Isolation of a polyketide synthase gene from *Dothistroma pini*

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Branwen Morgan

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ABSTRACT

Dothistromin is a polyketide derived mycotoxin, produced by *Dothistroma pini*, which is structurally related to aflatoxins produced by *Aspergillus parasiticus* and *Aspergillus flavus*. Southern blot analysis of *D. pini* genomic DNA was carried out using a probe (KS-2) encoding the highly conserved β keto-acyl synthase domain from the polyketide synthase gene (pksL1) of *A. parasiticus*, which indicated the presence of a homologous gene in strain Dp 2 of *D. pini*. Subsequently, KS-2 hybridising lambda clones were isolated from a *D. pini* genomic library. A 2411 bp fragment was subcloned and sequenced. Sequence analysis recognised two functional protein domains, β keto-acyl synthase (KS) and acyl transferase (AT), both of which are present in fatty acid and polyketide synthases. The sequence exhibited high homology with *A. nidulans wA* and *A. parasiticus* PKSL1 (62.3% and 59.9%) respectively, but only slight homology with the 6-MSA gene from *Penicillium patulum* and the *atX* gene from *Aspergillus tereus*. Additionally, a BLASTX search revealed some similarities with a number of FASs, although PKS genes had the highest scoring segment pairs. On the basis of these results, it is proposed that the 2.4 kb subcloned fragment encodes part of the *D. pini* PKS (pksDp) which synthesises the backbone polyketide and initiates dothistromin biosynthesis.

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ABBREVIATIONS

≈	approximately
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumen
CHEF	contour clamped homogenous electric field
CTAB	hexadecyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dp	decimal place
EtBr	ethidium bromide
EtOH	ethanol
fd	freeze dried
hr	hour (s)
IAA	iso-amyl alcohol
kb	kilobase pair(s)
KS	β–ketoacyl synthase domain
min	minute (s)
nt	nucleotides
OD	optical density
PCR	Polymerase Chain Reaction
PFGE	pulsed field gel electrophoresis
pfu	plaque forming units
PKS	polyketide synthase
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
sf	significant figure
SLS	sodium lauroyl sarkosine (Sigma) = Sarkosyl
TE	Tris/EDTA buffer
TEMED	N,N,N^{1},N^{1} - tetramethylethylenediamine

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1. INTRODUCTION

1.1 SETTING THE SCENE

Fungi are a diverse group of organisms which exist in either free living, commensal, symbiotic or parasitic relationships with other species. The key role of fungi in the environment is that of decomposition, i.e. the break down of dead or living organic matter. Fungi also produce a number of complex molecules including antibiotics, mycotoxins, and many pigments found in nature (Mayorga and Timberlake, 1992).

This research focuses on *Dothistroma pini*, a fungal pathogen which carries out part of its lifecycle in a parasitic relationship with *Pinus radiata* and other related pine species. *D. pini* belongs to the class Ascomytina, and the order Dothideales (Evans, 1984). Other Ascomycete fungi include the intensively studied *Aspergillus* which share a number of similarities with *D. pini* (in particular, the production of mycotoxin compounds) which provide a means for unlocking the mechanism of pathogenesis in *D. pini*.

1.2 *Dothistroma pini* : THE FUNGUS AND THE DISEASE

Dothistroma pini Hulbary is a filamentous pathogenic fungus which produces a red-coloured toxin known as dothistromin. The toxin is thought to induce needle blight in pine leading to a reduction in photosynthesis and wood yield, occasionally resulting in tree death. *D. pini* is the anamorphic (asexual) form of *Mycosphaerella pini* (also known as *Scirrhia pini*). The sexual form has not been found in New Zealand. *D. pini* is found in most countries, originating in Central America (Gadgil, 1984). It has recently been suggested that geographically diverse *D. pini* isolates in New Zealand originate from one isolate, as all appear genetically uniform (Hirst, 1996). This has implications for potentiatl control methods. One advantage of genetic uniformity of the pathogen population is that methods developed to control infection with a laboratory culture, should be equally effective with *D. pini* isolates nationwide. However, if an exotic strain was introduced the effect of treatment may differ.

Pinus radiata is the most predominant species in N.Z. forest plantations (Table 1-1). Young pine trees, between 2 and 15 years old, comprise 75% of existing pine plantations

(Inc., 1996). *D. pini* infects 35% of all trees, with young trees being more susceptible to invasion. In comparison with overseas plantations, New Zealand has very few significant disease or insect problems, but losses due to *Dothistroma* needle blight exceed $7/ha/year in the North Island (New, 1989). More recent figures state costs attributed to *D. pini* infection, in terms of fungicide spraying programmes, as $1.3 million per year; with respect to wood yield loss, in excess of $7.2 million per year (Bulman, 1996).

