CHARACTERIZATION OF SCYTALONE DEHYDRATASE AND REDUCTASE GENES AND EXPRESSION OF MELANIN BIOSYNTHESIS GENES IN *OPHIOSTOMA FLOCCOSUM*

by

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Abstract

Wood sapstain is a significant economic problem for the lumber industry. The discoloration of sapwood is mainly caused by sapstain fungi, which grow on wood and produce dark or brown pigment. The aim of the thesis was to obtain some molecular information about the pigmentation of sapstain fungi by cloning and characterizing the major melanin genes.

A transformation system is the prerequisite for conducting both gene disruption and genetic complementation of an organism. Transformation systems were set up for *Ophiostoma floccosum* 387N and other major sapstain fungal species such as *Ophiostoma piceae* using the transformation vectors pAN7-1 and pCB1004. This transformation system was applied to attempt to disrupt the cloned genes, *THN1* encoding a melanin pathway reductase gene and *OSD1* encoding a scytalone dehydrates gene in 387N. Unfortunately, no disruptant was identified by screening more than 2,000 transformants. We concluded that homologous DNA integration in *O. floccosum* 378 would be a rare event.

We isolated and characterized a putative scytalone dehydratase gene (*OSD1*) from *O. floccosum* 387N encoding a predicted polypeptide sequence of 216 amino acids that shared high homology to other fungal melanin scytalone dehydratases. The function of *OSD1* was determined by complementing a *Colletotrichum lagenarium* scytalone dehydratase deficient mutant. *OSD1* was able to restore the melanization and pathogenicity of the mutants. A reductase gene (*THN2*) encoding a protein of 284 amino acids was isolated, and it shared a 44% amino acid identity to the *O. floccosum THN1* genes' deduced protein sequence. We confirmed the function of the *THN2* gene by complementing the DHN melanin deficient, non-pathogenic mutants of *C. lagenarium* and *Magnaporthe grisea* that lack the 1,3,8-trihydroxynaphthalene reductase gene. Sequence analysis of all available fungal melanin reductases showed that two groups of the reductases are present in fungal DHN melanin biosynthetic pathway. *THN1* and *THN2* belonged to different groups. We tried to complement a double mutant of *M. grisea*, where the 1,3,6,8-tetrahydroxynaphthalene reductase gene and the 1,3,8-trihydroxynaphthalene reductase gene have been knocked out, using *THN1, THN2* and the combination of *THN1* and *THN2*, respectively. The results indicated that both reductases can not function as the 1,3,6,8-tetrahydroxynaphthalene reductase. However, whether they function in a similar way in *O. floccosum* remains unknown.

A partial melanin PKS gene (*OPKS1*) was cloned in *O. floccosum*. The expression of the melanin genes, *OPKS1, THN1, OSD1* and *THN2* was associated with the mycelial differentiation and affected by nutrients.

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List of Abbreviations

5', 3'	denotes 5'-hydroxy or 3'-phosphate end of sequence
AaTHN	*A. alternata* 1,3,8-THN reductase
A, C. G, T	nucleotides adenosine, cytosine, guanosine, thymidine
ACP	acyl carrier protein
AfTHN	*A. fumigatus* 1,3,6,8-THN reductase
amp	ampicillin
AT	acetyl/malonyl transferase domain
B	brown
BC	British Columbia
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
ChTHN	*C. heterostrophus* 1,3,8-THN reductase
Ci	Curie
ClTHN	*C. lagenarium* 1,3,8-THN reductase
CM	complete medium
DB	dark brown
DDAC	didecyl dimethyl ammonium chloride
DDN	3,4-dihydro-4,8-dihydroxy-1 naphthalenone
DHN/1,8-DHN	dihydroxynaphthalene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DOPA	dihydroxyphenylalanine
EDTA	ethylene diamine tetra acetic acid
est	Expression sequence tag
EtBr	ethidium bromide
EtOH	ethanol
E-Value	Expected value
GDHB	glutaminyl-3,4-dihydroxybenzene
GHB	γ-glutaminyl-4-hydroxybenzene
h	hour
4HNR	*M. grisea* 1,3,6,8-THN reductase
hph	hygromycin phosphotransferase
HR	ratio of hydrophilicity to hydrophobicity of a protein
IR	infrared
kb	kilobase
kDa	kilodalton
KS	β-ketoacyl synthase domain
LB	Light Brown
μ	micro
m	milli
MEA	malt extract agar
MgTHNA/T4HN	*M. grisea* 1,3,6,8-THN reductase

MgTHNB/T3HN	*M. grisea* 1,3,8-THN reductase
min	minute(s)
minus-RT	RNA samples without reverse transcriptase treatment
mm	millimeters
mRNA	messenger ribonucleic acid
MSAS	6-methylsalicylic acid synthase
NMR	nuclear magnetic resonance
NCBI	National Center of Biotechnology Information
No.	number
Novo-PCR	Novozym-aid PCR
nr	non-redundant
OD_{600}	optical density at 600 nm
OfTHNA	*O. floccosum* 1,3,6,8-THN reductase
OfTHNB	*O. floccosum* 1,3,8-THN reductase
ORF	open reading frame
PCR	polymerase chain reaction
PCP	polychlorophenates
PEG	polyethylene glycol
pfu	plaque-forming unit(s)
PM	proline medium
PKS	polyketide synthase
PDA	potato dextrose agar
PS	potato dextrose broth

PSA	potato sucrose agar
RM	regeneration medium
rDNA	DNA coding for rRNA
RNase	ribonuclease
rRNA	ribosomal RNA
rpm	round per minute
S	protein solubility
SADH	short chain alcohol dehydrogenase
SD	scytalone dehydratase
SDS	sodium dodecyl sulfate
sp	species
SSC	0.15 M NaCl/0.015 M Na3_citrate, pH 7.6
STC buffer	1 M sorbitol, 50 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.5
TAE	Tris-acetate EDTA
TC buffer	50 mM $CaCl_2$, 2.5 mM Tris-Cl, pH 7.5
TCMM	2-(2-thiocyanomethylthio) benzothiozole
TE buffer	10 mM Tris–HCl pH 7.5; 1 mM EDTA
1,3,6,8-THN	1,3,6,8-tetrahydroxynaphthalene
1,3,8-THN	1,3,8-trihydroxynaphthalene
Tris	tris-(hydroxymethyl)-aminoethane
UV	utraviolet
vol	volume
W	white

w/v	weight by volume
YM	mixed culture of yeast mycelia

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Chapter 1

Introduction: fungal melanin and the DHN melanin biosynthetic pathway

Melanins, generally described as dark-brown to black pigments, are biological macromolecules composed of various types of phenolic or indolic monomers, usually complexed with protein, or carbohydrates (Butler and Day, 1998). These pigments are produced by a variety of bacteria, fungi, protozoans, plants and animals. Because of their insolubility, melanins are difficult to characterize bio-chemically, however, utraviolet (UV), visible, infrared (IR) and nuclear magnetic resonance (NMR) spectrographic data are available for melanin from a few species (Bell and Wheeler, 1986). In this chapter, the different types of fungal melanins, the function of melanin in sapstaining fungi, and the biochemical and molecular aspects of the dihydroxynaphthalene (DHN) melanin biosynthetic pathway are described.

1.1 Fungal melanin types

Melanins are found in many fungi and can be synthesized by a variety of metabolic pathways. The types of melanins produced vary from organism to organism. Four types of fungal melanins have been reported based on their monomers: dihydroxyphenylalanine (DOPA) melanin, glutaminyl-3,4-dihydroxybenzene (GDHB) melanin, catechol melanin and dihydroxynaphthalene (DHN) melanin.

1.1.1 Dihydroxyphenylalanine (DOPA) melanin

Tyrosine is the starting monomer of DOPA melanin. DOPA is formed by the oxidation of tyrosine by a tyrosinase, as shown in Figure 1.1 (Mason, 1948). DOPA can then be oxidized to form melanin by many different enzymes, such as laccases, polyphenol oxidases, peroxidases and catalases that are found in the cell wall. Animal melanin is normally DOPA melanin, but DOPA melanin is also produced by yeasts, mushrooms and other fungi, such as *Cryptococcus neoformans*, *Neurospora crassa* and *Aspergillus nidulans* (Wang and Casadevall, 1994; Prota, 1998; Brown and Salvo, 1994). The tyrosinases used to produce DOPA in these fungi have been characterized (Horowitz and Shen, 1952; Bull and Carter, 1973), and found to contain a single copper ion necessary to bind the phenol group of the substrate.

1.1.2 Glutaminyl-3,4-dihydroxybenzene (GDHB) melanin

Glutaminyl-3,4-dihydroxybenzene (GDHB) melanin is synthesized from γ-glutaminyl-4-hydroxybenzene (GHB), via the shikimate pathway shown in Figure 1.2 (Rast et al., 1980). In this pathway shikimic acid can form GHB, which is then converted to GDHB, which undergoes a non-enzymatic polymerization to melanin. GHB has been found in fungal mycelia as well as in fruiting bodies, GDHB, on the other hand, has been isolated from the cell wall of *Agaricus bisporus* basidiospores (Rast et al., 1980). *Agaricus* produces a tyrosinase that oxidizes GHB to melanin, which is restricted to the

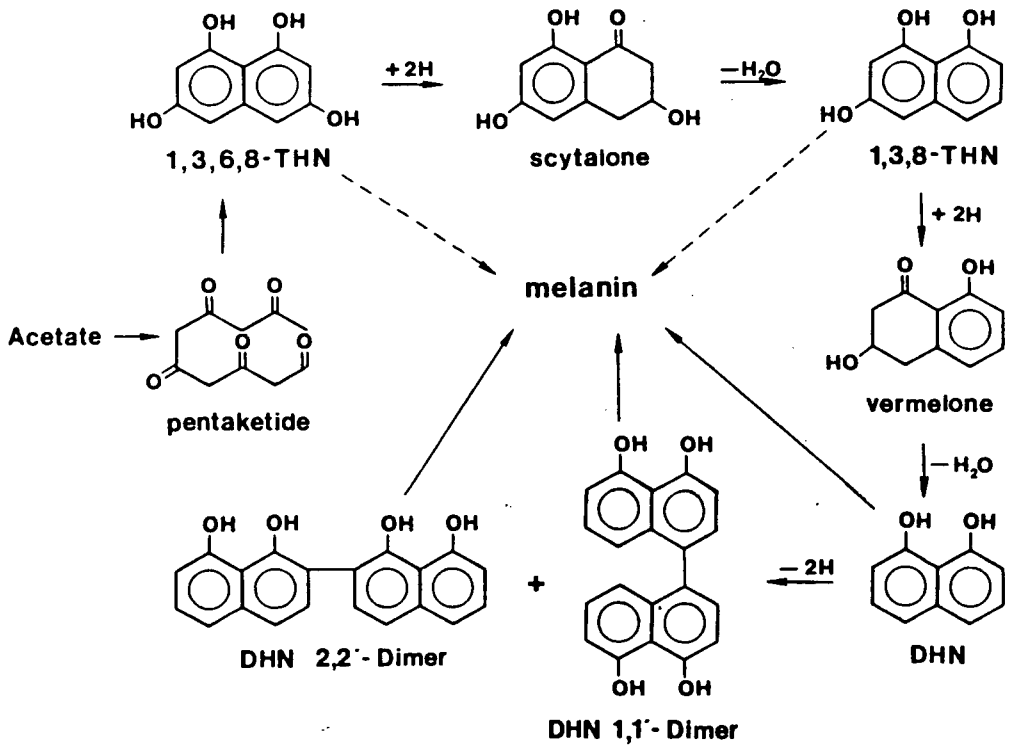

Figure 1.1. DOPA melanin biosynthesis (Bell and Wheeler, 1986).

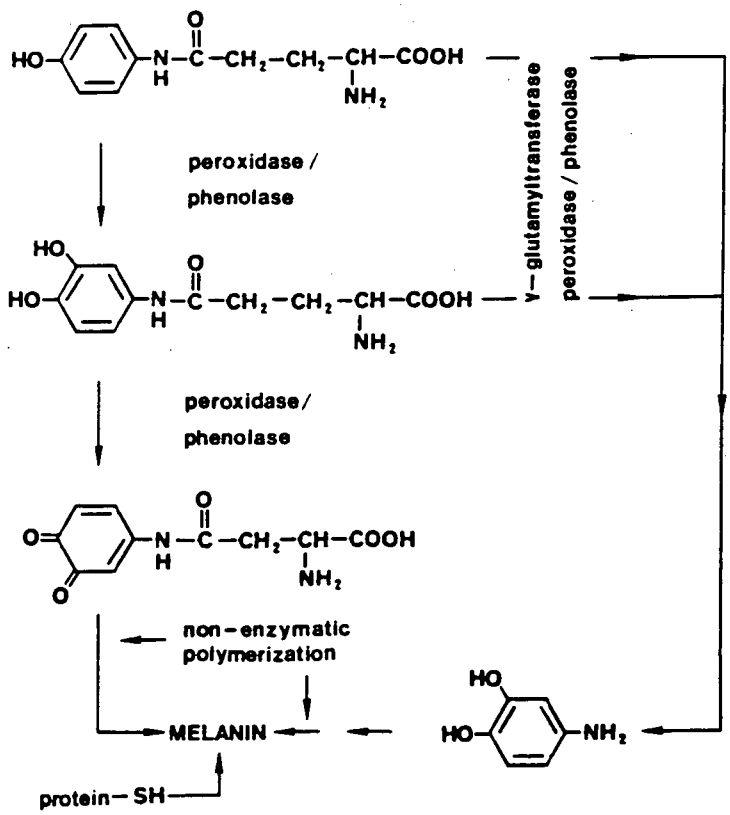

Figure 1.2. GDBH melanin biosynthesis (Bell and Wheeler, 1986).

reproductive hyphae that form melanized spores. GHB and GDHB have been found in several species of basidiomycetous fungi, but not in ascomycetous fungi, suggesting that GDHB melanins are produced only in the basidiomycetous fungi (Wheeler, 1983).

1.1.3 Catechol melanin

Catechol melanin lacks nitrogen and its polymerization proceeds through free radicals or quinone-catechol adducts (Figure 1.3), however, the biosynthetic origin of catechol is unknown. The analysis of *Ustilago maydis* teliospore melanin found that catechol is the precursor used to produce melanin in these structures (Piatelli et al., 1965; Banuett and Herskowitz, 1996).

1.1.4 Dihydroxynaphthalene (DHN) melanin

DHN melanin biosynthesis starts with the polymerization of acetate by a polyketide synthase (PKS) to form a pentaketide (Figure 1.4). 1,3,6,8-tetrahydroxy-naphthalene (1,3,6,8-THN) is then formed by the cyclization of the pentaketide. A reductase then converts 1,3,6,8-THN to scytalone, which is dehydrated by a scytalone dehydratase (SD) to form 1,3,8-trihydroxynaphthalene (1,3,8-THN). This compound is then converted to 1,8-dihydroxynaphthalene (1,8-DHN) after an additional reduction and dehydration step. Finally, the oxidative polymerization of 1,8-DHN produces DHN-melanin (Bell and Wheeler, 1986; Butler and Day, 1998). Although many fungi produce DHN melanin, only the melanin of a few species has been intensively studied, including *Magnaporthe grisea, Colletotrichum lagenarium, Exophiala dermatitidis (Wangiella dermatitidis),* and *Aspergillus fumigatus* (Butler and Day, 1998).

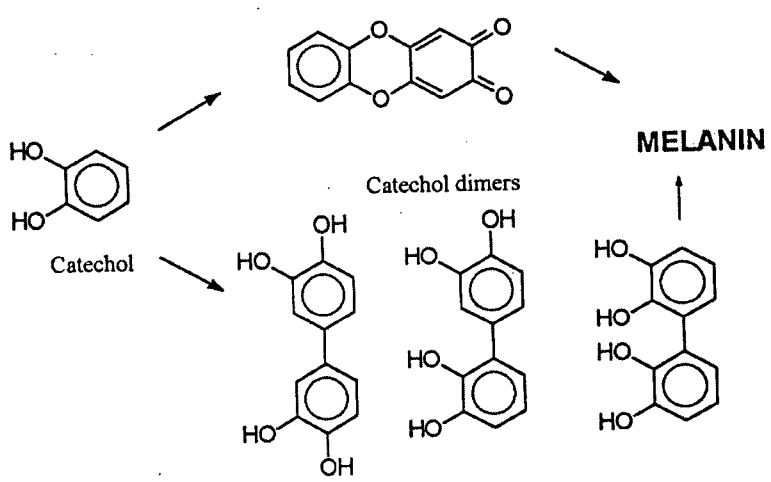

Figure 1.3. Catechol melanin biosynthesis (Bell and Wheeler, 1986).

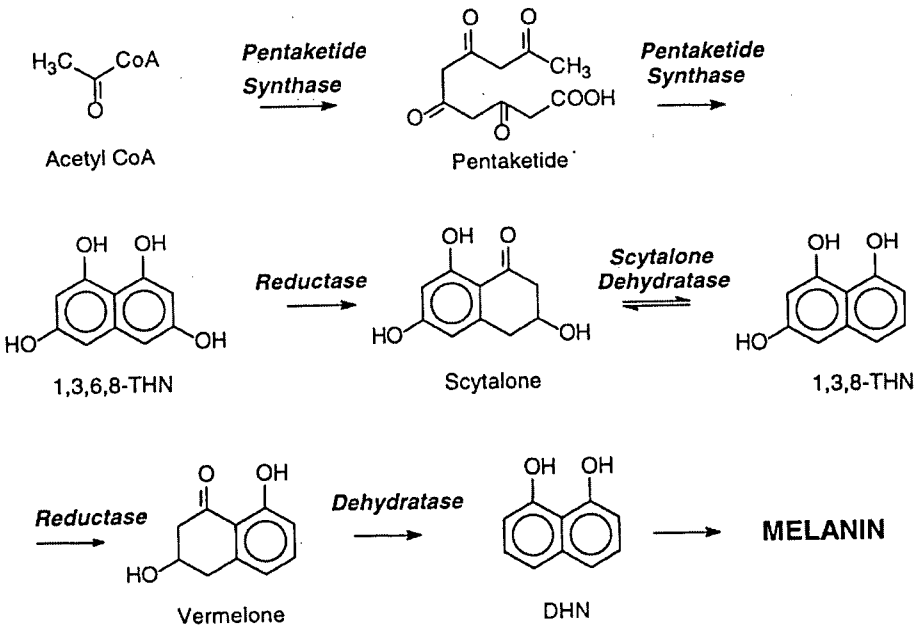

Figure 1.4. DHN melanin biosynthesis (Bell and Wheeler, 1986).

1.2 Molecular genetics of DHN melanin biosynthesis

Biochemical studies of the DHN pathway are difficult, because the intermediates of the pathway are unstable. Enzyme assays have to be performed under argon or nitrogen gas (Tajima et al., 1989; Vidal-Cros et al., 1994). Therefore, molecular approaches have been favored to study the pathway. Isolation and characterization of DHN melanin genes are necessary to conduct molecular analysis of the pathway. Several strategies have been successfully utilized in the cloning of DHN melanin biosynthetic genes.

1.2.1 Gene cloning strategies

The genetic complementation of melanin deficient mutants seems to be the most straightforward method to clone melanin synthesis genes. This method requires a well-established transformation system for the organism in which the complementation is to be performed. As well, a melanin-deficient mutant of the organism in which the complementation is to be performed has to be created. For example, in order to clone a SD gene in *A. fumigatus*, a melanin-deficient mutant with reddish pink conidia was first created and characterized. A genomic cosmid library of *A. fumigatus* was then used to transform the mutant. Cosmid-restored mutants producing bluish green conidia were then rescued from the complemented strains. Finally, the SD gene *arp1* was mapped in one of the complementing cosmids (Tsai et al., 1997). This approach has been

successful in the isolation of genes encoding the PKS, reductase and SD in *A. fumigatus* and *Alternaria alternata* (Kimura and Tsuge, 1993; Tsai et al., 1997, 1998, 1999).

The isolation of melanin genes using the reverse genetics cloning approaches relies on purified enzymes. Where purification is possible, production of antibodies to the enzyme, and isolation of the gene from a cDNA expression library can be accomplished. This procedure was used to clone a reductase gene from *M. grisea* (Vidal-Cros et al., 1994).

Melanin genes can also be isolated by PCR-based cloning approaches. For example, the PKS gene from *Nodulisporium sp.* and a reductase from *O. floccosum* have been cloned using this approach (Fulton et al., 1999; Eagen, 1999). For example, to clone the *Nodulisporium pks1* gene, degenerate PCR primers were developed based on the conserved regions of the *C. lagenarium PKS1* gene and its *Aspergillus* homolog. These primers were used to amplify and clone a fragment of the *Nodulisporium pks1* gene. A cosmid library of *Nodulisporium* genomic DNA was probed with this gene fragment, and a full-length copy of *pks1* was identified and cloned (Fulton et al., 1999).

Finally, melanin genes can be retrieved using cross-species hybridization. Once a gene has been cloned it can often be used as a probe for isolation of a cognate gene from a heterologous organism. The *SCD1* gene encoding a SD in *C. lagenarium* was cloned using this approach (Kubo et al., 1996). In this case the melanin-induced cDNA library of *C. lagenarium* was screened using a SD cDNA from *M. grisea* as a probe. Then to

retrieve the genomic copy of the gene, a cosmid library of *C. lagenarium* was screened using the cloned cDNA as a probe. Similarly, the *THR1* gene, encoding a reductase in *C. lagenarium* has been cloned using the heterologous probe, *BRM2*, a reductase gene involved in melanin biosynthesis in *A. alternata* (Perpetua et al., 1996).

1.2.2 Characterization and functions of the cloned DHN melanin synthesis genes and their products

Molecular studies of melanin biosynthesis have been mainly applied to three fungal species, *C. lagenarium, A. fumigatus* and *M. grisea*. In order to assign the functions of the isolated melanin genes in these species two gene transfer techniques were used. Firstly, genetic complementation was used, and secondly, gene disruption where 'knock-out' strains are developed was used. To date, molecular studies have identified 7 different genes involved in the DHN-melanin biosynthetic pathway in fungi: a pentaketide/pentaketide synthesis gene, two reductase genes, a scytalone dehydratase gene, an *ayg1* gene, an oxidase gene and a laccase gene.

1.2.2.1 Synthesis of 1,3,6,8-THN

The synthesis of 1,3,6,8-THN via a pentaketide intermediate has been confirmed using ^{2}H-, ^{13}C-, and ^{14}C-labelled acetate feeding experiments (McGovern and Bentley, 1975; Seto and Yonehara, 1977). A PKS participates in the formation of the naphthalene unit in DHN melanin by joining and cyclizing five acetate carbon units to form 1,3,6,8-THN.

The *PKS1* gene, encoding a PKS in *C. lagenarium*, has been cloned, and found to contain one open reading frame (ORF) encoding a polypeptide consisting of 2187 amino acids. This protein contains a β-ketoacyl synthase (KS), an acetyl/malonyl transferase (AT), and two acyl carrier (ACP) domains (Mayorga and Timberlake, 1992; Takano et al., 1995). This *PKS1* gene was over-expressed in the heterologous species, *Aspergillus oryzae*. Then the synthesis of 1,3,6,8-THN was successfully confirmed using in vitro cell-free extracts from *C. lagenarium* with ^{14}C-labeled acetyl CoA and/or ^{14}C-labeled malonyl CoA as substrates (Fujii et al., 1999, 2000). These results unambiguously identified malonyl CoA as the starter as well as the extender units in the formation of 1,3,6,8-THN by the *C. lagenarium PKS1*. However, other fungi may still use acetyl CoA (Tsai et al., 2001). Disrupted *PKS1* mutants do not synthesize melanin, but providing these mutants with scytalone can restore melanization.

Two functional homologues of the *C. lagenarium PKS1*, *A. fumigatus alb1* and *Nodulisporium sp. pks1* (Tsai et al., 1998; Fulton et al., 1999), have been isolated. *Nodulisporium pks1* encodes a putative protein of 2,159 amino acids. Inactivation of this gene resulted in melanin deficient transformants. The *A. fumigatus alb1* gene encodes a 7-kb transcript and a putative protein of 2,146 amino acids. An *alb1* disruptant lost its ability to produce 1,3,6,8-THN and its virulence to humans.

The amino acid sequences of *Nodulisporium pks1* product and *C. lagenarium* PKS are highly conserved (72% similarity), while *A. fumigatus* PKS amino acid sequence shares only 60% similarity with *C. lagenarium* PKS. However, the amino acid sequence of

A. fumigatus PKS has a significantly higher similarity, 80%, with an *A. nidulans* heptaketide synthase encoded by *wA*, indicating that *A. fumigatus* PKS is in fact a heptaketide synthase. (Mayorga and Timberlake, 1992). In *A. fumigatus*, the synthesis of the pentaketide 1,3,6,8-THN therefore occurs via a chain-length shortening of the heptaketide synthesized by PKS. This chain-length shortening occurs due to the protein product of a gene called *ayg1,* which encodes a putative protein of 406 amino acids (Tsai et al., 2001). At an early growth stage, *ayg1* deletants produce yellow conidia that gradually turn to a green color as the culture becomes older (Tsai et al., 1999).

Molecular analysis of bacterial polyketide biosynthesis has shown that PKSs can be divided into two types (Hopwood and Sherman, 1990; Hopwood and Khosla, 1992; Hutchinson and Fujii, 1995). Type I enzymes are large, multifunctional polypeptides, each encoding the necessary enzymatic motifs for one or more cycles of condensation, while type II enzymes consist of several single-function polypeptides associated in a complex. Fungal PKSs belong to the Type I PKS family, and are further divided into two subclasses. The WA subclass consists of PKSs involved in spore pigmentation, melanin, and aflatoxin biosynthesis. The *wA* protein from *A. nidulans* is an example of the WA subclass (Mayorga and Timberlake, 1992). The MSAS subclass consists of 6-methylsalicylic acid synthase (MSAS) enzymes which are found in *Penicillium patulum* (Beck et al., 1990) and *Penicillium freri* (Nicolaisen et al., 1997). MSAS catalyzes the biosynthesis of 6-methylsalicylic acid, the first intermediate in the pathway leading to the mycotoxin patulin.

1.2.2.2 Conversion of 1,3,6,8-THN to scytalone

The conversion of 1,3,6,8-THN to scytalone requires a tetrahydroxynaphthalene reductase (1,3,6,8-THN reductase). In *A. fumigatus*, the *arp2* gene has been cloned. This cDNA contains an 819-nucleotide ORF encoding a putative protein of 273 amino acids. An *arp2* disruptant produces reddish pink conidia and flaviolin abundantly (Tsai et al., 1997). Scytalone, a stable intermediate and branch product of the DHN-melanin pathway, has not been detected in *arp2* disruptant culture extracts. These data suggest that *arp2* encodes a 1,3,6,8-THN reductase.

A functional homologue of *arp2*, *HNR* encoding a 1,3,6,8-THN reductase (4HNR) has been isolated in *M. grisea* (Thompson et al., 2000). The *arp2* deduced protein shares 50% identity to 4HNR. The *HNR* gene has been expressed in *E. coli* and its product has been purified. The molecular mass of the purified enzyme is 28.4 kDa while the predicted molecular weight of the coding sequence is 28.6 kDa. The enzyme is NADPH-dependent and involved in converting 1,3,6,8-THN to scytalone. Unlike *arp2* disruptants, *HNR* gene disruptants produce melanin. These results suggest that another reductase is involved in this step of the melanin synthesis pathway of *M. grisea* (Section 1.2.2.4).

1.2.2.3 Conversion of scytalone to 1,3,8-THN

A SD involved in the conversion of scytalone to 1,3,8-THN, has been detected in cell-free homogenates of *Cochliobolus carbonum*, and *Verticillium dahliae* (Wheeler, 1981). The *SCD1* gene encoding a SD has been cloned in *C. lagenarium*, this gene contains one ORF composed of 188 codons (Kubo et al., 1996). A *SCD1* disruptant forms reddish-brown colonies and accumulates scytalone. The *SCD1* gene complements the SD deficient *C. lagenarium* mutant 9201Y and restores the wild-type phenotype.

A. fumigatus arp1, the apparent homologue of *C. lagenarium SCD1*, has been cloned by complementing a reddish-pink conidial mutant with a *A. fumigatus* genomic library (Tsai et al., 1997). This gene encodes a putative protein of 168 amino acids. Disruption of *arp1* results in the production of reddish pink conidia and the accumulation of scytalone and flaviolin. Accumulation of these compounds indicates that the *arp1* disruptant is unable to convert scytalone to 1,3,8-THN. A cDNA encoding a SD has also been cloned from *M. grisea* (Motoyama et al., 1998), this transcript is 740 bases long encoding a predicted protein with a mass of 23 kDa. This predicted protein shares 51% identity and 68% similarity with the predicted protein encoded by *arp1*, which in turn shares 58% identity and 75% similarity with the SD of *C. lagenarium*.

SDs has been purified and characterized in *Phaeococcomyces sp., M. grisea* and *Cochliobolus miyabeanus* (Butler et al., 1988; Lundqvist et al., 1993, 1994; Tajima et al., 1989). In these enzymes the catalyzing mechanism has been found to involve substrate

recognition and then assisting in the enolization of the substrate and finally the dehydration of the substrate by the removal of an hydroxyl radical (Basarab et al., 1999).

1.2.2.4 Conversion of 1,3,8-THN to vermelone

The conversion of 1,3,8-THN to vermelone involves a 1,3,8-THN reductase (3HNR). In *M. grisea*, a reductase gene encoding a 3HNR has been isolated from a cDNA library, and was found to contain an 846-bp ORF. Translation of the DNA sequence gives a 282-residue amino acid sequence with a predicted molecular mass of 29.9 kDa (Vidal-Cros et al., 1994). The encoded reductase exhibits the characteristics of the short-chain alcohol dehydrogenase family. This reductase is also NADPH-dependent, and is therefore, a class B dehydrogenase (Wheeler, 1982; Viviani et al., 1990). In the reductase-catalyzed reaction, the protonation of the substrate's carbonyl oxygen has been proposed to occur through a proton shuttling mechanism that proceeds through the hydroxyl group of Tyr-178, the 3'-hydroxyl group of NADPH, and a crystallographic water molecule, the ultimate donor (Liao et al., 2001a). This reductase, from *M. grisea*, is both able to convert 1,3,6,8-THN to scytalone and convert 1,3,8-THN to vermelone (Vidal-Cros et al., 1994; Thompson et al., 2000). However, this reductase prefers 1,3,8-THN as a substrate over 1,3,6,8-THN by a factor of 4.2. A 3-D structural analysis of 1,3,8-THN and 1,3,6,8-THN in the active site of the 1,3,8-THN reductase suggests a favorable interaction of the sulfur atom of the C-terminal Met-283 with the C6 CH group of 1,3,8-THN, and an unfavorable interaction with the C6 hydroxyl group of

1,3,6,8-THN (Liao et al., 2001b). Met is the N-terminal residue for 1,3,8-THN reductases but not for 1,3,6,8-THN reductases.

The functional homologues of the *M. grisea* reductase gene, *THR1*, *BRM2*, *THN1* and *brn1* have been cloned from *C. lagenarium*, *A. alternata*, *O. floccosum* and *Cochliobolus heterostrophus*, respectively (Perpetua et al., 1996; Kawamura et al., 1999; Eagen et al., 2000; Shimizu et al., 1997). *THR1* contains an ORF that encodes a protein of 282 amino acids and is able to complement and restore the melanization of the *C. lagenarium* mutant 9141 which is unable to convert 1,3,8-THN to vermelone (Perpetua et al., 1996). Sequence analysis of the *BRM2* gene finds one ORF of 801 bp, which potentially encodes a 267 amino acid protein. *BRM2* can complement and restore the melanization of the *A. alternate* brm2 mutant, which accumulates 2-hydroxyjuglone, a shunt product of 1,3,8-THN (Kimura and Tsuge, 1993; Takano et al., 1997a; Kawamura et al., 1997). Likewise, *brn1* has been demonstrated to be able to convert 1,3,8-THN to vermelone. The *O. floccosum THN1* gene contains one ORF and encodes for a protein of 268 amino acids. This gene has been demonstrated to complement a 1,3,8-reductase deficient mutant of *M. grisea*. However, none of these reductases have been demonstrated to be able to convert 1,3,6,8-THN to scytalone, unlike the 3HNR from *M. grisea*.

Sequence comparison demonstrated that the predicted *BRM2* product is closely related to the 1,3,8-THN reductases of *C. heterostrophus* (*Brn1* 95% identity; Shimizu et al., 1997), *C. lagenarium* (*THR1* 67% identity; Perpetua et al., 1996), *M. grisea* (69%

identity; Vidal-Cros et al., 1994) and to a lesser degree *O. floccosum* (*THN1* 44% identity, Eagen et al., 2000). These reductases share about 40% identities with 1,3,6,8-THN reductases (Tsai et al., 1999; Thompson et al., 2000), but do have high homology with other biosynthetic pathway enzymes. They share 50–70% similarities with an *Aspergillus parasiticus* putative ketoreductase, encoded by the *ver1* gene, which is involved in the conversion of versicolofin A to sterigmatocystin in aflatoxin biosynthesis (Skory et al., 1992).

1.2.2.5 Conversion of vermelone to DHN

The conversion of vermelone to DHN involves the same SD that dehydrates scytalone to 1,3,8-THN (Butler et al., 1988). The Ser-129 mutant of the *M. grisea* SD causes the enzyme to favor vermelone over scytalone as a substrate (Basarab et al., 1999). The sequence of SD from *A. fumigatus* places an Ala in the position corresponding to the Ser-129 of *M. grisea* SD (Tsai et al., 1997). This change may make the *A. fumigatus* SD more vermelone-specific.

1.2.2.6 Polymerization of DHN to melanin

The final step of the DHN melanin pathway is the polymerization of DHN molecules to form the melanin polymer. It is believed that several enzymes could be involved in polymerizing and oxidizing DHN into melanin. In previous studies, oxidases and laccases have been proposed as the polymerizing enzymes in the pathway (Bell and

Wheeler, 1986). Two genes, *abr1* and *abr2* have been sequenced in *A. fumigatus*. Sequence analysis reveals that *abr1* encodes a putative protein of 664 amino acids that possesses two multicopper oxidase signatures and has 34% identity and 43% similarity to an iron multicopper oxidase from *Candida albicans* (Tsai et al., 1999). *Abr2*, on the other hand, encodes a protein of 587 amino acids which has 41% identity to a laccase encoded by *yA* of *A. nidulans* (Aramayo and Timberlake, 1990). The disruption of either *abr1* or *abr2* in *A. fumigatus* results in an alteration of the conidial color phenotype. These results indicate that the *abr1* and *abr2* deduced proteins are possibly involved in polymerization of DHN to form melanin.

1.2.3 Genetic organization of the DHN melanin synthesis genes

Studies to date indicate that the DHN melanin synthesis genes are clustered in some fungal species, but not in others. Gene clustering is thought to be beneficial for gene regulation and facilitate the horizontal transfer of the cluster (Keller and Hohn, 1997). DHN melanin synthesis gene clusters have been identified in *A. alternata* and *A. fumigatus* (Kimura and Tsuge, 1993; Tsai et al., 1999). The *A. alternata* gene cluster (about 30 kb) contains three genes, *ALM*, *BRMI*, and *BRM2*, which encode a PKS, a SD, and a 1,3,8-THN reductase, respectively (Kimura and Tsuge, 1993). The *A. fumigatus* gene cluster includes the 6 genes previously described (Section 1.2.2), *alb1*, *arp1*, *arp2*, *abr1*, *abr2* and *ayg1* in a 19 kb genomic DNA fragment. *A. alternata ALM*, *BRMI*, and *BRM2*, and *A. fumigatus alb1*, *arp1* and *arp2*, are all conserved among the known DHN-melanin pathways in other fungi. The DHN melanin synthesis genes,

however, are dispersed in the genome of *C. lagenarium* (Kubo et al., 1996). Genetic analysis of pigmentation mutants from *M. grisea* has shown that the DHN melanin synthesis genes are unlinked in *M. grisea*, as well (Chumley and Valent, 1990).

1.3 Inhibitors of the DHN melanin biosynthetic pathway

Many metabolic inhibitors have been found or developed for the DHN melanin synthesis pathway. The PKS involved in this pathway is believed to be similar to fatty acid synthetases. Therefore, cerulenin, the fatty acid synthetase specific inhibitor, produced by *Cephalosporium caerulens*, has been used to test whether it can block melanin production. In these studies cerulenin was found to inhibit melanin formation in the appressoria of *Pyricularia sp.* (Chida and Sisler, 1987), *C. lagenarium* (Kubo et al., 1987) and *A. alternata* (Hiltunen and Soderhall, 1992).

(N-phenoxypropyl)-careoxamide, and carpropamid have been developed as SD inhibitors to control rice blast disease (Jennings et al., 1999; Jordan et al., 1999; Kurahashi et al., 1999). These inhibitors may be specific to the DHN melanin pathway, because SD has no known functional counterparts in plants or animals (Jordan et al., 2000). It has been shown that carpropamid treated cultures of *C. lagenarium* accumulated scytalone (Kurahashi et al., 1997; Tsuji et al., 1997). Analyses of the interaction between a SD inhibitor, carboxamide and *M. grisea* SD mutants show that the His-85, His-110, Val-75, Phe-158 and Phe-162 of SD have significant influences on inhibitor binding (Jordan et al., 2000).

The fungicides tricyclazole and pyroquilon, used to control rice blast disease caused by fungi such as *M. grisea*, inhibit the reductases of the DHN melanin synthesis pathway (Woloshuk et al., 1980; Yamaguchi et al., 1982). The interaction of the inhibitors with the reductase is as follows: the hydroxyl groups of Ser-164 and Tyr-178 are hydrogen bonded to the inhibitors' carbonyl oxygen, and the phenyl ring of Tyr-223 is stacked with the inhibitors' ring system (Liao et al., 2001a). The 30-fold lower affinity of the 1,3,6,8-THN reductase for pyroquilon in contrast to that of the 1,3,8-THN reductase can be explained by unfavorable interactions between the anionic carboxyl group of the C-terminal Ile-282 of the 1,3,6,8-THN reductase and CH and CH_2 groups of the inhibitor (Liao et al., 2001b).

Numerous studies have confirmed that tricyclazole specifically inhibits the reductases of the DHN melanin synthesis pathway in various fungi such as *Sclerotinia sclerotiorum*, *V. dahliae*, and *W. dermatitidis* (Buchenauer et al., 1985; Wheeler, 1981, 1982; Wheeler and Stipanovic, 1985). Therefore, tricyclazole was suggested to be a specific inhibitor of the fungal DHN melanin synthesis pathway (Wheeler and Greenblatt, 1988). However, it has since been demonstrated to inhibit reductase activities in other polyketide synthesis pathways including the biosynthesis of the non-melanin pigment cynodontin in *Pyrenochaeta terrestris* (Lazarovits et al., 1989) and aflatoxin biosynthesis in *Aspergillus parasiticus* (Wheeler et al., 1989). Butler and Day (1998) have listed many other similar inhibitors of the reductases in the DHN melanin synthesis pathway, such as chlobenthiazone (Wheeler et al., 1989), phthalide (Uehara et

al., 1995; Wheeler and Klich, 1995; Chida, 1989), 2,3,4,5,6-pentachloro-benzyl alcohol and coumarin (Woloshuk and Sisler, 1982; Inoue et al., 1984a, 1984b). These inhibitors are used in either preventing fungal diseases or in studying the fungal DHN biosynthetic pathway.

1.4 The functions of fungal melanin

Fungi produce melanin in cell walls, conidial walls and other complex fungal structures, such as perithecia, sclerotia and ascocarps. In fungi, melanins have been associated with virulence, protection from environmental stresses and fungal development (Bell and Wheeler, 1986; Butler and Day, 1998).

1.4.1 Melanin's importance to fungal virulence

Melanin is essential for the virulence of some fungi. For examples, the cucumber pathogen, *C. lagenarium*, and the rice blast *M. grisea*, lose their virulence when their melanin production is blocked (Howard and Valent, 1996; Kubo et al., 1985; Kubo and Furusawa, 1986). In melanized appressoria cell wall permeability is limited, facilitating the accumulation of glycerol thereby allowing the generation of high turgor pressures required for host tissue penetration (Money, 1997). Without these high turgor pressures, the infection peg of the appressorium cannot mechanically penetrate the underlying host tissue. When melanin production is blocked, fungal pathogens, like *Colletotrichum* and *Magnaporthe* species, form colorless appressoria, which have a reduced ability to

penetrate the hosts' leaves. Similarly, disruption of the *alb1* gene encoding PKS in *A. fumigatus,* results in an albino conidia phenotype which has reduced virulence in humans (Tsai et al., 1998). Thus, conidial melanization in *A. fumigatus* appears to be important in the establishment of infection.

1.4.2 Melanin protects fungi from environmental stress

Melanin is essential for protecting fungi from environmental stresses, such as desiccation, extreme temperatures, UV irradiation, hydrolytic enzymes, microbial attack, and oxidization. Microsclerotia from mutants of *V. dahliae* blocked in melanin production cannot survive desiccation in soil, whereas wild-type microsclerotia are able to germinate when the soil is rehydrated for about 15 days (Bell and Wheeler, 1986). Conidia of an albino strain *Monilinia fructicola* were destroyed by heat treatment at 40°C for 30 min, while 50% of the wild-type conidia were still alive after this treatment (Rehnstrom and Free, 1996), indicating that in this fungus melanin is playing a role in resistance to high temperatures. Similarly, the melanized hyphae of *Gaeumannomyces graminis* were reported to resist higher temperatures than their non-melanized counterparts (Frederick et al., 1999). Melanin also provides UV protection for hyphae, conidia (Gadd, 1980), and yeast cells (Marbach et al., 1984). The conidia of an *A. alternata* melanin-deficient mutant lost their ability to germinate after an exposure to UV irradiation for 120 seconds, while the germination rate in the wild type was 57% after this treatment, indicating that the non-melanized conidia were more susceptible to UV (Kawamura et al., 1999). Similar results were observed with *Cladosporium pp.* and

Oidiodendron cerealis (Zhdanova et al., 1973), and in *C. neoformans* yeast cells, melanin also seems to provide some protection against UV (Wang and Casadevall, 1994). Fungal melanin also provides protection against oxidizing agents. Melanized cells of *A. alternata* and *W. dermatitidis* were 1000 times more resistant to hydrogen peroxide treatment than albino cells (Jacobson et al., 1995). Melanin deficient mutants of *A. nidulans* and *Cochliobolus sativus* were highly susceptible to digestion by β-1,3 and β-1,6-glucanase (Kuo and Alexander, 1967; Old and Robertson, 1970), indicating that melanin may play an important role in resistance to hydrolytic enzymes or microbial attack. Melanin also protects certain fungi like *C. sativus* against lysis in natural soils (Old and Robertson, 1970).

