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**IDENTIFICATION OF DOTHISTROMIN  
BIOSYNTHETIC PATHWAY GENES**

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## ABSTRACT

Dothistromin is a polyketide-derived toxic secondary metabolite produced by the filamentous fungus *Dothistroma pini* which causes the disease *Dothistroma* needle blight in *Pinus radiata*. Dothistromin is considered to be an important component in the disease process, although its exact function is yet to be identified. By isolating and identifying genes involved in dothistromin biosynthesis, and subsequently obtaining mutants blocked or altered in the synthesis of dothistromin, the role of this toxin in pathogenicity will be able to be assessed. Dothistromin is structurally related to the mycotoxins, aflatoxin (AF) from *Aspergillus parasiticus* and *A. flavus*, and sterigmatocystin (ST) from *A. nidulans*. Three intermediates in the ST and AF biosynthetic pathways (averantin, averufin, and versicolorin B) are thought to also be intermediates dothistromin biosynthesis. Due to these similarities, cloned AF pathway genes were used as heterologous probes in Southern hybridisation analysis to provide a direct method for identifying dothistromin biosynthetic genes.

A fragment of the *A. parasiticus nor-1* gene, encoding a reductase involved in the conversion of norsolorinic acid (NA) to averantin (AVN) in the AF biosynthetic pathway, was used as a probe to detect a region of sequence similarity to *D. pini* genomic DNA. A *D. pini* genomic library was then constructed and screened, resulting in clone  $\lambda$ CGN2. However, Southern hybridisation analysis suggested that this clone did not contain a homologue of the *nor-1* gene from *A. parasiticus*.

A fragment of the *Aspergillus parasiticus ver-1* gene, encoding a reductase involved in the conversion of versicolorin A (VA) to ST in the AF biosynthetic pathway, was also used as a probe to detect a region of sequence similarity to *D. pini* genomic DNA. The *D. pini* genomic library was then screened. Two clones,  $\lambda$ CGV1 and  $\lambda$ CGV2, were isolated and Southern hybridisation analysis confirmed that these clones contained sequences hybridising to the *A. parasiticus ver-1* gene fragment. Fragments of these clones which hybridised were then sequenced and compared to the GenBank database. The amino acid coding sequence of a 0.8 kb *SalI* region from clone  $\lambda$ CGV1 exhibited a high degree of similarity with the *A. nidulans verA* and *A. parasiticus ver-1* genes, involved in the ST and AF biosynthetic pathways, and the *Magnaporthe grisea ThnR*, and *Colletotrichum lagenarium Thr1* genes, involved in melanin biosynthesis. This data suggested a *ver-1* homologue is present in the *D. pini* genome. Limited sequence analysis of a 2.1 kb region from clone  $\lambda$ CGV2 suggested that a second independent copy of a *ver-1*-like gene may also be present in the genome.

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## 1.0 INTRODUCTION

### 1.1 General Features

The filamentous fungus *Dothistroma pini* is a major pathogen of *Pinus radiata* and other pine species. It causes *Dothistroma* needle blight, a necrosis associated with red lesions or bands on the needles, often followed by premature needle cast (initially at the base of the crown) and consequent reduction of photosynthesis and wood yield. Sometimes this is followed by tree death (Gallagher 1971, Franich *et al.* 1982, Gadgil 1984).

*D. pini* is of the order Dothideales in the Ascomycotina class. It is the anamorphic (asexual) form of *Mycosphaerella pini*. Differences in pathogenicity between the two forms have not been reported in any of the wide number of pine species that they infect (Evans 1984). *D. pini* is a necrotrophic pathogen which is believed to kill plant tissue and then live saprophytically (Peterson and Walla 1978).

*D. pini* has caused serious defoliation in plantations of *P. radiata*, which is by far the most important susceptible tree species from an economic viewpoint (Phillips and Burdekin 1982). The disease is widely distributed throughout the world, having been recorded in Europe, South and East Africa, North and South America, India, Russia, and Australasia. *D. pini* was first identified in New Zealand in the central North Island in 1964. It is now found in all of the North Island except the Northern tip and Great Barrier Island. In the South Island it is found in Nelson, Marlborough, North of the Wairou river, Westland, Southland, and Otago (Gadgil, 1984). It is of commercial concern in New Zealand in that it affects the health and vigour of extensively grown exotic pine species *P. radiata*, *P. ponderosa* and *P. nigra*, which are unfortunately all highly susceptible to the disease (Elliot *et al.* 1989). Because of the important role that forestry plays in the New Zealand economy, the disease is of economic significance (Gallagher 1971).

Infection appears as chlorosis and necrosis of needles on the main stem and base of lower branches. Under favourable conditions, and on a susceptible host, defoliation may be so severe that only the needles at the extremities of the branches remain. Needle infections are first evident as yellow flecks which extend to become bands around the needle. As necrosis develops, these bands take on a characteristic red tinge, this has led to the commonly applied name 'red band' (Phillips and Burdekin 1982). These band-like lesions bear 1-12 black stromata (asexual fruiting bodies). The stromata vary in size

from 300-750 x 150-400  $\mu\text{m}$ . Conidia from these fruiting bodies are then released into a film of water on the needle surface following rainfall (Gadgil 1984). Rain splash dispersal of conidia usually occurs within a single tree, although wider distribution may occur when mist clouds are present. The perfect stage of *D. pini* has been identified as *Mycosphaerella pini* on a range of pine hosts including *P. radiata* on Vancouver Island (Phillips and Burdekin 1982). Sexual wind dispersed spores are also formed in stromata, but these have never been observed in New Zealand (P. Gadgil, pers. comm.) and are never seen in culture (D. Morrison, pers. comm.). Conidia are multicellular, and it is unknown whether each cell is derived from the same origin. Multiple germ tubes, up to one per compartment, produced from these conidia, penetrate the needles through stomata. This is followed by both inter- and intra-cellular hyphal growth, a process taking three days or longer, depending on temperature and humidity. Within the needle, disruption of the mesophyll tissue occurs well in advance of the developing hyphae as a result of the pathogen toxin, dothistromin. Macroscopic symptoms do not appear on the needles for 5-10 weeks. This relatively slow rate of growth is also a feature of the fungus in culture (Gadgil 1967, Phillips and Burdekin 1982).

## 1.2 Infection

Though *P. radiata* is susceptible to the pathogen when young, in general mature trees older than 15-20 years show little infection suggesting increased resistance. Several factors have been proposed to be involved in this resistance. The stomata on mature-tree needles have a smaller pore (10-15  $\mu\text{m}$ ) than the young-tree needles (15-20  $\mu\text{m}$ ). Stomata of young susceptible trees are open pores, the guard cells and subsidiary cells of which have an epidermis covered with fine rodlet or microtubular wax. Contact of hyphae with the guard cell wax apparently causes them to expand to form an appressorium, a specific infection structure, prior to a hyphal peg penetrating between the guard cells. However, the majority of stomata on needles from mature *Dothistroma* needle blight-resistant trees were shown to be occluded with resinous material which frequently closes the pore between the overarched stomatal lips. Such material could present a mechanical barrier to ingress of hyphae, or possibly mask chemotropic or chemotactic stimuli experienced by the hyphae during stages of stomatal penetration (Franich *et al.* 1977, Franich *et al.* 1983).

The chemical nature of the occluding material may also be important. Detailed analysis of needle epicuticular wax from young and old *P. radiata* trees has shown quantitative differences in the acidic fraction of the wax, from young trees it consists mainly of dehydroabietic acid, whereas from mature trees it comprises mainly a complex mixture

of oxygenated resin acid derivatives (Franich *et al.* 1978). An *in vitro* test on *D. pini* showed these oxygenated resin acid derivatives inhibited both conidia germination and mycelium growth (Franich *et al.* 1982). An *in vivo* test of artificial inoculation showed plants treated with acetone (which depletes epicuticular and stomatal pore fatty and resin acids) had a mean infection level about twice that of the control (Shain and Franich 1981). These experiments suggest that the presence of oxidised resin acids at the needle surface around, or occluding stomata could therefore be a preinfection chemical fungistasis factor partly responsible for the observed *D. pini* resistance of mature *P. radiata* trees. Once stomatal penetration and hyphal colonisation of mesophyll has occurred, the extent of tissue damage and the rate of fruiting body formation appears dependent on other resistance factors, among these being sensitivity of needle tissue to the dothistromin (Franich *et al.* 1983).

Attempts have been made to correlate differences in monoterpene profiles (Franich *et al.* 1982) and pH buffering capacities of foliage extracts (Franich and Wells 1977) from young and mature trees with differences in susceptibility to blight. A mixture of volatile compounds (consisting mainly of monoterpene hydrocarbons) from *P. radiata* foliage populations of young *Dothistroma* needle blight-susceptible and mature-resistant trees was shown to stimulate germination of *D. pini* spores and mycelial growth at specific concentrations. *D. pini* was found to be a pathogenic fungus well-adapted to growing in a monoterpene flux at the surface of, and within the pine needle. Differences in needle monoterpene hydrocarbon composition did not bear any simple relationship to mature-tree resistance. Nor did the high buffer capacity of mature-tree needle homogenates appear to be directly related to *Dothistroma* needle blight resistance.

Rainfall and other environmental factors have been observed to influence the disease intensity. Experiments on leaf wetness period and infection by *D. pini* suggest that if hydrated conidia are deposited on the surface of a susceptible plant host, germination and penetration will occur regardless of the length of wetness period that follows deposition, provided that suitable temperatures prevail. However, the severity of infection depends on the length of the dry period which follows deposition of conidia - the longer the dry period, the lower the severity (Gadgil 1977). Germination of conidia does not vary greatly with temperature but stomata appear sooner with higher temperatures. Warm temperatures (optimally 16-18°C) combined with prolonged periods of high humidity (> 96%) or the existence of free water films on needle surfaces have been observed to favour serious disease outbreaks (Sheridan and Yen 1970, Gadgil 1974). Infection of *P. radiata* requires 100 conidia/mm<sup>2</sup>, with even mature trees susceptible to infection if the inoculum is high enough. In addition, soil deficiencies (in boron or sulphur) have been associated with increased susceptibility to *D. pini* (Ades and Simpson 1989).

### 1.3 Chemical Control

It has been predicted that forestry will provide 25% of New Zealand's exports by the year 2010, *P. radiata* currently comprises 93% of our 1,330,000 hectare commercial forest plantations (L. Bulman, pers. comm.). Although *P. radiata* in New Zealand has few diseases, *Dothistroma* needle blight (the most significant disease) has been estimated to cost between \$6.1 million (New 1989) and \$20 million per year (P. Carter, pers. comm.). From this cost, 10% is due to chemical control, and 90% is due to wood loss. In the absence of management of *Dothistroma* needle blight, wood volume increment loss is directly proportional to the disease severity, so control of the disease is important.

Control of *Dothistroma* needle blight in New Zealand is based upon the aerial application of copper fungicides to *P. radiata* plantations during the susceptible ages of 2-15 years. Stands are sprayed when stand infection levels are estimated at 25% or greater. This has been successfully carried out for more than two decades. Spraying every three to four years reduces the mean disease severity thus keeping the disease in check (Shain and Franich 1981, Dick 1989).

Copper fungicide (as 50% cuprous oxide,  $\text{Cu}_2\text{O}$ ) can react with aqueous-exudates on *P. radiata* needles, and to a lesser extent, with *D. pini* metabolites, to form free or complexed  $\text{Cu}^{2+}$  in aqueous solution at concentrations sufficient to inhibit the germination of *D. pini* conidia. The interaction of  $\text{Cu}_2\text{O}$  and geothermal  $\text{H}_2\text{S}$  to produce  $\text{CuS}$  and subsequently  $\text{CuSO}_4$  can contribute to solubilising the fungicide. Low  $\text{Cu}^{2+}$  concentrations effectively reduce germ-tube growth and inhibit the production of secondary conidia as well as stimulating dothistromin biosynthesis. It is the combination of solubilisation and redistribution of  $\text{Cu}^{2+}$ , or its complexes, and their ready uptake by *D. pini* conidia, which can explain the good control of *Dothistroma* needle blight by copper fungicides (Franich 1988).

Although copper-based fungicide treatment is effective, and the development of more efficient application methods have lead to a reduction in treatment costs (from over \$60/ha to under \$15/ha in the twenty years to 1988), it is still expensive, costing \$1.6 million per year to spray the 35% of New Zealand's forests which are affected by *D. pini*, as well as a further \$4.5 million residual growth loss per year (L. Bulman, pers. comm., New 1989). As a consequence of this expense, a supplementary or long term alternative to copper fungicide spraying, particularly on high disease risk sites which require multiple spraying to achieve control, is obviously desirable (Shain and Franich 1981, Franich *et al.* 1988).

## 1.4 Resistant Strains

Early observations indicated that certain *D. pini* infected trees possess naturally increased resistance to *Dothistroma* needle blight. Thus it was thought that a good method of combating the disease would lie in the production of *Dothistroma* needle blight-resistant *P. radiata* somatic seedling stock with which to replenish milled forest (Gallagher 1971).

The tree breeding programme at the New Zealand Forest Research Institute (NZ FRI) is producing a *Dothistroma* needle blight-resistant (DR) breed of *P. radiata* for growth in high risk sites. Over the last ten years breeding population parents have been screened and selected for increased *Dothistroma* needle blight resistance and improved growth and form (GF) (FRI 1987). The best DR seedlots are expected to reduce mean stand infection by at least 15%. The effects of spraying and resistance are expected to be additive. This resistance, however, will only be effective for as long as *D. pini* maintains its current levels of virulence (Carson and Carson 1991). Given that the pathogen has a far shorter life cycle than its host, it is likely that strains may evolve which are capable of overcoming current plant resistance mechanisms.

In order to obtain reduction in *D. pini* infection that is economically and genetically meaningful it is necessary to simultaneously improve DR and GF. However, selection with heavy emphasis on *Dothistroma* needle blight resistance reduces the gain in growth rate on non-*Dothistroma* needle blight sites. So separate breeds are therefore used on sites with and without a *D. pini* problem. The nature of *Dothistroma* needle blight resistance remains unknown, although it has been suggested to be a complex trait involving at least three different resistance mechanisms (Carson and Carson 1991).

The New Zealand *P. radiata* population is derived from three localities on the central coast of mainland California in North America and from two islands near Mexico. Despite this small natural range, considerable variation between provenances has been demonstrated (Ades and Simpson 1991). For effective selection of disease resistance and other traits it is favourable to have a narrow genetic variability base to capture high genetic gains. Current selection, aimed at the eventual clonal propagation of superior breeds, is focusing on improving growth rate, stem form, and wood properties (principally wood density), as well as disease resistance (Carson and Carson 1991). Thus facilitating the production of a greater wood volume and more clearwood (less branching), resulting in the need for minimal pruning and allowing uniform milling. However, a reduction in genetic variation means an increase in biological risk factors. If a more virulent *D. pini* strain evolved, a monoclonal host population could be entirely destroyed. Thus, maintaining

genetic diversity is recognised as being very important. A range of parent material from diverse genetic backgrounds is being used in the breeding programme to ensure that separate batches of somatic seedlings are genetically variable.

Although the tree breeding programme is proving successful, difficulties remain in effectively and economically controlling *Dothistroma* needle blight. DR trees will only control the disease, not eliminate it. Investigation into the molecular biology of the *D. pini* disease process may provide a valuable approach to reduce or eliminate the infection.

## 1.5 Dothistromin Toxin

The red pigment in the *Dothistroma* needle blight lesions is due to the presence of the mycotoxin dothistromin, which is found in high concentrations in *D. pini* infected *P. radiata* needles (Gallagher 1971). The toxin has also been isolated from several *Cercospora* species and *Mycosphaerella laricina* (Stoessl *et al.* 1990) as well as from axenic cultures of *D. pini*. The capacity to induce *Dothistroma* needle blight symptoms artificially with purified dothistromin has been demonstrated, thus strongly supporting the hypothesis that dothistromin plays a significant role in pathogenesis and should be considered a toxin (Shain and Franich 1981). The exact function of dothistromin in the disease process is yet to be identified. It may be a pathogenicity factor enabling the fungus to breach the physical and chemical barriers presented by the tree and thus infect tree tissues or it may be a virulence factor acting as a specific elicitor of plant defence responses.

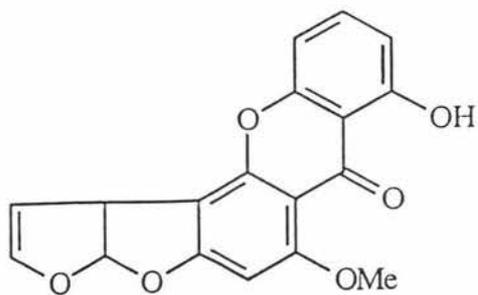
Extensive needle death in the presence of dothistromin is not thought to be due to the toxin itself, but due to benzoic acid synthesised and accumulated in cells adjacent to those initially killed by the toxin. Benzoic acid is found in a dark green region adjacent to the necrotic region of the infected needles where no hyphae are present and is bound to lignin polymers which are also present in disproportionately high amounts. Benzoic acid is highly toxic to *D. pini* (Gadgil 1967). This suggests that one purpose of the benzoic acid response is to restrict hyphal extension within the needle, leading to the proposal that it is a phytoalexin. Needles which respond by producing long lesions also create within the plant tissue a highly fungistatic environment. In the needles dothistromin is metabolised or phytolytically degraded to CO<sub>2</sub> and oxalic acid, a mechanism thought to involve peroxidase catalysed oxidation of the toxin by hydrogen peroxide (Franich *et al.* 1986). Despite these considerations, dothistromin is considered to be an important component in the disease process. The comparatively rapid production of dothistromin in culture, i.e., 6-10 days (Gallagher and Hodges 1972, Harvey *et al.* 1976) suggests that it is not a

staling product of old cultures. Furthermore, histological studies (Gadgil 1967) which demonstrated that host tissue was killed in advance of hyphal penetration, suggest the diffusion of a toxin from hyphae to uninfected tissue. Another point of evidence favouring the toxic role of dothistromin, is that in a limited trial of clonal material of susceptible age, susceptibility was correlated with sensitivity to dothistromin (Franich *et al.* 1977). However, in other limited tests a correlation between decreased sensitivity to dothistromin and mature-tree resistance was not obtained (Franich *et al.* 1983). This could reflect a continuing sensitivity of mature trees to dothistromin from the juvenile stage and their later acquisition of other mechanisms which retard needle penetration. A capacity to induce lesions artificially with dothistromin (Shain and Franich 1981) suggested a relationship between short lesion length and *D. pini* resistance.

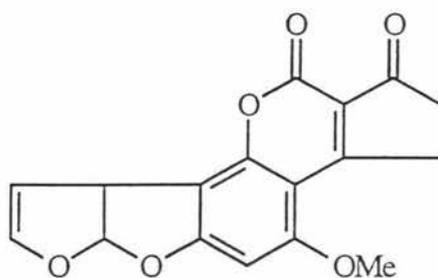
Dothistromin is the major metabolic by-product of *D. pini* cultures (Harvey *et al.* 1976, Danks and Hodges 1974). It has been shown using chemical, spectroscopic (Bassett *et al.* 1970, Gallagher and Hodges 1972), and crystallographic evidence (Bear *et al.* 1972, Assante *et al.* 1977) to be a difuroanthraquinone which is fused to the same tetrahydro-2-hydro-bisfuran ring system as the mycotoxins, aflatoxin (AF) from *Aspergillus parasiticus* and *Aspergillus flavus* and sterigmatocystin (ST) from *Aspergillus nidulans* (Fig. 1, Bassett *et al.* 1970, Shaw 1975, Shaw *et al.* 1978). It is the furan ring structural feature of AF and ST (which are difuranocourain secondary metabolites) that is considered to be responsible for their toxicity (Elliot *et al.* 1989, Harvey *et al.* 1976). In view of this, Shaw *et al.* (1978) performed nuclear magnetic resonance (NMR) and mass-spectrophotometric analysis of the reaction products of *D. pini* mycelia grown with [<sup>13</sup>C]-labelled acetate. The resulting labelling pattern strongly suggested dothistromin to be the product of a biosynthetic pathway involving at least one acetate-polymalonate intermediate, therefore showing similarities to AF. Dothistromin has been identified as the phytotoxic metabolite in some *Cercospora* species (Stoessl 1984). Subsequent work with *Cercospora arachidicola* (Stoessl and Stothers 1984) has identified several other anthraquinones. Three of these compounds (averantin, averufin, and versicolorin B) are intermediates in the ST and AF biosynthetic pathways in *Aspergillus* species, and are likely to also be intermediates in dothistromin biosynthesis (Fig. 2).

On the strength of the remarkable structural similarity between dothistromin and AF, and because forest workers might be exposed to high levels of dothistromin during the course of their work, mutagenic studies were initiated with dothistromin to confirm its proposed role as a toxin. At growth inhibitory concentrations, dothistromin strongly inhibits incorporation of [<sup>3</sup>H] uridine into RNA of *Chlorella pyrenoidosa* and *Bacillus megaterium* (Harvey *et al.* 1976). The similarity between the effects of dothistromin and

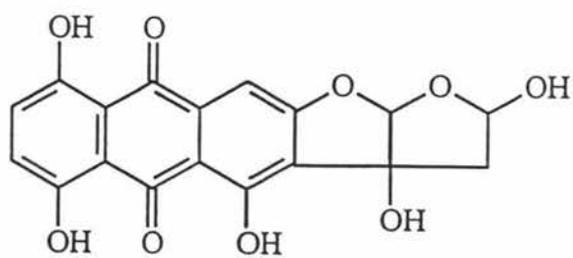
**Fig. 1**                      **Structures of sterigmatocystin, aflatoxin B1, and dothistromin**



Sterigmatocystin



Aflatoxin B1



Dothistromin

actinomycin D, which is known to inhibit the transcriptional process (Reich *et al.* 1967), supports the view that this may be a primary site of action of the toxin in inhibiting microbial growth. The inhibition of RNA synthesis by dothistromin is not surprising in view of its close structural similarity to AF. AF inhibits RNA synthesis in liver cells (Clifford and Rees 1966), and it has been shown to bind to DNA and thereby impair RNA polymerase-mediated transcription (Neely *et al.* 1970). AF is considered to be responsible for hepatotoxicity and potential human carcinogenicity (Ames *et al.* 1987). This information initiated a further series of mutagenicity studies of dothistromin including field sampling, environmental monitoring and epidemiological research which were coordinated by both the New Zealand Department of Health and the Dothistromin Advisory Working Group. Dothistromin was tested for mutagenicity in a wide variety of *in vitro* bioassays, most which were positive, including chromosome damage in human peripheral blood lymphocyte cultures (Elliot *et al.* 1989, Stoessl *et al.* 1990). In a mouse *in vivo* mutagenicity assay dothistromin appeared to be just as genotoxic as AF, causing a significant increase in the number of abnormal erythrocytes as a result of damage to the spindle apparatus or chromosome damage (Elliot *et al.* 1989). However, in a separate *in vitro* study, metaphase chromosome damage in human peripheral blood lymphocytes required high doses of dothistromin (Ferguson *et al.* 1986). This is in contrast to AF where aberrations were seen at low dose levels. These studies suggest that although AF and dothistromin have similar toxic properties, the toxicity of dothistromin is weaker.

## 1.6 Inactivation of Dothistromin

Copper fungicide treatment is only a limited control mechanism, and the resistant varieties are only successful as long as the pathogen retains its present virulence levels. For this reason different approaches to *Dothistroma* needle blight control are being investigated focusing on the mode of action of dothistromin.

One method of analysis of the role of dothistromin in the plant/fungal interaction is immunoassay. Immunoassays have been developed for other small molecules such as aflatoxins (Chu 1991). Three years ago at HortResearch (Palmerston North), the production of monoclonal antibodies (MAbs) to dothistromin was reported (Jones *et al.* 1993). They utilised dothistromin-carrier protein conjugates to prepare these MAbs for use in the development of an ELISA for dothistromin which has the required sensitivity and specificity to monitor development of the toxin in *D. pini* cultures and pine leaf lesions caused by *D. pini*. In *in vitro* experiments so far the antibody competes quite well for dothistromin binding, but preincubation of the antibody with dothistromin prior to challenging the plant cells is required (P. Reynolds, pers. comm.). As an alternative

strategy, they have also obtained single-chain antibodies to the bifuran ring of dothistromin. Anti-idiotypic antibodies are currently being made against these with a view to isolating dothistromin binding proteins (W. Jones, pers. comm.). They aim to produce transgenic plants which express the anti-dothistromin antibody, thus inactivating the dothistromin toxin if *D. pini* infects them and hence eliminating the need for spraying by copper fungicides. The fundamental assumption underlying this approach is that dothistromin is the primary cause of disease symptoms.

In the past five years a number of fungal genes have been isolated which were anticipated to have a function in pathogenicity (VanEtten *et al.* 1994). The roles of many of these genes in pathogenicity have been evaluated by the construction of mutants that lack a functional wild type gene through transformation-mediated gene disruption or UV mutagenesis. For example, in the Dutch elm disease (DED) pathogen *Ophiostoma ulmi* and the fungal soybean pathogen, *Cercospora kikuchii*.

*O. ulmi* produces a low molecular weight polypeptide toxin called cerato-ulmin (CU) which has been implicated as an important factor in virulence (Takai 1974). CU is an extracellular hydrophobin and is thought to act by accumulating at, and plugging intercellular openings in, the xylem (Russo *et al.* 1982), or by direct interaction with host parenchyma cells resulting in enhanced respiration and electrolyte loss (Richards and Takai 1984). The evidence that CU plays a key role in DED pathogenesis has come largely from physiological studies. Purified CU induces both internal and external symptoms similar to those of DED, when applied to elm saplings (Richards and Takai 1984). CU has been detected by immunocytochemistry and scanning electron microscopy only in aggressive (virulent) strains (Svircev *et al.* 1988), and isolate aggressiveness of the pathogen correlates with levels of CU production *in vitro* (Takai 1974). However, classical genetics gave only a weak correlation between CU expression and virulence towards the host elm. To directly test the role of CU in DED, Bowden *et al.* (1994) recently cloned and characterised the *cu* gene from a highly virulent isolate of *O. ulmi*. More recently, Bowden *et al.* (1996) generated a CU-minus mutant by transformation mediated gene disruption of the *cu* gene. This disruption mutant produced no detectable CU mRNA or detectable CU protein, but in greenhouse trials it retained full pathogenicity. These unexpected findings suggest that CU is not a primary pathogenicity factor in the development of DED symptoms. The biological role of CU has yet to be determined. It has been suggested (Bowden *et al.* 1996) that CU may be involved in surface hydrophobicity of aerial hyphae and conidiospores. The validity of this hypothesis is undergoing experimentation.

*C. kikuchii* produces the necrosis inducing polyketide toxin cercosporin. Cercosporin is a photosensitising toxin which requires light for both synthesis (Fajola 1978, Daub 1982, Ehrenshaft *et al.* 1991) and toxin activity (Daub 1982). Upon exposure to light, cercosporin interacts with molecular oxygen to produce highly toxic singlet oxygen which has been shown to cause membrane damage and cell death by the peroxidation of plant membrane lipids (Daub and Briggs 1983, Daub and Hangarter 1983). It is postulated that both tissue colonisation and nutrient acquisition by the fungus are facilitated by the action of cercosporin. Treatment of plant tissue with pure cercosporin reproduces the ultra-structural changes that are both consistent with the known mode of action of cercosporin and similar to the disease symptoms caused by the pathogen itself (Bails and Payne 1971). Also, cercosporin has been isolated from the necrotic lesions of several infected plant hosts (Fajola 1978, Venkataramani 1967). Although this evidence suggests that cercosporin plays an important role in diseases caused by *Cercospora* species, until recently, little was known about the actual mechanisms of pathogenicity. To contribute to a better understanding, Upchurch *et al.* (1991) isolated UV-induced cercosporin blocked mutants. These cercosporin-blocked mutants were used to inoculate soybeans which were shown to be non-pathogenic. This provided direct evidence that cercosporin is a crucial pathogenicity factor (Upchurch *et al.* 1991).

The ability to obtain mutants altered or blocked in the synthesis of toxins (such as CU and cercosporin in *O. ulmi* and *C. kikuchii*, respectively) offers considerable potential for use in elucidating the molecular basis of pathogenicity in *D. pini*. Although, indirect evidence has implicated the importance of dothistromin in *Dothistroma* needle blight, the true role of the toxin as a pathogenicity or virulence factor has yet to be determined. The multiseptate and multinucleate nature of *D. pini* conidia make it difficult to employ conventional UV mutagenesis for the isolation of a loss-of-function mutation, so dothistromin-mutants will be most easily isolated by disrupting the gene. Evaluation of the pathogenicity of dothistromin-minus *D. pini* strains could then be performed. This is vital information for the HortResearch programme, since if the toxin-minus mutants are still pathogenic there will be no resistance conferred by the production of anti-dothistromin antibodies in the host trees. Moreover, if dothistromin is a virulence factor then, potentially, in the absence of toxin, the severity of the disease may increase due to the lack of a hypersensitive-type defence response in the tree. The effect of dothistromin in the disease process may become apparent only after dothistromin biosynthesis is better understood.

## 1.7 Aflatoxin Biosynthesis

The polyketide-derived secondary metabolites, aflatoxin (AF) and sterigmatocystin (ST), are among the most toxic, mutagenic, and carcinogenic natural products known. AFs are produced only by certain *Aspergillus parasiticus*, *A. flavus*, and *A. nomius* strains, while ST, the penultimate intermediate in the AF pathway is synthesised as an end product by numerous ascomycetes and deuteromycetes including *A. nidulans* (Brown *et al.* 1996). These ubiquitous fungi are capable of infecting a wide variety of crops such as corn, peanuts, and cottonseed, which, under the proper environmental conditions, can become contaminated with this potent mycotoxin (Cary *et al.* 1996, Trail *et al.* 1995a). Because of their potent carcinogenic effects on laboratory animals including rats, ducks, and monkeys, aflatoxins are considered to be a potential threat to human health and are an economic problem in many areas of the world. Due to the difficulty in effectively and economically controlling aflatoxin contamination of food and feed by traditional agricultural methods (i.e. irrigation, application of fungicides or insecticides, and use of resistant crop varieties), recent efforts in several laboratories have focused on developing an in depth understanding of the molecular biology of the aflatoxin biosynthetic pathway. An understanding of the aflatoxin biosynthetic pathway may result in the identification of strategies to inhibit aflatoxin contamination of plant-derived products at the pre-harvest level. These strategies are focused on two main areas: (i) genetically modified crops to reduce fungal growth or inhibit aflatoxin biosynthesis (long-term approach); and (ii) utilisation of biological control organisms to competitively exclude the toxigenic fungus from infecting the crop (short-term approach). A detailed understanding of the aflatoxin biosynthetic pathway at the molecular level will aid in the pursuit of these approaches of control.

The ST/AF pathway was elucidated through the isolation and characterisation of *A. flavus* and *A. parasiticus* mutants blocked in AF production, radiolabelled-precursor feeding experiments, enzyme inhibitor studies, and biochemical characterisation of enzymatic activities. AF/ST biosynthesis is proposed to begin with the condensation of acetyl coenzyme A and malonyl coenzyme A via polyketide synthetase (PKS) to form the decaketide noranthrone. Alternatively, a six-carbon fatty acid, hexanoate, is first synthesised by a fatty acid synthetase and then extended by a PKS to generate noranthrone. Noranthrone is oxidised to norsolorinic acid (NA), which is converted to aflatoxin B1 (AFB1) through a series of pathway intermediates, including averantin (AVN), averufanin (AVNN), averufin (AVF), versiconal hemiacetal acetate (VHA), versiconal (VAL), versicolorin B (VB), versicolorin A (VA), demethylsterigmatocystin, sterigmatocystin (ST), *O*-methylsterigmatocystin (OMST), and AFB1. The generally

accepted pathway is illustrated in Fig. 2. As many as 17 different enzymes are proposed to be involved in aflatoxin biosynthesis (Trail *et al.* 1995a, Mahanti *et al.* 1996).