	Hectares (000)	% of total
Radiata pine	1338	90.5
Douglas fir	66	4.5
Other exotic softwoods	33	2.2
All exotic hardwoods	41	2.8
Total	1478	100

Table 1-1. Planted Production Forest Area By Species in N.Z.
(Inc., 1996)

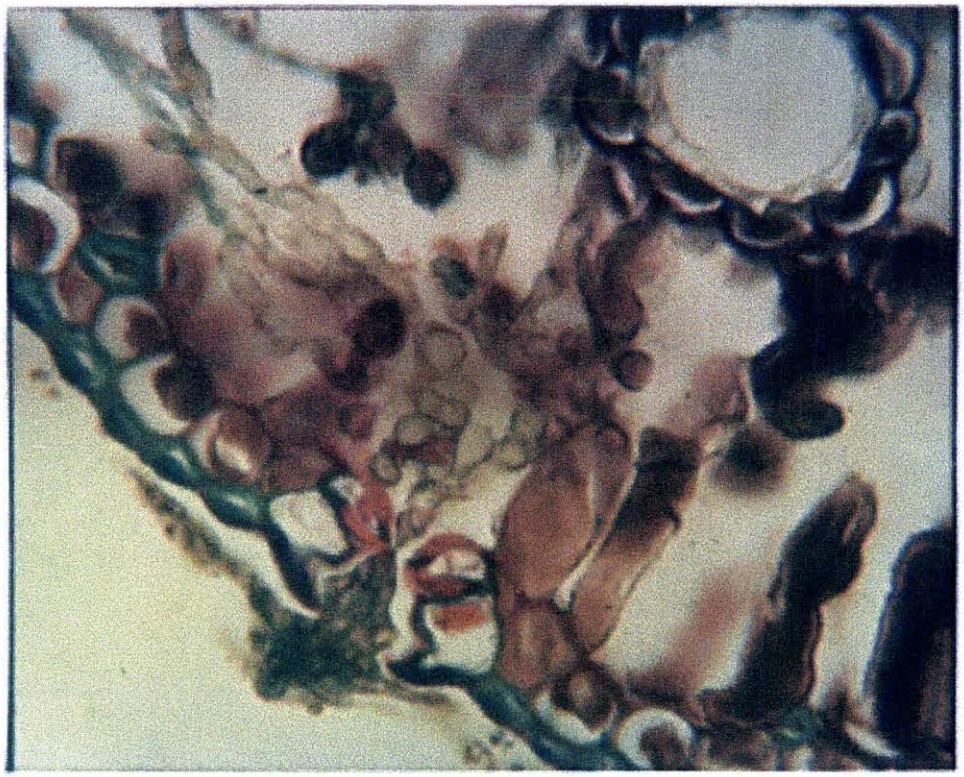
Infection begins in the lower branches of a tree and spreads to the crown. Infection spread is favoured under moist, warm, light and sheltered conditions. Some pines become more resistant to infection by *D. pini* as they mature, while others retain the same susceptibility independent of age. At the age of 8-9 years *P. radiata* are pruned of their lower branches, eliminating the microclimate favouring *D. pini* infection (Gadgil, 1984).

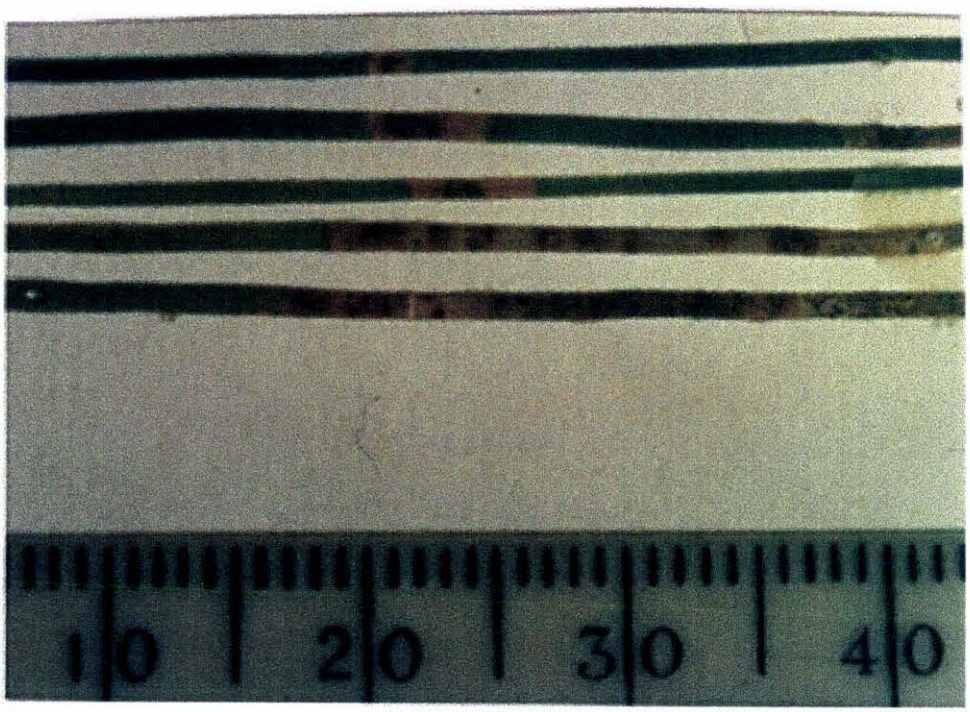
Conidia (asexual spores) produced by *D. pini* adhere to the surface of the pine needles, this process being more effective when needles are wet. Invasion proceeds through development of conidial germ tubes which penetrate the stoma. Conidia frequently produce more than one germ tube, with the initial invasive process taking up to three days. Infection then proceeds through both inter and intracellular hyphal growth, the fungus living saprophytically on pine tissue (Figure 1-1) (Gadgil, 1967). Mycelial growth appears to be confined to mesophyll tissue which contains chloroplasts. Infection is initially detected visually through the formation of yellow areas (formed through chlorosis) which develop into characteristic red banding through necrotic lesions (Figure 1-2). Stromata (irregularly shaped, small, black fruiting bodies produced by the asexual stage) form in the lesion and, following rainfall, release spores into water on the needle surface (Gadgil, 1984).