1.4.3 Melanin is involved in fungal development

In some fungi, melanin is involved in the development of perithecia and conidia. An albino mutant of *Ophiostoma piliferum* produces immature perithecia with non-viable ascospores. However, when scytalone is added to the media, melanin production is restored, and mature perithecia can be obtained (Zimmerman et al., 1996). However, other fungal species such as *C. heterostrophus* can develop lightly pigmented mature perithecia without melanin (Tanaka et al., 1991). Melanin also seems to be involved in *A. alternata* conidial development, as *A. alternata* melanin deficient mutants develop small conidia with fewer septa. The septa of the conidia in this species are melanized in the wild-type strain (Kawamura et al., 1999). In *A. fumigatus* the surface morphology of conidia lacking melanin is altered In this case, the unmelanized mutants produce

smooth conidia, and the wild type strain produces conidia with echinulate surfaces that facilitate the attachment of the spores to human tissues (Tsai et al., 1998; Jahn et al., 1997; Langfelder et al., 1998). Melanin has also been reported to facilitate invasive hyphal growth in *W. dermatitidis*, as wild-type strains of *W. dermatitidis* grow through 8% agar much faster than melanin-deficient mutants (Brush and Money, 1999).

1.5 Sapstaining fungi

Sapstaining fungi produce black or brown melanin and cause the discoloration of wood. There are three major groups of sapstain fungi. The first group includes the telomorphs and anamorphs of species from the ascomyceteous genera *Ophiostoma, Ceratocystis* and *Ceratocystiopsis*. Members of this group produce melanized mycelia and perithecia, or melanized conidia. The second major group comprises the black yeasts and includes fungi such as *Hormonema dematioides* and *Aureobasidium pullulans*. Melanin is produced in and outside the cell walls of these fungi. The third group includes molds that produce masses of conidia on wood surfaces and can also cause deep stain. *A. alternata*, *Cladosporium sphaerospermum* and *C. cladospoploides* are examples of this third group (Seifert, 1993). Other molds such as *Penicillium sp.* and *Trichoderma sp.* can also cause superficial discoloration on the surface of wood by producing pigmented spores (Zabel and Morrell, 1993). Our group conducted a Canada wide survey of sapstain fungi in 1997-98 and found that *O. floccosum* was frequently isolated across Canada. This fungus is a member of the *Ophiostomatoid* group (Uzunovic et al., 1999).

The first group of sapstaining fungi can colonize wood from wounded standing trees, during harvesting, or during final wood processing. Spores of sapstaining fungi germinate on wood and fungal hyphae then can grow radially through the ray parenchyma cells in sapwood (Ballard and Walsh, 1982). These rays provide fungi with easily assimilable substrates including soluble sugars, nitrogen and lipids (Subramanian, 1983; Gao et al., 1993; Merrill and Cowling, 1966; Terziev et al., 1993). Sapstaining fungi are unable to utilize the structural components of wood cell walls, such as cellulose and lignin. Fungi move from tracheid to tracheid via bordered pits or direct mechanical penetration of the cell wall using appressoria (Seifert, 1993). Melanin may play a role in the mechanical strengthening of both modified appressoria and mycelia to penetrate wood cell walls and the torus of bordered pits (Ballard and Walsh, 1982; Gibbs, 1993). Sapstain fungi grow best on wood at temperatures between 18 and 30°C, but serious staining also occurs on wood stored at temperatures as low as 3-8°C (Gibbs, 1993).

Sapstaining fungi produce melanin that causes cosmetic defects in wood that are costly for the wood industry. Melanized hyphae and conidiophores contribute to the appearance of the wood stain (Wheeler, 1983; Zink and Fengel, 1988). Wood discolorations or stain lead to wood product devaluation and insurance claims. Canada is the world's prime exporter of softwood lumber products and these exports contributed $36.2 billion to the Canadian trade balance (COFI, 2001). The Canadian lumber industry relies heavily on export markets and must spend millions of dollars annually to control the stain problem.

In Canada, lumber sapstain is controlled by either kiln drying the wood or by using anti-sapstain chemical treatments. Kiln drying is a process that reduces the moisture content of the wood to less than 20%. At this low moisture content fungal growth is inhibited as without water many fungal biochemical and enzymatic reactions are prevented (Zabel and Morrell, 1993). In British Columbia kiln drying is not always appropriate since some offshore markets demand green lumber, and large dimension timber is difficult to dry and may suffer drying defects. Furthermore, during ocean freighter shipments to offshore markets, lumber can re-wet and fungal growth and pigmentation can occur. Anti-stain chemicals inhibit fungal growth usually by the formation of a thin barrier that prevents the germination of fungal spores on the wood substrate (Zabel and Morrell, 1993). The chemicals are applied to the surface of lumber by dipping into tanks or by spraying. Before 1990, the polychlorophenates (PCP) were used. These chemicals were highly effective in controlling sapstain, but they were also environmentally hazardous and they have been banned in Canada and many other countries (Smith, 1991). Currently, several anti-sapstain controlling agents such as 2-(2-thiocyanomethylthio) benzothiozole (TCMM), sodium borate, and didecyl dimethyl ammonium chloride (DDAC) are being used in North America. However, they have a broad spectrum of action against organisms other than wood staining fungi and are not as effective as PCP for controlling stain. Thus, there is an urgent need for a new class of anti-fungal agents that demonstrates low environmental toxicity, known environmental fate, and high target specificity.

1.6 Research objectives

In order to implement methods of controlling wood stain, it is necessary to acquire detailed information regarding the mechanisms of growth and pigmentation of sapstain fungi on wood. However, specific knowledge concerning the biology, biochemistry, genetics and molecular biology of sapstaining species such as the *Ophiostoma* species is scarce.

Some physiological and biochemical aspects of *O. floccosum* 387N have been investigated (Abraham, 1995; Gao, 1996). Several fungal enzymes, which play key roles in the utilization of the nutritional resources found in wood, were identified and characterized using biochemical methods (Abraham et al., 1993; Gao and Breuil, 1995a, 1995b; Gao et al., 1993; Gao, 1996). Preliminary inhibition studies show that tricyclazole and cerulenin cause the reduction of pigment production in this fungus (Eagen, 1999). Genomic Southern analysis indicated that *O. floccosum* possesses sequences that are homologous to other fungal DHN melanin genes. Furthermore, a gene encoding a HN reductase has been cloned and characterized in *O. floccosum* (Eagen, 1999). These studies suggest that *O. floccosum* may utilize the DHN melanin biosynthetic pathway to produce pigment. As well, Eagen (1999) has demonstrated that nutrients affect fungal pigmentation. Further molecular studies of pigment production would allow us to understand the structures, functions, expression and regulation of the melanin genes in this fungus. This information would help researchers to either find specific targets to block pigment production or create albino mutants. For example,

specific chemicals could be designed to target key enzymes in melanin biosynthesis in these fungi, which could then be used to control wood stain. Albino mutants could be used in biological control of sapstaining fungi particularly at the harvest site. Since sapstaining fungi cause minimal structural damage to timber, a possible control method could be to simply prevent the growth of sapstain fungi by pre-inoculating susceptible wood with albino strains of the same species. The albino strains would then use up all of the available nutrients preventing the growth of the staining fungi. Inoculation of colorless mutants of *O. piliferum* on wood has been shown as a possible way to control wood stain in lab conditions (Uzunovic et al., 1999).

Since there is little information on melanin biosynthesis at a molecular level in *O. floccosum* 387N, the overall goal of this research project was to explore and understand the DHN melanin biosynthesis pathway in *O. floccosum* using a molecular approach, as biochemical techniques were likely to be difficult due to the instability of the pathway intermediates. In order to apply molecular approaches to study the functions of the genes in this pathway a genetic transformation system for this fungus was necessary. Therefore, the first objective of this research was to develop a genetic transformation system for *O. floccosum* 387N. An associated goal of this study was also to try to disrupt the reductase genes involved in melanin biosynthesis in this fungus. The results of this work are discussed in Chapter 2. SD is a key enzyme in the fungal DHN melanin pathway. Searching for sequence homology has shown that SD is only used in the fungal DHN melanin synthesis pathway. If we could isolate a SD from this fungus, we could provide more evidence that this fungus utilizes the DHN melanin pathway to

produce pigment. In addition, the *M. grisea* SD enzyme structure has been determined and several inhibitors have been developed to inhibit this enzyme. These inhibitors may be used to inhibit the SD in *O. floccosum*. Therefore, the second objective of this research was to isolate and characterize a SD gene in *O. floccosum*. The results of this work are discussed in Chapter 3. During a colleague's isolation of a reductase gene from *O. floccosum*, two DNA sequences, which were both reductase homologs, were isolated, however, only one was further characterized. Therefore, the third objective of this research was to characterize the second reductase gene and compare it with the first reductase gene. The associated goal of this work was to compare all fungal melanin reductases and find any association between their functions and their sequences. The results of this work are presented in Chapter 4. As we know that certain nutrients affected *O. floccosum* pigmentation, it was of interest to explore what happens at the transcription level to the melanin genes when the fungus is fed with different nutrients. The fourth objective of this study was therefore to determine the effect of nutrients on melanin gene expression. In order to include another important DHN melanin gene in the study, a partial PKS gene was isolated from the fungus. The results of this work are presented in Chapter 5. Finally, the conclusion and future directions of the study are discussed and presented in Chapter 6.

Chapter 2

Transformation of three *Ophiostoma* species and tentative genetic disruption of *O. floccosum* melanin genes

2.1 Introduction

Transformation is defined as a mechanism of genetic transfer whereby pure DNA extracted from one organism is able to induce permanent hereditary changes in the cells of a second organism, to which it is added (Finkelstein, 1991). Transformation techniques for the replacement, disruption or cloning of genes were first developed in the yeast, *Saccharomyces cerevisiae*. In the procedure used, yeast cells were first converted to protoplasts by enzyme treatment. After re-suspending the protoplasts in an osmotic support solution in the presence of $CaCl_2$, the exogenous DNA was added and the transformation effected by the addition of polyethylene glycol (PEG) (Hinnen et al., 1978). This technique is based on the idea that the transforming DNA integrates via crossover events at regions of shared homology between the incoming plasmid and the genome (Shortle et al., 1982; Rothstein, 1983). This PEG mediated transformation method has since been modified and adapted for the transformation of many species of filamentous fungi. Besides the PEG protocol, electroporation has also been employed to facilitate the uptake of foreign DNA. During electroporation, an electric pulse allows

fungal protoplasts, conidia, or yeast cells to absorb exogenous DNA (Van de Rhee et al., 1996; Yu et al., 1999; Chadha et al., 2000). Although this technique can be used to transform intact cells, it requires special equipment, such as a gene pulser transfection apparatus and a pulse controller.

Intact cells can also be transformed by biolistic bombardment. In this procedure, fungal cells are bombarded with microprojectiles, such as tungsten particles coated with a transformation vector, allowing the exogenous DNA to integrate into the fungal chromosomes (Armaleo et al., 1990; Durand et al., 1997). This method is easy to perform, but requires a gene gun. In addition, fungal cells of *Colletotrichum trifolii* treated with chemicals such as lithium acetate were shown to be amendable to transformation (Dickman, 1988). This technique is simple and fast, however, the transformation frequency of this method is lower than that of both the PEG mediated transformation and the electroporation methods. More recently, a transformation protocol with *Agrobacterium tumefaciens* has shown promising results with a few fungal species (Winans, 1992; de Groot et al., 1998; Gouka et al., 1999). *A. tumefaciens* has the natural ability to transfer a segment of DNA from its Ti plasmid, known as 'T-DNA' into plant and fungal cells so that the T-DNA integrates at random into the host's nuclear chromosomes. Several fungal species such as *Aspergillus awomori*, *Coccidioides immitis* and *Agaricus bisporus* have been successfully transformed by simply using a co-culture of fungal cells or spores with *A. tumefaciens* (de Groot et al., 1998; Gouka et al., 1999; Abuodeh et al., 2000). This method is simple, fast, and produces high transformation

rates. In addition, it improves the targeted integration of homologous DNA and produces transformants with a low copy number of integrated DNA.

Although many fungal transformation protocols have been developed, the transformation efficiencies of some fungi are still low depending mainly on the fungal species used and not the method used for transformation. Researchers have attempted to increase the transformation frequency of filamentous fungi by using restriction enzyme-mediated integration (Sanchez et al., 1998), where restriction enzymes are mixed with the transforming DNA and protoplasts during PEG mediated transformation. This technique has been shown to increase the transformation frequency in *A. nidulans* by between 20 - 60 fold, but the integration was random.

Among the transformation protocols, most researchers so far have used the PEG mediated transformation method to introduce foreign DNA, this protocol has also been applied to transform an Ophiostomatoid fungus *Ophiostoma ulmi* (Royer et al., 1991).

At the start of this thesis no system to transform any of the sapstaining fungi, including *O. floccosum*, had been developed. Development of such a system for sapstaining fungi would allow the examination of gene functions in more detail, for instance by enabling the construction of over-expression strains and knock-out mutants. As in this project we intended to isolate and characterise *O. floccosum* melanin genes to understand the functions of these genes, an efficient genetic transformation system for *O. floccosum* was critical. In this chapter, the development of a genetic transformation system for the

sapstaining fungi, *Ophiostoma quercus, O. floccosum,* and *O. piceae* with the hygromycin B resistance plasmids, pAN7-1 and pCB1004 is presented. We also described our efforts to disrupt two melanin genes, a reductase gene (*THN1;* isolated by Eagen, 1999) and a SD gene (*OSD1*; Chapter 3 in this thesis), which have been isolated in *O. floccosum* 387N.

2.2 Material and methods

2.2.1 Strains and culture conditions

O. floccosum 387N, which was previously misidentified as *O. piceae* 387N, was obtained from the Canadian Forintek culture collection. It belongs to the class Pyrenomycetes, the order Ophiostomales, and the genus *Ophiostoma*. In addition, three *O. piceae* strains (AU1, an isolate from this lab; H2009 and H2181; Brasier and Kirk, 1993) and three *O. quercus* strains (AU13, an isolate from this lab; H1039 and H1042; Brasier and Kirk, 1993) were used in this study.

Stock cultures were maintained on Oxoid malt extract agar (MEA: 33g malt extract agar and 10g technical agar/l of distilled water). To produce yeast-like cells for protoplast production, the isolates were grown either in complete medium (CM) or in proline medium (PM) at 22±1°C in rotating shakers at 230 rpm for 2-6 days (Harris and Taber, 1970; Kulkarni and Nickerson, 1981). For protoplast regeneration medium (RM), CM was supplemented with 0.6 M sucrose and the pH of the medium was adjusted to 6.1.

Except where noted, all chemicals and restriction enzymes were obtained from Sigma Chemical Company and Amersham Pharmacia, respectively.

2.2.2 Transformation vectors

The plasmids pAN7-1 and pCB1004 were generously provided by William Hintz (University of Victoria, Canada) and Louise Glass (University of California at Berkeley, USA), respectively. Both plasmids contain the *Escherichia coli hph* gene encoding a hygromycin B phosphotransferase under the control of the *Aspergillus nidulans trp*C promoter and terminator (Punt et al., 1987; Carroll et al., 1994). Plasmid pAN7-1 has an ampicillin resistance marker gene, while plasmid pCB1004 has a chloramphenicol resistance marker gene including a functional *lac*Z gene allowing blue/white screening (Carroll et al., 1994). The *E. coli* strain DH5α [F′ / *endA*1 *hsdR*17 (r_k-m_k^+) *supE*44 *thi*-1 *recA*1 *gyrA* (Nal^r) *relA*1 Δ (*lacZYA-argF*) *U*169 *deoR* (f80 *dlac* Δ (*lacZ*) *M*15] was grown in Luria broth (LB) at 37°C and used for bacterial transformation (Sambrook et al, 1989) and plasmid preparation. Plasmid DNA for fungal transformation was purified using a Plasmid Midi-Kit (QIAGEN) according to the manufacturer's instruction.

2.2.3 Protoplast production and separation

Protoplasts of *Ophiostoma* species were prepared from yeast-like cells through enzymatic digestion. Yeast-like cells were harvested from the fungal cultures by filtering through three layers of sterile cheesecloth, followed by centrifugation at 3000 rpm for 5 min. All

centrifugations in this experiment were done using a Sorvall RC24 centrifuge (Dupont) at 4°C. The cell pellet was washed twice with 40 ml sterile distilled water. The washed cells were then pre-treated for 20 min with 40 ml of 25 mM 2-mercaptoethanol, 5 mM Na_2EDTA at pH 8.0, then washed with 40 ml sterile distilled water, and digested for 2.5 h with 8 ml of filter sterilized Novozym 234 (20 mg/ml in 1 M $MgSO_4$). Protoplasts were separated from cell debris by centrifugation at 2000 rpm for 20 min. The supernatant containing the protoplasts was mixed with 42 ml of 0.6 M KCl and centrifuged at 640 rpm for 20 min. Then, the protoplasts were washed with STC buffer (1 M sorbitol, 50 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.5), and centrifuged at 640 rpm for 20 min. The protoplasts were then resuspended in STC buffer at a final density of 10^8 protoplasts/ml. Unless immediately used for transformation, the protoplasts were stored at −70°C with 60 μl of dimethyl sulfoxide (DMSO) and 10 μl of 2-mercaptoethanol until used for transformation.

2.2.4 Transformation

The method used was a modification of the transformation procedures described by Royer et al. (1991) and Wang et al. (1988). 1.0-1.5 x 10^7 protoplasts in 200 μl STC were mixed with 1 μl 2-mercaptoethanol, 2.5 ml of 40 or 66% PEG 3350 solution in TC buffer (50 mM $CaCl_2$, 2.5 mM Tris-Cl, pH 7.5) and 1, 3 or 5 μg vector DNA. After 40 min incubation at room temperature, 36 ml of STC buffer was added to the mixture of protoplasts and plasmid DNA, and the protoplasts were harvested by centrifugation at 640 rpm for 20 min. The harvested protoplasts were resuspended in 600 μl liquid RM.

After 4 h incubation at room temperature, aliquots were overlayed onto RM agar containing 200 μg/ml of hygromycin B. In all the transformation experiments, single spore isolations from positive transformants were performed to ensure single nuclear origin.

2.2.5 Disruption of a reductase and SD genes

To disrupt the reductase gene, the vector pANA476, based on the vector pAN7-1, was constructed by Eagan (1999). Two oligonucleotides, 2DVF1 and 2DVRI57, were designed and used in PCR reactions with pBSA4.2, a plasmid containing a full-length copy of the *O. floccosum* reductase gene (*THN1*). The forward primer, 2DVF1, was designed to add a 5' *Hind*III site, and the reverse primer, 2DVRI57 was to create stop codons in all of the reading frames and to add a 3' *Bam*HI site (Figure 2.1C, 2.1D). A product with the predicted size of 476 bp was obtained (Figure 2.1A and B) from PCR reactions with these primers. The PCR product obtained contained the first 157 codons of the putative N-terminal coenzyme-binding domain of *THN1*, the two incorporated restriction sites (*Hind*III and *Bam*HI) and stop codons. This PCR product and the plasmid pAN7-1 were digested with *Bam*HI/*Hind*III. The two generated *Bam*HI/*Hind*III fragments were then ligated together to form the vector pANA476 using T4 ligase (Amersham Pharmacia).

To disrupt the SD gene, the vector pBSD (Figure 2.2) was constructed by cloning a 4 kb *Bgl*II/*Hind*III pAN7-1 fragment containing the promoter region and the *hph* gene into the

*Bgl*II/*Hind*III site of the plasmid pBSU2 which contains a full length copy of the *O. floccosum* SD gene (*OSD1*). The *Bgl*II/*Hind*III digested pBSU2 contained a 5.5 kb *O. floccosum* genomic DNA fragment including a ~400 bp (5' region) of the *OSD1* ORF.

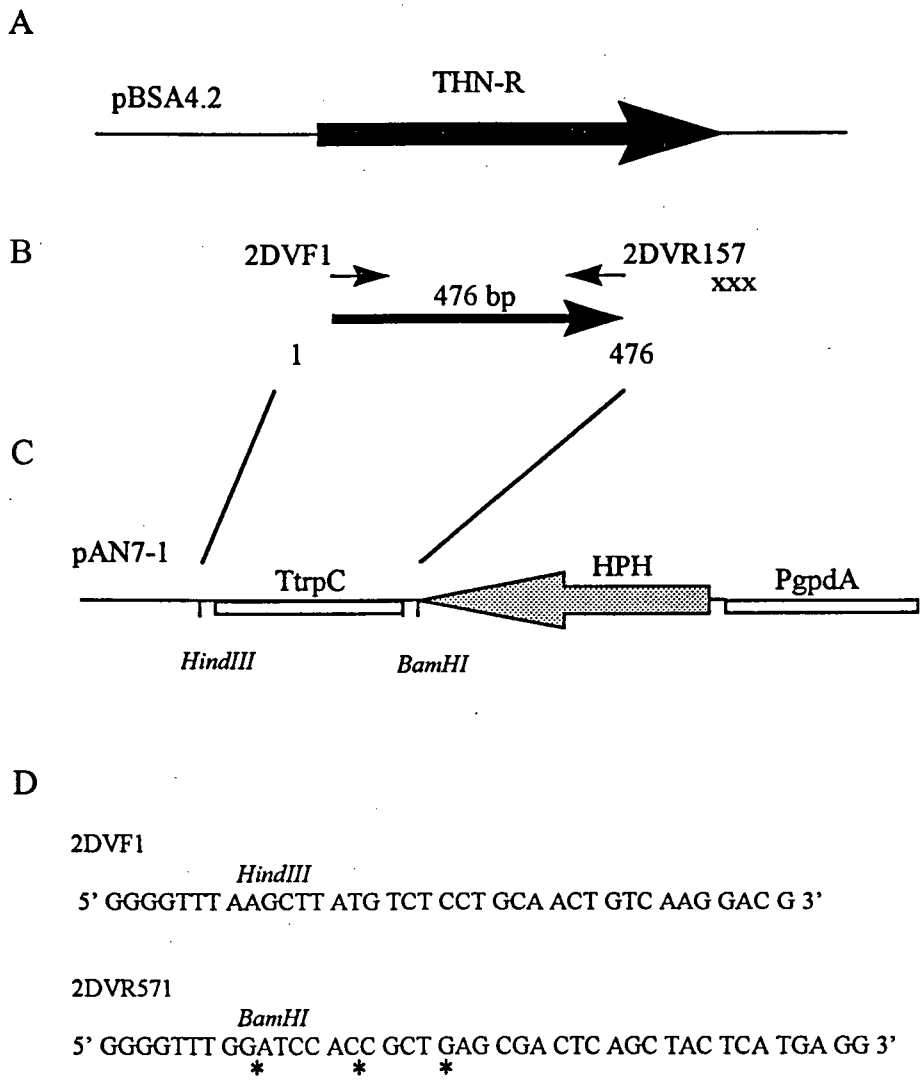

Figure 2.1. The construction of the *THN1* disruption vector pANA476. (A) The THN reductase gene in vector pBSA4.2 was used as template DNA with (B) disruption primers 2DVF I and 2DVRI 57. The PCR product was digested with *Bam*HI and *Hind*III and (C) ligated into the *Bam*HI/*Hind*III site of vector pAN7-1 to create pANA476. (D) The sequence of disruption primers showing the restriction sites and the location of the three stop codons as indicated by the asterisks.

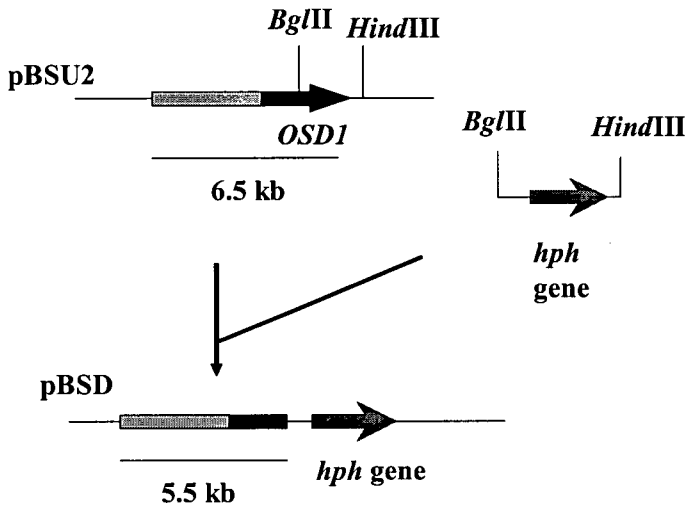

Figure 2.2. The construction of the *OSD1* disruption vector pBSD. The pBSU2 plasmid carrying a 6.5 kb *O. floccosum* genomic DNA containing the full-length *OSD1* gene was digested with *Hind*III/*Bgl*II, thus a 400 bp *OSD1* DNA fragment was removed. To construct the pBSD, the *Hind*III/*Bgl*II pAN7-1 fragment containing the *hph* gene and the expression promoter was cloned into the *Hind*III/*Bgl*II sites of the pBSU2.

Both disruption vectors, pANA476 and pBSD were used to transform *O. floccosum* 387N. The techniques for protoplasting and transformation of *O. floccosum* were as described above (2.2.3 and 2.2.4). Transformants were transferred onto 48-well cell culture clusters (Corning) containing B-medium agar (Abraham, 1995) supplemented with 200 μg/ml of hygromycin and 2% (w/v) filter-sterilized mannose. The transformants were incubated at 20-23°C for 30 days to observe their colour.

2.2.6 Genomic fungal DNA isolation

Genomic DNA was obtained from yeast-like cells of fungal transformants and non-transformants by using the glass bead shaking method of Ausubel et al. (1994). DNA concentrations and purities were determined by agarose gel electrophoresis or

spectrophotometrically using a GeneQuant DNA/RNA Calculator (Amersham Pharmacia).

Fungal genomic DNA from mycelia was prepared using a drilling method (Kim et al., 1999) as follows: approximately 200 mg of mycelium was collected by centrifugation or filtration using a Nitex membrane (Tetko) in 1.5 ml microcentrifuge tubes. The mycelium was broken in an extraction buffer (300 μl of 50 mM EDTA, 50 mM Tris-HCl, 3% SDS, pH 8.5) by drilling for 3 min at 600 rpm while on ice. After drilling 150 μl of 3 M sodium acetate was added and the samples were then frozen at -20°C for 20 min. The drilled mycelial extracts were then melted, centrifuged, and the DNA precipitated from the supernatant by adding an equal volume of isopropanol. The DNA was resuspended in 400 ml of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA), extracted twice with a mixture of phenol: chloroform: isoamyl alcohol (Amresco) and precipitated by the addition of 1 ml of EtOH (100%). The pellet was washed briefly by adding 400 μl of EtOH (70%) and dissolved in 100 μl of TE buffer.

2.2.7 Polymerase chain reaction (PCR)

PCR reactions were performed in 0.5 ml Omnitubes (Gordon Technologies) using a Hybaid TouchDown Thermal Cycler (InterScienecs). PCR reactions (50 μl) contained 100 ng of fungal genomic DNA, 40 pM of each primer, 50 μM dNTPs, 1X reaction buffer (10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl), and 1 unit of *Taq* polymerase (Appligene). Thermal cycling conditions were as follows: initial denaturation

(94°C, 4 min); followed by 30 cycles of denaturation (94°C, 50 sec), annealing (55°C, 50 sec) and primer extension (72°C, 50 sec); and one final cycle of primer extension (72°C, 5 min). Reaction products (15 μl) were analyzed by electrophoresis on 1.2 % agarose gels in 1X Tris-acetate EDTA (TAE) buffer including 1% ethidium bromide. The DNA was visualized under UV light and documented with an Image Analyzer (IS-500 Digital Image System, Alpha Innotech Co.). To amplify the *hph* gene, the PCR primer pair hphF (5'-ATG CCT GAA CTC ACC GCG AC-3') and hphR (5'-CT ATT CCT TTG CCC TCG GAC-3') was synthesized based on nucleotide sequences of the 5' and 3'- end of the coding region of the *hph* gene.

2.2.8 Novozym-aid PCR (Novo-PCR)

For PCR screening the *O. floccosum* transformants, spores (10 μl X 10^8 spores/ml) were inoculated into the wells of 96-well cell culture clusters (Corning) containing 200 ~ 300 μl CM in each well. The cultures were incubated 30 ~ 40 hours at 28°C without shaking. Mycelium was then transferred into a sterile eppendorf tube containing 50 μl of Novozym 234 (3 mg/ml in 0.8 M KCl, 10 mM Citric acid). After incubation for 60 min at 37°C, 150 μl of dilution buffer (10 mM Tris-HCl, pH7.5, 10 mM NaCl and 1 mM EDTA) and 20 μl of 2.5% sodium dodecyl sulfate (SDS) were added to the mixture. The mixture was then heated for 3 min at 100°C, cooled on ice for 5 min and then centrifuged at 13,000 rpm, for 5 min. 2~3 μl of the supernatant was removed for PCR as mentioned above but with the addition of 2% DMSO.

2.2.9 Genomic DNA dot blot and Southern hybridization analysis

hph DNA was PCR-amplified from the pAN7-1 plasmid, separated on a 1.2% agarose gel in 1X TAE buffer containing 1% ethidium bromide and purified using a GeneClean Spin Kit (Bio101). *hph* DNA was then labelled with 3000 Ci/mmole [^{32}P]α-dATP (Amersham Pharmacia) using the Random Primers DNA Labelling System (GIBCOBRL). Two μg of genomic DNAs from the transformants and non-transformants were then dot-blotted onto a Zeta-Probe GT membrane (Bio-Rad) using a BIO-RAD dot blot system as recommended by the manufacturer. The membrane was prehybridized at 65°C for 1 hour in 20 ml of hybridization solution (7% SDS, 0.25M Na_2HPO_4, pH7.2). Hybridization with the *hph* probe was performed overnight at 65°C in hybridization solution. The membrane was then washed twice for 30 min at 65°C first in 5% SDS, 20 mM Na_2HPO_4, pH 7.2, and then in 1% SDS, 20 mM Na_2HPO_4, pH 7.2, and finally subjected to autoradiography using Kodak X-OMAT film.

For performing Southern blot analyses, fungal genomic DNAs (10 μg each sample) were digested overnight at 37°C with *Hind*III, which does not digest *hph*, fractionated in a 1.0% agarose gel, denatured in 0.25M HCl for 15 min, and transferred onto Zeta-Probe GT blotting membrane (Bio-Rad) using 0.4M NaOH as a transfer solution. Southern blot analysis was performed with *hph* DNA as a probe. Probe labelling and hybridization conditions were the same as those described above.

2.3 Results

2.3.1 Transformation system

In order to transform *O. floccosum*, *O. piceae* and *O. quercus,* we produced protoplasts from fungal yeast-like cells (Figure 2.3). Once high quality protoplasts were obtained, the protoplasts were transformed using two different transformation vectors. Conditions for obtaining transformants, protoplast production and transformation efficiency were examined.

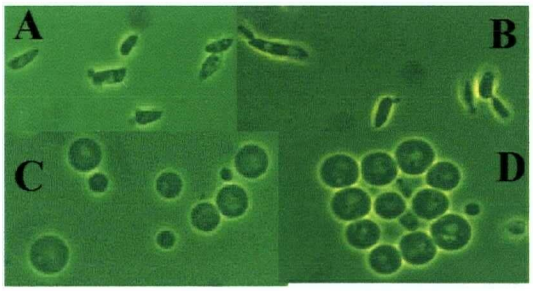

Figure 2.3. Yeast cells (A, X 100) and budding cells (B, X 100) and protoplasts (C, X 400 and D, X 400) of *O. floccosum* after treating the yeast cells with Novozym 234.

2.3.1.1 Optimization of protoplast production conditions

Several important variables such as cell age and Novozym 234 concentration were tested to determine the optimum conditions for protoplast release and regeneration in *O. floccosum* 387N. It was found that in cultures with OD_{600} values from 0.6 to 0.8, budding cells (Figure 2.3B) were actively produced and were most sensitive to digestion by Novozym 234. The production of budding cells and protoplast yield decreased when the OD_{600} value of the cultures reached or was above an OD of 1.0 (Table 2.1).

Table 2.1. The relation among OD_{600} values, yeast-like cell budding rates, and protoplasting rates of *O. floccosum* 387N cultured in proline media.

OD_{600} value	Cell budding rate (%)	Protoplasting rate (%)
0.6	76	83
0.8	70	80
1.0	33	28

In preliminary tests, Novozym 234 concentrations of less than 10 mg/ml did not release protoplasts from *O. floccosum* yeast-like cells, while Novozym 234 concentrations of 30 mg/ml destroyed most cells within 1.5 h digestion. Novozym 234 concentrations of 20 mg/ml resulted in the release of large numbers of protoplasts in a 2 h digestion (Figure 2.3 C and D; Table 2.2). The obtained protoplasting rates were found to vary with the different batches of Novozym 234 used, and Lot number of 96H0503 was determined to be the most effective for protoplasting yeast cells.

Table 2.2. Production of protoplasts of *O. floccosum* 387N using different enzyme concentrations and digestion time.

Concentration of Novozyme 234 (mg ml^{-1})	Digestion time (h)	Protoplasting rate (%)
10	1.0	20
	1.5	52
	2.0	57
20	1.0	50
	1.5	76
	2.0	83

Fungal cells pre-treated with 25 mM 2-mercaptethanol for 20 min released 5 times more protoplasts than cells without pre-treatment. However, increasing pre-treatment time to 40 min or increasing concentrations of 2-mercaptethanol to 50 mM did not improve the protoplasting rate. With 100 ml cultures of yeast-like cells grown in PM, about 9.5 x 10^6 protoplasts was generally obtained under the optimum conditions.

The optimum conditions for protoplasting *O. floccosum* 387N were then applied to the two fungal species, *O. piceae* (strains H2009, H2181, and AU1) and *O. quercus* (strains H1039, H1042, and AU13). Protoplast production from the *O. piceae* isolates was similar to 387N, with 70-90% of the total cells forming protoplasts. However, protoplast production from the *O. quercus* isolates reached only 20-30% as these isolates did not produce actively budding cells in PM. These results led us to examine the growth form of these fungi in CM.

All the isolates of *O. floccosum, O. piceae* and *O. quercus* produced yeast-like cells in CM, and it was found that yeast-like cells at OD_{600} values ranging from 1.4 to 1.9 were most suitable for protoplasting, with protoplast production of 90-100% within 2 h of digestion. Protoplast yields reached around 5 x 10^7 protoplasts from 30 ml cultures of the cells. These results showed that to obtain high protoplast yields, CM is better than PM. Interestingly, there was no apparent difference in the protoplasting rates among *O. floccosum, O. piceae* and *O. quercus* when the isolates of these three species were grown in CM.

2.3.1.2 Transformation conditions and regeneration

Before performing transformation of the protoplasts, their sensitivity to hygromycin was tested. All the isolates were sensitive to 100 μg/ml hygromycin, except isolate H2009 which was sensitive to 150 μg/ml. Consequently, to ensure dominant selection for the hygromycin resistance marker, the regeneration medium was supplemented with 200 μg/ml hygromycin. Regeneration rates of protoplasts on RM agar without hygromycin were about 60-70% for all the isolates tested.

To examine the effects of several parameters on the transformation efficiency initial experiments were performed with *O. floccosum* 387N using either 3 or 5 μg of pAN7-1. In preliminary tests with 40% PEG, only ~20 transformants were obtained for each experiment. Thus, the PEG concentration used was increased up to 50% or 66%. Both concentrations produced high transformation rates displaying above 10^3 transformants per μg of vector DNA, however, a 30 - 90 times higher transformation rate was obtained with 66% PEG than with 50% PEG (Table 2.3). Testing with different molecular weights of PEG molecules, PEG 3350 and PEG 8000, did not have much effect on the transformation rate. The transformation rate decreased by 10% when the PEG used was pre-warmed at 37°C. Decreases in transformation rates were also observed when the protoplasts were stored overnight at 4°C or at −70°C. The transformation rate of freshly prepared protoplasts was 1.1×10^5 transformants/μg vector DNA, while the transformation rates of 4°C and −70°C stored protoplasts were 7.0×10^4 and 2.7×10^3 transformants/μg vector DNA, respectively.

Table 2.3. The effects of PEG concentrations on transformation rates of *O. floccosum* 387N transformed with different amounts of pAN7-1 DNA.

PEG (%)	Transformants/ μg DNA	Transformants/ μg DNA
	3 μg DNA	5 μg DNA
50	1.3×10^3	1.2×10^3
66	3.7×10^4	1.1×10^5

2.3.1.3 Transformation of various *O. piceae* and *O. quercus* isolates with pAN7-1 and pCB1004

The optimum transformation conditions determined for 387N were applied to the *O. piceae* and *O. quercus* isolates with both pAN7-1 and pCB1004. Transformants were obtained from all the isolates. The effects of different concentrations of transformation vector on the transformation rates of these isolates were examined. The transformation rates were 10 times lower with 8 or 10 μg of DNA than with 1, 3, or 5 μg of DNA from either plasmid. Therefore, all the isolates were transformed with 1, 3, or 5 μg of either pAN7-1 or pCB1004. Three rounds of transformation were performed and the results are summarized in Table 2.4.

For all the isolates tested, the transformation rates were about 10^4~10^5 transformants/μg DNA for either pAN7-1 or pCB1004. For *O. quercus*, all three isolates yielded high transformation rates when 1 μg of either plasmid was used in the transformation. All of the *O. floccosum* and *O. piceae* isolates yielded the highest transformation rates when 1 μg of the pCB1004 DNA was used.

Table 2.4. The effects of DNA amount on the transformation rates of *O. floccosum, O. piceae* and *O. quercus* isolates transformed with pAN7-1 or pCB1004.

Isolates	Transformatants/ μg DNA pAN 7-1 (μg)			Transformatants/ μg DNA pCB1004(μg)		
	1	3	5	1	3	5
387N	1.3×10^3	3.7×10^4	1.1×10^5	6.0×10^4	1.2×10^3	1.2×10^3
H2009	8.2×10^5	3.7×10^5	1.9×10^5	7.7×10^5	2.6×10^5	2.0×10^5
H2181	5.2×10^4	2.0×10^4	9.0×10^4	7.5×10^4	2.3×10^4	3.6×10^4
AU1	3.3×10^4	5.0×10^4	2.4×10^4	3.3×10^4	3.4×10^4	1.5×10^4
H1042	4.8×10^5	1.6×10^5	1.5×10^5	4.7×10^5	2.5×10^5	1.9×10^5
H1039	4.0×10^5	2.4×10^5	8.8×10^4	5.7×10^5	1.6×10^5	8.5×10^4
AU13	1.2×10^5	4.8×10^4	5.3×10^4	1.0×10^5	5.8×10^4	3.9×10^4

Notes: 387N: *O. floccosum* 387N strain; H2009, H2181 and AU1: *O. piceae* strains; H1042, H1039 and AU13: *O. quercus* strains.

2.3.1.4 Analysis of transformants

Transformants from each isolate with either pAN7-1 or pCB1004 were observed as pinhead colonies after 2 or 3 days of incubation at 23°C on RM agar containing 200 μg/ml of hygromycin B. Approximately two percent of the transformants grew with a normal growth rate, whereas the rest of the transformants grew slowly. Sixty transformants for each isolate of *O. floccosum, O. piceae* and *O. quercus* were randomly selected and grown on CM medium containing 300 μg/ml of hygromycin. Among these transformants, 65% were unable to grow, suggesting that only 35% of the transformants were stable. From the transformants that grew seven transformants were randomly selected for each isolate and used for molecular analysis. Genomic DNA was isolated from both the selected transformants and the wild type strains of *O. floccosum, O. piceae*

and *O. quercus* and subjected to PCR analysis. *hph* DNA was successfully amplified from all the transformants through PCR, whereas no amplification of *hph* DNA was observed from the wild type isolates (Figure 2.4). Dot blot analysis using a radiolabelled *hph* gene fragment confirmed that the transformants contained the *hph* gene sequences (Figure 2.5). However, differences in signal intensity in the dot blot hybridization among the transformed isolates were observed.

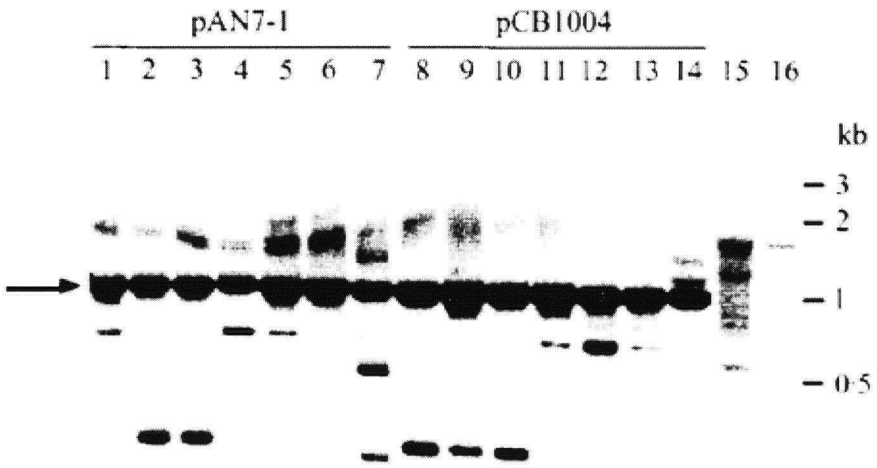

Figure 2.4. PCR analysis of *Ophiostoma* isolates transformed with plasmids pAN7-1 and pCB1004 using primers specific for hygromycin B phosphotransferase gene. PCR products (10 μl) were separated electrophoretically in a 1.2 % agarose gel and stained with ethidium bromide. Lanes 1 and 8: AU13; 2 and 9: H1042; 3 and 10: H1039; 4 and 11: AU1; 5 and 12: H2181; 6 and 13: H2009; 7 and 14: 387N; 15: wild type 387N; 16: 1kb DNA ladder. Arrow indicates location of hygromycin B phosphotransferase DNA bands. 387N: *O. floccosum* 387N strain; H2009, H2181 and AU1: *O. piceae* strains; H1042, H1039 and AU13: *O. quercus* strains.

To evaluate the mitotic stability of the transformants, the seven randomly selected transformants from each isolate were transferred six times onto CM agar at 5-day intervals and transferred back onto CM agar containing 300 μg/ml and 800 μg/ml hygromycin B. All transformants retained resistance to hygromycin, indicating good mitotic stability.

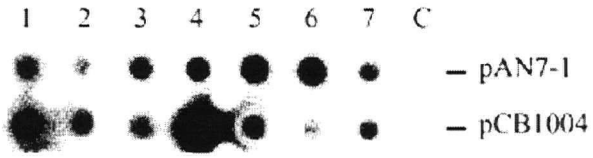

Figure 2.5. Dot blot analysis of *Ophiostoma* isolates transformed with plasmids pAN7-1 and pCB1004. Genomic DNA (2 μg) of each isolate was blotted on Zeta-Probe membrane and hybridized with ^{32}P-dATP-labeled hygromycin B phosphotransferase DNA. Lanes 1: AU13; 2: H1042; 3: H1039; 4: AU1; 5: H2181; 6: H2009; 7: 387N; C: wild type 387N. 387N: *O. floccosum* 387N strain; H2009, H2181 and AU1: *O. piceae* strains; H1042, H1039 and AU13: *O. quercus* strains.

2.3.2 Genetic disruption of melanin genes in *O. floccosum*

After establishing the transformation system, we attempted to disrupt a reductase and a SD gene in *O. floccosum*. The objective of this work was to elucidate the functions of these genes in *O. floccosum* melanin biosynthesis.