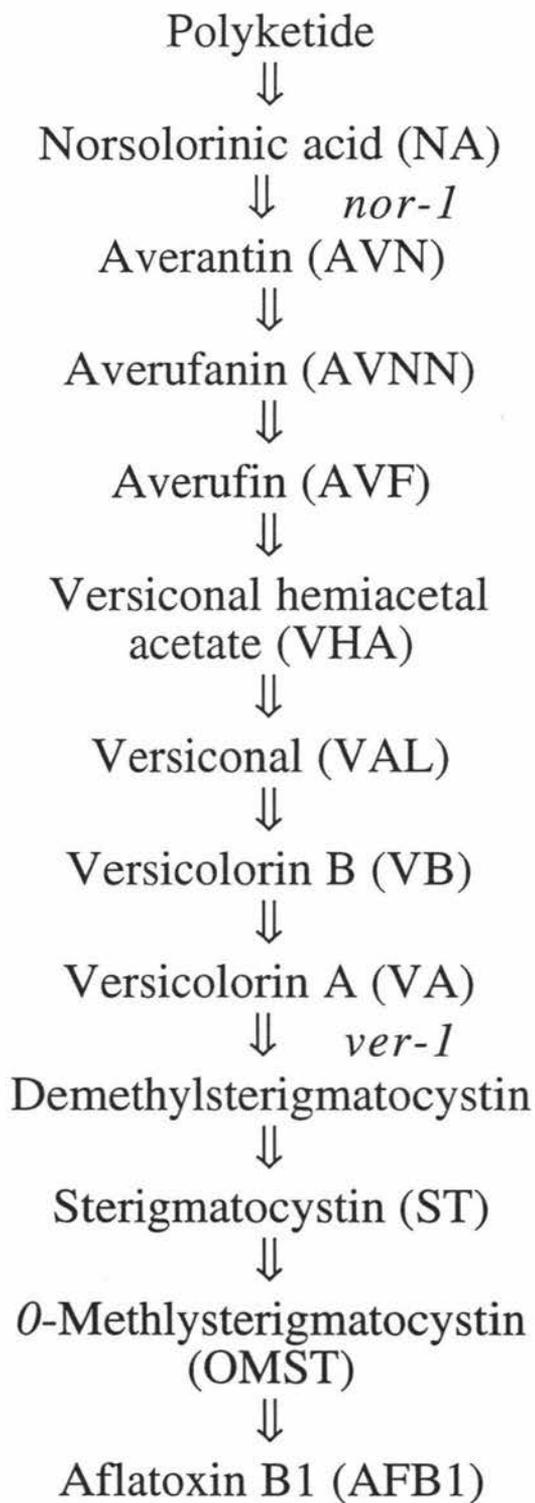
## 1.8 Gene Cloning Strategies in *Aspergillus* species

Aflatoxin-blocked mutants and purified enzymes have been used to clone several genes involved in the aflatoxin biosynthetic pathway. The cloning of these genes has been the key to increasing understanding of the molecular biology of the pathway. Cloned genes are useful probes for elucidating the molecular mechanisms that regulate the timing and expression of these genes. Two different strategies have been successfully utilised in the cloning of several genes encoding enzymes involved in AF biosynthesis in *A. parasiticus* and *A. flavus* and ST biosynthesis in *A. nidulans*. These genes include *pksA*, which codes for a polyketide synthase (Chang *et al.* 1995b, Trail *et al.* 1995b), *nor-1*, which codes for a reductase that reversibly converts NA to averantin (Chang *et al.* 1992, Trail *et al.* 1994), *ver-1*, which is involved in the conversion of VA to ST (Keller *et al.* 1994, Skory *et al.* 1992), and *omtA*, which codes for an S-adenosylmethionine-dependent O-methyltransferase that converts ST to OMST and dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin (Yabe *et al.* 1989, Yu *et al.* 1992). In addition to these structural genes, a regulatory gene, *aflR*, that codes for a regulatory factor (AFLR protein) has been cloned and was shown to be involved in the activation of pathway gene transcription (Chang *et al.* 1993, Payne *et al.* 1993). Also, a putative fatty acid synthase gene, *umv8*, potentially involved in polyketide backbone synthesis, and a gene, *aad*, homologous to aryl-alcohol dehydrogenase which may be involved in an intermediate step of AF biosynthesis have also been cloned (Yu *et al.* 1995). For introduction of DNA into the fungus, transformation systems were developed for *A. parasiticus* (Skory *et al.* 1990) and *A. flavus* (Woloshuk *et al.* 1989).

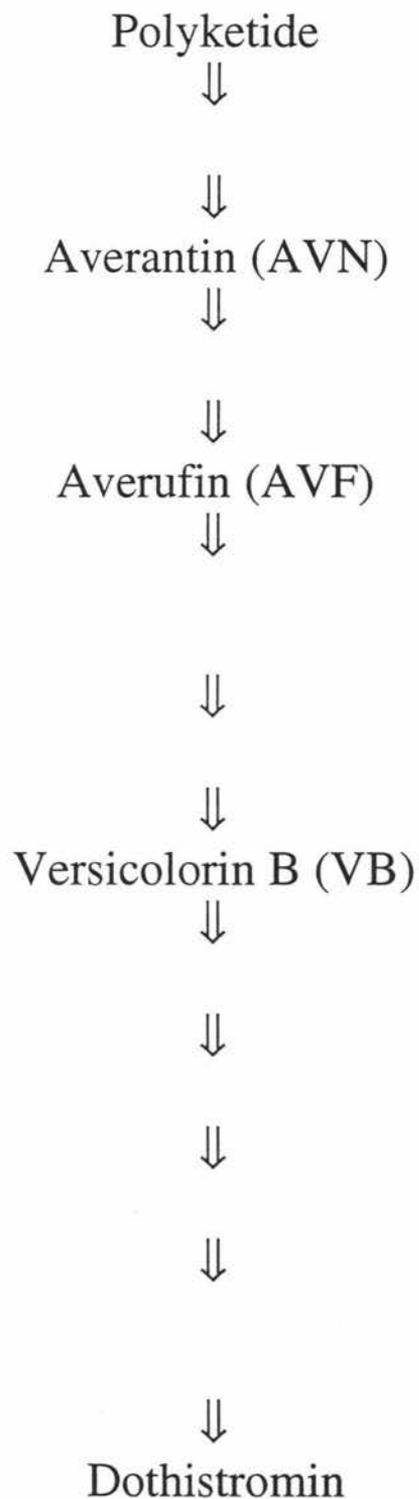
The *nor-1* (Chang *et al.* 1992) and *ver-1* (Skory *et al.* 1992) genes were cloned by complementation of aflatoxin blocked mutants which accumulate the brightly coloured pathway intermediates NA (brick-red) and VA (yellow), respectively. Complementation was achieved by introduction of a cosmid DNA library using genomic DNA from a wild type aflatoxin-producing strain of *A. parasiticus*. The functionally homologous *verA* gene of *A. nidulans* was isolated by hybridisation of *ver-1* to an *A. nidulans* genomic DNA library (Keller *et al.* 1994). The predicted amino acid sequences of the *ver-1* and *verA* gene products are nearly identical (Keller *et al.* 1994), illustrating the high degree of identity between aflatoxin biosynthetic genes among these *Aspergillus* species. The predicted amino acid sequences of *nor-1*, *ver-1*, and *verA* contain an NAD(P)H binding

**Fig. 2**                      **Comparison of the aflatoxin biosynthetic pathway with the proposed dothistromin biosynthetic pathway**

### Aflatoxin B1 Biosynthetic Pathway



### Proposed Dothistromin Biosynthetic Pathway



motif near the amino terminus and show significant identity (33% for *ver-1/verA*, 23% for *nor-1*) to several NADPH- and NADH-dependent reductase/dehydrogenase enzymes found in other polyketide biosynthetic pathways. Each sequence also contains a short-chain alcohol dehydrogenase motif (Trail *et al.* 1994).

To confirm the role of these genes in AF biosynthesis, gene disruption was conducted in toxigenic strains of *A. parasiticus* (*nor-1*, Trail *et al.* 1994; *ver-1*, Trail *et al.* 1995b) and *A. nidulans* (*ver-A*, Keller *et al.* 1994). Disruption of the *ver-A* gene resulted in loss of detectable ST and accumulation of VA by *A. nidulans*, confirming its role in conversion of VA to ST. Similarly, disruption of *ver-1* blocked the AF pathway, resulting in VA accumulation. Disruption of *nor-1* resulted in accumulation of large quantities of norsolorinic acid (NA). However, disrupted *nor-1* strains retained their ability to produce low levels of aflatoxin, supporting the hypothesis that there are one or more alternative routes (or enzymatic activities) in the AF pathway to synthesise averufin from NA (Yabe *et al.* 1993).

The *umv8* gene was cloned by complementation of an aflatoxin blocked mutant, *umv8*, derived by UV mutagenesis (Mahanti *et al.* 1994). Metabolite conversion studies confirmed that *umv8* has two blocks in the pathway, one block at *nor-1* and the other one prior to *nor-1*. Nucleotide sequence analysis of this gene revealed that the predicted protein contains a high degree of similarity (67%) and identity (48%) to the enoyl-reductase and malonyl/palmitoyl transferase domains in the  $\beta$  subunit of yeast fatty acid synthase (FAS1, Kottig *et al.* 1991) from *Saccharomyces cerevisiae* (Mahanti *et al.* 1996, Trail *et al.* 1995a). *umv8* was therefore thought to encode an FAS activity necessary for synthesis of the hexanoate starter. Consequently, its name was changed to *fas-1*. It is possible that synthesis of the hexanoate starter requires two FAS subunits ( $\alpha$  and  $\beta$ ; encoded by unique genes) analogous to those of yeast. Gene disruption combined with feeding studies will allow this hypothesis to be tested.

A second approach for isolating genes, reverse genetics, relied on the purified pathway enzymes discussed above. Where purification has been possible, production of antibodies to the enzyme, and isolation of the gene from a cDNA expression library in *E. coli*, can be accomplished. This procedure was utilised to clone the *omt-1* gene from *A. parasiticus* encoding the *O*-methyltransferase activity responsible for conversion of ST to *O*-sterigmatocystin (Yu *et al.* 1993). The predicted amino acid sequence derived from the cloned cDNA contained a motif found in other *S*-adenosylmethionine-methyl-dependent methyltransferases. More recently, Cary *et al.* (1996) reported the isolation of a full-length cDNA from *A. parasiticus* harbouring a gene designated *norA*. The *norA* cDNA

clone was isolated with monoclonal antibodies (MAbs) raised against a purified *A. parasiticus* enzyme demonstrating norsolorinic acid reductase (NOR) activity. They also identified and sequenced the *norA* homologue in *A. flavus* (Cary *et al.* 1996). So far, these genes are the only pathway genes cloned by the reverse genetics approach. However, this approach should be successful in cloning several other genes encoding the purified pathway enzymes.

Another molecular genetic approach for gene cloning, differential screening, has been used by Feng *et al.* (1992) in an attempt to isolate genes whose pattern of expression coincides with aflatoxin production in *A. parasiticus*. This method is not based on specific knowledge of the function of the gene product, as in the two previous methods, and can therefore be advantageous when the timing of induction of gene expression is known but pure enzymes or blocked pathway mutants are not available. With this technique Woloshuk and Payne (1994) were successful in isolating an alcohol dehydrogenase gene, *adh1*, from *A. flavus* that was induced under growth conditions conducive to aflatoxin biosynthesis. However, to date, this method has failed to identify conclusively any genes directly involved in the aflatoxin biosynthetic pathway.

## 1.9 Organisation and Arrangement of the AF/ST Pathway Genes

Parasexual analysis using *A. flavus* and *A. parasiticus* suggested that the genes involved in AF biosynthesis were linked (Papa 1978). Attempts to demonstrate linkage of *nor-1* and *ver-1* genes in *A. parasiticus* by parasexual analyses, however, gave conflicting results (Bradshaw *et al.* 1983, Lennox *et al.* 1983, Papa 1984). Molecular and genetic analyses have now provided proof that many of the genes involved in AF biosynthesis in *A. parasiticus* and *A. flavus* are physically clustered on one chromosome. During the cloning and characterisation of the *nor-1* and *ver-1* genes from *A. parasiticus*, one cosmid, NorA, was identified that hybridised to probes of both genes (Skory *et al.* 1992). This tentative evidence for linkage was later confirmed by physical mapping of the corresponding region in the fungal genome in *A. parasiticus* (Trail *et al.* 1995b). *aflR*, *umv8*, and *omt-1* were recently mapped to this cluster and to a similar cluster of AF genes in *A. flavus* (Yu *et al.* 1995, Trail *et al.* 1995b).

Since as many as 17 different enzymes are thought to be required to complete AF biosynthesis it was hypothesised that the cosmid NorA (and the corresponding region in *A. flavus*) encoded several other pathway enzymes. To determine the size, location, and pattern of expression of other genes in the cluster, a transcriptional map of the 35 kb

genomic DNA insert in cosmid NorA was completed (Trail *et al.* 1995b). Twelve unique RNA transcripts localised to this cluster. Eight of these transcripts, previously unidentified, showed a pattern of expression similar to that of *nor-1* and *ver-1*, suggesting that the genes encoding them are also involved in AF biosynthesis. To directly test this hypothesis, gene-1 (tentatively named because of its position at the far left end of the cluster), encoding one of the eight transcripts, was disrupted in an VA-accumulating mutant of *A. parasiticus*. Thin-layer chromatography revealed that gene-1 disruptant clones no longer accumulated VA. Southern hybridisation analysis of the disruptant clones confirmed that gene-1 is directly involved in AF biosynthesis. Nucleotide sequence analysis of two regions within gene-1 showed a high degree of identity and similarity with the  $\beta$ -ketoacyl-synthase and acyltransferase functional domains of the *wA* gene product in *A. nidulans* (which encodes a PKS involved in conidial pigment production, Mayorga and Timberlake 1992) and other polyketide synthases (Trail *et al.* 1995b). It is possible that this putative aflatoxin PKS is involved in extending the hexanoate starter unit synthesised by the *umv8* gene product.

Recently, it has been shown that the *A. nidulans* ST pathway is conserved at the functional, regulatory, and physical levels with the AF pathway in *A. flavus* and *A. parasiticus* (Keller *et al.* 1994, Keller *et al.* 1995, Yu *et al.* 1995). For example, *ver-A*, a homolog of the *A. parasiticus ver-1* gene is required for the same bioconversion in *A. nidulans* (Keller *et al.* 1994, Skory *et al.* 1992). In addition, the forced expression of the *A. flavus* AF regulatory gene, *aflR*, in *A. nidulans* induces expression of the *ver-A* transcript, indicating that regulation of the ST/AF pathway is functionally conserved (Chang *et al.* 1993, Woloshuk *et al.* 1994). Moreover, a putative PKS required for ST biosynthesis is located within approximately 48 kb of *ver-A* in *A. nidulans*, which is similar to the grouping of homologous genes in *A. parasiticus* and *A. flavus* (Yu *et al.* 1995, Keller *et al.* 1994, Yu and Leonard 1995). More recently, Brown *et al.* (1996) presented the entire sequence of a 60 kb region in the *A. nidulans* genome and found it to contain many, if not all, of the proposed genes needed for ST biosynthesis. This was based on three observations: (i) A total of eight of the genes, *stcA* (previously *pksST*, Yu *et al.* 1995), *stcS* (previously *verB*, Keller *et al.* 1995), *stcU* (previously *verA*, Keller *et al.* 1994), *aflR* (Yu and Leonard 1995), *stcJ*, *stcK*, *stcL*, and *stcN* (Brown *et al.* 1996) have been shown to be necessary for ST biosynthesis. (ii) All 25 transcripts corresponding to the proposed genes are coordinately regulated. (iii) The disruption of *aflR*, a putative pathway-specific regulator, resulted in loss or greatly reduced accumulation of all 25 transcripts (Yu *et al.* 1995). Functional assignment of the remaining cluster genes will require gene disruption followed by structural characterisation of any accumulating materials. It is possible that these studies will result in a revised ST/AF pathway.

Due to this increasing body of evidence that gene sequences are highly conserved among *A. parasiticus*, *A. flavus*, and *A. nidulans* it is probable that cloned AF and ST pathway genes will provide a direct method for identifying dothistromin biosynthetic genes via their use as heterologous probes in Southern hybridisation analysis.

### 1.10 Aims and Objectives

The primary focus of current research is to obtain mutants blocked or altered in the synthesis of dothistromin. This will allow the application of molecular biology approaches to the study of dothistromin synthesis and regulation, and the ability to more directly assess the role of dothistromin in the pathogenicity of *D. pini*. Biochemical and molecular genetic studies will allow the isolation and identification of genes involved in dothistromin biosynthesis and will contribute to a better understanding of the structure, function, and organisation of the dothistromin biosynthetic pathway genes, as well as elucidation of the molecular control mechanisms that regulate dothistromin production (i.e. regulatory genes). This study will focus on the isolation and cloning of genes required for dothistromin biosynthesis.

Clones of aflatoxin biosynthetic genes from *A. parasiticus* will be used as hybridisation probes in the hope that they will share sufficient sequence identity to *D. pini* dothistromin biosynthetic genes to permit their detection. Genes involved in dothistromin biosynthesis can then be isolated and cloned by screening a *D. pini* genomic library.

The eventual characterisation of several genes will allow testing of the fundamental assumption that pathogenesis is primarily dothistromin-mediated, by producing isolates of *D. pini* which no longer produce the toxin and assessing if they are capable of invoking the benzoic acid response from the plant. Hence we can ascertain the precise roles of the fungal mycelium and the toxin in the disease process. A transformation system has recently been developed for *D. pini* (Bidlake 1996) using a positive selection system based on the *E. coli* hygromycin resistance gene (*hph*). This will enable targeted disruption of wild type dothistromin biosynthetic genes.

The production of genetically stable atoxigenic strains of *D. pini* which, by disruption of several pathway genes (or a complete cluster), are rendered completely disabled in dothistromin production, could then be used for competitive exclusion of dothistromin-producing strains in the field. Biocompetition of this type has proven to be quite

successful in laboratory and field tests with mutant aflatoxin strains (Skory *et al.* 1990, Skory *et al.* 1992). Furthermore, an understanding of the molecular control mechanisms involved in dothistromin biosynthesis may lead to the development of agents or genetically engineered plants that inhibit toxin production.

## **2.0 MATERIALS AND METHODS**

### **2.1 Bacterial and Fungal Strains, $\lambda$ Clones and Vectors**

The bacterial and fungal strains,  $\lambda$  clones, and plasmids used in this study are listed in Table 1.

### **2.2 Media**

All media was prepared using MilliQ water. After preparation the media was sterilised by autoclaving at 121°C and 15 psi for 20 min. Liquid media was cooled to room temperature before addition of antibiotic(s) and inoculation. Solid media was cooled to approximately 50°C prior to antibiotic addition and pouring. Uninoculated plates were stored at 4°C.

#### **2.2.1 Fungal media**

##### **2.2.1.1 *D. pini* Media (DM) Broth**

DM broth contained (g/l): malt extract (Oxoid), 50.0 (5%); and nutrient broth (Oxoid), 28.0. When stated, malt extract content was altered to (g/l): either, 30.0 (3%) or 10.0 (1%).

##### **2.2.1.2 *D. pini* Media (DM) Agar**

DM agar contained (g/l): malt extract (Oxoid), 50.0; nutrient agar (Oxoid), 28.0; glucose (BDH), 20.0 (if required)

##### **2.2.1.3 Malt Extract Agar (MEA)**

MEA contained (g/l): malt extract (Oxoid), 30.0, peptone (Oxoid), 5.0; agar (Davis), 20.0; glucose (BDH), 20.0; uracil (Sigma), 2.2 (if required)

#### **2.2.1.4 Malt Yeast Glucose (MYG) Agar**

MYG contained (g/l): malt extract (Oxoid), 5.0; yeast extract (Oxoid), 2.5; agar (Davis) 20.0; glucose (BDH), 20.0;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , trace.

#### **2.2.1.5 Yeast Peptone Glycerol (YPG) Agar**

YPG contained (g/l): yeast extract (Oxoid), 10.0; peptone (Oxoid), 20.0; agar (Davis), 20.0; and (ml/l): glycerol (BDH), 30.0.

#### **2.2.1.6 Potato Dextrose Agar (PDA)**

PDA contained (g/l): potato dextrose broth (Difco), 24.0; agar (Davis) 20.0; glucose (BDH), 20.0 (if required).

#### **2.2.1.7 Nutrient Malt Yeast (NMY) Agar**

NMY agar contained (g/l): nutrient agar (Oxoid), 28; malt extract (Oxoid), 10.0; yeast extract (Oxoid), 10.0; glucose (BDH), 20.0 (if required).

#### **2.2.1.8 Minimal Medium (MM) Agar**

MM agar contained (g/l):  $\text{NaNO}_3$  (BDH), 6.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (BDH), 0.52; KCl (BDH), 0.52;  $\text{KH}_2\text{PO}_4$  (BDH), 1.52;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (BDH), trace;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (BDH), trace; glucose (BDH), 20.0; agar (Davis), 20.0.

#### **2.2.1.9 Nutrient Yeast (NY) Broth**

NY broth contained (g/l): nutrient broth (Oxoid), 28.0; yeast extract (Oxoid), 10.0.

#### **2.2.1.10 Nutrient Malt Yeast (NMY) Broth**

NMY broth contained (g/l): nutrient broth (Oxoid), 28.0; malt extract (Oxoid), 50.0; yeast extract (Oxoid), 10.0.

**Table 1** Fungal and Bacterial Strains,  $\lambda$  Clones and Vectors

Strain, $\lambda$ Clone or Plasmid	Relevant Characteristics	Source or Reference
<b>Fungal Strains</b>		
<i>Aspergillus nidulans</i> R21	<i>pabaA1 yA1</i>	Waldron and Roberts 1973
<i>Dothistroma pini</i> Dp1	wild type strain (forest isolate, Long Mile Road, Rotorua)	P. Debnam 1993
<b>Bacterial Strains</b>		
<i>Escherichia coli</i> KW251	F <sup>-</sup> <i>supE44 supF58 galK2 galT22 metB1 hsdR2 mcrB1 mcrA<sup>-</sup> argA81:Tn10 recD1014</i>	Murray <i>et al.</i> 1977
XL1-Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac<sup>-</sup> F<sup>-</sup> [proAB<sup>+</sup> lacZ<math>\Delta</math>M15 Tn10 (tet<sup>r</sup>)]</i>	Bullock <i>et al.</i> 1987
<b><math>\lambda</math> Clones</b>		
$\lambda$ CGV1 and $\lambda$ CGV2	$\lambda$ GEM-12 clones containing <i>D. pini</i> genomic DNA hybridising to the <i>A. parasiticus ver-1</i> gene	This study
$\lambda$ CGN1	$\lambda$ GEM-12 clone containing <i>D. pini</i> genomic DNA hybridising to the <i>A. paraciticus nor-1</i> gene	This study

**Table 1** (Continued)**Vectors**

pNA17	pUC19 containing a 1.7 kb <i>SphI/BglII</i> fragment containing the <i>A. parasiticus nor-1</i> gene	Chang <i>et al.</i> 1992
pBVer-1	pBR322 containing a 2.35 kb <i>ClaI/HindIII</i> fragment containing the <i>A. parasiticus ver-1</i> gene	Skory <i>et al.</i> 1992
pUC118	3.2 kb amp <sup>r</sup>	Messing 1983
λGEM-12	lambda genomic cloning vector	Frischauf <i>et al.</i> 1983
pCG1	pUC118 containing a 0.8 kb <i>SalI</i> fragment from λCGV1	This study
pCG2	M13mp18 containing a 2.1 kb <i>BamHI/SalI</i> fragment from λCGV2	This study

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## 2.2.2 Bacterial Media

### 2.2.2.1 Luria-Bertaini (LB) Media

LB media contained (g/l): tryptone (Difco), 10.0; NaCl, 5.0; yeast extract (Oxoid), 5.0. The pH was adjusted to 7.0 prior to autoclaving. For solid media, agar (Davis) was added to 15.0 g/l.

When required LB was supplemented after autoclaving to give final concentrations of: ampicillin, 100 µg/ml from a stock solution of 100 mg/ml; tetracycline, 10 µg/ml from a stock solution of 10 mg/ml in methanol; isopropylthio-β-D-galactoside (IPTG), 30 mg/ml and 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide (X-gal), 60 µg/ml.

### 2.2.2.2 TB Top Agar

TB top agar contained (g/l): tryptone (Difco), 10.0; NaCl, 5.0; agar (Davis), 8.0. This was cooled to 45-50°C following autoclaving and supplemented with 10 mM MgSO<sub>4</sub>.

### 2.2.2.3 NZCYM

NZCYM contained (g/l): NZ amine, 10.0; NaCl, 5.0; Casamino acids (Difco), 1.0; yeast extract, 5.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.0. NaOH was added until the pH was 7.5.

## 2.3 Growth and Maintenance of Cultures

### 2.3.1 Fungal Cultures

A (8.0 mm x 8.0 mm) chunk of *D. pini* mycelia (cut with a scalpel blade) was ground in 1ml of sterile MilliQ water using a plastic grinder in an eppendorf tube. From this, 200 µl was spread onto DM plates with sterile cellophane discs which were sealed with parafilm. *D. pini* fungal cultures were grown at 20°C for 7 days, and then stored at 4°C for up to 6 months before subculturing. When stated, two other methods of inoculation were also used: ground up mycelia were streaked onto agar plates, or 3-4 pieces of mycelia (approximately 3 mm x 3 mm) were placed on the agar plate. DM broth cultures were inoculated in the same way with 1 ml of inoculum/100 ml of DM broth in a 1 litre siliconised flask. These were grown at 20°C with gentle shaking (100 rpm) for up to 7

days. Mycelial growth was quantitated as follows. The entire contents of each broth culture was vacuum filtered through pre-dried and -weighed Whatman no. 1 filter paper, using a Buchner funnel, and the filtrate discarded. The mycelial extracts were washed, then dried in a hot air oven at 80°C for 2 hr, weighed, and expressed as mycelial dry weight (g/100 ml).

Note: the inoculum size is not strictly quantitative due to varying sizes (5.0 mm-10.0 mm x 5.0 mm-10.0 mm) of mycelia chunks being unavoidably cut depending on the morphology and thickness of the mycelial material.

### **2.3.2 Bacterial Cultures**

*E. coli* cultures were maintained on LB plates supplemented with the appropriate selective antibiotics. Cultures were grown at 37°C, sealed with parafilm, and then stored at 4°C. They were regularly restreaked onto fresh LB plates.

## **2.4 Buffers and Solutions**

### **2.4.1 TEG**

TEG contained: 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA.

### **2.4.2 Tris-Equilibrated Phenol**

Tris-equilibrated phenol was prepared by melting solid phenol at 50°C. An equal volume of 100 mM Tris-HCl (pH 8.0) was added at room temperature and stirred for 15 min, then the phases left to separate for 15-30 mins. The aqueous phase was then decanted, and the phenolic phase retained and repeatedly washed with 100 mM Tris-HCl until the pH reached 8.0. After equilibration the phenolic phase was retained and an equal volume of 100 mM Tris-HCl (pH 8.0) was added. Hydroxyquinoline was added to a final concentration on 0.1% (w/v). The equilibrated phenol was stored in a brown bottle at 4°C.

### **2.4.3 TE Buffer**

Tris EDTA buffer (10 mM Tris-HCl/1mM Na<sub>2</sub>EDTA (10/1) or 10 mM Tris-HCl/0.1 mM Na<sub>2</sub>EDTA(10/0.1)) was prepared to the required concentration from 1 M Tris-HCl (pH 7.5) and 0.5 M Na<sub>2</sub>EDTA (pH 8.5) stock solutions.

### **2.4.4 DNase free RNase**

DNase free RNase contained: 10 mg/ml pancreatic RNase A, 10 mM Tris-HCl (pH 7.5), and 15 mM NaCl. This was heated to 100°C for 15 min to inactivate the DNase, allowed to cool slowly to room temperature, checked for residual DNase activity, dispensed into aliquots, and stored at -20°C.

### **2.4.5 10 x TAE Buffer**

10 x Tris acetate EDTA buffer contained: 400 mM Tris, 11.4 ml/l glacial acetic acid, and 20 mM EDTA. The pH was adjusted to 8.5.

### **2.4.6 10 x Gel Loading Dye**

10 x gel loading dye contained 50% (w/v) glycerol, 1 x TAE buffer (see Section 2.4.5), 12% (w/v) urea and 0.4% (w/v) bromophenol blue.

### **2.4.7 20 x SSC**

20 x standard saline citrate contained (g/l): NaCl, 175.4 g; and trisodium citrate, 88.2 g to give final concentrations of 3 M NaCl and 0.3 M sodium citrate. The pH was adjusted to 7.0.

### **2.4.8 TES (10/1/100)**

TES buffer (10/1/100) contained 10 mM Tris-HCl (pH 8.5), 1mM Na<sub>2</sub>EDTA (pH 7.5) and 100 mM NaCl.

#### **2.4.9 50 x Denhardt's**

Denhardt's solution (50 x) contained (g/l): Ficoll, 10; PVP, 10; and BSA, 10. This was made up to 1 litre with sterile MilliQ water, filter sterilised, and stored in 5 ml aliquots at -20°C.

#### **2.4.10 SM Buffer**

SM buffer contained (g/l): NaCl, 5.8; MgSO<sub>4</sub>·7H<sub>2</sub>O, 2; and 50 ml/l 1 M Tris-HCl (pH 7.5).

#### **2.4.11 Acrylamide mix**

Acrylamide mix contained (g/l): urea, 288; acrylamide, 34.2; and bis-acrylamide, 1.8. This mix was made up to about 500 ml and deionised with 1 g of Amberlite MB-3 (Sigma), then filtered through a sintered glass funnel (porosity 1), 60 ml of 10 x TBE sequencing buffer (see Section 2.4.12) was then added and the volume made up to 600 ml with MilliQ water. This was stored at 4°C.

#### **2.4.12 10 x TBE Sequencing Buffer**

10 x TBE sequencing buffer contained (g/l): Tris, 162.0; Na<sub>2</sub>EDTA, 9.2; and boric acid, 27.5. The pH was adjusted to 8.8. For running sequencing gels this buffer was diluted 10 x with MilliQ water to give final 1 x concentrations of 134 mM Tris, 2.5 mM Na<sub>2</sub>EDTA and 45 mM boric acid.

### **2.5 DNA Preparations**

#### **2.5.1 Alkaline Lysis *E. coli* Plasmid Preparation**

This protocol is based on a procedure described by Sambrook *et al.* (1989) which is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowitz and Burke (1981). A 2 ml volume of LB (Section 2.2.2.1) supplemented with ampicillin was inoculated with a single bacterial colony, and incubated overnight on a shaker at 37°C. From this overnight culture, 1.5 ml was transferred into an eppendorf tube, and the cells pelleted by centrifugation for 1 min. The media was removed by aspiration, leaving the pellet as dry as possible. The cells were resuspended by vortexing in 100 µl of TEG

(Section 2.4.1). This mixture was kept at room temperature for 5 min. A 200  $\mu$ l aliquot of a freshly prepared solution of 0.2 M NaOH and 1% SDS was added, and mixing was carried out by several rapid inversions. This was stored on ice for 5 min. Then 150  $\mu$ l of ice cold 5 M potassium acetate was added, and the mixture was vortexed gently for 10 seconds, then stored on ice for 5 min. The mixture was centrifuged for 5 min, and the supernatant was transferred to a clean tube. An equal volume of Tris-equilibrated phenol/chloroform was added, and the mixture vortexed and stored at room temperature for 2 min. The phases were separated by centrifugation for 5 min. The aqueous phase was transferred to a clean tube, and 2 volumes of 100% ethanol was added. The mixture was vortexed and stored at room temperature for 2 min. The DNA was collected by a 5 min centrifugation, and the ethanol was drained from the tube. The pellet was washed with 70% ethanol, centrifuged for 5 min, and the ethanol drained from the tube. The pellet was dried, then resuspended in 25  $\mu$ l of TE buffer (Section 2.4.3). RNase (10 mg/ml, Section 2.4.4) was incorporated in restriction digests at 0.5  $\mu$ g/ $\mu$ l

### 2.5.2 Large Scale *D. pini* Genomic DNA Preparation

DNA was extracted from *D. pini* using a modification of the method described by Raeder and Broda (1985). *D. pini* was grown on DM agar (Section 2.2.1.2) plates overlaid with sterile cellophane discs. Mycelia from four plates were removed from the cellophane discs, and freeze-dried. This was ground to a powder in liquid N<sub>2</sub> using a mortar and pestle, and suspended in 4 ml of extraction buffer (200 mM Tris-HCl (pH 8.0), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS) per 100 mg of dry weight mycelium which was vortexed thoroughly. For each 4 ml of extraction buffer, 2.8 ml of phenol equilibrated with extraction buffer was added and mixed thoroughly. To this, 1.2 ml of chloroform:isoamyl alcohol (24:1) was added, then mixed, and centrifuged at 17,300 g (12,000 rpm, SS34) for 40 min at 4°C. The supernatant was transferred to a clean corex tube and re-extracted with phenol/chloroform in the same proportions, then centrifuged for 15 min. DNase free RNase (10 mg/ml, Section 2.4.4) was added to the supernatant at 100  $\mu$ g/ml, and incubated at 37°C for 30 min. Phenol/chloroform (1:1) was added and the mixture was centrifuged 15 min. An equal volume of chloroform was then added to the aqueous phase which was centrifuged for 20 min. The DNA was precipitated by adding 0.54 volume of isopropanol to the aqueous phase and centrifuged for 5 min. The supernatant was then discarded. The pellet was washed with 70% ethanol, centrifuged as before and the supernatant discarded. The DNA was vacuum dried and resuspended in 100  $\mu$ l TE buffer 10/1 (Section 2.4.3), and the concentration of the DNA determined (Section 2.6.4).

### 2.5.3 Preparation of Genomic DNA for *D. pini* Library Construction

DNA was extracted from *D. pini* using a modification of a method designed to obtain high molecular weight DNA from plant tissue (Ausubel *et al.* 1990). The basis of this method is that fungal cells are lysed by the detergent Sarkosyl and the lysates digested with Proteinase K. After clearing the insoluble debris from the lysate the nucleic acids are precipitated and the DNA purified on a caesium chloride gradient.

Fresh fungal tissue (10-50 g) was harvested, then frozen with liquid nitrogen and ground to a fine powder in a mortar and pestle. The frozen powder was transferred to 2 x 250 ml Nalgene centrifuge bottles and extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 250 mM NaCl, 100 µg/ml Proteinase K) at 20 ml/g was immediately added. An appropriate volume of Sarkosyl (10% (w/v) *N*-lauryl sarcosine) was added to achieve a final concentration of 1%. The mixture was incubated for 2 h at 55°C, and the lysate centrifuged for 10 min at 5,860 x *g* (6,000 rpm, GSA) at 4°C, to pellet the debris. To the supernatant, 0.6 volume of isopropanol was added, mixed gently, then placed at -20°C for 30 min. The DNA was then precipitated by centrifugation for 15 min at 10,400 x *g* (8,000 rpm, GSA) and the supernatant discarded. The pellet was resuspended in 9 ml of TE buffer (Section 2.4.3), then 9.7 g of solid caesium chloride was added and mixed gently until dissolved. The lysates were incubated on ice for 30 min, then centrifuged for 10 min at 7,500 x *g* at 4°C. A 0.5 ml volume of ethidium bromide (10 mg/ml) was added and the lysates incubated on ice for 30 min, then centrifuged for 10 min at 7,500 x *g* at 4°C. The supernatant was transferred to two 5 ml quick-seal ultracentrifuge tubes which were balanced and well sealed, then centrifuged overnight at 300,000 x *g* (60,000 rpm, Sorvall Ultracentrifuge) at 20°C. Using a large-bore needle (15-G) and syringe the DNA band was collected. To do this a hole was first punched into the top of the tube using the needle, and the DNA band was removed by inserting the collecting needle/syringe through the tube wall directly below the band. By repeatedly extracting the collected DNA with CsCl-saturated isopropanol the ethidium bromide was removed. Two volumes of water and 6 volumes of ethanol was added to the DNA solution and mixed, then centrifuged for 10 min at 7,710 x *g* (8,000 rpm, SS34) at 4°C. The DNA pellet was resuspended in TE buffer, reprecipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol, then incubated at -20°C for 30 min and centrifuged briefly. The DNA pellet was air dried, resuspended in 500 µl of TE buffer, and the concentration of the DNA was determined (Section 2.6.4).

