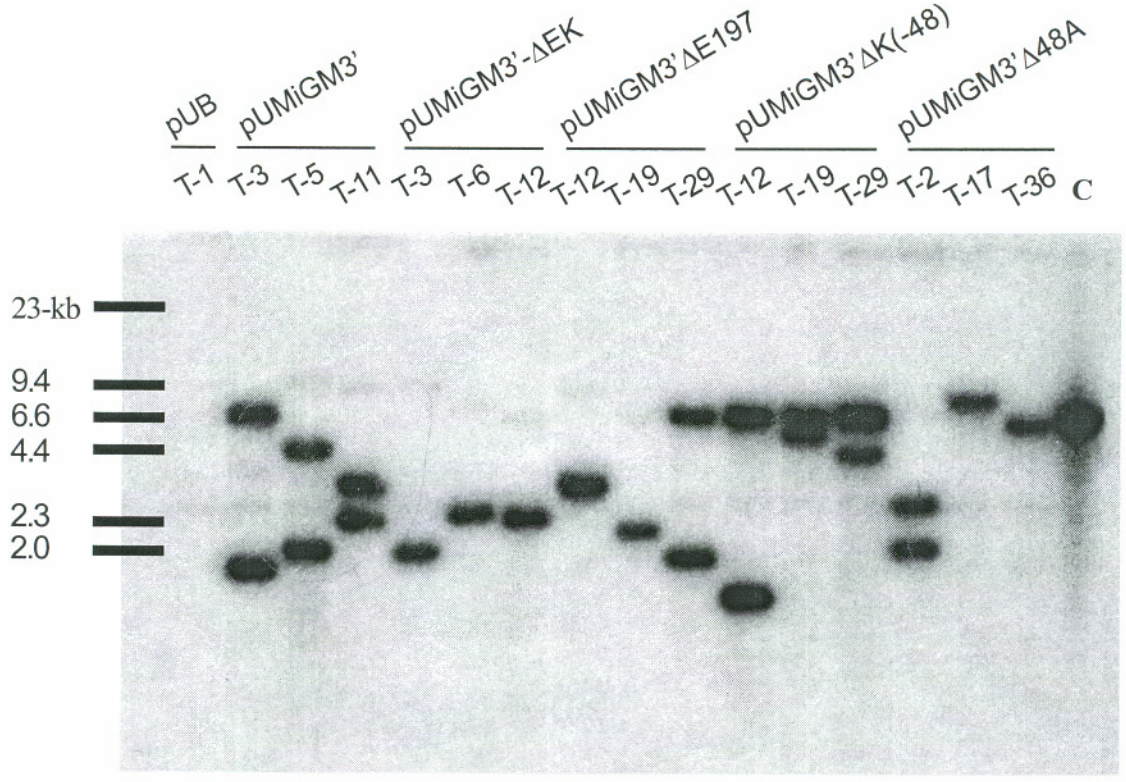


Fig. 3.5 Southern blot analysis of genomic DNA from transformants carrying pUMiGM3′ΔEK, pUMiGM3′ΔE197, pUMiGM3′ΔK(-48), and pUMiGM3′Δ48A. 1 mg of genomic DNA extracted from each specified transformant was digested with *Sac*I, resolved on a 0.8% agarose gel, transferred to a nylon membrane, and probed with the ^{32}P-labeled *egfp* coding region DNA. C (control), 100 pg of *Sac*I-linearized pUMiGM3′. The DNA molecular weight markers are indicated on the left.

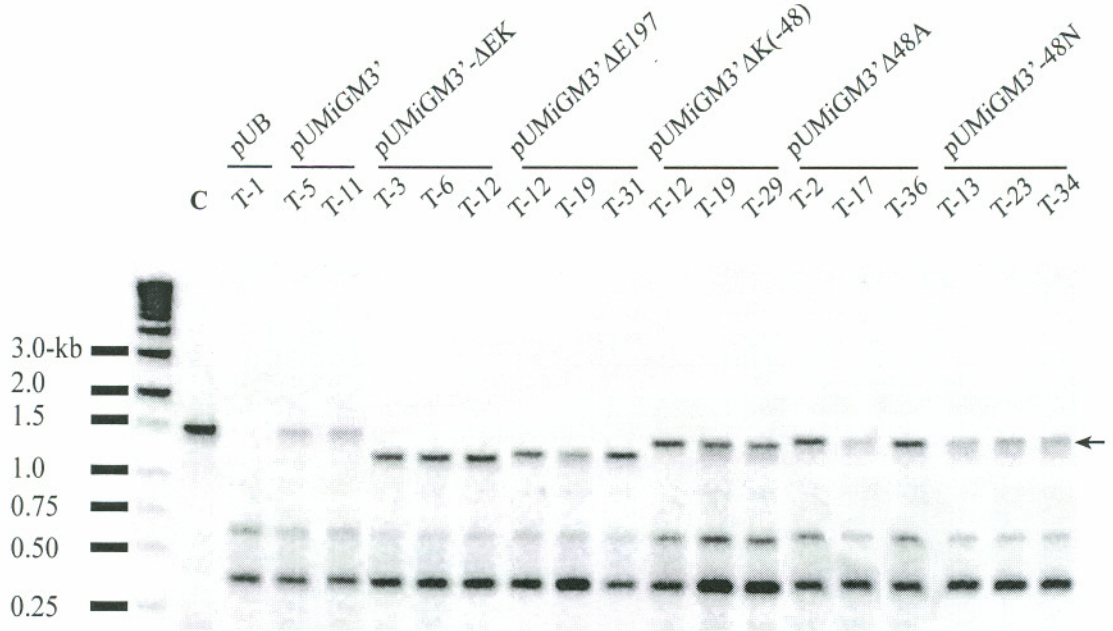


Fig. 3.6 Genomic PCR products of transformants carrying pUMiGM3′ΔEK, pUMiGM3′ΔE197, pUMiGM3′ΔK(-48), pUMiGM3′Δ48A, and pUMiGM3′-48N. A fragment (indicated by the arrow on the right) that spans from 1.4 kb upstream of the translation start codon in the *mnp1* promoter region to 40 bp downstream of the start codon in the *egfp* coding region were amplified, using 100 ng of genomic DNA extracted from each specified transformant as the template. The PCR products were resolved on a 1.0% agarose gel and visualized after ethidium bromide staining. The lengths of the amplified fragments in individual transformants as estimated from their mobility agree with their predicted lengths. C (control), PCR product using 50 ng of pUMiGM3′ as the template. The DNA molecular weight markers are indicated on the left.

3.3.5 GFP Expression in pUMiGM3′-48N

The 48-bp DNA immediately upstream of the *Kpn*I site in the *mnp1* promoter was deleted at its original location and inserted into a unique *Nsi*I site that resides 120 bp downstream of the *Kpn*I site, yielding pUMiGM3′-48N (Fig. 3.7). Significant GFP fluorescence was observed in transformants carrying pUMiGM3′-48N when grown in HCLN media supplemented with 180 μM Mn^{2+}. However, only low levels of fluorescence were observed in these transformants when grown in HCLN media without exogenous Mn. The [GFP(+Mn)/GFP(–Mn)] ratio ranged from 12 to 34 in transformants carrying pUMiGM3′-48N (Table 3.1). As shown in Fig. 3.7, the three most fluorescent transformants carrying pUMiGM3′-48N (T-13, T-23 and T-34) and the three carrying pUMiGM3′ (T-3, T-5 and T-11) exhibited similar GFP expression profiles in HCLN cultures without Mn and supplemented with 180 μM Mn^{2+}. This expression profile was different from that shown in transformants carrying pUMiGM3′ΔK(-48). Genomic PCR was performed on transformants carrying pUMiGM3′-48N as described above (Fig. 3.6) and the sequencing result of the PCR products confirmed the location change of the 48-bp fragment in the *mnp1* promoter [data not shown]. These data suggest that the 48-bp sequence itself contributes to Mn^{2+}-dependent regulation of *mnp1* promoter activity.