2.3.2.1 Genetic disruption of an *O. floccosum* 387N reductase gene

To disrupt the reductase gene, *O. floccosum* 387N was transformed using the reductase gene disruption vector, pAN-A467. Three rounds of transformation were performed using pAN-A467 DNA, and about 150 transformants were retrieved from CM agar containing 200 μg/ml hygromycin. Most of the transformants were brownish after 3 weeks of incubation. Around 10% of the transformants were dark-brown in colour.

To find any reductase gene disruptants we screened the transformants using a PCR approach. The primer combinations of 414F/hphF, and 414F/1756R (414F: 5' CCT TTC GGA CTT CAG AAT GC 3' and 1756R: 5' ATT GTC CGA GGT GGC AAT AAC G 3') were used to screen the transformants (Figure 2.6). In the first round of screening, five light-brown and 11 dark-brown *O. floccosum* transformants were screened. With the primer combination of 414F/hphF, no PCR product was amplified in these transformants, but PCR products of about 1.4 kb were amplified with the primer pair of 414F/1756R (Figure 2.7). This size of PCR product was the same as that of the PCR product amplified from the wild type strain with the same primers. This suggested that no homologous integration had occurred in these transformants. Due to the difficulty of visually determining the colour of these fungi, we further PCR-screened the remaining *O. floccosum* transformants. However, no reductase gene disruptant was found.

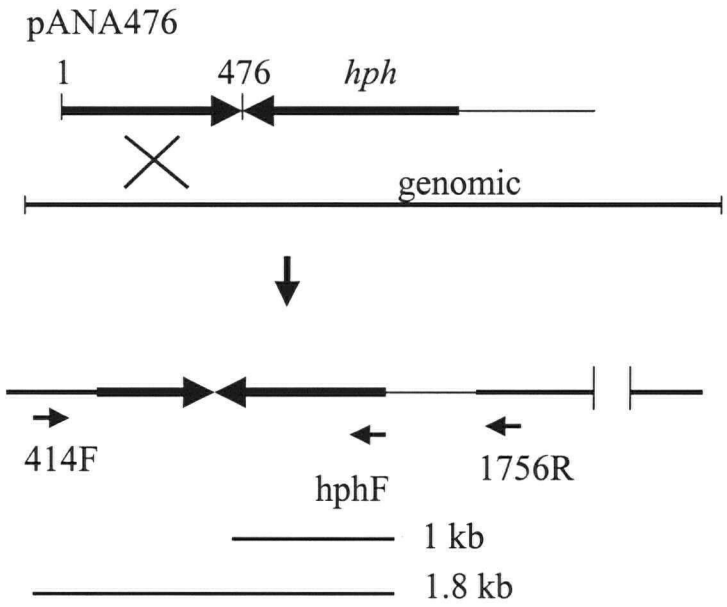

Figure 2.6. The assumed integration event by homologous recombination of the pANA476 into genomic 387N DNA. The location of PCR primers and the sizes of the predicted products if occurs as in the model.

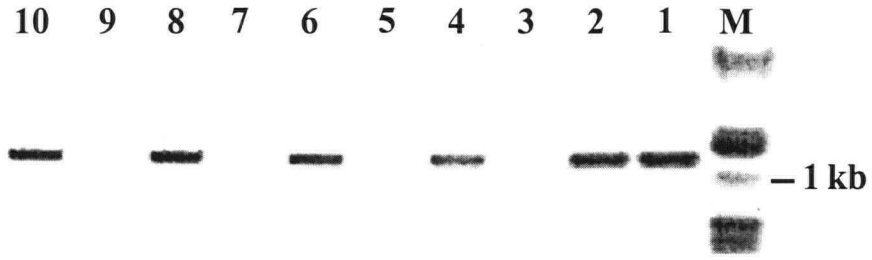

Figure 2.7. PCR screening pANA476 transformed *O. floccosum* transformants. PCR products (15 μl) were separated electrophoretically in a 1.2 % agarose gel and stained with ethidium bromide. Lanes 1: wild type strain, 387N; lanes 2, 4, 6, 8 and 10: transformants used to perform PCR using primers 414F/1756R; lanes 3, 5, 7 and 9: transformants used to perform PCR using primers 414F/hphF; lane M, 1kb DNA ladder.

2.3.2.2 Genetic disruption of an *O. floccosum* 387N SD gene

We retrieved 1392 and 808 transformants after transforming 387N with a circular or *Sac*I-digested linear *OSD1* disruption vector (pBSD), respectively. To observe the colour of the transformants, they were transferred onto B-medium supplemented with 2% mannose. After a 4-week incubation the transformants were divided into five groups based on their colour and growth patterns (Table 2.5). Among the 2100 transformants, two produced mixed culture of yeast mycelia with white colour (YM), while the others produced mycelia only. Twelve transformants were white (W) and one hundred and twenty eight transformants were white but changed to slightly brownish after 20-day incubation (LB). Many transformants (1050) produced a brown colour similar to the wild type strain (B), whereas 868 transformants were dark brown (DB). PCR was used to search for the integration of the target vector into the wild type *OSD1* gene in the transformants. One hundred seventy transformants belonging to different phenotypic groups (2 YM, 12 W, 20 LB, 70 B, and 66 DB) were screened using PCR. In the PCR reactions, the primer pair SD23/SD24 (SD23: 5' GGC CTC AAT ATC AGC AGC CTC A 3'; SD24: 5' AAC CAG CGG ATC TTG GGA TCG A 3') was used to detect whether the whole or partial vector DNA was inserted into the transformants. Using this primer pair, a 0.6 kb fragment could be amplified from the wild type strain. At the same time, the primer pair PANS/SD24 (PANS: 5' ACT CGT CCG AGG GCA AAG GAA 3') was used to detect junctions between the recipient *OSD1* and the integrated vector DNA (Figure 2.8). If integration into the genomic *OSD1* gene had occurred, a PCR product of

1.0 kb would be amplified. However, the 170 transformants screened produced a 0.6 kb fragment with the primers SD23/SD24 and no product when the primers PANS/SD24 were used (Figure 2.9). This suggested that the *OSD1* gene was not disrupted in these transformants.

Table 2.5. Different phenotypes of the transformants transformed using the *OSD1* disruption vector pBSD.

Phenotype	Number of the transformants	Characteristics
White (W)	12	Mycelia with white colour
Mixed culture of yeast mycelia (YM)	2	Mycelia and yeast-like cells with white colour
Light Brown (LB)	168	Mycelia with white colour after 20-day incubation but turned to slightly brownish after 30-day incubation
Brown (B)	1050	Mycelia with light brown colour which is similar to the wild type strain
Dark brown (DB)	868	Mycelia with dark brown colour

Because the *OSD1* disruptant was not found it was desirable to screen the remaining transformants (~1,940). However, screening of fungal transformants using PCR is limited by the difficulty of extracting genomic DNA. Therefore, we examined several different fungal genomic DNA extraction methods to determine which method was the most efficient. When using the drilling method, we could extract genomic DNA from 12-20 samples per day, while we could work on 40 samples per day using the glass-bead method. However, fungal yeast-like cells were required in the glass-bead DNA extraction

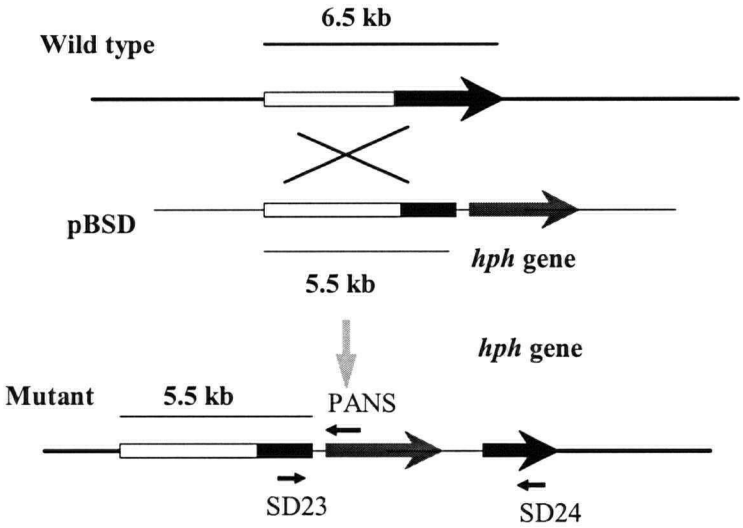

Figure 2.8. The assumed integration event by homologous recombination of the pBSD into genomic 387N DNA. The location of PCR primers and the sizes of the predicted products if occurs as in the model.

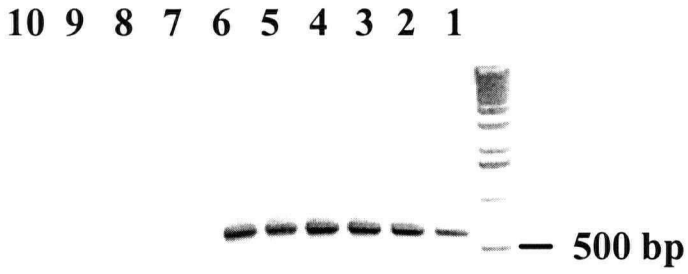

Figure 2.9. PCR screening pBSD transformed *O. floccosum* transformants. PCR products (15 μl) were separated electrophoretically in a 1.2 % agarose gel and stained with ethidium bromide. Lanes 1: wild type strain, 387N; 2-6: transformants used to perform PCR using primers SD23/SD24; 7-10: transformants used to perform PCR using primers SD23/PANS; M: 1kb DNA ladder.

method and 387N did not produce many yeast-like cells. Therefore, we developed a Novozym-aid PCR (Novo-PCR) protocol to screen the remaining fungal transformants. In this protocol, Novozym 234 is used to release the genomic DNA from the fungal cells as it removes fungal cell walls.

Several conditions were examined to improve the efficiency of the protocol. In Novo-PCR reactions (20 μl), different final concentrations of Triton X-100, 0.1%, 0.5% and 1% were tested. PCR products were easily amplified when Triton X-100 was added at 0.5% or 1%, while no PCR products were observed when Triton X-100 was added at 0.1% (Figure 2.10). Final concentrations of 2%, 5%, 8% and 10% of DMSO were also tested in the PCR reactions. A strong band was observed when the reaction contained 2% DMSO (Figure 2.11). This protocol allowed us to screen 100 samples per day. Therefore, we screened ~1,940 transformants using Novo-PCR and the primers mentioned above.

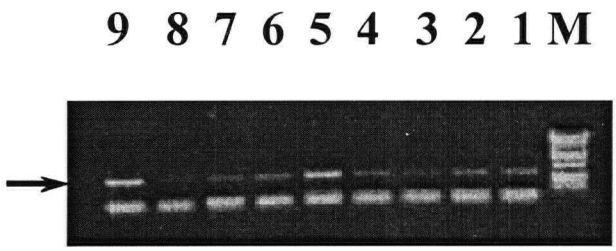

Figure 2.10. Novo-PCR with Triton-100. PCR products (15 μl) were separated electrophoretically in a 1.2 % agarose gel and stained with ethidium bromide. Lanes 1: wild type strain, 387N; 2-9: pBSD transformed *O. floccosum* transformants used to carry on PCR with primers SD23/SD24. Triton-100 was added in the PCR reaction up to 1% (lanes 5, 9), 0.5% (lanes 1, 2, 4, 6, 7) and 0.1% (lanes 3, 8 respectively).

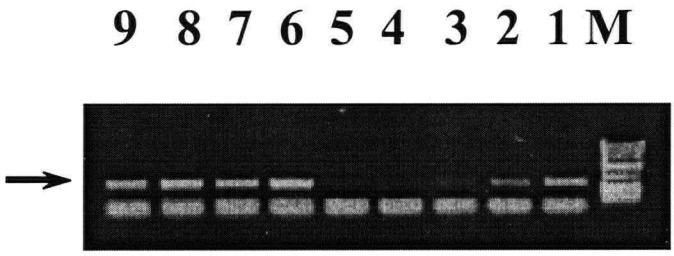

Figure 2.11. Novo-PCR with DMSO. PCR products (15 μl) were separated electrophoretically in a 1.2 % agarose gel and stained with ethidium bromide. Lanes 1: wild type strain, 387N; 2-9: pBSD transformed *O. floccosum* transformants used to carry on PCR with primers SD23/SD24. DMSO was added in the PCR reaction up to 2% (lanes 1, 6-8), 5% (lane 2), 8% (lane 3) and 10% (lanes 4-5).

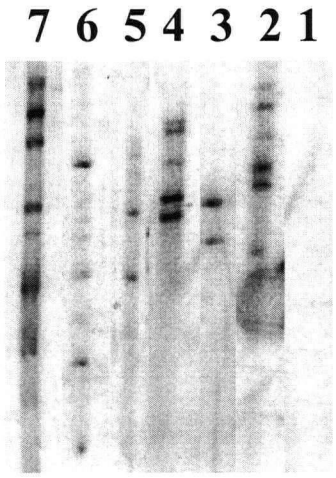

Figure 2.12. Southern hybridization of the *O. floccosum* transformants with pBSD. Genomic DNA (10 μg) of each isolate was blotted on Zeta-Probe membrane and hybridized with ^{32}P-dATP-labeled *hph* DNA. Lanes 1: 387N and 2-7: transformants.

Unfortunately, no *OSD1* gene disruptant was found by PCR-screening the 1,940 transformants. To confirm the integration of the disruption vector into the fungal genome,

20 transformants were selected to perform Southern blot analysis using *hph* DNA as a probe. The wild type 387N strain was included as a negative control. No band was visible in the control, but 2-8 bands of at least 12 kb were seen in the transformants (Figure 2.12).

2.4 Discussion

2.4.1 Transformation

In the transformation study, Novozym 234, a multi-enzyme extract of *Trichoderma* was used as it has been shown to be effective in digesting the fungal cell walls of many fungi (Beach & Nurse, 1981; Brygoo & Debuchy, 1985; Ballance et al., 1983, 1985; Yelton et al., 1984; Vollmer & Yanofsky, 1986). This enzyme preparation contains a complex mixture of hydrolytic enzymes, with high levels of 1,3-glucanases and chitinases. Novozym 234 concentrations of less than 10 mg/ml, which worked in other filamentous fungi, did not release protoplasts well in 387N yeast-like cells. A Novozym 234 concentration of 20 mg/ml produced the highest protoplasting rates with the *Ophiostoma* isolates tested. It is known that the *Ophiostoma* species have cellulose in addition to chitin in their cell wall (Jewell, 1974). In addition, chitin contents in fungal cell wall are affected by culture media, culture conditions and cell age (Ouellette et al., 1995). These findings might explain why a higher concentration of enzyme was necessary to obtain protoplasts in our studies.

We also found that different batches of Novozym 234 affected the protoplasting rates of our isolates, similar findings have been reported by other researchers (Akins & Lambowitz, 1985; Kinnaird et al., 1982; Kinsey & Rambosek, 1984). This is probably due to the lack of specificity of the enzyme preparation, since these preparations are not marketed specifically for fungal protoplasting. It was found that PEG concentrations influenced the transformation rate. Higher concentrations of PEG increased the transformation efficiency, as high concentrations of PEG likely cause the treated protoplasts to clump, and this may facilitate the trapping of DNA (Fincham, 1989). We observed that the incubation of PEG, DNA and protoplasts at 37°C decreased the transformation efficiency. This may be due to protoplast damage occurring at these temperatures as it has been reported that temperatures above 30°C damage protoplasts (Shirahama et al., 1981). Transformations of freshly made protoplasts were more efficient than transformations of protoplasts that were kept overnight at 4°C or 1 month at -70°C. These data suggested that low temperatures influenced the stability of 387N protoplasts, subsequently reducing the transformation rate. As well, different vectors and their amounts affected the transformation rates of the isolates. When we used pCB1004 to perform the transformations, the transformation rates were higher at lower pCB1004 concentrations. These results were in agreement with the data reported for *A. nidulans* transformation (Yelton et al., 1984).

In the DNA dot blot analysis, differences in signal intensity of dot hybridization were apparent among the transformed isolates, suggesting that different numbers of *hph* gene copies were present in the transformants. The Southern blot analysis of the transformants

confirmed that several copies of the transformation vector were integrated into the fungal genome. It seems that these fungi are good recipients of foreign DNA, and this could explain why the transformation rates of our isolates were 100 to 1000 times higher than the transformation rates in other fungi, which reach value of about $10 - 10^3$ transformants per μg of DNA (Fincham, 1989).

2.4.2 Gene disruption

Disruption of the melanin genes *THN1* and *OSD1* from 387N, was attempted using the established transformation system. The transformants were screened using a PCR approach but no disruptants were found. The attempts to disrupt *THN1* in 387N by a colleague also failed (Eagan, 1999). In her attempts, five putative *THN1* disruptants were obtained by PCR screening. However, hybridization studies of these putative disruptants suggested that targeted integration of the disruption vector into the genomic gene had not occurred and that the PCR results were artefacts.

Our attempts to disrupt the *THN1* and *OSD1* genes and Eagen's attempts to disrupt the *THN1* gene suggested that homologous recombination in *O. floccosum* might be a rare event, although the transformation efficiency of *O. floccosum* was high. Homologous recombination is also a rare event in *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Coccidioides immitis* transformants (Lodge et al., 1994; Woods and Heinecke, 1996; Yu and Cole, 1998). Phenotypic characterization of the transformants, however, showed that some of the transformants were significantly different from the wild type strain, as

some of the transformants had reduced or increased pigmentation. A possible explanation for these differences may be that the transformation event, while not appearing to affect the *OSD1* or *THN1* genes directly, may have randomly integrated into other genes, which may be involved in the regulation of melanin biosynthesis. Our Southern blot analyses of the transformants showed that multiple copies of *hph* were integrated into the *O. floccosum* genome. Heterologous DNA integration has been reported in the transformation of species from *Trichoderma, Gliocladium*, *Cryptococcus* and *Coccidioides* (Lorito et al., 1993; Toffaletti et al., 1993; Yu and Cole, 1998). Furthermore, the relative level of non-homologous integration versus integration at a site of homology in filamentous fungi depends on both the gene being utilized and the specific recipient strain that is employed (Kim and Marzluf, 1988).

Recently, a gene was successfully disrupted in another *Ophiostoma* species, *O. ulmi* (Hintz, 1999). The transformation rate of *O. ulmi* is similar to that of our *Ophiostoma* isolates. In their work, the disruption cassette was constructed so that the target gene flanked a dominant selectable marker on both sides. This type of disruption vector may facilitate homologous integration. In our attempts, we tried to construct a similar disruption vector but we could not find the appropriate enzyme digestion sites to place the dominant selectable marker in the middle of *OSD1*.

In conclusion, a transformation system has been established in several *Ophiostoma* fungi including *O. floccosum*. The optimum conditions for protoplasting and transformation, such as cell age and Novozym 234 and PEG concentrations have been determined. This

information will help other researchers to perform transformation of sapstaining fungi. The attempts to disrupt two melanin genes in *O. floccosum* have failed. Molecular analyses of the transformants in our experiments and others suggest that homologous recombination in *O. floccosum* may be a rare event.

Chapter 3

Isolation and characterization of an *O. floccosum OSD1* gene that complements a *Colletotrichum lagenarium* melanin-deficient mutant

3.1 Introduction

SD is one of the key enzymes in the DHN melanin biosynthetic pathway. This enzyme converts scytalone into 1,3,8-THN and is probably also involved in dehydrating vermelone into 1,8-DHN (Butler and Day, 1998). It was the first enzyme of the fungal DHN-melanin pathway to be isolated and characterized, from both *Phaeococcomyces sp.* and *C. miyabeanus* (Butler et al., 1988; Tajima et al., 1989). The studies of the crystal structure of *M. grisea* SD show that the enzyme has a novel folding pattern that buries the hydrophobic active site in its interior when the substrate is bound (Lundqvist et al., 1993, 1994). A detailed mechanism of catalysis for the *M. grisea* SD was proposed following site-directed mutagenesis of its active-site residues (Zheng and Bruice, 1998; Basarab et al., 1999). Active site residues Tyr-30, Asp-31, Tyr-50, His-85, His-110, Ser-129, and Asn-131 were all found to be important in substrate binding and catalysis. Ser-129 participates in the orientation of the substrate within the active site, while Asn-131 is involved in positioning the substrate for binding and in the protonation of the substrates' carbonyl through donation of hydrogen to its' phenolic oxygen atom. Tyr-30 and Tyr-50 assist the protonation of the substrates' carbonyl through the water molecule. His-85 provides a general base and a general acid in the reaction. Asp-31 and His-85 form a

dyad that increases the basicity of the His-85 imidazole. His-110 is thought to have a role in stretching the C3 carbon-oxygen bond by sharing a hydrogen bond with the hydroxyl group.

Furthermore, *M. grisea* SD has been used as a molecular target for designing inhibitors to block disease-causing fungal melanin production (Jennings et al., 1999; Basarab et al., 1999; Jordan et al., 1999). Several inhibitors such as amids and carpropamid have been developed. SD has no known functional counterparts in plants or animals, therefore inhibitors for this enzyme should decrease the risk of deleterious effects occurring in off-target organisms (Jordan et al., 2000). These inhibitors can block fungal melanin production and thus, protect plants from fungal diseases caused by melanin producing pathogens.

The SD genes of *C. lagenarium* (Kubo et al., 1996), *M. grisea* and *A. fumigatus* (Tsai et al., 1997) have been isolated and characterized. Both *C. lagenarium* and *M. grisea* are phytopathogenic fungi and cause diseases on cucumber and rice, respectively. *A. fumigatus* is a human pathogen which causes invasive pulmonary aspergilliosis (Tsai et al., 1997). Disruption of *C. lagenarium* SD gene blocks the conversion of scytalone to 1,3,8-THN in the melanin biosynthetic pathway. Scytalone accumulates in these disruptants which produce a reddish pigment on potato sucrose agar (PSA), form non-melanized appressoria on glass slides, and do not have the ability to infect cucumber leaves (Kubo et al., 1996). Likewise, disruption of *M. grisea* and *A. fumigatus* SD genes results in the disruptants accumulating scytalone and producing reddish pigment (Tsai et

al., 1997; Butler and Day, 1998; Thompson et al., 2000). As mentioned in Section 1.4.1 melanized appressoria are essential for host penetration and thus these disruptants are not able to penetrate host tissues (Kubo et al., 1985, 1996).

In this chapter, we present the isolation of the *OSD1*, a SD gene of *O. floccosum*, and the complementation of a SD deficient mutant of a distantly related pathogenic fungus, *C. lagenarium* using the cloned *O. floccosum* SD gene.

3.2 Materials and methods

3.2.1 Fungal and bacterial strains, culture media, vectors and PCR primers

C. lagenarium wild type strain 104-T and mutant 9201Y were provided by Dr. Kubo at Kyoto Prefectural University, Japan. The fungal strains were grown on potato dextrose agar (PDA; Difco). Culture storage procedures were the same as those described previously (Section 2.2.1). Mycelia of *C. lagenarium* were produced from two cores of *C. lagenarium* macerated in potato dextrose broth (PS) using a blender. Cultures were incubated in a rotary shaker at 20°C for 7 days. *Escherichia coli* strains DH5α (Life Technologies) and LE392 (Promega) were used for the propagation of plasmids and bacteriophage lambda EMBL3 (Promega) clones, respectively.

The plasmid pBSKII+ (Stratagene) was used for general DNA manipulation. The plasmid, pBSU2 was produced by subcloning a 6.5 kb *Bam*HI fragment of *O. floccosum*

genomic DNA containing the SD gene into pBSKII+. The transformation vector, pESD was used for complementing the *C. lagenarium* SD deficient mutant 9201Y. This vector was constructed by ligating the 6.5 kb *Bam*HI genomic DNA fragment containing the SD gene into *Bam*HI digested pAN7-1, which contains a hygromycin-resistance *hph* gene as a selective marker. PCR and DNA sequencing primers are listed in Table 3.1

3.2.2 Nucleic acid manipulations and genomic DNA library amplification

Bacterial plasmid DNAs were purified using the Plasmid Midi Kit (QIAGEN) according to the manufacturer's instruction. Phage DNA was isolated using the QIAGENE lambda DNA isolation kit. For the genomic DNA extraction from *C. lagenarium* and *O. floccosum* the drilling method described in Section 2.2.6 was used. Restriction digestions and ligations were performed according to manufactures' instructions using commercially supplied enzymes and buffers. Mycelia harvested and frozen at -80°C were used for RNA extraction. Total RNA was extracted using the RNeasy Plant Extraction kit (QIAGEN) after drilling the frozen mycelia at 600 rpm for 3 min on ice or smashing liquid nitrogen-frozen conidial or yeast cells.

An *O. floccosum* 387N genomic DNA library was constructed by Eagen (1999) using the EMBL3 replacement vector (Promega) and the manufacturers' protocol. The library was amplified as follows: an aliquot of the library containing 10^5 plaque forming unit (pfu)/ml phage was used to infect the host bacteria LE 392. The mixture was plated on a 150 mm LB plate, and incubated for 8 hrs at 37°C. The amplified library was titered (10^{11} pfu/ml) and stored at -80°C.

Table 3.1. List of the oligonucleotides synthesized for use in sequencing and as PCR primers.

Name	Sequence	Use	Remark
SD1	5' GAG TGG GCI GA(T/C) (T/A) (C/G)IT A(C/T) GA 3'	PCR	F
SD2	5' CCI GC(G/A) AA(C/T) TTC CAI ACI CC 3'	PCR	R
SD5	5' GAT TCC AAG GAC TGG GAC CGT C 3'	PCR	F
SD6	5' CGT CGA CCT TGC GGT ACC AGT G 3'	PCR	R
SD3	5' CTC ACA CAA GTT GCC GTC AAG 3'	Sequencing	F
SD10	5' CTG TCT GTT AGC AAG AAG ATC 3'	Sequencing	R
M13F	5' TGT AAA ACG ACG GCC AGT 3'	Sequencing	F
M13R	5' CAG GAA ACA GCT ATG ACC 3'	Sequencing	R
SD23	5' GGC CTC AAT ATC AGC AGC CTC A 3'	PCR	F
SD24	5' AAC CAG CGG ATC TTG GGA TCG A 3'	PCR	R
SD26	5' TGC GCC GAT GCA ATG GCA CT 3'	PCR	R

Note: F represents forward primer, while R represents reverse primer

3.2.3 DNA hybridization using fluorescein-labelled probes

DNA fragments were labelled with fluorescein using the Gene Images Random Prime Labelling System (Amersham Pharmacia) according to the manufacturers' instructions. The labelling reaction (50 μl) used 150 ng of probe DNA and was incubated for 4 hours at 37°C. The reaction was terminated by adding EDTA to a final concentration of 20 mM. The DNA transferred membranes were hybridized with 100 ng of the fluorescein-labelled DNA overnight at 60°C in 0.1% SDS/5% SSC (0.3 M Na_3 citrate, 3M

NaCl)/5%(w/v) dextran sulphate/20 fold dilution of the block solution (Amersham Pharmacia). The membranes were first washed in 0.1% SDS/1% SSC for 15 min at 60°C, and then in 0.1% SDS/0.5% SSC for 15 min at 60°C. Signal detection was performed using a Gene Images Detection Kit (Amersham Pharmacia).

3.2.4 PCR-based cloning of the *OSD1* gene

Several conserved regions of the SD amino acid sequences of *C. lagenarium* (GenBank accession number: D86079) and *M. grisea* (GenBank accession number: AB004741) were used to create SD degenerate primers. The regions chosen were based on the codon usage weight and preference. A codon frequency table (based on several genes from various filamentous fungi) was used to help determine which nucleotide to substitute in the third codon position. Furthermore, the primers were evaluated to avoid any direct repeats, internal hairpin structures or dimerization. Two degenerate oligonucleotides, SD1 and SD2, were designed. SD1 is fully degenerate to the conserved amino-acid sequence, EWADSYD, and SD2 is fully degenerate to the conserved amino-acid sequence, GVWKFAG (Figure 3.1).

SD1 and SD2 were used as primers with genomic DNA from 387N in order to amplify SD sequences from this fungus. Reaction products (30 μl) were analyzed on 1.2% agarose gels. The desired PCR-amplified DNA fragments were excised, and purified using the GeneClean kit (Bio 101). The purified PCR amplified products were ligated into pBSKII+ using T4 ligase and then transformed into competent DH5α *E. coli* cells

```
ClSD : MASPAGN---ITFEDYLGLNAALFEWADSYDSKDWDRLRKCIAPELRIDYRSFLDKIWEAMP :  59
MgSD : MGSQVQKSDEITFSDYLGLMTCVYEWADSYDSKDWDRLRKVIAPTLRIDYRSFLDKLWEAMP :  62
                              EWADSYD

ClSD : AEEFIAMISDKSVLGNPLLKTQHFIGGSRWEKVSDTEVIGHHQLRVPHQKYTDASRTEVAVK : 121
MgSD : AEEFVGMVSSKQVLGDPTLRTQHFIGGTRWEKVSEDEVIGYHQLRVPHQRYKDTTMKEVTMK : 124

ClSD : GHAHSYNMHWYRKVNGVWKFAGLNPEIRWSEYDFDAVFADGRDSYGTEDQKTDVKVVEKEIK : 183
MgSD : GHAHSANLHWYKKIDGVWKFAGLKPDIRWGEFDFDRIFEDGRETFGDK-------------- : 172
                      GVWKFAG

ClSD : FAAAH : 188
MgSD : ----- :   -
```

Figure 3.1. An amino acid sequence alignment of the SDs of *C. lagenarium* and *M. grisea*. Conserved regions are black shaded and locations of the degenerate oligonucleotides are indicated by the arrows. Dashes indicate absent amino acids necessary for alignment purposes.

using the calcium chloride procedure described by Sambrook et al. (1989). Transformed colonies were screened using white/blue selection based on the insertional inactivation of β-galactosidase. Five white colonies were selected from LB/amp/Xgal plates for plasmid DNA preparations. Plasmids were checked by PCR using the primers SD1 and SD2 to ensure that the appropriate insert was present. DNAs were purified from the confirmed clones and sequenced.

To clone the full-length gene, an obtained fragment, showing high sequence homology to fungal SDs, was used as a probe to screen an *O. floccosum* genomic DNA library. For screening the library, an aliquot of the amplified library was used to infect LE 392 bacteria and plated on 150 mm LB plates at approximately 10^5 pfu/plate. Plaques were transferred onto a Hybond-H+ nylon membrane (Amersham Pharmacia). The membranes were then hybridized with the fluorescein-labelled PCR fragment as described above. Six

positive plaques were picked up and the recombinant lambda phage DNAs purified and digested with restriction enzymes. The digested DNAs were fractionated in a 1.0% agarose gel, and transferred onto a Hybond-H+ membrane. Southern blot analysis was performed with the fluorescein-labelled probe. The identified DNA fragment containing the putative *OSD1* was cloned into pBSKII+ and sequenced.

3.2.5 Complementation of *C. lagenarium* mutant 9201Y

Mycelia of 2-day old *C. lagenarium* mutant 9201Y were grown in liquid culture (100 ml) for 2 days then harvested by filtration using a Nitex membrane (Tetko) and treated with Novozym 234. The procedures of protoplast production and transformation were the same as those described in Section 2.2.3. Approximately, 1×10^7 protoplasts of *C. lagenarium* mutant 9201Y were transformed with 20 μg of the transformation vector pESD containing the full length *OSD1* gene sequence. Transformants were selected on PDA plates with 1 M glucose and 200 mg/ml of hygromycin. Single spore isolations were then performed to ensure single nuclear origin.

3.2.6 RT-PCR analysis of *OSD1* expression in transformants

The first strand of cDNAs was synthesized using 6 μg of DNase treated total RNA, a Oligo(dT) primer and AMV Reverse Transcriptase (Promega) according to the manufacturer's instruction. PCR conditions were the same as described in Section 2.2.7. Detection of the *OSD1* transcript from the first strand of cDNAs was performed by PCR using the gene-specific primers, SD23 and SD26, which were designed based on the

OSD1 DNA sequence. A 0.6 kb fragment would be amplified if *OSD1* was expressed in the transformants. RT-PCR was conducted on cDNA from wild-type strain, the Scd$^-$ mutant 9201Y, the obtained melanin-restored transformants of *C. lagenarium,* and *O. floccosum* 387N as a positive control. RNA samples from *O. floccosum* not treated with reverse transcriptase were included as a negative control. The PCR products were resolved on a 1.2 % agarose gel by electrophoresis.

3.2.7 Pathogenicity test of transformants

Inoculation and pathogenecity assays on cucumber leaves were performed as described by Kubo et al., 1982. Cucumber (*Cucumis sativus* L.; "Suyo") seeds were kindly provided by Dr. Y. Kubo. Cucumber leaves were excised from one-month old plants. Conidia were collected in water by brushing culture plates of *C. lagenarium* wild-type strain, mutant 9201Y and 4 melanin-restored transformants. The obtained conidial suspensions were centrifuged at 2,000 g for 2 min. 20 μl of resuspended conidia (10^5 conidia/ml) was spotted on the surface of the cucumber leaves. After inoculation, the leaves were incubated in humid petri dishes at 24°C for a week before observation.

3.2.8 DNA sequencing and computer analysis of sequence data

DNA sequencing was performed at the Nucleic Acid Protein Service of University of British Columbia. AmpliTag FS DyeDeoxy™ terminator (Applied Biosystems) was used for the sequencing. Sequencing primers are listed in Table 3.1.

PCR primer design was performed using the PCR primer design program, GeneFisher (http://bibiserv.techfak.uni-bielefeld.de/genefisger/). Database searches were performed using the BLAST server at the National Centre of Biotechnology Information (NCBI; Altschul et al., 1990). DNA and protein sequences were used as to search the nr or est databases at the NCBI web server. The parameters used in the searches were as follows: BLOSUM-62 matrix, ungapped search, E (Expected)-value of 1.0 and masking was turned on. The ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and GenScan (Burge and Karlin, 1997) programs were used for protein translation and ORF and intron prediction. PepTool 2.0 (BioTool) was used to predict protein chemical and physical properties. The PC-GENE software package (IntelliGenetics) and clustalW (Thompson et al., 1994) server at European Bioinformatics Institute and GeneDoc program (Nicholas & Nicholas, 1997) were used to conduct the protein sequence alignment and analysis. Phylograms were constructed from alignments using the PAUP* 4.0b6 for Windows/DOS (Sinauer Association Inc). Multiple sequence alignments were converted into distance matrixes and then converted into trees using the neighbor-joining method of clustering. The resulting trees were displayed using TreeView (Page, 1996). A bootstrapped protein distance analysis was also performed on the same sequence alignments to generate 1000 resampled datasets and CONSENSE was used to construct a consensus tree (Brinkman and Leipe, 2001). The search for potential transcription factor binding sites was performed with the matrix search program MatInspector (Quandt et al., 1995) using the TRANSFAC 4.0 matrices (Wingender et al., 2000). ProtComp

(http://www.softberry.com/protein.html) was used to predict the probability of the subcellular localization of proteins, and has a prediction accuracy of 70-90%.

3.3 Results

3.3.1 Isolation and characterization of a putative SD gene

3.3.1.1 Cloning of the *OSD1* gene

In order to isolate a SD DNA sequence from 387N, a PCR-based cloning method was used. The degenerate primers, SD1 and SD2 were designed based on two highly conserved regions of amino acid alignment between the SDs of *C. lagenarium* and *M. grisea*. In the PCR reactions with 387N genomic DNA and the primers SD1 and SD2 only one amplicon of approximately 420 bp was produced (Figure 3.2, lane 1). No amplification occurred in the control reactions (Figure 3.2, lanes 2, 3). The PCR-amplified DNA fragment was subcloned and five transformants were tested to check whether they contained the 420 bp insert. PCR using SD1 and SD2 as primers showed that the 420 bp band was present in all the transformants (Figure 3.3, lanes 1-5). One of the positive clones was designated as OCD and chosen for sequencing.

A BLASTX search of the OCD sequence in the NCBI nr database found 2 hits, one with a *C. lagenarium* and the other with *M. grisea* SD amino acid sequences, with significant E value ($1e^{-50}$). The deduced protein sequence (Figure 3.4) from OCD shared 83% identity and 91% similarity to the *C. lagenarium* SD amino acid sequence, and

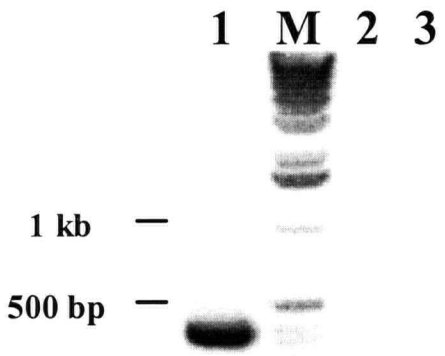

Figure 3.2. PCR products of genomic DNA of *O. floccosum* using SD1/SD2 (lanes 1), SD1 (lanes 2) and SD2 (lanes 3) as primers. The lane labeled 'M' is the one kilobase ladder (BRL).

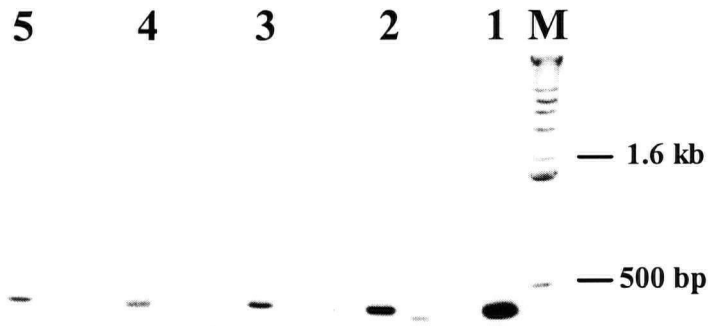

Figure 3.3. PCR products amplified with plasmid DNA from bacterial transformants with the primer combinations of SD1/SD2. The lane labeled 'M' is the one kilobase ladder (BRL).

77% identity and 88% similarity with *M. grisea* sequence. These results suggested that the 420 bp OCD sequence was a fragment of the SD homolog.

Before screening the library, we checked whether the library contained the putative SD gene by PCR. One, two, five, ten and twenty μl of aliquots of the library were used as templates in the PCR reactions. The nested primers SD5 and SD6, designed based on OCD sequence information were used in the reactions. A specific band of 400 bp was observed in each sample (Figure 3.5), indicating that the putative SD gene was in the library. The library was screened by colony blot hybridization using the fluorescein-labelled 420 bp OCD sequence. Six positive plaques were found. Crude phage plugs were picked up, subdivided, and amplified in *E. coli* LE392 to perform another round of screening. Finally, three putative clones were recovered.

The DNA of one of the positive clones was analyzed by restriction digestion with *Bam*HI, *Hind*III, *Hind*III/*Sac*I, *Rsa*I, and *Eco*RI, respectively (Figure 3.6A). Digested DNAs were transferred onto a Hybond N+ membrane and a Southern blot analysis was performed using fluorescein-labelled OCD sequence as a probe. A 6.5 kb *Bam*HI fragment containing the putative SD gene (Figure 3.6B) was then subcloned into the *Bam*HI site of pBSKII+ to produce pBSU2 (Figure 3.7). The plasmid was checked by digestion with *Bam*HI to ensure that a fragment of the appropriate size was present. The *Bam*HI fragment from the plasmid was sequenced using the specific primers (SD3 and SD10) designed based on the OCD sequence information.

```
  1  gagtgggccgacagctatgattccaaggactgggaccgtctgcgc
     E  W  A  D  S  Y  D  S  K  D  W  D  R  L  R

 46  aagtgcattgctcccactctgcgaatcGTACGTCTTATACAACCC
     K  C  I  A  P  T  L  R  I

 91  TGCTTTCTCTATTAACCCATAAAGATCTTCTTTGCTAACAGACAG

136  gactaccgctcgttcctgaacaagctgtgggaggccatgccggcc
     D  Y  R  S  F  L  N  K  L  W  E  A  M  P  A

181  gaagagtttatcggcatgatctccgaccccagcgttctcggcaac
     E  E  F  I  G  M  I  S  D  P  S  V  L  G  N

226  cccctgctgcgcacacaacacttcttcggcgcctcgcgctgggag
     P  L  L  R  T  Q  H  F  F  G  A  S  R  W  E

271  cgcatctccgacaccgaggtcgtgggctaccatcagctgcgcgtc
     R  I  S  D  T  E  V  V  G  Y  H  Q  L  R  V

316  ccccaccaggtctacacagataccactctcacacaagttgccgtc
     P  H  Q  V  Y  T  D  T  T  L  T  Q  V  A  V

361  aagggccacgcccactcggccaacacccactggtaccgcaaggtc
     K  G  H  A  H  S  A  N  T  H  W  Y  R  K  V

406  gacggcgtctggaag
     D  G  V  W  K
```

Figure 3.4. DNA sequences and deducted amino acid sequence of the 420-bp PCR product. The DNA sequence is shown in the upper strand, and the amino acid sequence is shown in the lower strand. Intron is depicted in italic letters.

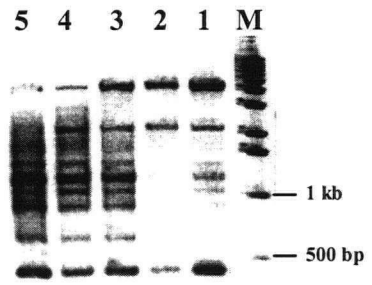

Figure 3.5. PCR products amplified with 387N genomic library aliquots (lanes 1-5: 20 μl, 1 μl, 5 μl, 2 μl and 10 μl,) with the nested primer combinations SD5/SD6.

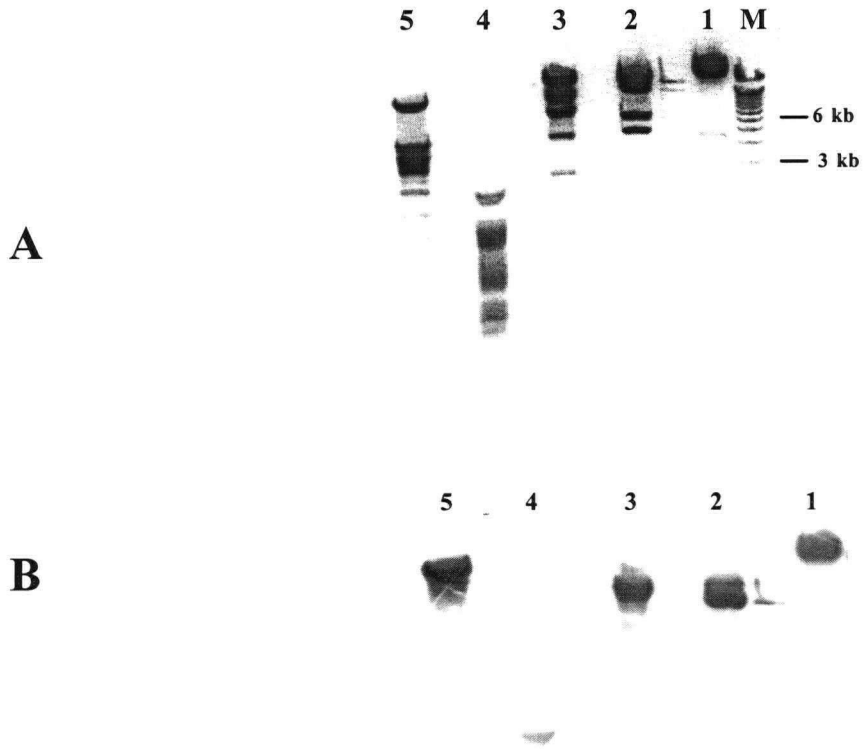

Figure 3.6. Identification of the DNA fragment containing *OSD1*. (A) Gel patterns of the positive phage DNA digested with *Hind*III (lane 1), *Bam*HI (lane 2), *Hind*III/*Sac*I (lane 3), *Rsa*I (lane 4) and *Eco*RI (lane 5), and (B) identical blots hybridized with OCD fragment.

