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**Development of an Efficient Transformation System for
*Dothistromin pini***

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of the requirements for the degree of
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Abstract

A transformation system has been developed for the plant pathogenic fungus *Dothistroma pini* using a positive selection system based on the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*). After optimising the conditions under which protoplasts were isolated a transformation protocol was determined. The system developed gave large, stable transformants at frequencies between 1 and 48 transformants per μg of DNA. A second type of colony also grew on the selective plates. These grew in higher numbers but less vigorously, and did not grow when subcultured onto plates containing hygromycin B. These are believed to be abortive transformants. Southern analysis indicated that transformation takes place via the integration of the plasmid DNA into the fungal chromosomal DNA. The DNA integrated at a single site in 88% of the transformants, with all of the sites containing only a single copy of pAN7-1. Propagation of two of the transformants through single spore analysis indicated that they were homokaryons, though molecular results of another transformant indicated that it was a heterokaryon. Placing the transformants on increasing concentrations of hygromycin B indicated that the copy number of the integrated plasmid was not related to hygromycin resistance. In order to try and enhance the transformation rate of *Dothistroma pini*, by using a homologous promoter, the β -tubulin gene was isolated from a genomic library using the β -tubulin gene from *Neurospora crassa* as a probe. A restriction map was made and the gene was sequenced and shown to closely resemble β -tubulin genes from other fungi.

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Chapter 1. Introduction

1.1 General Characteristics of *Dothistroma pini*

The fungus *D. pini* is a needle pathogen found mainly on many pine species including *Pinus radiata*. Infection begins with chlorosis and necrosis at the base of the crown (Philips and Burdekin, 1982) and is seen as red tinged lesions or bands. Necrosis often extends throughout the needle leading to premature defoliation, followed by a reduction in photosynthesis and eventually wood yield (Franich *et al.*, 1982). In extreme cases this can be followed by death of the tree (Gallagher, 1971).

D. pini has been recorded in Europe, South and East Africa, North and South America and Australasia. It was first identified in the central North Island of New Zealand in 1962 and is now found in all of the North Island except the Northern tip and Great Barrier Island. In the South Island Nelson, Marlborough, North of the Wairou river, Westland, Southland and Otago are all sites of infection (Gadgil, 1984). Most species of genus *Pinus* are susceptible to *D. pini* attack including *Pinus radiata*, *P. ponderosa*, *P. nigra* (Gallagher, 1971) although other species such as *Pseudotsuga menziesii* and *Larix decidua* are also slightly susceptible (Philips and Burdekin, 1982).

Indigenous New Zealand trees are slow growing and would take rotations of hundreds of years to achieve the desired crop. Consequently introduced softwoods, which are fast growing are used in our commercial plantations, of which radiata pine makes up 93%. Accordingly because of the important role the forestry industry has in New Zealand and the role *P. radiata* plays in it, *D. pini* infection is of major economic significance to New Zealand. In fact plantation losses in New Zealand by disease and insects is only exceeded by losses caused by wind. Direct costs in controlling *D. pini* which can cause 10-25% periodic advancement of growth loss, since 1967 in New Zealand have totalled \$35.3 million (1988) (New, 1989).

D. pini caused lesions are band-like and contain 1-12 stromata which vary in size from 300-750 x 150-400µm. Asexual sporation inside the stromata is good. The black conidia, which are produced as sticky masses vary in size and are septate when mature. Below the stromata in the host mesophyll are septate, many branched hyphae. The perfect (sexual) stage is *Scirrhia pini* or *Mycosphaerella pini* and is in the order Dothideales of the Ascomycotina.

Fortunately for New Zealand the perfect stage is only found in North America (Vancouver) and Europe for its readily produced airborne spores would have dire consequences for our industry (Gadgil, 1984).