3.3.6 GFP Expression in pUMiGM3′Δ24 and pUMiGM3′Δ33

Two plasmids, pUMiGM3′Δ24 and pUMiGM3′Δ33, containing the internal deletions in the 48-bp DNA fragment immediately upstream of the *Kpn*I site in the *mnp1* promoter, were constructed. pUMiGM3′Δ24 contains deletion of the 24-bp DNA, which resides between positions that are 48 bp and 25 bp upstream of the *Kpn*I site, while pUMiGM3′Δ33 contains a deletion of the 33-bp DNA immediately upstream of the *Kpn*I site (Fig. 3.8). In HCLN cultures supplemented with 180 μM Mn^{2+}, the transformants carrying either pUMiGM3′Δ24 or pUMiGM3′Δ33 exhibited significant GFP fluorescence. In HCLN cultures without exogenous Mn, significant GFP fluorescence was only observed in transformants carrying pUMiGM3′Δ33, but not in those carrying pUMiGM3′Δ24. The [GFP(+Mn)/GFP(–Mn)] ratio ranged

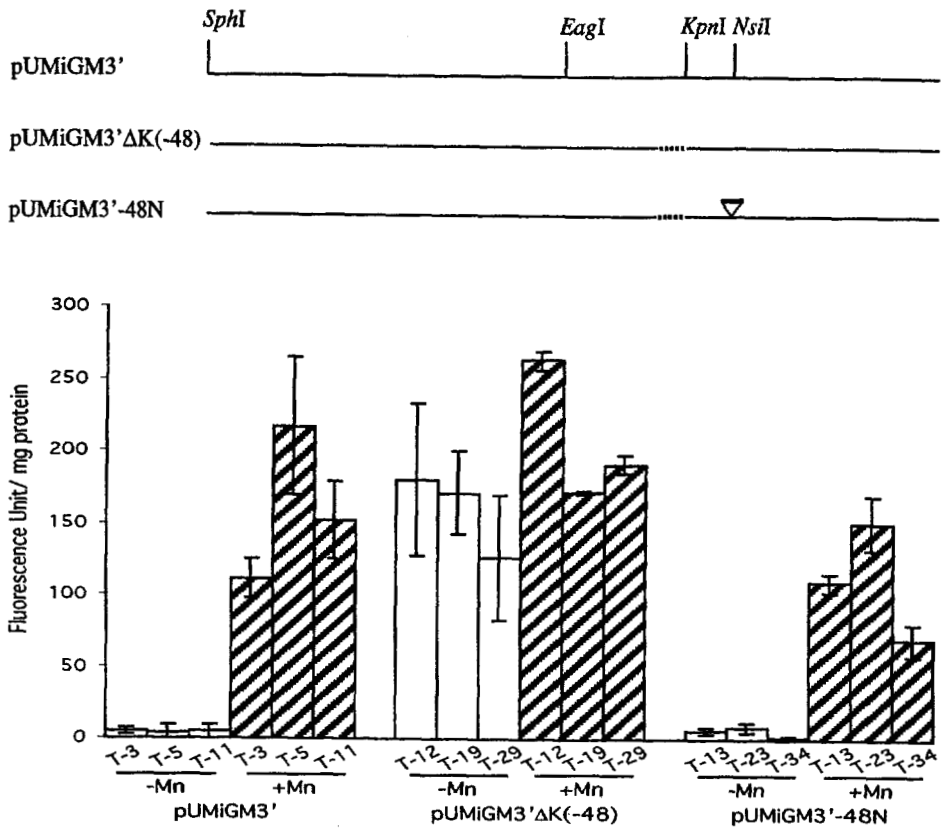


Fig. 3.7 GFP expression in transformants carrying pUMiGM3′ΔK(-48) and pUMiGM3′-48N. The upper panel shows the modified *mnp1* promoter regions (deleted sequence, dashed line; inserted sequence, triangle) in pUMiGM3′ΔK(-48) and pUMiGM3′-48N. The lower panel shows the fluorescence intensity of the three most fluorescent transformants carrying each specified plasmid. Data were calculated as described in Fig. 3.2.

```
pUMiGM3'        ...tggtCTGAGGAACTGTGCTCCCTT
                   GCGTTGGGCTTGGCGTTGGGTCCA
                   CGGTaccg...

pUMiGM3'Δ24     ...tggA********************
                   TCCTTGGGCTTGGCGTTGGGTCCA
                   CGGTaccg...

pUMiGM3'Δ33     ...tggtCTGAGGAACTGTGGT*****
                   ************************
                   ****accg...
```

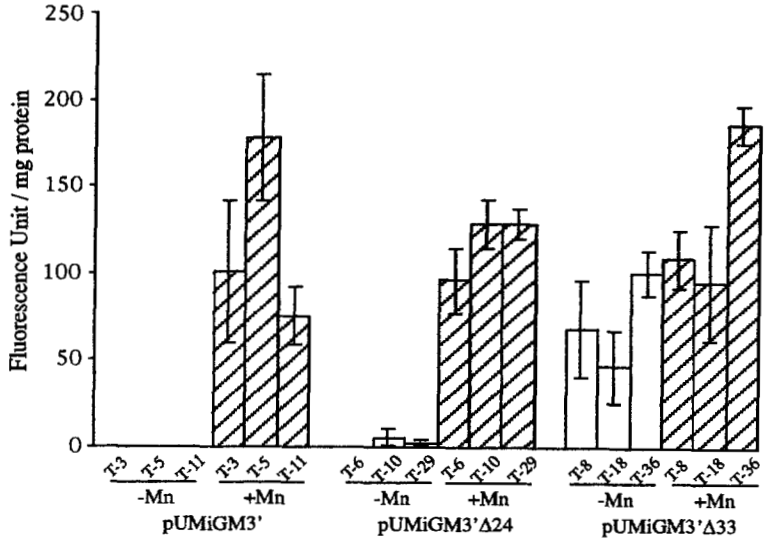


Fig. 3.8 GFP expression in transformants carrying pUMiGM3′Δ24 and pUMiGM3′Δ33. The upper panel shows the DNA sequence of the 48-bp fragment (in capital letters) immediately upstream of the *Kpn*I site in the *mnp1* promoter and its mutated derivatives in pUMiGM3′Δ24 and pUMiGM3′Δ33. The stars represent the deleted nucleotides. The putative AP-2 binding site is shown in bold. The nucleotides that were mutated are shown in italics and underlined. The lower panel shows the fluorescence intensity of the three most fluorescent transformants carrying each specified plasmid. Data were calculated as described in Fig. 3.2.

from 1.6 to 7 in transformants carrying pUMiGM3′Δ33, whereas the ratio was more than 15 in transformants carrying pUMiGM3′Δ24 (Table 3.1). The levels of GFP fluorescence exhibited by the three most fluorescent transformants carrying pUMiGM3′Δ24 (T-6, T-10 and T-29) and the three most fluorescent transformants carrying pUMiGM3′Δ33 (T-8, T-18 and T-36) in HCLN cultures supplemented with 180 μM Mn^{2+} and without Mn are shown in Fig. 3.8. These results suggest that the 33-bp fragment immediately upstream of the *Kpn*I site in the *mnp1* promoter plays an important role in Mn^{2+}-dependent regulation of *mnp1* promoter activity.

3.4 DISCUSSION

The transcription of *P. chrysosporium mnp* genes is activated by nitrogen-depletion and Mn^{2+}-supplementation [Pribnow et al., 1989; Brown et al., 1990, 1991; Gettemy et al., 1997, 1998]. Sequence analysis has revealed that both *P. chrysosporium mnp1* and *mnp2* genes [Lundquist and Kristersson, 1985; Godfrey et al., 1990; Gold and Alic, 1993; Mayfield et al., 1994a] contain short promoter sequences matching the consensus sequence of *MRE*s (metal regulatory elements), which were defined in mammalian metallothionein (MT) genes [Culotta and Hamer, 1989; Faist and Meyer, 1992]. *MT*s are small, cysteine-rich proteins that bind and store transition metal ions such as Cd^{2+}, Zn^{2+} and Cu^{+} in mammalian cells [Palmiter, 1998]. The activation of *MT* genes transcription by transition metals depends on the presence of *MRE*s in their promoters, and on the binding capacity of MRE-binding transcription factor-1 (MTF-1) to these *MRE*s in the presence of Zn [Thiele, 1992; Radtke et al., 1993; Heuchel et al., 1994]. Six putative *MRE*s were found in sequenced *mnp*1 promoter and among them, four *MRE*s located near the translation start codon are arranged into two pairs with 4 bp palindromes [Gold and Alic, 1993; Gold et al., 2000]. These two pairs are designated *MRE*d (with sequence GCGTGCACGC) and *MRE*p (with sequence GTGTGCACGC), which reside at 315 bp and 91 bp upstream of the start codon, respectively (Figs. 1.5 and 1.6). Point mutations (changing the center four bases TGCA to TTAA) were constructed in either

*MRE*d or *MRE*p, or in both *MRE* pairs within *mnp1* promoter in pUMiGM3′. In addition, insertion mutations were constructed by inserting 10-bp linkers (with sequence GCTGATCACG) into the center of either *MRE*p or *MRE*d, or into both MRE pairs. Transformants carrying the *egfp* expression plasmids that contain the above mutations showed the regulation of GFP expression by Mn^{2+}-supplementation and nitrogen limitation in the same manner as transformants carrying pUMiGM3′ [unpublished data]. These data suggest that promoter sequences other than the putative *MRE*s are responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity. Recently, it was reported that in the basidiomycete *Trametes versicolor*, addition of 200 μM Mn^{2+} to the Mn-deficient cultures resulted in an 8-fold and a 250-fold increase of *mnp1* and *mnp2* transcript concentrations, respectively [Johansson et al., 2002]. In this fungus, putative *MRE* was only found in *mnp1* gene, but not in *mnp2* gene. This also suggests that in *T. versicolor*, the Mn^{2+}-dependent regulation of *mnp* expression may not require *MRE*.