Figure 1-1. Hyphal growth into pine needle tissue*

Figure 1-2. Pine needles infected with *D. pini* *

* Reproduced with kind permission of P.D. Gadgil, Forestry Research Institute, Rotorua, N.Z.

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1.2.1 THE TOXIN DOTHISTROMIN

1.2.1.1 Possible role in disease

Dothistromin is thought to be the primary cause of pine needle blight symptoms. Histological studies have demonstrated that host tissue is killed in advance of hyphal penetration, indicating diffusion of a toxic substance from hyphae to uninfected tissue (Gadgil, 1967). Although these findings suggest dothistromin to be a key player in pathogenesis, the exact role of the mycotoxin is unknown. The disease symptoms may be a direct result of dothistromin toxicity, or an indirect result of the plants defence response to pathogen attack. This may include rapid localised cell death (hypersensitive response), increased cell wall lignification and/or phytoalexin production e.g. benzoic acid. All of these symptoms occur on infection by *D. pini*, with dothistromin induced lesions usually being terminated by narrow areas of dark green highly lignified tissue (Franich *et al.*, 1986).

D. pini needle blight symptoms have been induced artificially with purified dothistromin, suggesting the toxin plays an important role in pathogenesis (Shain andFranich, 1981). However, a review by Van Etten *et al.* (1994) reported pathogenicity to be unaffected in two out of five cases where toxin production was disrupted, in separate fungal species. Similar findings were made with *Ophiostoma novo-ulmi*. This fungus synthesises the toxin cerato-ulmin (CU), and is the causative agent of Dutch elm disease. Purified toxin is capable of producing similar disease symptoms; but there appears to be no association between cerato-ulmin production and virulence in *O. ulmi* mutants, with CU^- mutants retaining ability to produce symptoms of Dutch elm disease (Bowden *et al.*, 1996). A key question is whether the dothistromin or the fungal mycelium elicits the defence response.

Purified fungal cell wall extracts are reported to initiate a defence response (Dr. Grant Hotter, *pers. comm.*). This suggests dothistromin functions as a pathogenicity factor; allowing the fungus to overcome host physical and chemical barriers, rather than an avirulence factor which acts as a specific elicitor of plant defence responses. As additional mechanisms for pathogenicity may exist, it is possible that targeting dothistromin to combat the disease will not solve the problem, i.e. *D. pini* may still be pathogenic without the toxin. It is also possible that dothistromin minus mutants will be more pathogenic if the tree does not recognise it has been infected, and mycelial invasion does not initiate all the defence responses required to contain infection.

To determine if blight symptoms are observed in the absence of dothistromin requires infection by a non dothistromin producing isolate. One approach is to isolate and characterise fungal toxin genes thought to confer pathogenicity to the organism which can then be disrupted by transformation-mediated methods.

1.2.1.2 Properties of dothistromin

Dothistromin is a difuroanthroquinone. The red pigment dothistromin is a mixture of two epimers, $C_{18}H_{12}O_9$ (dothistromin 80-90%) and $C_{18}H_{12}O_8$ (deoxy-dothistromin). Dothistromin possesses a furobenzofuran moiety which is a common feature of many compounds which are potent carcinogens. These compounds include the aflatoxins, sterigmatocystin and versicolorins (Gallagher and Hodges, 1972).