#### 2.5.4 Mini-prep of $\lambda$ Phage DNA

This method is a modification of the Liquid Lysate method of phage preparation (Sambrook *et al.* 1989) which was originally described by Leder *et al.* (1967). A 50 ml volume of LB (Section 2.2.2.1) supplemented with 0.2% maltose and 10 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  was inoculated with a single colony of KW251 and shaken at 37°C overnight. Volumes of 100  $\mu\text{l}$  of titred eluted phage (diluted to give  $10^6$ - $10^7$  phage/100  $\mu\text{l}$ ) and 100  $\mu\text{l}$  of the overnight bacterial culture were combined and incubated at 37°C for 30 min. The phage mixture was transferred to 50 ml of NZCYM (Section 2.2.2.3) in 500 ml flasks and shaken vigorously at 37°C for 6-8 h until lysis occurred. The culture was harvested immediately upon clearing. A few drops of chloroform were added and the culture shaken for a further 15 min to lyse any remaining cells, then transferred to sterile 250 ml Nalgene bottles and centrifuged for 10 min at 16,300  $g$  (10,000 rpm, GSA) to pellet the debris. The lysate was transferred to a fresh tube and stored at 4°C.

DNase free RNase (10 mg/ml, Section 2.4.4) and DNase (10 mg/ml, Sigma) were added to the liquid lysate to give final concentrations of 10  $\mu\text{g/ml}$ , and incubated for 1 h at 37°C. NaCl and PEG 6000 were added to give final concentrations of 0.5 M and 10% w/v, respectively, and dissolved using a magnetic stirrer, then precipitated on ice for two hours. The phage were pelleted by centrifugation at 4,920  $g$  (5,500 rpm, GSA) for 10 min at 4°C, the supernatant discarded and the bottles left upside down for 20 min to drain. The pellet was resuspended in 1 ml of SM buffer (Section 2.4.10), transferred to an eppendorf, then microcentrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a fresh tube and Proteinase K was added (final concentration 0.1 mg/ml) and incubated at 37°C for 30 min. The phage suspension was extracted twice with an equal volume of phenol/chloroform, vortexed for 20 min and centrifuged at 16,300  $g$  for 5 min. An equal volume of chloroform was then added, vortexed for 10 min and centrifuged for 5 min. The aqueous phase was transferred to a clean tube and 2 volumes of ethanol + 0.3 M ammonium acetate were added, the mixture centrifuged for 10 min, and the supernatant was discarded. The DNA pellet was washed with 70% ethanol and centrifuged for 5 min, the supernatant discarded, then recentrifuged briefly to remove excess liquid. The pellet was air dried and resuspended in TE buffer 10/0.1 (Section 2.4.3) for 20 min at 65°C, then centrifuged to remove debris. The supernatant was transferred to a fresh eppendorf and stored at 4°C.

### **2.5.5 Extraction of DNA from Seaplaque Agarose**

Restriction enzyme digested DNA (Section 2.6.1.1) and a size marker used to determine the molecular weight of the DNA fragments (Section 2.6.3) were size fractionated by electrophoresis (Section 2.6.2) on a 1% Seaplaque agarose (Biorad) gel (150 x 200 x 8 mm or 140 x 110 x 8 mm) in 1 x TAE buffer (Section 2.4.5) at 34 V for 16-20 h. The gel was stained for 30 min in ethidium bromide solution, and destained in MilliQ water. The DNA fragments to be isolated were visualised under U.V. light, photographed, and the appropriate band was excised from the gel with a sterile scalpel blade. The excess agarose was trimmed away, then the DNA containing the band was placed in a 1.5 ml eppendorf tube. To extract the DNA from the agarose, one of the following two kit based methods below were used, according to the manufacturers instructions.

#### **2.5.5.1 Bio 101 Geneclean kit**

The Bio 101 Inc. Geneclean DNA purification process (Labsupply Pierce) is based on a procedure described by Vogelstein and Gillespie (1979). To the excised DNA band 2.5 to 3 volumes of NaI stock solution was added and incubated for 5 min at 50°C to melt the agarose. The GLASSMILK was vortexed vigorously for a min, then 5 µl was added to the NaI/DNA solution, mixed, and incubated on ice for 5 min to allow binding of the DNA to the silica matrix, mixing every 1-2 min to ensure that the GLASSMILK stayed in suspension. The GLASSMILK/DNA complex was microcentrifuged for 5 seconds, and the supernatant removed and set aside until the DNA recovery was confirmed. 500 µl of ice cold NEW wash (7 ml NEW stock concentrate diluted with 140 ml sterile MilliQ water and 155 ml 100% ethanol) was added to the GLASSMILK/DNA pellet, and the pellet resuspended by pipetting up and down. This solution was microcentrifuged for 5 seconds and the supernatant was discarded. The wash procedure was repeated twice. Following removal of the supernatant from the third wash, the tube was spun for 10 seconds and the liquid remnants removed. The GLASSMILK/DNA pellet was then resuspended in 20 µl of TE buffer, incubated at 50°C for 5 min, then centrifuged for 30 seconds, and the supernatant containing the eluted DNA was placed in a new tube.

#### **2.5.5.2 Gibco BRL GlassMAX DNA Isolation Spin Cartridge System kit**

The GlassMAX Spin Cartridge purification process (Life Technologies) is based on a procedure described by Vogelstein and Gillespie (1979). A stock of wash buffer was prepared by transferring 2.5 ml of Wash Buffer Concentrate to a labelled 200 ml screw-cap container, adding 45 ml of distilled water and 52.5 ml of absolute ethanol, then

mixing thoroughly. This was stored at 4°C. The excised DNA band was weighed in a preweighed eppendorf tube, and 0.45 ml of Binding Solution per 0.1 g of agarose gel was added and incubated at 50°C for 5-7 min until the agarose was fully melted. Up to 550 µl of DNA/Binding Solution mixture was added to the GlassMAX Spin Cartridge. Volumes greater than 550 µl required more than one loading. The cartridge was centrifuged at 13,000 x *g* for 20 seconds and the tube emptied. The solution was saved until recovery of the DNA was confirmed. A 0.4 ml volume of cold wash buffer was added to the spin cartridge and centrifuged at 13,000 x *g* for 20 seconds, and the tube emptied. This step was repeated twice. After removal of the final wash buffer, the tube was centrifuged at 13,000 x *g* for 1 min. The spin cartridge was inserted into a fresh Sample Recovery Tube and 20 µl of TE preheated to 65°C was added, then the spin cartridge centrifuged at 13,000 x *g* for 20 seconds to elute the DNA.

### **2.5.6 Purification of DNA**

This method was based on that of Sambrook *et al.* (1989). A known volume of a solution of DNA was transferred to a centrifuge tube. One volume of Tris-equilibrated phenol was added to the DNA solution and vortexed thoroughly. This was stored on ice for 5 min, and then microcentrifuged for 5 min. The aqueous phase was transferred to a clean eppendorf tube. This process was repeated, first with an equal volume of phenol:chloroform (1:1 v/v), and then with an equal volume of chloroform. Either, 2.5 volumes of 95% ethanol with 0.1 volumes of 3 M sodium acetate was added, or 0.6 volumes of isopropanol was added. This mixture was vortexed thoroughly, and stored at -70°C for 30 min or left at -20°C for at least 2 h to precipitate the DNA. The DNA was pelleted by microcentrifugation for 15 min, and washed twice with an equal volume of ice cold 70% ethanol. After centrifugation for 1 min, the ethanol was drained off, and the DNA pellet was vacuum dried, then resuspended in TE buffer.

## **2.6 DNA Manipulations**

### **2.6.1 Restriction Enzyme Digests**

#### **2.6.1.1 Plasmid Digests**

Plasmid DNA (typically 50-250 ng) was digested in a total volume of 10-25 µl. Commercially prepared buffer (1x) was specifically matched to the appropriate restriction enzyme to give the salt concentration recommended by the manufacturer. When a higher

salt concentration was required for addition of an enzyme to a double digest, 1 M NaCl was used to adjust the salt concentration accordingly. A 0.5  $\mu$ l volume of restriction enzyme (10 u/ $\mu$ l) was used per 10  $\mu$ l reaction volume. Digestion was generally performed for 2 h at 37°C. An aliquot of the digest was then run on a minigel (Section 2.6.2.1) to check that the digestion was complete.

#### **2.6.1.2 Genomic Digests**

Genomic DNA (typically 3-10  $\mu$ g) was digested in a 30-60  $\mu$ l reaction volume. Commercially-prepared buffer (1x) was specifically matched to the appropriate restriction enzyme to give the salt concentration recommended by the manufacturer. A 0.5  $\mu$ l volume of restriction enzyme (10 u/ $\mu$ l) was used per 10  $\mu$ l digest volume, along with 100  $\mu$ g/ml acetylated BSA (Promega) and 500  $\mu$ g/ml DNase free RNase (Section 2.4.4). Digestion was performed overnight at 37°C. An aliquot of the digest was then run on a minigel (Section 2.6.2.1) to check that the digestion was complete. If incomplete digestion was observed, a second quantity of enzyme was added, and the mixture was incubated further at 37°C.

### **2.6.2 Agarose Gel Electrophoresis**

#### **2.6.2.1 Minigels**

Agarose was dissolved in 1 x TAE buffer (Section 2.4.5) to give a 0.7%-1.0% (w/v) gel, cooled, and poured into a Horizon 58 gel apparatus (83 x 57 x 4 mm). When set, the gel was covered with 1 x TAE buffer. Gel loading dye (Section 2.4.6) was added to an aliquot of digested DNA to give a final concentration of 10%, then this mixture was loaded onto the gel. The DNA was size fractionated at 80 V for 30 to 60 min, stained in 0.01% ethidium bromide solution for 10 min, destained in MilliQ water, visualised on a UV transilluminator and photographed using Polaroid film 667.

#### **2.6.2.2 Overnight Gels**

Agarose was dissolved in 1 x TAE buffer (Section 2.4.5) to give a 0.6%-1.0% (w/v) gel, cooled and poured into a Biorad DNA sub-cell (150 x 200 x 8 mm) or Horizon 11.14 (140 x 110 x 8 mm) gel apparatus. Digested DNA (including 0.1 volume of gel loading dye) was size fractionated in 1 x TAE electrophoresis buffer at 34 V for 16-20 h. Gels were stained in 0.01% ethidium bromide solution for 30 min, and destained in

MilliQ water. They were visualised on a UV transilluminator and photographed with a ruler alongside using Polaroid film 667 or 665.

### **2.6.3 Determination of Fragment Sizes**

Samples of *HindIII/EcoRI*-digested  $\lambda$  DNA and/or *HindIII*-digested  $\lambda$  DNA or BRL 1 kb ladder standard markers were run alongside DNA samples on agarose gels. The relative mobility (mm) of each fragment was measured as the distance migrated from the centre of the well to the centre of the band. The molecular weight was then calculated by interpolation from a plot of the distance migrated in the same gel by suitable size (molecular weight) markers, against the logarithm of the molecular weight (kb or bp) of the size markers, or by use of the computer program Cricket Graph, or the molecular weight tool box of the IS-1000 Digital Imaging System.

### **2.6.4 Determination of DNA Concentration**

DNA was quantified by three methods, by intensity of ethidium bromide fluorescence in a gel for samples of very low concentration, spectrophotometrically for pure solutions of high concentrations, or fluorometrically for impure samples of high or low concentration.

#### **2.6.4.1 Concentration Standards**

*EcoRI/HindIII*-digested  $\lambda$  DNA (200 ng), and linearised pBR322 concentration standards representing 2.5 ng/5 $\mu$ l, 5.0 ng/5 $\mu$ l, 10.0 ng/5 $\mu$ l, and 20 ng/5 $\mu$ l were run on 1% agarose minigels (Section 2.6.2.1) alongside a known volume of the DNA sample. The concentration of the DNA sample was estimated by visual comparison with the concentration standards.

#### **2.6.4.2 Spectrophotometric Method**

To 995  $\mu$ l of TE buffer, 5 $\mu$ l of DNA was added, and mixed gently. The absorbance of the solutions in quartz cuvettes with a 1 cm light path was determined at both 260 nm and 280 nm using the Shimadzu automatic spectrophotometer zeroed with TE buffer for a blank. The reading at 260 nm allowed calculation of the concentration of nucleic acid present in the sample since an OD of 1 corresponds to approximately 50  $\mu$ g/ml of double stranded DNA. The ratio of readings at 260 nm and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>) was used as an estimate of the DNA purity. Pure DNA has an OD<sub>260</sub>/OD<sub>280</sub> value of 1.8-2.0

### 2.6.4.3 Fluorometric Method

For impure DNA samples, or pure samples of low concentration, DNA was quantitated on a Hoefer Scientific TKO 100 Fluorometer. This method was suitable for quantitating down to 10 ng/ $\mu$ l and only 2  $\mu$ l of sample was needed. The fluorometer was turned on at least 15 min before use. 10 ml of 10 x TNE buffer stock solution (100 mM Tris-HCl, 10 mM EDTA, and 1.0 mM NaCl, pH 7.4) was diluted with 100  $\mu$ l of Hoescht 33258 dye (1 mg/ml) and 90 ml of sterile MilliQ water to give a 1 x TNE working solution (Working Dye Solution B). The TKO 105 glass fluorometry cuvette was filled with 2 ml of Working Dye Solution B. The sides of the cuvette were wiped clean and the cuvette was placed in the sample chamber - always in the same orientation ("G" imprint to the front). The fluorometer was adjusted to zero with the scale knob at 50% sensitivity (i.e. 5 clockwise turns of the knob from the counter clockwise position). A 2  $\mu$ l aliquot of the reference standard calf thymus DNA (1 mg/ml in 10 mM Tris-HCl, 50 mM EDTA, pH 8.0) was delivered into the 2 ml of dye solution in the cuvette and mixed by pipetting up and down without introducing bubbles into the solution. The cuvette chamber was closed and the "scale" knob adjusted until the display read "1000" indicating 1000 ng/ml. This was repeated twice more, or until the reference standard read "1000" reproducibly. The cuvette was emptied and drained thoroughly before samples. DNA samples of *D. pini* were measured in the same manner in units of ng/ml. Each sample was blanked by adjusting the "zero" control knob each time 2 ml of Working Dye Solution B was loaded. The "scale" control knob was not adjusted.

## 2.7 Subcloning

The process of subcloning typically involved recovery of DNA fragments from agarose gels (Section 2.5.5), ligation of DNA fragments into a suitable host vector (Section 2.7.3), transformation of ligation mixtures into a suitable *E. coli* host (Section 2.8.2), and then screening for recombinants by gel electrophoresis (Section 2.2.6.2.1) of diagnostic restriction digests (Section 2.6.1.1) of plasmid DNA isolated from transformants by the alkaline lysis method (Section 2.5.1). Blue/white selection ( $\alpha$ -complementation) was employed to screen for putative recombinants in a suitable *E. coli* background (strain XL1-Blue, Table 1). Transformed cells were then plated onto medium containing ampicillin, IPTG and X-gal (Section 2.2.2.1) and white colonies were screened for recombinants as outlined above.

### 2.7.1 Preparation of Insert DNA

DNA was digested with the appropriate restriction enzyme(s) (Section 2.6.1.1) and electrophoresed through a Seaplaque agarose gel (Section 2.6.2) to separate DNA fragments. The fragment(s) of interest were excised from the gel and then purified (Section 2.5.5).

### 2.7.2 Linearisation and CAP-Treatment of Vector DNA

Approximately 5.0 µg of vector DNA was digested to completion by the appropriate restriction endonuclease (Section 2.6.1.1), dephosphorylated by addition of 1.0 µl of calf alkaline phosphatase (CAP, Boehringer 1U/µl) and the mixture incubated for 30 min at 37°C. The reaction was terminated by the addition of 5 mM EDTA, 0.5% (w/v) SDS, and 50 µg/ml Proteinase K (final concentrations), mixed by inversion, and incubated for 1 h at 37°C. A phenol/chloroform extraction (Section 2.5.6) and ethanol precipitation (Section 2.5.6) were then performed and the precipitated DNA resuspended in TE (10/1) at a concentration of 200 ng/µl. This method was based on that of Sambrook *et al.* (1989).

### 2.7.3 Ligation

Ligation mixtures contained 2.0 µl of the manufacturers (New England Biolabs) 5 x ligation buffer, a 2-3 times molar excess of insert:vector (at least 20 ng of DNA insert and at least 20 ng of vector DNA), 1 µl of a 10-fold dilution (40 units) of T4 DNA ligase (New England Biolabs), and MilliQ water to 10 µl. Ligation mixtures were incubated overnight at 4°C. To check that ligation had occurred, a 1.0 µl aliquot of the ligation mix was removed prior to addition of T4 DNA ligase and the sample was examined on an agarose gel (Section 2.6.2.1) alongside a 1.0 µl sample removed after addition of T4 DNA ligase and overnight ligation. This method was based on a modification of the method proposed by Dugaiczky *et al.* (1975).

## **2.8 Transformation of *E. coli***

### **2.8.1 Calcium Chloride Transformation**

This procedure from Sambrook *et al.* (1989) is a variation of that developed by Cohen *et al.* (1972)

#### **2.8.1.1 Preparation of CaCl<sub>2</sub> Competent Cells**

A 50 ml volume of LB broth (Section 2.2.2.1) in a 250 ml flask was inoculated with the desired *E. coli* strain and grown at 37°C on a shaking platform at 225 rpm to mid log phase (OD<sub>600</sub> 0.45-0.60, about 2 h). The cells were chilled on ice for 10 min then harvested by centrifugation at 3,000 rpm for 5 min at 4°C. The cells were resuspended in 0.5 volume of ice cold 100 mM CaCl<sub>2</sub> chilled on ice for 10 min, then pelleted by centrifugation and resuspended in 0.05 volume of 100 mM CaCl<sub>2</sub>. The cells were kept on ice and used within 48 h, or 0.3 volume of 50% glycerol/100 mM CaCl<sub>2</sub> was added and the mixture dispensed into 200 µl aliquots, frozen on dry ice, and stored at -80°C.

#### **2.8.1.2 Transformation**

Competent cells (200 µl, stored on ice for 30 min before use) were mixed with 1-2 µl of DNA (Section 2.5.1) and left on ice for 30 min. Following heat shock at 42°C for 2 min, 5 ml of prewarmed (37°C) LB broth was added and the cells incubated for 30 min at 37°C. The cells were then centrifuged at 270 x *g* (1,500 rpm, SS34) for 5 min, the supernatant decanted and the cells resuspended in about 100 µl LB broth (amount remaining in tube). The cells were then spread onto a fresh LB + amp plate and incubated at 37°C for 16 h. Ampicillin resistant colonies were picked and grown for DNA extraction.

### **2.8.2 Transformation of *E. coli* by Electroporation**

This method from Sambrook *et al.* (1989) was based on a procedure developed by Dower *et al.* (1988).

#### **2.8.2.1 Preparation of Electro-Competent *E. coli* Cells**

One litre of LB broth was inoculated (1/100) with the desired *E. coli* strain and grown at 37°C with vigorous shaking to mid-log phase (OD<sub>600</sub> 0.5-1.0, about 3 h). The cells

were chilled on ice for 20 min then harvested by centrifugation at 4,000 g for 10 min (all centrifugations were at 4°C). The cells were washed sequentially (by resuspension, centrifugation at 4,000 x g to pellet, then draining supernatant) in ice cold water (1 litre followed by 500 ml) and then ice cold 10% glycerol (20 ml then finally resuspended in 4 ml). Cell suspensions were stored at -70°C in 200 µl aliquots.

### **2.8.2.2 Electroporation**

Electrocompetent *E. coli* cells (Section 2.8.2.1) were thawed gently at room temperature, divided into 40 µl aliquots in ice cold microcentrifuge tubes, 1-2 µl of DNA added (generally ligations from Section 2.7.3), mixed and the DNA/cell mixture left on ice for 1 min. The Biorad Gene Pulser Transfection Apparatus was set to 25 µF and 2.5 V and the pulse controller to 200 Ω resistance, in parallel with the sample chamber. The mixture of DNA and cells was transferred to an ice-cold 0.2 ml cuvette, shaken to the bottom, then pulsed at the above settings and the time constant checked. When a time constant of 4-5 mseconds was obtained the cells were immediately resuspended in 1 ml of LB medium (Section 2.2.2.1) and incubated at 37°C for 1 h to aid recovery of transformed *E. coli*. A positive (typically 2 ng of pUC118) and negative (water only) control was always employed. Cells were plated at suitable dilutions onto selective LB plates (Section 2.2.2.1).

Transformants were screened via informative restriction digests (Section 2.6.1.1) of plasmid DNA isolated by alkaline lysis (Section 2.5.1) followed by gel electrophoresis (Section 2.6.2.1).

## **2.9 DNA Hybridisations**

### **2.9.1 Southern Blotting**

DNA from overnight electrophoresis gels (Section 2.6.2.2) was transferred to Hybond-N nylon filters (Amersham) by a modification of the method described by Southern (1975). The DNA was denatured by gently shaking the gel in denaturing solution (0.5 M NaOH/1.5 M NaCl) for 2 x 10 min. This was followed by shaking in neutralising solution (1.5 M NaCl/0.5 M Tris-HCl, pH 7.2) for 2 x 15 min. The gel was equilibrated in 20 x SSC (Section 2.4.7) for 15 min, then the blotting apparatus assembled. Four thin pieces of sponge larger in size than the gel to be blotted, were placed in a plastic container and saturated with 20 x SSC. Two pieces of Whatmann 3 mm filter paper, the same size as

the gel were soaked in 20 x SSC, and placed on top of the sponges. Parafilm was laid around the edges of the filter paper, covering the sponges, and the gel inverted and placed onto the exposed filter paper. This was to ensure that 20 x SSC was drawn up only through the gel and membrane. A sheet of Hybond-N nylon membrane, the same size as the gel and wet with 3 x SSC was placed on top. The lower left hand corner of the gel and membrane were removed for orientation purposes. Three more pieces of Whatmann 3 mm filter paper the same size as the gel and soaked in 3 x SSC, then four dry pieces, were placed on top of the membrane. During assembly of the apparatus care was taken to remove all air bubbles. A stack of dry paper towels was placed on top and a weight upon this to assist transfer. This was left overnight at 4°C, to blot by capillary action. The apparatus was disassembled down to the membrane, onto which the position of the gel wells were marked. The membrane was then lifted from the gel, and the area above the wells was cut off using a clean scalpel. The membrane was labelled on the non-DNA side and left to air dry. The DNA was fixed to the membrane either by exposing it to UV light from a transilluminator for 2 min or by oven baking at 80°C for 2 h. The membrane was wrapped in Gladwrap and stored at 4°C until required.

### **2.9.2 Random-Primer Labelling of Probes**

The DNA to be used as a probe was digested with the appropriate enzyme (Section 2.6.1.1). Fragments were isolated from a Seaplaque agarose gel (Section 2.5.5) and the concentration of the DNA determined (Section 2.6.4). Two alternative commercial kits were used to radioactively label the DNA using the random-primer method (Feinberg and Vogelstein 1983).

DNA (25-50 ng) in a volume of no greater than 45 µl was denatured in a boiling water bath for 3 min, cooled on ice for 2 min, then centrifuged for 10 seconds. The denatured DNA, 3-5 µl of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mol, 10 µCi/µl), and sterile MilliQ water to make a total volume of 50 µl were added to a tube containing the Ready-To-Go (Pharmacia, cat. no. 27-9251-01) reagent mix, and mixed by gently pipetting up and down, then spun briefly in a microcentrifuge.

Using the High Prime DNA Labelling Kit (Boehringer Mannheim, cat. no. 1585 584) the following components were mixed on ice: 25 ng (8 µl) denatured DNA, 4 µl High Prime reaction mixture, 3 µl dATP, dGTP, dTTP mixture, 3-5 µl [ $\alpha$ -<sup>32</sup>P]dCTP, and sterile MilliQ water to give a total volume of 30 µl.

For both kits the reaction was incubated at 37°C for 15-60 min. Unincorporated nucleotides were separated from labelled DNA on a minispin Sephadex G-50 column (Section 2.9.3) equilibrated with TES (10/1/100, Section 2.4.8). The probe was denatured by boiling for 3 min and rapidly cooling on ice for 2 min. Incorporation was checked by pipetting 1 µl of the labelled probe onto a strip of polyethyleneimine paper, followed by chromatography in 2N HCl. A Geiger counter was used to estimate the ratio of incorporated [ $\alpha$ -<sup>32</sup>P]dCTP, (located at the lower end of the chromatography strip) to unincorporated [ $\alpha$ -<sup>32</sup>P]dCTP, (located at the top end of the chromatography strip).

### **2.9.3 Separation of Unincorporated Nucleotides by Minispin Column Chromatography**

Minispin columns were constructed by plugging the bottom of a 1 ml plastic, disposable, Terumo, Tuberculin syringe with sterile siliconised glass wool. The syringe was filled with Sephadex G-50 resin, equilibrated in TES (10/1/100, Section 2.4.8). Additional resin was added until the syringe was full to the 1 ml mark. The end of the syringe was inserted into the perforated cap of a 1.5 ml microcentrifuge tube. The assembly was inserted into a plastic 50 ml Falcon tube and centrifuged at approximately 1 500 x g in a BTL bench centrifuge for 3 min at room temperature in a swinging bucket rotor. Additional resin was added until the volume of resin in the syringe, after centrifugation as before, was unchanged at approximately 0.9 ml. A 100 µl volume of TES (10/1/100) was then added to the column, which was recentrifuged, this step was repeated twice. The radiolabelled DNA was added to the column in 100 µl of TES (10/1/100) and recentrifuged as before into an empty 1.5 ml microcentrifuge tube.

### **2.9.4 Hybridisation of Probe DNA to Southern Blots**

Nylon membranes were placed into glass hybridisation tubes, and 30 ml of hybridisation solution (3 x SSC, 0.02 % Denhardt's, 0.5% SDS, 50 µl/ml salmon sperm DNA, MilliQ water) was added. The membranes were rotated in a hybridisation oven at the appropriate temperature (45°C-65°C) to prehybridise for at least 2 h. The hybridisation solution was tipped out of the tube, and 7 ml of fresh solution added. Denatured radiolabelled probe was added to the appropriate tube, and hybridised with the membrane for 20-28 h.

### 2.9.5 Autoradiography of Southern Blots

The membranes were washed three times in washing solution (3 x SSC, 0.2 % SDS) for 20 min at hybridisation temperature, then the membrane was covered in Gladwrap while still damp, placed in a cassette, and exposed to Kodak or Fuji X-ray film in the presence of Cronex intensifying screens at -70°C for a length of time appropriate to the signal intensity. After exposure, the film was developed for 3 min, fixed for 3 min, rinsed with water and dried at 37°C. When a more stringent wash was necessary, washing was performed at 65°C in 1 x SSC, 0.2% SDS.

### 2.9.6 Stripping the Filters

Filters which were to be probed more than once had the hybridised DNA removed by immersion in boiling stripping solution (1 mM EDTA, 0.1% SDS) which was left to cool, shaking, at room temperature. The membrane was then exposed to Kodak or Fuji X-ray film in the presence of Cronex intensifying screens at -70°C for 24 h to ensure that all the radiolabelled DNA had been removed. The filter must remain moist after hybridisation to ensure successful stripping. If stripping was incomplete this process was repeated.

## 2.10 Genomic Library Construction

This procedure is a modification of that outlined in the Promega Protocols and Applications Guide (1991).

### 2.10.1 Establishing Conditions for Partial Digestion of High Molecular Weight Genomic DNA

Once high molecular weight DNA was isolated (Section 2.5.3) it was necessary to make fragments of the correct sizes (15-23 kb), with suitable sticky ends compatible with the cloning site, to ensure a successful, representative genomic library. This was done by partial digestion with a restriction enzyme (Section 2.6.1.2). In order to establish the optimum enzyme concentration, to generate fragment sizes of 15-23 kb, small scale reactions were performed with *Mbo*I (10 u/μl, Gibco BRL). To ensure that *Mbo*I cut the DNA a 10 μl trial digest was first performed with 100 ng of genomic DNA, 5.0 u of *Mbo*I, and 10 x buffer M (Boehringer Mannheim). This was digested for 2 h and an aliquot was run on a minigel (Section 2.6.2.1) to check digestion. A reaction mixture

containing: 5 µg of DNA, 10 x buffer M, and MilliQ water to a final volume of 150 µl was prepared. From this mixture serial dilutions were performed on ice by numbering 9 individual eppendorf tubes, and adding 30 µl to tube 1 and 15 µl to each of tubes 2-9. To tube 1, 10 u of *Mbo*I was added, and 2-fold serial dilutions were then performed to tubes 2-9. The reactions were incubated at 37°C for 2 h then run overnight on 30 V on a 1% Seaplaque agarose gel (Section 2.6.2.1) at 4°C. Uncut DNA was loaded as a control, and *Eco*RI/*Hind*III-digested lambda DNA and *Hind*III-digested lambda DNA were included on either side as size markers. By observing the gel polaroid, the amount of enzyme needed to produce the maximum number of molecules in the desired size range was determined. The intensity of fluorescence is related to the mass distribution of the DNA. To obtain the maximum number of molecules in the desired size range, half the amount of enzyme that produced the maximum amount of fluorescence was used in the large scale digests (Seed *et al.* 1982).

### **2.10.2 Large Scale Preparation of Partially Digested DNA**

Using the optimised conditions determined in Section 3.2, digests using 5 µg of high molecular weight DNA were carried out using identical enzyme concentrations, time, temperature, materials and DNA concentrations as in the small scale digests. A 100 ng aliquot was checked on a minigel (Section 2.6.2.1) to ensure digestion, and to check the size distribution. The reaction was stopped by heating the digest at 65°C for 15 min. Two volumes of 95% ethanol and 0.1 volume of 3M sodium acetate was added, vortexed gently then incubated at -20°C overnight. The sodium acetate/ethanol mixture was centrifuged for 20 min, the supernatant removed, and the pellet recentrifuged briefly to remove excess fluid. The pellet was then washed with 70% ethanol, centrifuged for 5 min, the supernatant removed, and recentrifuged briefly to remove excess ethanol, then air dried. Each pellet was then resuspended at 4°C overnight in 10.5 µl of TE (10/1) to give a combined volume of 21 µl. Two 0.5 µl aliquots (controls 1 and 2) were removed for a control gel (Section 2.10.4.1). The DNA was stored at -20°C.

### **2.10.3 Partial Fill-in Reaction for Genomic DNA**

This procedure used the LambdaGEM-12 *Xho*I Half-Site Arms Cloning System and was performed as outlined in the Promega Technical Bulletin 005. The cloning strategy used with this system relies on the high specificity with which partially filled-in *Xho*I half-site arms can be combined with partially filled-in genomic DNA digested with *Mbo*I. The only ligation products possible are single copies of genomic inserts with arms, since the partial fill-in prevents self-ligation reactions of vector arms, central stuffer (which is still

present in the vector preparation), and genomic fragments. A 50  $\mu$ l reaction mixture was set up containing: 11  $\mu$ l of MilliQ water, 5  $\mu$ l of 10 x BRL REact buffer 4, 20  $\mu$ l of digested genomic DNA prepared as described above (8  $\mu$ g, first heated to 65°C for 15 min, then put on ice), 5.0  $\mu$ l each of dATP and dGTP (to give a final concentration of 1 mM), and 4.0  $\mu$ l of Klenow (to give a final concentration of 1 u Klenow/ $\mu$ g DNA). This was incubated at 37°C for 30 min, then extracted twice with 1 volume of phenol:chloroform (1:1) saturated with TE buffer (pH 8.0), vortexed for 1 min and centrifuged at 12,000 rpm for 5 min. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1) added, vortexed for 1 min and centrifuged. This step was then repeated. The upper aqueous phase was transferred to a fresh tube and 0.1 volume of 3M sodium acetate and 2 volumes of ethanol were added. The mixture was then incubated at -70°C for 30 min and following this centrifuged at 12,000 rpm for 15 min. The supernatant was then carefully poured off and the pellet washed with 1 ml of 70% ethanol, centrifuged for 5 min, the supernatant removed and the pellet recentrifuged to remove the excess liquid. The pellet was air dried and resuspended in 15  $\mu$ l TE (10/1) overnight at 4°C. Two 0.5  $\mu$ l aliquots (controls 3 and 4) were removed for a control gel (Section 2.10.4.1).

## **2.10.4 Ligation of Insert to Vector Arms**

### **2.10.4.1 Klenow/Ligation Controls**

Using the control aliquots, labelled 1-4, removed in Sections 2.10.2 and 2.10.3, ligation reactions were performed to ascertain whether efficient end-filling had been achieved in the Klenow reaction, and to ensure that ligation reagents were working correctly. Reactions 1 and 4 contained DNA (Sections 2.10.2 and 2.10.3, respectively) and MilliQ water to a volume of 5  $\mu$ l. Reactions 2 and 3 contained DNA (Sections 2.10.2 and 2.10.3, respectively), 1 x ligase buffer, 1 mM ATP, T<sub>4</sub> ligase, and MilliQ water to a final volume of 5  $\mu$ l. The reactions were incubated at 25°C for 2 h and then overnight at 4°C. Controls 1-4 represented: cut only DNA (1), cut and ligated DNA (2), cut, end-filled and ligated DNA (3), and cut and end-filled DNA (4). The reactions were heated at 65°C for 10 min then examined on an agarose gel (Section 2.6.2.1) with concentration standards. If end-filling had been achieved, self-ligation should not occur, since the vector ends were no longer complementary.