In this chapter, I describe the characterization of the *mnp1* promoter by introducing deletion, replacement and translocation mutations within its sequence and examining GFP expression directed by the mutated *mnp1* promoter under both Mn^{2+}-sufficient and Mn^{2+}-deficient conditions. The 1,528-bp fragment containing *mnp1* promoter in pUMiGM3′ is sufficient for promoting GFP expression, but only under Mn^{2+}-sufficient and nitrogen-limited condition, which is similar to that required for endogenous MnP production. However, deletion of a 253-bp *Eag*I–*Kpn*I fragment in the *mnp1* promoter region of pUMiGM3′ resulted in the accumulation of GFP transcripts and the significant expression of GFP even under Mn-deficient condition. The same phenotype was observed from deletion of the 48-bp DNA fragment immediately upstream of the *Kpn*I site, but not the remaining fragment within the *Eag*I–*Kpn*I region. This suggests that the 48-bp DNA is responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity. Since the sequence deletions may cause a DNA configuration change that might affect promoter activity, we constructed a replacement mutation of the 48-bp DNA and maintained the length of *mnp1* promoter. This mutation also resulted in a GFP expression profile in response to

Mn^{2+}-status that is similar to that observed from deletion of the entire *Eag*I-*Kpn*I fragment and of the 48-bp fragment. In addition, the 48-bp DNA was moved 120 bp downstream from its original location and the *mnp1* promoter containing this mutation could efficiently promote GFP expression under the Mn^{2+}-sufficient condition, but not under the Mn^{2+}-deficient condition. These data again suggest that the nucleotide sequence of the 48-bp DNA is responsible for Mn^{2+}-dependent regulation of *mnp1* promoter activity. Since the deletion and the replacement of the 48-bp DNA resulted in a partial de-repression of GFP expression under Mn^{2+}-deficient condition, it can be proposed that negative control is exerted at a site within the 48-bp DNA and this control is relieved upon Mn^{2+}-supplementation. Negative control mechanism has also been found in the regulation of genes involving in siderophore biosynthesis and siderophore-mediated iron-uptake in response to iron-status in *Aspergillus nidulans* [Haas et al., 1999], *Neurospora crassa* [Zhou et al., 1998], *Ustilago maydis* [Voisard et al., 1993], and *Penicillium chrysogenum* [Haas et al., 1999]. Moreover, the GFP fluorescence intensity exhibited by transformants containing the deletion of the *Eag*I-*Kpn*I fragment, the deletion of the 48-bp DNA and the replacement of the 48-bp DNA under Mn^{2+}-deficient condition were approximately 40–60% of those observed under Mn^{2+}-sufficient condition (Fig. 3.3). This suggests there may be other regulatory element(s) in *mnp1* promoter besides that residing in the 48-bp DNA fragment functioning in Mn^{2+}-dependent regulation of *mnp1* promoter activity.

Two internal deletions within the 48-bp DNA fragment were constructed and their effects on *mnp1* promoter activity were examined. Deletion of 33 bp residing at its 3′ half resulted in the significant GFP expression under Mn^{2+}-deficient condition, whereas deletion of 24 bp residing at its 5′ half did not result in such an effect. These data suggest that the 33-bp DNA sequence immediately upstream of the *Kpn*I site contain the Mn^{2+}-responsive element. To our knowledge this is the first promoter sequence containing Mn^{2+}-responsive element being characterized in eukaryotes.

In our *P. chrysosporium* transformation experiments, the plasmids that enter the fungus integrate into the chromosomes, predominantly at ectopic sites [Alic et al., 1990, 1993; Akileswaran et al., 1993; Gold and Alic, 1993]. The locations where the

plasmids integrate may affect the expression of genes carried on the plasmid. This may explain why only ~20% of the transformants carrying various *egfp* expression plasmids exhibited significant GFP fluorescence in our study. Among the transformants that showed significant GFP fluorescence, the fluorescence intensity varies under both Mn^{2+}-sufficient and Mn^{2+}-deficient conditions. However, the ratios of GFP fluorescence intensity in 7-day-old HCLN cultures supplemented with 180 μM Mn^{2+} versus without Mn^{2+}, [GFP(+Mn)/GFP(-Mn)], were consistently high or low among transformants carrying the same plasmid (Table 3.1). As shown, the [GFP(+Mn)/GFP(-Mn)] ratios among transformants carrying pUMiGM3′-ΔEK, pUMiGM3′ΔK(-48) pUMiGM3′Δ48A or pUMiGM3′Δ33 were low, and variations of the ratio values were relatively small. In contrast, the [GFP(+Mn)/GFP(-Mn)] ratios among transformants carrying pUMiGM3′, pUMiGM3′ΔE197, pUMiGM3′-48N or pUMiGM3′Δ24 were generally high. In several transformants carrying pUMiGM3′, pUMiGM3′ΔE197 or pUMiGM3′Δ24, the fluorescence intensity exhibited under Mn^{2+}-deficient condition was less than or equal to the background fluorescence intensity showed in transformants carrying the blank vector, pUB. In these cases, the [GFP(+Mn)/GFP(-Mn)] ratio is not calculated. Most importantly, the difference between the [GFP(+Mn)/GFP(-Mn)] ratio calculated for transformants carrying pUMiGM3′, pUMiGM3′ΔE197, pUMiGM3′48N or pUMiGM3′Δ24 and the ratio calculated for transformants carrying pUMiGM3′-ΔEK, pUMiGM3′ΔK(-48) pUMiGM3′Δ48A or pUMiGM3′Δ33 is significant. This marked difference should result from the various mutations constructed in the *mnp1* promoter region of individual *egfp* expression plasmids.

All the mutations constructed within the *Eag*I–*Kpn*I region had no or very little effect on the high transcription activity of *mnp1* promoter under Mn^{2+}-sufficient and nitrogen-limited conditions. However, deletion of the *mnp1* promoter sequence upstream of the *Eag*I or the *Kpn*I site resulted in low levels of GFP expression under such conditions. Deletion of the *mnp1* promoter sequence to a position at 80 bp upstream of the start codon resulted in no GFP expression. These data suggest that promoter elements residing outside of the *Eag*I–*Kpn*I region also contribute to the

modulation of *mnp1* promoter activity. Furthermore, no transformant characterized in this study showed significant GFP fluorescence in nitrogen-sufficient cultures, suggesting the nitrogen-dependent regulation of *mnp1* promoter activity requires elements other than the 48-bp DNA fragment that is responsible for the Mn^{2+}-dependent regulation.

Finally, the 33-bp DNA immediately upstream of the *Kpn*I site in *mnp1* promoter possesses a high (G+C) content (67%). There are two identical repeating sequences (GCGTTGGG) in this fragment that conform to the binding site for AP-2 [Godfrey et al., 1990; Faist and Meyer, 1992; Gold and Alic, 1993] (Fig. 3.3 and 3.8). In vertebrates, AP-2 is a family of cell type-specific and developmentally regulated transcription factors, which are critical regulators of gene expression during development and carcinogenesis [Kannan et al., 1994; Nottoli et al., 1998]. Sequences with high homology to the 33-bp fragment have not been found in the 5′ UTR of *P. chrysosporium mnp2* [Mayfield et al., 1994a] and *mnp3* genes [Alic et al., 1997]. Future experiments will focus on the characterization of nucleotides in the 33-bp fragment that are essential for the regulation of *mnp1* promoter activity in response to Mn^{2+}, and also the characterization of *trans*-acting transcription factor(s) that interact with this element.

CHAPTER 4
BIALAPHOS RESISTANCE AS A DOMINANT SELECTABLE MARKER IN *PHANEROCHAETE CHRYSOSPORIUM*

4.1 INTRODUCTION

The white-rot basidiomycete, *Phanerochaete chrysosporium,* has been the focus of numerous studies on lignin [Kirk and Farrell, 1987; Gold et al., 1989] and aromatic pollutant degradation [Bumpus and Aust, 1987; Hammel, 1989; Reddy et al., 1998]. The major components of the lignin degradation system in this fungus are two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), along with an extracellular H_2O_2-generating system [Kirk and Farrell, 1987; Gold et al., 1989; Gold and Alic, 1993]. Both MnP and LiP have been characterized by a variety of biochemical and kinetic techniques [Gold et al., 1989, 2000; Gold and Alic, 1993]. Crystal structures of these two peroxidases have been reported [Piontek et al., 1993; Poulos et al., 1993; Sundaramoorthy et al., 1994]. The genes encoding various LiPs and MnPs from *P. chrysosporium* have been isolated and sequenced for over the past 15 years [Gold and Alic, 1993; Cullen, 1997]. The regulation of MnP gene expression has also been studied using promoter-reporter systems [Godfrey et al., 1994; Gettemy et al., 1997; Ma et al., 2001].