Natural and artificially induced dothistromin lesions are favoured by high light intensity. Shain *et al.* (1981) attributed necrosis to an interaction between dothistromin and photosynthetically active tissue. This is possibly due to interference of the quinone moiety of dothistromin with electron transport in photosynthetic tissue (Shain and Franich, 1981). Furthermore, there is tentative evidence for a dothistromin binding protein in chloroplasts (Paul Reynolds, *pers. comm.*). The toxin breaks down the photosynthetic pigments causing reductive oxygen activation, leading to super oxide and hydrogen peroxide formation (H_2O_2)(Stoessl *et al.*, 1990). Super oxide and H_2O_2 may also act as phytoalexin elicitors, leading to the formation of benzoic acid in regions adjacent to necrotic lesions. Benzoic acid inhibits growth of *D. pini*, possibly through restricting hyphal extension within the pine needle. However, high concentrations of benzoic acid also have a damaging effect on needle tissue (Gadgil, 1967).

The antimicrobial and membrane disruptive activity of dothistromin is due to photo-oxidative action (Stoessl *et al.*, 1990). Dothistromin also exhibits antimicrobial activity through inhibition of RNA synthesis, and can cause chromosome damage in human blood lymphocyte cultures. If the toxin concentration is high enough, red blood cell lysis may ensue; although dothistromin is less potent than its relative, aflatoxin B1. (Ferguson *et al.*, 1986) (Stoessl *et al.*, 1990).

1.3 CONTROL OF INFECTION

Large scale aerial spraying of pine forests with fungicide is the only current control programme to combat infection by *D. pini*. Aerial spraying programmes were first used in 1967 in an attempt to control *D. pini* needle blight. Copper based compounds (e.g. copper-oxy chloride and cuprous oxide) have proven to be effective in inhibiting

germination of *D. pini* conidia, the only drawback being cost. Plantations of less than 15 years of age are aerially assessed for foliage damage every 2-3 years and treated when necessary (i.e. when the mean stand infection is 25% or greater) (Dick, 1989).

Reproducible and reliable screening techniques are required to enable the amount or rate of development of a disease to be assessed. Disease impact is best assessed through percentage of pine needles infected, which is directly proportional to loss in wood yield (Carson and Carson, 1989). Breeding for *D. pini* resistance has been reasonably effective, and selective breeding programmes have been carried out at the Forestry Research Institute (FRI), Rotorua, N.Z. for the past thirty years. The basis for increased resistance to *D. pini* needle blight is unknown but is thought to involve several different mechanisms. Selection of *P. radiata* families most resistant to *D. pini* are predicted to reduce crown infection by 16% in diseased stands (Carson and Carson, 1989). It is estimated if these strains had been planted in the Kinleith forest, that spraying costs would have been reduced by 56% (Carson and Carson, 1991). Due to the long life spans involved in pine breeding generations, it is extremely important to take great care in the choice and use of selection traits. As the pathogen has a much shorter life cycle than its host, it is expected that strains will evolve which will be capable of overcoming resistance mechanisms. With breeding and planning strategies, there is not much room for natural genetic/evolutionary advantages to evolve. Therefore, it is of utmost importance to maintain genetic variability among separate batches of seedlings.

1.4 SECONDARY METABOLITE PRODUCTION

1.4.1 POLYKETIDES

Polyketides are a large and diverse class of compounds and include antibiotics, pigments, and immunosupressants. They are among the most abundant secondary metabolites produced by fungi, and are also produced by organisms as diverse as plants, insects, bacteria and marine organisms. The polyketide biosynthetic pathway resembles that of fatty acid synthesis; however, total reduction of the keto groups is rare, giving rise to the name polyketides (Hopwood and Sherman, 1990). Most polyketides contain structural complexities that can be accounted for by the use of different extender units at various steps, and by variations in the extent of processing of the β-carbon (β-ketoreduction, dehydration, enoyl reduction). This leads to a vast array of end products.