#### 2.10.4.2 Determination of Optimum Ligation Conditions

To determine the optimal ligation conditions for a genomic insert, three small scale reactions were performed containing: 1  $\mu$ l of 10 x ligase buffer, 1  $\mu$ l of 10 mM ATP, 2.0  $\mu$ l of 0.5  $\mu$ g/ $\mu$ l vector DNA (Lambda GEM-12 *Xho*I half site arms), 1  $\mu$ l of T<sub>4</sub> ligase, and MilliQ water to a final volume of 10  $\mu$ l. Reactions B and C, also contained 2.0  $\mu$ l and 0.5  $\mu$ l of 0.5  $\mu$ g/ $\mu$ l insert DNA, respectively. Reaction A represented a negative control lacking insert DNA (which determined any background levels of religated arms), reaction B a 1:1 vector to insert ratio, and reaction C a 1:4 vector to insert ratio. The reactions were ligated at 25°C for 2 h and then overnight at 4°C.

#### 2.10.5 Packaging of Ligated DNA and Titration of Recombinant Phage

This procedure used the Packagene Lambda DNA Packaging System kit derived from a method described by Rosenberg *et al.* (1985) and Rosenberg (1987), and was performed as outlined in the Promega Technical Bulletin 005.

##### 2.10.5.1 Packaging of Ligated DNA

Two Packagene extracts were removed from -80°C and placed directly onto ice to thaw. 25  $\mu$ l of each 50  $\mu$ l extract was removed immediately after thawing and transferred to labelled eppendorf tubes to give 4 x 25  $\mu$ l extracts (A, B, C, and D). To extracts A-C, 2.5  $\mu$ l of the corresponding ligation mixture was added (Section 2.10.5). To extract D, 0.25  $\mu$ g (0.64  $\mu$ l) of control DNA ( $\lambda$ c1857 *Sam*7, 392  $\mu$ g/ $\mu$ l) was added. The extract/DNA mixtures were then incubated at 22°C for 3 h. After incubation, 222.5  $\mu$ l of SM buffer was added to each tube, mixed, and 12.5  $\mu$ l of chloroform added and mixed by gentle inversion. Packaged phage could be stored at 4°C for 7 days with no drop in titre, or up to 3 weeks, although the titre may drop several-fold.

##### 2.10.5.2 Titration of Packaged Phage on LB Plates

Two days previous to the ligation and packaging being performed, a single colony of *E. coli* strain KW251 bacteria was used to inoculate 3 ml of LB medium supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O and incubated with shaking overnight at 37°C. This was done in duplicate. The following day, 500  $\mu$ l of the overnight culture was subcultured into 50 ml of LB media supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O. This was shaken at 37°C for approximately 2.5 h or until the OD<sub>600</sub> had reached 0.6-0.8. This could be stored at 4°C for up to 24 h.

Ten-fold serial dilutions of  $10^{-1}$  to  $10^{-4}$  of each of the 4 tubes of packaged phage were made in SM buffer. To 100  $\mu$ l of the overnight KW251 culture in a sterile 5 ml test tube, 100  $\mu$ l of the diluted phage was added, and the phage allowed to absorb to the cells by incubation at 37°C for 30 min. A 1 ml volume of 1 M  $MgSO_4$  was added to 100 ml of TB top agar that had been melted and cooled to 45°C in a waterbath. To each tube containing infected cultures, 3 ml of the molten TB top agar was then added, mixed, and immediately poured onto LB plates prewarmed to 37°C. The top agar was allowed to harden and the inverted plates incubated at 37°C overnight.

The next day, plaques were counted and the titre of the phage calculated in plaque forming units (pfu) per ml, as described below:

$$\frac{\text{number of plaques} \times \text{dilution factor}}{\text{volume of extract plated (ml)}} \\ = \text{pfu/ml}$$

The packaging efficiency of the arms was calculated by dividing the pfu/ml by the concentration of the vector DNA packaged (1  $\mu$ g/ml) to give recombinants per  $\mu$ g of  $\lambda$  DNA.

Titres were compared to determine the optimal relative concentrations of vector arms and genomic DNA insert for ligation. The yield of recombinant plaques (from tubes B and C) should be at least 100- to 1,000 fold greater than the yield of non-recombinant plaques on the negative control plate (Section 2.10.4). Large-scale reactions were then performed based on the optimal ratio of insert to vector DNA determined above. The expected packaging efficiency for the LambdaGEM-12 *Xho*I Half-Site Arms positive control was  $2 \times 10^6$  recombinants/ $\mu$ g DNA and that of the negative control (background nonrecombinant Half-Site Arms) was  $\leq 100$  pfu/ $\mu$ g.

#### **2.10.6 Large Scale Packaging of Ligated DNA**

The optimal ratio of vector arms and genomic DNA insert for ligation was found to be 1:4 (ligation C) (Section 2.10.5). The remaining 7.5  $\mu$ l of ligation C was divided into 2.5  $\mu$ l and 5.0  $\mu$ l in eppendorfs to which 25  $\mu$ l and 50  $\mu$ l of Packagene extract was added respectively. These reactions were named C<sub>1</sub> and C<sub>2</sub>, respectively. The reactions were mixed by gently tapping the bottom of the tube several times. Conditions and times of incubation were identical to those outlined in Section 2.10.6. After incubation 222.5  $\mu$ l of SM buffer and 12.5  $\mu$ l of chloroform was added to packaging reaction C<sub>1</sub> and 445  $\mu$ l

of SM buffer and 25  $\mu\text{l}$  of chloroform was added to packaging reaction  $C_2$ . The packaged phage (i.e.  $C$ ,  $C_1$ , and  $C_2$ ) were combined, 150  $\mu\text{l}$  of which was stored temporarily at  $4^\circ\text{C}$  and 279  $\mu\text{l}$  of which (in 3 x 93  $\mu\text{l}$  aliquots) was stored at  $-80^\circ\text{C}$  with dimethyl sulphoxide (DMSO) to a final concentration of 7%. Titration of the packaged phage was then performed as outlined in Section 2.10.5.2.

To determine whether enough clones were present in the library as to include all sequences the following equation derived by Clarke and Carbon (1976), which relates the probability ( $P$ ) of including any DNA sequence in a random library of  $N$  independent recombinants, from a genome size of  $n$ , was used:

$$N = \frac{\ln(1 - P)}{\ln(1 - 1/n)}$$

Therefore, to determine a 99% probability ( $P = 0.99$ ) of including any particular sequence in a random *D. pini* library (assuming the *D. pini* genome to be  $4 \times 10^4$  kb) the following calculation was performed:

$$\begin{aligned} N &= \frac{\ln(1 - 0.99)}{\ln(1 - 1/4 \times 10^4)} \\ &= 1.2 \times 10^4 \end{aligned}$$

### 2.10.7 Amplification of the Library

This procedure is based a method described by Sambrook *et al.* (1989). Bacterial cells for plating were prepared as described in Section 2.10.5.2. The titred packaged phage containing the library to be amplified was gently mixed and then briefly microcentrifuged to separate out the chloroform. The chloroform was then removed and the packaged phage added to plating bacteria (0.25 ml of host cells for every  $1 \times 10^5$  phage). The host/phage mixture was incubated at  $37^\circ\text{C}$  for 30 min to allow the phage to absorb the bacteria. Molten ( $45^\circ\text{C}$ ) TB top agar (3 ml per 200  $\mu\text{l}$  host/phage mixture) was then added to the tube, mixed, and then poured onto a prewarmed ( $37^\circ\text{C}$ ) LB plate. The top agar was allowed to harden and the inverted plate incubated at  $37^\circ\text{C}$  overnight. The following day 10 ml of SM buffer was added to the plate lawn placed on a level surface and incubated at  $4^\circ\text{C}$  overnight to elute the phage. Using a pipette the SM buffer was removed and transferred into a polypropylene Nunc tube. A 0.5 ml volume of chloroform was added and mixed by inverting several times. The amplified library was

then titred as described in Section 2.10.6 (a titre of  $10^{10}$  to  $10^{11}$  PFU/ml was expected). DMSO to a final concentration of 7% was added and the amplified library stored at  $-80^{\circ}\text{C}$ .

## 2.11 Library Screening by Plaque Hybridisation

Recombinant phage were screened by plating phage (Section 2.11.1), taking filter lifts off the resulting plaques (Section 2.11.2), and hybridisation of an appropriate probe to the filters (Section 2.11.3). Plugs containing positive plaques were stored in SM buffer (Section 2.4.10) containing a drop of chloroform. The phage from these plaques were purified twice more by screening as outlined above except only 30-300 phage per plate were plated on the second and third screens. DNA was then extracted from the positive plaques (Section 2.5.4). This method was based on that of Sambrook *et al.* (1989).

### 2.11.1 Plating Phage $\lambda$

*E. coli* strain KW251 was used as a host for  $\lambda$  phage. The phage population to be screened was diluted to  $1.5 \times 10^4$  pfu/ml in SM buffer (2.4.10) and 100  $\mu\text{l}$  of phage mixed with 100  $\mu\text{l}$  of an overnight culture of KW251 grown in LB medium (Section 2.2.2.1) supplemented with 0.2 % maltose and 10 mM  $\text{MgSO}_4$ . The host/phage mixture was incubated at  $37^{\circ}\text{C}$  for 30 min, 3 ml of molten ( $45^{\circ}\text{C}$ ) TB top agar (Section 2.2.2.2) was then added to the tube, mixed, and poured onto a prewarmed ( $37^{\circ}\text{C}$ ) LB plate (see Section 2.2.2.1). The top agar was allowed to set and the inverted plates were incubated at  $37^{\circ}\text{C}$  overnight. The plates were stored at  $4^{\circ}\text{C}$ .

### 2.11.2 Filter Lifts

Nylon filters (Biorad 162-0162 or Biotechnology Systems WEF-978) were placed on the KW251/phage lawn (Section 2.11.1) and marked asymmetrically so the position of the filters was known for later reference. Four lifts were taken from each plate with the filter left on the plate for 1 min once completely wet, the second for 2 min, the third for 4 min and the fourth for 7 min. The filters were then removed and placed DNA side up on 3 layers of 3 MM filter paper soaked in 500 mM NaOH, 500 mM NaCl (2 min) then 500 mM Tris-HCl (pH 7.4), 2 M NaCl (5 min) and finally 2 x SSC (2 min). The filters were then air dried and baked in a vacuum oven at  $80^{\circ}\text{C}$  for 2 h. On the second and third screens only two lifts were taken from each plate. This procedure is adapted from that of Ausubel *et al.* (1990).

### **2.11.3 Hybridisation of Phage $\lambda$ DNA [ $\alpha$ - $^{32}\text{P}$ ]dCTP Labelled DNA Probe**

Labelling of the DNA to be used as a probe, hybridisation of filters to labelled DNA and autoradiography was performed as detailed in Sections 2.9.1-2.9.5. The hybridisation temperature was 65°C. Positive plaques were identified as signals on autoradiographs in identical positions on duplicate filters. The positions of the positive plaques on the KW251 lawn were established by alignment of the asymmetric markings on the plates and filters with the autoradiograph, and plaques corresponding to signals on the autoradiographs were picked. Positive plaques were removed with the pipette tip of a 1 ml Gilson autopipettor from which the end 5-10 mm had been removed and were placed in an eppendorf with 1 ml of SM buffer and 20  $\mu\text{l}$  of chloroform for 2 h at room temperature to allow the phage to elute, then stored at 4°C.

## **2.12 DNA Sequencing**

Two different sequencing protocols were employed, the Sequenase Version 2.0 DNA Sequencing Kit from USB and the AmpliCycle Sequencing Kit from Perkin Elmer. Both methods are based on the dideoxy-mediated chain termination method of Sanger (1977). One major difference between these two methods was in the DNA polymerase used. The AmpliCycle Sequencing Kit used a recombinant form of a modified thermostable DNA polymerase. This AmpliTaq DNA polymerase allowed sequencing reactions to be performed at high temperatures (chain extension/termination reactions were performed at 72°C), and thermal cycling of the sequencing reactions, allowing multiple cycles of template melting, primer annealing and extension, hence only a very small amount of target DNA was needed before readable sequence was obtained.

### **2.12.1 Preparation of DNA for Sequencing**

#### **2.12.1.1 Preparation of Single Stranded M13 Template DNA**

Single stranded recombinant M13 DNA for sequencing with the Sequenase Sequencing Kit was prepared according to a procedure described by Sambrook *et al.* (1989). A  $\lambda$  DNA fragment was subcloned into M13mp18 and transformed into *E. coli* XL1-Blue cells (Section 2.8.1) from which DNA was extracted by the following method. A 5 ml volume of LB (Section 2.2.2.1) was inoculated with a single bacterial colony of *E. coli* strain XL1-Blue, and incubated overnight on a shaker at 37°C. From this overnight

culture, 500  $\mu$ l was added to 50 ml of LB and the mixture transferred in 1.5 ml aliquots to 5 ml Kimax tubes. Using sterile toothpicks recombinant plaques were stabbed, and the bottom half of the toothpick broken off and immersed in the LB culture. The tubes were shaken vigorously at 300 rpm at 37°C for 6 hours. The cells were then transferred into eppendorfs and pelleted by centrifugation for 5 min. The supernatant, containing the phage, was decanted into 200  $\mu$ l of a solution containing 20% (w/v) PEG 6000 and 2.5 M NaCl. The mixture was kept for 30 min at room temperature, then the phage were pelleted by centrifugation for 5 min. The supernatant was discarded, and any remaining traces of the supernatant was removed by wiping the inside of the tube with tissue. The phage pellet was resuspended in 100 TE buffer (10/0.1), vortexed, and 50  $\mu$ l of phenol added, the mixture vortexed again, and centrifuged for 3 min. The aqueous phase was transferred to a fresh tube and extracted with an equal volume of phenol:chloroform (1:1), vortexed for 20 min, and centrifuged for 3 min. To the aqueous phage an equal volume of chloroform was then added, vortexed for 10 seconds and centrifuged for 5 min. An equal volume of chloroform was then added to the aqueous phase and centrifuged for 1 min. To this, 2.5 volumes of 95% ethanol + 0.3 M sodium acetate were added and the mixture left at -80°C for 30 min. Following this, the mixture was centrifuged for 5 min, and the supernatant discarded. The DNA pellet was washed with 70% ethanol, then centrifuged briefly to remove excess liquid. The pellet was air dried and resuspended in 30  $\mu$ l of TE buffer 10/0.1 (Section 2.4.3). A 2  $\mu$ l aliquot was electrophoresed through a 1 % (w/v) agarose gel (Section 2.6.2.1) to determine the concentration of the DNA. The templates were stored at -20°C.

#### **2.12.1.2 Preparation of Template for AmpliCycle Sequencing**

DNA for sequencing with the AmpliCycle Sequencing Kit was prepared by subcloning a  $\lambda$  DNA fragment into pUC118 and transforming it into *E. coli* XL1-Blue cells (Section 2.8.2) from which DNA was extracted by an alkaline lysis preparation (Section 2.5.1). This procedure was performed by N. Forester.

#### **2.12.2 Sequenase Version 2.0 Protocol**

For each set of sequencing reactions a 10  $\mu$ l cocktail was prepared containing: 1  $\mu$ l of universal primer, 2  $\mu$ l of 5 x sequenase sequencing buffer, 7  $\mu$ l of single-stranded template DNA (0.2-1.0  $\mu$ g), and 1  $\mu$ l of MilliQ water. The solution was mixed, heated to 65°C, and the primer left to anneal by cooling slowly to 30°C over 30 min. While the annealing mixes were incubating the chain termination mixes were set up on a microtitre

plate: one set of four termination mixes per sequencing reaction; 2.5  $\mu\text{l}$  of the appropriate d/ddNTP mix per well. Following the 30 min incubation of the annealing mixes, 1.0  $\mu\text{l}$  of 0.1 M dithiothreitol, 2.0  $\mu\text{l}$  of labelling mix (diluted 5-fold in MilliQ water), 0.5  $\mu\text{l}$  of [ $\alpha$ - $^{35}\text{S}$ ]dATP (3000 Ci/mol, 10  $\mu\text{Ci}/\mu\text{l}$ ), and 2.0  $\mu\text{l}$  of Sequenase Polymerase (diluted 8-fold in enzyme dilution buffer, pH 7.4) were added to the annealing mix. These labelling reactions were incubated at room temperature for 2-5 min, then 3.5  $\mu\text{l}$  of the labelling reactions were transferred to each of four termination mixes on the microtitre dish and incubated at 37°C for 5 min. Sequenase stop solution (4  $\mu\text{l}$ ) was then added to each well and the reactions stored at -20°C for up to 1 week.

### 2.12.3 AmpliCycle Sequencing Protocol

Four different primers were used to sequence one template in order to get complete coverage (Table 2). For each set of four sequencing reactions a 30  $\mu\text{l}$  cocktail was prepared containing: 22  $\mu\text{l}$  of sterile MilliQ water, 2  $\mu\text{l}$  of DNA template (20 ng), 1  $\mu\text{l}$  of primer (20  $\mu\text{M}$ ), 4  $\mu\text{l}$  of 10 x cycling mix, and 1  $\mu\text{l}$  [ $\alpha$ - $^{33}\text{P}$ ]dCTP (3000 Ci/mol, 10  $\mu\text{Ci}/\mu\text{l}$ ). For each set of sequencing reactions four 0.2 ml thin wall PCR tubes (Biotek) were labelled G, A, C, and T and 2  $\mu\text{l}$  of the appropriate d/ddNTP termination mix was added to each tube and the tubes left on ice. To each tube containing the d/dNTPs, 6 $\mu\text{l}$  of the enzyme/primer/template cocktail was then added and mixed gently. The reaction tubes were then placed in a Corbett FTS-960 thermal cycler preheated to 95°C, and after an initial 2 min melt at 95°C, subjected to 25 cycles of 95°C for 30 sec (denaturing), 60°C for 30 sec (annealing), and 72°C for 1 min (extension). After the cycles were complete 4  $\mu\text{l}$  of stop solution was added to each tube and the reactions were stored at -20°C for up to 1 week.

### 2.12.4 Polyacrylamide Gel Electrophoresis (PAGE) of Sequencing Reactions

Sequencing gels were poured with 70 ml of acrylamide mix (Section 2.4.11) containing 42  $\mu\text{l}$  of TEMED and 420  $\mu\text{l}$  of 10% (w/v) ammonium persulphate. Once set the gel was pre-run for 15-60 min with constant power (65 W) in 1 x TBE sequencing buffer (Section 2.4.12). Sequencing reactions were then denatured at 75°C for 2 min and 3  $\mu\text{l}$  loaded onto the sequencing gel. These reactions were run until the second dye front (the xylene cyanol FF) had run off the end of the gel (typically 4 h) then the same set of reactions were loaded onto the gel and run until the first dye front from these reactions (the bromophenol blue) had run off the gel (typically 2 h). If necessary medium runs were performed where the same sequencing reactions were loaded onto the gel after 2 h,

**Table 2 Primers used in Sequencing Reactions**

Primer	Size	Tm°C <sup>a</sup>	Sequence <sup>b</sup>	Source
M13 (-40)	17 mer	52	GTTTTCCCAGTCAC GAC	USB
Forward M13 ( <i>lacZ</i> )	22 mer	70	GCCAGGGTTTTCC CAGTCACGA	Perkin Elmer
Reverse M13 ( <i>lacZ</i> )	24 mer	70	GAGCGGATAACAA TTTCACACAGG	Perkin Elmer
VRA 1	24 mer	66	GGTGATGTACGTT GTAAACATTTG	This Study <sup>c</sup>
VRA 2	22 mer	70	GGAAGACGCGGTC GAATTCCTG	This Study <sup>c</sup>

<sup>a</sup> Calculated as  $T_m = 2(A+T) + 4(G+C)$  from Hakura *et al.*, (1984)

<sup>b</sup> 5' to 3' sequence

<sup>c</sup> Gibco BRL custom made

giving runs of 2 h (short), 4 h (medium) and 6 h (long). The gel was then disassembled, fixed in a solution containing 10% acetic acid and 10% ethanol for 30 min, transferred onto Whatman 3 mm filter paper, dried for 30 min under vacuum at 80°C, and then autoradiographed overnight.

## 3 RESULTS

### 3.1 Determining Optimal Mycelial Growth Conditions in Culture

Previous studies on the effects of temperature and medium on *D. pini* cultures have aimed to optimise *in vitro* conditions for dothistromin production (Gallagher 1971, Shaw 1975). These studies found temperature and growth medium components to have marked effects on dothistromin accumulation. Malt was shown to be a satisfactory medium for dothistromin production and mycelial growth. Nutrient agar/broth was also suggested as a favourable media component. However, because these studies gave limited consideration to mycelial growth, further investigation was required to establish a clearly defined procedure for culture conditions. Based on these previous studies, and for the purpose of further experiments in this study, a growth media containing malt extract and nutrient agar/broth was designated *D. pini* media (DM) (Section 2.2.1).

#### 3.1.1 Determining Optimal Solid Media for Mycelial Growth

DM agar, and ten other media omitting malt extract and/or nutrient agar, and/or including additional, ingredients (Section 2.2.1) were tested for their ability to support mycelial growth. The agar cultures were inoculated in triplicate by three different methods (Section 2.3.1) which were then incubated at 20°C, 25°C, and 30°C for 21 days.

##### 3.1.1.1 Temperature Effect on Mycelial Growth

Comparison of the amount of mycelial growth on the same media, showed that temperature conditions markedly affect mycelial growth. Satisfactory growth was observed only at 20°C. The temperatures of 25°C and 30°C either prevented or severely reduced growth.

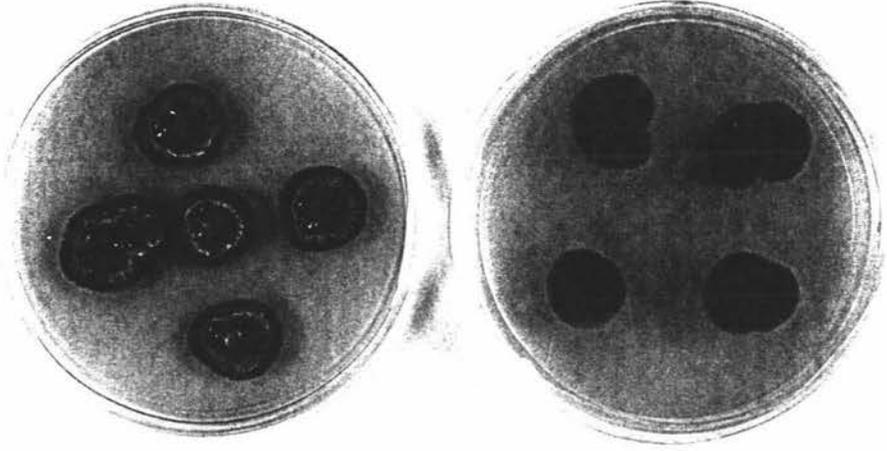
##### 3.1.1.2 Media Effect on Mycelial Growth

Observation of the cultures showed that different media affect the amount of mycelial growth (Fig. 3A-F). Colony morphology and colour, and dothistromin production also varied significantly (Fig. 3A-F). Production of dothistromin was indicated by a red-black colouring of the surrounding media, most obvious on the underside of the plate.

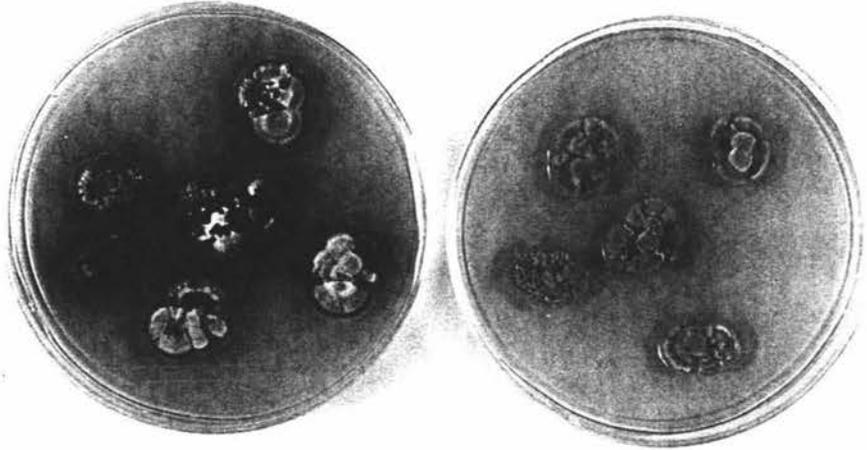
**Fig. 3A-F      Demonstration of media differences on mycelial growth and appearance**

Agar plates (where <sup>a</sup> = pieces, <sup>b</sup> = streak, and <sup>c</sup> = spread, referring to different inoculation methods), left to right: **A** MEA<sup>a</sup>, MEA + ura<sup>a</sup> (underside); **B** MYG<sup>a</sup>, YPG<sup>a</sup>; **C** PDA<sup>a</sup>, DM + glu<sup>a</sup>; **D** DM + glu<sup>b</sup>, DM<sup>b</sup> (underside); **E** NMY<sup>b</sup>, NMY + glu<sup>b</sup>; **F** MM<sup>c</sup>, MM<sup>b</sup>.

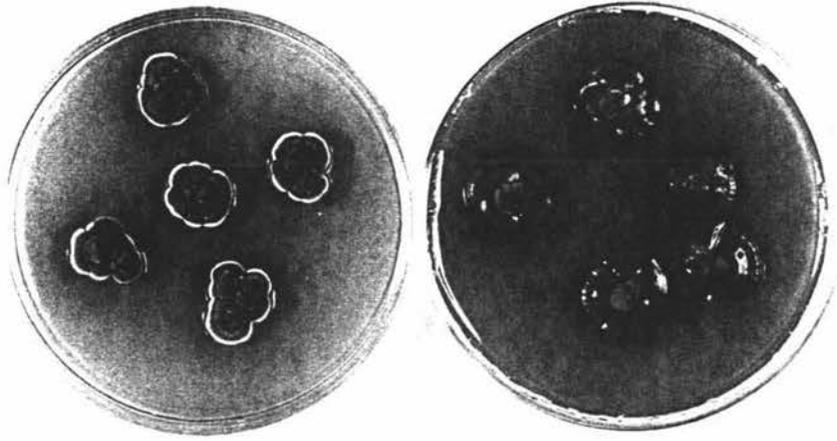
A



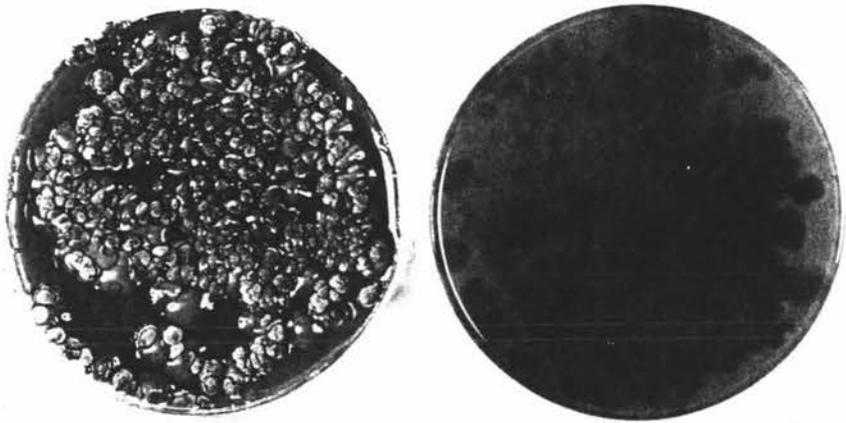
B



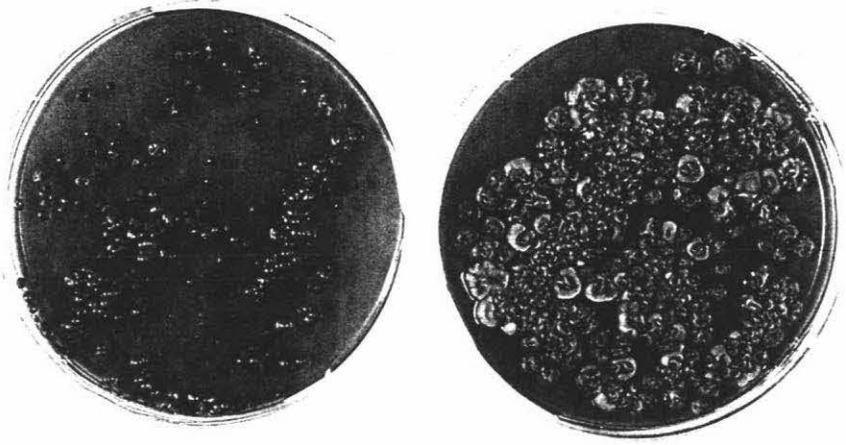
C



D



E



F

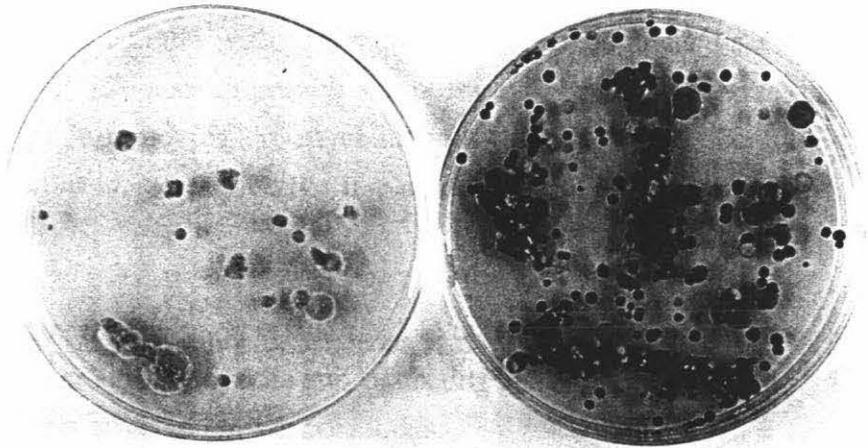


Table 3 summarises the phenotypic data for cultures grown at 20°C. It was found that the amount of mycelial growth was high for those media containing malt extract, and even higher for those containing malt and nutrient agar. Cultures grown on media without malt did not grow well. The most favourable media for mycelial growth was DM agar and the least favourable were YPG and MM. Dothistromin production was mostly associated with high-moderate growth levels. These observations support the earlier studies which suggest that a media containing malt extract and nutrient agar should be used for culturing *D. pini*.

### **3.1.1.3 Effect of Inoculation Method and Incubation Period on Mycelial Growth**

Due to the inability to achieve sporulation of *D. pini* strain Dp1, three different inoculation methods were performed to determine an effective and convenient inoculation method. Over the growth period of 21 days the cultures were observed every 3 days and the mycelial growth compared with their original inoculum size. The spread method of inoculation was observed to be most effective with the highest level of growth, and the streak method the least effective with very low growth. Mycelia inoculated as pieces produced a moderate-low level of growth. The optimum time for incubating cultures was assessed to be between 7-10 days, after which mycelial growth and any associated dothistromin production appeared to slow markedly.

A comparison of DM agar cultures with, and without, sterile cellophane discs was also performed. No obvious difference in growth was observed. But the presence of discs provided an advantage in removing the mycelia from the plates for subculturing or harvesting. It also allowed more effective implementation of the spread method of inoculation.

Overall, the above observations suggest that media containing malt and nutrient agar, inoculated by spreading ground up mycelia and incubating at a temperature of 20°C for 7-10 days, provides optimal mycelial growth conditions.

### **3.1.2 Quantitation of Mycelial Growth in Liquid Media**

To provide a quantitative indication of the importance of malt for mycelial growth (observed in Section 3.1.1.2), the mycelial dry weight of duplicate liquid cultures, with nil to 5% malt (see Appendix 1), were averaged and compared (Table 4). All cultures contained nutrient agar. It was found that mycelial dry weight was high for those

**Table 3 Optimisation of Media and Method of Inoculation at 20°C**

Media	Pieces	Spread	Streak
DM	1	1	2
DM + glu	1	1	2
MEA	2	6	2
MEA + ura	4	4	4
MYG	4	4	4
YPG	5	5	5
PDA	3	6	3
PDA + glu	4	6	4
NMY	2	2	2
NMY + glu	2	2	6
MM	5	5	6

1 high growth

2 moderate growth

3 low growth, but still same form

4 low growth with altered morphology and colour, dothistromin produced

5 low growth with altered morphology and colour, no dothistromin produced

6 nil growth

**Table 4 Quantitation of Mycelial Growth with Malt Extract Concentration**

Media <sup>a</sup>	Mycelial Dry Weight <sup>b</sup> (g/100 ml)
1% malt	0.088
1% yeast	0.486
3% malt	0.575
5% malt (i.e. DM broth)	0.665
5% malt + 1% yeast	0.617

<sup>a</sup> all media contained nutrient broth

<sup>b</sup> results are the mean from duplicate flasks

cultures containing 5% malt and 1% yeast (NMY broth), and even higher for those cultures containing 5% malt with no added yeast (DM broth). The lowest mycelial dry weight was for media containing 1% malt (altered DM broth) and next lowest for media containing 1% yeast (NY broth). These results suggest that liquid media containing 5% malt extract and nutrient broth allow for optimal mycelial growth. This supports the observations in Section 3.1.1. DM agar/broth was subsequently used throughout this study as a growth media.

## 3.2 Southern Hybridisations

Southern blot analysis of *D. pini* genomic DNA was used to detect regions of the *D. pini* genome potentially heterologous to aflatoxin biosynthetic genes using clones of *Aspergillus parasiticus* *nor-1* and *ver-1* genes.