An efficient DNA transformation system is required for studying both structure-function relationships and gene expression regulation. Two strategies have been employed for selection of transformed *P. chrysosporium* strains. The first method, pioneered in our lab, involves selection of prototrophic transformants after complementation of auxotrophic *P. chrysosporium* strains with wild-type genes. We have reported integrative transformation systems using both heterologous and homologous *ade* [Alic et al., 1989, 1990] and *ura* [Akileswaran et al., 1993] genes as

the selectable markers. The *ade* and *ura* transformation systems have also been employed for homologous production of recombinant *P. chrysosporium* MnP [Mayfield et al., 1994b], LiP [Gelpke et al., 1999], and cellobiose dehydrogenase (CDH) [Li et al., 2000]. A second method for selection of *P. chrysosporium* transformants uses dominant selectable markers, which are genes introduced into wild-type strains and conferring drug-resistance. A bacterial kanamycin resistance determinant of Tn903 has been reported to confer G418 resistance in transformed *P. chrysosporium* [Randall et al., 1989, 1991; Randall and Reddy, 1991, 1992]. Recently, transformation systems, utilizing the phleomycin resistance (Ph^R) gene from *Streptoallotiechus hindustanus* as the selectable marker, have been reported [Gessner and Raeder, 1994; Birch et al., 1998]. In both cases, the transforming plasmids were apparently maintained extrachromosomally. However, the transformation frequency of these systems was too low (6–10 transformants/μg of plasmid DNA) for constructing strains used for efficient expression of recombinant protein and other applications.

Since many *P. chrysosporium* auxotrophic strains grow less robustly than the wild-type strain and reversion of some auxotrophic mutations (e.g., *ura11* that was isolated after mutating wild type strain OGC101 by UV irradiation) is a theoretical possibility, a transformation system using a dominant selectable marker with high transformation frequency is advantageous. This system could also aid in gene disruption experiments in *P. chrysosporium*. In this Chapter, I describe a new, integrative transformation system of *P. chrysosporium* with relatively high transformation frequency, using the Bialaphos-resistance gene (*bar*) from the bacterium *Streptomyces hygroscopicus* as a selectable marker. Bialaphos is a potent, nonselective herbicide produced by *S. hygroscopicus*, which is composed of two L-alanine residues and an analogue of glutamic acid, phosphinothricin (PPT) [Thompson et al., 1987]. PPT, the active component of bialaphos, is a potent inhibitor of glutamine synthetase. The Bialaphos-resistance gene (*bar*) was isolated from *S. hygroscopicus* and encodes an acetyl-transferase that acetylates PPT [Thompson et al., 1987]. The *bar* gene has been introduced into plants including tobacco, tomato and

potato [De Block et al., 1987] as well as fungi including *Neurospora crassa* [Avalos et al., 1989], *Erysiphe graminis* [Chaure et al., 2000], *Cercospora kikuchii* [Upchurch et al., 1994] and *Pleurotus ostreatus* [Yanai et al., 1996], resulting in high level resistance to bialaphos. At last, I report the construction of a relatively efficient homologous expression system for recombinant *P. chrysosporium* MnP isozyme 1 (rMnP1) using the *bar* gene as a selectable marker.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

PPT (also known as glufosinate) was obtained from Sigma-Aldrich Laborchemikalien GmbH, Germany.

4.2.2 Organisms

Homokaryon 8, a homokaryon derivative of *P. chrysosporium* strain OGC101, was isolated by fruiting and subsequently selecting single basidiospore as described previously [Gold and Cheng, 1978; Gold and Cheng, 1979; Alic et al., 1987]. *Escherichia coli* strain DH5α was used as host for plasmid construction and preparation.

4.2.3 Construction of pGBar and pGBar(i)

pCB1635, containing *bar*, was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City. pGBar contains a *bar* expression cassette, which includes, from 5′ to 3′, the 1.1-kb *P. chrysosporium gpd* promoter [Mayfield et al., 1994b], the *bar* coding sequence (CDS) and the 250-bp *P. chrysosporium mnp1* 3′ untranslated region (UTR), subcloned into pUC18 (New England Biolabs) (Fig. 4.1). The *gpd* promoter was joined with the *bar* CDS at the translation start codon using a megaprimer PCR method [Barik, 1997]. In the first PCR for constructing the megaprimer, the template was pGPDstu1.8, which contains 1.8 kb of *gpd* DNA (including the 1.1-kb *gpd* promoter and the 0.8-kb *gpd* CDS)

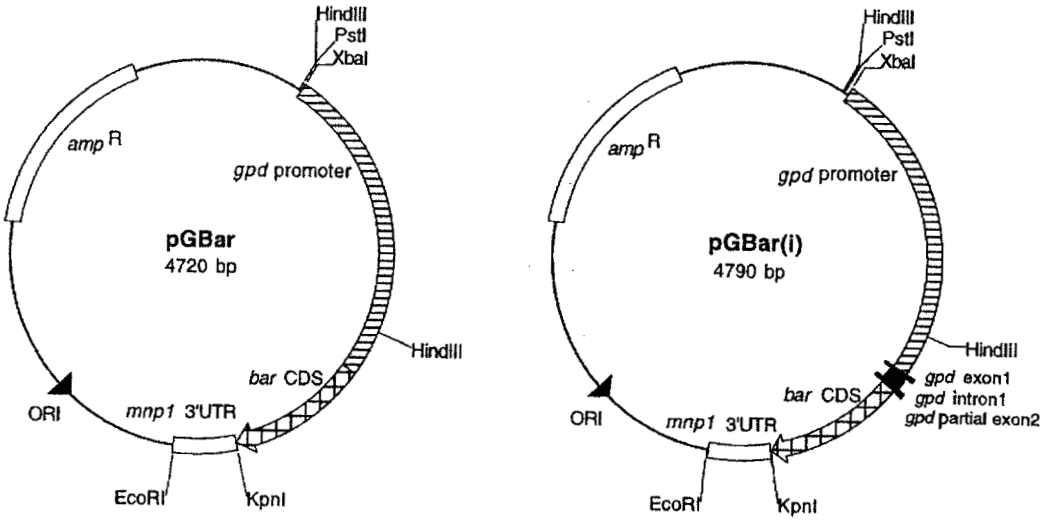


Fig. 4.1 Restriction maps of pGBar and pGBar(i). The relative locations of the *gpd* promoter, the *bar* coding sequence (CDS), and *the mnp1* 3′ untranslated region (UTR) are indicated. In the map of pGBar(i), the short intron-containing sequence is also indicated.

subcloned as a *Xba*I–*Eco*RI fragment into pUC18 [Mayfield et al., 1994b]. The 18-nt forward primer corresponds to the *gpd* promoter sequence, which resides 170 bp upstream of the start codon and contains a *Hin*dIII site. The 34-nt reverse primer comprises 17-nt of the 5′ end of *bar* CDS followed by 17-nt of the 3′ end of *gpd* promoter. The amplified megaprimer was purified by agarose gel electrophoresis and extracted using a gel extraction kit (Qiagen). The second PCR mixture included the forward primer used in the first PCR, the megaprimer and pCB1635 as the template. The reverse primer in this PCR includes the 3′ end of the *bar* CDS and a *Kpn*I site at its 5′ end. The final PCR product was a 0.7-kb fragment (*gpd-bar* fragment) containing the 170-bp *gpd* promoter joined with the entire *bar* CDS. This fragment was subsequently digested with *Hin*dIII and *Kpn*I. The 250-bp *mnp1* 3′ UTR with an introduced *Kpn*I site at its 5′ end was amplified by PCR using *mnp1* genomic DNA as the template and was subsequently digested with *Kpn*I and *Eco*RI. Finally, the *Hin*dIII–*Kpn*I *gpd-bar* fragment and the *Kpn*I–*Eco*RI *mnp1* 3′ UTR fragment were ligated to *Hin*dIII–*Eco*RI digested pGPDstu1.8, yielding pGBar. pGBar(i) is similar to pGBar, except that the *bar* expression cassette in pGBar(i) contains the 1.1-kb *gpd* promoter together with the *gpd* exon 1 (6 bp), intron 1 (55 bp) and the 5′ end of exon 2 (9 bp) joined with the ATG-less *bar* CDS (Fig. 4.1). The same materials and megaprimer PCR procedure for constructing pGBar were used to construct pGBar(i), with the exception of the 34-nt reverse primer used in the first PCR. For constructing pGBar(i), this primer comprises 17-nt of the 5′ end of *bar* CDS (excluding the start codon) followed by 9-nt of the 5′ end of the *gpd* exon 2 and 8-nt of the 3′ end of the *gpd* intron 1.