The toxin dothistromin is a polyketide derived secondary metabolite, produced by some *Cercospora* sp., *Mycosphaerella laricini* and *D. pini* (Stoessl *et al.*, 1990). Dothistromin bears structural similarity to other polyketide-derived, toxic secondary metabolites, e.g. the aflatoxins, which are produced by some *Aspergillus* sp. (Figure 1-3). Most polyketides are a result of secondary metabolic processes. Secondary metabolism occurs predominantly in idiophase, upon deletion of nutrients required for primary metabolism. It is thought to provide a pathway for the removal of intermediates which would otherwise accumulate and lead to inhibition of primary processes during times of stress (Trail *et al.*, 1994)(Turner, 1971). Secondary metabolites are derived from precursors which are intermediates of primary metabolic pathways. The most important of these intermediates is acetyl CoA, a pivotal intermediate in three major pathways of primary metabolism. Acetyl CoA is derived from either carbohydrate metabolism via the glycolytic pathway, via β–oxidation of long chain fatty acids or catabolism of several amino acids (Shaw *et al.*, 1978). Condensation of acetyl CoA and its derivatives, e.g. malonyl CoA (formed by carboxylation of acetyl CoA) results in the formation of polyketide precursors for secondary metabolite production. Alternatively a fatty acid starter unit may be the starting molecule for polyketide synthesis, with extension by a polyketide synthase to form noranthrone, which is oxidised to norsolorinic acid. Polyketide synthases are generally one of two types: type II PKSs consist of several monofunctional proteins, whereas type I PKSs are large multifunctional enzymes (encoded by 5-10 kb of nucleotide sequence), found in fungi and eukaryotes (Yu and Leonard, 1995). For example, the type I PKS of *Penicillium patulum* catalyses the initial seven reactions in the biosynthesis of the mycotoxin patulin (Beck *et al.*, 1990). Many of the characterised PKSs contain conserved functional/ catalytic domains which are also found in fatty acid synthases (FAS)(Table 1-2), the most highly conserved being the β-keto acyl carrier protein synthase (KS) domain (Feng and Leonard, 1995). However, fungal PKS genes have a amino acid homology with vertebrate FAS genes (49%) than with fungal FAS genes (15%).

FAS are also classified as type I or type II FAS, with type II containing separate associating polypeptides, and type I with multifunctional domains for each enzymatic step. The β-ketoacyl-ACP synthase domain, found in both FASs and PKSs has been well characterised, and the average amino acid identity between fungal and bacterial FAS and PKS genes isolated to date is 49%. KR and ACP domains of FAS and PKS genes

Figure 1-3 Comparison of Aflatoxin and Dothistromin biosynthesis

Names of precursor intermediates involved in aflatoxin B1 and dothistromin synthesis are depicted in green and corresponding enzymes in brown.

Outline of Aflatoxin B1 Biosynthesis in *Aspergillus sp.*

Potential Dothistromin Intermediates found in *Dothistroma* and *Cercospora* sp.

(PKS)

polyketide — polyketide

↓

norsolorinic acid

nor-1 ↓

averantin — averantin

↓

5' hydroxyaverantin

↓

averufin — averufin

↓

versiconal acetate

↓

versicolorin B — versicolorin B

↓

versicolorin A

ver-1 ↓

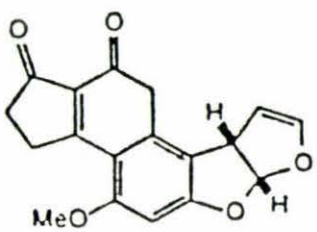
sterigmatocystin

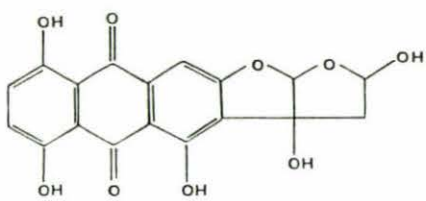
↓

O-methylsterigmatocystin

↓

aflatoxin B1 — **dothistromin**





from the same organisms exhibit 30% and 24% identity respectively. The function of the KS domain is to catalyse the condensation of malonyl-ACP with the growing fatty acid chain. Although all PKS enzymes appear to have an ACP domain (but not necessarily a KR domain), the amino acid homology of ACPs is suprisingly low.

Table 1-2. Comparison of functional domains of type I FASs and PKSs

(√ denotes presence of domain, - denotes uncertainty)

Functional domains	Fatty acid synthase (type I)	Polyketide synthase (type I)
Acetyl/malonyl transferase (AT/MT)	√	√
Acyl carrier protein (ACP)	√	√
Enoyl reductase (ER)	√	some
Dehydratase (DH)	√	some
Thioesterase	√	some
β-keto-ACP synthase (KS)	√	√
β-keto-ACP reductase (KR)	√	some

Table 1-3. Homology of fungal PKS comparisons

	A. parasiticus pksA
A. nidulans pksST	
% identity	64%
% similarity	77%
A. nidulans wA	
% similarity	61%

Polyketide formation precedes aflatoxin formation, so isolation of PKS genes is a rational starting point for dissection of polyketide based pathways. Comparisons of the *pksA* gene (also called *pksL1*) from *A. parasiticus*, the *pksST* gene (from the sterigmatocystin gene cluster in *A. nidulans*) and the *wA* gene of *A. nidulans* (which is thought to be a PKS for conidial pigmentation) show the overall conserved nature of PKS (Feng and Leonard, 1995)(Table 1-3). As the KS domain is the most highly conserved, identification of corresponding PKS genes in additional fungal species should be most successful through use of a KS domain as a heterologous probe. Successful identification and isolation of a putative PKS from *A. terreus* has been achieved using a heterologous gene fragment containing the KS domain from the 6-methylsalicylic acid synthase (MSAS) of *P. patulum* as a probe (Fujii *et al.*, 1996).