Water is required for rain-splash dispersal, as asexual spores are liberated from fruiting bodies in a film of water on the needle surface. As the water droplets hit the ground the spores are released to the air where they normally infect only the neighbouring trees. Germination of the spores is nearly complete within 48 hours. Mycelium then grows on the surface of the needle for 7-10 days, producing secondary conidia, before penetrating the stomata between the guard cells where its lateral spread is limited to a few millimeters from the point of infection (Gadgil, 1967, 1984). The first macroscopic sign of needle infection is yellow flecks which extend to become "red bands". Three to four months after the initial inoculation stroma primordia develop and push there way to the needle surface within these bands. Necrosis develops eventually leading to premature shedding of infected needles (so the crowns may appear thin with tufts of needles at the tips of the branches) and to a litter layer bearing a large number of *D. pini* stromata (Gadgil, 1970).

1.2 *Dothistroma pini* Infection

The sources of inoculum and the factors which influence infection by *Dothistroma* have undergone extensive investigation. In one study Gadgil (1970) looked at the survival of *D. pini* on fallen needles of *P. radiata*. The litter layers around infected trees are comprised of needles bearing large numbers of *D. pini* stromata. If *D. pini* could survive for long periods in competition with other micro-organisms, then the litter layer could be a major source of inoculum of fresh foliage. However it was found that *D. pini* was a poor saprophyte because the proportion of *D. pini* conidia compared to other fungi decreased rapidly over time. The viability period of the stromata was shown to differ throughout the year. The infective period was shown to depend on the position of the infected needles and whether the stand was thinned or left untreated, not on actual seasons (Gadgil, 1970).

It has been observed that along with the actual number of the infective spores landing on the leaf surface (usually several thousand) rainfall and other environmental parameters influence the rate and intensity of *Dothistroma* infection. In unpublished work Gadgil used a Hirst spore trap (which efficiently collects dry spores), operating continuously for six months (1974-1975), in a heavily infected *P. radiata* stand but caught very few conidia. This supports his theory (1984) that the asexual spores are released in a film of water so are

therefore hydrated when they fall on to a susceptible host. It has therefore being shown that germination and penetration of the host by the fungi will occur with relatively short wetness periods, as the conidia which are deposited on the surface of the host are always hydrated (Sheridan *et al.*, 1970; Gadgil, 1977). However stomatal development was shown to be reduced until the foliage was moistened and the longer the dry period following deposition of conidia the lower the severity of the infection.

If the inoculum is sufficient infection will occur at a wide range of temperatures, between 5 and 30°C, with an optimum at 17°C. A study of meteorological data shows that the temperatures suitable for infection occur from November to March in all radiata pine growing areas in New Zealand (Gadgil, 1974; Sheridan *et al.*, 1970). Field observations indicated that shaded foliage of radiata pine is markedly less infected than foliage exposed to the full light. Though neither germination or growth of the fungus on needle surfaces was affected by light intensities, this was believed to be a response of the host to low light intensities that reduced the infection (Gadgil and Holden, 1976).

The genus *Pinus* shows varying resistance to the pathogen. *P. radiata* is susceptible when young, but mature trees older than 15 to 20 years show little infection, suggesting increased resistance (Franich *et al.*, 1982). Other species for example *P. ponderosa* remain equally susceptible at all age (Philips and Burdekin, 1982). Entry of the pathogen was examined and direct entry of the epidermis was only seen when macerated mycelium was sprayed as the inoculum in the absence of spores. So it seems penetration of the stomata by hyphae between the guard cells, although not common (seen 5 times in about 20000 sections of inoculated needles) is the only way for infection to develop inside the needles naturally (Gadgil, 1967).

Scanning electron microscopy testifies that stomata of young, vulnerable trees to be open pores, 15-20µm long of which the guard and subsidiary cells have an epidermis covered with fine microtubular wax. In contrast the mature trees have stomatal of 10-15µm which are often occluded by an amorphous wax. It has been suggested that this wax could present a mechanical barrier to ingress of hyphae, or act by masking possible chemotactic or chemotrophic stimuli experienced by the hyphae during stomatal penetration (Franich *et al.*, 1977; 1983).