### 3.2.1 Detection of a *D. pini* Region Heterologous to the *A. parasiticus* *nor-1* Gene

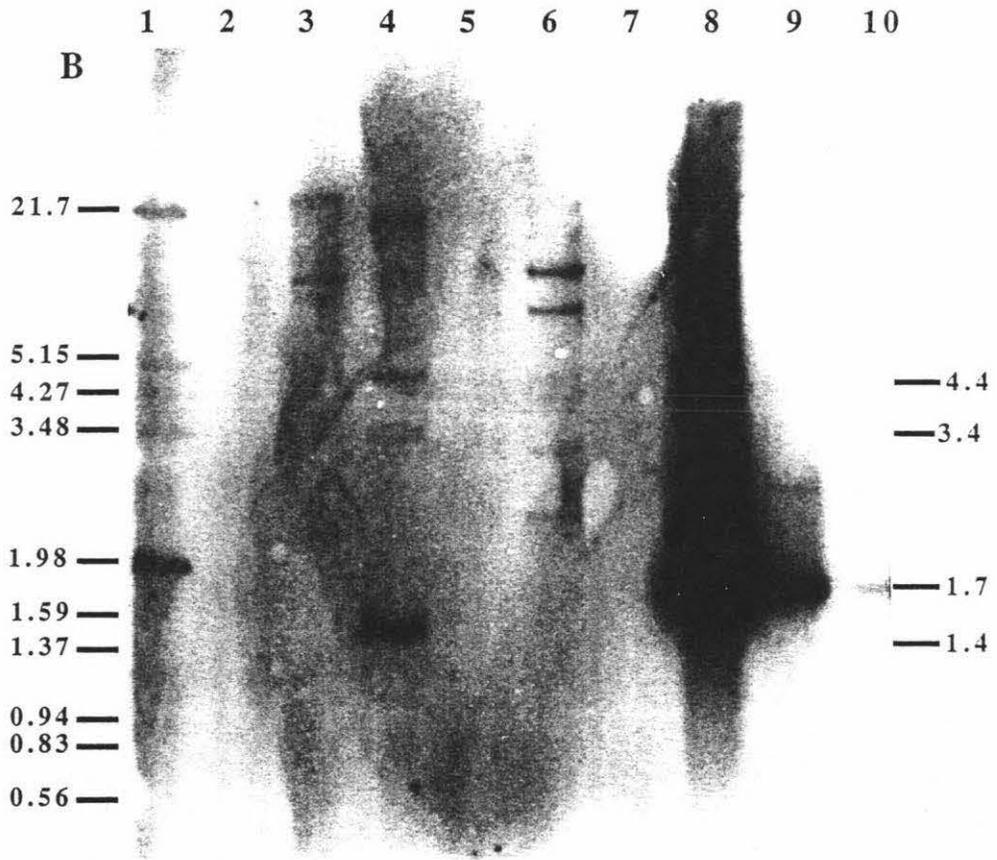
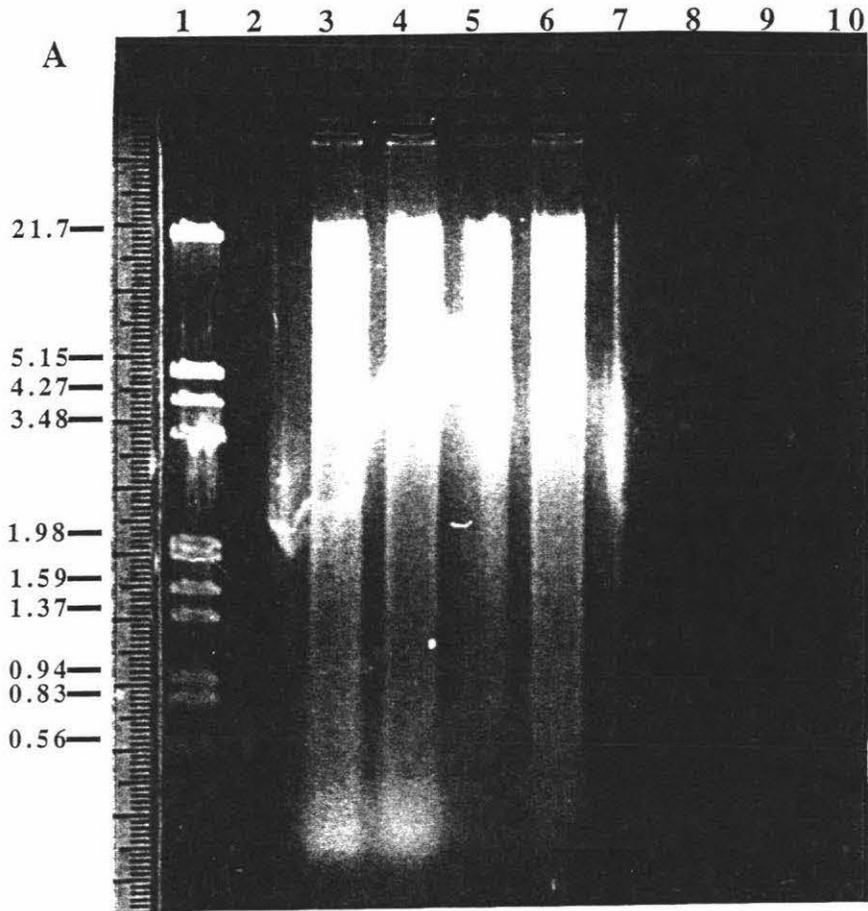
A Southern blot containing *EcoRI/HindIII* digested  $\lambda$  DNA, 10  $\mu$ g *BamHI* digested *D. pini* DNA, 10  $\mu$ g *EcoRI* digested *D. pini* DNA, 10  $\mu$ g *BamHI* digested *A. nidulans* DNA, and 5 pg, 50 pg, and 500 pg dilutions of *SphI/EcoRI* digested pNA17 (Fig. 4A) was hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP-labelled 1.7 kb *nor-1* gene fragment from *A. parasiticus* *SphI/EcoRI* digested pNA17 (see Appendix 1.0 for map) at 60°C. Washes were carried out at hybridisation temperature in 3 x SSC, 0.2% SDS and the membranes autoradiographed overnight. The 1.7 kb *nor-1* probe hybridised strongly to a 1.4 kb *EcoRI* *D. pini* fragment (Fig. 4B, lane 4). Weaker hybridisation was also observed to larger *EcoRI* fragments of sizes 4.4 kb and 3.4 kb (Fig. 4B, lane 4). No hybridisation was observed to *BamHI* digested *D. pini* DNA. Hybridisation of the *nor-1* probe occurred as expected to the *BamHI* digested *A. nidulans* DNA heterologous control (Fig. 4B, lane 6). Further, more stringent, washes were carried out in 1 x SSC, 0.2% SDS at 65°C and autoradiographed for 72 h. All hybridisation signals remained (Fig. 4C). This suggested that 60°C is a suitable temperature for hybridisation of the *nor-1* probe.

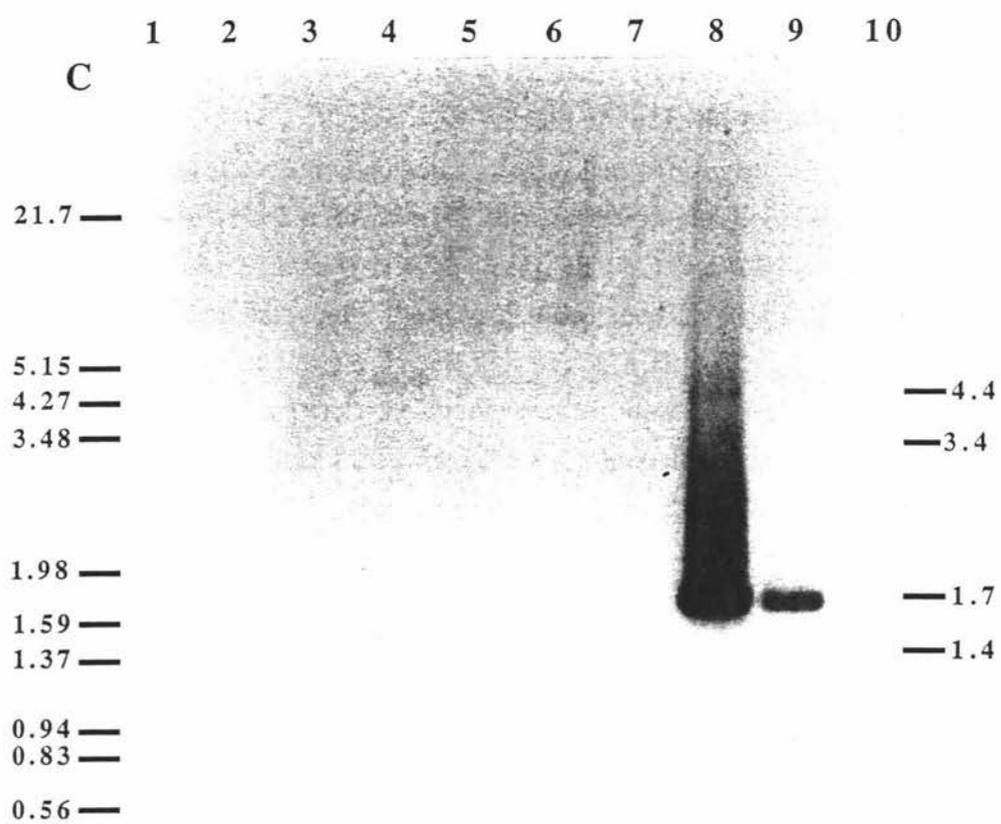
### 3.2.2 Detection of a *D. pini* Region Heterologous to the *A. parasiticus* *ver-1* Gene

A Southern blot containing *EcoRI/HindIII* digested  $\lambda$  DNA, 10  $\mu$ g *BamHI* digested *D. pini* DNA, 10  $\mu$ g *EcoRI* digested *D. pini* DNA, 10  $\mu$ g *BamHI* digested *A. nidulans* DNA, and 5 pg, 50 pg, and 500 pg dilutions of *HindIII/EcoRI* digested pBVer-1 was hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP-labelled 1.8 kb *ver-1* gene fragment from *A. parasiticus*

**Fig. 4A-C Southern blot of *D. pini* genomic DNA probed with *nor-1***

**A** Restriction digestion profile of *EcoRI/HindIII* digested  $\lambda$  DNA (lane 1), 10  $\mu$ g *BamHI* (lane 3) and 10  $\mu$ g *EcoRI* (lane 4) digested *D. pini* genomic DNA, 10  $\mu$ g *BamHI* digested *A. nidulans* DNA (lane 6), and 500 pg (lane 8), 50 pg (lane 9), and 5 pg (lane 10) *SphI/EcoRI* digested pNA17. **B** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ - $^{32}$ P]dCTP labelled 1.7 kb pNA17 *BamHI/EcoRI* fragment washed in 3 x SSC, 0.1% SDS at hybridisation temperature (60°C). **C** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ - $^{32}$ P]dCTP labelled 1.7 kb pNA17 *BamHI/EcoRI* fragment washed in 1 x SSC, 0.1% SDS at 65°C.





*EcoRI/HindIII* digested pBVer-1 (see Appendix 1.0 for map) at 60°C. Washes were carried out at hybridisation temperature in 3 x SSC, 0.2% SDS and the membranes autoradiographed overnight. No *D. pini* hybridisation was detected. Hybridisation of the *ver-1* probe occurred as expected to the *BamHI* digested *A. nidulans* DNA heterologous control (results not shown). This suggested that 60°C is too high for the *ver-1* probe to hybridise to *D. pini* DNA.

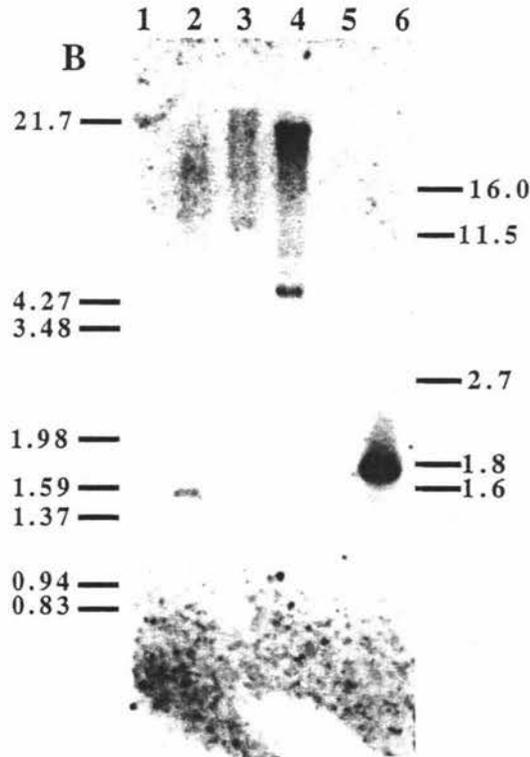
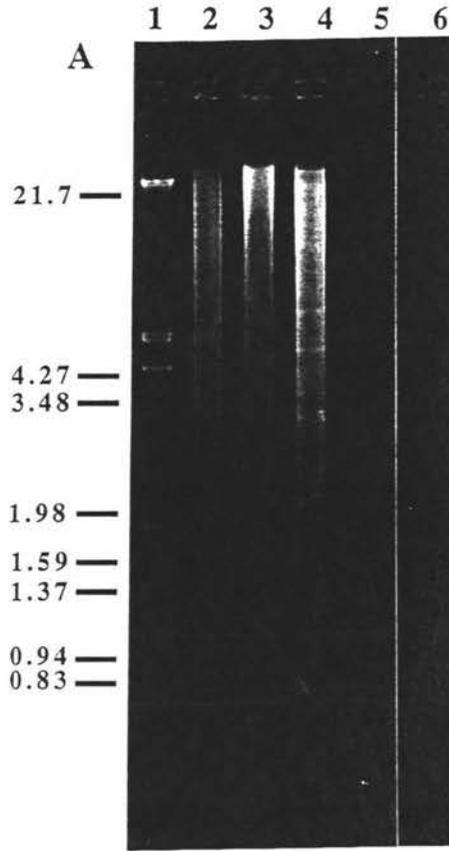
To facilitate the detection of a heterologous *ver-1* hybridisation signal a new Southern blot containing *EcoRI/HindIII* digested  $\lambda$  DNA, 6  $\mu$ g *BamHI* digested *D. pini* DNA, 6  $\mu$ g *EcoRI* digested *D. pini* DNA, 6  $\mu$ g *BamHI* digested *A. nidulans* genomic DNA, and 10 pg and 100 pg dilutions of *EcoRI/HindIII* digested pBVer-1 (Fig. 5A) was hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP-labelled 1.8 kb *ver-1* gene fragment from *A. parasiticus* *EcoRI/HindIII* digested pBVer-1 at 55°C. Washes were carried out at hybridisation temperature in 3 x SSC, 0.2% SDS and the membrane autoradiographed overnight. The 1.8 kb *ver-1* probe hybridised strongly to a 1.6 kb *BamHI* *D. pini* fragment (Fig. 5B, lane 2). Weaker hybridisation was also observed to larger fragments in the *EcoRI* digested *D. pini* DNA of sizes 16.0 kb, 11.5 kb, and 2.7 kb (Fig. 5B, lane 3). Hybridisation of the *ver-1* probe occurred as expected to the *BamHI* digested *A. nidulans* DNA heterologous control (Fig. 5B, lane 4). Further, more stringent, washes were carried out in 1 x SSC, 0.2% SDS at 65°C and autoradiographed for 72 h. All hybridisation signals remained (Fig. 5C). This suggests that 55°C is the optimal temperature for *ver-1* hybridisation.

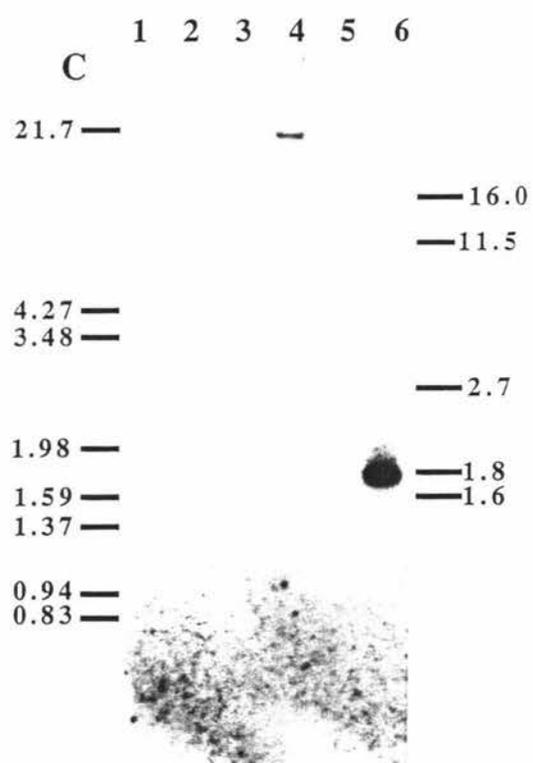
### 3.3 Library Construction

Following extraction of *D. pini* genomic DNA (Section 2.9.1) an *MboI* digest was performed to ensure that *MboI* cuts *D. pini* DNA. Trial digestions (Section 2.10.1) were then carried out to establish the optimum enzyme concentration to generate fragment sizes of 15-23 kb suitable for cloning into the  $\lambda$ GEM-12 vector (Fig. 6A). The optimum enzyme concentration was defined by the amount of enzyme needed to produce the maximum intensity of fluorescence in the desired size range. Tubes 8 ( $7.8 \times 10^{-2}$  u/ $\mu$ g DNA) and 9 ( $3.9 \times 10^{-2}$  u/ $\mu$ g DNA) filled this criteria. To obtain the maximum number of molecules in this size range half the amount of enzyme that produces the maximum amount of fluorescence was used. Therefore the large scale reactions (Section 2.10.2) were performed using the concentrations  $3.9 \times 10^{-2}$  u/ $\mu$ g DNA and  $1.95 \times 10^{-2}$  u/ $\mu$ g DNA (Fig. 6B). The DNA concentration, time, and temperature of digestion were identical to those used in the optimised small scale reactions. The digestion products were end-filled (Section 2.10.3) and ligated (Section 2.10.4) into the  $\lambda$ GEM-12 vector

**Fig. 5A-C Southern blot of *D. pini* genomic DNA probed with *ver-1***

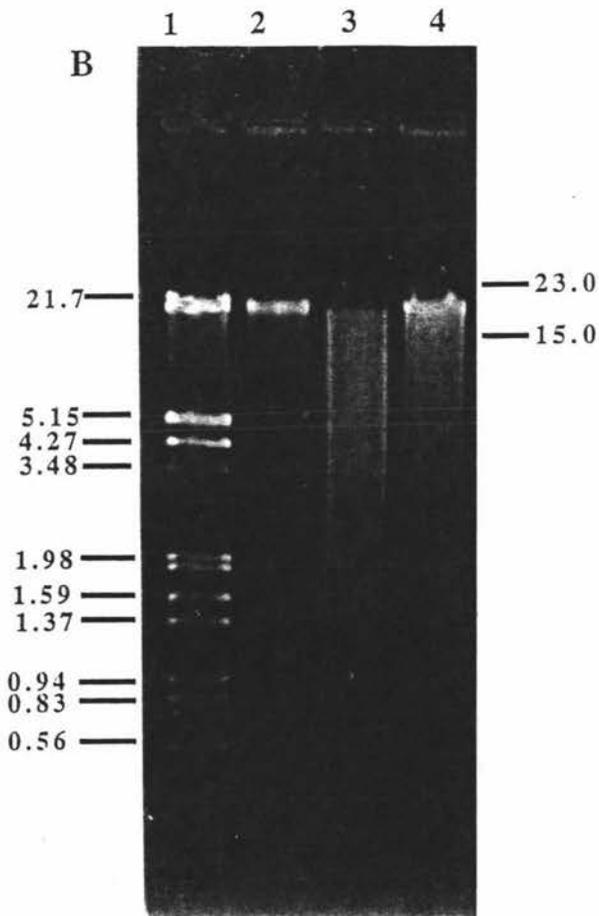
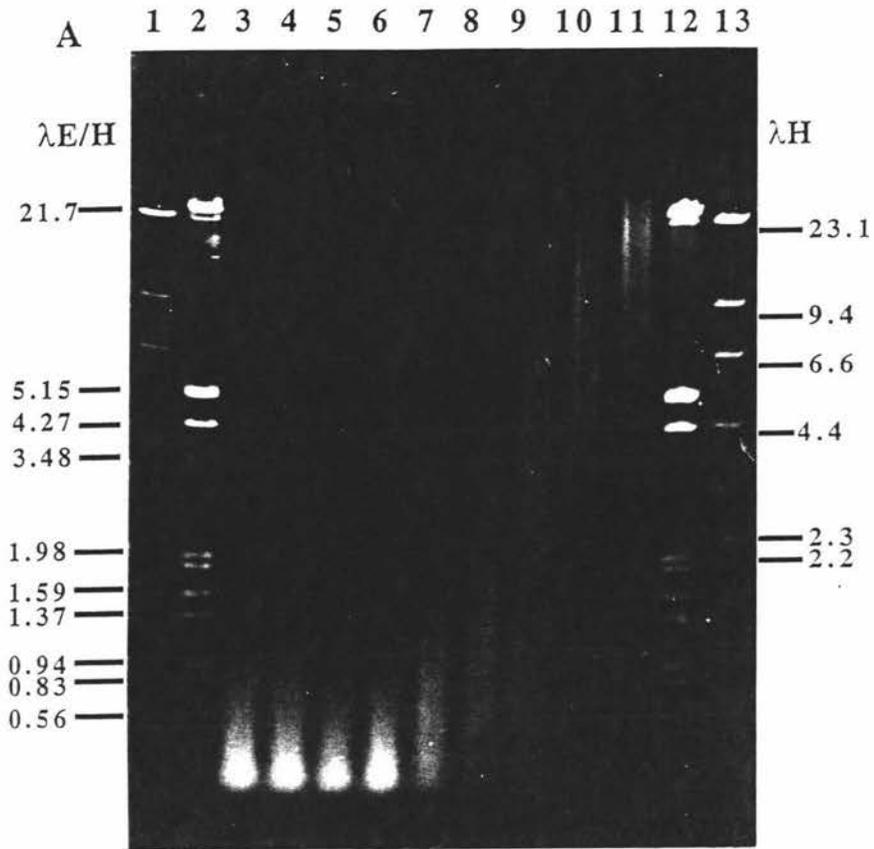
**A** Restriction digestion profile of *D. pini* genomic DNA *EcoRI/HindIII* digested  $\lambda$  DNA (lane 1), 6  $\mu$ g *Bam*HI (lane 2) and 6  $\mu$ g *Eco*RI (lane 3) digested *D. pini* genomic DNA, 6  $\mu$ g *Bam*HI digested *A. nidulans* genomic DNA (lane 4), 10 pg (lane 5) and 100 pg (lane 6) *Eco*RI/*Hind*III digested pBVer-1. **B** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP labelled 1.8 kb pBVer-1 *Eco*RI/*Hind*III fragment washed in 3 x SSC, 0.2% SDS at hybridisation temperature (55°C). **C** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP labelled 1.8 kb pBVer-1 *Eco*RI/*Hind*III fragment washed 1 x SSC, 0.2% SDS at 65°C.





**Fig. 6A-B Profiles of partial *MboI* digestions of genomic DNA from *D. pini***

**A** Profiles of 100 ng of small scale partial *MboI* digests of: 10 enzyme u/  $\mu\text{g}$  DNA (lane 3), 5 enzyme u/ $\mu\text{g}$  DNA (lane 4), 2.5 enzyme u/ $\mu\text{g}$  DNA (lane 5), 1.25 enzyme u/ $\mu\text{g}$  DNA (lane 6),  $6.25 \times 10^{-1}$  enzyme u/ $\mu\text{g}$  DNA (lane 7),  $3.125 \times 10^{-1}$  enzyme u/ $\mu\text{g}$  DNA (lane 8),  $1.56 \times 10^{-1}$  enzyme u/ $\mu\text{g}$  DNA (lane 9),  $7.8 \times 10^{-2}$  enzyme u/ $\mu\text{g}$  DNA (lane 10), and  $3.9 \times 10^{-2}$  enzyme u/ $\mu\text{g}$  DNA (lane 11), flanked by *HindIII* digested  $\lambda$  DNA (lanes 1 and 13) and *EcoRI/HindIII* digested  $\lambda$  DNA (lanes 2 and 12). **B** Profiles of 100 ng of partial large scale *MboI* digests of:  $3.9 \times 10^{-2}$  enzyme U/ $\mu\text{g}$  (lane 3), and  $1.95 \times 10^{-2}$  enzyme U/ $\mu\text{g}$  (lane 4), alongside *EcoRI/HindIII* digested  $\lambda$  DNA (lane 1), and 100 ng uncut *D. pini* DNA (lane 2).



(see Appendix 1.0 for map). DNA was packaged *in vitro* and the resulting phage were titred by plating on *E. coli* strain KW251. Under these conditions, only phage containing insert DNA were able to form plaques. Preliminary packaging (Section 2.10.5) was conducted on a small scale, along with a negative and positive controls (reaction A and D, respectively) to determine the optimum ratio of insert to  $\lambda$  DNA. The positive control did not work, this may be because the control DNA has a strict specificity for *E. coli* strain LE392. However, reactions B and C (1:1 and 1:4 vector to insert ratios) gave phage titres of  $4.2 \times 10^4$  pfu/ml and  $4.7 \times 10^5$  pfu/ml, respectively (Table 5). Large scale packaging (Section 2.10.6) was then performed using the remaining 4:1 ligation (reactions C<sub>1</sub> + C<sub>2</sub>) with a negative control (Table 5). This packaging gave titres of  $1.16 \times 10^5$  pfu/ml (reaction C<sub>1</sub>) and  $1.22 \times 10^5$  pfu/ml (reaction C<sub>2</sub>). The optimally packaged DNA (C + C<sub>1</sub> + C<sub>2</sub>) was then combined and titred (Table 5). A 93  $\mu$ l aliquot ( $1.36 \times 10^4$  pfu/ml) of this library was amplified, titred (Table 5), and stored for future use.

### **3.4 Isolation of $\lambda$ Clones Hybridising to the *A. parasiticus* *ver-1* Gene**

#### **3.4.1 Library Screening**

Filters containing approximately 50 000 pfu from the non-amplified *D. pini* genomic library were screened by plaque hybridisation (Section 2.1.1) with [ $\alpha$ -<sup>32</sup>P]dCTP-labelled 1.8 kb *ver-1* gene fragment from *A. parasiticus* *Hind*III/*Eco*RI digested pBVer-1 (Section 2.9.2) using the conditions determined in Section 3.2.2. The filters were washed at 65°C in 3 x SSC and 0.2% SDS. Initially 3 positive plaques hybridised with the probe, however on the second screen only 2 of these plaques remained positive ( $\lambda$ CGV1 and  $\lambda$ CGV2). A further round of plaque purification using filters containing approximately 100 pfu was performed revealing numerous positive plaques. Two plaques were taken of each positive clone and DNA was isolated (Section 2.5.4).

#### **3.4.2 Restriction Digestion of $\lambda$ Clones and Southern Hybridisation**

Inserts in the 2 positive clones were digested (Section 2.6.1) with all single and double combinations of *Bam*HI, *Eco*RI, *Sal*I, and *Xho*I. These enzymes were chosen as *Bam*HI and *Eco*RI cut cloned inserts out of  $\lambda$ GEM-12 arms, leaving the arms essentially intact (see Appendix 1.0 for map), whilst *Sal*I and *Xho*I do not cut within  $\lambda$ GEM-12 arms. Digested DNA was separated by gel electrophoresis on a 1% agarose gel (Section 2.6.2.2, Figs. 7A and 8A). Information from these digests is summarised in Tables 6

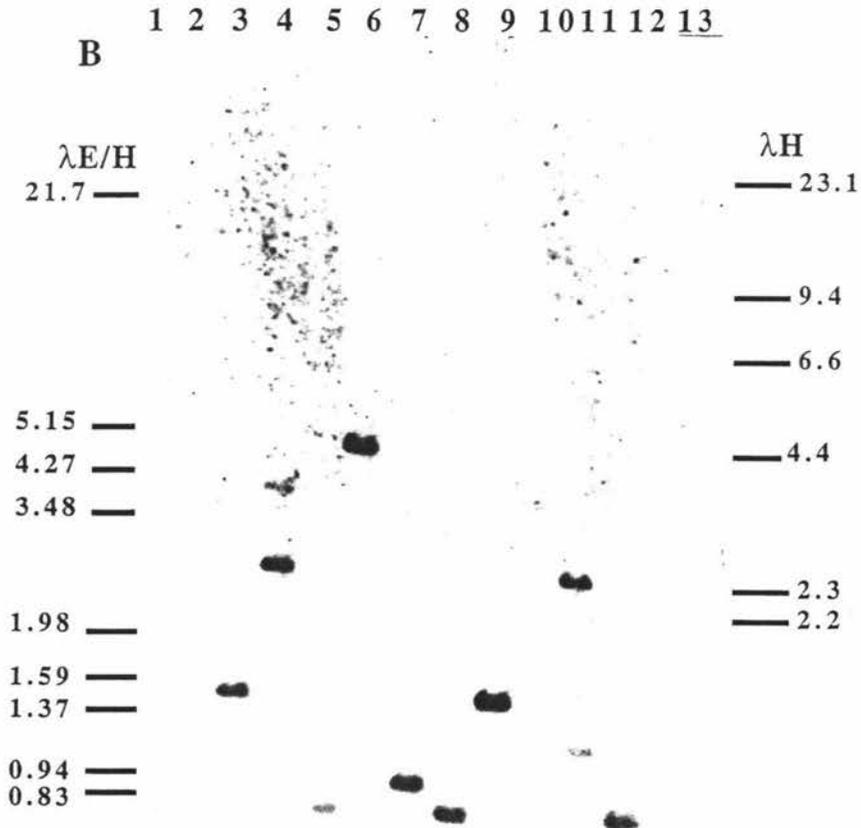
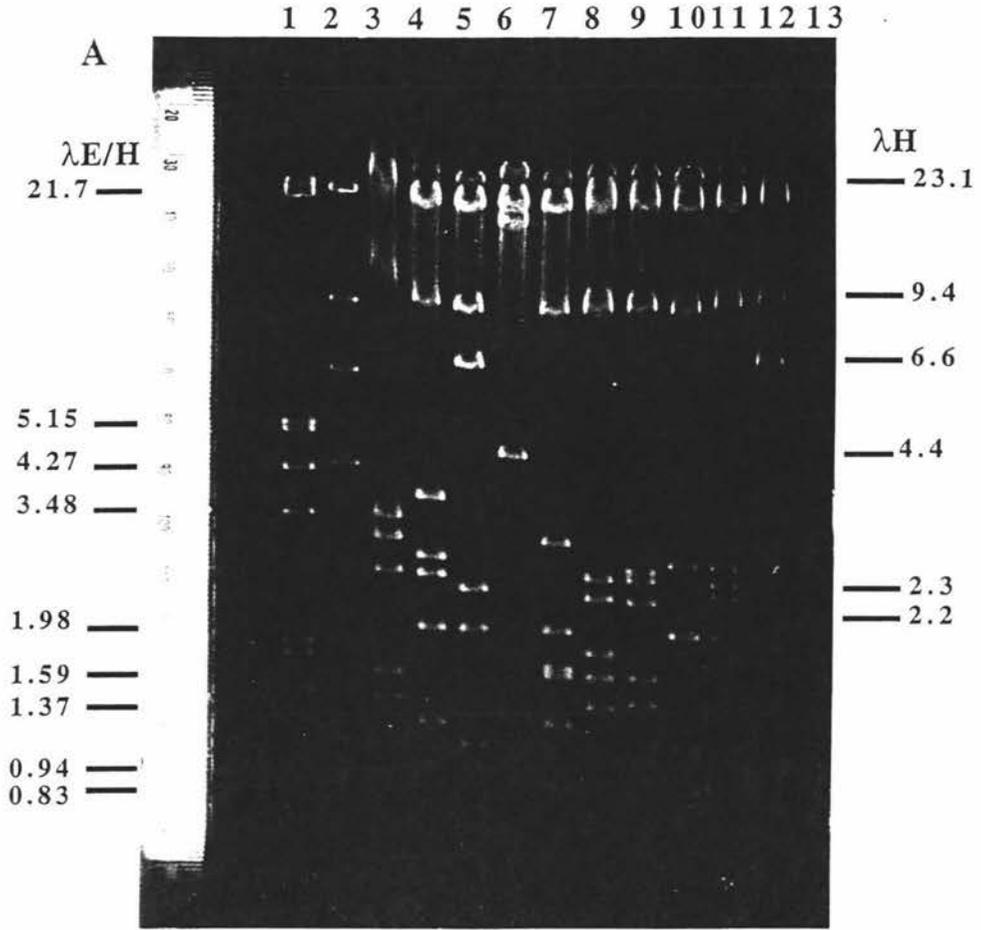
Table 5 Titres of Small Scale, Large Scale, and Amplified Libraries

Reaction	Phage Titre (pfu/ml)	Number of Recombinants/ $\mu$ g $\lambda$ DNA
<b>Small Scale</b>		
A (negative control)	0	0
B (1:1 vector to insert)	$4.2 \times 10^4$	$4.2 \times 10^4$
C (1:4 vector to insert)	$4.7 \times 10^5$	$4.7 \times 10^5$
D (positive control)	0	0
<b>Large Scale</b>		
A (negative control)	0	0
C <sub>1</sub>	$1.16 \times 10^5$	$1.16 \times 10^5$
C <sub>2</sub>	$1.22 \times 10^5$	$1.22 \times 10^5$
C + C <sub>1</sub> + C <sub>2</sub> <sup>a</sup>	$1.43 \times 10^5$	$1.43 \times 10^5$
<b>Amplified</b>		
C + C <sub>1</sub> + C <sub>2</sub>	$2.3 \times 10^{10}$	

<sup>a</sup> Combined and re-titred

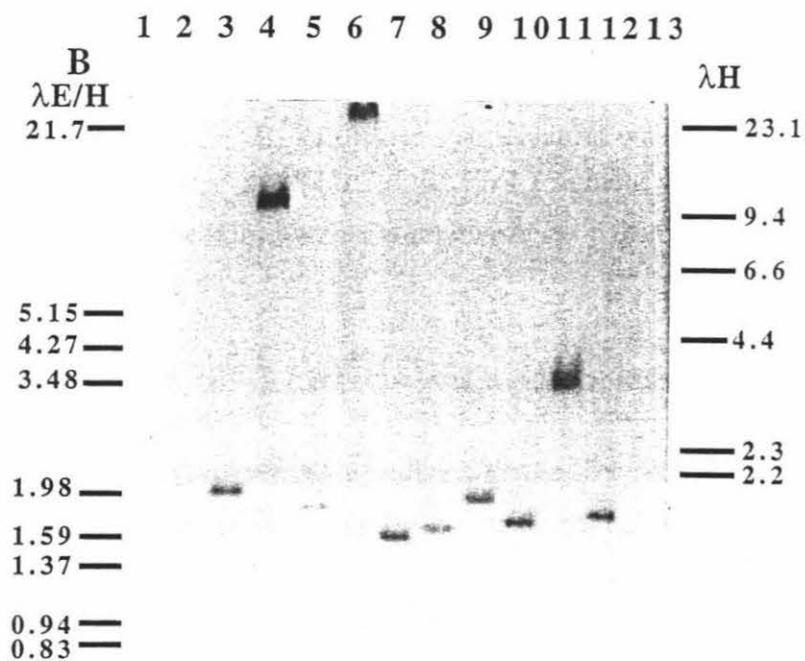
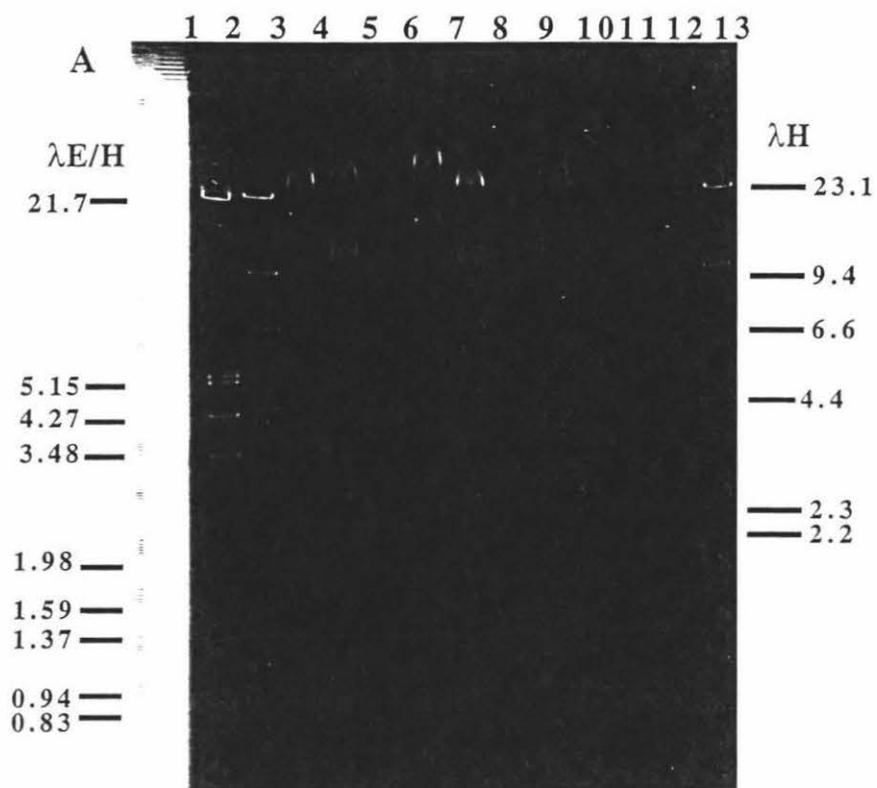
**Fig. 7A-B Mapping the position of *ver-1* on clone  $\lambda$ CGV1**

**A** Restriction digestion profile of clone  $\lambda$ CGV1 with: *Bam*HI (lane 3), *Eco*RI (lane 4), *Sal*I (lane 5), *Xho*I (lane 6), *Bam*HI and *Eco*RI (lane 7), *Bam*HI and *Sal*I (lane 8), *Bam*HI and *Xho*I (lane 9), *Eco*RI and *Sal*I (lane 10), *Eco*RI and *Xho*I (lane 11), and *Sal*I and *Xho*I (lane 12) digests flanked by *Eco*RI/*Hind*III digested  $\lambda$  DNA (lanes 1 and 13) and *Hind*III digested  $\lambda$  DNA (lane 2). **B** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP labelled 1.8 kb pBVer-1 *Eco*RI/*Hind*III fragment.