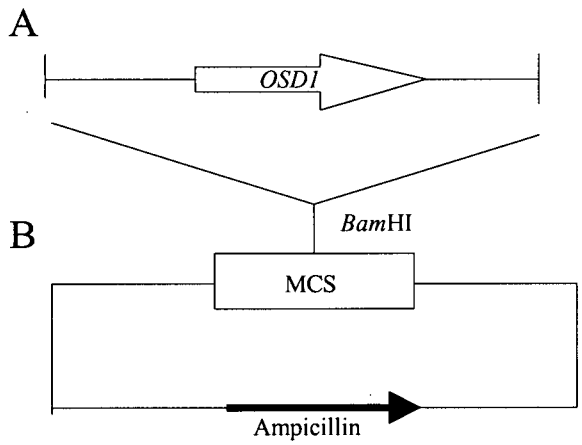

Figure 3.7. The construction of the vector pBSU2. (A) The 6.5 kb *Bam*HI fragment of a genomic λ phage clone showing the orientation and approximate location of the *OSD1* gene, (B) The cloning vector pBSKII+ used to accommodate the fragment.

3.3.1.2 Nucleotide sequence and deduced amino acid sequence of *O. floccosum OSD1*

A total of 1477 nucleotides designated as *OSD1* (GenBank accession Number: AF316575) was obtained by sequencing pBSU2. *OSD1* contained 315 bp upstream sequence of the translation initiation codon (ATG), an ORF with 648 bp, and 372 bp downstream sequence of the stop codon (TAG) (Figure 3.8). A short sequence characteristic of a potential Hogness box (TATAAAA) was observed. The first ATG occurred 140 nucleotides downstream of the Hogness box and was the most likely translation initiation codon. Sequences sharing significant homology with several fungal transcription factor binding sites such as ABAA, NIT-2, and HAP234 were detected upstream from the putative start codon. Among these elements, the nitrogen regulatory binding site NIT-2 protein (Fu and Marzluf, 1990) was present at 3 locations in the 5' upstream region (Figure 3.8). A potential polyadenylation signal, AATAA, was present

112 bp downstream from the stop codon. However, the significance of these detected sites remains to be elucidated. The GC content of the coding sequence was 61%. Two introns were found in *OSD1*; one of 76 bp and the other of 63 bp in length were located at the nucleotide positions 79-154 and 257-319, respectively. The organization of introns and exons in *OSD1* started with a 78 nucleotide exon followed by a 76 nucleotide intron, then a 102 nucleotide exon followed by a 63 intron and a 478 nucleotide exon.

The putative spliced *OSD1* sequence encodes a predicted polypeptide sequence of 216 amino acids (Figure 3.8). The predicted molecular weight and theoretical isoelectric point of the deduced *OSD1* protein were 24.2 kDa and 6.07, respectively. Ala, Asp and Ser appeared as the three major amino acids comprising the protein sequence. The frequencies of these amino acids in the protein sequence were ~ 2% higher than their frequencies in an average protein, while the frequencies of Asn, Cys and Gly were ~ 2% lower than their frequencies in an average protein (Table 3.2). The percentage of hydrophilic amino acids comprising the sequence was 51.4% and the ratio of hydrophilicity to hydrophobicity (HR) of the sequence was 1.3. Protein solubility (S) was 1.56. The *OSD1* deduced protein was predicted to locate in the cytoplasm.

```
-315  TGGTCTATTGTTAGTAGCTATATGTATTTCTAATGATAGGCATAT
-270  GGCATACTGAGTTACTTCTTTTTTGATTGTCTTGCTTACTTGGC
-225  ACGTCTCGACAAGGTGTGACCAGACTCTCGGGCTCCTCCATGGGA
-180  AGGCCGTAGTGTCTGAGACCAGACTCTTGATATAAAAGTAAAGCT
-135  GTGACGATCTAGTTTCTAGATTTCATCTCATTCAAAAGTTCTTCT
 -90  TTTCTTCCTTTTTTCGACGATCGAGGACAAGCTGACTAGTATCGA
 -45  CAAGACACATTTTTAACGGATTCATACTCTCACCAACCAGCACAA
   1  atggcctcaatatcagcagcctcacatcgaccacgtcgagcgcg
   1  M  G  L  N  T  S  S  L  T  S  T  T  S  S  A

  46  cccaagacaaccggcagcgacatctcctttgagGGTATTTCCTAT
  16  P  K  T  T  G  S  D  I  S  F  E

  91  TCTCCTCAAAATGCTCGCCAAATAAAAAAACAAAACAAGAAAACA

 136  AGAAGGACACTAATAGCTAgactacatgggcctctgcagtgccgc
  27                      D  Y  M  G  L  C  S  A  A

 181  ctacgagtgggccgacagctatgattccaaggactgggaccgtct
  36   Y  E  W  A  D  S  Y  D  S  K  D  W  D  R  L

 226  gcgcaagtgcattgctcccactctgcgaatcGTACGTCTTATACA
  51   R  K  C  I  A  P  T  L  R  I

 271  ACCCTGCTTTCTCTATTAACCCATAAAGATCTTCTTTGCTAACAG

 316  ACAGgactaccgctcgttcctgaacaagctgtgggaggccatgcc
  61      D  Y  R  S  F  L  N  K  L  W  E  A  M  P

 361  ggccgaagagtttatcggcatgatctccgaccccagcgttctcgg
  75   A  E  E  F  I  G  M  I  S  D  P  S  V  L  G

 406  caaccccctgctgcgcacacaacacttcttcggcgcctcgcgctg
  90   N  P  L  L  R  T  Q  H  F  F  G  A  S  R  W

 451  ggagcgcatctccgacaccgaggtcgtgggctaccatcagctgcg
 105   E  R  I  S  D  T  E  V  V  G  Y  H  Q  L  R

 496  cgtcccccaccaggtctacacagataccactctcacacaagttgc
 120   V  P  H  Q  V  Y  T  D  T  T  L  T  Q  V  A

 541  cgtcaagggccacgcccactcggccaacacccactggtaccgcaa
 135   V  K  G  H  A  H  S  A  N  T  H  W  Y  R  K

 586  ggtcgacggcgtctggaagtttgccggcctcgatcccaagatccg
```

```
150   V  D  G  V  W  K  F  A  G  L  D  P  K  I  R

631   ctggttcgaatacgattttgataaggtgtttgccagcggccgcga
165   W  F  E  Y  D  F  D  K  V  F  A  S  G  R  D

676   ccagtttggcaccgaggagaaggcggcagcaactgccggaccaga
180   Q  F  G  T  E  E  K  A  A  A  T  A  G  P  E

721   actcctcgccaaggacaaggtgcagagtgccattgcatcggcgca
195   L  L  A  K  D  K  V  Q  S  A  I  A  S  A  Q

766   gagagccgtggccgtcagtgcttagTTTGTCTATTGTAATTTAGT
210   R  A  V  A  V  S  A  STOP

811   TTGTTGTTTTCTTTTGCTAAACGCGTATGGGAGCTGTTTTGTTCG
856   ACACCTTCTCAAGTCTACTTGTCCATCTTATTTATTGCACTTGAA
901   AGAATAACCAGGCCGTTTACCTTGTTGTATGAATAGAGTAATCAC
946   ATTGAGCACATTGAGCGCACTTGAGAAAGACTTTAGGAAAAATCA
991   AATTCTAAATGAAATACATATCGGTATCTGAGCAGTAATTGGGTA
1036  ATCCGTAAGGCCAAACGCAAAAAAGACAAAGCAGACAGACATAGA
1081  AGCAAAGAACCTAAACTGGAAACAACAGAGAACAGAAAACGTTAA
1126  AAAGTAAAAGTGAATGAAAAGAAACGAAATGTCATGC
```

Figure 3.8. Nucleotide and deduced amino acid sequences of *OSD1* from *O. floccosum*. Numbers in the left margin indicate position relative to the nucleotide of the start codon, or amino acid position. The start codon (atg) and stop codon (tag) are bolded. Upper case nucleotides in the coding region indicate the introns. The potential Hogness box is bolded and underlined. The putative binding site of transcription factor nit2 in the 5' untranslated leader sequence is underlined. The potential polyadenylation signal sequence is in italic and bolded.

Table 3.2. Inferred amino acid composition of *O. floccosum OSD1* deduced protein.

Amino acid	No.	Real frequency (%)	Expected frequency (%)
Ala (A)	24	11.1	8.8
Arg (R)	12	5.6	4.2
Asn (N)	4	1.9	4.6
Asp (D)	16	7.4	5.9
Cys (C)	2	0.9	2.1
Gln (Q)	7	3.2	3.7
Glu (E)	11	5.1	5.9
Gly (G)	13	6.0	8.3
His (H)	6	2.8	2.1
Ile (I)	9	4.2	5.4
Leu (L)	15	6.9	8.0
Lys (K)	12	5.6	6.3
Met (M)	4	1.9	2.0
Phe (F)	10	4.6	3.7
Pro (P)	8	3.7	4.5
Ser (S)	20	9.3	6.6
Thr (T)	15	6.9	6.0
Trp (W)	7	3.2	1.4
Tyr (Y)	8	3.7	3.7
Val (V)	13	6.0	7.1

3.3.1.3 Comparison of the *OSD1* deduced protein sequence with other fungal SDs

A BLASTX scan of the NCBI nr database found 3 hits: SDs of *C. lagenarium* with an E-value at 10^{-74}, *M. grisea* with an E-value at 10^{-69} and *A. fumigatus* with an E-value at 10^{-51} (GenBank accession number: AAC49843). *OSD1* deduced protein sequence showed a high degree of identity and similarity with the SDs of *C. lagenarium* (69% and 87%), *M. grisea* (70% and 89%) and *A. fumigatus* (51% and 78%). The alignment of these sequences revealed the presence of four regions that were conserved in all of the fungal SD sequences (Figure 3.9).

```
                                                  **
OfSD : MGLNISSLTSTTSSAPKTTGSDISFEDYMGLCSAAYEWADSYDSKDWDRLRKCIAP :  56
MgSD : MG----------SQVQKSD--EITFSDYLGLMTCVYEWADSYDSKDWDRLRKVIAP :  44
ClSD : -------------MASPAG--NITFEDYLGLNAALFEWADSYDSKDWDRLRKCIAP :  41
AfSD : -------------MVEKKPNLTLEFHDYLALKKVLFDWADSYDAKDWDRLRSIIAP :  43

            *                                 *
OfSD : TLRIDYRSFLNKLWEAMPAEEFIGMISDPSVLGNPLLRTQHFFGASRWERISDTEV : 112
MgSD : TLRIDYRSFLDKLWEAMPAEEFVGMVSSKQVLGDPTLRTQHFIGGTRWEKVSEDEV : 100
ClSD : ELRIDYRSFLDKIWEAMPAEEFIAMISDKSVLGNPLLKTQHFIGGSRWEKVSDTEV :  97
AfSD : TLTVDYRQIGLRKWDDMPAEDYMAMISDMDFLGDPTVKTQHLLGESWWEKISDTEV :  99
                                        @         @

                  *                         *
OfSD : VGYHQLRVPHQVYTDTTLTQVAVKGHAHSANTHWYRKVDGVWKFAGLDPKIRWFEY : 168
MgSD : IGYHQLRVPHQRYKDTTMKEVTMKGHAHSANLHWYKKIDGVWKFAGLKPDIRWGEF : 156
ClSD : IGHHQLRVPHQKYTDASRTEVAVKGHAHSYNMHWYRKVNGVWKFAGLNPEIRWSEY : 153
AfSD : IGHHQLRAAHQVYTDSTLQTVKLKGHGHATNEHYYRKVDGVWKFAGLKPTVRWNEY : 155
                 @                         @

OfSD : DFDKVFASGRDQFGTEEKAAATAGPELLAKDKVQSAIASAQRAVAVSA : 216
MgSD : DFDRIFEDGRETFG----------------DK---------------- : 172
ClSD : DFDAVFADGRDSYGTED-------------QKTDVKVVEKEIKFAAAH : 188
AfSD : QFEDVFRAAKPSV------------------------------------ : 168
        @   @
```

Figure 3.9. Comparison of the deduced amino acid sequence of *O. floccosum OSD1* (OFSD) with those of *C. lagenarium* (CLSD), *M. grisea* (MGSD), and *A. fumigatus* (AFSD). Dashes indicate gaps introduced to maintain the alignment. Perfectly conserved and well-conserved positions in the alignment are shaded with black and grey colours respectively. The residues comprising the active site are indicated as *.

The predicted *OSD1* amino acid sequence showed a C-terminal extension and a N-terminal extension. The active site residues Tyr-30, Asp-31, Tyr-50, His-85, His-110, Ser-129, and Asn-131 in *M. grisea* SD, and *C. lagenarium* SD as Tyr-27, Asp-28, Tyr-47, His-82, His-107, Ser-126, and Asn-128 were well conserved in the *O. floccosum OSD1* deduced protein as Tyr-42, Asp-43, Tyr-62, His-97, His-122, Ser-141, and Asn-143 (Figure 3.9).

3.3.2 Functional characterization of *OSD1* gene

3.3.2.1 Complementation of the *C. lagenarium* mutant 9201Y with *O. floccosum OSD1*

As the *OSD1* disruption failed, the functional analysis of the gene product was conducted by genetic complementation. The vector pESD (Figure 3.10) was used to transform the *C. lagenarium* mutant 9201Y. The plasmid pAN7-1 was included as a control for the transformation. Two transformation experiments were conducted using 10 μg of the vectors in each transformation. Transformants were selected from PDA plates containing 200 μg/ml hygromycin. Fifty five hygromycin-resistant transformants were retrieved and transferred onto 24-well cell culture cluster (Corning) plates containing PDA agar and 600 μg/ml hygromycin for further characterization.

3.3.2.2 Characterization of *C. lagenarium* transformants

The colour of the transformants was used as an indication that complementation had occurred. Approximately, 24 out of 52 hygromycin-resistant transformants produced a greenish-black color on PDA plates. The restored color was slightly lighter than that of wild type strain 104-T (Figure 3.11).

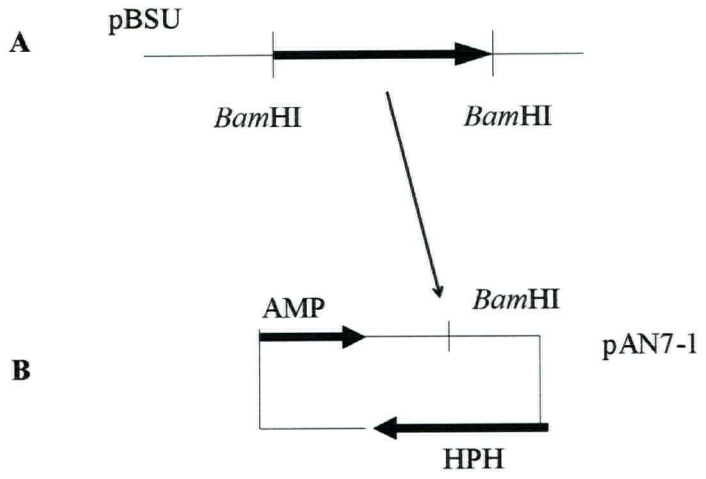

Figure 3.10. The construction of the transformation vector, pESD. The *Bam*HI fragment (A) from pBSU which was inserted into the *Bam*HI site of pAN7-1 (B). HPH: hygromycin phosphotransferase gene, AMP: ampicillin resistance gene.

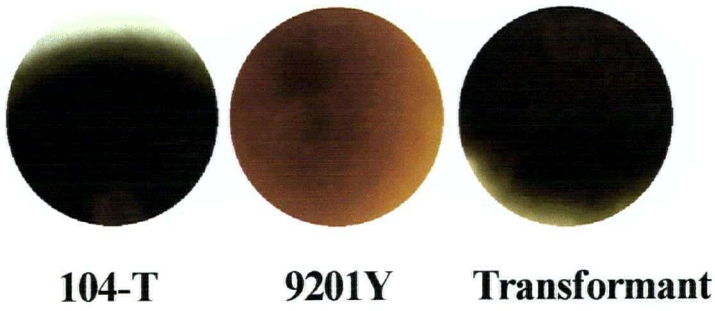

Figure 3.11. The colour of *C. lagenarium* cultures. 104-T: wild type, 9201Y: Scd$^-$ mutant, and transformant: a melanin-restored *O. floccosum OSD1* gene transformant.

The integration of *OSD1* into the genome of the melanin-restored transformants was examined by Southern blot analysis. Genomic DNA, extracted from wild type strain 104-

T, mutant 9201Y, 5 melanin-restored transformants of *C. lagenarium* and *O. floccosum* 378N, was digested with *Eco*RI. The blots were hybridised with the full length *OSD1* sequence. There is no *Eco*RI site in *OSD1*. All the transformants displayed at least one band in the blot. Five bands were observed in one of the transformants (Figure 3.12). No band was detected in either *C. lagenarium* wild-type strain or mutant 9201Y. A single band of *OSD1* gene was detected in *O. floccosum* 378N (Figure 3.12).

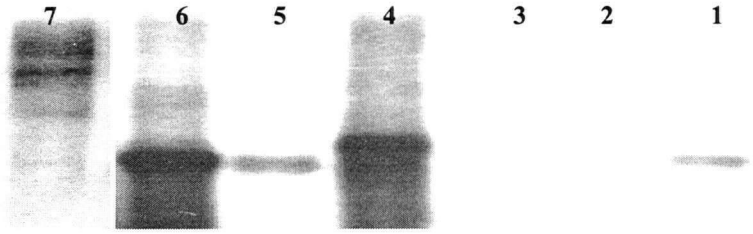

Figure 3.12. Genomic Southern blot analysis of the *C. lagenarium* Scd⁻ mutant transformed with *O. floccosum OSD1*gene carrying plasmid pESD. Lanel: *O. floccosum*, lanes2-3: *C. lagenarium* wild type strain and Scd⁻ mutant 9201Y, lanes 4-7: melanin-restored transformants.

The expression of *OSD1* in the melanin-restored transformants was examined. RT-PCR was performed using total RNA extracted from the mycelia of the *C. lagenarium* wild type strain, mutant 9201Y, 4 melanin restored transformants and *O. floccosum* 387N. The primer pair, SD23/SD26, which is specific for the *O. floccosum OSD1* sequence, was used in the PCR reactions. An expected fragment of 0.6 kb was produced in the four transformants and *O. floccosum*, but not in the mutant 9201Y and the wild type strain of *C. lagenarium* (Figure 3.13). No signal was detected in RNA samples without the treatment with reverse transcriptase.

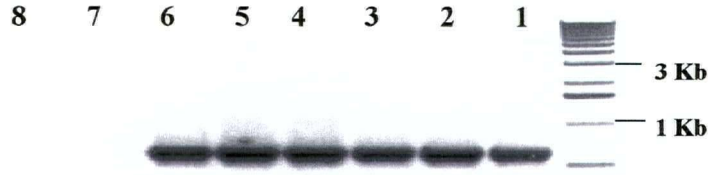

Figure 3.13. RT-PCR detection of the *O. floccosum OSD1* gene transcript from the *C. lagenarium* Scd⁻ mutant transformed with the *O. floccosum OSD1* gene. Lane M: 1 kb DNA ladder, lane 1: *O. floccosum*, lanes 2-6: melanin-restored transformants, lane 7: Scd⁻ mutant 9201Y, lane8: wild rype strain of *C. lagenarium*.

The pathogenicity of the *C. lagenarium* melanin-restored transformants was examined. Conidia of *C. lagenarium* wild type strain, mutant 9201Y and 4 melanin-restored transformants were inoculated on cucumber leaves. After 7-day incubation, the wild-type strain and the melanin restored transformants caused necrotic lesions on the cucumber leaves. In contrast, the mutant 9201Y did not cause any necrotic lesions on the cucumber leaves (Figure 3.14).

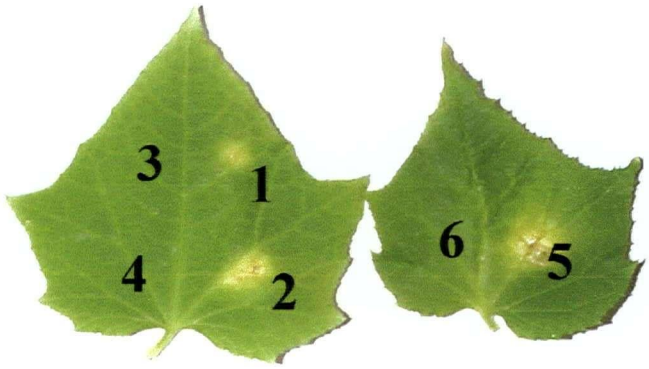

Figure 3.14. Pathogenicity test of the melanin-restored transformant complemented with *O. floccosum OSD1* gene. Two cucumber leaves were inoculated with the transformant, mutant 9201Y, and wild type strain 104-T of *C. lagenarium*. Mutant 9201Y could not form necrotic lesion (spots 3, 4, 6), wild type strain 104-T (spot 5) and the melanin-restored transformant (spots 1, 2) form necrotic lesions.

3.4. Discussion

3.4.1 Characterization of *OSD1* gene

An *OSD1* gene from *O. floccosum* 387N was isolated by a PCR-based cloning approach. Database searches using this gene sequence retrieved three fungal SDs that had very low BLAST E-values between 10^{-51} and 10^{-71}. E-values below 10^{-50} indicate that the query sequence is a match to the target in the database and if the alignment covers the whole of both proteins, then there is a good chance that they share the same or a related function (Brenner et al., 1998). These results suggested that the *OSD1* is a structural gene encoding a SD. This conclusion was confirmed by the complementation of a *C. lagenarium* SD deficient mutant using *OSD1*.

The number and position of introns in *OSD1* and SD genes from *C. lagenarium* and *A. fumigatus* were similar. The organisation of the SD gene in *M. grisea* is not known because only cDNA sequence encoding the SD is available. In *OSD1* two introns of 76 bp and 63 bp were identified. In *C. lagenarium* the introns were 57 bp and 67 bp while in *A. fumigatus* the introns varied from 49 to 54 nucleotides. The size of these introns was in agreement with the concept that fungal introns have an average length of less than 100 bp (Gurr et al., 1987). The first and third exons in *OSD1* were larger than those of SD genes from *C. lagenarium*, *M. grisea* and *A. fumigatus*. Thus *OSD1* encoded the longest SD protein reported so far, with 216 amino acids. The signal sequences in the putative intron matched the consensus sequences of the *N. crassa* 5' splice signal

GGT(A/G/T)(A/C/T)G(T/C), and the 3' splice signal (A/T)(T/C)AG (Bruchez et al., 1993), suggesting that introns in this gene conformed to filamentous fungal consensus splice sequences.

The *OSD1* protein's predicted molecular weight was similar to that of the purified SD (23 kDa) of *C. miyabeanus* (Tajima et al., 1989). The percentage (51.4%) of hydrophilic amino acids of the *OSD1* deduced protein is higher than the average percentage of hydrophilic amino acids (47.6%) of a naturally occurring soluble protein, suggesting it is hydrophilic. This is consistent with the fact that *M. grisea* SD is a hydrophilic protein (Lundqvist et al., 1993, 1994). It is reported that highly hydrophilic enzymes have lower thermal stability, for example, lipase at 50°C loses 75% activity (Longo and Combes, 1997). This suggests that high temperatures may decrease the activity of or inactivate the SD in this fungus and may thereby block melanin production.

The predicted SD protein from *O. floccosum* was more closely related to the *M. grisea* SD than the *C. lagenarium* and *A. fumigatus* SDs. It is important to notice that active site residues of *M. grisea* SD are well conserved in the *O. floccosum OSD1* deduced protein and *C. lagenarium* SD, suggesting that the *OSD1* deduced protein has the same catalytic function as the *M. grisea* and *C. lagenarium* SDs.

The study of inhibitor binding interactions with the *M. grisea* SD showed that Val-75, His-85, His-110, Asn-131, Phe-158, Phe-162 are major amino acid residues that contribute to binding of the inhibitor (Jordan et al., 2000). These amino acids were well

conserved in the *OSD1* deduced amino acid sequence (Figure 3.9; marked by @). Therefore, inhibitors of the *M. grisea* SD may inhibit the *O. floccosum* SD and reduce pigmentation, due to the high sequence similarity between the two enzymes. In fact Fleet (2001) applied the *M. grisea* SD inhibitor carpropamid to *O. floccosum* and found that the inhibitor did reduce the melanization of this fungus. The ProtComp program predicted that the *OSD1* protein was located in the cytoplasm, as well the locations of the other three fungal SDs were also predicted to be in the cytoplasm. This is in agreement with the hypothesis that the melanin precursors were synthesized in cytoplasmic organelles or in the cytoplasm (Bell and Wheeler, 1986).

3.4.2 Confirmation of *O. floccosum OSD1* gene function

In order to confirm the function of *OSD1* in *O. floccosum*, genetic disruption of this gene was attempted, but failed probably due to the very low frequency of homologous recombination that occurs in this organism. Therefore, the functional analysis of *OSD1* was conducted by genetic complementation. In brown or black plant pathogenic fungi, melanin genes from the DHN pathway of one species are often able to replace their homologues in another species. For example the *A. alternata ALM* gene encoding a PKS can restore the melanin production and pathogenicity of the *C. lagenarium* mutant Pks$^-$ (Takano et al., 1997a) and the *M. grisea* mutant Alb$^-$ (Kawamura et al., 1997), both of which lack PKS activity. A reductase gene of *A. alternata* was also able to restore the melanin production and pathogenicity of the *M. grisea* mutant buf$^-$ defective in a HN

reductase activity (Kawamura et al., 1997). However, this work is the first demonstration of a SD gene from one species replacing its homologue in another species.

The complementation experiment showed that *OSD1* restored the mutants' melanization and pathogenicity. The *OSD1* gene was determined to be integrated into the genomes of the transformants and its transcripts were detected in the melanin-restored transformants. The data also suggested that the *OSD1* gene product had SD activity and that its' function was similar to that of the *C. lagenarium* SD. Southern blot analysis indicated that random integration of the complementation vector occurred in the genomes of the pigment-restored transformants. Since the band intensities were different for each transformant, tandem integration may have occurred. The presence of the *OSD1* gene and gene transcripts suggested that the *OSD1* gene can be expressed in *C. lagenarium* during vegetative growth and was functional in hyphal melanization, and that its product functioned in appressorial melanization and restored the pathogenicity in *C. lagenarium*.

SDs of *M. grisea*, *C. lagenarium* and *A. fumigatus* are known to be involved in the DHN-melanin pathway responsible for fungal pigmentation. A scan of NCBI nr database and est database using *OSD1* did not result in finding other homologues except the three reported DHN-melanin SDs. Thus, SD gene sequences may be unique and be used as a genetic marker of the DHN melanin biosynthetic pathway. Cloning of a SD in *O. floccosum* suggests that the presence of a biosynthetic pathway for pigment production in 387N similar to the DHN-melanin pathway. The following evidence also supports this conclusion: 1. the recent cloning of a reductase gene which is able to complement and

restore the melanization of a *M. grisea* reductase deficient mutant (Eagen, 1999). 2. the inhibition of pigmentation of 387N by tricyclazole, a DHN-melanin pathway inhibitor (Eagen, 1999). Actually, many sapstaining fungi may use the DHN pathway to synthesize melanin. Recently, a colleague has identified three major DHN melanin genes, PKS, SD and HN reductase genes in 6 other sapstaining species (Fleet, 2001).

In conclusion, a gene involved in melanin biosynthesis was isolated and characterized from the sapstaining fungus, *O. floccosum*. Comparative sequence analysis shows that the cloned *O. floccosum* gene encodes for a protein highly homologous to other fungal SD sequences. The isolated *OSD1* gene restored the wild-type phenotype of the *C. lagenarium* mutant 9201Y, which is defective in SD activity. This indicates that the *O. floccosum* gene product has the same function as the SD in *C. lagenarium*.

Chapter 4

Function analysis of the second melanin reductase gene in *O. floccosum* 387N

4.1 Introduction

In the DHN melanin biosynthetic pathway 1,8-DHN is formed after two reduction and dehydration reactions of the starting molecule, 1,3,6,8-THN (Figure 1.4). Among the two reduction reactions, one reaction converts 1,3,6,8-THN to scytalone while the other converts 1,3,8-THN to vermelone (Figure 1.4). However, in most fungi, it is not known whether one or two reductases are necessary to catalyze these two reactions. The functional expression of the β-galactosidase-fused reductase of *M. grisea* shows that the fusion enzyme is able to reduce both 1,3,6,8-THN and 1,3,8-THN (Vidal-Cros et al., 1994). A few years later, a reductase purified from *M. grisea* was shown to be able to reduce both 1,3,6,8-THN and 1,3,8-THN (Andersson et al., 1996; Andersson et al., 1997a, 1997b). The authors stated that these results support the one-reductase hypothesis. However, this hypothesis cannot be generalized to all the fungi producing melanin through the DHN pathway. In *V. dahliae*, two reductase mutants have been isolated; one mutant lacks both reductase activities whereas the other lacks only 1,3,8-THN reductase activity (Bell and Wheeler, 1986).

Similarly, molecular data about the melanin pathway reductases does not support the one-reductase hypothesis. Genes encoding 1,3,8-THN reductases have been cloned in *M.*

grisea, *A. alternata*, *C. lagenarium* and *C. miyabeanus* (Vidal-Cros et al., 1994; Shimizu et al., 1997; Kawamura et al., 1999). Disruption of the *C. lagenarium THR1* gene resulted in the accumulation of a shunt product, 3,4-dihydro-4,8-dihydroxy-1 naphthalenone (DDN), which is derived from 1,3,8-THN. This suggested that the mutant synthesized 1,3,8-THN, even though *THR1* was not functional, and therefore another reductase catalyzed the reduction of 1,3,6,8-THN (see Figure 1.4). In *A. fumigatus,* the disruption of the reductase gene *arp2* blocked the production of scytalone, indicating that this gene codes for a 1,3,6,8-THN reductase (Tsai et al., 1999). Chemical feeding and analysis of *arp2* deletants have suggested that two reductase genes are present in this pathway, although the second reductase gene, converting 1,3,8-THN into vermelone, has not yet been isolated in this fungus.

Recently, a *M. grisea* reductase gene encoding a 1,3,6,8-THN reductase (4HNR) has been reported (Thompson et al., 2000). Mutant analyses showed that this 4HNR was only able to convert 1,3,6,8-THN to scytalone, while the 1,3,8-THN reductase (3HNR) of this fungus reduced both 1,3,8-THN to vermelone and 1,3,6,8-THN to scytalone. In *O. floccosum*, a reductase gene (*THN1*) has been isolated using a PCR-based cloning strategy. The isolated gene was found to be able to complement and restore the melanization of the *M. grisea buf* mutant, which lacks the gene encoding the 1,3,8-THN reductase (Eagen, 1999). During the isolation of the *O. floccosum THN1*, two different sequences were obtained from the amplification of the *O. floccosum* genomic DNA using the degenerate primers (Eagen, 1999). Both sequences showed high homology to known

fungal reductase genes, suggesting that two reductase genes may be present in *O. floccosum*.

In this chapter, the isolation and characterization of the second *O. floccosum* reductase gene (*THN2*) is presented. The role of this gene in melanin production is demonstrated by complementing the *M. grisea buf* mutant, a double mutant and a *C. lagenarium THR1* reductase deficient mutant.

4.2 Materials and methods

4.2.1 Mutants used for complementation studies

The *M grisea buf* mutant IC4-1 was received from Dr. M. Farman, Department of Plant Pathology, University of Kentucky. This mutant was recovered from crosses between parental strains of Guy11 and 2638. The *buf* locus on chromosome 2 near the marker CH3-24H (Nitta et al., 1997) of strain 2638 is unstable due to the presence of many transposable elements. This locus is spontaneously deleted during mating with Guy11, and mutations are caused by deletions of approximately 50 kb that remove all of the 1,3,8-THN reductase gene sequence. These strains were cultured on oatmeal agar at 25-28°C in the dark. The *M. grisea* double mutant 444-5-3 lacking both 1,3,6,8-THN reductase and 1,3,8-THN reductase was provided by Dr. B. Valent from DuPont Agricultural Products, USA. The Thr$^-$ mutant 9141 of *C. lagenarium* was obtained from

Dr. Y. Kubo at Kyoto Prefectural University, Japan. It cannot convert 1,3,8-THN to vermelone in the melanin biosynthetic pathway (Perpetua et al., 1996).

4.2.2 Plasmids

The plasmid pTHN2 was produced by ligating a 3 kb *Pst*I *O. floccosum* genomic fragment containing the putative reductase gene into pBSKII+. As well the 3 kb *Pst*I genomic DNA fragment containing the putative reductase gene was cloned into *Pst*I digested pCB1004 plasmid to produce the hygromycin-resistant transformation vector, pETHN. The transformation vector, pCB1.9X, which contains the full length *THN1* gene was obtained from Eagen (this lab). pETHN was used to complement the *M. grisea* and *C. lagenarium* mutants, while pCB1.9X was used to complement the *M. grisea* mutants.

4.2.3 Molecular techniques

DNA and RNA manipulations were the same as described in Sections 2.2.6 and 3.2.2. RT-PCR analyses, DNA sequencing and sequence data analysis using bioinformatics tools were the same as described in Sections 2.2.9, 3.2.3, 3.2.6 and 3.2.8. RT-PCR detection of the *O. floccosum* reductase genes' transcripts in transformants used the primer sets RTHN01 (5' GAA GTT GTC AAG CTG TTC GA 3') and RTHN02 (5' CGT ACA TGT CCG TCT TGA CA 3') for *THN1* and THNRT1 (5' TCC CAG CAA GTA CGA CGC CAT TC 3') and THNRT2 (5' CTG GCC ATC CTG CGA CGC TAG 3') for the second reductase gene.

4.2.4 Cloning of the second reductase gene (*THN2*) in *O. floccosum*

To retrieve the second putative reductase gene from 387N, we aligned and compared the two insert sequences from pGT2 and pGT5 (Eagen, 1999) using the BLAST2 program at the National Center of Biotechnology Information (NCBI). Specific primers based on the unique region of the pGT2 insert sequence were designed and named as THN3 (5' GAC TCG TCG GCC CAG GAG GTT GTC GA 3') and THN4 (5' GAA CTG GCC GCG GGT GTT GAC GGA GA 3'). These primers and *O. floccosum* genomic DNA were used to perform PCR reactions (Section 2.2.7). The obtained amplicon was then used to screen the genomic library and clone the gene using procedures described previously (Section 3.2.4).

4.2.5 *M. grisea* and *C. lagenarium* protoplast formation and transformation

The protocol for *Ophiostoma* protoplasting and transformation (see Sections 2.2.3 and 2.2.4) was used for the transformation of *M. grisea* and *C. lagenarium*. About 40 μg of the transformation vector, pETHN, was used to transform both *C. lagenarium* and *M. grisea buf* mutants. The *M. grisea* double mutant 444-5-3 was transformed with pETHN, pCB1.9X and with both of these vectors together. For color observation, transformants of *M. grisea* and *C. lagenarium* were transferred onto MEA and PDA, respectively. Pathogenicity tests of *C. lagenarium* transformants were the same as described in Section 3.2.7.

4.2.6 Genomic Southern hybridization analysis

Genomic DNA from four *M. grisea* and three *C. lagenarium* melanin-restored transformants was digested with *Eco*RI and *Hind*III and transferred onto Zeta-Probe GT blotting membrane (Bio-Rad). *Eco*RI does not cut the putative second reductase gene, while *Hind*III has one cleavage site in the gene. The putative second reductase gene was used as a probe to perform the Southern blot analysis. DNA hybridization was performed under conditions of high stringency using the procedure described in Section 2.2.9. *Eco*RI digested genomic DNA of *O. floccosum* wild type strain, *M. grisea buf* mutant and *C. lagenarium* mutant 9141 were included as controls. The genomic DNA from 6 transformed *M. grisea* double mutants was *Eco*RI-digested and hybridized with both *O. floccosum THN1* and *THN2*. *Eco*RI does not cut either *THN1* or *THN2*.

4.2.7 Assay of appressorium formation

To examine conidial germination and appressorium formation, conidia were harvested from 7-day-old cultures grown on PDA medium for *C. lagenarium* and 5-day-old cultures grown on oatmeal agar for *M. grisea*. Twenty microliters of conidial suspension (10^5 conidia/ml for *C. lagenarium* and 10^3 conidia/ml for *M. grisea*) were placed onto a glass slide and incubated in a humid environment at 24°C in the dark. After 24 hr of incubation, samples were observed by microscopy (Axioplan, Carl Zeiss). Pictures were taken using a digital video camera (MDS 100, Kodak). The percentage of germinated conidia and appressoria formed were recorded for the three slides.

4.3 Results

4.3.1 Isolation of the putative second reductase gene in *O. floccosum* 387N

We compared the insert sequences of pGT2 and pGT5 and found that ~100 bp of the 400 bp sequences were identical (Figure 4.1). Therefore, specific primers based on the unique regions of the pGT2 insert sequence were designed and named as THN3 and THN4. PCR reactions with these primers and *O. floccosum* genomic DNA amplified only one band of about 170 bp (Figure 4.2). To confirm that the PCR-amplified fragment was a portion of the pGT2 insert, the PCR product was hybridized with the chemical fluorescein-labelled pGT2 insert. The PCR fragment was found to hybridize with the pGT2 insert. This suggested that the amplified PCR product was a portion of the putative second reductase gene. To isolate the full-length gene, the PCR-amplified DNA fragment was used as a probe to screen the *O. floccosum* genomic library. Three positive recombinant lambda phage plaques were retrieved. DNA purified from one positive recombinant lambda phage was digested using *Xba*I, *Pst*I, *Eco*RI, *Eco*RV and *Bam*HI. Southern analysis performed on the digested DNA using the PCR product as a probe identified a 3 kb *Pst*I fragment containing the putative reductase gene (Figure 4.3A and 4.3B). This fragment was therefore sub-cloned into pBSKII+ (designed as pTHN2) and sequenced.

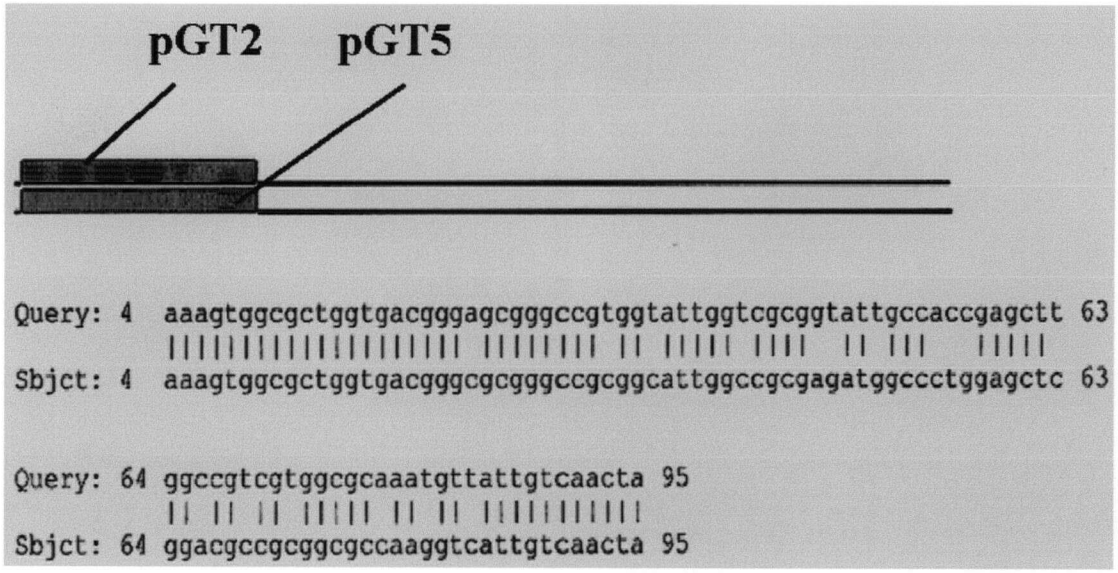

Figure 4.1. A nucleotide sequence comparison of the two insert sequences of the plasmids, pGT2 and pGT5 using the BLAST2 program. Conserved regions are indicated by boxes and the alignment is shown. Query and Subject represents the insert sequences of the pGT2 and pGT5, respectively.

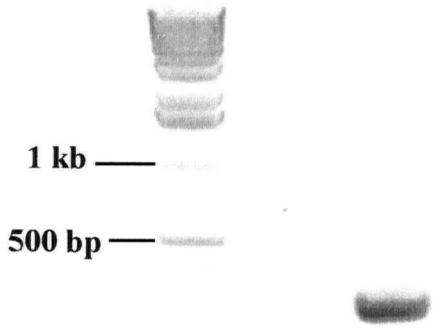

Figure 4.2. PCR products from *O. floccosum* 387N genomic DNA using THN3 and THN4 as primers. The lane labeled 'M' is the one kilobase ladder (BRL).

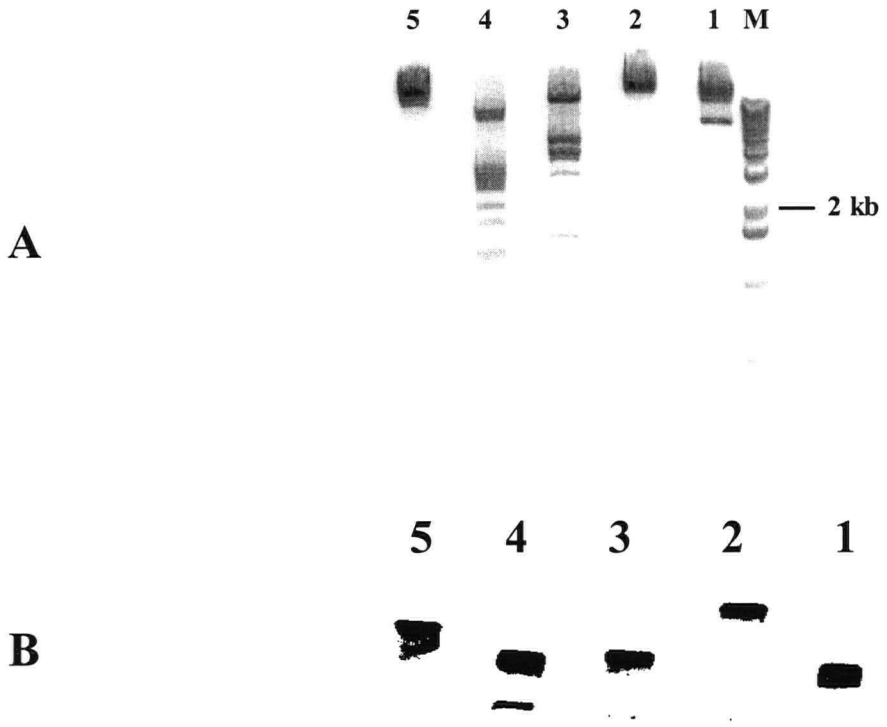

Figure 4.3. Identification of the DNA fragments containing *THN2*. (A) Gel patterns of the positive phage DNA digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Eco*RV (lane 3), *Pst*I (lane 4), and *Xba*I (lane 5), and (B) identical blots hybridized with the PCR-amplified fragment.