The actual chemical nature of this epicuticular wax may be of importance. In young trees it is made up of dehydroabietic acid while in mature trees it comprises a mix of oxygenated

resin acid derivatives. An *in vitro* test on *D. pini* showed these oxygenated resin acids inhibited both pore germination and mycelium growth. An *in vivo* test of artificial inoculation showed plants treated with acetone (depletes epicuticular and stomatal pore fatty and resin acids) had a mean infection level about two times that of the control. These experiments suggest that the resin acid derivatives could be pre-infection factors contributing to resistance in mature trees and once stomatal penetration and hyphae growth in the mesophyll has occurred the extent of the tissue damage and the rate of fruiting body formation is dependent on other factors such as sensitivity of the needle tissue to the toxin dothistromin (Franich *et al.*, 1983).

The pH buffering capacity and monoterpene (volatile compounds consisting mainly of 13 monoterpene hydrogen compounds) levels in young and mature trees have been looked at in attempts to associate them to differences in the level of resistance to *D. pini*. Measuring the pH buffering capacity, (at pH 6.2) of one-year-old needles of trees increasing in age, showed the level of buffering capacity increased with maturity. But because *Dothistroma* in culture tolerates a wide pH range, the pH buffering capacity of the *P. radiata* needles was thought unlikely to be directly linked to increased mature tree resistance (Franich and Wells, 1977). As with monoterpenes, although the yields of volatile compounds from young trees were twice that from 20 to 40 year-old trees, and the volatile mixes stimulated the germination of spores and growth of the mycelium in liquid culture; monoterpene concentration does not bear any simple relationship to mature tree resistance (Franich *et al.*, 1982).

1.3 Chemical Control

East African work earlier showed that the needle blight by *D. pini* could be controlled by copper-based fungicides and subsequent New Zealand work showed that the disease could be controlled by aerial applications of copper oxychloride (Dick, 1989). Consequently aerial application of copper fungicide has been the main method for control of *D. pini* in New Zealand over the last three decades. Pruning of the branches of the infected foliage also helps and can postpone the need for a fungal application for several years (Gadgil, 1984).

Stands in the age class susceptible to the blight (less than 16 years) are surveyed from the air, every 2 to 3 years in mid-winter and the average percent of crown infection in each stand is observed. This is important as 23% of the areas of susceptible age classes of *P. radiata* are infected. The major consequence of this is that it leads to a loss in wood volume. On each tree the reduction in increment is proportional to the level of crown infection (for

example 10% infection is equal to 10% loss). The overall effect of infection is apparent after about 2 years (Gadgil, 1984).

Copper fungicide as 50% cuprous oxide (Cu_2O) reacts with aqueous substances on the needles and with *D. pini* metabolites to give in aqueous solution cupric ion (Cu^{2+}) at concentrations sufficient to inhibit germination of *Dothistroma* conidia. The cupric ions also stimulate dothistromin toxin biosynthesis, which forms a brown water-soluble complex with Cu^{2+} probably making the toxin unavailable to needle tissue and also removing some of the Cu^{2+} on the needle surface by chelation. Interaction of Cu_2O fungicide with geothermal sulphur (H_2S) gases in the air gives CuS and CuSO_4 which also contribute to solubilising the fungicide. It is this combination of solubilisation and redistribution of Cu^{2+} and its complexes, along with their ready uptake of *D. pini* conidia which explains why copper fungicides give good control of this needle blight (Franich, 1988).

Spraying is usually undertaken in November which kills most of the inoculum at the time it is multiplying (Gadgil, 1984). After a couple of months of rain in the North Island the copper is washed off but reinfection is quite slow due to the rapid reduction with time in the amount of inoculum, produced by the stromata, on fallen needles. This short viable conidia time (4-6 months) has other benefits because as trees over 20 years are seldom infected, and the shortest possible rotation for *P. radiata* in New Zealand is 22 years, there should be no danger that regenerating seedlings will become infected as a direct result of growing on an area where a previous crop was infected (Gadgil, 1970).