**Fig. 8A-B Mapping the position of *ver-1* on clone  $\lambda$ CGV2**

**A** Restriction digestion profile of clone  $\lambda$ CGV2 with: *Bam*HI (lane 3), *Eco*RI (lane 4), *Sal*I (lane 5), *Xho*I (lane 6), *Bam*HI and *Eco*RI (lane 7), *Bam*HI and *Sal*I (lane 8), *Bam*HI and *Xho*I (lane 9), *Eco*RI and *Sal*I (lane 10), *Eco*RI and *Xho*I (lane 11), and *Sal*I and *Xho*I (lane 12) digests flanked by *Eco*RI/*Hind*III digested  $\lambda$  DNA (lane 1) and *Hind*III digested  $\lambda$  DNA (lanes 2 and 13). **B** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP labelled 1.8 kb pBVer-1 *Eco*RI/*Hind*III fragment.



and 7. Clones  $\lambda$ CGV1 and  $\lambda$ CGV2 shared no common restriction fragments. Southern blots of these gels were hybridised to the [ $\alpha$ - $^{32}$ P]dCTP-labelled (Section 2.9) 1.8 kb *ver-1* *A. parasiticus* gene fragment. These blots revealed that the two clones  $\lambda$ CGV1 and  $\lambda$ CGV2 contained fragments hybridising to the *ver-1* probe (fragments that hybridised are identified by a superscript a in Tables 6 and 7), however fragments from these clones which hybridised differed in size (Figs. 7B and 8B, Tables 6 and 7) suggesting they are from different genetic regions. A 1.6 kb *Bam*HI fragment and a 2.7 kb *Eco*RI fragment of clone  $\lambda$ CGV1 hybridised to the *ver-1* probe. These signals were the same size as those of the corresponding genomic DNA digests from the original hybridisation (Section 3.2.2). An 11.5 kb *Eco*RI fragment from clone  $\lambda$ CGV2 hybridised to the *ver-1* probe. This signal was the same size as that of the corresponding genomic DNA digest from the original hybridisation (Section 3.2.2). This suggested that the chromosomal region containing the *D. pini ver-1* gene is located on these clones. Because clone  $\lambda$ CGV1 appeared to contain a fragment corresponding to the most distinctly hybridising band (1.6 kb *Bam*HI) on the genomic Southern blot (*ver-1* probed), complete characterisation of this clone was the primary focus of further study.

### 3.5 Further Characterisation of Clone $\lambda$ CGV1

#### 3.5.1 Mapping Clone $\lambda$ CGV1 Further

The complexity of fragments from restriction digests (summarised in Table 6) made mapping of this clone difficult, so further hybridisation was performed. Since initial hybridisation suggested that a 0.8 kb *Sal*I  $\lambda$ CGV1 fragment was flanked by 1.1 kb and 2.1 kb *Sal*I fragments these fragments were chosen as hybridisation probes for further mapping.

The Southern blot of the  $\lambda$ CGV1 gel (Fig. 7A) was hybridised to [ $\alpha$ - $^{32}$ P]dCTP-labelled (Section 2.9) 1.1 kb and 2.1 kb *Sal*I  $\lambda$ CGV1 *D. pini* fragments. This blot revealed which of the  $\lambda$ CGV1 fragments hybridised to the *D. pini* 1.1 kb probe (Fig. 9A, fragments that hybridised are identified by a superscript b in Table 6) and the *D. pini* 2.1 kb probe (Fig. 9B, fragments that hybridised are identified by a superscript c in Table 6).

The information in Table 6 was used to construct an *Eco*RI and *Sal*I restriction map of the  $\lambda$ CGV1 clone. Data from the double digest with *Eco*RI/*Sal*I (Table 6) was used to further refine the map. Information from the *Bam*HI and *Xho*I single digests, and *Bam*HI/*Eco*RI, *Bam*HI/*Sal*I, *Bam*HI/*Xho*I, *Eco*RI/*Xho*I, and *Sal*I/*Xho*I double digests

**Table 6** Data from Restriction Mapping of Clone  $\lambda$ CGV1

Restriction digest	Fragment size (kb)
<i>Bam</i> HI	18.2, 10.7, 3.6 <sup>c</sup> , 3.3, 2.8, 1.6 <sup>a</sup> , 1.4
<i>Eco</i> RI	18.2, 10.7, 3.9 <sup>ac</sup> , 3.0, 2.7 <sup>ab</sup> , 2.1, 1.3
<i>Sal</i> I	18.2, 10.1, 7.7, 2.8 <sup>c</sup> , 2.1 <sup>ac</sup> , 1.1 <sup>a</sup> , 0.8 <sup>a</sup>
<i>Xho</i> I	18.2, 16.5, 4.8 <sup>abc</sup>
<i>Bam</i> HI/ <i>Eco</i> RI	18.2, 10.1, 3.2 <sup>c</sup> , 2.1 <sup>b</sup> , 1.65, 1.6, 1.3, 0.9 <sup>a</sup>
<i>Bam</i> HI/ <i>Sal</i> I	18.2, 10.1, 2.7, 2.4, 1.8 <sup>c</sup> , 1.6, 1.4, 0.8 <sup>a</sup>
<i>Bam</i> HI/ <i>Xho</i> I	18.2, 10.1, 2.8 <sup>c</sup> , 2.7, 2.4 <sup>b</sup> , 1.6 <sup>abc</sup> , 1.4
<i>Eco</i> RI/ <i>Sal</i> I	18.2, 10.1, 2.9, 2.0 <sup>c</sup> , 1.3, 1.1 <sup>ab</sup>
<i>Eco</i> RI/ <i>Xho</i> I	18.2, 10.1, 2.9, 2.6 <sup>ab</sup> , 2.4 <sup>c</sup> , 2.0, 1.1 <sup>ac</sup>
<i>Sal</i> I/ <i>Xho</i> I	18.2, 10.7, 10.1, 1.6, 1.3 <sup>c</sup> , 1.1 <sup>ab</sup> , 0.8 <sup>ac</sup>

<sup>a</sup> Indicates fragments hybridising to [ $\alpha$ -<sup>32</sup>P]-labelled *A. parasiticus* 1.8 kb *Eco*HI/*Hind*III gene fragment (see Fig. 7B).

<sup>b</sup> Indicates fragments hybridising to [ $\alpha$ -<sup>32</sup>P]-labelled *D. pini* 1.1 kb *Sal*I fragment (see Fig. 9A).

<sup>c</sup> Indicates fragments hybridising to [ $\alpha$ -<sup>32</sup>P]-labelled *D. pini* 2.1 kb *Sal*I fragment (see Fig. 9B).

**Table 7 Data from Southern Hybridisation Analysis of Clone  $\lambda$ CGV2**

Restriction digest	Fragment size (kb)
<i>Bam</i> HI	17.1, 11.4, 7.9, 4.4, 2.45 <sup>ab</sup> , 1.0 <sup>b</sup>
<i>Eco</i> RI	17.6, 11.4 <sup>ab*</sup> , 3.9
<i>Sal</i> I	2.35 <sup>ab**</sup>
<i>Xho</i> I	18.1, 13.4, 3.0, 1.1, 1.0
<i>Bam</i> HI/ <i>Eco</i> RI	16.7, 11.1, 7.9, 3.9, 2.0 <sup>ab</sup> , 1.0 <sup>b</sup>
<i>Bam</i> HI/ <i>Sal</i> I	17.6, 11.7, 6.8, 4.6, 2.1 <sup>ab</sup>
<i>Bam</i> HI/ <i>Xho</i> I	17.6, 11.7, 3.0, , 2.45 <sup>ab</sup> , 2.3, 1.1, 1.0 <sup>b</sup> , 0.9
<i>Eco</i> RI/ <i>Sal</i> I	8.0, 3.9, 2.2 <sup>ab</sup> , 1.1
<i>Eco</i> RI/ <i>Xho</i> I	18.6, 12.4, 4.7 <sup>ab</sup> , 3.0, 2.25, 1.1, 0.9
<i>Sal</i> I/ <i>Xho</i> I	3.0, 2.4 <sup>ab</sup> , 1.7, 1.1, 0.9

<sup>a</sup> Indicates fragments hybridising to [ $\alpha$ -<sup>32</sup>P]-labelled *A. paraciticus* 1.8 kb *Eco*RI/*Hind*III *ver-1* gene fragment (see Fig. 8B).

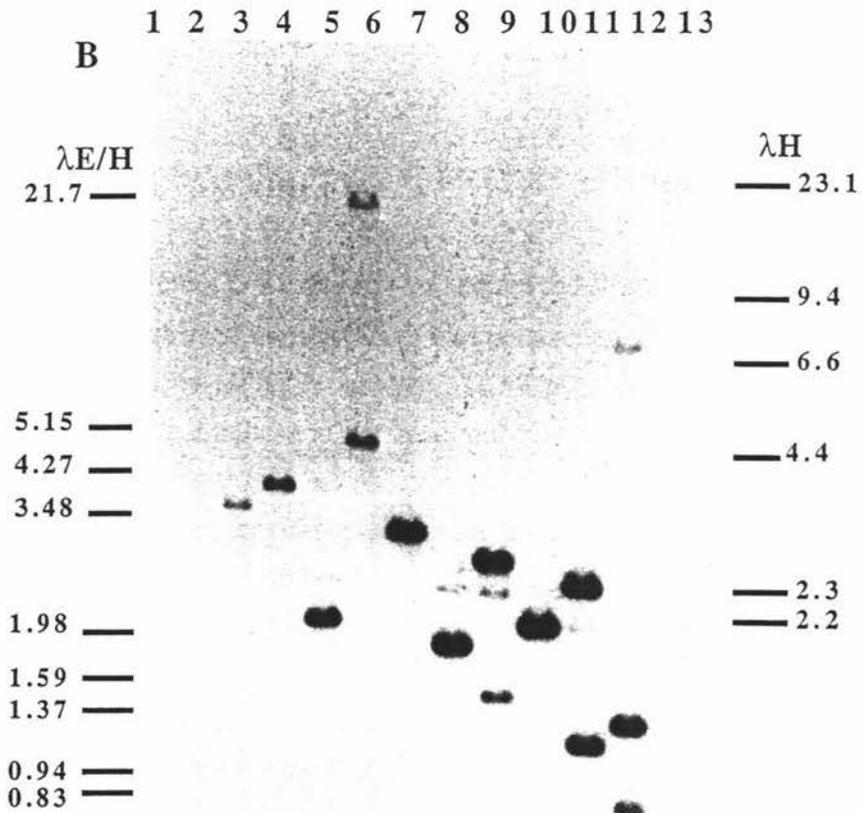
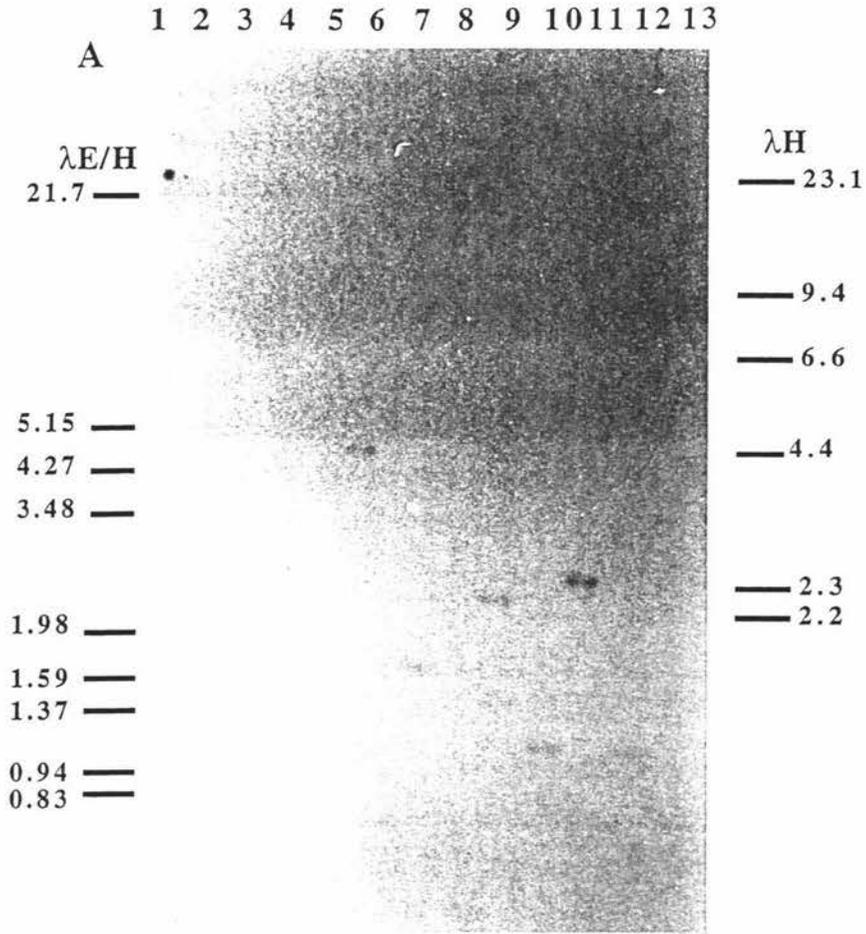
<sup>b</sup> Indicates fragments hybridising to [ $\alpha$ -<sup>32</sup>P]-labelled *D. pini* 0.8 kb *Sal*I fragment (see Fig. 13).

\* This fragment is presumably a doublet (see Fig. 8A).

\*\* This fragment was not visible on the gel (see Fig. 8A).

**Fig. 9A-B Further mapping analysis of clone  $\lambda$ CGV1**

**A** Autoradiograph of Southern blot of gel shown in Fig. 7A hybridised to [ $\alpha$ - $^{32}$ P]dCTP labelled 1.1 kb *D. pini SalI* fragment. **B** Autoradiograph of Southern blot of gel shown in Fig. 7A hybridised to [ $\alpha$ - $^{32}$ P]dCTP labelled 2.1 kb *D. pini SalI* fragment.



(Table 6) was used to map the *Bam*HI and *Xho*I sites. In this way a complete map of clone  $\lambda$ CGV1 was obtained for restriction enzymes *Bam*HI, *Eco*RI, *Sal*I, and *Xho*I (Fig. 10A). This map was consistent with all information in Table 6. Using the information in Table 6 the insert size was estimated to be 13 kb.

### 3.5.2 Subcloning of a $\lambda$ CGV1 Region Required for Sequencing

To further characterise the cloned genomic region, the 0.8 kb *Sal*I fragment that hybridised to the *A. parasiticus* 1.8 kb pBVer-1 *Eco*RI/*Hind*III fragment (Table 6) was subcloned (Section 2.7) into pUC118 to give plasmid pCG1 (Table 1). The identity of the fragment subcloned was confirmed by restriction digestion (Section 2.6.1.1) and gel electrophoresis (Section 2.6.2.1).

### 3.5.3 Sequence Analysis of Clone $\lambda$ CGV1

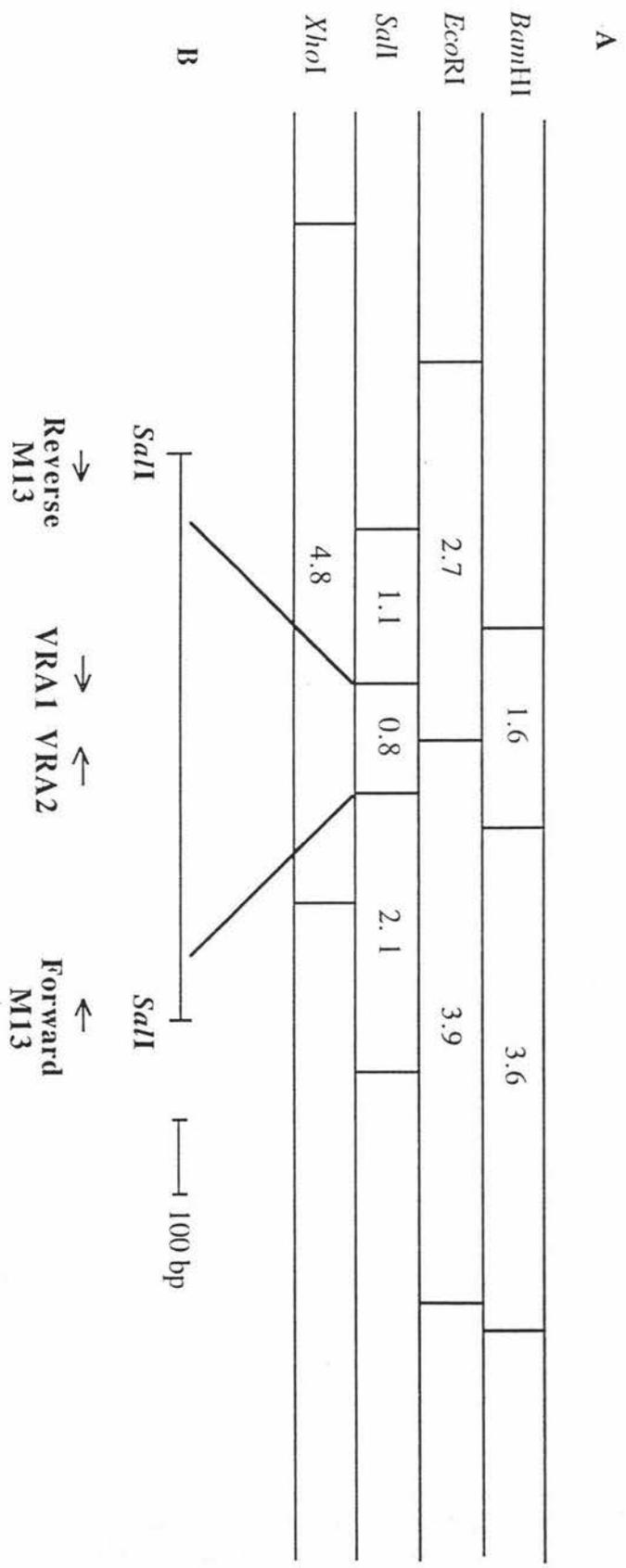
DNA from the plasmid subclone (Section 3.5.1) was isolated by the alkaline lysis method (Section 2.5.1), and sequenced using the AmpliCycle sequencing system (Section 2.12). M13 forward and reverse primers (Table 2) were used to obtain insert sequence. This information was used to design primers (Table 2) for further sequencing of the 0.8 kb *Sal*I fragment from clone  $\lambda$ CGV1. Sequences through the restriction sites used for subcloning enabled the complete sequence of the 0.8 kb *Sal*I fragment to be obtained (Fig. 10B).

### 3.5.4 Sequence Identification

A total of 757 bp of the  $\lambda$ CGV1 region was sequenced (Fig. 11). A six frame translation of this nucleotide sequence (Section 3.5.2) was performed using the MAP program of the Genetics Computer Group (GCG) package. The amino acid sequence was compared to the GenBank sequence database at the National Centre for Biotechnology Information with NCSA Mosaic using the BLASTX 1.4 program. This search revealed a high degree of sequence similarity of the 757bp sequence with the *A. nidulans ver-A* and the *A. parasiticus ver-1* genes involved in the conversion of VA to ST. The *ThnR* gene from *Magnaporthe grisea* and the *ThnI* gene from *Colletotrichum lagenarium*, both involved in melanin biosynthesis, also showed a high degree of sequence similarity. The location of two introns of 65 bp (nucleotides 319 to 384) and 57 bp (nucleotides 574 to 631) was predicted (Fig. 11) using information provided by the sequences. The signal sequences in the presumptive introns matched the consensus sequences for fungal 5' splice sites [GT(A/G/T)(A/C/T)G(T/C)] and 3' splice sites [(C/T)AG] (Ballance, 1986).

**Fig. 10A-B** Restriction map of clone  $\lambda$ CGV1 from a *D. pini* genomic library that hybridised to *Aspergillus parasiticus ver-1*

**A** Restriction map of clone  $\lambda$ CGV1. **B** Region of this clone sequenced and primers used for sequencing.



**Fig. 11**      **Partial sequence of the putative *D. pini ver-1* gene from clone  $\lambda$ CGV1**

DNA sequence of a 0.8 kb *SalI* region of the *D. pini ver-1* gene from  $\lambda$ CGV1 and deduced amino acid sequence. For the region shown the nucleotide sequence of both strands was determined. Sequences of the primers are underlined. Intron sequences are shown in lower case.

1 AACTTCCGCCTCGACGGCAAGGTCGCCCTCGTTACTGGATCTGGCCGTGGTATCGGTGCT 60  
 N F R L D G K V A L V T G S G R G I G A

61 GCCATCGCCATCGAGCTCGGCAAGCGTGGCGCAAACGTCGTCGTCAACTACTCGCGAGCC 120  
 A I A I E L G K R G A N V V V N Y S R A

121 GTCGCAGAGGCCAACAAAGTCGTCGAGACCATCATCGCCAACGGCACCAAGGCCATCGCC 180  
 V A E A N K V V E T I I A N G T K A I A

181 ATCAAGGCCGATGTCGGTGAGATTGACCAGGTCGCGAAGATGATGGACCAGGCTGTTGAG 240  
 I K A D V G E I D Q V A K M M D Q A V E

241 CACTTTGGTCAACTCGATATCGTCTCTTCGAACGCTGGGCTTGTTTCTTTTGGACATTTG 300  
 H F G Q L D I V S S N A G L V S F G H L

301 AAGGATGTCACTGGTGATtatacattgataaacatttgcttttcgtttgaatggatgagagc 360  
 K D V T G D

361 acagatggctgatttatcaaacaGAATTTCGACCGCGTCTTCCGTGTCAACACCCGAGGT 420  
 E F D R V F R V N T R G

421 CAATTCTTTGTCGCTCGCGAGGGCTACCGCCACCTCAGCGTCGGCGGCCGCATCATTCTC 480  
 Q F F V A R E A Y R H L S V G G R I I L

481 ACATCCTCAAACACCGCCTCCATCAAGGGTGTTCCTCAAGCAGCCATCTACTCTGGCTCC 540  
 T S S N T A S I K G V P K H A I Y S G S

541 AAGGGCGCCATCGACACCTTTGTCCGCTGCATGgtatgtccccaatcacctcgaaacc 600  
 K G A I D T F V R C M

601 tcgcaaaccaccccaagctaatacatacctagGCCATCGACGCCGGTGACAAAAAATCAC 660  
 A I D A G D K K I T

661 CGTCAACGCCGTGCTCCCGGCCATTAAGACTGACATGTACGCGCCGTGCGCGCGGA 720  
 V N A V A P G A I K T D M Y A A V A R E

721 GTACCTCCCGGGTGGCGACAAGTTCACTGATGAGCAA 757  
 Y L P G G D K F T D E Q

### 3.5.5 Sequence Comparison

Sequences from *A. nidulans* (Keller *et al.* 1994), *A. parasiticus* (Skory *et al.* 1992), *M. grisea* (Vidal-Cros *et al.* 1994), and *C. lagenarium* (Perpetua *et al.* 1996) were retrieved using Entrez and compared to the deduced *D. pini* amino acid coding sequence using the CLUSTAL W 1.5 multiple sequence alignment program (Thompson *et al.* 1994). The *D. pini* amino acid coding sequence exhibited 78% identity with the *A. nidulans ver-A* sequence, 78% identity with the *A. parasiticus ver-1* sequence, 62% identity with the *M. grisea ThnR* sequence, and 64% identity with the *C. lagenarium Thr1* sequence (Fig. 12). The *D. pini* amino acid sequence identity also included conservation of the postulated NADPH-dependent keto-reductase binding site (GXGXXA, residues 15 to 20) near the amino terminus (Fig. 12). The alignments suggested that a *ver-1* like gene had been cloned, and that the 0.8 kb *Sall* fragment contains most of the coding region, except for approximately 3-5 amino acids at the N-terminal and approximately 48 amino acids at the C-terminal.

### 3.5.6 Comparison of Intron Positions

The positions of the introns in the region sequenced were compared to the positions of introns in the *ver-1* region from *A. parasiticus* and the *ver-A* region from *A. nidulans*. The first intron (65 bp) was found to be in exactly the same position as the first intron in the *ver-1* (53 bp) and *ver-A* (51 bp) sequences. The second *D. pini* intron was in a unique place. The second intron in the *ver-1* and *ver-A* sequences (51 bp and 62 bp, respectively) occurred three amino acids after the end of the *D. pini* region sequenced.

### 3.5.7 Comparison of GC Content

The GC content of the region sequenced was determined with the COMPOSITION program of the GCG package. The CG content of the *D. pini* sequence is similar to the *ver-1* region from *A. parasiticus* and the *ver-A* region from *A. nidulans*. The *D. pini ver-1* region has a 56% GC content over 757 bp, while the *ver-1* and *ver-A* regions have 53% and 55% CG contents over 1330 bp and 1386 bp, respectively. Intron sequences showed a preference for AT. The first intron sequence has a 38% GC content, and the second intron sequence has a 49% GC content. The first introns of the *ver-1* and *ver-A* sequences had 38% and 51% GC content, respectively. The second intron in these sequences had 50% (*ver-1*) and 49% (*ver-A*) GC content.

**Fig. 12**            **Comparison of *D. pini* sequence to other amino acid sequences**

Alignment of the amino acid sequences of *ver-A* from *Aspergillus nidulans* (An), *ver-1* from *A. parasiticus* (Ap), *ThnR* from *Magnaporthe grisea* (Mg), and *Thr1* from *Colletotrichum lagenarium* (Cl) with the partial predicted *Dothistroma pini ver-1* sequence. Dashes (-) correspond to alignment gaps, asterisks (\*) correspond to invariant sites, and dots (.) correspond to conservative changes within the sequences. The *ver-A*, *ver-1*, *ThnR*, and *Thr1* sequences were retrieved from the GenBank database using the accession numbers U34740, A48950, S41412, and D83988, respectively.

Dp *ver-1* -----NFRLDGKVALVTGSGRGIGAAIAIELGKRGANVVVNY  
 An *ver-A* -----MSSSDNYRLDGKVALVTGAGRGIGAAIAVALGQRGAKVVVNY  
 Ap *ver-1* -----MSDNHRLDGKVALVTGAGRGIGAAIAVALGERGAKVVVNY  
 Mg *ThnR* MPAVTQPRGESKYDAIPGPLGPQSASLEGGKVALVTGAGRGIGREMAMELGRRGCKVIVNY  
 Cl *Thr1* MPGVTSQSAGSKYDAIPGPLGLASASLMGKVALVTGAGRGIGREMAMELGRRGAKVIVNY  
 \* \* \* \* \* . \* \* \* \* . \* . \* \* \* \* . \* \* \* \*

Dp *ver-1* SRAVAEANKVVETIIANGTKAIAIKADVGEIDQVAKMMDQAVEHFGQLDIVSSNAGLVSF  
 An *ver-A* ANSREAAEKVVDEIKSNAQTALSIQADVGDPAVTKLMDQAVEHFGYLDIVSSNAGIVSF  
 Ap *ver-1* AHSREAAEKVVEQIKANGTDAIAIQADVGDPEATAKLMAETVRHFGYLDIVSSNAGIVSF  
 Mg *ThnR* ANSTESAEEVVAAIKKNGSDAACVKANVGVEDIVRMFEEAVKIFGKLDIVCSNSGVVVF  
 Cl *Thr1* ANSAETAEEVVQAIKKSGSDAASIKANVSDVDQIVKMFGEAKQIWGRLDIVCSNSGVVVF  
 . . \*

Dp *ver-1* GHLKDVTGDEFDRVFRVNTRGQFFVAREAYRHLVGGRIILTSSNTASIKGVPKHAIYSG  
 An *ver-A* GHVKDVTPEDEFDRVFRVNTRGQFFVAREAYRHLREGGRIILTSSNTASVKGVPKHAVYSG  
 Ap *ver-1* GHLKDVTPEEFDRVFRVNTRGQFFVAREAYRHMREGGRIILTSSNTACVKGVPKHAVYSG  
 Mg *ThnR* GHVKDVTPEEFDRVFTINTRGQFFVAREAYKHLEIGGRLILMGSITGQAKAVPKHAVYSG  
 Cl *Thr1* GHVKDVTPEEFDRVFAINTRGQFFVAREAYKHLEVGGRLILMGSITGQAKGVPKHAVYSG  
 \*\* . \* \* \* \* . \* \* \* \* \* . \*

Dp *ver-1* SKGAIDTFVRCMAIDAGDKKITVNAVAPGAIKTDMYAAVAREYLPGGDKFTDEQ-----  
 An *ver-A* SKGAIDTFVRCLAIDCGDKKITVNAVAPGAIKTDMFSLVSREYIPNGETFTDEQVDECAA  
 Ap *ver-1* SKGAIDTFVRCMAIDCGDKKITVNAVAPGAIKTDMFSLVSREYIPNGETFTDEQVDECAA  
 Mg *ThnR* SKGAIETFARCMAIDMADKKITVNVVAPGGIKTDMYHAVCREYIPNGENLSNEEVDEYAA  
 Cl *Thr1* SKGTIETFVRCMAIDFGDKKITVNAVAPGGIKTDMYRDVCREYIPNGGELDDEGVDEFAA  
 \*\*\* . \*

Dp *ver-1* -----  
 An *ver-A* W-LSPLNRVGLPVDVARVVSFLASDAAEWISGKIIGVDGGAFR  
 Ap *ver-1* W-LSPLNRVGLPVDVARVVSFLASDTAEWVSGKIIGVDGGAFR  
 Mg *ThnR* SAWSPLHRVGLPIDIARVVCFLASNDGGWVTGKVIIGIDGGACM  
 Cl *Thr1* G-WSPMHRVGLPIDIARVVCFLASQDGEWINGKVLGIDGAACM

### 3.5.8 Comparison of Codon Usage

Codon usage of the *D. pini* sequence was measured with the CODONFREQUENCY program of the GCG package and was clearly biased. There was a preference against A in the first and third positions, and G in the third position, with codons GTG, AGG, AGA, AGT, ATA, ACG, TAG, CTG, CCG not being used at all (Table 8). For *ver-A* and *ver-I* there was no obvious codon bias (see Appendix 2.0), and all the codons were used.

## 3.6 Further Characterisation of Clone $\lambda$ CGV2

### 3.6.1 Further Hybridisation Analysis of Clone $\lambda$ CGV2

In order to determine the relationship between  $\lambda$ CGV1 and  $\lambda$ CGV2, a Southern blot of the  $\lambda$ CGV2 gel (Fig. 8A) was hybridised to the [ $\alpha$ - $^{32}$ P]dCTP-labelled (Section 2.9) 0.8 kb *Sal*I  $\lambda$ CGV1 *D. pini* fragment. This blot revealed that clone  $\lambda$ CGV2 contained fragments hybridising to the *D. pini* 0.8 kb  $\lambda$ CGV1 probe (Fig. 13, fragments that hybridised are identified by a superscript b in Table 7). Using the information in Table 7 the insert size was estimated to be 14 kb.