1.4.2 AFLATOXIN AND DOTHISTROMIN BIOSYNTHESIS

Aflatoxins are mycotoxins produced by *Aspergillus parasiticus and A. flavus*, which infect common foods e.g. peanuts, corn and cottonseed. Aflatoxins are among the most toxic, mutagenic and carcinogenic natural compounds known to man. Contamination of agriculturally important crops is both a health and economic problem, hence understanding the molecular biology of aflatoxin biosynthesis has been a major focus of many laboratories. This has led to production of several genetically modified fungal species disrupted at various stages in the aflatoxin biosynthetic pathway.

The aflatoxin biosynthetic pathway is very complex, consisting of at least 40 conversions, the main ones of which are shown in Figure 1-4, along with key enzymes that have been identified. Up to seventeen different enzymes are proposed to have a role in aflatoxin biosynthesis (Mahanti *et al.*, 1996). The origins of parts of the carbon skeleton in dothistromin production are the same as in aflatoxin production, and some known aflatoxin intermediates are found in both *D. pini* and *Aspergillus* sp. (Figure 1-3) (Shaw, 1975). These include averantin, averufin and versicolorin B. Enzymes which catalyse these conversions may also be found in *D. pini*. Genes for synthesis of microbial secondary metabolites have long been known to be clustered. More recently, genes involved in fungal toxin biosynthetic pathways have been shown to be physically linked on a single large piece of chromosome. Isolation of *nor-1* and *ver-1* aflatoxin genes from a single cosmid provided initial evidence of clustering; this was confirmed by karyotyping studies involving additional putative toxin biosynthetic genes (Skory *et al.*, 1992) (Yu *et al.*, 1995). Restriction endonuclease and transcript mapping has been used to determine

Figure 1-4 Aflatoxin pathway gene cluster*

This diagram represents the generally accepted aflatoxin B_1 and B_2 biosynthetic pathway in *A. parasiticus* and *A. flavus*. The schematic representation only shows identified enzymes for some specific conversion steps, others may exist. The regulatory gene, *afl*R, codes for the pathway regulator factor, which controls expression of the structural genes at the transcriptional level. The genes for *pksA* and *nor-1* enzymes are located at the start of the cluster, which is the region targeted for isolation from *D. pini*.

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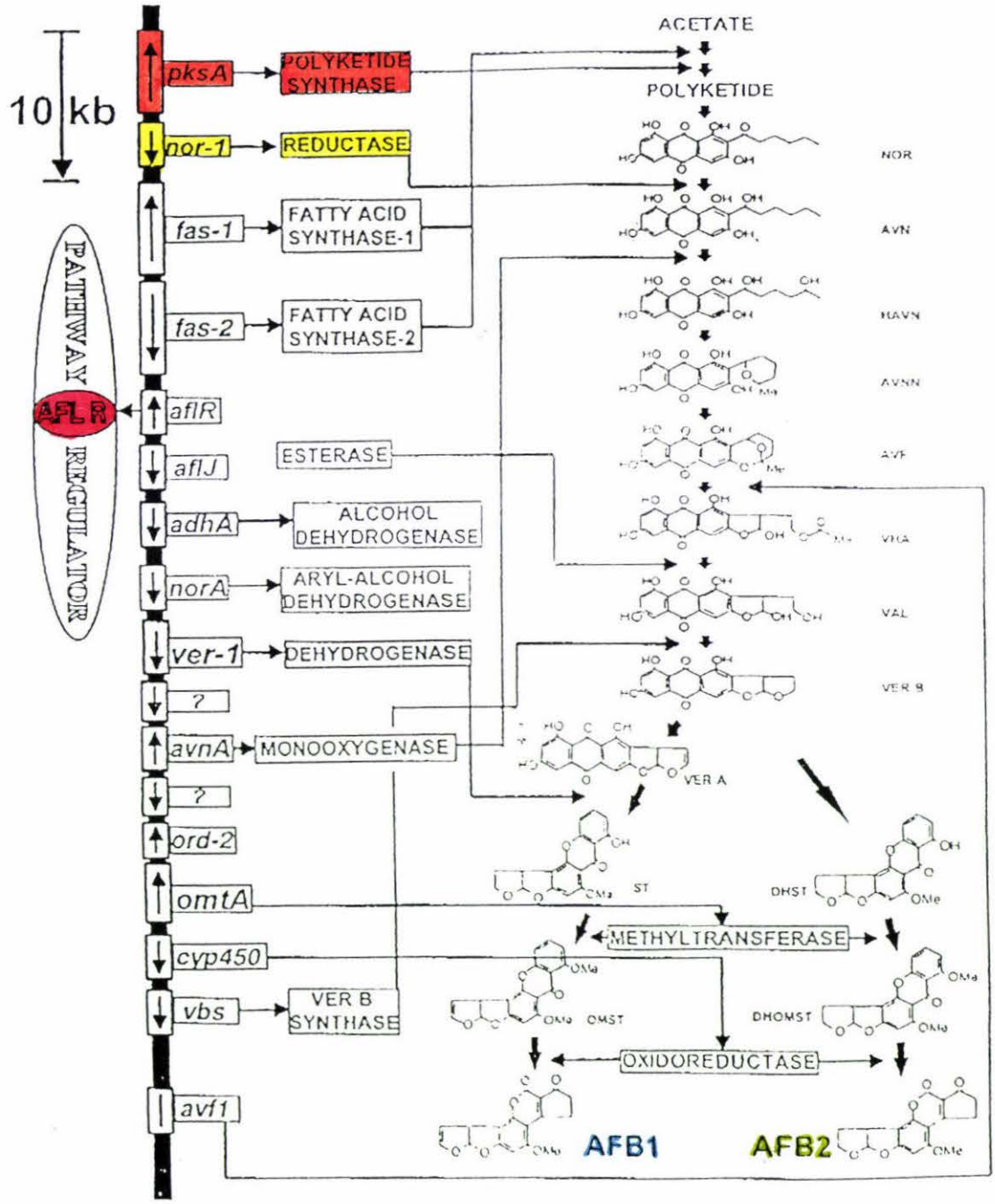
10 kb
PATHWAY REGULATOR
AFLR
pksA
POLYKETIDE SYNTHASE
nor-1
REDUCTASE
fas-1
FATTY ACID SYNTHASE-1
fas-2
FATTY ACID SYNTHASE-2
aflR
aflJ
ESTERASE
adhA
ALCOHOL DEHYDROGENASE
norA
ARYL-ALCOHOL DEHYDROGENASE
ver-1
DEHYDROGENASE
?
avnA
MONOOXYGENASE
?
ord-2
omtA
cyp450
vbs
VER B SYNTHASE
avf1
ACETATE
POLYKETIDE
NOR
AVN
HAVN
AVNN
AVF
VHA
VAL
VER B
VER A
ST
DHST
METHYLTRANSFERASE
OMST
DHOMST
OXIDOREDUCTASE
AFB1
AFB2