4.2.4 Construction of pBar3.8 and pBarTM1

A shorter version of the *bar* expression cassette from pGBar, including only 300 bp of *gpd* promoter followed by the *bar* CDS and the *mnp1* 3′ UTR, was amplified by PCR. This fragment was ligated to *Ssp*I digested pUC18, yielding pBar3.8 (Fig. 3.2). pBarTM1 was constructed by inserting a *mnp1* expression cassette into pBar3.8 between two multiple cloning sites, *Xba*I and *Eco*RI. The *mnp1*

expression cassette contains an 870-bp promoter region of *P. chrysosporium* translation elongation factor (TEF) gene, followed by a 2.6-kb *P. chrysosporium* *mnp1* genomic DNA (including 1.5 kb of the *mnp1* CDS and 1.0 kb of the *mnp1* 3′ UTR) (Fig. 4.2). The TEF gene promoter was isolated as follows. The deduced amino acid sequence of translation elongation factor 1α (TEF-1α) from *Aureobasidium pullulans* [Thornewell et al., 1995] was used to search for its homologue in translated *P. chrysosporium* genomic nucleotide sequence (http://www.jgi.doe.gov/programs/whiterot.html), using the BLAST program [Altschul et al., 1997]. A putative *P. chrysosporium* TEF coding region was located on Scaffold 2 from nt 202805 to 204593. An 870-bp fragment containing the *P. chrysosporium* TEF gene promoter was amplified by PCR using the genomic DNA of Homokaryon 8 (a homokaryon derivative of *P. chrysosporium* OGC 101) as the template. The forward primer and the reverse primer contain the following sequences: 5′ TTGGTCTAGAGACAGGACGGTAAGTG 3′ and 5′ AGCTAAGCTTTTTTGAAGCTGGAGGAAGG 3′, respectively. The TEF gene promoter was joined with the *mnp1* genomic sequence at the translation start codon using the megaprimer PCR method. In the first PCR, a megaprimer containing 18 bp of the TEF gene promoter 3′ end followed by 100 bp of the *mnp1* CDS 5′ end was prepared. The second PCR used the 870-bp TEF gene promoter DNA as the template, and yielded a 1.0-kb fragment (*tef-mnp* fragment) containing the 870-bp TEF gene promoter joined with the 100-bp *mnp1* CDS. This fragment was digested at an *Xba*I site introduced at its 5′ end and a *Not*I site residing at 80 bp downstream of the start codon in the *mnp1* CDS. Finally, the *Xba*I–*Not*I *tef-mnp* fragment, the *Not*I–*Eco*RI *mnp1* fragment (containing the *mnp1* CDS and 1.0 kb of 3′ UTR) and the *Xba*I–*Eco*RI-digested pBar3.8 were ligated in a three-way ligation, yielding pBarTM1.

4.2.5 Culture Medium

The GK medium contains Kirk's salt [Kirk et al., 1978], 1% glucose and 12 mM ammonium tartrate in 10 mM K-phosphate buffer, pH 5.0. The minimal medium contains 2% glucose, 0.2% asparagine and 3 μM thiamine in 10 mM K-

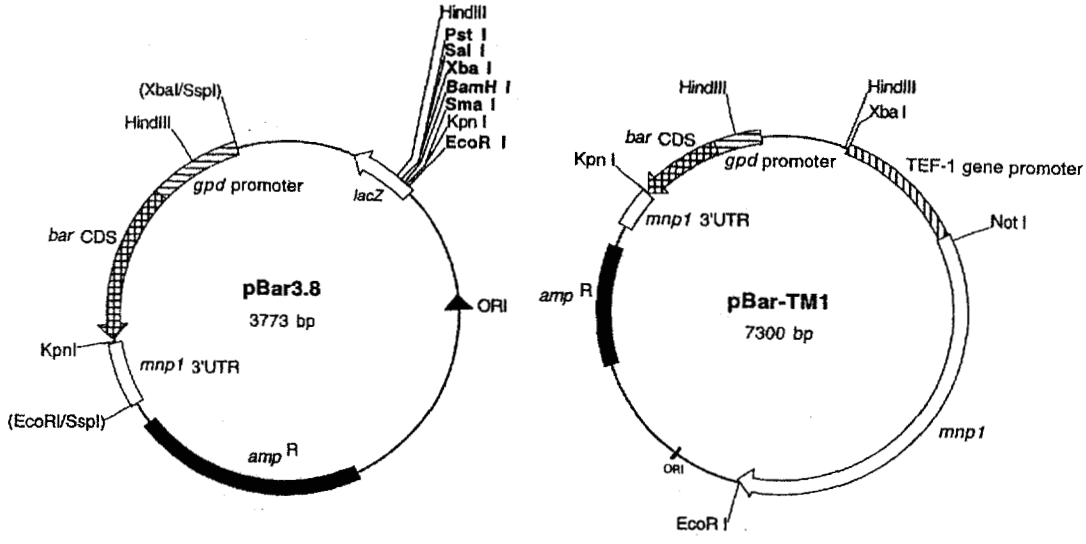


Fig. 4.2 Restriction maps of pBar3.8 and pBarTM1. The relative locations of the *gpd* promoter, the *bar* CDS, the *mnp1* 3′ UTR, the TEF-1 gene promoter, and the *mnp1* sequence are indicated. The restriction sites in parentheses are not present in pBar3.8. The unique restriction sites located in the multiple cloning sites (MCS) are in bold.

phosphate buffer (pH 5.0) [Alic et al., 1989]. GK agar and GK top agar media are the basic GK medium supplemented with 0.5 M $MgSO_4$ as well as 1.5% agar and 0.4 M $MgSO_4$ as well as 1% agar, respectively. The high-carbon, high-nitrogen (HCHN) medium contains Kirk's salts, 2% glucose and 12 mM ammonium tartrate in 20 mM 2,2-dimethyl succinate buffer (pH 4.5).

4.2.6 Fungal Transformations

1 μg of plasmid containing the *bar* expression cassette was linearized at the unique *Eco*RI site and used to transform protoplasts (2×10^6) generated from the basidiospores of Homokaryon 8 as described previously [Alic et al., 1991]. The following modifications were made in the transformation procedure. The diluted protoplast-DNA mixture, containing approximately 4×10^4 protoplasts and 25 ng DNA, was added to 3 ml of melted GK top agar medium and immediately spread onto a plate containing 20 ml of solid GK agar medium. After the plates were incubated at 37°C for 2 hrs, PPT was diluted in another 3 ml of melted GK top agar medium and spread on each plate to a final concentration of 30 μg/ml.

4.2.7 Southern Blot Analysis

Genomic DNA from various transformants was extracted as described previously [Alic et al., 1989]. 1 μg of genomic DNA from each transformant was digested with a selected restriction enzyme. The resulting fragments were resolved on a 0.7% agarose gel and subsequently transferred to a nylon membrane. The *bar* CDS was used as the template to synthesize random primed [α-^{32}P] dCTP labeled probes. Southern hybridization and autoradiography were performed as described previously [Alic et al., 1989].

4.2.8 Production of rMnP1

Approximately 40 PPT-resistant transformants carrying pBarTM1 were assayed for rMnP activity by the *o*-anisidine plate assay [Mayfield et al., 1994b]. The transformant exhibiting the highest rMnP activity in the plate assay was purified further by fruiting and selecting single basidiospore isolates. The purified

transformant strain was then grown from a conidial inoculum in 20-ml stationary, HCHN cultures (with the addition of 0.2% tryptone) at 37°C for 3 days. Subsequently the mycelial mat from two stationary cultures was homogenized for 30 s in a blender and used to inoculate 500 ml of HCHN medium in 1-liter flasks. The cultures were grown at 37°C on a rotary shaker at 150 rpm and MnP activity from the extracellular fluid was measured by monitoring the formation of Mn^{3+}-malonate at 270 nm, using a Shimadzu model UV-260 spectrophotometer, as described previously [Glenn and Gold, 1985; Wariishi et al., 1992]. The reaction mixtures (1 ml) contained 0.1 ml extracellular fluid, 5 mM $MnSO_4$ and 0.1 mM H_2O_2 in 50 mM sodium malonate, pH 4.5.

4.2.9 Western Blot Analysis

The extracellular fluid from 3-day-old HCHN shake culture of various transformants was resolved on a SDS-polyacrylamide gel (SDS-PAGE) and then transferred to a nitrocellulose membrane. A polyclonal antibody against MnP [Pribnow et al., 1989] and the protocol for the Western-light™ protein detection kit (Tropix, Bedford, MA) was used to detect the MnP signal.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Transforming *P. chrysosporium* to PPT-Resistance

The sensitivity of *P. chrysosporium* strain OGC101 to PPT was examined in minimal medium, which was used in transformation studies with *ade* or *ura* genes as the selectable markers [Alic et al., 1989, 1991; Akileswaran et al., 1993], and also in GK medium. 40 μg /ml of PPT completely inhibited the germination of conidia spores from strain OGC101 on solid GK medium (GK supplemented with 1.5% agar). In contrast, 80 μg /ml of PPT was required to inhibit conidia spore germination on solid minimal medium (minimal medium supplemented with 1.5% agar). To provide genetic homogeneity, a homokaryon derivative of strain OGC101 was chosen as the host for this transformation study. 30 μg /ml of PPT completely inhibited the germination of basidiospores from Homokaryon 8 on solid GK medium. Thus, the

GK medium containing 30 μg/ml of PPT were utilized in the following transformation experiments.