### 3.6.2 Subcloning of a $\lambda$ CGV2 Region Required for Sequencing

To further characterise the cloned genomic region, the 2.1 kb *Bam*HI/*Sal*I fragment that hybridised to the *A. parasiticus* 1.8 kb pBVer-1 *Eco*RI/*Hind*III fragment (Table 7) was subcloned (Section 2.7) into M13mp18 give plasmid pCG2 (Table 1). The identity of the fragment subcloned was confirmed by restriction digestion (Section 2.6.1.1) and gel electrophoresis (Section 2.6.2.1).

### 3.6.3 Sequence Analysis of Clone $\lambda$ CGV2

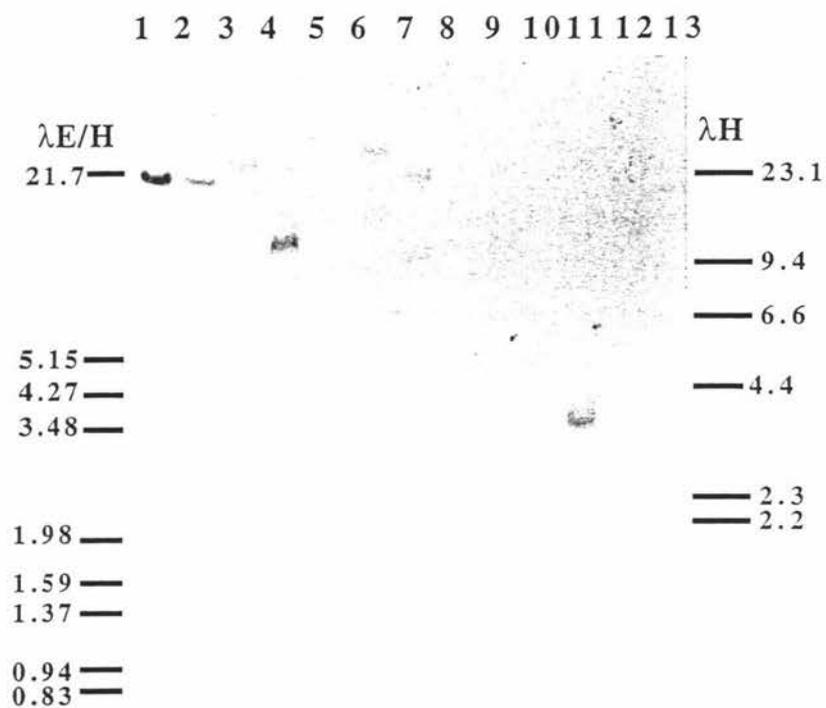
DNA from the plasmid subclones (Section 3.5.1) was isolated by the alkaline lysis method (Section 2.5.1), and sequenced using the Sequenase Version 2.0 (Section 2.12) sequencing system. An M13 universal primer (Table 2) was used to obtain insert sequence. Sequence from one end of the subclone enabled partial sequence of one end of the 2.1 kb *Bam*HI/*Sal*I fragment to be obtained.

Table 8 Codon Bias Table

<u>AmAcid Codon Number Fraction</u>				<u>AmAcid Codon Number Fraction</u>			
Gly	GGG	2.00	0.09	Ser	TCG	4.00	0.22
Gly	GGA	2.00	0.09	Ser	TCA	2.00	0.11
Gly	GGT	7.00	0.30	Ser	TCT	5.00	0.28
Gly	GGC	12.00	0.52	Ser	TCC	4.00	0.22
Glu	GAG	7.00	0.88	Arg	CGG	2.00	0.08
Glu	GAA	1.00	0.13	Arg	CGA	5.00	0.19
Asp	GAT	6.00	0.50	Arg	CGT	7.00	0.27
Asp	GAC	6.00	0.50	Arg	CGC	12.00	0.46
Val	GTG	0.00	0.00	Gln	CAG	3.00	0.30
Val	GTA	4.00	0.15	Gln	CAA	7.00	0.70
Val	GTT	6.00	0.23	His	CAT	5.00	0.50
Val	GTC	16.00	0.62	His	CAC	5.00	0.50
Ala	GCG	3.00	0.14	Leu	CTG	0.00	0.00
Ala	GCA	3.00	0.14	Leu	CTA	1.00	0.10
Ala	GCT	4.00	0.18	Leu	CTT	1.00	0.10
Ala	GCC	12.00	0.55	Leu	CTC	6.00	0.60
Arg	AGG	0.00	0.00	Pro	CCG	0.00	0.00
Arg	AGA	0.00	0.00	Pro	CCA	1.00	0.17
Ser	AGT	0.00	0.00	Pro	CCT	1.00	0.17
Ser	AGC	3.00	0.17	Pro	CCC	4.00	0.67
Lys	AAG	9.00	0.64				
Lys	AAA	5.00	0.36				
Asn	AAT	1.00	0.11				
Asn	AAC	8.00	0.89				
Met	ATG	4.00	1.00				
Ile	ATA	0.00	0.00				
Ile	ATT	2.00	0.13				
Ile	ATC	14.00	0.88				
Thr	ACG	0.00	0.00				
Thr	ACA	2.00	0.17				
Thr	ACT	2.00	0.17				
Thr	ACC	8.00	0.67				
Trp	TGG	1.00	1.00				
End	TGA	6.00	0.75				
Cys	TGT	2.00	0.67				
Cys	TGC	1.00	0.33				
End	TAG	0.00	0.00				
End	TAA	2.00	0.25				
Tyr	TAT	0.00	0.00				
Tyr	TAC	4.00	1.00				
Leu	TTG	2.00	0.20				
Leu	TTA	0.00	0.00				
Phe	TTT	5.00	0.50				
Phe	TTC	5.00	0.50				

**Fig. 13**      **Southern blot of  $\lambda$ CGV2 probed with the *D. pini ver-1*  
fragment**

Autoradiograph of Southern blot of gel shown in Fig. 8A hybridised to [ $\alpha$ - $^{32}$ P]dCTP labelled 0.8 kb *D. pini SalI*  $\lambda$ CGV1 fragment.



### 3.6.4 Sequence Identification

Limited nucleotide sequence analysis of the  $\lambda$ CGV2 region gave a total of 436 bp (Fig. 14). The amino acid sequences from all six possible translation frames were compared to the GenBank sequence database at the National Centre for Biotechnology Information with NCSA Mosaic using the BLASTX 1.4 program. This search revealed no significant similarity with any sequences on the GenBank database.

### 3.6.5 Comparison Between *D. pini* Sequences

Comparisons of the partial *D. pini* nucleotide sequences obtained from  $\lambda$ CGV1 and  $\lambda$ CGV2 were performed using the BESTFIT and GAP programs of the GCG package with a gap weight of 5.0 and a gap length weight of 0.10. There were no significant similarities (data not shown). Comparison of the  $\lambda$ CGV2 sequence, using the CLUSTAL W programme, with the *A. nidulans stcS* gene (previously *ver-B*, a second gene required for conversion of VA to ST, Keller *et al.* 1995) revealed no significant similarity (data not shown). The sequence was also compared to other genes (*stcT*, *stcV*, and *stcW*) adjacent to *ver-A* (i.e. *stcU*) in the *A. nidulans* ST gene cluster (Brown *et al.* 1996), all of which indicated no significant alignment.

### 3.6.6 Comparison of GC Content

The GC content of the region sequenced was determined with the COMPOSITION program of the GCG package. The GC content was 49%, thus showing a preference to AT. This GC content is similar to that of the intron regions of the *ver-1* gene from *A. parasiticus* (38% and 50%), *ver-A* gene from *A. nidulans* (51% and 49%), and the putative *D. pini ver-1* gene (38% and 49%). Therefore, this indicates that the 436 bp sequence may be a non coding region.

## 3.7 Isolation and Characterisation of $\lambda$ Clone Hybridising to the *A. parasiticus nor-1* Gene

### 3.7.1 Library Screening

Filters containing approximately 50 000 pfu from the *D. pini* genomic library were screened by plaque hybridisation (Section 2.11) with the [ $\alpha$ - $^{32}$ P]dCTP-labelled 1.7 kb

**Fig. 14**            **Partial sequence of a *ver-1* hybridising fragment from clone  $\lambda$ CGV2**

Partial DNA sequence of a 2.1 kb *Bam*HI/*Sal*I fragment from clone  $\lambda$ CGV2 which hybridised to the *A. parasiticus ver-1* gene and the putative *D. pini ver-1* gene (from clone  $\lambda$ CGV1) .

1 GTTGTGTAATGCTGGGTAAGCGCGCAGGAATCTGGTAGTCCACCATAAGTTGCCAACTGT 60  
61 GAATTCCATGTCCGTAGAGGTTGTTTGC GGCTTGCAGTAGCATATGATCTATGCCATAGA 120  
121 GGCAGCCCAAGCGCTGATCTCTTCTGCAATATCGGCATCGCAATGGGACGAAATCGCCTG 180  
181 ACTTCAGACTTGCTGGCAATGAGCAGGCTGGGAGTTGCCATTGCGAGGGATAGGCGCTAC 240  
241 GTGTGCGTGTCCACACACATTCTAATCAAGCCTCTGTCGCCATCTCCCGGAGACATTTAA 300  
301 GCCTTCTTGATTCTCGATGAAGGtaGCGATATCAAGAAAaCATATTCATCAGTCGTCTGT 360  
361 GAGTATCTTCTAATTGCGTGCTGTTGACATACAGATACGTTGTCTGTTGCTGATCCAAA 420  
421 ACATCGAACCACGAAC 436

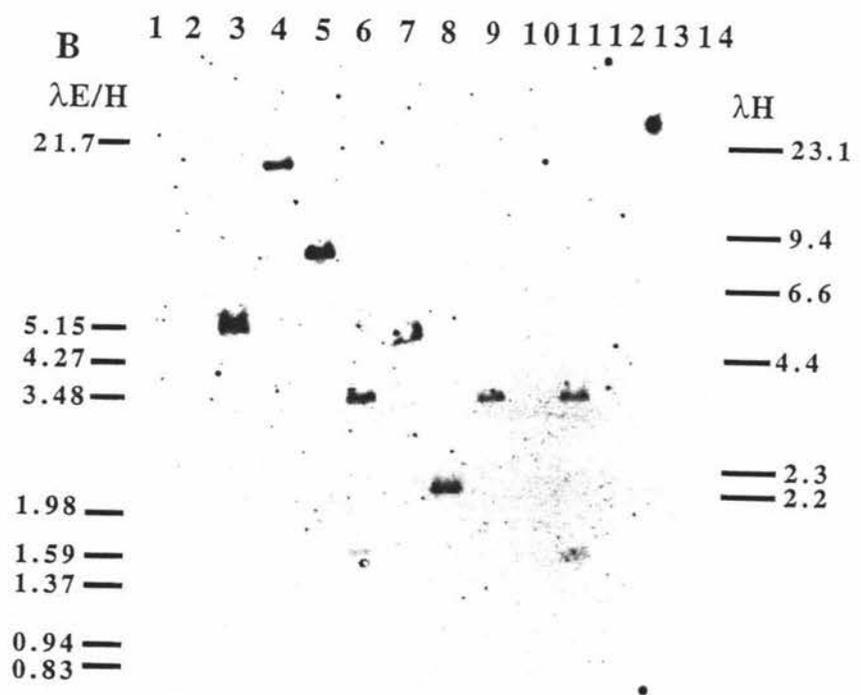
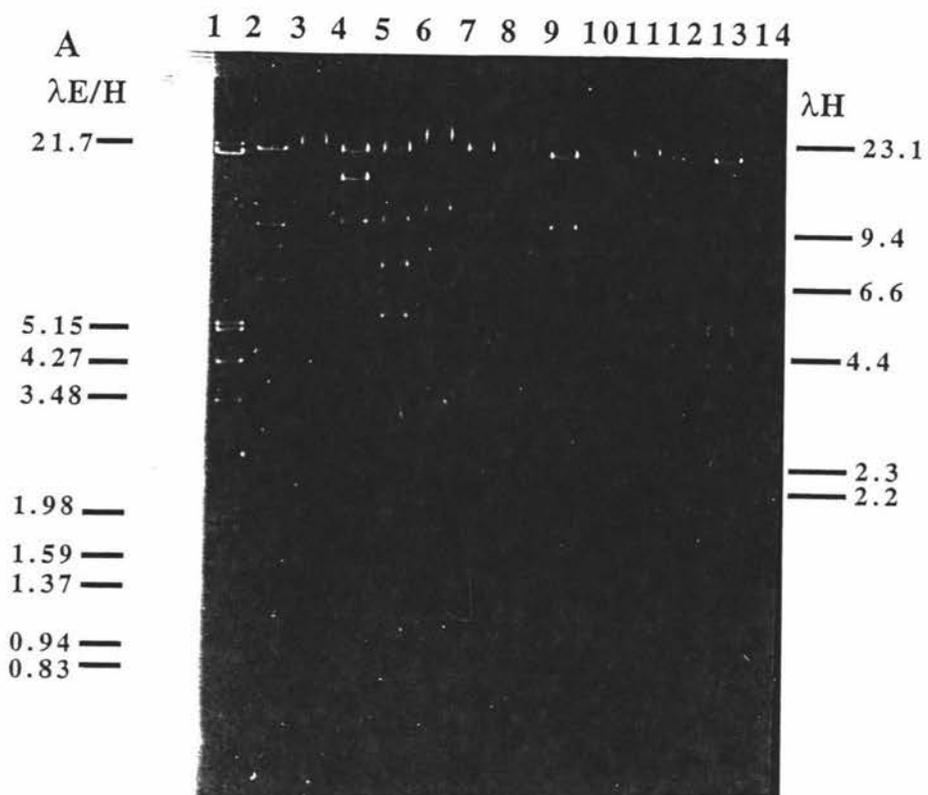
*nor-1* gene fragment from *A. paraciticus* *SphI/EcoRI* digested pNA17 (Section 2.9.2) using the conditions determined in Section 3.1 (i.e. 55°C). The filters were washed at 65°C in 3 x SSC and 0.2% SDS. Initially 1 positive plaque hybridised with the probe, however on the second screen this plaque did not remain positive. The *nor-1* primary membranes were rescreened and revealed 1 new positive plaque ( $\lambda$ CGN2). On the second screen this plaque remained positive. A further round of plaque purification using filters containing approximately 100 pfu was performed revealing numerous positive plaques. Two plaques were taken of this positive clone and DNA was isolated (Section 2.5.4).

### 3.7.2 Restriction Digestion of Clone $\lambda$ CGN2 and Southern Hybridisation

Inserts in the positive clone were digested and separated by gel electrophoresis as in Section 3.4.2 (Fig. 15A). Information from these digests is summarised in Table 9. A Southern blot of this gel was hybridised to the [ $\alpha$ -<sup>32</sup>P]dCTP-labelled (Section 2.9) 1.7 kb *nor-1* gene fragment from *A. paraciticus* *SphI/EcoRI* digested pNA17. This blot revealed that clone  $\lambda$ CGN2 contained fragments hybridising to the *nor-1* probe (Fig. 15B, fragments that hybridised are identified by a superscript a in Table 9). No fragments from clone  $\lambda$ CGN2 which hybridised were the same size as those in the corresponding genomic digest from the original hybridisation (Section 3.2.1, Fig. 4B). The probe hybridised to a 6.2 kb *BamHI* fragment of clone  $\lambda$ CGN2, but no signals remained in the *BamHI* genomic DNA hybridisation analysis following stringent washes. Also, in the  $\lambda$ CGN2 *EcoRI* digest, the probe hybridised to a 14.1 kb fragment. Since the size of the insert can be estimated to be about 14 kb, it is likely that there are no *EcoRI* sites in this fragment. This is opposed to the genomic DNA hybridisation which gave *EcoRI* signals of 5.2 kb, 4.4 kb, 3.4 kb, and 1.4 kb. Therefore, further analysis of clone  $\lambda$ CGN2 was not performed.

**Fig. 15A-B Southern blot of  $\lambda$ CGN2 probed with *nor-1***

**A** Restriction digestion profile of clone  $\lambda$ CGN2 where: *Bam*HI (lane 3), *Eco*RI (lane 4), *Sal*I (lane 5), *Xho*I (lane 6), *Bam*HI and *Eco*RI (lane 7), *Bam*HI and *Sal*I (lane 8), *Bam*HI and *Xho*I (lane 9), *Eco*RI and *Sal*I (lane 10), *Eco*RI and *Xho*I (lane 11), and *Sal*I and *Xho*I (lane 12) digests flanked by *Eco*RI/*Hind*III digested  $\lambda$  DNA (lanes 1 and 13) and *Hind*III digested  $\lambda$  DNA (lanes 2 and 14). **B** Autoradiograph of Southern blot of gel shown in A hybridised to [ $\alpha$ -<sup>32</sup>P]dCTP labelled 1.7 kb pNA17 *Sph*I/*Eco*RI fragment.



**Table 9** Data from Southern Hybridisation Analysis of Clone  $\lambda$ CGN2

Restriction digest	Fragment size (kb)
<i>Bam</i> HI	16.7, 11.3, 6.2 <sup>a</sup> , 3.8, 2.0, 1.8, 1.6
<i>Eco</i> RI	16.7, 14.1 <sup>a</sup> , 11.0
<i>Sal</i> I	16.7, 11.0, 8.6 <sup>a</sup> , 6.5, 1.3, 1.0
<i>Xho</i> I	17.2, 11.7, 3.8 <sup>a</sup> , 2.2, 1.6 <sup>a</sup>
<i>Bam</i> HI/ <i>Eco</i> RI	16.7, 11.3, 5.5 <sup>a</sup> , 3.9, 1.8, 1.6, 1.2, 0.8
<i>Bam</i> HI/ <i>Sal</i> I	16.7, 11.3, 2.8, 2.4 <sup>a</sup> , 2.0, 1.8, 1.6, 1.3, 1.0
<i>Bam</i> HI/ <i>Xho</i> I	16.7, 11.0, 3.8 <sup>a</sup> , 2.5, 2.0, 1.6, 1.1, 1.0, 0.8
<i>Eco</i> RI/ <i>Sal</i> I	*
<i>Eco</i> RI/ <i>Xho</i> I	16.7, 11.3, 7.3, 3.9 <sup>a</sup> , 2.2, 1.7 <sup>a</sup>
<i>Sal</i> I/ <i>Xho</i> I	*

<sup>a</sup> Indicates fragments hybridising to [ $\alpha$ -<sup>32</sup>P]-labelled *A. paraciticus* 1.7 kb *SphI/EcoRI* *nor-1* gene fragment (see Fig. 15B).

\* no bands were visible in these digests, possibly due to degradation.

## 4.0 DISCUSSION

### 4.1 Identification of Clone $\lambda$ CGV1

The basic assumption that determined the experimental approach in this study was that genes for dothistromin biosynthesis can be identified and cloned by hybridisation of heterologous genes from *Aspergillus parasiticus*. The results show that this is possible. During Southern blot analysis of *Bam*HI and *Eco*RI digested *D. pini* genomic DNA, using a fragment of the *A. parasiticus ver-1* gene as a heterologous probe, the presence of a region of similar sequence was indicated by five hybridisation signals (Section 3.2.2). Following high-stringency washes (1 x SDS, 0.2% SSC) four of these signals remained: a 1.6 kb *Bam*HI fragment, and 16.0 kb, 11.5 kb, and 2.7 kb *Eco*RI fragments. The 1.6 kb *Bam*HI signal hybridised strongly, while the other three were very faint signals (Section 3.2.2). This region was then cloned by isolation and characterisation of the  $\lambda$ CGV1 library clone (Section 3.4), and a 0.8 kb *Sal*I fragment sequenced (Section 3.6.3). Comparison of the deduced amino acid sequence of this fragment (from clone  $\lambda$ CGV1) with GenBank amino acid sequences revealed a high level of similarity with the *A. parasiticus ver-1* (78% identity), *A. nidulans ver-A* (78% identity), *M. grisea ThnR* (62% identity), and *C. lagenarium Thr1* (64% identity) genes (Section 3.6.5). This similarity included homology with the postulated NADPH-dependent keto-reductase active site amino acid sequence binding domain (GXGXXA) which contains homologies with NADPH-dependent reductases found in other polyketide biosynthetic pathways. The sequence similarities suggest that the *A. parasiticus ver-1* gene and the *A. nidulans ver-A* (the *ver-1* homologue) gene have a homologue *ver-1* in *D. pini* (hereafter called *Dpver-1* in this discussion to avoid confusion with the *A. parasiticus ver-1* gene).

### 4.2 Role of the *ver-1*-like Genes

#### 4.2.1 The Role of the *Aspergillus ver-1* and *ver-A* Genes

Over the past few years intensive efforts have been directed at delineating the biochemistry leading to ST/AF. However, this complex and important pathway in fungal secondary metabolism remains incompletely characterised. It is unclear how many enzymatic activities are needed to produce each intermediate from its intermediate precursor after the initial polyketide backbone is formed. The molecular rearrangements needed to convert each intermediate to the next known metabolite in the pathway are often

complex, and it is possible that more than one enzyme is required for each step or that some enzymes have more than one function. The identification of the gene(s) required for each bioconversion will contribute significantly to its final characterisation.

According to their complementation properties and to the high similarity between their deduced amino acid sequences with those of reductases, the *ver-1* and *ver-A* gene products have been precisely associated with the transformation of the anthraquinone versicolorin A (VA) to sterigmatocystin (ST). The structural changes needed for the conversion of VA to ST suggested that several enzyme-mediated steps were involved (Keller *et al.* 1995). The complete set of enzymes which catalyse this conversion have not yet been identified, probably because of their short half-life and their instability. The lack of purified enzyme activity together with the absence of identified intermediates between VA and ST make it difficult to elucidate the exact function of the *ver* genes. The conversion of VA to ST has been proposed to contain at least four enzymatic steps including keto-reduction, oxidation, decarboxylation, and methylation (Bhatnagar *et al.* 1992, Dutton 1988). Nucleotide sequence analysis of the *ver-1* gene showed that it probably encodes a NADPH-dependent ketoreductase (Skory *et al.* 1992). When the predicted amino acid sequence of *ver-1* was compared with the EMBL and GenBank databases it revealed a striking similarity (30% identity) with the polypeptide sequence for the *Streptomyces coelicolor actIII* gene (Hallam *et al.* 1988), which encodes a ketoreductase associated with the biosynthesis of the polyketide antinorhodin. Based on this result the *ver-1* gene was proposed to be responsible for a keto-reduction. In *Aspergillus versicolor*, a metabolite called 6-deoxyversicolorin A (6-deoxy VA) has been identified which produces ST (Elsworthy *et al.* 1970). This prompted Liang *et al.* (1996) to propose, more specifically, that the *ver-1* gene is involved in the keto-reduction of VA to form 6-deoxy VA. In support of this, a polyhydroxyhaphthalene reductase involved in melanin biosynthesis in *Magnaporthe grisea* was recently purified to homogeneity, and shown to display a 65% identity and 82% similarity with the deduced amino acid sequence of the *ver-1* gene product. This dehydroxylation reaction, in part catalysed by this reductase, is entirely analogous to the proposed keto-reduction (i.e. deoxygenation) of VA. A further analogous reaction is the reduction of emodin to chrysophanol at an early stage of the biosynthesis of ergochromes (fungal pigments produced by *Claviceps purpurea*; Anderson 1986). This conversion, which requires NADPH, is believed to consist of two steps, reduction and dehydration (Ichinose *et al.* 1993). Based on these observations, Liang *et al.* (1996) hypothesise that VA is processed by two successively operating enzymes, the product of the *ver-1* gene and a dehydratase, to form 6-deoxy VA. Recently, Keller *et al.* (1995) isolated a second gene, *stcS*, involved in the

conversion of VA to ST in *A. nidulans*. *stcS* is located within 2 kb of *ver-A* (renamed *stcU* in their publication) in the ST gene cluster. They showed that disruption of *stcS* also results in failure to convert VA to ST. Sequence analysis of *stcS* shows that it contains the conserved heme-binding motif found in all cytochrome P-450 monooxygenases, thus it is likely that *stcS* catalyses the proposed oxidation step necessary to convert VA to ST. This is the first genetic proof that this step requires more than one enzymatic activity. Before completely understanding the conversion of VA to ST, however, it is necessary to clone several other genes involved in this complex reaction. The close spatial relationship between these genes could lead to future studies focused on the isolation and characterisation of other genes involved in the conversion of VA to ST in order to more clearly understand this process.

#### 4.2.2 The Role of the *ThnR* and *ThrI* Genes

As mentioned above, examination of the fungal melanin biosynthetic pathway in *M. grisea* and *C. lagenarium* reveals the existence of a similar two-step dehydroxylation conversion. *M. grisea* is the agent of rice blast disease, and *C. lagenarium* causes anthracnose of cucumber. During melanin biosynthesis, the tetrahydroxynaphthalene reductase catalyses the NADPH-dependent reduction of 1,3,6,8-tetrahydroxynaphthalene (T<sub>4</sub>HN) into (+)scytalone, and 1,3,8-trihydroxynaphthalene into (-)vermelone (Wheeler *et al.* 1976).

The involvement of fungal melanin in pathogenicity has been well established in these fungi. Melanin is a high-molecular-mass black pigment, which is synthesised by numerous pathogenic fungi. These fungi produce melanised appressoria during the infection process, which has been implicated as an important factor for penetration and pathogenicity. It is envisaged that melanin mediates the build-up of pressure in the appressorium and that this high pressure provides the essential driving force for mechanical penetration (Takano *et al.* 1995). The importance of appressorial melanisation in penetration and pathogenicity was first noted in a *C. lagenarium* albino mutant (Pks<sup>-</sup>). Following this, the *PKS1* gene was cloned by complementation of this mutant to the wild type phenotype (Kubo *et al.* 1991). Recently, the *PKS1* gene and its flanking regions were sequenced (Takano *et al.* 1995). The predicted PKS1 polypeptide of *C. lagenarium* shares highly significant homology with type 1 polyketide synthase enzymes (PKSs). The *PKS1* gene contains the highly conserved  $\beta$ -ketoacyl synthase, acetyl/malonyl transferase, and acyl carrier protein domains present in other PKSs. This result strongly suggests that the *PKS1* gene encodes a polyketide synthase involved in melanin biosynthesis in *C. lagenarium*.

The fungal melanin biosynthetic pathway has been established by genetic studies using melanin-deficient mutants which accumulate shunt products and exhibit pigmentation phenotypes (Bell and Wheeler 1986). These mutants, lacking the capacity to synthesise melanin, lose their ability to penetrate the host leaf and, consequently, their pathogenicity. The biosynthetic pathway for melanin starts from pentaketide synthesis which is cyclised to 1,3,6,8-tetrahydroxynaphthalene (T<sub>4</sub>HN). The subsequent steps consist of a series of reductions and dehydrations leading to 1,8-dihydroxynaphthalene (DHN) via scytalone, 1,3,8-trihydroxynaphthalene (T<sub>3</sub>HN) and vermeline. Polymerisation and oxidation of DHN yields melanin (Wheeler *et al.* 1976). In contrast with the intermediates, the enzymes carrying out these transformations are much less characterised. Biochemical analysis of the two enzymes, dehydratase and reductase, has been reported in *Verticillium dahliae*, *Cochliobolus miyabeanus*, and *M. grisea* (Wheeler *et al.* 1982, Tajima *et al.* 1989, Vidal-Cros *et al.* 1994). Recently, the genes encoding this reductase in *M. grisea* and *C. lagenarium*, *ThnR* and *ThrI*, respectively, have recently been cloned and characterised (Vidal-Cros *et al.* 1994, Perpetua *et al.* 1996). Their deduced amino acid sequences are almost identical (81%). They also show significant similarity (65% identity each) to the *ver-1* gene of *A. parasiticus*. This high sequence identity between the *ver-1*, *ThnR*, and *ThrI* genes, coupled with the biochemical characterisation of the polyhydroxynaphthalene reductase (Vidal-Cros *et al.* 1994), suggests that the proteins encoded by *ThnR* and *ThnI* are the counterparts of *ver-1* for the melanin biosynthetic pathway. The development of future disruption strains coupled with biochemical, labelling, and cross-feeding studies, will enable precise identification of the order and number of enzymatic steps in this conversion and the similar conversion of VA to ST.

### 4.3 Duplication of the *ver-1* Gene

Southern hybridisation analysis has indicated that there are at least two copies of the *ver-1* gene, designated *ver1A* and *ver1B*, located in separate regions in the *A. parasiticus* (strain SU-1) genome (Skory *et al.* 1992). By comparing the restriction enzyme polymorphisms present in these two chromosomal copies with the cloned *ver-1* gene, it was shown that the gene originally cloned was *ver-1A* (Trail *et al.* 1995a). *ver-1B* was subsequently cloned and its nucleotide sequence determined (Liang *et al.* 1996). These genes were found to share 93% nucleotide sequence identity. A translational stop codon was identified near the middle of the *ver-1B* gene coding region suggesting that it encodes a truncated polypeptide. Gene disruption and genetic complementation experiments recently confirmed that *ver-1A* is the only functional copy of *ver-1* in *A. parasiticus* and

that its gene product is directly involved in the conversion of VA to ST (Liang *et al.* 1996). This duplication of *ver-1* genes is thought to be the consequence of a duplicated chromosomal region (>12 kb) which also contains an additional copy of *nor-1* and *aflR* (Trail *et al.* 1995a, Yu *et al.* 1995). To date the presence of only one copy of these genes has been demonstrated in *A. flavus* (Skory *et al.* 1992). The duplication of a portion of the AF gene cluster in *A. parasiticus* (especially the duplication of one of the pathway regulators) but not in *A. flavus* may help explain the observation that more than 90% of *A. parasiticus* strains isolated produce high levels of aflatoxins, whereas many *A. flavus* isolates (up to 40% or more) produce no aflatoxins (Bennett and Papa 1988).

#### 4.4 Identification of Clone $\lambda$ CGV2

During the cloning of the region containing similar sequence to the *A. parasiticus ver-1* gene, a second clone ( $\lambda$ CGV2) was also identified (Section 3.4.1). Southern hybridisation analysis of this clone (Section 3.4.2), using the same probe, revealed many hybridisation signals. One of these, an 11.4 kb *EcoRI* signal, was the same size as a signal on the corresponding digest of genomic DNA indicated by Southern hybridisation analysis. This suggests that the chromosomal region containing the sequence similar to the *A. parasiticus ver-1* gene is located on this clone. This 11.4 kb *EcoRI* signal also corresponds to a  $\lambda$ GEM-12 arm (Table 7). Observation of the gel for the  $\lambda$ CGV2 Southern blot, along with calculations of  $\lambda$  insert size, suggests that the 11.4 kb fragment may be a doublet (Fig 8A) of  $\lambda$  arm + insert fragments. The other digest corresponding to a genomic DNA digest, *BamHI*, did not indicate a hybridisation signal of the same size: genomic DNA Southern hybridisation gave a strong 1.6 kb signal and a weak 16.0 kb signal, whereas the  $\lambda$  clone DNA Southern hybridisation gave a 2.45 kb signal. This is therefore inconsistent with the result indicated in the *EcoRI* digests. Because there was a  $\lambda$  DNA signal the same size as that indicated in the *EcoRI* genomic digest, then the *BamHI* digest signals should also correspond. However, under low-stringency washing conditions (3 x SSC, 0.2% SDS) the *A. parasiticus ver-1* probe hybridised to 16.0 kb and 1.6 kb genomic DNA fragments of the *EcoRI* digest. Only the 1.6 kb signal remained following more stringent washes (1 x SSC, 0.2% SDS). But because during screening of the *D. pini* library, the filters were washed under the low-stringency conditions, clones containing part of this 16.0 kb signal would have remained. Therefore, it is postulated that the 2.45 kb *BamHI* fragment seen in clone  $\lambda$ CGV2 may have been derived from the 16.0 kb genomic *BamHI* fragment by being cut by *MboI* digestion during library construction. In this case, the hybridising 2.45 kb *BamHI*

fragment is expected to lie next to one of the arms in the  $\lambda$ CGV2 clone. Unfortunately, due to loss of resolution of fragments (particularly those digested with *SalI*), a meaningful restriction map of clone  $\lambda$ CGV2 could not be worked out from the available data.

Restriction digestion and hybridisation analysis of clones  $\lambda$ CGV1 and  $\lambda$ CGV2 suggests that they share regions of similar sequence. When the 0.8 kb fragment of clone  $\lambda$ CGV1 was hybridised to clone  $\lambda$ CGV2 distinct hybridisation signals were observed. Interestingly, the signals corresponded exactly with the signals obtained when the same  $\lambda$ CGV2 blot was hybridised to the *A. parasiticus ver-1* gene fragment. This supports the sequence evidence suggesting that clone  $\lambda$ CGV1 contains a *D. pini ver-1* gene. But because none of the sizes of hybridising fragments corresponded to any of those observed during analysis of clone  $\lambda$ CGV1, clone  $\lambda$ CGV2 must therefore be located in a different genetic region. Also, the different sizes of the hybridising fragments suggest that the clones are not overlapping; restriction mapping analysis shows the 0.8 kb *SalI* fragment of  $\lambda$ CGV1 to be located in the centre of the clone. The only corresponding (hybridising) *SalI* fragment in clone  $\lambda$ CGV2 was 2.35 kb in size. Considering all the above evidence, it is difficult to come to a firm conclusion as to what clone  $\lambda$ CGV2 contains. By taking the evidence that the clones are located in different genetic regions, two hypotheses can be made. (i) Clone  $\lambda$ CGV2 may contain a further *D. pini ver-1* gene. This hypothesis is supported by the recent finding that there are two *ver-1* genes present in different regions in the *A. parasiticus* genome (Section 4.3). (ii) Clone  $\lambda$ CGV2 may contain a different gene encoding a further enzyme required for a similar conversion step in the same, or a related, pathway. Given that the step involving the conversion of VA to ST has been proposed to contain at least five enzymatic steps (Section 4.2.1), a similar conversion in *D. pini* may also involve multiple enzymes. Moreover, in the conversion of norsolorinic acid (NA) to averantin (AVN) there are believed to be up to three parallel pathways for this conversion (Bhatnagar *et al.* 1992). So far two genes, *nor-1* (Chang *et al.* 1992) and *norA* (Cary *et al.* 1996), each separately able to convert NA to AVN have been identified and shown to be located in different regions of the genome. However, these genes share only a low (22%) sequence identity.