4.3.2 Sequence characterization of the second reductase gene in *O. floccosum*

A total of 1444 nucleotides were sequenced from the 3 kb *Pst*I fragment and designated as *O. floccosum* reductase gene, *THN2* (GenBank accession number: AF317668), after the first reductase gene isolated by Eagen (1999), *THN1*. *THN2* contained 395 bp of 5' flanking DNA, the upstream sequence from the translation initiation codon (ATG), a 855 bp open reading frame (ORF), and 194 bp of 3' downstream sequence from the stop codon (TAG) (Fig. 4.4). The GC content of the coding sequence was 63%. Several motifs

possibly involved in the transcription of the gene were identified in the promoter region. Putative TATA boxes were predicted at positions -240, -254 and -341. Three putative binding sites for the transcription factor Nit2 were found at positions -106, -137, and -364. Another motif, CCAAT box was found at position -381. A potential polyadenylation signal, AATATATAAA, was present 73 bp downstream from the stop codon.

THN2 consisted of an ORF without any introns and encoded a predicted protein of 284 amino acids (Figure 4.4), having a theoretical isoelectric point of 7.0 and a predicted molecular mass of 30.5 kDa. Ala, Gly and Val appeared to be the three major amino acids in the protein sequence. The frequencies of these amino acids in the *THN2* deduced protein sequence were about 2-4% higher than in an average protein, while the frequencies of Glu and Leu were around 2% lower than in an average protein (Table 4.1). The percentage of hydrophilic amino acids comprising the sequence was 44.7%. Protein solubility (S) was 1.46. The *THN2* deduced protein was predicted to be located in the cytoplasm.

4.3.3 Comparison of the deduced *THN2* protein with other fungal melanin reductases

To determine the association of the *O. floccosum THN2* to other melanin reductases, an amino acid sequence identity matrix was constructed for the eight complete melanin reductase sequences available for the ascomycetous fungi using the clustalW alignment program and GeneDoc program (Table 4.2). The BLOSUM62 scoring matrix (Henikoff

and Henikoff, 1992) was used to calculate the global identity between each pair of sequences.

```
-390            tagagctcaccaatagcagcaccacaagagttatc
-360 cgacttggcagacgtgcaagtataagtagacagaaatacatctcag
-315 atccatcatgggcttctccaacaccgtccgcggctctccatgctc
-270 ttccatgaagagacggatataccggtggctgtataccagtaagccc
-225 tggcgcgggtgtaaataaaagaggctctcccctccaatattgttt
-180 tctcgatactcgtattattcacagcctcacatcttcatcaacata
-135 tcctctcaacctcttcaacacctcagcactatcaccgtcatcctg
 -90 gcgagcttgatagactacctcatacaggccttcctccgtctgtac
 -45 cactcgctcttcacaacatccccttcatcacataccaccgccaaa
   1 atggttaccacaaagtcagacaaggcccatactcccagcaagtac
     M  V  T  T  K  S  D  K  A  H  T  P  S  K  Y
  46 gacgccattccgggcccgctcggtctgccgtcggcgtcgctggcc
     D  A  I  P  G  P  L  G  L  P  S  A  S  L  A
  91 ggcaaggttgccctcgtcacgggcgcgggccgcggcattggccgc
     G  K  V  A  L  V  T  G  A  G  R  G  I  G  R
 136 gagatggccctggagctcggacgccgcggcgccaaggtcattgtc
     E  M  A  L  E  L  G  R  R  G  A  K  V  I  V
 181 aactatgccaacagcgactcgtcggcccaggaggttgtcgatgcc
     N  Y  A  N  S  D  S  S  A  Q  E  V  V  D  A
 226 atcaaggcggccggctccgacgccgccgctattaaggccaacgtc
     I  K  A  A  G  S  D  A  A  A  I  K  A  N  V
 271 tccgacgtcgaccagattgtcaccctctttgaaaagaccaagcag
     S  D  V  D  Q  I  V  T  L  F  E  K  T  K  Q
 316 cagtggggcaagcttgacattgtgtgctccaactcgggcgtcgtc
     Q  W  G  K  L  D  I  V  C  S  N  S  G  V  V
 361 agctttggccatgtcaaggatgtcacgcccgaggagtttgaccgc
     S  F  G  H  V  K  D  V  T  P  E  E  F  D  R
 406 gtcttctccgtcaacacccgcggccagttctttgtcgcgcgcgag
     V  F  S  V  N  T  R  G  Q  F  F  V  A  R  E
 451 gcctacaagcacctcgagattggcggccgtctgatcctcatgggc
     A  Y  K  H  L  E  I  G  G  R  L  I  L  M  G
 496 tctattaccggccaggccaagatggtcccgcggcacgccgtctac
     S  I  T  G  Q  A  K  M  V  P  R  H  A  V  Y
 541 tcggctagcaagggcgccatcgagacctttgtgcgctgcatggcc
     S  A  S  K  G  A  I  E  T  F  V  R  C  M  A
 586 gtcgactttggcgacaagaagatcactgtcaacgccgtcgcgcct
     V  D  F  G  D  K  K  I  T  V  N  A  V  A  P
 631 ggcggtatcaagacggacatgtaccacgctgtttgccgcgagtac
     G  G  I  K  T  D  M  Y  H  A  V  C  R  E  Y
 676 atccccaacggcctgactctcaacgacgacgagacggatgagtac
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```
     I  P  N  G  L  T  L  N  D  D  E  T  D  E  Y
 721 gctgctggctggtcgcccattcaccgcgtcggcctgcccattgat
     A  A  G  W  S  P  I  H  R  V  G  L  P  I  D
 766 gtggcgcgcgtcgttgcgttcctagcgtcgcaggatggccagtgg
     V  A  R  V  V  A  F  L  A  S  Q  D  G  Q  W
 811 atcaacggcaaggtccttggtgtcgacggcggtgcctgcatgtag
     I  N  G  K  V  L  G  V  D  G  G  A  C  M  *

 856 Acaatgaagggtatcacgccatggtttcgcagacaagcagtttat
 901 aaatgaatatacgtttagcaagatttgaatatataaagttacata
 946 agtgaaccataaatgtttctatacgctgctgatacaaaattctaa
 991 tatattttaacccttttaaaacttttgaacgctttttcgcccaat
1036 atacaaaccagaca
```

Figure 4.4. Nucleotide and deduced amino acid sequences of the *O. floccosum THN2*. Numbers in the left margin indicate position relative to the nucleotide of the start codon, or amino acid position. The start codon (atg) and stop codon (tag) are bolded. The putative binding site of transcription factor nit2 in the 5' untranslated leader sequence is in italic, bolded and underlined. Putative TATA box is bolded and underlined. CCAAT box is underlined. The potential polyadenylation signal sequence is in italic and bolded.

Pairwise comparisons of the reductase sequences of *O. floccosum THN2* (OfTHNB), *C. lagenarium THR1* (ClTHN; Perpetua et al., 1996), *C. heterostrophus Brn1* (ChTHN; Shimizu et al., 1997), *A. alternata BRM2* (AaTHN; Kawamura et al., 1999), and *M. grisea* 3HNR (MgTHNB) yielded 62-95% identity. In contrast, pairwise comparisons of each of these 5 sequences to the *O. floccosum THN1* (OfTHNA), *A. fumigatus arp2* (AfTHN; Tsai et al., 1999) and *M. grisea* 4HNR (MgTHNA) reductases yielded 42-46% identity. Therefore, these 8 reductases were divided into two groups; Group I consisted of the 3 sequences, OfTHNA, AfTHN, and MgTHNA, sharing 50-74% identity to each other, while Group II consisted of the other 5 sequences sharing 62-95% identity to each other. From this grouping it was deduced that the *THN2* reductase belonged to Group II, while the *THN1* reductase belonged to Group I.

Table 4.1. Inferred amino acid composition of *O. floccosum THN2* deduced protein.

Amino acid	No.	Real frequency (%)	Expected frequency (%)
Ala (A)	33	11.6	8.8
Arg (R)	13	4.6	4.2
Asn (N)	9	3.1	4.6
Asp (D)	19	6.7	5.9
Cys (C)	4	1.4	2.1
Gln (Q)	8	2.8	3.7
Glu (E)	12	3.2	5.9
Gly (G)	30	10.1	8.3
His (H)	6	2.1	2.1
Ile (I)	17	6.0	5.4
Leu (L)	16	5.6	8.0
Lys (K)	18	6.3	6.3
Met (M)	7	2.5	2.0
Phe (F)	9	3.2	3.7
Pro (P)	10	3.5	4.5
Ser (S)	18	6.3	6.6
Thr (T)	14	4.9	6.0
Trp (W)	3	1.1	1.4
Tyr (Y)	7	2.5	3.7
Val (V)	31	10.9	7.1

To further examine the relationships of these reductases, a cladogram was constructed using these reductase sequences and a reductase of *Streptomyces cyanogenus* (GenBank accession number: AAD13552.1) as an outgroup to root the tree. *S. cyanogenus* reductase was assumed to have diverged early in the evolution of these reductases.

Table 4.2. Amino acid sequence identity matrix (%) of fungal reductases of *O. floccosum THN1* (OfTHNA) and *THN2* (OfTHNB) with *C. lagenarium THR1* (ClTHN), *M. grisea* 3HNR (MgTHNB) and 4HNR (MgTHNA), *A. alternata BRM2* (AaTHN), *C. heterostrophus Brn1* (ChTHN) and *A. fumigatus arp2* (AfTHN).

Sequences	OfTHNB	ClTHN	MgTHNB	ChTHN	AaTHN	OfTHNA	MgTHNB	AfTHN
OfTHNB	-	78	77	66	65	44	44	44
ClTHN	78	-	78	62	62	43	43	42
MgTHNB	77	78	-	69	69	43	44	44
ChTHN	66	62	69	-	95	46	45	45
AaTHN	65	62	69	95	-	46	44	44
OfTHNA	44	43	43	46	46	-	74	50
MgTHNA	44	43	44	45	44	74	-	51
AfTHN	44	42	43	45	44	50	51	-

Notes: Group I reductases are shaded and their identity scores are boxed and shaded.

Two clusters were clearly distinguished in the phylogenetic tree (Figure 4.5). One cluster contained the three sequences, while the other contained the other 5 sequences. In the group I reductase cluster, *O. floccosum* OfTHNA fell into a clad with *M. grisea* MgTHNA, while *A. fumigatus* AfTHN reductase diverged from this clad. In the group II reductase cluster the *C. heterostrophus* ChTHN and *A. alternata* AaTHN reductases formed a clad that diverged from a clad consisting of the *M. grisea* MgTHNB, *O. floccosum* OfTHNB and *C. lagenarium* ClTHN reductases.

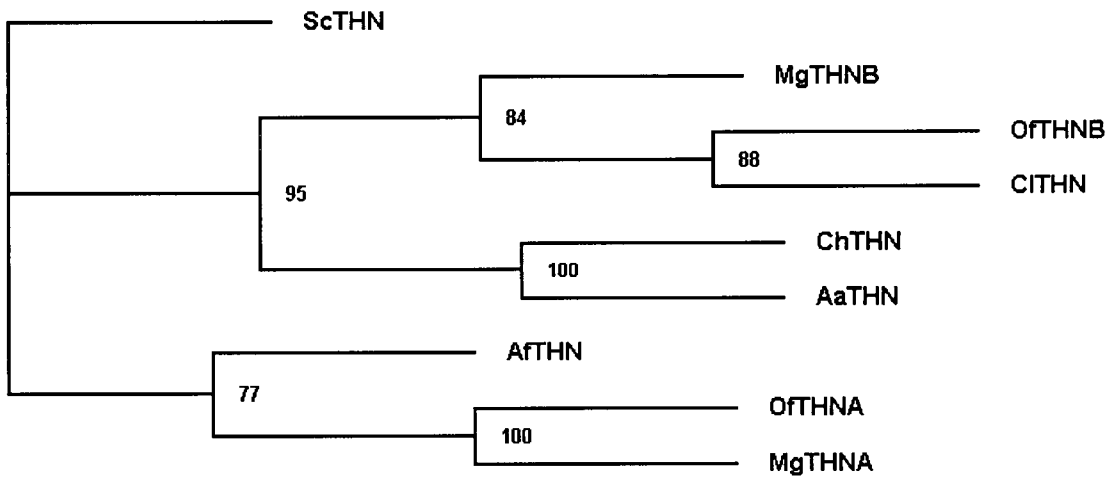


Figure 4.5. The relationships of fungal melanin reductases. The reductase sequence of *Streptomyces cyanogenus* (ScTHN) was used as an outgroup. The sequences were aligned using ClustalW and the cladogram was generated using Paup 4.0b6. Bootstrap values based on 1000 replicates are shown within branch nodes. Fungal reductase sequences are: *O. floccosum THN1* protein (OfTHNA), *M. grisea* 4HNR (MgTHNA), *A. fumigatus arp2* protein (AfTHN), *C. heterostrophus Brn1* protein (ChTHN), *A. alternata BRM2* protein (AaTHN), *O. floccosum THN2* protein (OfTHNB), *M. grisea* 3HNR (MgTHNB), and *C. lagenarium THR1* protein (ClTHN).

4.3.4 Complementation of the reductase defective mutants with *O. floccosum THN2*

To confirm that *THN2* encoded a melanin reductase, genetic complementation of reductase deficient mutants using *THN2* was performed. Putative transformants were selected on culture medium containing 200 μg/ml of hygromycin. Transformants were selected and transferred onto hygromycin-free plates for color evaluation. Six out of ~240 *M. grisea buf* transformants, and eight out of ~240 *C. lagenarium* transformants produced black color similar to their wild type strains (Figure 4.6). Spore germination, appressorium formation and appressorium pigmentation of these transformants were observed. *C. lagenarium* and *M. grisea*

which were similar to their wild-type strains, while the mutants formed slightly less pigmented appressoria (Figures 4.7 and 4.8). The pathogenicity of the *C. lagenarium* melanized-transformants was tested. One-month old cucumber leaves were tested with three transformants and the mutant 9141. The three melanized-transformants caused lesions on the cucumber leaves, while the melanin-deficient mutant 9141 did not cause any lesions on the leaves (Figure 4.9). These results indicated that the *O. floccosum THN2* gene was acting in *C. lagenarium* appressorium melanization and the restoration of the pathogenicity of the *C. lagenarium* mutant. So far, it appeared that both *O. floccosum THN1* and *THN2* can function as a 1,3,8-THN reductase. We did not investigate the pathogenicity of the *M. grisea* melanized-transformants due to the lack of rice seed.

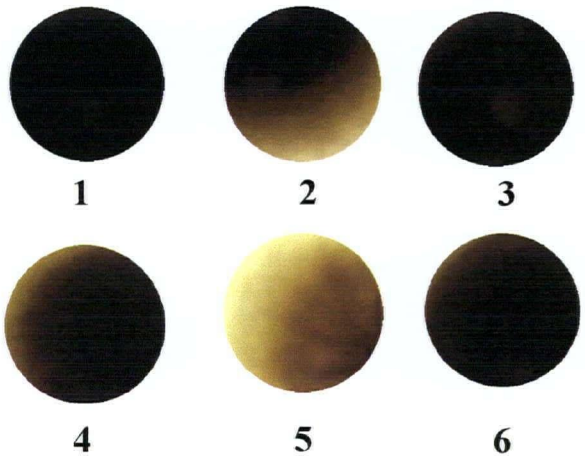


Figure 4.6. The colour of the *C. lagenarium* and *M. grisea* cultures. 1, 2, and 3: wild type strain104-T, mutant 9141 and a transformant of *C. lagenarium*; 4, 5, and 6: wild type strain Guy 11, *buf* mutant and a transformant of *M. grisea.*

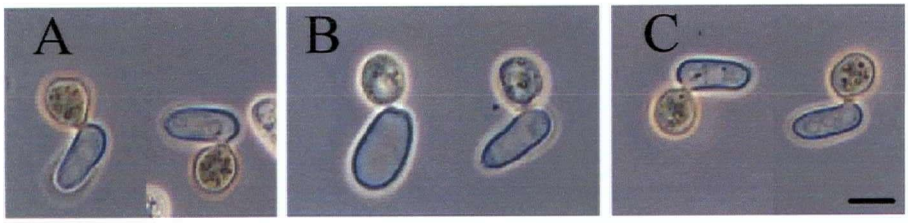


Figure 4.7. Appressorium pigmentation of the melanin-restored transformants of *C. lagenarium*. A, B and C: appressoria of the wild type, mutant 9141 and melanized transformants, respectively; bar in C = 10 μm for A to C.

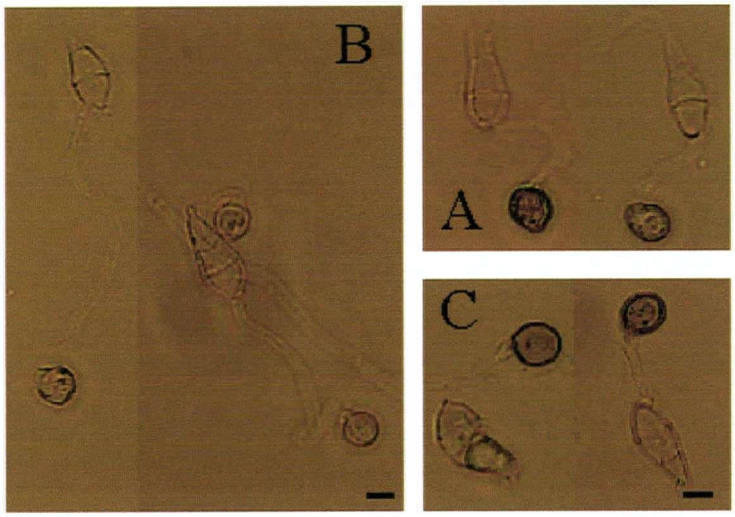


Figure 4.8. Appressorium pigmentation of the melanin-restored transformants of *M. grisea*. A, B and C: appressoria of the wild type, *buf* mutant and melanized transformants, respectively; bar in B = 10 μm; bar in C = 10 μm for A and C.

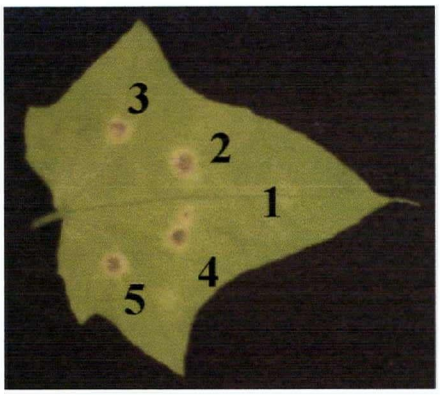


Figure 4.9. Pathogenicity test of melanin restored transformants of *C. lagenarium*, complemented with *O. floccosum THN2*. The cucumber leaf was inoculated with the mutant 9141 (spot 1), wild type strain 104-T (spot 2), transformants (spots 3-5) of *C. lagenarium*. The mutant did not cause necrotic lesions while the transformants and the wild type strain caused necrotic lesions on the cucumber leaf.

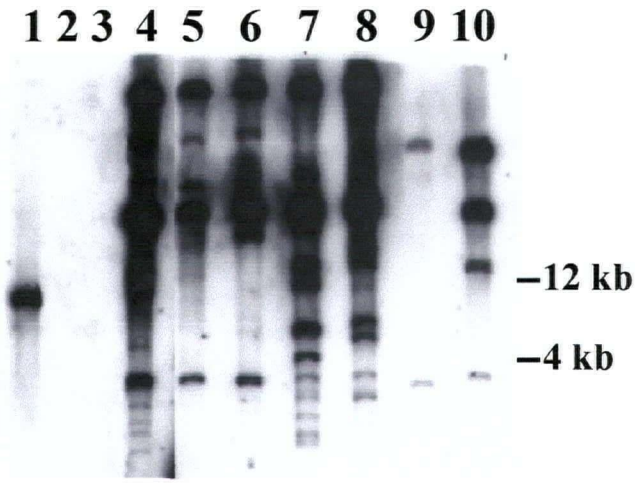


Figure 4.10. Genomic Southern blot analysis of the *C. lagenarium* and *M. grisea* mutants transformed with *O. floccosum THN2* gene. Lane 1: *O. floccosum* 387N; lane 2: *C. lagenarium* mutant 9141; lane 3: *M. grisea buf* mutant; lanes 4-6: *C. lagenarium* melanin-restored transformants; lanes 7-10: *M. grisea* melanin-restored transformants. *THN2* was used as a probe for the hybridization.

Southern blot analysis was performed to determine whether the complementation vectors were present in the transformants' genome. Only one band was seen in *O. floccosum* 387N, while no band was visible in the *M. grisea buf* mutant or the *C. lagenarium* mutant (Figure 4.10). Two to ten bands corresponding to one to five copies of the *O. floccosum THN2* gene were detected in both *M. grisea* and *C. lagenarium* melanized transformants (Figure 4.10). *THN2* gene expression in the melanized-transformants was investigated by RT-PCR. Total RNAs were extracted from 2-day old cultures of three *C. lagenarium* and *M. grisea* melanized-transformants. Total RNAs of the *O. floccosum* wild type strain, *M. grisea buf* and *C. lagenarium* mutants were included as controls. RNA samples without reverse transcriptase treatment (minus-RT) were included to detect genomic DNA contamination. The primer pair (THNRT1/THNRT2) specific for the *O. floccosum THN2* sequence was used in these PCR reactions. Expected fragments of 0.7 kb were produced in the six transformants and *O. floccosum*, but not in the *C. lagenarium* mutant 9141, *M. grisea buf* mutant and minus-RT samples (Figure 4.11).

M. grisea double mutant 444-5-3 was transformed with *O. floccosum THN1*, *THN2* and both genes together to investigate whether *O. floccosum THN1* or *THN2* can function as 1,3,6,8-THN reductase. Three rounds of complementation were conducted and around 1,200 transformants (about 400 for each treatment) were retrieved. All the transformants produced color similar to the double mutant (Figure 4. 12). Southern blot analysis was performed to determine whether the complementation vectors were present in the transformants' genome. Two transformants from each treatment were chosen to conduct the experiment. The double mutant 444-5-3 was included as a control.

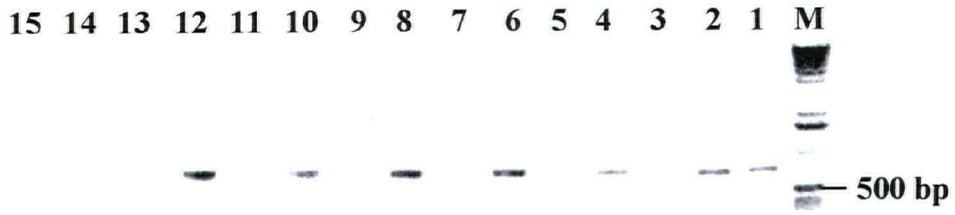
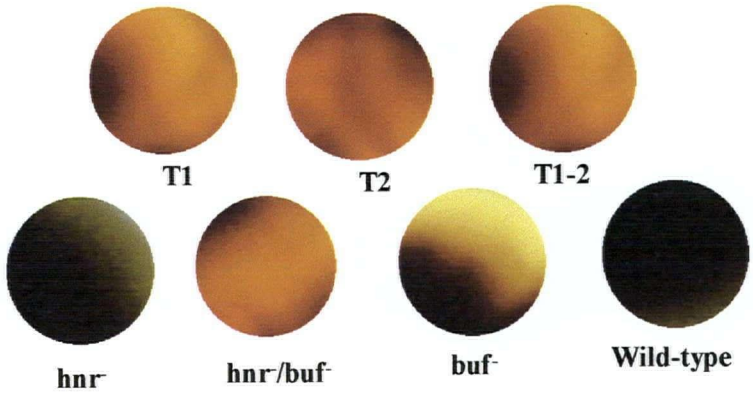


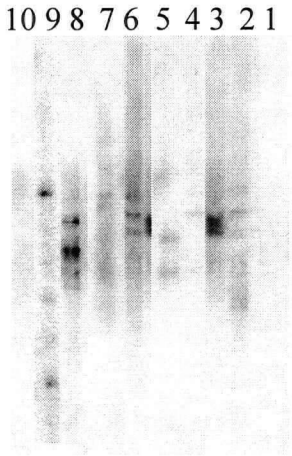
Figure 4.11. RT-PCR detection of the *O. floccosum THN2* gene transcript from the *C. lagenarium* and *M. grisea* mutants transformed with *O. floccosum THN2*. *O. floccosum* (lane 1); *C. lagenarium* melanin-restored transformants (lanes 2, 4 and 6), their negative controls (lanes 3, 5 and 7) and mutant 9141 (lane 14); *M. grisea* melanin-restored transformants (lanes 8, 10 and 12), their negative controls (lanes 9, 11 and 13) and *buf* mutant (lane 15).



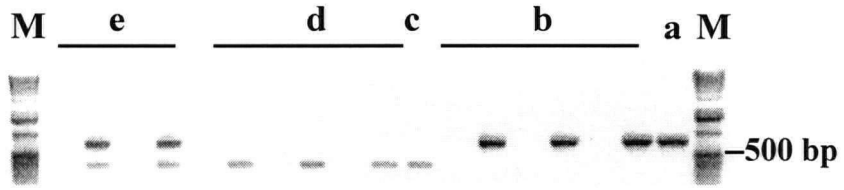
4.12. The colour of the *M. grisea* double mutant cultures. T1, T2, and T1-2: transformants of the double mutants transformed with *THN1, THN2* and both *THN1* and *THN2*; hnr⁻, hnr⁻/buf⁻, buf⁻, and wild type: hnr⁻, hnr⁻/buf⁻ and *buf* mutants, and wild type strain of the *M. grisea.*

One or more bands were observed in each transformant (Figure 4.13). *THN1* and *THN2* expression in the transformants was investigated by RT-PCR. Total RNAs were extracted from 2-day old cultures of the six transformants. Total RNAs of the *O. floccosum* wild

type strain and the double mutant were included as controls. RNA samples without reverse transcriptase treatment (minus-RT) were included to detect the genomic DNA contamination. The primer pairs, RTHN01/RTHN02 for *THN1* and THNRT1/THNRT2 for *THN2* were used in these PCR reactions. No signal was produced in the mutant and minus-RT samples. An expected fragment of 0.7 kb or 0.6 kb was produced in the transformants transformed with *THN2*, or with *THN1* from *O. floccosum*. Both fragments were detected in the transformants transformed with both *THN1* and *THN2* (Figure 4.14).



4.13. Genomic Southern blot analysis of the *M. grisea* double mutant 444-5-3 transformed with *O. floccosum THN1*, *THN2* and both. Lane 1: *M. grisea* double mutant 444-5-3 hybridized with *THN1*; lanes 2-3: transformants transformed with *THN1* hybridized with *THN1*; lane 4-5: transformants transformed with *THN2* hybridized with *THN2*, lanes 6-7: transformants transformed with both *THN1* and *THN2* and hybridized with *THN1*; lanes 8-9: transformants transformed with both *THN1* and *THN2* and hybridized with *THN2*; lane 10: *M. grisea* double mutant 444-5-3 hybridized with *THN2*.



4.14. RT-PCR detection of the *O. floccosum* reductase gene transcripts from the *M. grisea* double mutants transformed with the *O. floccosum THN1*, *THN2* and both. Lanes a and c: transcripts of *THN2* and *THN1* from *O. floccosum*; b: *THN2* transcripts from three *THN2* transformed transformants and their minus-RT controls, d: *THN1* transcripts from three *THN1* transformed transformants and their minus-RT controls; e: *THN1* and *THN2* transcripts from two *THN1* and *THN2* transformed transformants and their minus-RT controls.

4.4 Discussion

4.4.1 Characterization of *O. floccosum THN2*

Two different DNA sequences sharing high homology with other fungal reductase genes were amplified by PCR from *O. floccosum* (Eagan, 1999). This suggested that two reductase genes might be utilized in the melanin biosynthetic pathway of this fungus. Eagan (1999) isolated one reductase gene, *THN1* from *O. floccosum* and showed that it was involved in melanin biosynthesis. In the work presented here, the isolation of the second reductase gene, *THN2*, is described. *THN2* encodes 284 amino acids and is the longest sequence among the known fungal reductases. In contrast, *THN1* reductase encoded 269 amino acids and was one of the three shortest fungal reductases.

THN2 did not contain any introns, while *THN1* contained 1 intron. For comparison, the other melanin reductase genes in *C. lagenarium, A. fumigatus, A. alternata, C. heterostrophus* and *M. grisea* contained either two or four introns. These results are consistent with the observation that intron numbers are highly variable in filamentous fungi (Gurr et al., 1987). Three putative binding sites for the transcription factor Nit2 were found in the upstream sequence of *THN2*. Nit2 is involved in the regulation of the nitrogen metabolism. Similarly, several putative Nit2 protein binding sites were detected in other *O. floccosum* melanin genes, *THN1* and *OSD1* (Eagen et al., 2000; Section 3.3.1.2). This suggested that nitrogen might regulate melanin gene expression and affect *O. floccosum* pigmentation. However, the Nit2 protein binding sites remain to be verified. The percentage of hydrophilic amino acids comprising the reductase sequence was 44.7%, which is lower than the average percentage of hydrophilic amino acids (47.6%) of a naturally occurring soluble protein, suggesting that the encoded reductase is a hydrophobic enzyme. It has been reported that enzymes with higher hydrophobicity have higher thermal stability (Longo and Combes, 1997). This suggested that the reductase is more stable than hydrophilic enzymes at a high temperature.

4.4.2 Two groups of fungal melanin reductases

Two groups of fungal melanin reductases have been identified using an amino acid sequence identity matrix analysis. *O. floccosum* OfTHNB belongs to Group II reductases; these enzymes are able to convert 1,3,8-THN to vermelone in the DHN melanin

biosynthetic pathway. These results are in agreement with our results showing that the *O. floccosum THN2* complemented and restored the color of *M. grisea* and *C. lagenarium* mutants that lack 1,3,8-THN reductase activity. *O. floccosum* OfTHNA belongs to Group I, along with *M. grisea* MgTHNA and *A. fumigatus* AfTHN which were able to convert 1,3,6,8-THN to scytalone in the melanin biosynthetic pathway.

A cladogram of these fungal reductases indicates their possible functional relationships. As expected, the fungal reductase genes fell into two distinct clusters that were well supported by the amino acid sequence identity matrix analysis. Proteins in each cluster might share a common progenitor. OfTHNA was more closely related to *M. grisea* MgTHNA than to *A. fumigatus* AfTHN, while *O. floccosum* OfTHNB was more closely related to the *M. grisea* MgTHNB and *C. lagenarium* ClTHN than to the *C. heterostrophus* ChTHN and *A. alternata* AaTHN.

An alignment of the Group I and Group II reductase sequences shows that 6 residues (marked by asterisks) are strictly conserved in all the sequences (Figure 4.15). These residues are also conserved in the short chain alcohol dehydrogenase (SADH) family (Persson et al., 1991). This suggests that these fungal reductases belong to the SADH family. These enzymes have broad substrate specificity and some of them have high thermal stability (Secundo and Phillips, 1996). Among the 6 amino acid residues, Ser-164, Tyr-178, and Lys-182 in *M. grisea* MgTHNB were believed to form the active site and operate as the catalytic domain for the transfer of hydrogen from the coenzyme to the

naphthol substrate (Persson et al., 1991; Andersson et al., 1996). Thus, it is likely that the other melanin reductases have a similar catalytic mechanism.

Nine amino acid residues from *O. floccosum* OfTHNB, Ser-166, Ile-167, Tyr-180, Met-217, Tyr-218, Cys-222, Tyr-225, Trp-244 and Met-284 (marked by .; Figure 4.15) formed the enzyme binding site (Andersson et al., 1996) and are completely conserved in Group II sequences, except for the *C. lagenarium* ClTHN, in which Met-284 was not present. Five of the nine amino acid residues (Ile-167, Tyr-218, Cys-222, Trp-244 and Met-284) are not present or changed to other amino acid residues in Group I sequences. In *M. grisea*, it was determined that the MgTHNB reductase (group II) prefers 1,3,8-THN to 1,3,6,8-THN by a factor of 4.2, and that the C-terminal residue (Met-283) of this reductase determines the substrate specificity (Thompson et al., 2000; Liao et al., 2001a).

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                                                  *       *       *
OfTHNB : MVTTKSDKAHTPSKYDAIPGPLGLPSASLAGKVALVTGAGRGIGREMALELGR :  53
ClTHN  : MPGVTSQSAG--SKYDAIPGPLGLASASLMGKVALVTGAGRGIGREMAMELGR :  51
MgTHNB : MPAVTQPRGE--SKYDAIPGPLGPQSASLEGKVALVTGAGRGIGREMAMELGR :  51
ChTHN  : MANIEQ-------------------TWSLAGKVAVVTGSGRGIGKAMAIELAK :  34
AaTHN  : MASIEQ-------------------TWSLAGKVAVVTGSGRGIGKAMAIELAK :  34
OfTHNA : MSP------A----------TVKDAARPLAGKVAIITGAGRGIGRGIATELGR :  37
MgTHNA : MAPSADITSS----------GPSDASKPLAGKVALVTGAGRGIGRGIAIELGR :  43
AfTHN  : MVN-------------------TCTYLPLSGKVALVTGGGRGIGAGIALELAR :  34

OfTHNB : RGAKVIVNYANSDSSAQEVVDAIKA--AGSDAAAIKANVSDVDQIVTLFEKTK : 104
ClTHN  : RGAKVIVNYANSAETAEEVVQAIKK--SGSDAASIKANVSDVDQIVKMFGEAK : 102
MgTHNB : RGCKVIVNYANSTESAEEVVAAIKK--NGSDAACVKANVGVVEDIVRMFEEAV : 102
ChTHN  : RGAKVAVNYANAVEGAEQVVKEIKALGNGSDAHAFKANVGNVEESEKLMDDVV :  87
AaTHN  : RGAKVAVNYANAVEGAEQVVKEIKALGNGSDAAAFKANVGNVEESEKLMDDVV :  87
OfTHNA : RGANVIVNYGSSSAAAEEVVADLKA--LGTDAVAMQADISKPDEVVKLFDRAV :  88
MgTHNA : RGASVVVNYGSSSKAAEEVVAELKK--LGAQGVAIQADISKPSEVVALFDKAV :  94
AfTHN  : RGASVAINYGHSAKSAQEVVEAIQA--IGRQAVAIQADLTCVPNIESLIQEVV :  85
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OfTHNB  :  QQWGKLDIVCSNSGVVSFGHVKDVTPEEFDRVFSVNTRGQFFVAREAYKHLEI  :  157
ClTHN   :  QIWGRLDIVCSNSGVVSFGHVKDVTPEEFDRVFAINTRGQFFVAREAYKHLEV  :  155
MgTHNB  :  KIFGKLDIVCSNSGVVSFGHVKDVTPEEFDRVFTINTRGQFFVAREAYKHLEI  :  155
ChTHN   :  KHFGKLDICCSNSGVVSFGHFKDVTPEEFDRVFNINTRGQFFVAKAAYKRMEM  :  140
AaTHN   :  AHFGKLDICCSNSGVVSFGHFKDVTPEEFDRVFTINTRGQFFVAKAAYKRMEM  :  140
OfTHNA  :  AHFGGIDIVVSNSGMEVWSSELDVTQELFDKVFNLNCRGQFFVAQQGLKHCRR  :  141
MgTHNA  :  SHFGGLDFVMSNSGMEVWCDELEVTQELFDKVFNLNTRGQFFVAQQGLKHCRR  :  147
AfTHN   :  RHFGRLDIVVSNSGMEKFKPLEETTLEDFNEVFNLNTRAQMFVARYAYDHIQP  :  138

                    *              *   *
OfTHNB  :  GGRLILMGSITGQAKMVPRHAVYSASKGAIETFVRCMAVDFGDKKITVNAVAP  :  210
ClTHN   :  GGRLILMGSITGQAKGVPKHAVYSGSKGTIETFVRCMAIDFGDKKITVNAVAP  :  208
MgTHNB  :  GGRLILMGSITGQAKAVPKHAVYSGSKGAIETFARCMAIDMADKKITVNVVAP  :  208
ChTHN   :  GGRIILMGSITGQAKGVPKHAVYSGSKGAIETFTRCMAVDAGEKKVTVNCVAP  :  193
AaTHN   :  GGRIILMGSITGQAKGVPKHAVYSGSKGAIETFTRCMAIDAGEKRITVNCVAP  :  193
OfTHNA  :  GGSIILTSSVAASLSGIPNHALYAGSKAAVEGFTRAFSVDCGEKGVTVNAIAP  :  194
MgTHNA  :  GGRIILTSSIAAVMTGIPNHALYAGSKAAVEGFCRAFAVDCGAKGVTVNCIAP  :  200
AfTHN   :  GGRVILMSSIAAGL-GVPGHALYAGSKAAIEGFTRCLAADFGRKGCTVNAIAP  :  190
                  #..##          .#   #

OfTHNB  :  GGIKTDMYHAVCREYIPNGLT-LNDDETDEYAAG-WSPIHRVGLPIDVARVVA  :  261
ClTHN   :  GGIKTDMYRDVCREYIPNGGE-LDDEGVDEFAAG-WSPMHRVGLPIDIARVVC  :  259
MgTHNB  :  GGIKTDMYHAVCREYIPNGEN-LSNEEVDEYAASAWSPLHRVGLPIDIARVVC  :  260
ChTHN   :  GGIKTDMYHAVCREYIPNGDQ-LSDDQVDEYACT-WSPHNRVGQPIDIARVVC  :  244
AaTHN   :  GGIKTDMYHAVCREYIPGGEK-LSDDQVDEYACT-WSPHNRVGQPVDIARVVC  :  244
OfTHNA  :  GGVKTDMYDENSWHYVPGGYKGMSQDVIDEGILK-ACPLKRVGTPSDIGKAVA  :  246
MgTHNA  :  GGVKTDMFDENSWHYAPGGYKGIPQEKIDEGLAN-MNPLKRIGYPADIGRAVS  :  252
AfTHN   :  AGVKSDMWRENAWRYAPGCDKSSSLEEIETALAS-GSPLKRCGVPEDIGKVVS  :  242
                 ..  #.# .                     .