Two plasmids, pGBar and pGBar(i), which carry bar-expression cassettes were constructed. The bar-expression cassette in pGBar contains the 1.1-kb *P. chrysosporium gpd* promoter followed by the *bar* coding sequence (CDS) and the 250-bp *P. chrysosporium mnp1* 3′ untranslated region (UTR) (Fig. 4.1). The bar-expression cassette in pGBar(i) is similar to that in pGBar, except that an extra sequence, containing the *gpd* exon 1 (6 bp), intron 1 (55 bp) and the 5′ end of exon 2 (9 bp), was inserted at the 5′ end of the *bar* CDS (Fig. 4.1). Transformation of the Homokaryon 8 protoplasts with 1 μg of linearized pGBar or pGBar(i) yielded 80–100 PPT-resistant transformants and approximately 0.1% of the viable protoplasts were transformed to PPT-resistance. In contrast, no PPT-resistant transformants were observed in minus DNA controls. The transformants first appeared after incubation at 37°C for three days, and continued to emerge until day 7. During this period, the background growth of non-transformants was much weaker than the growth of PPT-resistant transformants; thus, the transformants could readily be distinguished. 40 transformants carrying either pGBar or pGBar(i) were stably maintained on slants containing 2 ml GK solid medium supplemented with 60 μg/ml PPT for 6 generations, suggesting that they are mitotically stable. Moreover, 5 transformants carrying either pGBar or pGBar(i) were fruited and over 80% of the isolated basidiospore progeny of each transformant grew robustly on GK slants supplemented with 60 μg/ml PPT, suggesting the transformants are meiotically stable. Since the DNA-treated *P. chrysosporium* protoplasts were plated in a high concentration on plates and the protoplasts may undergo fusion during the transformation process, some of the isolated transformants are likely to be heterokaryons, containing both transformed and untransformed nuclei

In Chapter 2, I demonstrated that in *P. chrysosporium*, the significant expression of the enhanced green fluorescent protein gene (*egfp*) driven by either the *gpd* or the *mnp1* promoter was observed only when an intron-containing sequence was inserted at the 5′ end of *egfp* [Ma et al., 2001]. In addition, in *Schizophyllum*

commune, inserted introns were required for the efficient expression of several homologous and heterologous genes [Lugones et al., 1999; Scholtmeijer et al., 2001], including the bacterial Hygromycin B resistance gene. However, our present study shows that both intron-less and intron-containing *bar*-expressing plasmids could transform *P. chrysosporium* to PPT resistance with equal transformation frequency. One possible explanation is that even a low level of *bar* expression might still be sufficient to confer resistance to PPT at the concentration of 30 μg/ml. Furthermore, the transformation frequency obtained with both pGBar and pGBar(i) is about 80–100 transformants per μg of DNA. This frequency is comparable to those obtained with plasmids containing *ade* (100–300 transformants per μg of DNA) or *ura* genes (100–600 transformants per μg of DNA) from either *P. chrysosporium* or *S. commune* as the selectable markers [Alic et al., 1989, 1990, 1991; Akileswaran et al., 1993]. However, these frequencies are significantly higher than those of reported plasmid constructs containing either the bacterial kanamycin resistance gene or Ph^R gene as the selectable markers (6–10 transformants per μg of DNA) [Randall et al., 1989, 1991; Randall and Reddy, 1991, 1992; Gessner and Raeder, 1994; Birch et al., 1998].

The *gpd* promoters with varying lengths were tested for their ability to drive *bar* expression in *P. chrysosporium*. A minimum of 300 bp of the *gpd* promoter is sufficient for *bar* expression, as no reduction in transformation frequency was observed with a plasmid containing this short promoter. For subsequent subcloning, the *bar* expression cassette, containing the 300-bp *gpd* promoter, the *bar* CDS and the *mnp*1 3′ UTR was inserted at a unique *Ssp*I site in pUC18, freeing the multiple cloning site for additional inserts. This plasmid was named pBar3.8 (Fig. 4.2). Southern blot analysis was performed on DNA extracted from the homokaryon derivatives of three randomly chosen PPT-resistant transformants carrying pBar3.8 using the ^{32}P-labeled *bar* CDS as the probe (Fig. 4.3). The absence of rapidly migrating bands with undigested transformant DNA (lanes 4, 5, and 6) indicates the transforming plasmids have integrated into the chromosomes rather than being carried in autonomously replicating form. This contrasts with previous reports of the transformation systems using the bacterial kanamycin resistance gene or PhR gene as

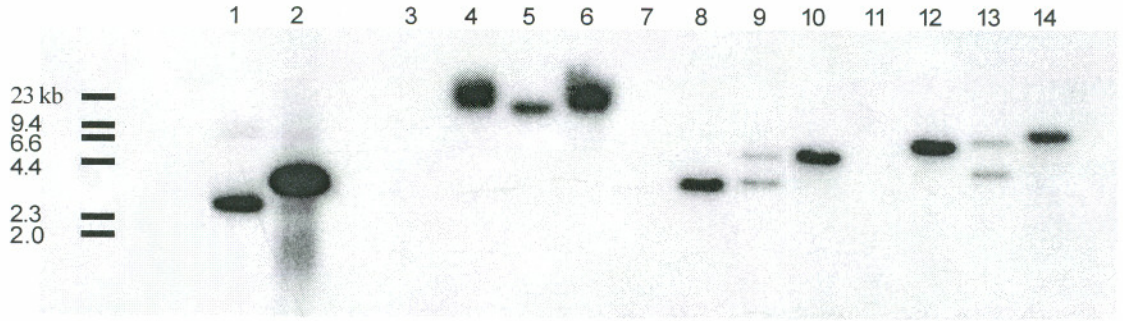


Fig. 4.3 Southern blot analysis of DNA from Homokaryon 8 and transformants carrying pBar3.8. Samples of genomic DNA were electrophoresed on a 0.7% agarose gel. The blot was hybridized with the ^{32}P-labeled *bar* coding region fragment. Lane 1, 100 pg of undigested pBar3.8; lane 2, 100 pg of *Eco*RI-linearized pBar3.8; lanes 3–6, 1 mg of undigested genomic DNA from Homokaryon 8 and transformants 2, 3, and 4 carrying pBar3.8, respectively; lanes 7–10, the same genomic DNA as in lanes 3–6 digested with *Pst*I; lanes 11–14, the same genomic DNA digested with *Hin*dIII. The positions of molecular weight standards are shown on the left.

the selectable markers [Randall et al., 1989; Randall and Reddy, 1991, 1992; Birch et al., 1998]. In those earlier studies, the transforming plasmids contained characterized sequences [Rao and Reddy, 1984; Randall et al., 1989, 1991] or some unidentified sequences [Randall and Reddy, 1991; Birch, 1998] that apparently allow autonomous and extrachromosomal replication. Such sequences are lacking in our *bar*-expressing plasmids. The Southern blot also shows that the three transformants carry one or more copies of integrated pBar3.8 (Fig. 4.3, lane 8–10 and lane 12–14). Multiple copy integration is frequently observed in fungal transformations [Fincham, 1989; Gold and Alic, 1993]. In conclusion, an efficient and integrative transformation system utilizing the *bar* gene as selectable marker has been developed for *P. chrysosporium*.

4.3.2 Homologous Expression of rMnP1 in *P. chrysosporium* Using *bar* as a Selectable Marker

The plasmid, pBarTM1, containing the 870-bp promoter region of the *P. chrysosporium* translation elongation factor (TEF) gene joined with the 2.6-kb fragment encompassing the *P. chrysosporium mnp1* CDS and 3′ UTR, was constructed using pBar3.8 for homologous expression of rMnP1 in *P. chrysosporium* (Fig. 4.2). In this fungus, the endogenous *mnp* genes are only expressed during the secondary metabolic phase, which is triggered by the depletion of nutrient nitrogen [Kirk and Farrell, 1987; Gold and Alic, 1993]. Coupling the *mnp1* CDS to the TEF gene promoter enables the production of rMnP1 during the primary metabolic phase when nutrient nitrogen is sufficient and the endogenous *mnp* genes are not expressed. Using a similar strategy, we previously constructed pAGM1 in which the 1.1-kb *gpd* promoter was joined with the *mnp1* genomic sequence and the *ade5 gene* was used as the selectable marker [Mayfield et al., 1994b]. The transformant carrying pAGM1 (transformant T15) that exhibited highest rMnP1 activity produces approximately 2 mg of rMnP1 per liter of extracellular culture fluid. The rMnP1 produced in this transformant exhibits identical physical, kinetic and spectral characteristics as the native MnP1.