the physical distance between aflatoxin genes (Skory *et al.*, 1992) (Trail *et al.*, 1995). In *A. parasiticus*, the *pksA* and *nor-1* genes are contained within a 10 kb region (Figure 1-4). These two genes are divergently transcribed from a 1.5 kb intergenic region (Chang *et al.*, 1995). All genes involved in aflatoxin biosynthesis in *Aspergillus* sp. appear to be contained within a 60-75 kb fragment, with some of the genes being duplicated in *A. parasiticus* (e.g. *ver-1A* and *ver-1B*) (Cary *et al.*, 1996)(Brown *et al.*, 1996). Comparisons between the sterigmatocystin pathway in *A. nidulans* and the aflatoxin pathway in *A. parasiticus* and *A. flavus*, also indicate conservation at the functional and regulatory level. In each cluster, in all species examined to date, there is a positively acting regulatory gene, *aflR*, which encodes a sequence specific DNA binding protein required for cluster gene expression (Feng and Leonard, 1995). The ability of *aflR* to activate expression is thought to be linked to regulation of asexual sporulation. This is thought to occur through a requirement for inactivation of a heterotrimeric G protein mediated signal transduction pathway (Keller and Adams, 1997). Expression of cluster genes is also influenced by three other main factors; medium components, growth phase-related physical conditions, and culture temperature. Aflatoxin gene clustering does not appear to confer a selective advantage to the host organism. Aflatoxin production does not deter growth of competing organisms or increase the producer organisms' invasive ability. However, clustering does allow co-ordinate gene regulation and expression, and rapid onset of aflatoxin production is seen after 18-20 hour of mycelial growth (Yu *et al.*, 1995).

1.5 GENETIC APPROACH TO COMBAT *D. pini* INFECTION

Determining how the plant responds to infection is one avenue of research to find a commercially viable control method. Peroxide catalysed oxidation of dothistromin by hydrogen peroxide is the predicted mechanism of phytolytic toxin degradation to produce CO_2 and oxalic acid (Franich *et al.*, 1986). Antibodies against the toxin are being developed with the aim of producing transgenic pine seedlings capable of antibody production. This will render the dothistromin toxin ineffectual on infection by *D. pini* (P. Reynolds, *pers. comm.*). This approach assumes dothistromin is the primary causal agent of Dothistroma needle blight disease.