OfTHNB  :  FLASQDGQWINGKVLG-------VDGGACM-  :  284
ClTHN   :  FLASQDGESR----LE-------L-------  :  272
MgTHNB  :  FLASNDGGWVTGKVIG-------IDGGACM-  :  283
ChTHN   :  FLASQDGDWVNGKVIG-------IDGAACM-  :  267
AaTHN   :  FLASQDGDWVNGKVIG-------IDGAACM-  :  267
OfTHNA  :  LLVSEEGEWINGQIIK-------LSGGSAV-  :  269
MgTHNA  :  ALCQEESEWINGQVIK-------LTGGGI--  :  274
AfTHN   :  FLASPDAEWVNGEFFSPPPCKGPLPGGDLEC  :  273
                                        .|
```

Figure 4.15. Comparison of the deduced amino acid sequence of fungal reductases. Dashes indicate gaps introduced to maintain the alignment. Sequence identity of 80% and more in a column is indicated by black shading of the conserved residues and sequence similarity is indicated by grey shading. The residues conserved in short chain alcohol dehydrogenases family are indicated as *. The residues considered to be comprising the enzyme binding site are marked by . . The residues positioned near the enzyme binding site residues and showed clear difference between Group I and Group II sequences are indicated as #. Fungal reductase sequences are: *O. floccosum THN1* protein (OfTHNA), *M. grisea* 4HNR (MgTHNA), *A. fumigatus arp2* protein (AfTHN), *C. heterostrophus Brn1* protein (ChTHN), *A. alternata BRM2* protein (AaTHN), *O. floccosum THN2*

protein (OfTHNB), *M. grisea* 3HNR (MgTHNB), and *C. lagenarium THR1* protein (ClTHN).

It is highly probable that all the reductases from Group II could prefer 1,3,8-THN to 1,3,6,8-THN as a substrate. In group I reductase sequences, the five amino acid residues Ile-167, Tyr-218, Cys-222, Trp-244, and Met-284 are changed or not present. This might contribute to their preference for 1,3,6,8-THN.

Seven amino acid residues of *O. floccosum* OfTHNB, Gly-165, Thr-168, Gly-169, Ser-181, Gly-185, Val-221, Arg-223 (marked by #) were completely conserved in Group II sequences, and changed to Ser, Ala, Ala, Ala, Ala, Asp and Try in Group I sequences. Interestingly, these amino acid residues are the neighbors of some of the substrate binding residues (marked by .; Figure 4.15). These changes may also have a contribution for the substrate preference of the enzymes. However, it is not known whether this preference is caused by the above changes, and further 3-D structure analyses of Group I reductases are needed to provide this information.

4.4.3 *O. floccosum THN2* protein function

The function of *O. floccosum THN2* was confirmed by complementing *C. lagenarium* and *M. grisea* reductase deficient mutants using *THN2*. The accumulation of a shunt metabolite, 3,4-dihydro-4,8-dihydroxy-1 naphthalenone (DDN), derived from 1,3,8-THN, was observed in the medium of the *C. lagenarium* mutant 9141 (Perpetua et al., 1996). This mutant produces a brown phenotype on PSA, forms non-melanized

appressoria on glass slides, and has lost the ability to infect cucumber leaves (Perpetua et al., 1996). The mutant 9141 can be complemented by the *C. lagenarium THR1* reductase gene that restores its melanization and pathogenicity (Perpetua et al., 1996). The 1,3,8-THN reductase gene sequence was deleted from the *M. grisea* mutant which produces a brown phenotype on MEA. The absence of this gene sequence was confirmed by hybridization studies (Eagen, 1999).

In complementation experiments, some of the transformants produced melanized mycelia and appressoria that were similar to the wild type strains. This indicated that the genomic clone of the *THN2* complemented the *C. lagenarium* and *M. grisea* reductase deficient mutants and functioned in mycelial and the appressorial melanization. This provides evidence that *THN2* does in fact encode a homologous reductase. Furthermore, *C. lagenarium* melanin-restored transformants showed pathogenicity on cucumber leaves. Similarly, *O. floccosum OSD1* encoded a SD that was able to restore *C. lagenarium* melanization and pathogenicity. It is apparent that the melanin genes from *O. floccosum* can replace and function as melanin genes in *C. lagenarium*. This phenomenon was found in other fungal DHN melanin pathways, as the reductase gene (*BRM2*) of *A. alternata* was able to restore the melanin production and pathogenicity of the *M. grisea* mutant buf$^-$ mutant (Kawamura et al., 1997); *A. alternata ALM* gene encoding a PKS restored the melanin production and pathogenicity of PKS deficient mutant of *C. lagenarium* (mutant Pks$^-$; Takano et al., 1997a) and *M. grisea* (mutant Alb$^-$; Kawamura et al., 1997). These data suggested that the melanin genes were able to replace their counterparts in other fungal species.

Southern blot analysis suggested that only one copy of the *THN2* gene existed in the *O. floccosum* genome. In the transformants two to ten bands were observed, representing one to five copies of integrated *THN2*, since the transformant genomic DNAs were digested with *Eco*RI and *Hind*III that cut *THN2* into two fragments. RT-PCR results suggested that *THN2* was being expressed in *C. lagenarium* and *M. grisea* during vegetative growth. The analysis of *THN2* function suggested that the enzyme encoded by *THN2* might be involved in the conversion of 1,3,8-THN to vermelone in *O. floccosum.* Furthermore, the *THN2* product was predicted to be located in the cytoplasm, suggesting that the conversion of 1,3,8-THN to vermelone in *O. floccosum* might occur in the cytoplasm.

Because *THN1* complemented the *buf* mutant as well (Eagan, 1999), both *O. floccosum THN1* and *THN2* products could function as 1,3,8-THN reductases. However, both *THN1* and *THN2* could not complement the *M. grisea* double mutant lacking MgTHNA and MgTHNB, although they integrated into the transformants' genome and were being expressed in the transformants. These results suggested that neither OfTHNA nor OfTHNB can function as MgTHNA in *M. grisea*, although our sequence analysis suggested that OfTHNA could have a function similar to MgTHNA. However, improper posttranslational modifications of transformed OfTHNA might not allow functionality in *M. grisea*, as it is known that a posttranslational modification is made in *M. grisea* MgTHNA that lacked the N-terminal Met and Ala residues of the primary translation product (Thompson et al., 2000). Therefore, proper posttranslational modifications might

be required for OfTHNA to have proper functionality when it is expressed and translated in *M. grisea*. These modifications might be involved in contributing to the enzymes stability, its' correct localization within the cells, or its' enzymatic optimal activity (Shaw et al., 1990).

In addition, the localization of the reactions such as the conversion of 1,3,6,8-THN to scytalone could take place in different organelles in different fungal species. ProtComp was used to predict the probability of the subcellular localization of these reductases. The program can be used to predict the subcellular localization of fungal proteins with a predictive accuracy of 70-90%. *O. floccosum* OfTHNA and *A. fumigatus* AfTHN were predicted to be located in the mitochondria, while *M. grisea* MgTHNA was predicted to be located in the cytoplasm. In addition, all Group II reductases were predicted to be located in the cytoplasm. This suggests that both *M. grisea*, MgTHNA and MgTHNB are located in the cytoplasm, which might explain why the *M. grisea* mutant lacking MgTHNA produced melanin. However, it is different in *A. fumigatus*, the *A. fumigatus* mutant lacking AfTHN did not produce melanin, this might be due to AfTHN being located in the mitochondria and the other potential reductase being in the cytoplasm.

In conclusion, a second reductase gene has been isolated from *O. floccosum*, and thus two reductases are involved in the *O. floccosum* melanin synthesis. Based on an amino acid sequence identity matrix analysis, it was shown that both *O. floccosum THN1* and *THN2* reductases belong to two different groups of melanin reductases. Although, both reductases appeared to function as 1,3,8-THN reductase in *M. grisea*, it was not possible

to determine whether the two reductases catalyzed the same or different reduction reactions in *O. floccosum*.

Chapter 5

Cloning a partial PKS gene and analyzing transcriptional patterns of melanin genes in *O. floccosum* 387N

5.1 Introduction

Nutrients play an important role in the pigmentation of sapstain fungi, including *O. floccosum* (Eagen, 1999; Fleet, 2001). The color of fungal mycelia ranges from white, to grey, to brown and black when *O. floccosum* is grown on media supplemented with different carbon and nitrogen sources (Eagen, 1999). With respect to the nitrogen source, brown hyphae were observed with globulins, while white, beige or grey hyphae were observed with ammonium chloride or tyrosine. When skim milk was used as a nitrogen source, the fungus consistently produced white mycelia (Abraham, 1995). However, when the media were supplemented with asparagine the color of fungal hyphae ranged from white to black depending on the carbon source. For example, mannose addition resulted in the darkest color while the addition of starch and glucose gave light-brownish fungal mycelia. Similarly, other sapstain fungi such as *O. piceae* when grown on olive oil or glycerol produced brownish mycelia (Fleet, 2001). We also observed that in *O. floccosum* melanin was only produced in the mycelia and not in its conidia and yeast-like cells. These observations led us to question whether the expression of melanin genes in *O. floccosum* is affected by the nutrients or by fungal development.

Except for the work on melanin gene expression in *C. lagenarium* there is little information about fungal melanin gene expression systems (Takano et al., 1997b). In *C. lagenarium*, the transcripts of the three melanin genes, *PKS1, SCD1* and *THR1* accumulated one or two hours after the start of conidial germination. This transcriptional pattern was observed both in differentiating and non-differentiating conidia, although no appressorium or pigment were produced from the non-differentiating conidia.

Four genes, encoding a PKS, a SD, and two reductases, are involved in the fungal melanin DHN biosynthesis pathway (Figure 1.4). So far, only three melanin genes, a SD gene (*OSD1*) and two reductase genes (*THN1* and *THN2*) have been isolated in *O. floccosum*. To include the fourth melanin gene in the gene expression study, we had to isolate at least a partial PKS gene in *O. floccosum*.

Fungal PKSs are large multifunctional proteins that possess up to eight types of functional domains (acyl transferase, acyl carrier protein, ketosynthase, ketoreductase, enoyl reductase, dehydratase, thioesterase, and methyltransferase). The acyl transferase domain initiates the polyketide chain-building process; ketosynthase and acyl carrier protein process chain elongation; ketoreductase and dehydratase reduce and process the keto group; and thioesterase and meth-yltransferase release the full-length chain (Khosla, 1997). Fungal melanin PKS genes have been isolated in *C. lagenarium*, *A. fumigatus* and *Nodulisporium sp* (Takano et al., 1995; Tsai et al., 1998; Fulton et al., 1999). *C. lagenarium* PKS uses malonyl CoA to produce 1,3,6,8-THN (Fujii et al., 1999, 2000),

while *A. fumigatus* PKS synthesizes a heptaketide from malonyl CoA, which is then converted to 1,3,6,8-THN by the gene product of *ayg1* (Watanabe et al., 2000; Tsai et al., 2001). *C. lagenarium* and *Nodulisporium* PKSs are more similar to each other than to the *A. fumigatus* melanin PKS. PKS disruptants for these three fungal species are not able to produce pigment and are white in culture. In this chapter, we present the isolation and characterization of a partial melanin PKS gene (*OPKS1*), and the transcriptional regulation of the four *O. floccosum* melanin genes (*OPKS1*, *OSD1, THN1* and *THN2*) when grown on different media.

5.2 Materials and methods

5.2.1 PCR primers and molecular techniques

PCR primers used in this study are listed in Table 5.1. DNA and RNA manipulations were the same as described in Sections 2.2.6 and 3.2.2. RT-PCR analysis, DNA sequencing and sequence data analyses using bioinformatics tools were the same as described in Sections 2.2.9, 3.2.3, 3.2.6 and 3.2.8. Protein motif analysis was carried out with the ScanProsite software at the ExPASy proteomics tool server (www.expasy.org).

5.2.2 Cloning of a partial PKS gene in *O. floccosum*

To isolate a portion of the PKS gene (*OPKS1*) in *O. floccosum*, two degenerate oligonucleotides, PKS3 and PKS6, were designed based on the amino acid sequences of

the PKSs of *C. lagenarium* (GenBank accession number: S60224) and *A. nidulans* (GenBank accession number: Q03149). The primer PKS3 is degenerate to the conserved amino acid sequence FFNMSPRE, and the PKS6 is fully degenerate to the conserved amino acid sequence MHGTGTQ. To amplify a partial PKS gene from 387N,

Table 5.1. List of the oligonucleotides synthesized for PCR reactions.

Primer name	Sequence	Remark
PKS3	5' TTCTTCAACATG TC(A/T)CC(C/T)CGIGA 3'	F, for *OPKS1*
PKS6	5' CTGGGTACC(T/G)GT(T/G)CC(A/G)TGCA 3'	R, for *OPKS1*
RTPKS1	5' CGTCGACACCTACTATATCAC 3'	F, for *OPKS1*
RTPKS2	5' GTCAATGTCAAGGGGATCGA 3'	R, for *OPKS1*
THNRT1	5' TCCCAGCAAGTACGACGCCATTC 3'	F, for *THN2*
THNRT2	5' CTGGCCATCCTGCGACGCTAG 3'	R, for *THN2*
RTHN01	5' GAAGTTGTCAAGCTGTTCGA 3'	F, for *THN1*
RTHN02	5' CGTACATGTCCGTCTTGACA 3'	R, for *THN1*
SD23	5' GGCCTCAATATCAGCAGCCTCA 3'	F, for *OSD1*
SD26	5' TGCGCCGATGCAATGGCACT 3'	R, for *OSD1*
NL1*	5' GCATATCAATAAGCGGAGGAAAAG 3'	F, for 25S rDNA
NL2*	5' CTCTCTTTTCAAAGTGCTTTTCATCT 3'	R, for 25S rDNA

Notes: 'I' represents inosine. F represents a forward primer, while R represents a reverse primer. * courtesy of S. K. Kim.

its genomic DNA was used as a template in the PCR reactions with the primers PKS3 and PKS6 (Section 2.2.7). Purification, subcloning and sequencing of the PCR-amplified DNA fragments were performed as described in Sections 3.2.4 and 3.2.8.

5.2.3 Culture of *O. floccosum*

O. floccosum conidia were produced by inoculating mycelial fragments onto sterile wood blocks and incubating for 7-10 days at 20°C. Conidia were harvested by washing the wood block surface with sterile, deionized water containing 0.01% (vol/vol) Tween 20. The solution was diluted to obtain 10^6 conidia per ml, and 600-μl of the conidial suspension was inoculated onto cellophane (BioRad) overlaid B-media agar plates (Section 2.2.5) supplemented with different carbon and nitrogen sources. The plates were incubated at 20°C. B-media with 0.1% asparagine was supplemented with 2% (w/v) glycerol (medium 1), 2% (w/v) mannose (medium 2), 2% (w/v) olive oil (medium 3). In medium 4, the B-medium was supplemented with 2% (w/v) skim milk (Defico) only. All the supplemented nutrients were filter-sterilized before being added to the media. Yeast cells were produced in PM medium (see Section 2.2.1) by inoculating the germinated conidia collected from media 2 after one-day incubation. All the experiments were repeated three times.

5.2.4 Reverse-transcription PCR

For RNA preparation the procedures described in Section 3.2.2 were followed. After treatment with DNAase I, the RNA samples were used as templates to perform PCR using the 26S rDNA primer set NL1-NL2. If no PCR products were amplified, the samples were considered as free of DNA contamination, and reverse-transcription reactions were preformed as described in Section 3.2.6 using 2 μg of total RNA. PCR reactions were performed using the genomic DNA free reverse-transcribed samples, and melanin gene target primer sets, RTPKS1-RTPKS2 for *OPKS1*, RTHN01-RTHN02 for *THN1*, SD23-SD26 for *OSD1*, THNRT1-THNRT2 for *THN2* and the 26S rDNA primer set NL1-NL2 as an internal control. As the PCR primer pair for the *OSD1* spans across the two introns of the gene it was used to detect genomic DNA contamination in each sample. PCR conditions were the same as described in Section 2.2.7. Experiments were carried out to examine the levels of the melanin genes' transcripts in conidia, yeast cells and mycelia. For transcriptional analysis, samples were collected and analyzed from mycelia grown on media 1, 2, 3, and 4 for four days, and from mycelia grown for 1, 2, 3, and 4 days on media 2. In each sample, the target genes and the rDNA were amplified using 16–30 cycles in the PCR reactions. The cycle number that corresponded to the linear phase of this PCR reaction was determined and used in a subsequent PCR of all treatments. The cycle number was determined using the following procedure: reverse-transcribed treatments (Day 4 samples) were diluted 1:30 with water, then 500 μl of a randomly chosen reverse-transcribed treatment was PCR amplified using the rDNA primer set by 6–30 cycles, The PCR products were gel electrophoresed by loading the same volumes of the PCR products. In order to compare the relative transcript levels of

the target genes the gel loading volumes of the target gene PCR reactions were standardized by loading the same relative amount of rDNA PCR product for each sample.

5.3 Results

5.3.1 Isolation of a partial PKS gene in *O. floccosum* 387N

With the primers PKS3 and PKS6 and *O. floccosum* genomic DNA, a fragment of ~720 bp in length was amplified, subcloned and sequenced (Figure 5.1; GenBank accession number: AF411603). The sequence, designated *OPKS1*, contained 717 bp of DNA and encoded 239 amino acids. No introns were found in the sequence. A BLASTX scan of the sequence in the NCBI nr database found 14 hits with significant E value ($1e^{-129}$ – $1e^{-72}$). The deduced protein sequence shared 62-91% identity and 73-94% similarity to the PKSs of *C. lagenarium*, *A. fumigatus, Nodulisporium sp*, and *A. nidulans*. This strongly suggests that the *OPKS1* sequence is a fragment of a PKS homolog. Furthermore, the motif analysis revealed that the *OPKS1* predicted protein sequence contain a β-ketoacyl synthase motif.

Figure 5.2 shows the β-ketoacyl synthase motif of the *OPKS1* predicted protein aligned with the motifs of available fungal PKSs and a PKS from *Streptomyces coelicolor*. Two subclasses of PKSs, WA-type and MSAS-type have been described in the fungal kingdom. The *OPKS1* β-ketoacyl synthase motif is conserved throughout most of the sequence with WA type fungal PKS sequences, and the extent of conservation with the

```
  1 ttcttcaacatgtcacctcgggaggctctgcagacggatcccatgcagcgcatggcaatc
    F  F  N  M  S  P  R  E  A  L  Q  T  D  P  M  Q  R  M  A  I
 61 accacggcatttgaggctctggagatgtctggctacgtgccaaaccgtacgccctcgacg
    T  T  A  F  E  A  L  E  M  S  G  Y  V  P  N  R  T  P  S  T
121 cgtctcgaccgcattggcactttctacggccagacgtcggatgactggcgcgagatcaac
    R  L  D  R  I  G  T  F  Y  G  Q  T  S  D  D  W  R  E  I  N
181 gccgcacagtccgtcgacacctactatatcacaggtggtgtgcgcgccttcggccccggc
    A  A  Q  S  V  D  T  Y  Y  I  T  G  G  V  R  A  F  G  P  G
241 cgcactaattatcactttggcttcagtggccctagtctcaacattgacacggcctgctca
    R  T  N  Y  H  F  G  F  S  G  P  S  L  N  I  D  T  A  C  S
301 tcgagtgctgctgccatgaacgtcgcctgcacatctctctgggcacgtgactgtgacacc
    S  S  A  A  A  M  N  V  A  C  T  S  L  W  A  R  D  C  D  T
361 gccattatcggcggtctgtcctgtatgaccaattcggacatctttgccggcctgagtcgc
    A  I  I  G  G  L  S  C  M  T  N  S  D  I  F  A  G  L  S  R
421 ggccagttcctatccaagacgggcccctgcgccacatttgacaacgccgctgacggctac
    G  Q  F  L  S  K  T  G  P  C  A  T  F  D  N  A  A  D  G  Y
481 tgccgtggtgatggctgtgcgtcggtcattgtcaagcgcctcgatgatgccgaggctgat
    C  R  G  D  G  C  A  S  V  I  V  K  R  L  D  D  A  E  A  D
541 ggtgacaatatcctggcagttatcttaggcaccgccactaaccactcggccgatgctatt
    G  D  N  I  L  A  V  I  L  G  T  A  T  N  H  S  A  D  A  I
601 tccatcacgcacccgcacggtcctacccagtcaatcctttcgtcagccatcttggatgat
    S  I  T  H  P  H  G  P  T  Q  S  I  L  S  S  A  I  L  D  D
661 gccggtgtcgatccccttgacattgactatgttgagatgcacggcaccggtactcag
    A  G  V  D  P  L  D  I  D  Y  V  E  M  H  G  T  G  T  Q
```

Figure 5.1. Nucleotide and deduced amino acid sequences of *O. floccosum* *OPKS1*.

MSAS-type PKS motifs is much lower. This alignment shows that Cys-21, the active site, is conserved among all the fungal β-ketoacyl synthase motifs. To examine the association of the *OPKS1* protein to other PKSs, a cladogram (Figure 5.3) was constructed based on the motif sequence alignment. The PKS gene from *S. coelicolor* was used as an out-group. The fungal PKS proteins grouped into two distinct clusters, one cluster contained all MSAS-type PKSs including *Penicillium patulum* PpMSAS, *Phoma sp.* PspMSAS and *Penicillium freii* AfMSAS, and the other cluster contained the WA-type PKS sequences.

```
                                   *
AfPKS    : PGRINYYFKFSGPSVSVDTACSSSLAAIHLACNAIWRNDCDTAISGGVNLLTNPD : 55
AnWA     : PGRINYYFKFSGPSVSVDTACSSSLAAIHLACNSIWRNDCDTAITGGVNILTNPD : 55
ApPKS    : PGRINYYFKFSGPSVSVDTACSSSLAAIHMACNSIWRNDCDAAIAGGVNILTNPD : 55
ApPKSL   : PGRINFCFEFAGPSYTNDTACSSSLAAIHLACNSLWRGDCDTAVAGGTNMIYTPD : 55
AnST     : PGRINFCFEFSGPSYSNDTACSSSLAAIHLACNSLWRGDCDTAVAGGTNMIFTPD : 55
OpPKS    : PGRTNYHFGFSGPSLNIDTACSSSAAAMNVACTSLWARDCDTAIIGGLSCMTNSD : 55
NspPKS   : PGRINYHFGFSGPSLNIDTACSSSAAALQVACTSLRAKECDTAIVGGLSCMTNSD : 55
PspPKS   : PGKINYHFGFSGPSLNIDTACSSSAAVLQIACTSFWAKDCDTAVVGGLSCMTNPD : 55
ClPKS    : PGRINYHFGFSGPSLNVDTACSSSAAALNVACNSLWQKDCDTAIVGGLSCMTNPD : 55
PspMSAS  : PNRISYHLNLMGPSAAVDAACASSLVAVNSGQQAILAGKSRVAIVGGVNVCLSPA : 55
AfMSAS   : PNRISYHLNLMGPSTAVDAACASSLVAIHHGRQAILQGESKVAIVGGVNALCGPG : 55
PpMSAS   : PSRISYLLDLMGPSVALDAACASSLVAVHHARQAIRAGETDLAIAGGVNALLGPG : 55
ScPKS    : PGRLSYFFDLTGPSLALDTACSSSLAAVHTALRSLRDGECGVALAGGVNLMLTPG : 55
```

Figure 5.2. Alignment of the β-ketoacyl synthase motif of the cloned *OPKS1* PCR product with other PKS β-ketoacyl synthase motifs. Sequence identity of 80% and more is indicated by black shading of the conserved residues and sequence similarity is indicated by grey shading. The active site cysteine is indicated by an asterisk. Sequence identification codes are: OpPKS, *O. floccosum OPKS1* (see Figure 5.1); ClPKS: *C. lagenarium PKS1* product for melanin biosynthesis (Takano et al., 1995); PspPKS: putative melanin PKS of *Phoma sp.* C2932 (Bingle et al., 1999); NspPKS: *Nodulisporium sp.* ATCC74245 pks for melanin biosynthesis (Fulton et al., 1999); ApPKS: putative WA type ketosynthase domain of *Aspergillus parasiticus* (Bingle et al., 1999); AfPKS: PKS for *A. fumigatus* melanin biosynthesis (Tsai et al., 1998); ApPKSL: PKS for *Aspergillus parasiticus* aflatoxin biosynthesis (Feng and Leonard, 1995); AnWA: conidial green pigment synthase of *A. nidulans* (Mayorga and Timberlake, 1992); AnST: *A. nidulans* pksST for sterigmatocystin biosynthesis (Yu and Leonard, 1995); PpMSAS: *Penicillium patulum* MSAS for 6-methylsalicylic acid synthesis (Beck et al., 1990); PspMSAS: putative MSAS-type ketosynthase domain of *Phoma sp.* C2932 (Bingle et al., 1999); AfMSAS: *Penicillium freii* Pfks for 6-methylsalicylic acid synthesis (Nicolaisen et al., 1997); ScPKS: *Streptomyces coelicolor* PKS for antibiotic actinorhodin (Fernandes-Moreno et al., 1992).

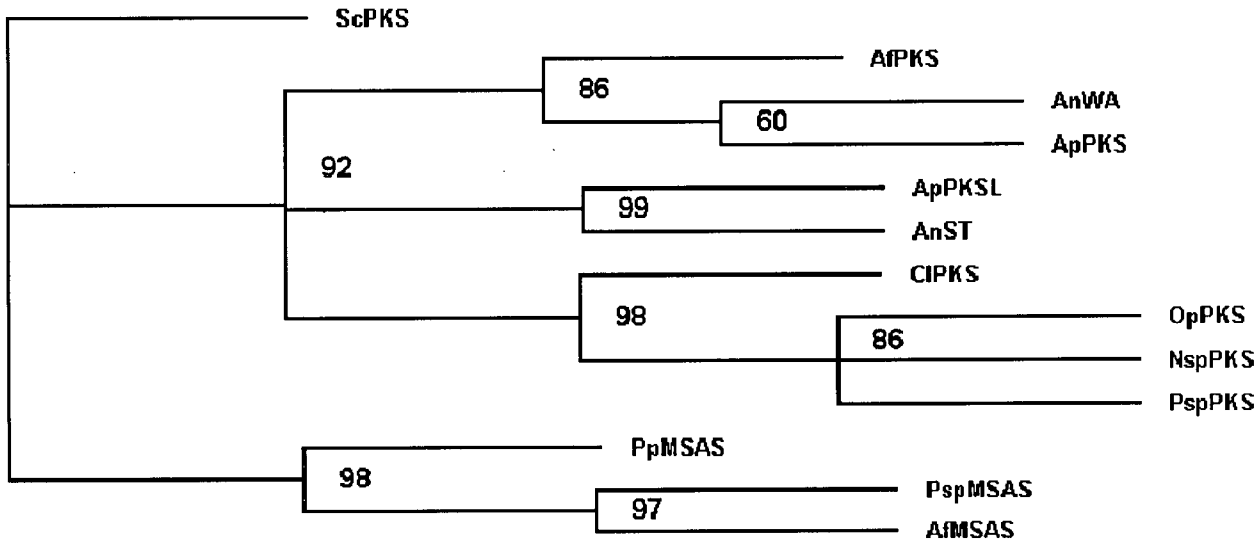

Figure 5.3. The relationships of the β-ketoacyl synthase motif of fungal PKSs. The PKS sequence of *Streptomyces coelicolor* was used as an outgroup. The cladogram was generated using Paup 4.0b6. Bootstrap values based on 1000 replicates are shown within branch nodes. Sequence identification codes are: OpPKS, *O. floccosum OPKS1* (see Figure 5.1); ClPKS: *C. lagenarium PKS1* product for melanin biosynthesis (Takano et al., 1995); PspPKS: putative melanin PKS of *Phoma sp.* C2932 (Bingle et al., 1999); NspPKS: *Nodulisporium sp.* ATCC74245 pks for melanin biosynthesis (Fulton et al., 1999); ApPKS: putative WA type ketosynthase domain of *Aspergillus parasiticus* (Bingle et al., 1999); AfPKS: PKS for *A. fumigatus* melanin biosynthesis (Tsai et al., 1998); ApPKSL: PKS for *Aspergillus parasiticus* aflatoxin biosynthesis (Feng and Leonard, 1995); AnWA: conidial green pigment synthase of *A. nidulans* (Mayorga and Timberlake, 1992); AnST: *A. nidulans* pksST for sterigmatocystin biosynthesis (Yu and Leonard, 1995); PpMSAS: *Penicillium patulum* MSAS for 6-methylsalicylic acid synthesis (Beck et al., 1990); PspMSAS: putative MSAS-type ketosynthase domain of *Phoma sp.* C2932 (Bingle et al., 1999); AfMSAS: *Penicillium freii* Pfks for 6-methylsalicylic acid synthesis (Nicolaisen et al., 1997); ScPKS: *Streptomyces coelicolor* PKS for antibiotic actinorhodin (Fernandes-Moreno et al., 1992).

In the latter, three sub-clusters were formed; one sub-cluster contained three PKSs including *A. parasiticus* ApPKS, *A. fumigatus* AfPKS and *A. nidulans* AnWA; the second sub-cluster contained *A. nidulans* AnST PKS and *A. parasiticus* ApPKSL; and finally the

last subcluster contained *O. floccosum OPKS1*, *Nodulisporium sp.* NspPKS, *C. lagenarium* ClPKS and *Phoma sp.* PspPKS.

5.3.2 Melanin gene expression in *O floccosum 387N*

The melanization of *O. floccosum* mycelia on different media (media 1, 2, 3, and 4) was examined. Table 5.2 showed that after one or two days of incubation the mycelia were white on all the media. However, at day 3, slight pigmentation was observed on media 1 and 2. At day 4, the mycelia were brown or light brown in color on media 1, 2 and 3, while the hyphae were still white on media 4. The fungus had no pigmentation on media 4 even after one month of incubation.

Table 5.2. Color of *O. floccosum* mycelia on different nutrient media.

Culture medium	Fungal color at different incubation days			
	Day 1	Day 2	Day 3	Day 4
Medium1	White	White	Very light brown	Light brown
Medium 2	White	White	Light brown	Brown
Medium 3	White	White	White	Light brown
Medium 4	White	White	White	White

Note: B-media with 0.1% asparagine supplemented with 2% (w/v) glycerol (medium 1), 2% (w/v) mannose (medium 2), 2% (w/v) olive oil (medium 3). Medium 4: B-medium supplemented with 2% (w/v) skim milk.

To examine the presence or absence of the melanin gene transcripts in *O. floccosum* conidia, mycelia and yeast cells RT-PCR reactions were carried out using the primer sets for the four melanin genes and the rDNA. In these experiments, the rDNA was used as a positive control. The results showed that although transcripts of the rDNA were detected in conidia and yeast cells, no transcripts for the four melanin genes were detected in these samples. Transcripts of the four melanin genes were, however, detected in the mycelial samples (Figure 5.4).

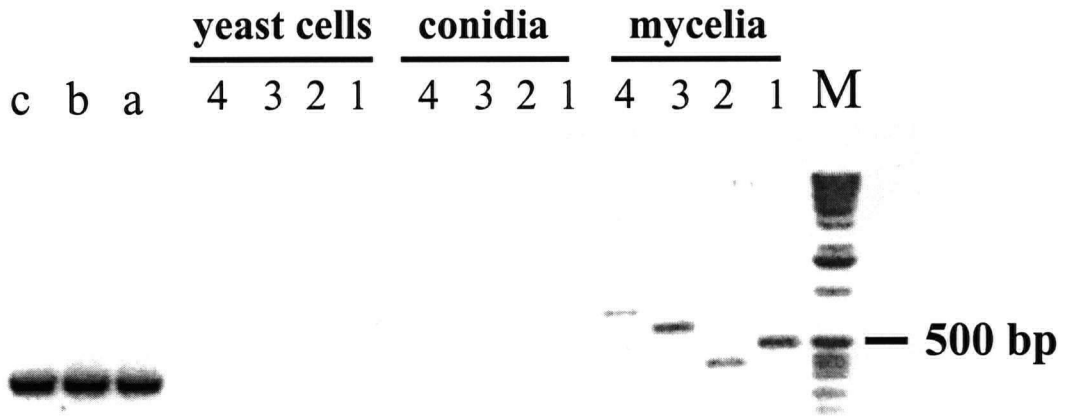

Figure 5.4. RT-PCR detection of the transcripts of the four melanin genes in conidia, yeast cells and mycelia of *O. floccosum*. Lane M: 1 kb DNA ladder, four lanes 1, 2, 3 and 4 in each sample: *OPKS1*, *THN1*, *OSD1* and *THN2*; a-c: mycelia, conidia and yeast cells using 26S rDNA PCR primer set.

The melanin genes' transcription in mycelia grown on media 2 at 1, 2, 3, and 4 days was examined using RT-PCR (Figure 5.5). At Day 1, among the four gene transcripts, *OPKS1* accumulated to the highest level, *THN2* to the lowest level, and *OSD1* transcripts were produced at a higher concentration than *THN1* transcripts. At Day 2, all gene transcripts were more prevalent than at Day 1; *OSD1* and *OPKS1* transcripts were the highest and

THN2 transcripts were the lowest among the four genes' transcripts. At Day 3, the transcripts of the four genes were similar and higher than at Day 2. At Day 4, the transcripts of the four genes were similar to those at Day 3.

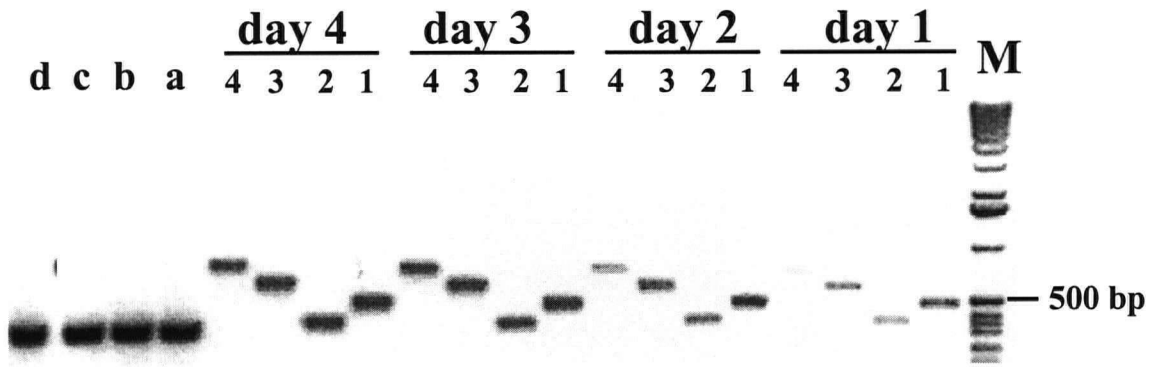

Figure 5.5. Time course of expression of the *OPKS1, THN1, OSD1* and *THN2* genes during the growth of *O. floccosum* on media 2. Lane M: 1 kb DNA ladder, four lanes 1, 2, 3 and 4 in each sample: *OPKS1*, *THN1*, *OSD1* and *THN2*; 26S rDNA was used as an internal control for the 4 samples, a-d: day 1-4.

The transcriptional patterns of the melanin genes in mycelia grown on media 1, 2, 3, and 4 at Day 4 were investigated using RT-PCR (Figure 5.6). The transcripts of the four genes on media 1, 2, 4 were similar and higher than the transcripts of the four genes of the fungus grown on medium 3.

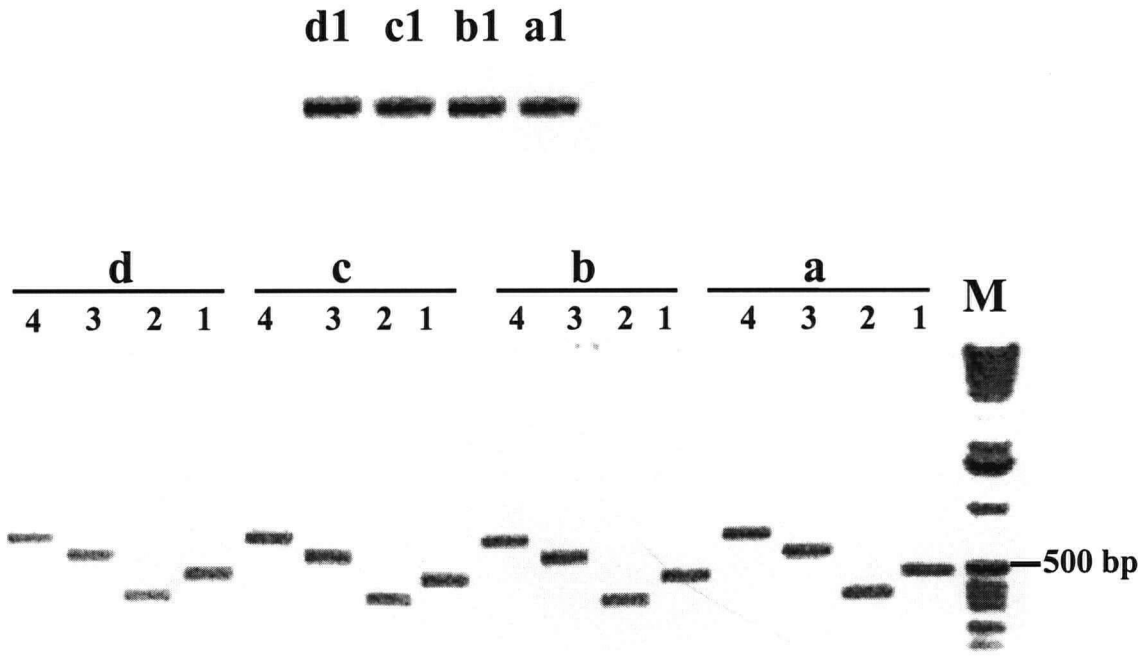

Figure 5.6. The expression of the *OPKS1, THN1, OSD1* and *THN2* genes during the growth of *O. floccosum* on B-media supplemented with different combinations of carbon and nitrogen sources. Lane M: 1 kb DNA ladder, a: medium 4, b: medium 2, c: medium 1, d: medium 3, four lanes 1, 2, 3 and 4 in each sample: *OPKS1*, *THN1*, *OSD1* and *THN2*; 26S rDNA was used as an internal control for the 4 samples, a1: medium 4, b1: medium 2, c1: medium 1, d1: medium 3.

5.4 Discussion

5.4.1 *OPKS1* is a partial DHN melanin PKS gene

The β-ketoacyl synthase motif is one of the components of a number of multifunctional enzymes including fatty acid synthetases and PKS's. Fatty acid synthetases catalyze the formation of long-chain fatty acids, while PKSs catalyze the formation of 6-

methysalicylic acid in *P. patulum*, melanin in *C. lagenarium* and green conidial pigment in *A. nidulans* (Beck et al., 1990; Mayorga and Timberlake, 1992). Among the fungal PKSs two subclasses, WA-type and MSAS-type PKS, have been proposed. WA-type PKSs initiate the synthesis of green spore pigment, melanin, and aflatoxin (Mayorga and Timberlake, 1992). The MSAS-type PKSs catalyze the biosynthesis of 6-methylsalicylic acid, the first intermediate in the pathway leading to the mycotoxin patulin in *P. patulum* (Beck et al., 1990).

Because we had cloned several melanin genes in the DHN pathway in *O. floccosum*, we assumed that a melanin PKS gene would be present in this fungus. For *O. floccosum*, a BLASTX scan of the NCBI nr database using the *OPKS1* sequence found a dozen hits of PKSs with significant E values. This indicated that the *OPKS1* protein belonged to the PKS family instead of the fatty acid synthetase family. However, it has been reported that multiple PKS genes could be present in one fungal genome. For example, two PKS genes had been isolated in *Phoma sp.*, one PKS is involved in the DHN melanin biosynthesis, and the other encoded a MSAS type PKS (Bingle et al., 1999). As well this situation was reported by Geisen (1996), who used a multiplex PCR method to identify sequences homologous to aflatoxin biosynthesis genes in non-aflatoxin-producing species of *Aspergillus* and *Penicillium.* This observation is not unexpected, as some genera of filamentous fungi are known to produce several polyketide metabolites, requiring more than one class of polyketide backbone.

Although PKSs are highly divergent in sequence, the β-ketoacyl synthase motif is the most highly conserved of the PKS motifs (Bingle et al., 1999). Therefore, this motif is particularly suitable for grouping fungal PKSs. The alignment of the motifs of the *OPKS1* protein and the available fungal PKSs showed high conservation with WA-type PKSs but not with MSAS-type PKSs, suggesting that *OPKS1* is a WA-type PKSs gene. This motifs' sequence determines one of the enzyme functions; therefore, the cladogram (Figure 5.3) indicated the possible function relationship between the different fungal PKSs. In the diagram the WA-type PKS and MSAS-type PKS clusters were clearly identified. This was consistent with previous studies that type I PKSs are divided into two subclasses (Hopwood and Khosla, 1992; Bingle et al., 1999). In the WA-type PKS cluster, *OPKS1* falls into a sub-cluster that contained three other PKSs, *Nodulisporium sp* NspPKS, *C. lagenarium* ClPKS and *Phoma sp* PspPKS. NspPKS, ClPKS and PspPKS are known to be involved in the DHN melanin biosynthesis in *Nodulisporium sp.*, *C. lagenarium* and *Phoma sp.*, respectively (Fulton et al., 1999; Takano et al., 1995; Bingle et al., 1999). Furthermore, *C. lagenarium* ClPKS is a pentaketide synthase (Takano et al., 1995). This strongly suggested that *OPKS1* is part of a gene that encodes a pentaketide synthase involved in DHN melanin biosynthesis. *A. nidulans* AnWA (Mayorga and Timberlake, 1992), *A. parasiticus* ApPKS (Bingle et al., 1999) and the *A. fumigatus* AfPKS (Tsai et al., 1998) are involved in fungal conidial pigmentation and are in another sub-cluster. It was noticed that *A. fumigatus* AfPKS is involved in the DHN melanin biosynthetic pathway. Unlike *C. lagenarium* ClPKS, *A. fumigatus* AfPKS is not a pentaketide synthase, but a heptaketide synthase, although *A. fumigatus* still utilizes the DHN pathway to produce melanin (Tsai et al., 2001). *A. nidulans* AnWA is a heptaketide

synthase as well, although *A. nidulans* does not utilize the DHN pentaketide pathway (Tsai et al., 2001). Therefore, it is understandable that *A. fumigatus* AfPKS falls into a sub-cluster with *A. nidulans* AnWA instead of with *C. lagenarium* ClPKS. *A. parasiticus* ApPKSL which is involved in aflatoxin biosynthesis (Feng and Leonard, 1995) and *A. nidulans* AnST which is involved in sterigmatocystin biosynthesis (Yu and Leonard, 1995) were in the same clade.

5.4.2 Melanin gene expression

Fungal development has been correlated with the production of secondary metabolites, for example in the fungal aflatoxin biosynthetic pathway researchers observed that serial transfers of macerated mycelium of *Aspergillus flavus* or *A. parasiticus* led to the appearance of morphological variants which also lacked the ability to produce aflatoxin (Bennett et al., 1986). Kale et al. (1994, 1996) further examined the morphological variants of *A. parasiticus* and observed that these mutants had an abundance of vegetative mycelia and reduced numbers of condiophores and conidia. These mutants lacked transcripts for several aflatoxin synthesis pathway genes such as *nor-1,* and *omtA,* and failed to accumulate any of the pathway intermediates. Guzman-de-Pena and Ruiz-Herrera (1997) also associated the loss of sporulation with loss of aflatoxin production. Melanin genes are not expressed in *C. lagenarium* conidia but in mycelium and appressorium (Takano et al., 1997b). Similarly, *O. floccosum* melanin genes' transcripts were not detected in conidia and yeast cells but in mycelia. As well it was found that pigmentation occurred in mycelia but not in conidia or yeast cells of this fungus. This

suggests that melanin production and the melanin genes' expression in *O. floccosum* are probably associated with fungal development, specifically with mycelial differentiation.

In fungal secondary product biosynthesis pathways, common regulation in pathway gene expression was observed. For example, in *A. nidulans*, the expression of penicillin biosynthesis genes was assumed to be coordinated by some common factors, although these factors have not been yet identified (Brakhage, 1998). In fungal aflatoxin synthesis, the expression of the pathway genes was co-regulated by a regulatory gene, *aflR* (Brown et al., 1999). Common regulation in *C. lagenarium* melanin genes' expression was also suggested (Takano et al., 1997b). In the time course study, the four melanin genes of *O. floccosum* were expressed as the mycelium grew. In media 2 (mannose as a carbon source) the accumulation of the melanin genes' transcripts suggested that some common regulatory mechanisms coordinated the expression of the four-melanin genes in this fungus. It seems reasonable to assume that the expression of these genes is coordinated to ensure the concomitant appearance of all of the gene products in *O. floccosum*.

The transcripts of the four melanin genes of the fungus grown on medium 1 (glycerol) and 2 (mannose) were slightly more abundant than on medium 3 (olive oil). This was consistent with their pigmentation. *O. floccosum* had a darker pigment on medium 2 than on medium 3. This suggested that different carbon sources affected melanin gene expression. Mannose and glycerol led to a slightly higher expression of the genes than olive oil. However, the statistical significant of the expression differences remains to be

verified by some methods such as competitive RT-PCR that can monitor gene expression quantitatively.

In fungal secondary metabolite pathways, the transcription of pathway genes is not sufficient to produce the final pathway products. In *A. flavus* and *A. parasiticus*, the transcripts of the pathway regulatory gene, *aflR* or pathway genes were also detected without the aflatoxin biosynthesis (Feng and Leonard, 1995; Liu and Chu, 1998). In *P. chrysogenum* penicillin biosynthesis, mRNA levels of all the biosynthesis genes were highest during rapid growth when no penicillin was produced (Renno et al., 1992). It was suggested that posttranslational regulation of the pathway genes occurred, although the mechanism of this posttranslational regulation was not clear (Brakhage, 1998; Brown et al., 1999). A similar observation has been reported with *C. lagenarium*. Three melanin genes encoding a PKS, a SD and a reductase were expressed in colorless *C. lagenarium* appressoria (Takano et al., 1997b). In our study, the transcripts of all four melanin genes were observed in mycelia grown on medium 4, in which skim milk was used as the nitrogen and carbon source, which does not support melanin production. In *O. floccosum*, melanin biosynthesis seems not to be repressed by the skim milk through the transcriptional regulation of the melanin genes, but probably through the posttranslational regulation of the melanin genes or the inhibition of the activities of the enzymes in this pathway.

Overall, in *O. floccosum*, the carbon source, mannose, may support higher melanin gene expression, while skim milk repressed melanin production but not melanin gene

transcription. The four melanin genes' expression patterns were similar as *O. floccosum* grew, suggesting some common mechanisms regulate the pathway gene expression.

Chapter 6

Conclusions and future work

6.1. Conclusions

In order to address an important economic problem for the wood industry, wood sapstain, we proposed to explore the genetic information on the pigmentation in sapstain fungi. The discoloration of sapwood is mainly caused by sapstain fungi, which grow on wood and produce dark or brown pigment. The ultimate goal of the research program is to develop sapstain control methods by using physiological, chemical and molecular information about the growth and pigmentation of sapstain fungi.

Information was available on the physiology and biochemistry of some aspects of growth and pigmentation in the sapstain fungi. The fungal utilization of wood nutrients and the effects of some nutrients on fungal pigmentation were well described for the model organism *O. floccosum* 387N (Abraham et al., 1993; Abraham, 1995; Gao, 1996; Eagen, 1999). The pigment produced by the sapstain fungi has been characterized and identified as melanin. Preliminary investigations on *O. floccosum* melanin production included inhibitor studies and cloning of a melanin reductase gene (*THN1*) (Eagen, 1999). DNA sequence analyses and the genetic complementation studies indicated that *O. floccosum* synthesizes its melanin through a pathway similar to the DHN-melanin pathway found in many brown and black fungi. The notion of the presence of a DHN melanin biosynthetic

pathway in *O. floccosum* was strengthened by the previous observations that the color of the wild-type strain was reduced when treated with DHN pathway inhibitors, tricyclazole, carpropamid and cerulenin (Eagen, 1999; Fleet, 2001). However, the information on the pathway was incomplete, and it was necessary to generate more information on the other genes and enzymes involved in pigment production. We anticipated that information at the molecular level would help us determine whether the pigment could be controlled by different means such as designing chemicals to inhibit the pathway enzymes, or preventing wild type strain growth by inoculating albino strains, which could be produced by knocking out some pathway genes.

In this project, we isolated and characterized in *O. floccosum* a scytalone dehydratase gene, a second reductase gene, and a partial polyketide synthase gene. At present four genes designated *OPKS1*, *OSD1*, *THN1* and *THN2* have been identified and characterized. According to the deduced amino acid sequence data, the *OPKS1* and *OSD1* gene products are homologs of polyketide synthase and scytalone dehydratase, respectively; *THN1* and *THN2* gene products are homologs of HN reductase. Furthermore, the functions of *OSD1*, *THN1* and *THN2* were determined by genetic complementation of appropriate melanin deficient mutants. The *OSD1* gene product was able to convert scytalone to 1,3,8-THN, and then restored the pigmentation of a fungal mutant lacking scytalone dehydratase activity; meanwhile, the *THN1* and *THN2* gene products were involved in another DHN melanin reaction, converting 1,3,8-THN to vermelone and restoring the pigmentation of fungal mutants lacking 1,3,8-THN reductase

activity. The expression of *O. floccosum* melanin genes, *OPKS1, THN1, OSD1* and *THN2* may be associated with mycelial differentiation.

Two reductase genes have been isolated in *O. floccosum*. In complementation studies, both the reductases of *O. floccosum* function to convert 1,3,8-THN to vermelone in *M. grisea*, but neither convert 1,3,6,8-THN to scytalone. Whether they would function in a similar way in *O. floccosum* remains unknown. Nevertheless, *O. floccosum* provides the material to study two reductases in the DHN melanin pathway.

6.2 Future directions

Targeted disruption of either and both reductase genes in *O. floccosum* would provide a way to elucidate the functions of both reductases in vivo. A transformation system has been set up for *O. floccosum* 387N, however, target gene disruption in *O. floccosum* failed. Therefore, further improvements of the target gene disruption in *O. floccosum* are necessary. Constructing a different gene disruption vector or using the *A. tumefaciens*-facilitated transformation could achieve the improvements. *Agrobacterium*-mediated transformation has been demonstrated to facilitate homologous DNA integration (Abuodeh et al., 2000); for example, *Coccidioides immitis* was successfully transformed by *A. tumefaciens* but not by other transformation methods (Yu and Cole, 1998). Furthermore, most *A. tumefaciens*-facilitated transformants contain only a single copy of integrated T-DNA (de Groot et al., 1998).

Direct testing of the substrate specificities of both reductases with respect to 1,3,6,8-THN and 1,3,8-THN would provide another way to understand the gene functions *of O. floccosum* reductases. However, these two substrates are unstable (Bell and Wheeler, 1986) and this makes it difficult to conduct these tests. Recently, the reverse reactions of the reductases using scytalone and vermelone have been demonstrated in *M. grisea* (Thompson et al., 2000). In this study, the kinetic parameters of both reductases using scytalone and vermelone as substrates were measured and used to calculate the substrate preference for 1,3,6,8-THN and 1,3,8-THN. This approach could be used in *O. floccosum* reductases in future studies. To conduct these tests, it is necessary to express both reductase genes, purify the enzymes, synthesize scytalone and vermelone, and set up the reaction conditions and the reaction rate detection system.

The preliminary studies of melanin gene expression in *O. floccosum* raise many interesting questions: are the melanin genes co-regulated? Do some factors regulate mycelium differentiation and melanin gene expression? How might the melanin gene expression correlate with other metabolite pathways, which are involved in nutrient utilization? With current microarray technology, it should be possible to identify the genes responsible for these regulations and relations through analyzing and clustering the microarray profiles (Jagota, 2001). For example, using DNA array technology, a genomic wide study of *S. cerevisiea* gene expression profiles revealed many uncharacterized genes associated with regulating the metabolic shift from fermentation to respiration (Joseph et al., 1997). Given the knowledge of exogenously supplied nutrients which affect melanin biosynthesis, we could artificially perturb the biosynthesis of melanin using these

nutrients and use the microarray technique to look for novel genes which are altered in their regulation. We can classify and cluster the microarray gene expression profiles, which are response for melanin biosynthesis and fungal development to study the relationships between fungal development genes or their regulation genes with melanin genes.

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Appendix

Fungal DHN melanin gene system and modeling

> *Biology in the 21st century is being transformed from a purely laboratory-based science to an information science as well. One of the crucial steps in this transformation will be training a new generation of biologists who are both computational scientists and laboratory scientists.*
>
> *- Eric S. Lander (MIT-Whitehead Center for Genome Research)*

A.1 Motivation

In the last ten years or so biology has become a data-rich science. One question that I am interested in involves biological modules - by that I mean groups of genes, proteins or other molecules that work together to accomplish some function at biochemical, cellular or physiological level. The fungal DHN melanin pathway is an example of the biological module. Functional genomics provide tools such as microarray analysis to allow reconstructing the biological modules (sometime called networks). This project is aimed at integrating genetic/biochemical/phenotypic information of the fungal DHN melanin pathway into the fungal genomic and microarray data (*M. grisea* and *A. fumigatus* genome sequencing are in progress, these fungi have the DHN melanin pathway) to reconstruct the whole pathway. The long-term goal of the project is to reconstruct and model the whole fungal biological networks (biological modules in the fungi) based on the fungal genomic and functional genomic data.

A.2 A brief description of the concepts and models

The levels of the knowledge abstraction are the following: DNA sequence to function; interaction to network to function. Graphs are used to present the networks. G = (V, E), G, V, E represent graph (network), vertices (genes, enzymes, or metabolites) and edges (the links between vertices, or the relations between genes, enzymes, or metabolites). Therefore, we could construct a genome network representing gene locations and sequence information based on genome sequencing information, a genetic network representing the gene interactions based on functional genomic data such as microarray data, and a metabolite network representing the pathway based on the known genetic and biochemical data. We can predict or reconstruct the metabolite network by aligning genome network, genetic network to metabolite network in one organism. We also can align genome network-genome network, genetic network-genetic network and metabolite network-metabolite network across species to infer knowledge. By modeling the pathway using the gene knock out data or gene expression data, we can predict the function of the network.

A.3 Results

As a preliminary effort, a software program (Fungal Melanin Gene System, Version: 1.0.0) for managing the fungal DHN melanin gene and gene knock-out information, reconstructing metabolite network and modeling the pathway has been developed. The

program allows searching fungal melanin gene/enzyme/gene-knock-out information, modeling and visualizing the DHN melanin pathway and the melanin gene disruption events (Figure A.1). For information searching, there are three choices by searching gene, searching enzyme, or searching species. In the gene and enzyme search, the user can enter the enzyme/gene name and search for either selected species or among all the species. In the species search, the user does not need to enter any keyword; he/she should select the species name and the list for enzyme or gene (Figures A.2 and A.3). For the pathway modeling and visualization/melanin-gene-disruption, the user can select which gene will be disrupted (or none is disrupted), the pathway and the color of the phenotype will be displayed (Figures A.4 and A.5). Future improvements involve database integration such as genome and microarray data, algorithm development for data analysis, network reconstruction and visualization, and reconstruction of all biological blocks in fungi.

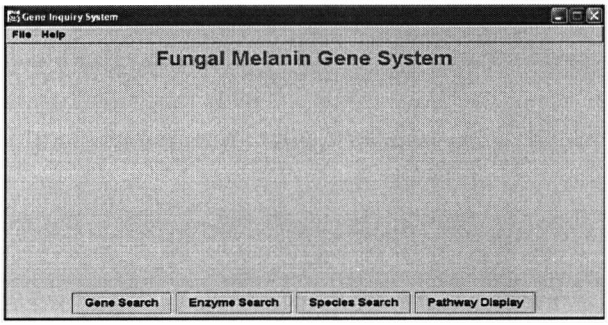

Figure A.1. The main interface of the system.

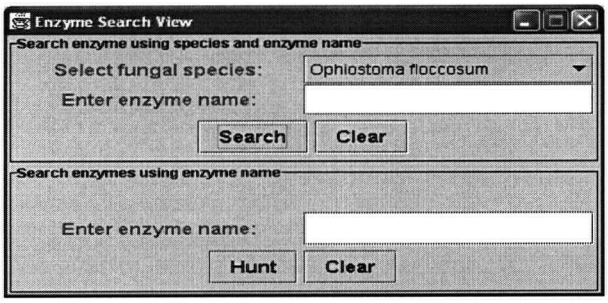

Figure A.2. An example interface for information searching.

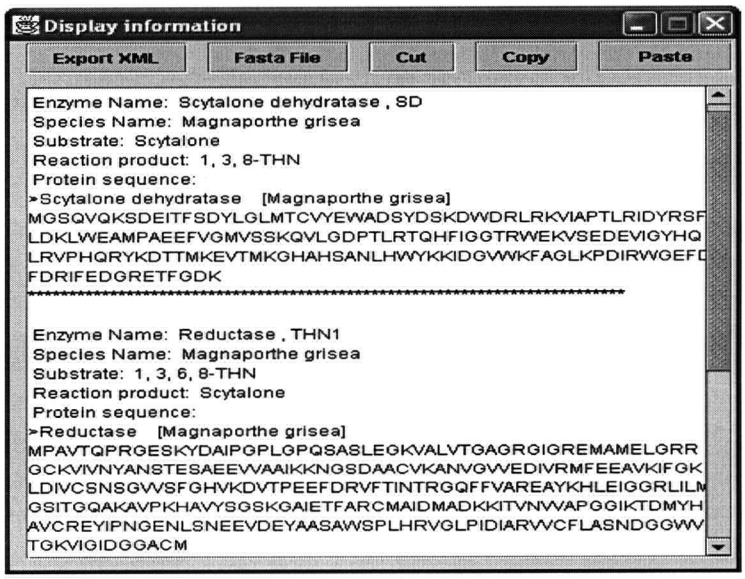

Figure A.3. A searching result example.

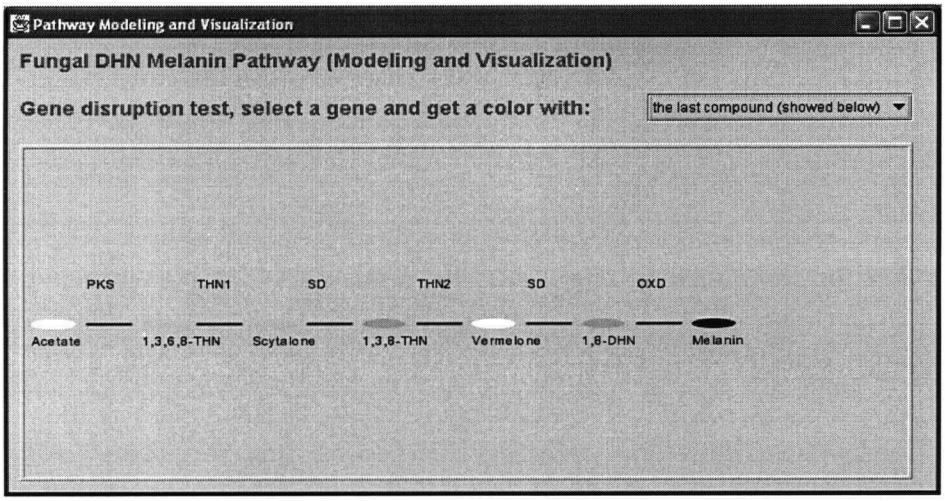

Figure A.4. A presentation of the genetic and metabolite networks of the fungal DHN melanin pathway.

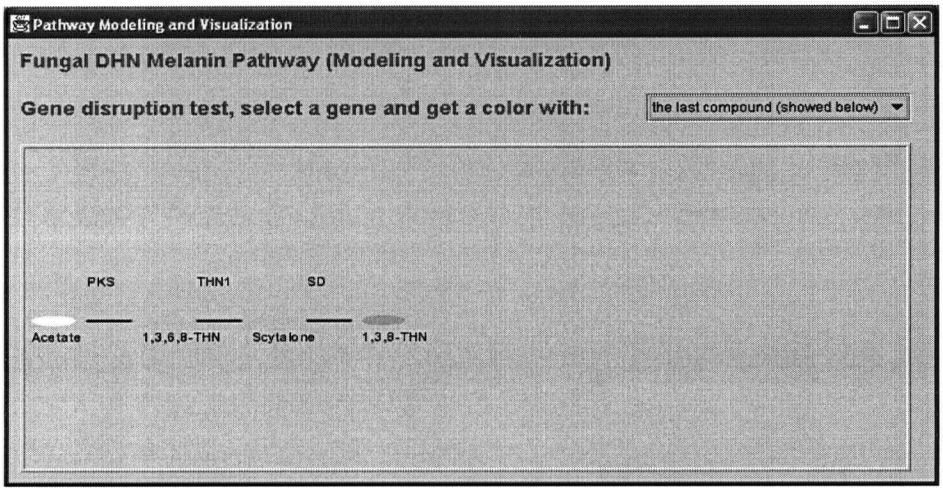

Figure A.5. Pathway modeling and function inference using the gene disruption data.

A.4 How to run the software and the Java source code of the software

The program has been tested on Windows 98, Windows 2000 and Windows XP. To execute the program, a Sun Microsystems Java™ 2 Runtime Environment, Standard Edition (JRE), v 1.2.2 or higher versions such as 1.3.x and 1.4.x are needed. To trigger and run the program, using the "java Model" command. Alternatively, users can double click the executable jar file (with an icon) to run the program. Beside the Java source code for the program, the following supporting files are also necessary. The gene0.txt ~ gene8.txt files for the fungal melanin gene sequence and knock out information; the enzy0.txt ~ enzy8.txt files for the fungal melanin enzyme sequence information, and the duke0.gif ~ duke3.gif files for animation.