Forty PPT-resistant transformants carrying pBarTM1 were examined for rMnP activity using the *o*-anisidine plate assay [Mayfield et al., 1994b]. Among these transformants, 80% exhibited rMnP activity. In contrast, transformants carrying the blank vector, pBar3.8, or the untransformed Homokaryon 8 exhibited undetectable rMnP activity. This indicates that the rMnP activity in transformants carrying pBarTM1 results from expression of *mnp1* driven by the TEF gene promoter. The level of rMnP activity varies among the transformants carrying pBarTM1, as observed for transformants carrying pAGM1 [Mayfield et al., 1994b]. Transformant T1 carrying pBarTM1, exhibiting the highest rMnP activity in the plate assay, was purified by isolating single basidiospores and analyzed further in liquid, shake cultures. Fig. 4.4 shows a time course for rMnP activity in shake cultures of T1 (carrying pBarTM1), T15 (carrying pAGM1), and T1 (carrying pBar3.8 as a control). While no MnP activity was observed in control cultures during the experimental period, similar rMnP activity profiles were exhibited in T1 (pBarTM1) and T15 (pAGM1). As previously shown for T15 (pAGM1) [Mayfield et al., 1994b], the rMnP activity from T1 (pBarTM1) is highest when grown in nitrogen-sufficient medium with an initial pH of 6.5, while the endogenous MnP is most abundant when grown in nitrogen-limited medium with an initial pH of 4.5 [data not shown]. These data again suggest the activity measured is indeed from recombinant, but not endogenous MnP. Differences exist between the rMnP activity profiles exhibited by T1 (pBarTM1) and by T15 (pAGM1). As shown in Fig. 4.4, at pH 4.5, rMnP activity peaked on day 2, and increased again on day 5 in both the T1 (pBarTM1) and T15 (pAGM1) cultures. On day 6, the rMnP activity of T1 (pBarTM1) was 5-fold higher than that for T15 (pAGM1). At pH 6.5, similar rMnP activity was reached in both the T1 (pBarTM1) and T15 (pAGM1) cultures; however, T1 (pBarTM1) peaked at day 3 and T15 (pAGM1) peaked at day 4. Fig. 4.5 shows a western blot of purified *P. chrysosporium* native MnP1 and the extracellular fluid from 3-day-old shake cultures of T1 (pBar3.8), T1 (pBarTM1), and T15 (pAGM1). As shown, the rMnP1 expressed in T1 (pBarTM1) and T15 (pAGM1) co-migrates with the purified native MnP1. Approximately 2–3 mg rMnP1 per liter of extracellular culture fluid was produced in either T1 (pBarTM1) or T15 (pAGM1) cultures. This yield is

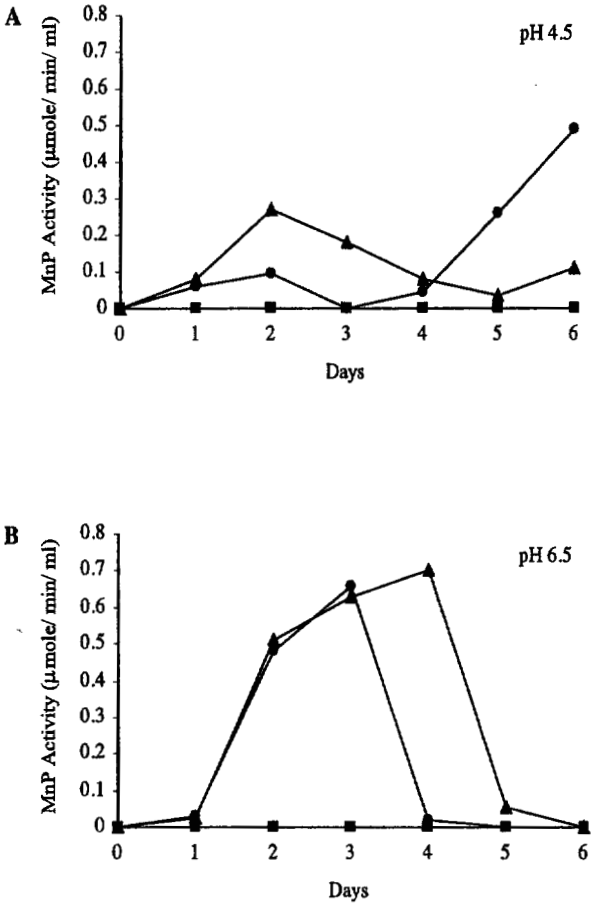


Fig. 4.4 MnP activity in extracellular fluid of *P. chrysosporium* transformants carrying pBarTM1 and pAGM1. All transformants were grown in HCHN media with the initial pH adjusted to 4.5 or 6.5. The culture condition is described in the text. The data from one of two independent experiments (with similar result) is shown. Transformant 1 (T 1) carrying pBar3.8 (■); transformant 15 (T 15) carrying pAGM1 (▲); transformant 1 (T 1) carrying pBarTM1 (•).

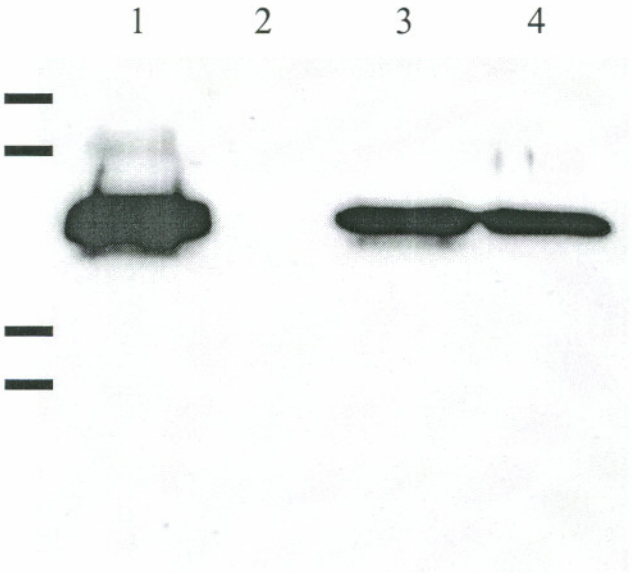


Fig. 4.5 Western blot analysis of rMnP expressed in T1 carrying pBarTM1 and in T15 carrying pAGM1. Lane 1, 100 ng of purified *P. chrysosporium* MnP; lane 2, extracellular media containing 15 mg of total protein from 3-day-old agitated HCHN cultures of T1 (pBar3.8); lane 3, T1 (pBarTM1); lane 4, T15 (pAGM1). All samples were separated on 12% SDS-PAGE and blotted with polyclonal antibody raised against MnP, as described in the text. The positions of the following molecular weight standards are indicated on the left: from the top, bovine serum albumin (molecular weight, 66,300), glutamate dehydrogenase (55,440), lactate dehydrogenase (36,500), and carbonic anhydrase (31,000).

approximately 30% of the endogenous MnP produced in wild type strain OGC101 from multiple *mnp* genes under nitrogen-limited conditions.

The production of rMnP has also been examined in other organisms including *E. coli* [Whitwam et al., 1995] and *Aspergillus niger* [Conesa et al., 2000, 2002]. In *E. coli*, the rMnP was expressed in the form of inclusion bodies. Through the process of reconstitution, low yield of active rMnP could be obtained. In *A. niger*, the active rMnP was secreted into the culture medium and upon hemoglobin supplementation, the production of rMnP could reach 100 mg per liter of extracellular culture fluid. However, the rMnP produced by *A. niger* exhibited a slightly lower mobility on SDS-PAGE than the native MnP, suggesting different posttranslational modification in *A. niger* compared to that in *P. chrysosporium*. The advantage of a homologous expression system is that the produced rMnP is essentially identical to native MnP. However, the improvement of rMnP yield from the homologous expression system must be addressed. In our present study, both homologous rMnP1 expression systems that employ different transfromation systems (with *ade5* and *bar* as the selectable markers) and different promoters (*gpd* and TEF gene promoter) to drive the *mnp1* expression exhibited the same levels of rMnP1 production. Future experiments on reducing proteolytic activity and/or enhancing protein secretion may hold the key of improving rMnP production in *P. chrysosporium*.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 HETEROLOGOUS GENE EXPRESSION IN *PHANEROCHAETE CHRYSOSPORIUM*

Two foreign genes, *egfp* and *bar*, have been successfully expressed in *Phanerochaete chrysosporium*. *egfp* was shown to be an efficient and faithful reporter for monitoring the activity of both *P. chrysosporium gpd* and *mnp1* promoters. In the future, plasmid constructs containing *egfp* joined with other promoters, such as those of the *P. chrysosporium mnp2*, *mnp3*, *mnp4* and various *lip* genes, can be made to study the regulation of the activity of these promoters. In addition, translational fusions of *egfp* and various *P. chrysosporium* genes can be constructed to monitor protein localization and translocation in this fungus. For instance, using MnP-GFP or LiP-GFP fusion proteins, the distribution of these extracellular ligninolytic enzymes in woody tissues may be detected. In Chapter 2, I have shown that the GFP expressed in transformants carrying pUGiGM3', in which the expression of *egfp* was directed by the *gpd* promoter, distributed evenly in the cytoplasm of *P. chrysosporium* mycelium. Thus, such a transformant can be used to monitor the growth of *P. chrysosporium* on natural media, such as wood chips and straw.

bar was utilized as a dominant selectable marker for *P. chrysosporium* transformation. This gene can also be used as a screening marker for gene disruption experiments in *P. chrysosporium*. Specifically, the *bar* expression cassette (as in pBar3.8), containing the 300-bp *gpd* promoter followed by the *bar* coding sequence and the 250-bp *mnp1* 3' untranslated region, can partially replace or be inserted into a particular *P. chrysosporium* gene sequence that has been cloned into a plasmid. Transformation of *P. chrysosporium* protoplasts generated from basidiospores with the

resulting plasmids will yield bialaphos-resistance transformants. A small percentage of these transformants will contain the *bar*-disrupted gene sequence replacing its chromosomal copy through a double crossover mechanism. These transformants containing gene disruption can be confirmed by Southern blot analysis.