One long term aim of this project is to disrupt the dothistromin biosynthetic pathway, leading to the production of non-dothistromin producing *D. pini* isolates, so that pathogenicity of the transformants can be assessed and the mode of action of dothistromin elucidated. The task of creating a dothistromin-minus mutant involves isolation and characterisation of *D. pini* dothistromin biosynthetic genes. In addition, to enable targeted

gene disruption a transformation system for *D. pini* is required. Hygromycin resistance genes were used as selectable markers in the development of this system (Bidlake, 1996). Gene targeting in filamentous fungi is a difficult task, and considerable work in optimising this process has recently been completed for the model fungus *A. nidulans* (Bird, 1997). It is hoped these results can be projected to the *D. pini* system. Obtaining a stable dothistromin-minus mutant may lead to another alternative for fungicide treatment in the control of *D. pini* needle blight, through use of mutant isolates as biological competitors. Isolation of toxin genes for use in targeted disruption studies is also interesting from a gene regulation and gene conservation perspective, perhaps providing insight into the phylogenetic relationship between *D. pini* and other ascomycetes e.g. *Aspergillus* sp. It is possible that aflatoxin and sterigmatocystin genes have evolved from fungal pigment biosynthetic pathway genes. This hypothesis is supported by the finding that *ver-1* and *ver-A* gene products are related to the *thnR* and *thr1* gene products in the melanin producing fungus *Magnaporthe grisea* (Chang *et al.*, 1995). These gene products all have functional motifs characteristic of ketoreductases.

Toxin deficient fungal isolates have traditionally been isolated through screening of mutants produced by conventional mutagenesis (e.g. UV induced); or by examining large numbers of natural isolates in a hit or miss approach. Genes can then be cloned by complementation of toxin blocked mutants (e.g. *nor-1* and *ver-1* genes) (Skory *et al.*, 1992). However, it is not easy to identify loss of function mutants in *D. pini* due to the multinucleate nature of the *D. pini* conidia. In addition, filamentous fungi are reknowned for their morphological and metabolic variability, especially when the strains have been maintained in laboratory culture for extended periods (Bennett, 1981). Toxin production may cease as a result of this. If toxin production ability is lost through a random mutagenesis event, the difficulty is then determining which gene has been affected. It may be that toxin production is lowered below a detectable level, or that alternative pathways exist which are then induced when the organism returns to the natural environment. Trail *et al.* (1994) found disruption at the *nor-1* site lowered, but did not inhibit aflatoxin production in *Aspergillus* sp. This supports the hypothesis that at least one alternative pathway exists for the conversion of norsolorinic acid (NA) to averantin (AVN) (Figure 1-4). Another possibility is that there are two or more enzymes present with similar activity, only one of which has been disrupted. Therefore, it would be preferable to achieve disruption at the earlier stages of dothistromin biosynthesis, i.e. at the polyketide synthesis stage (Trail *et al.*, 1994).

1.6 AIMS AND OBJECTIVES

The availability of cloned aflatoxin genes from *Aspergillus parasiticus*, and other fungal species which contain genes for polyketide production, provided an opportunity to locate related genes in the dothistromin biosynthetic pathway. These aflatoxin related genes were used as probes to aid isolation of heterologous toxin genes from *D. pini*. Two of the probes were gene sequences from *Aspergillus parasiticus*, named *nor-1* and KS-2 (a gene segment encoding the highly conserved β-ketoacyl-ACP synthase domain from *pksA*, a polyketide synthase gene. KS-2 is the abbreviation given to the probe in this study). Each of these enzyme encoding genes acts at a different step in the production pathway of aflatoxin B1(Chang *et al.*, 1995)(Chang *et al.*, 1992). Another probe originated from the MSAS gene, found in *Penicillium patulum*, which is also a polyketide synthase (KS-1) (Wang *et al.*, 1991). Both PKS probes (KS-1 and KS-2) contained the highly conserved β-ketoacyl-ACP synthase (KS) domain .

Previous work in our laboratory involving the screening of a Lambda GEM-12 *D. pini* genomic library has led to the isolation of several clones, hybridising to *nor-1* and *ver-1*. Characterisation of a clone which hybridised to the *ver-1* probe led to a partial nucleotide sequence, which revealed the presence of an open reading frame (ORF) with predicted amino acid similarity to that of *ver-1* (Gillman, 1996). Inconsistencies were noted with the isolated λ GEM 12 *D. pini* clone hybridising to *nor-1*. Therefore, a goal of this research was to construct a new genomic library in the hope of isolating clones hybridising to the KS probes, as well as renewing attempts to isolate another *nor-1* hybridising clone. Chromosome walking should then enable elucidation of additional biosynthetic genes, leading to characterisation of the proposed gene cluster. In addition, PCR primers based on the conserved PKS regions of *PksA* in *A. parasiticus* were constructed for a PCR based approach. to cloning dothistromin biosynthetic genes.

Production of specific dothistromin-minus mutants will allow us to confirm the essential role of dothistromin in the disease process, and the isolation of toxin genes takes us one step closer to this aim.