```
// ***********************************************************************
// Version: 1.0.0
//
// @Author: Edwin (Honglong) Wang
//
// Tested on Java™ 2 Platform
//
// Sun Microsystems, Inc.,
// 901 San Antonio Road,
// Palo Alto, CA 94303 USA.
//
// Copyright (c) 2000
// E. Wang
// University of British Columbia,
// Vancouver, Canada
//
// All Rights Reserved
//
// License: non-commercial
// ***********************************************************************

// Sequence.java

/** this class defines the information of a sequence,
 * the superclass of the classes: Gene and Enzyme */

public class  Sequence
{
        /** the name of the sequence */
        private String seqName;
        /** the sequence ID number */
        private String seqID;
        /** the sequence information */
        private String seqInfo;
        /** species name */
        private String speciesName;

        /** a constructor */
        public Sequence()
        {
                seqName         = "unknown";
                seqID    = "unknown";
                speciesName = "unknown";
                seqInfo  = "unknown";
        }

        /** a convenience constructor */
        public Sequence(String n, String i, String sn)
        {
                seqName         = n;
                seqID    = i;
                speciesName = sn;
                seqInfo  = "unknown";
```

```
        }

        /** the method is to set the name of the sequence */
        public void setName(String name)
        {
                seqName = name;
        }

        /** the method is to set the ID number of the sequence */
        public void setID(String id)
        {
                seqID = id;
        }

        /** the method is to read the sequence information */
        public void setSeq(String info)
        {
                seqInfo = info;
        }

        /** the method is to set the species name */
        public void setSpeciesName(String species)
        {
                speciesName = species;
        }

        /** the method is to obtain the name of the sequence */
        public String getName()
        {
                return seqName;
        }

        /** the method is to obtain the ID number of the sequence */
        public String getID()
        {
                return seqID;
        }

        /** the method is to obtain the information of the sequence */
        public String getSeq()
        {
                return seqInfo;
        }

        /** the method is to obtain the species name */
        public String getSpeciesName()
        {
                return speciesName;
        }
}

// ************************************************************************

// Gene.java
```

```
/** this class defines gene information, subclass of the class Sequence */
public class  Gene extends Sequence
{
        /** gene description */
        private static String description;

        /** a constructor */
        public Gene()
        {
                super();
                description = "A structure gene";
        }

        /** a convenience constructor */
        public Gene(String n, String i, String sn)
        {               super(n, i, sn);
                        description = "A structure gene";
        }
}

// ***********************************************************************

// Genelist.java

import java.util.*;

/** the class stores genes */
public class Genelist
{
        /** the Genelist varaible,type: ArrayList
         * Gene objects will be stored*/
        protected ArrayList geneCollection;

        /** the constructor */
        public Genelist ()
        {       geneCollection = new  ArrayList();
        }
        /** This method is to add Gene objects to the gene collection */
        public void addGene(Gene c)
        {
                if(c == null)
                        c = new Gene();
                else
                {if(geneCollection.contains(c) == false)
                        geneCollection.add(c);
                        }
        }
        /** This method is to remove Gene objects from the collection */
        public ArrayList removeGene(Gene c)
        {
                geneCollection.remove(geneCollection.indexOf(c));
                return geneCollection;
        }
        /** The method is to search the gene from the list
         *  the search attributes is the species name  */
```

```
	public ArrayList speciesSearch(String species)
	{
		ArrayList c = new ArrayList();
		int i = 0;
		Gene tempGene = null;
		for(i = 0; i < geneCollection.size(); i++){
			tempGene = (Gene) geneCollection.get(i);
			if (species.equalsIgnoreCase(tempGene.getSpeciesName()))
				c.add(tempGene);
		}
		return  c;
	}

	/** The method is to search the gene from the list
	 * the search attributes is the species name and the gene name */
	public Gene geneSearch(String species, String name)
	{
		int i = 0;
		Gene tempGene = null;
		for(i = 0; i < geneCollection.size(); i++){
			tempGene = (Gene) geneCollection.get(i);
			if (species.equalsIgnoreCase(tempGene.getSpeciesName())
				&& name.equalsIgnoreCase(tempGene.getName()))
				return (Gene) tempGene;
		}
		return null;
	}

	/** The method is to search the gene from the list
	 * the search attributes is the gene name */
	public Gene nameSearch(String name)
	{
		int i = 0;
		Gene tempGene = null;
		for(i = 0; i < geneCollection.size(); i++){
			tempGene = (Gene) geneCollection.get(i);
			if (name.equalsIgnoreCase(tempGene.getName()))
				return (Gene) tempGene;
		}
		return null;
	}
}


// ***********************************************************************

// GeneView.java

import javax.swing.*;
//import javax.swing.event.*;
import java.awt.*;
import java.awt.event.*;
import javax.swing.border.*;


/**
```

```
 * The class is responsible for providing an interface for gene search.
 */
public class GeneView extends JFrame {
  /** declare the references. */
  JComboBox cbox = null;
  Container c = null;
  JButton b1, b2, b3, b4;
  JTextField t1, t2;

  /** define an array of species name to be shown in the
   * combo box pull-down list, by default. */
  String[] defaultSpecies = {
      "Ophiostoma floccosum", "Magnaporthe grisea",
      "Colletotrichum lagenarium"};
  String defaultEdit = "Ophiostoma floccosum";

        /** model variable */
        protected Model mymodel;

        /** constructor */
        public GeneView (Model m)
        {
                super();
                setTitle("Gene Search View");
                mymodel = m;
                //configure the frame using its content pane
                c = this.getContentPane();
                c.setBackground(Color.lightGray);
                c.setLayout(new GridLayout(2,1));
                init();
                sizeandplaceWindow();
                addWindowListener(new WindowCloser());
                setDefaultCloseOperation(WindowConstants.DISPOSE_ON_CLOSE);
        }

        /** construct the view */
         public void init() {

         // prepare the container with layout
        JPanel p1 = new JPanel();
         p1.setLayout(new BorderLayout());

        // set the title border around the panel.
         TitledBorder border1 = new TitledBorder(
                 new LineBorder(Color.blue),
                 "Search gene using species and gene product names");

        border1.setTitleColor(Color.blue);
        p1.setBorder(border1);

        JPanel p2 = new JPanel();
         p2.setLayout(new BorderLayout());

        // set the title border around the panel.
        TitledBorder border2 = new TitledBorder(
                    new LineBorder(Color.blue),
```

```
                "Search genes using gene product names");

    border2.setTitleColor(Color.blue);
    p2.setBorder(border2);
    JPanel p3 = new JPanel();
    p3.setLayout(new GridLayout(1,2));
    JPanel p4 = new JPanel();
    p4.setLayout(new GridLayout(1,3));
    JPanel p5 = new JPanel();
    p5.setLayout(new GridLayout(1,2));
    JPanel p6 = new JPanel();
    JPanel p7 = new JPanel();
    JPanel p8 = new JPanel();


// create the combo box with the list of default sites.
cbox = new JComboBox(defaultSpecies);
cbox.setOpaque(true);

// make the combo box ineditable.
cbox.setEditable(false);

// configure the combo box editor.
cbox.configureEditor(cbox.getEditor(), defaultEdit);

// rows to be visible without scrollbars.
cbox.setMaximumRowCount(3);

// set the combo box editor colors and font.
ComboBoxEditor cboxEditor = cbox.getEditor();
Component editorComp = cboxEditor.getEditorComponent();
editorComp.setBackground(Color.white);
editorComp.setForeground(Color.blue);

Font f1 = new Font("Dialog", Font.PLAIN, 14);
editorComp.setFont(f1);

// font for the combo box popup list.
cbox.setFont(f1);

// label to indicate what to do.
JLabel label1 = new JLabel("Select  fungal  species:",
                JLabel.CENTER);
label1.setFont(new Font("SansSerif", Font.BOLD, 16));
            JLabel label2 = new JLabel("Enter gene product name:",
                JLabel.CENTER);
label2.setFont(new Font("SansSerif", Font.BOLD, 16));
            JLabel label3 = new JLabel("Enter gene product name:",
                JLabel.CENTER);
label3.setFont(new Font("SansSerif", Font.BOLD, 16));

            JLabel label4 = new JLabel("        ",
                JLabel.CENTER);

    // button for search
    b1 = new JButton("Find");
```

```
        b1.setFont(new Font("SansSerif", Font.BOLD, 16));
        b1.addActionListener(mymodel);
        b2 = new JButton("Go");
        b2.setFont(new Font("SansSerif", Font.BOLD, 16));
        b2.addActionListener(mymodel);
        b3 = new JButton("Clear");
        b3.setFont(new Font("SansSerif", Font.BOLD, 16));
        b3.addActionListener(new Listener());
        b4 = new JButton("Clear");
        b4.setFont(new Font("SansSerif", Font.BOLD, 16));
        b4.addActionListener(new Listener());

        // textfiels
          t1 = new JTextField(2);
                t1.setFont(f1);
          t2 = new JTextField(2);
                t2.setFont(f1);

        //construct the first search block
         p3.add(label1);
         p3.add(cbox);
        p4.add(label3);
        p4.add(t1);
        p7.add(b1);
        p7.add(b3);

                p1.add(p7, BorderLayout.SOUTH);
                p1.add(p4, BorderLayout.CENTER);
                p1.add(p3, BorderLayout.NORTH);

                //construct the 2nd search block
                p5.add(label2);
                p5.add(t2);
                p6.add(b2);
                p6.add(b4);
                p8.add(label4);

                p2.add(p6, BorderLayout.SOUTH);
                p2.add(p5, BorderLayout.CENTER);
                p2.add(p8, BorderLayout.NORTH);

                //add the blocks to the frame
                c.add(p1);
                c.add(p2);
}
        /** this inner class deals with closing the window */
        private class WindowCloser extends WindowAdapter
        {
                public void windowClosing(WindowEvent e){
                        dispose();
                        }
        }

        /** this class is to implement the ActionListener interface */
        private class Listener implements ActionListener
        {       /** the method is listening */
```

```
		public void actionPerformed(ActionEvent e){

		Object source = e.getSource();
		if (source == b3)
			t1.setText("");
			else if (source == b4)
				t2.setText("");
		}
	}

	/** this private method sizes and places the frame in the middle of the screen */
	private void sizeandplaceWindow()
	{
		this.setSize(480, 290);
		this.setResizable(false);
		Dimension frameD = new Dimension(480, 290);
		Dimension screenD = new Dimension();
		screenD = Toolkit.getDefaultToolkit().getScreenSize();
		if(frameD.width >= screenD.width)
			this.setLocation(1, 1);
		this.setLocation(((screenD.width - frameD.width)/2),
			((screenD.height - frameD.height)/2));
		this.setSize(frameD.width, frameD.height);
	}


	//testing
	public static void main(String arg[])
	{
		Model a = new Model();
		GeneView gv = new GeneView(a);
	}
}


// ***********************************************************************

//Structure.java

public class Structure{
public Structure(){}
}


// ***********************************************************************

// Enzyme.java


/** this class defines protein information, subclass of the class Sequence */
public class  Enzyme extends Sequence
{
	/** its gene */
	private Gene gene;
	/** the substrate name */
	private String subName;
```

```
        /** the raction product */
        private String productName;
        /** a constructor */
        public Enzyme()
        {
                super();
                subName  = "unknown";
                productName = "unknown";
        }

        /** a convenience constructor */
        public Enzyme(String a, String b, String c, Gene g, String sn, String pn )
        {
                super(a, b, c);
                gene = g;
                subName = sn;
                productName = pn;
        }

        /** the method is to set the gene */
        public void setGene(Gene ge)
        {
                gene = ge;
        }

        /** the method is to set the substrate name */
        public void setSubName(String substrate)
        {
                subName = substrate;
        }

        /** the method is to set the product name */
        public void setProductName(String product)
        {
                productName = product;
        }

        /** the method is to obtain the gene */
        public Gene getGene()
        {
                return gene;
        }

        /** the method is to obtain the substrate name */
        public String getSubName()
        {
                return subName;
        }

        /** the method is to obtain the product name */
        public String getProductName()
        {
                return productName;
        }
}
```

```
// ***********************************************************************

// Enzymelist.java

import java.util.*;

/** the class stores enzymes */
public class Enzymelist
{
        /** the Enzymelist varaible,type: ArrayList
         * Enzyme objects will be stored*/
        protected ArrayList enzyCollection;

        /** the constructor */
        public Enzymelist ()
        {
            enzyCollection = new  ArrayList();
        }

        /** This method is to add Enzyme objects to the enzyme collection */
        public void addEnzyme(Enzyme c)
        {
                if(c == null)
                    c = new Enzyme();
                else
                {if(enzyCollection.contains(c) == false)
                        enzyCollection.add(c);
                        }
        }

        /** This method is to remove Enzyme objects from the collection */
        public ArrayList removeEnzyme(Enzyme c)
        {
                enzyCollection.remove(enzyCollection.indexOf(c));
                return enzyCollection;
        }

        /** The method is to search the enzyme from the list
         *  the search attributes is the species name  */
        public ArrayList speciesSearch(String species)
        {
                ArrayList c = new ArrayList();
                Enzyme tempEnzyme = null;
                for(int i = 0; i < enzyCollection.size(); i++){
                        tempEnzyme = (Enzyme) enzyCollection.get(i);
                        if (species.equalsIgnoreCase(tempEnzyme.getSpeciesName()))
                                c.add(tempEnzyme);
                }
                return  c;
        }

        /** The method is to search the enzyme from the list
         *  the search attributes is the species name and the enzyme name */
        public Enzyme enzySearch(String species, String name)
        {
                Enzyme tempEnzyme = null;
```

```
		for(int i = 0; i < enzyCollection.size(); i++){
			tempEnzyme = (Enzyme) enzyCollection.get(i)
			if (species.equalsIgnoreCase(tempEnzyme.getSpeciesName())
				&& name.equalsIgnoreCase(tempEnzyme.getName()))
				return (Enzyme) tempEnzyme;
		}
		return null;
	}


	/** The method is to search the enzyme from the list
	 * the search attributes is the enzyme's name */
	public Enzyme nameSearch(String name)
	{
		Enzyme tempEnzyme = null;
		for(int i = 0; i < enzyCollection.size(); i++){
			tempEnzyme = (Enzyme) enzyCollection.get(i);
			if (name.equalsIgnoreCase(tempEnzyme.getName()))
				return (Enzyme) tempEnzyme;
				}
		return null;
	}

	/** The method is to search the enzyme from the list
	 * the search attributes is the enzyme's name */
	public Enzyme nameSearch1(String name)
	{
		Enzyme tempEnzyme = null;
		//for(int i = 0; i < enzyCollection.size(); i++){
			//tempEnzyme = (Enzyme) enzyCollection.get(i);
		//	if (name.equalsIgnoreCase(tempEnzyme.getName())){
			ListIterator counter =  enzyCollection.listIterator();
			while(counter.hasNext()){
				tempEnzyme = (Enzyme) counter.next();
				if (name.equalsIgnoreCase(tempEnzyme.getName())){
					//(Enzyme) tempEnzyme = counter.next();
				return tempEnzyme;}
				}
		return tempEnzyme;
	}

	public void print()
	{	Enzyme tempEnzyme = null;
		for (int k = 0; k < enzyCollection.size(); k++){
			tempEnzyme = (Enzyme) enzyCollection.get(k);
			System.out.println(tempEnzyme.getName());
			System.out.println(tempEnzyme.getSeq());
			}
	}
}

// ***********************************************************************

// EnzymeView.java

import javax.swing.*;
```

```
import java.awt.*;
import java.awt.event.*;
import javax.swing.border.*;
import javax.swing.event.*;


/**
 * The calass is responsible for providing an interface for enzyme search.
 */
public class EnzymeView extends JFrame {
   /** declare the references. */
   JComboBox cbox = null;
          Container c = null;
          JButton b1, b2, b3, b4;
          JTextField t1, t2;

   /** define an array of species name to be shown in the
    * combo box pull-down list, by default. */
   String[] defaultSpecies = {"Ophiostoma floccosum", "Magnaporthe grisea",
       "Colletotrichum lagenarium"};
   String defaultEdit = "Ophiostoma floccosum";

          /** model variable */
          protected Model mymodel;

          /** constructor */
          public EnzymeView (Model m)
          {
                    super();
                    setTitle("Enzyme Search View");
                    mymodel = m;
                    //configure the frame using its content pane
                    c = this.getContentPane();
                    c.setBackground(Color.lightGray);
                    c.setLayout(new GridLayout(2,1));
                    init();
                    sizeandplaceWindow();
                    addWindowListener(new WindowCloser());
                    setDefaultCloseOperation(WindowConstants.DISPOSE_ON_CLOSE);
          }

          /** construct the view */
          public void init() {

           // prepare the container with layout
          JPanel p1 = new JPanel();
           p1.setLayout(new BorderLayout());
          // set the title border around the panel.
           TitledBorder border1 = new TitledBorder(
                    new LineBorder(Color.blue),
                    "Search enzyme using species and enzyme name");
           border1.setTitleColor(Color.blue);
           p1.setBorder(border1);
          JPanel p2 = new JPanel();
           p2.setLayout(new BorderLayout());
          // set the title border around the panel.
```

```
TitledBorder border2 = new TitledBorder(
            new LineBorder(Color.blue),
            "Search enzymes using enzyme name");
border2.setTitleColor(Color.blue);
p2.setBorder(border2);

JPanel p3 = new JPanel();
p3.setLayout(new GridLayout(1,2));
JPanel p4 = new JPanel();
p4.setLayout(new GridLayout(1,3));
JPanel p5 = new JPanel();
p5.setLayout(new GridLayout(1,2));
JPanel p6 = new JPanel();
JPanel p7 = new JPanel();
JPanel p8 = new JPanel();

// create the combo box with the list of default species.
cbox = new JComboBox();
        for(int i = 0; i < 3; i++)
                cbox.addItem(defaultSpecies[i]);
                cbox.setOpaque(true);
// make the combo box ineditable.
cbox.setEditable(false);
Font f1 = new Font("Dialog", Font.PLAIN, 14);
// font for the combo box popup list.
cbox.setFont(f1);
// label to indicate what to do.
JLabel label1 = new JLabel("Select fungal species:",
            JLabel.CENTER);
label1.setFont(new Font("SansSerif", Font.BOLD, 16));
JLabel label2 = new JLabel("Enter enzyme name:",
            JLabel.CENTER);
label2.setFont(new Font("SansSerif", Font.BOLD, 16));
JLabel label3 = new JLabel("Enter enzyme name:",
            JLabel.CENTER);
label3.setFont(new Font("SansSerif", Font.BOLD, 16));
JLabel label4 = new JLabel("        ",
            JLabel.CENTER);

        // button for search
        b1 = new JButton("Search");
        b1.setFont(new Font("SansSerif", Font.BOLD, 16));
        b1.addActionListener(mymodel);
        b2 = new JButton("Hunt");
        b2.setFont(new Font("SansSerif", Font.BOLD, 16));
        b2.addActionListener(mymodel);
        b3 = new JButton("Clear");
        b3.setFont(new Font("SansSerif", Font.BOLD, 16));
        b3.addActionListener(new Listener());
        b4 = new JButton("Clear");
        b4.setFont(new Font("SansSerif", Font.BOLD, 16));
        b4.addActionListener(new Listener());

        // textfiels
        t1 = new JTextField(2);
        t1.setFont(f1);
```

```
            t2 = new JTextField(2);
            t2.setFont(f1);

            //construct the first search block
            p3.add(label1);
            p3.add(cbox);
            p4.add(label3);
            p4.add(t1);
            p7.add(b1);
            p7.add(b3);

            p1.add(p7, BorderLayout.SOUTH);
            p1.add(p4, BorderLayout.CENTER);
            p1.add(p3, BorderLayout.NORTH);

            //construct the 2nd search block
            p5.add(label2);
            p5.add(t2);
            p6.add(b2);
            p6.add(b4);
            p8.add(label4);
            p2.add(p6, BorderLayout.SOUTH);
            p2.add(p5, BorderLayout.CENTER);
            p2.add(p8, BorderLayout.NORTH);

            //add the blocks to the frame
            c.add(p1);
            c.add(p2);
}

    /** this inner class deals with closing the window */
    private class WindowCloser extends WindowAdapter
    {
            public void windowClosing(WindowEvent e){

                    dispose();
                    }
    }

    /** this class is to implement the ActionListener interface */
    private class Listener implements ActionListener
    {       /** the method is listening */
            public void actionPerformed(ActionEvent e){

            Object source = e.getSource();
            if (source == b3)
                    t1.setText("");
                    else if (source == b4)
                            t2.setText("");
            }
    }

    /** this private method sizes and places the frame in the middle of the screen */
    private void sizeandplaceWindow()
    {
            this.setSize(480, 290);
```

```
		this.setResizable(false);
		Dimension frameD = new Dimension(480, 290);
		Dimension screenD = new Dimension();
		screenD = Toolkit.getDefaultToolkit().getScreenSize();
		if(frameD.width >= screenD.width)
			this.setLocation(1, 1);
		this.setLocation(((screenD.width - frameD.width)/2),
			((screenD.height - frameD.height)/2));
		this.setSize(frameD.width, frameD.height);
	}

	//testing
	public static void main(String arg[])
	{
		Model a = new Model();
		EnzymeView gv = new EnzymeView(a);
	}
}

// ***********************************************************************
// Compound.java

import java.awt.*;

/** this class defines pathway compound information */
public class Compound
{
	/** the previous compound name */
	private String preName;
	/** the compound name */
	private String compName;
	/** the raction product */
	private String productName;
	/** the structure of the compound */
	private Structure compStruct;
	/** the compound position- integer */
	private int position;
	/** the genes associated*/
	private Gene gene;
	/** the color of the phenotype when the compound is blocked*/
	private Color color;

	/** a constructor */
	public Compound()
	{	preName = "unknown";
		compName  = "unknown";
		productName = "unknown";
		position = 0;
		gene = new Gene();
		color = new Color(1,1,1);
	}

	/** a convenience constructor */
	public Compound (String a, String sn, String pn, int n, Color c )
	{
```

```
                preName = a;
                compName = sn;
                productName = pn;
                position = n;
                color = c;
}

/** the method is to set the previous compound */
public void setPreName(String ge)
{
        preName = ge;
}

/** the method is to set the compound name */
public void setCompName(String comp)
{
        compName = comp;
}

/** the method is to set the product name */
public void setProductName(String product)
{
        productName = product;
}

/** the method is to set the gene obj */
public void setGene(Gene g)
{
        gene = g;
}

/** the method is to set the compound position with integer */
public void setPosition(int p)
{
        position = p;
}

/** the method is to set the color  */
public void setColor(Color c)
{
        color = c;
}

/** the method is to obtain the previous compound */
public String getPreName()
{
        return preName;
}

/** the method is to obtain the compound name */
public String getCompName()
{
        return compName;
}

/** the method is to obtain the product name */
```

```
        public String getProductName()
        {
                return productName;
        }

        /** the method is to get the gene obj */
        public Gene getGene()
        {
                return gene;
        }

        /** the method is to get the compound position with integer */
        public int getPosition()
        {
                return position;
        }

        /** the method is to get the color  */
        public Color getColor()
        {
                return color;
        }
}

// ***********************************************************************

// CompoundSet.java

import java.util.*;

/** the class stores Compounds */
public class CompoundSet
{
        /** the CompoundSet varaible,type: ArrayList
         * Compound objects will be stored*/
        protected ArrayList compCollection;

        /** the constructor */
        public CompoundSet ()
        {
                compCollection = new ArrayList();
        }

        /** This method is to add Compound objects to the Compound collection */
        public void addCompound(Compound c)
        {
                if(c == null)
                        c = new Compound();
                        else
                        compCollection.add(c);
        }

        /** This method is to remove Compound objects from the collection */
        public ArrayList removeCompound(Compound c)
        {
                compCollection.remove(compCollection.indexOf(c));
```

```
		return  compCollection;
	}

	/** The method is to search the Compound from the list
	 *  the search attributes is the gene's name */
	public Compound geneNameSearch(String name)
	{
		Compound tempCompound = null;
		ListIterator counter =  compCollection.listIterator();
			while(counter.hasNext()){
				tempCompound = (Compound) counter.next();
				if (name.equalsIgnoreCase(tempCompound.getGene().getName()))
				return tempCompound;
				}

			return tempCompound;
	}

	/** get the elements fron the first elem to the c elem*/
	public ArrayList getFirstPart(Compound c){

		int index = getIndex (c);
		Compound tempCompound = null;
		for(int i = index + 1; i < compCollection.size(); i++){
			tempCompound = (Compound) compCollection.get(i);
			removeCompound(tempCompound);

		}
			return  compCollection;
	}
	public int getSize(){
		return compCollection.size();
	}
	public Compound getElement(int i){
		Compound tempCompound = null;
		if (i <= compCollection.size())
			tempCompound = (Compound) compCollection.get(i);
		return tempCompound;
	}

	public int getIndex (Compound c){
		return (int) compCollection.indexOf(c);
	}
}


// ***********************************************************************

// Pathway.java

import javax.swing.event.*;
import javax.swing.*;
import java.util.*;
import java.awt.*;
import java.lang.*;
import java.io.*;
```

```
import java.awt.event.*;
import javax.swing.tree.*;
import java.util.*;

/** this class for building the DHN pathway */
public class Pathway {

        /** Compounds variable */
        protected Compound cp;
        //views will be triggered by this
        protected PathwayView pathwayview;
        String geneNames [] = {"Polyketide synthase gene (PKS)",
                "Reductase I gene (THN1)",
                "Scytalone dehydratase gene (SD)",
                "Reductase II gene (THN2)",
                "Scytalone dehydratase gene (SD)",
                "Oxidases"};
        //compund names in the pathway
        String compNames[] = {"Acetate", "1,3,6,8-THN", "Scytalone",
                                                "1,3,8-THN", "Vermelone", "1,8-DHN",
                                                "Melanin"};
        Gene genes [] = new Gene[6];
        Compound compounds[] = new Compound [compNames.length];
        Color colors [] = {Color.white, Color.pink, Color.pink, new Color(208,160,132),/*brown*/
                                Color.white, new Color(208,160,132),Color.black};
        CompoundSet comCollect;

        public Pathway(int z){
                comCollect = new CompoundSet(); //TreeSet is sorted/unrepeated
                pathwayBuilder(z);
                }

        /** build the compounds in the pathway */
        public CompoundSet pathwayBuilder(int len){
        //creat genes and assign gene names for them
        for (int i = 0; i < len -1; i++){
                genes[i] = new Gene();
                genes[i].setName(geneNames[i]);
                }

        //creat compounds and assign gene names and positions for them
        for (int j = 0; j < len; j++){
                compounds[j] = new Compound();
                compounds[j].setPosition(j*10);
                compounds[j].setCompName(compNames[j]);
                compounds[j].setColor(colors[j]);
                if (j == len -1) break;//no gene is associated with the last compound
                compounds[j].setGene(genes[j]);
                }

                for (int k = 0; k < len; k++){
                        comCollect.addCompound(compounds[k]);
                        }
                return comCollect;
                }
```

```
        public int getPathwaySize(){
                return comCollect.getSize();
        }

        public Compound getPathwayElement(int i){
                Compound tempCompound = null;
                if (i <= getPathwaySize())
                        tempCompound = (Compound) comCollect.getElement(i);
                return tempCompound;
        }

        public static void main (String [] args){
                new Pathway(7);
        }
}


// ***********************************************************************

// DrawPathway.java


import java.awt.*;
import java.awt.event.*;
import java.awt.geom.*;
import javax.swing.*;
import java.awt.event.ItemListener;
import java.awt.event.ItemEvent;
import java.awt.event.ActionListener;
import java.awt.event.ActionEvent;
import java.util.*;


  public class DrawPathway extends JPanel{
  final static int maxCharHeight = 15;
  final static int minFontSize = 11;
   final static Color bg = Color.white;
   final static Color fg = Color.black;
   final static Color red = Color.red;
   final static Color white = Color.white;
   final static BasicStroke stroke = new BasicStroke(2.0f);
   final static BasicStroke wideStroke = new BasicStroke(8.0f);
   final static float dash1[] = {10.0f};
   final static BasicStroke dashed = new BasicStroke(1.0f,
                                    BasicStroke.CAP_BUTT,
                                    BasicStroke.JOIN_MITER,
                                    10.0f, dash1, 0.0f);
   Dimension totalSize;
   FontMetrics fontMetrics;
   CompoundSet c;
   Pathway p;
   HashMap geneHash;

   public DrawPathway(Pathway p1) {
      //Initialize drawing colors
      setBackground(bg);
```

```
    setForeground(fg);
        geneHash = new HashMap();
    p = p1;

    // Compound pathwayCompounds [] = new Compound[]{ };
  }

//gene names in abbrev
public HashMap setGeneName(){

    geneHash.put ("Polyketide synthase gene (PKS)", "PKS");
    geneHash.put("Reductase I gene (THN1)", "THN1");
    geneHash.put("Scytalone dehydratase gene (SD)", "SD");
    geneHash.put("Reductase II gene (THN2)", "THN2");
    geneHash.put("Oxidases", "OXD");
    return geneHash;
    }