In the present study, we have found that the efficient expression of *egfp* in *P. chrysosporium* requires an intron inserted at its 5' end. However, both an intron-less and an intron-containing *bar*-expressing plasmid (pGBar and pGBar(i)), respectively) could transform *P. chrysosporium* to PPT resistance with equal transformation frequency when 30 μg/ml of PPT was added to the culture medium. It is likely that even a low level of *bar* expression could still be sufficient to confer resistance to PPT at the concentration of 30 μg/ml. To analyze the effect of the inserted intron on *bar* expression, the transformation frequency of pGBar and pGBar(i) should be compared when high concentrations of PPT (e.g., 50, 100, 200, 500 μg/ml) are utilized. Under these conditions, if the transformation frequency of pGBar is considerably lower than that of pGBar(i), it will be suggestive that the inserted intron does enhance *bar* expression in *P. chrysosporium*. In addition, using Northern blot analysis to measure *bar* transcript level in transformants carrying pGBar and pGBar(i) will also be informative of the intron's effect on *bar* expression.

To further study the requirement and the effect of introns on gene expression in *P. chrysosporium*, we can compare the efficiency of homologous expression of the cDNA and genomic DNA of various genes, such as different *mnp* and *lip* genes. Intron(s) can be inserted at various positions in the cDNA sequences of these genes and its effect can be examined. Furthermore, introns can be inserted into other widely used reporter genes, such as the *lacZ* and luciferase genes, as well as drug-resistance genes, such as the bacterial hygromycin-resistance gene. Such insertions might result in efficient expression of these genes in *P. chrysosporium*, which has not been achieved. As discussed in Chapter 1, modifying the codons in some foreign genes according to the codon preference of basidiomycetes and increasing the GC-content of AT-rich sequences can enhance the expression of these genes in some basidiomycete species. Such sequence modifications can be explored when expressing

various reporter genes and drug-resistance genes in *P. chrysosporium*. A selection of available reporter systems and selectable markers will facilitate the molecular and genetic research in *P. chrysosporium.*

A new homologous expression system of rMnP1 in *P. chrysosporium* using *bar* as the selectable marker was described in Chapter 4. We compared the production of rMnP1 directed by two different promoters from two constitutively expressed genes, the *gpd* and the translation elongation factor gene. Similar but only modest levels of rMnP1 ($\sim$2 mg active rMnP1 per liter of extracellular fluid) were produced in these two systems. This yield is approximately 30% of the endogenous MnP produced in wild type *P. chrysosporium* strain OGC101 from multiple *mnp* genes under ligninolytic conditions. The limitation of rMnP1 expression and secretion may occur at the posttranslational stage. *P. chrysosporium* MnPs are extracellular proteins that contain glycosylated residues, disulfide bonds and cofactors including heme, Mn^{2+} and Ca^{2+}. In eukaryotic cells, the secretory proteins mature during the passage through the endoplasmic reticulum, the Golgi apparatus and the secretory vesicles. In this process, nascent peptides obtain correct structure and modifications through the assistance of an array of proteins, i.e., chaperons and foldases [Conesa et al., 2001]. Overexpression of some chaperons and foldases in filamentous fungi has been shown to have an enhancing effect on the production of heterologous proteins. In *Aspergillus niger*, overexpression of a lectin-like chaperon, calnexin, which is involved in the folding of glycosylated proteins, resulted in a 5-fold increase of recombinant *P. chrysosporium* MnP1 production in this fungus [Conesa et al., 2002]. Using the completed *P. chrysosporium* genome sequence, the nucleotide sequences that encode some chaperons and foldases could be obtained by searching for conserved domains. Overexpression of these genes in rMnP-producing *P. chrysosporium* strains may be a strategy for increasing rMnP productions.

5.2 Mn^{2+}-DEPENDENT REGULATION OF *mnp* GENE EXPRESSION

Using *egfp* as the reporter gene, we have characterized a 33-bp DNA sequence residing 521 bp upstream of the translation start codon in the *mnp1* promoter that is responsible for Mn^{2+}-dependent gene regulation. We have proposed that negative control is exerted on this promoter element under Mn^{2+}-deficient conditions, while it is relieved upon Mn^{2+}-supplementation. To further examine the function of this Mn^{2+}-responsive element, it would be useful to place this element in a heterologous promoter, whose activity is not regulated in response to Mn^{2+}-status, and demonstrate that the element confers Mn^{2+}-dependent regulation to this promoter using GFP as the reporter. I have constructed insertion of the 33-bp *mnp1* promoter fragment into the 1.1-kb *P. chrysosporium gpd* promoter region at a position 820 bp upstream of the start codon. In addition, the 33-bp fragment was inserted at the 5' end of 300 bp of the *gpd* promoter. When each of these modified *gpd* promoters was used to direct *egfp* expression in *P. chrysosporium*, no difference in GFP activity level was observed under either Mn^{2+}-deficient or Mn^{2+}-sufficient conditions in both nitrogen-sufficient and nitrogen-limited cultures [data not shown]. In the future, the 33-bp putative Mn^{2+}-responsive element may be inserted into various other positions in the *gpd* promoter and its effect on reporter gene expression can be examined. In addition, this element can be inserted into different positions of other *P. chrysosporium* promoters, especially those that only promote gene transcription under nitrogen-limited conditions. For example, this element can be inserted into the promoters of various *lip* genes. If the putative Mn^{2+}-responsive element confers Mn^{2+}-dependent regulation to these heterologous promoters, it will confirm its essential role in Mn^{2+}-dependent gene regulation. The results obtained from these experiments will further prove if negative control is functioning through this Mn^{2+}-responsive element and if this element itself is sufficient for this control.

Future work will also focus on the identification of transcriptional regulator(s) that interact with the Mn^{2+}-responsive element characterized in our study. Electrophoretic mobility shift assay (EMSA) can be performed using the radiolabeled

Mn^{2+}-responsive element DNA and the crude cell-free extracts from *P. chrysosporium* cells cultured under either Mn^{2+}-deficient or Mn^{2+}-sufficient conditions. Proteins that bind specifically to the radiolabeled DNA fragment will retard the mobility of the fragment during electrophoresis, resulting in discrete bands corresponding to the individual protein–DNA complexes. The crude cell-free extracts can be separated into different fractions using various chromatography methods and the EMSA will be performed using these cell-extract fractions. The fraction that contains protein factors, which specifically bind to the Mn^{2+}-responsive element, could further be purified by several rounds of chromatography and EMSA. Finally, the specific protein–DNA complex that is responsive to Mn^{2+}-status can be obtained by cutting the corresponding band out of the gel. The DNA can be removed from the gel piece by eletroelution and the remaining proteins can be characterized by mass spectroscopy analysis. The information obtained from this assay will facilitate the cloning of Mn^{2+}-responsive transcriptional regulators in *P. chrysosporium*. Moreover, DNase 1-mediated or chemicals-mediated DNA footprint analysis can be performed on radiolabeled DNA fragments containing the Mn^{2+}-responsive element. In this experiment, the protein factors that specifically bind to the Mn^{2+}-responsive element will protect the phosphodiester backbone of this region from DNase 1 catalyzed or chemical-induced hydrolysis. Subsequently the binding site can be visualized by resolving the DNA fragments resulting from hydrolysis through electrophoresis on denaturing DNA sequencing gels and by the following autoradiography. Partially purified protein factors that bind to the Mn^{2+}-responsive element as characterized in EMSA can be used in footprint analysis. The result from this experiment will define the particular nucleotides in the Mn^{2+}-responsive element that interact with the transcriptional regulators.

Finally, promoter analysis similar to that described in Chapter 3 could be performed on *P. chrysosporium mnp2*, *mnp3* and *mnp4* gene promoters to identify the DNA sequences in these promoters that may contain Mn^{2+}-responsive elements. So far, by sequence comparison, no DNA sequences closely resembling the 33-bp DNA sequence identified in the *mnp1* promoter could be found in the promoter regions of *mnp2*, *mnp3* and *mnp4* genes. Our data presented in Chapter 3 suggest that there

might be other promoter element(s) besides that residing in the 33-bp fragment, which are also responsible for Mn^{2+}-dependent regulation of the *mnp1* promoter activity. Future work should also focus on identifying these putative Mn^{2+}-responsive elements in the *mnp1* promoter. The analysis of all the *P. chrysosporium mnp* gene promoters will tell us if there is a conserved promoter element sequence responsible for Mn^{2+}-dependent gene regulation. If such a Mn^{2+}-responsive element exists, most likely it should be found in *mnp* genes from other white-rot fungi whose expression are induced by Mn^{2+}-supplementation. A conserved Mn^{2+}-responsive element would also suggest that a common mechanism is responsible for Mn^{2+}-dependent gene regulation. As discussed in Chapter 1, a common *cis*-element and *trans*-regulator pair whose interaction is regulated by the status of a particular metal (such as Fe, Zn or Cu) controls the expression of a variety of genes that are involved in maintaining the homeostasis of this metal element in many species. This regulation formula may be true for Mn^{2+}-dependent regulation of *mnp* gene expression in white-rot fungi. The study of this regulation will enrich our knowledge of the physiology of MnP and the delignification process catalyzed by this enzyme in white-rot fungi.

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