Limited sequence analysis of a 2.1 kb *BamHI/SalI* fragment from clone  $\lambda$ CGV2 was performed, and 436 bp of sequence was obtained (Section 3.6.3). The deduced amino acid sequence was then compared to the GenBank database. No significant level of similarity was detected. Comparisons with the other *ver-1*-like gene sequences also failed to show any similarities. Further sequencing of the 2.1 kb region may allow detection of

any significant similarities. For example, similarities between the sequence from clone  $\lambda$ CGV1 and a more complete  $\lambda$ CGV2 sequence may be observed from the other end of the 2.1 kb region.

#### 4.5 Identification of Clone $\lambda$ CGN2

During Southern blot analysis of *Bam*HI and *Eco*RI digested *D. pini* genomic DNA, using a fragment of the *A. parasiticus nor-1* gene as a probe, the presence of an heterologous region of sequence similarity was indicated by several hybridisation signals (Section 3.2.1). Following high-stringency washes (1 x SDS, 0.2% SSC) only four of these signals, all in the *Eco*RI digested lane, remained. They were: 5.2 kb, 4.4 kb, 3.4 kb, and 1.4 kb in size; although only the 1.4 kb signal hybridised strongly. It is not clear why a signal did not remain in the *Bam*HI lane. Observation of the gel for the Southern blot does not indicate any less DNA than the *Eco*RI lane, or any partial digestion. It is possible that the DNA in the *Bam*HI lane did not transfer completely to the nylon membrane during Southern blotting, particularly as only faint large *Bam*HI fragments (of approximately 21.8 kb and 13.4 kb) were visible, following low stringency washes, in the genomic Southern hybridisation.

Upon screening of the *D. pini* genomic library a single clone ( $\lambda$ CGN2) was obtained (Section 3.8.1). Southern hybridisation analysis of this clone (Section 3.8.2), using the same *nor-1* probe, revealed specific hybridisation signals to fragments of the clone. However, none of the hybridisation fragments in the *Eco*RI digested lane were the same size as any of those in the corresponding genomic digest which remained after stringent washing. This suggests that this clone does not contain the *nor-1*-equivalent gene from *A. parasiticus*. Since there were so many weakly hybridising bands to *nor-1* in both the *Bam*HI and *Eco*RI digests of the genomic Southern blot, this does offer a possible explanation. The library filters were washed in 3 x SSC, 0.2% SDS, so because of this low-stringency washing, the clone derived may therefore represent a low-stringency hybridisation. Due to this evidence, further analysis of clone  $\lambda$ CGN2 was not performed. However, the existence of the distinct *Eco*RI 1.4 kb genomic signal indicates that there is a region of significant similarity to the *A. parasiticus nor-1* gene in the *D. pini* genome, therefore further investigation should be carried out. An additional point is that only one clone hybridising to the *nor-1* probe was obtained from the library. Therefore further investigation should begin by obtaining clones by screening a new *D. pini* library.

## 4.6 Potential Uses of the *D. pini ver-1* Gene

### 4.6.1 Cloning of Other Pathway Genes

Continued identification of dothistromin biosynthetic genes will aid in the elucidation of dothistromin biosynthesis. Genes of particular interest include those involved in the earlier steps of dothistromin biosynthesis since many of the subsequent pathway intermediates may themselves be toxic in nature. A current study is focusing on isolation of the *D. pini* polyketide synthase gene using the heterologous probe method employed in this study. Progress is already underway; *D. pini* genomic DNA Southern hybridisation analysis using a 1.3 kb fragment of the *6-MSA* gene from *Penicillium patulum* has indicated the presence of a region of sequence similarity. In addition, as mentioned above (Section 4.4) further investigation will be performed to obtain clones containing a possible *nor-1 D. pini* homologue. In confirmation of the result obtained in this study, which indicated a *D. pini* region of significant similarity to the *A. parasiticus nor-1* gene (Section 3.2.1), genomic DNA Southern hybridisation analysis has been reproduced in further investigation.

The size of *A. parasiticus/A. flavus* AF and *A. nidulans* ST clusters and the striking conservation of genes and cluster organisation (Section 1.9) suggests that dothistromin biosynthetic genes may also be located in a cluster. Clustering of the dothistromin biosynthetic genes will make available other molecular biology approaches, such as chromosome walking, for the isolation of further pathway genes. Chromosome walking will allow the isolation of gene sequences whose genetic location is approximately known. It is important that the starting point for the walk is as close as possible to the suspected destination point. A recently presented schematic diagram of the AF gene cluster depicts *ver-1* as located near the middle (Cary *et al.* 1996). The newly characterised gene, *norA* (Cary *et al.* 1996), thought to be involved in the conversion of norsoloric acid (NA) to averantin (AVN), is located directly upstream of *ver-1* within the AF gene cluster. This not only offers an ideal opportunity for chromosome walking but also a promising opportunity for cloning a gene involved in an earlier step in the pathway. By using fragments of the  $\lambda$ CGV1 clone as a starting point for the walk, a *D. pini* genomic library can be screened to identify overlapping clones. These clones can then be analysed by Southern hybridisation analysis and nucleotide sequence analysis. This walk can potentially occur in both directions along the chromosome, thereby allowing for the isolation of further genes.

NA represents the first identified stable intermediate in AF biosynthesis. Following the cloning and disruption of the *nor-1* gene (Trail *et al.* 1994) shown to be involved in this conversion, it was found that disruption of this gene failed to block completely the synthesis of AF. This result was consistent with earlier observations that NA-accumulating mutant strains were leaky because of the continued accumulation of much lower, yet significant, levels of AF (Detroy *et al.* 1973). The detectable levels of AF in both the blocked mutant and the *nor-1* disrupted strain indicated that there may be an alternative pathway(s) and consequently other enzymes that catalyse the conversion of AF to AVN and subsequently to averufin (AFN) (Yabe *et al.* 1991, Bhatnagar *et al.* 1992). In the Cary *et al.* (1996) study, the *norA* gene demonstrated a high degree of nucleotide (68%) and amino acid (63%) sequence similarity to an aryl-alcohol dehydrogenase (*aad*) gene from the white rot fungus *Phanerochaete chrysosporium*. The AAD enzyme is involved in lignin degradation and was shown to reduce aromatic benzaldehydes to their respective alcohols in the presence of NADP(H) (Muheim *et al.* 1991). But among the postulated enzymatic mechanisms involved in AF biosynthesis (Bhatnagar *et al.* 1992), none of the steps suggests an AAD-type activity would be required, especially with respect to the conversion of NA to AVN. Although the exact function of the *norA* gene product has yet to be determined, it is believed to be a dehydrogenase-type enzyme. This conclusion is based on the observations that the MAbs used to isolate the *norA* cDNA clone are capable of significantly inhibiting the conversion of NA to AVN (Cary *et al.* 1996). Thus the *A. parasiticus nor-A* gene is distinct from the *A. parasiticus nor-1* gene in both sequence, predicted function and location within the gene cluster. Since *nor-A* is adjacent to *ver-1* in the AF gene cluster it is possible that a *nor-A* homologue will be easily located in *D. pini*.

The clustering of dothistromin biosynthetic genes will not only facilitate the isolation and identification of genes but could have significant implications for how such structural organisation has evolved and is maintained in fungi. The significance (if any) of gene clustering in the function, regulation, or evolution of the AF biosynthetic pathway has not yet been elucidated. No evidence has been obtained to suggest that the presence of the genes organised in the cluster confers any selective advantage for the survival of the organism since aflatoxins do not deter the growth of competing organisms and also do not increase the ability of the organism to invade its hosts. However, the cluster of AF pathway genes may allow all of the pathway genes to be expressed rapidly upon onset of secondary metabolism since aflatoxins start to accumulate rapidly after 18-20 hours of mycelium growth. This may ensure that all of the aflatoxin pathway enzymes are available at the same time for efficient production, assuming that the fungus needs to produce aflatoxins at a rapid rate. There is no evidence that the toxin gene cluster is the

result of either horizontal gene transfer or vertical transmission from common ancestral genes since no pattern of homology is evident between aflatoxin pathway genes and the sources of their homologues (Yu *et al.* 1995). However, it has been suggested that if clustering is related to regulation of gene expression, there is probably a selective advantage to having genes of like function clustered together on a chromosome (Trail *et al.* 1995b). One possibility which is receiving further study is that the AF and ST pathway genes have evolved from genes of pigment biosynthetic pathways in fungi. In support of this notion is the similarity of the *ver-1* and *ver-A* gene products to the *ThnR* and *Thr1* gene products (Chang *et al.* 1995b).

Regardless of the origin or genetic reason behind cluster organisation, it is becoming increasingly clear that genes involved in secondary metabolism are often found in clusters. The clustering of pathway genes is also common for other microbial secondary metabolites. For example, different species of *Streptomyces* produce a variety of polyketide-derived antibiotics, including erythromycin and actinorhodin (Martin and Liras 1989). Several genes in these biosynthetic pathways show a high degree of identity with genes in analogous pathways and are clustered in similar patterns on the chromosome. The genes encoding enzymes in the penicillin and cephalosporin ( $\beta$ -lactam antibiotics) pathways of *Penicillium chrysogenum* (Diez *et al.* 1990) and *Cephalosporium acremonium* (Aharonowitz and Cohen 1992), as well as genes in the trichothecene pathway (toxic sesquiterpenes) in *Fusarium sporotrichoides* (Hohn *et al.* 1993), and the melanin pathway in *Alternaria alternata* (Kimura and Tsuge 1993) occur as gene clusters (Trail *et al.* 1995a). In view of this it seems likely that the dothistromin pathway genes may also be clustered. Comparing the dothistromin gene organisation to those of AF, ST, and melanin will be especially interesting from an evolutionary viewpoint.

#### 4.6.2 Gene Disruptions

In the last few years many new approaches to identify pathogenicity genes in fungi have begun to gain popularity. These techniques appear to offer great potential for the identification of pathogenicity genes and require no prior knowledge of the genes biochemical function. One approach, gene disruption, utilises fungal transformation vectors to both mutagenise and tag the mutated genes. The strategy is to inactivate a pathogenicity gene by integration of the vector into the gene and to detect this mutation by screening transformants for altered pathogenicity. This method has been used to successfully identify genes that affect pathogenicity in *M. grisea* (Van Etten *et al.* 1994).

Ongoing *Dpver-1* research is focused on gene disruption to help define the role that this gene plays in dothistromin biosynthesis, and subsequently pathogenicity. Although, before this is performed, to assess the number of copies of the *Dpver-1* gene homologue in the genome comparative Southern hybridisation analysis should be performed between the cloned fragment and *D. pini* genomic DNA digested with several different restriction enzymes. Targeted disruption of this gene and other future characterised genes will allow testing of the pathogenicity of mutants which are specifically blocked in dothistromin synthesis. Thereby establishing the functional role these genes play in the dothistromin biosynthetic pathway and allow testing of the fundamental assumption that pathogenesis is primarily dothistromin-mediated. The presence of a recently developed transformation system (Bidlake 1996) will enable targeted disruption of wild type dothistromin biosynthetic genes.

Indirect approaches are currently being used for reducing or eliminating *Dothistroma* needle blight. These strategies are designed to block fungal infection of *P. radiata* or block the ability of the fungal pathogen to grow or synthesise dothistromin on *P. radiata*. These costly approaches, including the application of copper fungicides and the breeding of resistant tree varieties, fall short of effective control. With the use of a more direct molecular genetics approach, genetically stable atoxigenic biocontrol strains of *D. pini*, that are known to compete well with toxigenic strains, can be generated by specific deletion of key genes in the dothistromin biosynthetic pathway. Using this gene disruption technology, at least one genetically engineered *Aspergillus* biocontrol strain (an *umv8* disruption strain) has so far been made available for field testing and has proven to be quite successful (Cotty and Bhatnagar 1994). Therefore, this approach holds promise. However, because it depends on survival and successful occupation of an ecological niche by the biocontrol strain, identification of the environmental factors that favour certain isolates of *D. pini* over others must also be understood.

#### **4.6.3 Identification of Molecular Mechanisms which Regulate Pathway Genes**

A further objective is to isolate and characterise regulatory genes involved in dothistromin biosynthesis. Knowledge gained from the cloning and analysis of dothistromin biosynthetic genes should aid in the identification of regulatory genes. If the genetic regulation of dothistromin biosynthesis is understood it may be possible to prevent synthesis of this toxin through repression of gene expression at critical points in the pathway. An understanding of the control mechanisms involved in dothistromin

biosynthesis may lead to the development of agents or genetically engineered plants that inhibit toxin production. This could possibly lead to field approaches for preventing dothistromin production.

Some emphasis has recently been focused on identifying the molecular control mechanisms which regulate AF production. The AF biosynthetic pathway involves several enzymatic steps which appear to be regulated by the *afl-2* gene in *A. flavus* and the *apa-2* (*aflR*) gene in *A. parasiticus*. The *afl-2* (*aflR*) gene was isolated by complementation of an aflatoxin-nonproducing mutant (blocked in AF biosynthesis at the *afl-2* allele) using a wild type genomic cosmid library from *A. flavus* (Payne *et al.* 1993). Genetic evidence, as well as metabolite feeding studies, suggested that *afl-2* is involved in aflatoxin biosynthesis before NA. For example, a mutant strain of *A. flavus* blocked at *afl-2* was unable to convert a number of exogenously supplied pathway intermediates to AF, indicating that key enzyme pathway enzymes were not present. Also, complementation of mutant strains with the wild type *afl-2* gene simultaneously restored expression of several AF pathway enzyme activities in crude cell extracts. This finding suggested that the entire AF pathway may be under the control of a common regulator.

Additional support for a regulatory role of *afl-2* comes from the research of Chang *et al.* (1993). They identified a gene in *A. parasiticus*, *apa-2*, that when transformed into a wild type strain caused overproduction (i.e. up-regulation) of AF biosynthesis and accumulation of pathway intermediates. No mutation comparable to *afl-2* is known in *A. parasiticus*, but sequence comparison of the *apa-2* gene with the *afl-2* gene has shown them to share greater than 96% nucleotide sequence identity. Furthermore, *apa-2* complements the *afl-2* mutation in *A. flavus* (Woloshuk *et al.* 1994). Therefore, *apa-2* appears to be a homologue of *afl-2*. Because the preponderance of data strongly suggested that *afl-2* and *apa-2* are positive regulators of AF biosynthesis, these homologues were renamed *aflR* (Woloshuk *et al.* 1994).

Further characterisation of the *afl-R* locus in *A. flavus* and *A. parasiticus*, by sequence analysis of genomic and cDNA clones, showed that *afl-R* codes for a protein, AFLR, that has a cysteine-rich zinc cluster motif (Chang *et al.* 1993, Woloshuk *et al.* 1994). This zinc cluster motif is a distinct feature of some of the pathway-specific regulatory proteins in fungi and yeasts. It is believed that continued isolation of AF biosynthetic and regulatory genes will aid in the elucidation of additional mechanisms that are involved in control of AF biosynthesis. Identification of regulatory genes and factors that modulate their expression should provide a basis for the development of strategies for the control of AF contamination of food and feeds.

## 5.0 SUMMARY AND CONCLUSIONS

Preliminary progress into elucidating the molecular biology of dothistromin biosynthesis is reported here. Comprehensive understanding will depend on continuing isolation and characterisation of genes involved in the pathway. In this study, the presence of a *D. pini ver-1* gene heterologous to the *A. parasiticus ver-1* and *A. nidulans ver-A* genes, involved in the conversion of versicolorin A (VA) to sterigmatocystin (ST) in the aflatoxin (AF) and ST biosynthetic pathways, is indicated. The presence of this gene was first suggested by Southern hybridisation analysis where *D. pini* genomic DNA probed with a fragment of the *A. parasiticus ver-1* gene resulted in the detection of a region of sequence similarity. This region was then isolated by construction and screening of a *D. pini* genomic library. Two clones,  $\lambda$ CGV1 and  $\lambda$ CGV2, were isolated and Southern hybridisation analysis confirmed that these clones contained sequences hybridising to the *A. parasiticus ver-1* gene fragment. The hybridisation signals were the same size as the genomic DNA Southern hybridisation signals in the corresponding digests. Southern hybridisation analysis also allowed construction of a restriction map for  $\lambda$ CGV1. Sequencing of a 0.8 kb *Sa*II region of this clone, followed by comparison of the deduced amino acid sequence to the GenBank database, revealed a high level of similarity with the *A. nidulans verA* (or *stcU*), *A. parasiticus ver-1*, *M. grisea ThnR*, and *C. lagenarium Thr1* genes. The *D. pini* amino acid coding sequence exhibited 78% identity with the ST/AF biosynthetic genes (*ver-A*, *ver-1*) and 62-64% identity with the melanin biosynthetic genes (*ThnR*, *Thr1*). This similarity included homology with the postulated NADPH-dependent keto-reductase amino acid sequence binding domain which is also found in other NADPH-dependent reductases involved in other polyketide biosynthetic pathways. This data suggested that the *D. pini* genome contains a gene heterologous to the *A. parasiticus ver-1* and *A. nidulans ver-A* genes. This *D. pini* gene was therefore designated *ver-1*.

Southern hybridisation analysis of the second clone,  $\lambda$ CGV2, also indicated that it contained sequence hybridising to the *A. parasiticus ver-1* gene fragment. However, clones  $\lambda$ CGV1 and  $\lambda$ CGV2 contained no common restriction fragments. Southern hybridisation of  $\lambda$ CGV2 digests with the 0.8 kb *Sa*II  $\lambda$ CGV1 fragment as a probe revealed cross-hybridisation between these two clones. Partial sequencing of a 2.1 kb *Bam*HI/*Sa*II fragment of  $\lambda$ CGV2, followed by comparison of the deduced amino acid sequence using GenBank, detected no significant similarity. Comparison of the

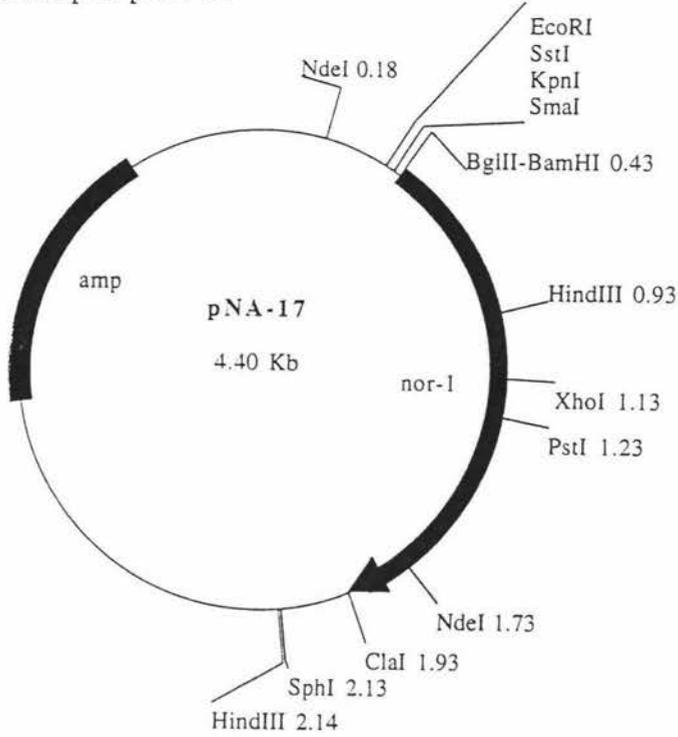
sequences from clones  $\lambda$ CGV1 and  $\lambda$ CGV2 with each other likewise did not show any significant similarity. The relationship between these clones remains unresolved and requires further sequence analysis.

Genomic DNA Southern hybridisation analysis also suggested the presence of a region of sequence similarity to the *A. parasiticus nor-1* gene, which is involved in the conversion of norsolorinic acid (NA) to averantin (AVN) early in the AF biosynthetic pathway. Subsequent screening of the *D. pini* genomic library with the heterologous *A. parasiticus nor-1* probe lead to the isolation of one clone,  $\lambda$ CGN2. Southern hybridisation analysis of this clone showed none of the fragments to be the same size as the corresponding genomic DNA fragments. Therefore, this data indicated that clone  $\lambda$ CGN2 does not contain the region of significant sequence similarity detected by the hybridisation of the *A. parasiticus nor-1* probe to genomic DNA. Consequently, further analysis of this clone was not performed.

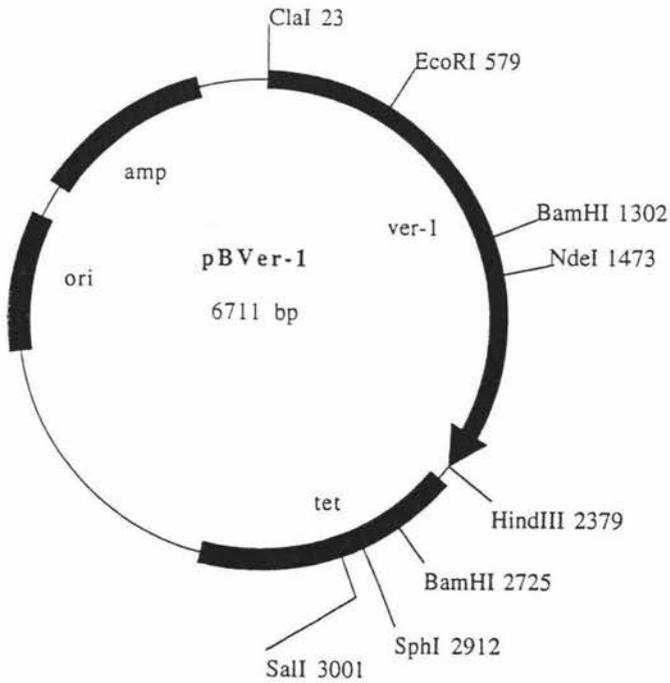
Recently, intensive efforts have been directed at delineating the biochemistry leading to ST/AF. The gene disruption of *verA* in *A. nidulans*, and *nor-1* and *ver-1* in *A. parasiticus*, as well as other genes, has firmly established the functional role of these genes in the AF and ST biosynthetic pathways. This approach has recently been facilitated by DNA sequence analysis and restriction enzyme mapping of cosmid and phage libraries from *A. parasiticus*, *A. flavus*, and *A. nidulans* which have shown that the genes required for AF and ST production are functionally conserved and tightly clustered within an approximately 60 kb region of the fungal genome. The study of ST and AF biosynthesis provides a model system for approaches in the isolation and identification of structural and regulatory genes from the *D. pini* dothistromin biosynthetic pathway. The isolation of the *Dpver-1* gene and the likelihood that the dothistromin biosynthetic genes are clustered means that other pathway genes are now within our reach.

## APPENDIX 1.0

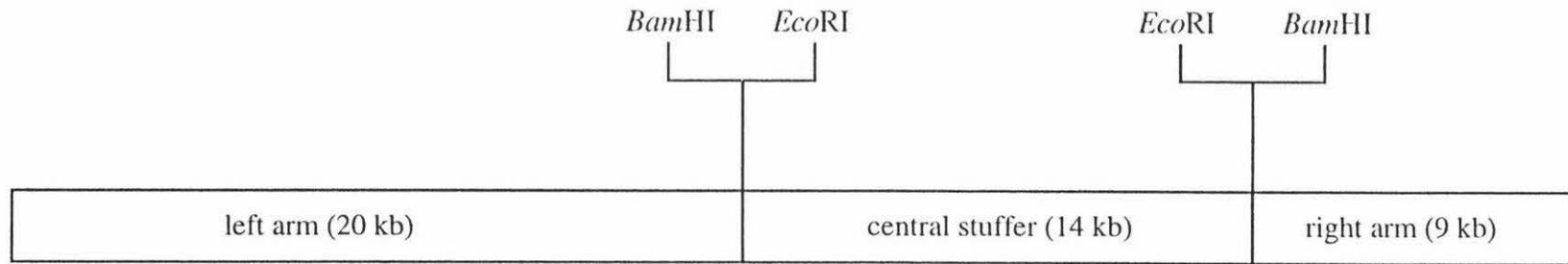
Restriction map of pNA-17



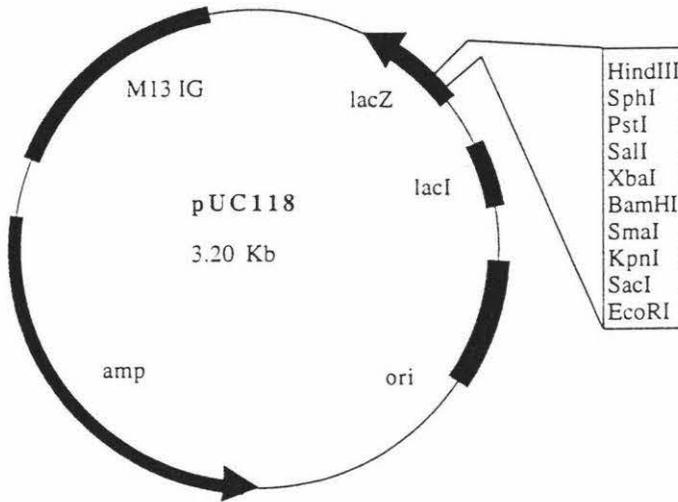
Restriction map of pBVer-1



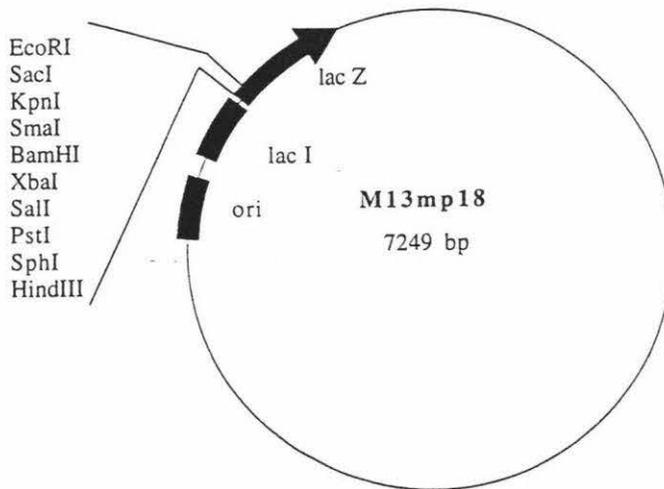
Restriction map of the  $\lambda$ GEM-12 vector showing *Bam*HI and *Eco*RI enzyme sites



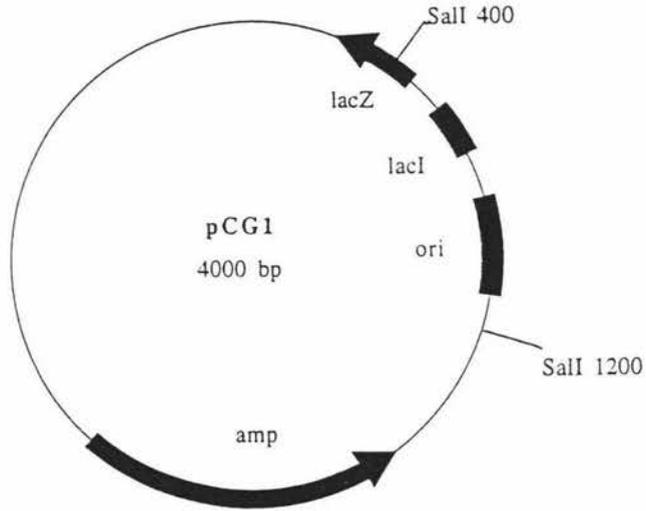
Restriction map of pUC118



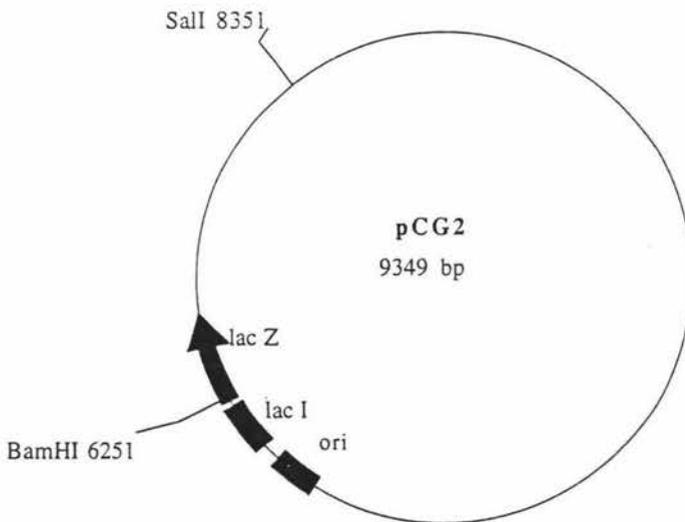
Restriction map of M13mp18



Restriction map of pCG1



Restriction map of pCG2



## APPENDIX 2.0

### Codon bias table for *ver-A*

<u>AmAcid Codon Number Fraction</u>				<u>AmAcid Codon Number Fraction</u>			
Gly	GGG	15.00	0.17	Leu	TTG	24.00	0.20
Gly	GGA	19.00	0.22	Leu	TTA	13.00	0.11
Gly	GGT	25.00	0.29	Phe	TTT	19.00	0.51
Gly	GGC	27.00	0.31	Phe	TTC	18.00	0.49
Glu	GAG	20.00	0.44	Ser	TCG	34.00	0.20
Glu	GAA	25.00	0.56	Ser	TCA	43.00	0.25
Asp	GAT	21.00	0.60	Ser	TCT	17.00	0.10
Asp	GAC	14.00	0.40	Ser	TCC	21.00	0.12
Val	GTG	13.00	0.20	Arg	CGG	29.00	0.19
Val	GTA	14.00	0.22	Arg	CGA	26.00	0.17
Val	GTT	18.00	0.28	Arg	CGT	14.00	0.09
Val	GTC	20.00	0.31	Arg	CGC	28.00	0.19
Ala	GCG	26.00	0.29	Gln	CAG	29.00	0.55
Ala	GCA	18.00	0.20	Gln	CAA	24.00	0.45
Ala	GCT	23.00	0.26	His	CAT	18.00	0.53
Ala	GCC	23.00	0.26	His	CAC	16.00	0.47
Arg	AGG	28.00	0.19	Leu	CTG	25.00	0.21
Arg	AGA	25.00	0.17	Leu	CTA	13.00	0.11
Ser	AGT	26.00	0.15	Leu	CTT	24.00	0.20
Ser	AGC	28.00	0.17	Leu	CTC	22.00	0.18
Lys	AAG	22.00	0.56	Pro	CCG	29.00	0.25
Lys	AAA	17.00	0.44	Pro	CCA	36.00	0.31
Asn	AAT	23.00	0.57	Pro	CCT	23.00	0.19
Asn	AAC	17.00	0.43	Pro	CCC	30.00	0.25
Met	ATG	26.00	1.00				
Ile	ATA	21.00	0.36				
Ile	ATT	16.00	0.27				
Ile	ATC	22.00	0.37				
Thr	ACG	15.00	0.19				
Thr	ACA	20.00	0.25				
Thr	ACT	22.00	0.28				
Thr	ACC	22.00	0.28				
Trp	TGG	22.00	1.00				
End	TGA	35.00	0.52				
Cys	TGT	22.00	0.45				
Cys	TGC	27.00	0.55				
End	TAG	21.00	0.31				
End	TAA	11.00	0.16				
Tyr	TAT	21.00	0.66				
Tyr	TAC	11.00	0.34				

Codon bias table for *ver-1*AmAcid Codon Number Fraction

Gly	GGG	12.00	0.32
Gly	GGA	7.00	0.18
Gly	GGT	10.00	0.26
Gly	GGC	9.00	0.24

Glu	GAG	8.00	0.53
Glu	GAA	7.00	0.47
Asp	GAT	13.00	0.62
Asp	GAC	8.00	0.38

Val	GTG	15.00	0.35
Val	GTA	12.00	0.28
Val	GTT	8.00	0.19
Val	GTC	8.00	0.19

Ala	GCG	7.00	0.14
Ala	GCA	7.00	0.14
Ala	GCT	14.00	0.28
Ala	GCC	22.00	0.44

Arg	AGG	10.00	0.22
Arg	AGA	12.00	0.26
Ser	AGT	7.00	0.12
Ser	AGC	6.00	0.11

Lys	AAG	7.00	0.41
Lys	AAA	10.00	0.59
Asn	AAT	4.00	0.22
Asn	AAC	14.00	0.78

Met	ATG	12.00	1.00
Ile	ATA	10.00	0.42
Ile	ATT	7.00	0.29
Ile	ATC	7.00	0.29

Thr	ACG	2.00	0.06
Thr	ACA	14.00	0.44
Thr	ACT	7.00	0.22
Thr	ACC	9.00	0.28

Trp	TGG	11.00	1.00
End	TGA	5.00	0.33
Cys	TGT	8.00	0.53
Cys	TGC	7.00	0.47

End	TAG	6.00	0.40
End	TAA	4.00	0.27
Tyr	TAT	9.00	0.60
Tyr	TAC	6.00	0.40

Leu	TTG	12.00	0.21
Leu	TTA	6.00	0.11
Phe	TTT	13.00	0.59
Phe	TTC	9.00	0.41

AmAcid Codon Number Fraction

Ser	TCG	8.00	0.14
Ser	TCA	15.00	0.26
Ser	TCT	9.00	0.16
Ser	TCC	12.00	0.21

Arg	CGG	4.00	0.09
Arg	CGA	6.00	0.13
Arg	CGT	5.00	0.11
Arg	CGC	9.00	0.20

Gln	CAG	5.00	0.42
Gln	CAA	7.00	0.58
His	CAT	8.00	0.53
His	CAC	7.00	0.47

Leu	CTG	10.00	0.18
Leu	CTA	7.00	0.13
Leu	CTT	10.00	0.18
Leu	CTC	11.00	0.20

Pro	CCG	8.00	0.24
Pro	CCA	14.00	0.41
Pro	CCT	8.00	0.24
Pro	CCC	4.00	0.12

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