  FontMetrics pickFont(Graphics2D g2,
                String longString,
                int xSpace) {
    boolean fontFits = false;
    Font font = g2.getFont();
    FontMetrics fontMetrics = g2.getFontMetrics();
    int size = font.getSize();
    String name = font.getName();
    int style = font.getStyle();

    while ( !fontFits ) {
      if ( (fontMetrics.getHeight() <= maxCharHeight)
          && (fontMetrics.stringWidth(longString) <= xSpace) ) {
        fontFits = true;
      }
      else {
        if ( size <= minFontSize ) {
          fontFits = true;
        }
        else {
          g2.setFont(font = new Font(name,
                          style,
                          --size));
          fontMetrics = g2.getFontMetrics();
        }
      }
    }

    return fontMetrics;
  }

  public void paint(Graphics g) {
    Graphics2D g2 = (Graphics2D) g;

        //compound number in the pathway
    int pathwaySize = p.getPathwaySize();

    HashMap hm;
```

```
    hm = this.setGeneName();
    g2.setRenderingHint(RenderingHints.KEY_ANTIALIASING,
RenderingHints.VALUE_ANTIALIAS_ON);
    Dimension d = getSize();
    int gridWidth = d.width / 8;
    int gridHeight = d.height / 8;
    fontMetrics = pickFont(g2, "Filled and Stroked GeneralPath",
                    gridWidth);
    Color fg3D = Color.lightGray;
    g2.setPaint(fg3D);
    g2.draw3DRect(0, 0, d.width - 1, d.height - 1, true);
    g2.draw3DRect(3, 3, d.width - 7, d.height - 7, false);
    g2.setPaint(fg);
    int x = 10;
    int y = 10;

   // y += gridHeight;
    int rectWidth = gridWidth - 2*x;
    int stringY = gridHeight - 3 - fontMetrics.getDescent();
    int rectHeight = stringY - fontMetrics.getMaxAscent() - y - 1;
                //stringY += gridHeight;

                //draw the pathway
                Shape compoundShpes[] = new Shape[pathwaySize];
        Shape lines[] = new Shape [pathwaySize];
    g2.setStroke(stroke);
   Compound myCompounds[] = new Compound [pathwaySize];
   String compName;
   String geneName;
   Color color;
   draw: for(int i = 0; i < pathwaySize; i++){

        compoundShpes[i] = new Ellipse2D.Double(x, y+150, rectWidth/2, rectHeight);
        myCompounds[i] = (Compound) p.getPathwayElement(i);
        compName = myCompounds[i].getCompName();
        String key = null;
        key = myCompounds[i].getGene().getName();
        geneName = (String) hm.get (key);
        color = myCompounds[i].getColor();

        g2.setPaint(color);
         g2.fill(compoundShpes[i]);
        g2.setPaint(fg);

    g2.drawString(compName, x, stringY+152);
    x += gridWidth/2
    int y1;
    y1 = y + 150 + rectHeight/2;
    // draw Line2D.Double
    lines[i] = new Line2D.Double(x, y1, x + rectWidth/2, y1);
   //the last line can not be appeared
    if (i == (pathwaySize-1)) break draw;
    g2.draw(lines[i]);
    g2.drawString(geneName, x, stringY+100);
    x += gridWidth/2;
```

```
                }
  }

}

// ***********************************************************************
//PathwayView.java

import java.awt.*;
import java.awt.event.*;
import java.awt.geom.*;
import javax.swing.*;
import java.awt.event.ItemListener;
import java.awt.event.ItemEvent;
import java.awt.event.ActionListener;
import java.awt.event.ActionEvent;

/*
 * This class for displaying the DHN pathway, it
 * uses the Java 2D APIs to define and render the graphics and text.
 */

public class PathwayView extends JFrame implements
ActionListener {
  String geneNames [] = new String [] {"the last compound (showed below)",
                    "Polyketide synthase gene (PKS)",
                    "Reductase I gene (THN1)",
                    "Scytalone dehydratase gene (SD)",
                    "Reductase II gene (THN2)",
                    "Oxidases"};
  String defaultEdit = "Currently, no gene is dirupted";
  Pathway pp, p;
  DrawPathway d;

  public PathwayView(int x){
        super("Pathway Modelling and Visualization");
        setSize(800, 450);
        addWindowListener(new WindowAdapter() {
       public void windowClosing(WindowEvent e) {dispose();}
        });
         pp = new Pathway(x);
         init(pp);
        setVisible(true);
  }

  public void init(Pathway p1){

                Container contentPane = this.getContentPane();
                Font labelFont = new Font("SanSerif", Font.BOLD, 18);
                p = p1;
                //set the container to the grid bag layout and
                //define the constarint obj
                 GridBagLayout gridbag = new  GridBagLayout();
                contentPane.setLayout(gridbag);
                GridBagConstraints c = new  GridBagConstraints();
                //setings for constraint obj instant varaibales
```

```
 c.fill =  c.BOTH;
//setting for label1
c.insets = new Insets(10,10,10,20);
c.gridx = 0;
c.gridy = 0;
c.gridwidth = 1; c.gridheight = 1;
c.anchor = c.WEST;
c.weightx = 0.0; c.weighty = 0.0;
JLabel l1 = new JLabel("Fungal DHN Melanin Pathway (Modelling and Visualization)");
l1.setFont(labelFont);
gridbag.setConstraints(l1, c);
contentPane.add(l1); //add the label1
//setting for label2
 c.gridx = 0;
c.gridy = 1;
c.gridwidth = 1; c.gridheight = 1;
c.anchor = c.WEST;
c.weightx = 0.0; c.weighty = 0.0;
 JLabel l2 = new JLabel("Gene disruption test, select a gene and get a color with:");
 l2.setFont(labelFont);
 gridbag.setConstraints(l2, c);
 contentPane.add(l2); //add the label2

 //add a combox, containing gene names

 c.gridx = 4;
 c.gridy = 1;
 c.gridwidth = 1; c.gridheight = 1;
 c.anchor = c.CENTER;
 c.weightx = 0.0; c.weighty = 0.0;
JComboBox genes = new JComboBox(geneNames);
 genes.setOpaque(true);
 genes.setEditable(false);
 // configure the combo box editor.
 genes.configureEditor(genes.getEditor(), defaultEdit);
 // rows to be visible without scrollbars.
 genes.setMaximumRowCount(6);
// set the combo box editor colors and font.
ComboBoxEditor cboxEditor = genes.getEditor();
Component editorComp = cboxEditor.getEditorComponent();
 editorComp.setBackground(Color.white);
 editorComp.setForeground(Color.blue);
Font f1 = new Font("Dialog", Font.PLAIN, 12);
 editorComp.setFont(f1);
// font for the combo box popup list.
 genes.setFont(f1);
//genes.addItemListener(this);
 genes.addActionListener(this);
 gridbag.setConstraints(genes, c);
 contentPane.add(genes);
//add the drawPathway
 c.gridx = 0;
 c.gridy = 3;
 c.gridwidth = 5; c.gridheight = 1;
 c.anchor = c.CENTER;
 c.weightx = 1.0; c.weighty = 1.0;
```

```
                    c.fill = c.BOTH;
                    d = new DrawPathway(p);
                   gridbag.setConstraints(d, c);
                   contentPane.add(d);
  }

 // public void itemStateChanged(ItemEvent e){}

  public void actionPerformed(ActionEvent e) {
     JComboBox source = (JComboBox) e.getSource();
     String item = (String) source.getSelectedItem();
     if (item.equals(geneNames[0])){
                              this.setVisible(false);
                              new PathwayView(7);
                              }
                    for (int j = 1; j < geneNames.length; j++) {
          if (item.equals(geneNames[j])){
                   this.setVisible(false);
                   //int length;
                   if (item.equals("Oxidases"))
                            j = j +1;
                   new PathwayView(j);
          }
                   }
  }

   public static void main(String s[]) {

     PathwayView f = new PathwayView(7);
    f.show();
  }
 }

//HelpItem.java

import java.awt.*;
import java.awt.event.*;

/** this class shows help information*/
public class  HelpItem extends Frame
{
          /** text Area */
          private TextArea ta1;
          /** panels */
          private Panel  p1, p2, p3, p4, p5;
          /** Constructor */
                    public HelpItem() {
                    super(); // call Frame's empty constructor
          setTitle("Help information");
          sizeandplaceWindow();
          setBackground(Color.lightGray);
          addWindowListener(new WindowCloser());
           init();
          buildHM();
          setVisible(true);
  }
```

```
/** this method is to instantiate variables */
public void init(){
        ta1 = new TextArea(8, 50);
        ta1.setFont(new Font("ARIAL", Font.BOLD, 14));
        p1 = new Panel();
        p2 = new Panel();
        p3 = new Panel();
        p4 = new Panel();
        p5 = new Panel();
}

/** build the view */
public void buildHM()
{ta1.setText(" This program contains the gene/enzyme/mutant\n" +
                " information about the fungal DHN-melanin pathway.\n\n" +
                " To search genes, click the Gene Search button.\n" +
                " To search enzymes, click the Enzyme Search button.\n" +
                " To search gene/enzyme using species as a seraching\n" +
                "   key, click Species Search button.\n"
                  );
        p1.add(ta1);
        add("North", p3);
        add("South", p2);
        add("Center", p1);
        add("East", p5);
        add("West", p4);
}


/** this inner class deals with closing the window */
private class WindowCloser extends WindowAdapter
{public void windowClosing(WindowEvent e){
                dispose();
                }
}
/** this private method sizes and places the frame in the right upper corner of the screen */
private void sizeandplaceWindow()
{
        this.setSize(500, 200);
        this.setResizable(false);
        this.setLocation(200, 200);
}

public static void main(String args[]){
        HelpItem hm = new HelpItem();
}

}

// ***********************************************************************
// MainFrame.java

import java.awt.*;
import java.awt.event.*;
import javax.swing.event.*;
```

```
import javax.swing.*;
import javax.swing.border.*;
import java.io.*;


/**
 * The calass is responsible for providing an interface to the services.
 */

public class MainFrame extends JFrame
{
/** the container reference */
Container c = null;
/** model variable */
protected Model mymodel;
/** file variable */
private File files;
/**label variable */
private JLabel l;
/** a menubar in the main view */
private JMenuBar mb;
/** menu items in the main view */
private JMenuItem findfile, content, about;
/** menu in the main view */
private JMenu file, help;
/**panel*/
private JPanel p, p1;
/** buttons */
private JButton gene, enzyme, species, pathway;
String s;
Frame myView;
/** a loadview variable */
//LoadView loadview;

/**constructor */

public MainFrame(Model m)
{
        super();
        mymodel = m;
        s = "frame";
        setTitle("Gene Inquiry System");
        //configure the frame using its content pane
        c = this.getContentPane();
        c.setBackground(Color.lightGray);
        sizeandplaceWindow();
        init();
        buildMenu();
        setVisible(true);
        addWindowListener(new WindowCloser());
}


/** this method is to initialize the variables */
public void init()
{
```

```
        mb = new JMenuBar();
        mb.setBorder(new BevelBorder(BevelBorder.RAISED));
        mb.setBorderPainted(true);
        file = new JMenu("File", true);
        file.setFont(new Font("ARIAL", Font.BOLD, 14));
        help = new JMenu("Help");
        help.setFont(new Font("ARIAL", Font.BOLD, 14));
        findfile = new JMenuItem ("Exit");
        findfile.setFont(new Font("ARIAL", Font.BOLD, 12));
        findfile.addActionListener(new Menulistener ());
        content = new JMenuItem("Help info");
        content.setFont(new Font("ARIAL", Font.BOLD, 12));
        content.addActionListener(new Menulistener ());
        about = new JMenuItem("About");
        about.setFont(new Font("ARIAL", Font.BOLD, 12));
        about.addActionListener(new Menulistener ());
        l = new JLabel("Fungal Melanin Gene System", JLabel.CENTER);
        l.setFont(new Font("ARIAL", Font.BOLD, 28));
        p = new JPanel ();
        p1 = new JPanel();
        gene = new JButton("Gene Search");
        gene.addActionListener(mymodel);
        gene.setFont(new Font("ARIAL", Font.BOLD, 16));
        enzyme = new JButton("Enzyme Search");
        enzyme.addActionListener(mymodel);
        enzyme.setFont(new Font("ARIAL", Font.BOLD, 16));
        species = new JButton("Species Search");
        species.addActionListener(mymodel);
        species.setFont(new Font("ARIAL", Font.BOLD, 16));
        pathway = new JButton("Pathway Display");
        pathway.addActionListener(new Menulistener ());
        pathway.setFont(new Font("ARIAL", Font.BOLD, 16));
}

/** build the menu structure */
public void buildMenu()
{
        c.add(mb, BorderLayout.NORTH);
        mb.add(file);
        file.add(findfile);
        p1.add(l);

        //menu "help"
        mb.add(help);
        help.addSeparator();
        help.add(content);
        help.addSeparator();
        help.add(about);

        //add buttons
        p.add(gene);
        p.add(enzyme);
        p.add(species);
        p.add(pathway);

        c.add("Center", p1);
```

```
        c.add("South", p);
}

/** this class is to implement the ActionListener interface */
private class Menulistener implements ActionListener
{       /** the method is listening */
        public void actionPerformed(ActionEvent e){
        Object source = e.getSource();
         if(source == about){
                AboutView av = new AboutView(myView, "About Gene System", true);
                        av.start();
                }else if (source == content){
                 HelpItem helpitem = new HelpItem();
                helpitem.setVisible(true);
                }else if (source == pathway){

                PathwayView p = new PathwayView(7);
                 p.setVisible(true);
                }else if (source == findfile)

                {
                        System.exit(0);
                }
        }
}

/** this inner class deals with closing the window */
private class WindowCloser extends WindowAdapter
{
        public void windowClosing(WindowEvent e){
                System.exit(0);
                }
}

/** this private method sizes and places the frame in the middle of the screen */
private void sizeandplaceWindow()
{
        this.setSize(800, 450);
        this.setResizable(false);
        Dimension frameD = new Dimension(800, 450);
        Dimension screenD = new Dimension();
        screenD = Toolkit.getDefaultToolkit().getScreenSize();
        if(frameD.width >= screenD.width)
        this.setLocation(1, 1);
        this.setLocation(((screenD.width - frameD.width)/2),
                ((screenD.height - frameD.height)/2));
        this.setSize(frameD.width, frameD.height);
}

// testing
public static void main(String arg[]){

        Model a = new Model();
        MainFrame mf = new MainFrame(a);
}
```

```
}

// ***********************************************************************
// SpeciesView.java

import javax.swing.*;
import java.awt.*;
import java.awt.event.*;
import javax.swing.border.*;


/**
 * The calass is responsible for providing an interface for species search.
 */
public class SpeciesView extends JFrame {
   /** declare the references. */
   JComboBox cbox = null;
   JComboBox cbox1 = null;
   Container c = null;
   /** define an array of species name to be shown in the
    * combo box pull-down list, by default. */
   String[] defaultSpecies = {
       "Ophiostoma floccosum", "Magnaporthe grisea",
       "Colletotrichum lagenarium"};
   String defaultEdit = "Ophiostoma floccosum";

         /** define an array of either gene or enzyme to be shown in the
    * combo box pull-down list, by default. */
   String[] defaultGen = {
       "Gene", "Enzyme"};
   String defaultEdit1 = "Gene";

         /** model variable */
         protected Model mymodel;

         /** constructor */
         public SpeciesView (Model m)
         {
                  super();
                  setTitle("Species Search View");
                  mymodel = m;

                  //configure the frame using its content pane
                  c = this.getContentPane();
                  c.setBackground(Color.lightGray);
                  c.setLayout(new GridLayout(1,1));
                  init();
                  sizeandplaceWindow();
                  addWindowListener(new WindowCloser());
                  setDefaultCloseOperation(WindowConstants.DISPOSE_ON_CLOSE);
         }

         /** construct the view */
         public void init() {

      // prepare the container with layout
```

```
JPanel p1 = new JPanel();
 p1.setLayout(new BorderLayout());
// set the title border around the panel.
 TitledBorder border1 = new TitledBorder(
          new LineBorder(Color.blue),
          "It will list all genes or enzymes for the selected species");
 border1.setTitleColor(Color.blue);
 p1.setBorder(border1);
 JPanel p3 = new JPanel();
 p3.setLayout(new GridLayout(1,2));
 JPanel p4 = new JPanel();
 p4.setLayout(new GridLayout(1,3));
JPanel p7 = new JPanel();

 // create the combo box with the list of default sites.
 cbox = new JComboBox(defaultSpecies);
 cbox.setOpaque(true);
 cbox1 = new JComboBox(defaultGen);
 cbox1.setOpaque(true);

 // make the combo box ineditable.
 cbox.setEditable(false);
 cbox1.setEditable(false);

 // configure the combo box editor.
 cbox.configureEditor(cbox.getEditor(), defaultEdit);
cbox1.configureEditor(cbox.getEditor(), defaultEdit1);

 // rows to be visible without scrollbars.
 cbox.setMaximumRowCount(3);
 cbox1.setMaximumRowCount(2);

 // set the combo box editor colors and font.
 ComboBoxEditor cboxEditor = cbox.getEditor();
 Component editorComp = cboxEditor.getEditorComponent();
 editorComp.setBackground(Color.white);
 editorComp.setForeground(Color.blue);

 Font f1 = new Font("Dialog", Font.PLAIN, 14);
 editorComp.setFont(f1);

 ComboBoxEditor cboxEditor1 = cbox1.getEditor();
 Component editorComp1 = cboxEditor1.getEditorComponent();
 editorComp1.setBackground(Color.white);
 editorComp1.setForeground(Color.blue);

 Font f2 = new Font("Dialog", Font.PLAIN, 14);
 editorComp1.setFont(f2);

 // font for the combo box popup list.
 cbox.setFont(f1);
cbox1.setFont(f2);

 // label to indicate what to do.
 JLabel label1 = new JLabel("Select fungal species:",
                    JLabel.CENTER);
```

```
    label1.setFont(new Font("SansSerif", Font.BOLD, 16));

   JLabel label2 = new JLabel("Select gene/enzyme:",
                    JLabel.CENTER);
    label2.setFont(new Font("SansSerif", Font.BOLD, 16));

    // button for search
    JButton b1 = new JButton("Seek");
    b1.setFont(new Font("SansSerif", Font.BOLD, 16));
    b1.addActionListener(mymodel);

   //construct the first search block
    p3.add(label1);
    p3.add(cbox);
  p4.add(label2);
  p4.add(cbox1);
  p7.add(b1);
  p1.add(p7, BorderLayout.SOUTH);
  p1.add(p3, BorderLayout.CENTER);
  p1.add(p4, BorderLayout.NORTH);

   //add the blocks to the frame
   c.add(p1);
 }
        /** this inner class deals with closing the window */
        private class WindowCloser extends WindowAdapter
        {
                public void windowClosing(WindowEvent e){
                        dispose();
                        }
        }

        /** this private method sizes and places the frame in the middle of the screen */
        private void sizeandplaceWindow()
        {
                this.setSize(400, 150);
                this.setResizable(false);
                Dimension frameD = new Dimension(400, 150);
                Dimension screenD = new Dimension();
                screenD = Toolkit.getDefaultToolkit().getScreenSize();
                if(frameD.width >= screenD.width)
                        this.setLocation(1, 1);
                this.setLocation(((screenD.width - frameD.width)/2),
                        ((screenD.height - frameD.height)/2));
                this.setSize(frameD.width, frameD.height);
        }


        //testing
        public static void main(String arg[])
        {
                Model a = new Model();
                SpeciesView gv = new SpeciesView(a);
        }
}
```

```
// ***********************************************************************
//AboutView.java

// This Class will display "About" window and play animation in a thread
// Toolkit has been used to fetch the images and loading them in array
// It is invoked, when menu option About is clicked

import java.awt.*;
import java.awt.event.*;
import java.util.*;
import java.awt.Toolkit;


public class AboutView extends Thread implements Runnable, ActionListener, WindowListener
{
//      Dialog d;
        Frame d;
        Button OK;
        Panel p1, p2, plabel ;
        Label heading,l1, l2, l3, l4, l5, l6;
        String g1= " ", g2= " ", g3= " ", g4= " ", g5= " ", g6= " ";
        Image images[];
         int totalImages = 4,      // total number of images
         currentImage = 0,                    // current image subscript
        sleepTime = 500;       // milliseconds to sleep
         String imageName = "duke"; // base name of images
        Thread animate;          // animation thread
         Graphics gContext;
        int height = 0, width = 0;
         AboutViewCanvas canvas;

        public AboutView(Frame parent, String s, boolean bvalue)
        {
                d = new Frame(s);
                d.setSize(400, 225);
                d.setLocation(200,150);
                d.setBackground(Color.lightGray);
                d.setResizable(false);
                init();
                startanimation();
        }

        public void init(){
                loadImages();
                addGUI();

                canvas = new AboutViewCanvas();
                d.add("North", p1);
                d.add("Center" , canvas);
                d.add("South", p2);

                // add the listeners
                OK.addActionListener(this);
                d.addWindowListener(this);

                d.setVisible(true);
```

```
        }

        public void loadImages()
        {  images = new Image[ totalImages ];
                for ( int i = 0; i < images.length; i++ )
                        images[ i ] = Toolkit.getDefaultToolkit().getImage(imageName + i + ".gif" );
        }

        // instantiate the Button, Label & Panel for our Dialog box
        public void addGUI(){
                p1 = new Panel();
                p1.setLayout(new BorderLayout());
                p1.setBackground(Color.lightGray);

                heading = new Label("Fungal Melanin Gene System", Label.CENTER);
                heading.setFont(new Font ("Sans Serif", Font.ITALIC, 18));
                heading.setForeground(Color.blue);

                 g1 = "Fungal Melanin Gene System, version 1.0.0" ;
                 g3 = "        All Rights Reserved              " ;

                l1 = new Label(g1, Label.CENTER);
                l1.setBackground(Color.lightGray);
                l1.setFont(new Font ("ARIAL", Font.BOLD, 14));

                l2 = new Label(g2, Label.CENTER);
                l2.setBackground(Color.lightGray);
                l2.setFont(new Font ("ARIAL", Font.BOLD, 14));

                l3 = new Label(g3, Label.CENTER);
                l3.setBackground(Color.lightGray);
                l3.setFont(new Font ("ARIAL", Font.BOLD, 14));

                plabel = new Panel();
                plabel.add(l1);

                p2 = new Panel();
                p2.add(OK = new Button("OK"));

                // now add the components to the frame
                p1.add("North", heading);
                p1.add("Center", plabel);
                p1.add("South", l3);
        }

        public Dimension getPreferredSize(){ return new Dimension(width, height);}
        public Dimension getMinimumSize(){ return getPreferredSize();}

// start the applet
public void startanimation()
{
   //always start with 1st image
    currentImage = 0;

  // create a new animation thread when user visits page
  if ( animate == null ) {
```

```
                animate = new Thread( this );
        animate.start();
      }
    }

    // override update to eliminate flicker
    public void run()
    {
          while ( true ){
          canvas.setPic(images[currentImage]);
          currentImage = ( currentImage + 1 ) % totalImages;
        try {
          Thread.sleep( sleepTime );
        }
        catch ( InterruptedException e ) {}

      }
    }
          /** since we are handling the event processing, do so */
          public void actionPerformed(ActionEvent e)
          {
                // check the cmd field to see if it's our button
                if(e.getActionCommand() == "OK")
                {
                      d.dispose();
                      animate = null;
                }
          }

          public void windowClosing(WindowEvent e){
          //animate.stop();
          animate = null;
          d.hide();
          d.dispose();
          //animate.destroy();
          }

          public void windowActivated(WindowEvent e){}
          public void windowClosed(WindowEvent e){}
          public void windowDeiconified(WindowEvent e){}
          public void windowIconified(WindowEvent e){}
          public void windowOpened(WindowEvent e){}
          public void windowDeactivated(WindowEvent e){}
}


// ***********************************************************************

// AboutViewCanvas.java


// This class is used to create a canvas for About Class inorder to draw animated object in it

import java.awt.*;
public class AboutViewCanvas extends Canvas {
 Image image;
```

```
public AboutViewCanvas () {}

public void setPic( Image imagefile )
 {
        image = imagefile;
        repaint();
 }

 public void paint( Graphics g )
  {
                g.drawImage(image, 150, 10, this );
  }
 }

// ***********************************************************************
//Display.java

import javax.swing.*;
import java.awt.event.*;
import java.awt.*;


/** this class displays information*/

public class  Display extends JFrame

{
 // Declare the references for the following objects.
   Container container;
   JLabel label = null;
   JTextArea textArea = null;
   JButton insertButton = null;
   JButton fastaButton = null;
   JButton cutButton = null;
   JButton copyButton = null;
   JButton pasteButton = null;
   JButton xmlButton = null;

        /** Constructor */
   public Display() {
        super("Display information");
        sizeandplaceWindow();
        addWindowListener(new WindowCloser());

         init();
        setVisible(true);
  }

  public void init () {

     // Assign a name to the frame and obtain a handle
     // on the frame's content pane.
     container = this.getContentPane();

     // Create the fonts for label and text area.
```

```
Font labelFont = new Font("SanSerif", Font.BOLD, 14);
Font textFont = new Font("Dialog", Font.PLAIN, 12);

// Use the gridbag layout for the applet.
GridBagLayout gridbag = new GridBagLayout();
container.setLayout(gridbag);
GridBagConstraints c = new GridBagConstraints();

c.insets = new Insets(2,10,10,2);

// Add the xml button.
c.gridx = 0; c.gridy = 0;
c.gridwidth = 1; c.gridheight = 1;
c.fill = c.BOTH;
xmlButton = new JButton("Export XML");
xmlButton.setBackground(Color.lightGray);
gridbag.setConstraints(xmlButton, c);
container.add(xmlButton); // add the paste button
ButtonListener xmlButtonListener = new ButtonListener();
xmlButton.addActionListener(xmlButtonListener);

// Add the Delete button.
c.gridx = 1; c.gridy = 0;
fastaButton = new JButton("Fasta File");
fastaButton.setBackground(Color.lightGray);
gridbag.setConstraints(fastaButton, c);
container.add(fastaButton); // add the delete button
ButtonListener dlButtonListener = new ButtonListener();
fastaButton.addActionListener(dlButtonListener);

// Add the Cut button.
c.gridx = 2; c.gridy = 0;
cutButton = new JButton("Cut");
cutButton.setBackground(Color.lightGray);
gridbag.setConstraints(cutButton, c);
container.add(cutButton); // add the cut button
ButtonListener ctButtonListener = new ButtonListener();
cutButton.addActionListener(ctButtonListener);

// Add the Copy button.
c.gridx = 3; c.gridy = 0;
copyButton = new JButton("Copy");
copyButton.setBackground(Color.lightGray);
gridbag.setConstraints(copyButton, c);
container.add(copyButton); // add the copy button
ButtonListener cpButtonListener = new ButtonListener();
copyButton.addActionListener(cpButtonListener);

// Add the Paste button.
c.gridx = 4; c.gridy = 0;
pasteButton = new JButton("Paste");
pasteButton.setBackground(Color.lightGray);
gridbag.setConstraints(pasteButton, c);
container.add(pasteButton); // add the paste button
ButtonListener psButtonListener = new ButtonListener();
pasteButton.addActionListener(psButtonListener);
```

```
    // Add the text area.
    c.gridx = 0; c.gridy = 1;
    c.gridwidth = 5; c.gridheight = 1;
    c.weightx = 1.0; c.weighty = 1.0;
    c.anchor = c.CENTER;
    c.fill = c.BOTH;

    textArea = new JTextArea(10, // Number of rows.
                    30); // Number of columns.

    JScrollPane sp = new JScrollPane(textArea);

    textArea.setFont(textFont);
    textArea.setBackground(Color.white);
    textArea.setSelectionColor(Color.yellow);
    textArea.setTabSize(5); // The tab size.
    textArea.setLineWrap(true); // Wrap the line at the container end.
    gridbag.setConstraints(sp, c);
    container.add(sp); // Add the text area.

    // Add the window listener to close the frame
    // and display it with the specified size.

    setDefaultCloseOperation(WindowConstants.DISPOSE_ON_CLOSE);
  }

        /** this inner class deals with closing the window */
        private class WindowCloser extends WindowAdapter
        {
        public void windowClosing(WindowEvent e){

                dispose();
                }
        }


//Button listener class.
 class ButtonListener implements ActionListener {
    public void actionPerformed(ActionEvent e) {
       JButton b = (JButton) e.getSource();
       if (b == fastaButton) {
         ; // Fasta file will be created
       }
       else if (b == cutButton) {
          textArea.cut(); // Cut operation.
       }
       else if (b == copyButton) {
          textArea.copy(); // Copy operation.
       }
       else if (b == pasteButton) {
          textArea.paste(); // Paste operation.
       }
        else if (b == xmlButton) {
         ; // export xml file
                }
```

```
        }
    }
        /** this private method sizes and places the frame in the right upper corner of the screen */
        private void sizeandplaceWindow()
        {
                this.setSize(450, 500);
                this.setResizable(false);
                this.setLocation(100, 50);
        }

        public static void main(String args[]){


                Display hm = new Display();
        }
}

// ***********************************************************************

// Phenotype.java

/** this class defines the information of a phenotype,
 * wild type and mutant which is defective in some gene */

public class  Phenotype
{
        /** the defective gene */
        private Gene g;
        /** the phenotype description */
        private String description;
        /** the accumulated compound */
        private String accumulator;
        /** species name */
        private String speciesName;

        /** a constructor */
        public Phenotype()
        {
                description        = "Unknown";
                accumulator = "Unknown";
        }

        /** a convenience constructor */
        public Phenotype(String i, String sn)
        {
                description        = i;
                accumulator = sn;
        }

        /** the method is to set the description */
        public void setDescription (String d)
        {
                description        = d;
        }

        /** the method is to set accumulator */
```

```
        public void setAccumulator(Enzyme e)
        {
                accumulator = e.getSubName();
        }

        /** the method is to set accumulator */
        public void setAccumulator(String e)
        {
                accumulator = e;
        }

        /** the method is to obtain the description */
        public String getDescription()
        {
                return description;
        }

        /** the method is to obtain the accumulator */
        public String getAccumulator()
        {
                return accumulator;
        }

}

// ***********************************************************************
// Version: 1.0.0
//
// @Author: Edwin (Honglong) Wang
//
// Tested on Java(TM) 2 Platform
//
// Sun Microsystems, Inc.,
// 901 San Antonio Road,
// Palo Alto, CA 94303 USA.
//
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// University of British Columbia,
// Vancouver, Canada
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// ***********************************************************************

// Model.java

import javax.swing.event.*;
import javax.swing.*;
import java.util.*;
import java.awt.*;
import java.lang.*;
import java.io.*;
import java.awt.event.*;
```

```
/** the Model class, provides all methods to processe data, trigger several major views  */
public class Model implements ActionListener
{
        /** Genelist variable */
        private static Genelist myGenelist;

        /** Enzymelist variable */
        private static Enzymelist myEnzymelist;

        /** collection of rnzymes */
        private ArrayList collection;

        //views will be triggered by Model
        protected GeneView geneview;
        protected EnzymeView enzyview;
        protected SpeciesView speciesview;
        protected MainFrame mf;

        /** HashMap contains gene-mutant pair */
        protected HashMap myHM;

        /** enzyme names */
        String [] enzyme_name = {"Polyketide synthase", "Reductase",
                                                "Scytalone dehydratase"};

        /** species names */
        String[] species_name = {"Ophiostoma floccosum", "Magnaporthe grisea",
            "Colletotrichum lagenarium"};

        /** the constructor of the model class */
        public Model()
        {
          //HashMap contains gene as key, phenotype as value
          myHM = new HashMap();
          collection = new ArrayList();
                geneview = new GeneView(this);
                enzyview = new EnzymeView(this);
                speciesview = new SpeciesView(this);
                mf = new MainFrame(this);
                this.setupData();
        }

        /** This methos is to set up the databases for gene, enzyme, and phenotype */
        public void setupData()
        {
                //data
                String[] gene_name = {"OSD1", "THN1", "THN2", "mgSD", "T4HR gene",
                                        "T3HR gene", "PKS1", "SCD1", "THR1"};
                String[] e_name = {"Scytalone dehydratase", "Reductase", "Reductase",
                                "Scytalone dehydratase", "Reductase", "Reductase","Polyketide
synthase",
                                "Scytalone dehydratase", "Reductase"};
                String[] enzyme_id = {"SD", "THN1", "THN2", "SD", "THN1", "THN2", "PKS", "SD",
                                "THN2"};
                for (int i = 0; i < species_name.length; i++ )
```

```
for (int j = 0; j < 3; j++)
{       int c = 0;
        Gene gc = new Gene();
        c = i * 3 + j;
        gc.setSpeciesName(species_name[i]);
        gc.setName(gene_name[c]);

        //open the file and read sequences
        // buffered input file
         try{
                        BufferedReader in =
                        new BufferedReader(
                        new FileReader(("gene" + c + ".txt")));

         String s, s2 = new String();
        while((s = in.readLine())!= null)
                s2 += s.toUpperCase().trim(); // change to upper case, trim
space,
                                        // and cancat;
                gc.setSeq(s2);
                in.close();

                } catch(FileNotFoundException e) {
                        System.out.println(
                         "File Not Found:" + "gene" + c + ".txt");
                } catch(IOException e) {
                        System.out.println("IO Exception");
                }

        Enzyme ec = new Enzyme();
        ec.setSpeciesName(species_name[i]);
        ec.setName(e_name[c]);
        ec.setID(enzyme_id[c]);
        ec.setGene(gc);
        //set the substrate and reaction product names
        if (ec.getID().equals("SD"))
        {
                ec.setSubName("Scytalone");
                ec.setProductName("1, 3, 8-THN");
        }else if(ec.getID().equals("THN1"))
                {
                        ec.setSubName("1, 3, 6, 8-THN");
                        ec.setProductName("Scytalone");
                }else if (ec.getID().equals("THN2"))
                {
                        ec.setSubName("1, 3, 8-THN");
                        ec.setProductName("Vermelone");
                }else if (ec.getID().equals("PKS"))
                {
                        ec.setSubName("Acetate");
                        ec.setProductName("1, 3, 6, 8-THN");
                }

        //open the file and read sequences
        // buffered input file
         try{           BufferedReader in =
```

```
                                        new BufferedReader(
                                        new FileReader(("enzy" + c + ".txt")));
                                        String s1, s21 = new String();
                                while((s1 = in.readLine())!= null)
                                        s21 += s1.toUpperCase().trim(); // change to upper case, trim
space,
                                                                // and cancat
                                        ec.setSeq(s21);
                                        in.close();
                                        } catch(FileNotFoundException e) {
                                                System.out.println(
                                                "File Not Found:" + "enzy" + c + ".txt");
                                        } catch(IOException e) {
                                                System.out.println("IO Exception");
                                        }
                                //add to the list
                                collection.add(ec);

                                //set up my HashMap
                                Phenotype pc = new Phenotype();

                                if(gc.getName().equals(gene_name[0]) ||
                                        gc.getName().equals(gene_name[1]) ||
                                        gc.getName().equals(gene_name[2]))
                                                ;
                                        else if (gc.getName().equals(gene_name[3]) ||
                                        gc.getName().equals(gene_name[7]))
                                        {
                                                pc.setDescription("Reddish color");
                                                pc.setAccumulator(ec);
                                        }else if (gc.getName().equals(gene_name[5]) ||
                                                gc.getName().equals(gene_name[8]))
                                                {
                                                pc.setDescription("Brown color");
                                                pc.setAccumulator(ec);
                                        }else if (gc.getName().equals(gene_name[4]))
                                        {
                                                pc.setDescription("Black color");
                                                pc.setAccumulator("Melanin");
                                        }else
                                                {
                                                        pc.setDescription("Albino color");
                                                        pc.setAccumulator(ec);
                                                }
                                myHM.put(gc, pc);

                        }
                }

        /** this method is display enzyme information */
        public String enzyDisplay(Enzyme tempEnzy)
        {
                return ("\n Enzyme Name:  " + tempEnzy.getName() + " , " +
                                tempEnzy.getID() + "\n Species Name:  " +
                                tempEnzy.getSpeciesName() + "\n Substrate:  " +
                                tempEnzy.getSubName() + "\n Reaction product:  " +
```

```
                                tempEnzy.getProductName() + "\n Protein sequence:  \n>"
                                + tempEnzy.getName() + "    [" + tempEnzy.getSpeciesName()
                                + "]\n" + tempEnzy.getSeq() + "\n" + "*******************"
                                + "*****************************************************"
+ "\n");
        }

        /** this method is display enzyme information */
        public String geneDisplay(Enzyme tempEnzy)
        {               String s1, s2 = null;
                        Phenotype p = null;

                        p = (Phenotype) myHM.get(tempEnzy.getGene());
                        s1 = p.getDescription();
                        s2 = p.getAccumulator();

                        return ("\n Gene Name:  " + tempEnzy.getGene().getName() +
                                "\n Its enzyme Name:  " + tempEnzy.getName()
                                + "\n Species Name:  " +
                                tempEnzy.getSpeciesName() +
                                "\n Gene mutant information:\n    Mutant color:  "
                                + s1 + "\n    Compound accumulated in the mutant's melanin pathway:
"
                                + s2 + "\n Gene sequence:  \n> " + tempEnzy.getName() + "   [" +
                                tempEnzy.getSpeciesName()
                                + "]\n" + tempEnzy.getGene().getSeq() + "\n" +
"*******************"
                                                        +
                        "*****************************************************" + "\n");
        }


        /** the method is listening */
        public void actionPerformed(ActionEvent e)

        { if (e.getActionCommand() == "Gene Search")
                        geneview.setVisible(true);
                else if (e.getActionCommand() == "Enzyme Search")
                {enzyview.setVisible(true);
                }else if (e.getActionCommand() == "Species Search")
                {       speciesview.setVisible(true);
                }else if (e.getActionCommand() == "Find"){
                if(geneview.t1.getText().equals(""))
                        geneview.t1.setText("please enter data.");
                else{Display d = new Display();
                        d.textArea.append("Nothing matches your search, please check and search
again\n");
                                d.textArea.append("The key words for searching are: Scytalone
                        dehydratase, Reductase, or Polyketide synthase");
                        Enzyme tempEnzy = null;
                        for(int i = 0; i < enzyme_name.length; i++){
                        if (geneview.t1.getText().equalsIgnoreCase(enzyme_name[i])){
                        for(int j = 0; j < species_name.length; j++){
                        if
(geneview.cbox.getSelectedItem().toString().equalsIgnoreCase(species_name[j])){
                                d.textArea.setText("");
```

```
                                for (int v = 0; v < collection.size(); v++ ){
                                        tempEnzy = (Enzyme) collection.get(v);
                                  if(tempEnzy.getSpeciesName().equals(species_name[j]) &&
                                        tempEnzy.getName().equals(enzyme_name[i])){
                                        d.textArea.append(geneDisplay(tempEnzy));
                                         }
                                        }
                                 }
                                }
                         }
                        }
                 }
                }else if (e.getActionCommand() == "Go"){
                        if(geneview.t2.getText().equals(""))
                                geneview.t2.setText("please enter data.");
                        else{
                                Display d = new Display();
                                d.textArea.append("Nothing matches your search, please check and
search again\n");
                                d.textArea.append("The key words for searching are: Scytalone
                        dehydratase, Reductase, or Polyketide synthase");
                                Enzyme tempEnzy = null;
                                for(int i = 0; i < enzyme_name.length; i++){
                                if (geneview.t2.getText().equalsIgnoreCase(enzyme_name[i])){
                                        d.textArea.setText("");
                                        for (int v = 0; v < collection.size(); v++ ){
                                                tempEnzy = (Enzyme) collection.get(v);
                if(tempEnzy.getName().equals(enzyme_name[i])){
                                        d.textArea.append(geneDisplay(tempEnzy));
                                                                }
                                                        }
                                                }
                                        }
                                }
                }else if (e.getActionCommand() == "Search"){
                if(enzyview.t1.getText().equals(""))
                        enzyview.t1.setText("please enter data.");
                else{
                        Display d = new Display();
                        d.textArea.append("Nothing matches your search, please check and search
again\n");
                                d.textArea.append("The key words for searching are: Scytalone
                        dehydratase, Reductase, or Polyketide synthase");
                        Enzyme tempEnzy = null;
                        for(int i = 0; i < enzyme_name.length; i++){
                        if (enzyview.t1.getText().equalsIgnoreCase(enzyme_name[i])){
                        d.textArea.setText("");
                        for(int j = 0; j < species_name.length; j++){
                        if
(enzyview.cbox.getSelectedItem().toString().equalsIgnoreCase(species_name[j])){
                                        for (int v = 0; v < collection.size(); v++ ){
                                                tempEnzy = (Enzyme) collection.get(v);

                if(tempEnzy.getSpeciesName().equals(species_name[j]) &&
                                                tempEnzy.getName().equals(enzyme_name[i])){
```

```
d.textArea.append(enzyDisplay(tempEnzy));
                                                                        }
                                                                }
                                                        }
                                                }
                                        }
                                }
                        }
                }else if (e.getActionCommand() == "Hunt"){
                        if(enzyview.t2.getText().equals(""))
                                enzyview.t2.setText("please enter data.");
                        else{
                                Display d = new Display();
                                d.textArea.append("Nothing matches your search, please check and
search again\n");
                                d.textArea.append("The key words for searching are: Scytalone
                                dehydratase, Reductase, or Polyketide synthase");
                                Enzyme tempEnzy = null;

                                for(int i = 0; i < enzyme_name.length; i++){
                                if (enzyview.t2.getText().equalsIgnoreCase(enzyme_name[i])){
                                        d.textArea.setText("");
                                        for (int v = 0; v < collection.size(); v++ ){
                                                tempEnzy = (Enzyme) collection.get(v);

        if(tempEnzy.getName().equals(enzyme_name[i])){
        d.textArea.append(enzyDisplay(tempEnzy));
                                                                        }
                                                                }
                                                        }
                                                }
                                        }
                                }
                }else if (e.getActionCommand() == "Seek"){
                        Display d = new Display();
                        Enzyme tempEnzy = null;
                        for(int j = 0; j < species_name.length; j++){
                         if (speciesview.cbox.getSelectedItem().toString().equals(species_name[j])){
                                if
(speciesview.cbox1.getSelectedItem().toString().equals("Enzyme")){
                                        for (int v = 0; v < collection.size(); v++ ){
                        tempEnzy = (Enzyme) collection.get(v);

                        if(tempEnzy.getSpeciesName().equals(species_name[j]))
                                d.textArea.append(enzyDisplay(tempEnzy));
                                                                        }
                        }else{
                                 for (int v = 0; v < collection.size(); v++ ){
                                tempEnzy = (Enzyme) collection.get(v);

                                if(tempEnzy.getSpeciesName().equals(species_name[j]))
                                        d.textArea.append(geneDisplay(tempEnzy));
                                                                        }
                                                                }
                                                        }
                                                }
```

```
        }
}

/** THIS IS THE STRAT POINT OF THE PROGRAM */
public static void main(String[] args)
{
        Model m = new Model ();

}

}
```