Even though the average spray frequency per stand is higher than initial research indicated because of the wet summers, deep gullies and extensive mists in infected areas (Dick, 1989) the cost per hectare for New Zealand spraying of copper fungicide has reduced from >\$60 (1960s) to \$15 (1988). This reduction is due to the careful assessment of the optimum time to spray, improvements in spraying techniques (therefore less spray and reduction in flying time) and reduction in fungicide doses (less chemical costs) (Dick, 1989; Franich, 1988). Further savings now have to be found by reducing disease levels using *Dothistroma* resistant breeds of trees.

1.4 Resistant Strains

Thirty years ago all seed for the industry in New Zealand was unimproved bulk seed. Improvements to radiata pine seed have largely been made by selected breeding at the Forest

Research Institute (FRI) in Rotorua, by selection for growth and form. The FRI maintain an extensive breeding population from which parent trees are selected and crossed and their progeny tested in trials. This has led to three main seed and plant classifications: GF (Growth and Form - improved growth and more merchantable volume), LI (Long Internode - more clearwood and minimal pruning) and DR (*Dothistroma* Resistant - better growth rate on high *D. pini* risk sites) (FRI, 1987).

The best *Dothistroma* resistant seedlots are expected to reduce stand mean infection by 15% and the effects of spraying and resistance are expected to be additive (Carson and Carson, 1991). As an example of estimated saving in terms of spraying costs, if the resistant breed of radiata pine is established in Kinleith forest, in place of existing breeds, savings of 56% are expected. But a prerequisite for improvement in disease resistance in a particular area, is the need to determine the economic significance of the disease relative to other selection traits. A 6% difference in growth-rate was suggested by Carson (1989) between the best general breed and her proposed resistant breed. Therefore where past history indicates frequent spraying, then the resistant breed should be established but on healthier sites, the standard breeds should be used (Dick, 1989).

D. pini resistance behaves as a classical additively inherited trait with little dominance variance. Estimates of genetic correlation of *D. pini* disease symptoms across sites have generally been high. Subsequently there appears no reason to regionalise a selection program for *Dothistroma* resistance as resistant seedlots will probably be resistant on all sites where *D. pini* infection is present (Carson and Carson, 1991).

The nature of resistance seems to be the property of the whole tree and variability in selecting reliable resistant selections could be due a number of reasons including disease escape, variable symptom expression and non-optimal time of original assessment for resistance. Due to different weather patterns field assessment needs to be carried out for 3 to 5 consecutive years (Frainch *et al.*, 1986). Earlier research showed cuttings overall seemed to have more resistance than seedlings hence chemical control could be reduced if new plantations were established with cuttings rather than seedlings in areas where needle blight appear prevalent (Gadgil and Holden, 1976).

P. radiata has a broad genetic variability base as the original trees are from 2 to 3 independent American populations. Monoclonal propagation of *P. radiata* could potentially become a hazard, for by breeding for individual characteristics, genetic variation could

decrease and more virulent strains of *Dothistroma* could arise and overcome current resistant mechanisms. Because of this possibility and since spraying with copper will only control the disease and not eliminate it, it seems necessary to know more about the pathogen - host relationship. In particular the mechanism resulting in needle death which involves the *D. pini* toxin, dothistromin.

1.5 Dothistromin Toxin

D. pini produces the mycotoxin dothistromin as a major metabolic byproduct (Gallagher and Hodges, 1972). This red pigmented metabolite was first isolated from *D. pini* and subsequently from several *Cercospora* spp. and *Mycosphaerella larcina*. Early evidence of phytotoxicity of dothistromin was demonstrated by the injection of acetone and dothistromin into pine needles: within 5 days dothistromin 'red band' symptoms typical of those produced by *D. pini* appeared (Franich *et al.*, 1986; Stoessl *et al.*, 1990).

Up to 90% of the injected dothistromin is metabolised by needle cells or phytolytically degraded to CO₂ and oxalic acid within the first 24 hours, while simultaneously the formation of a small necrotic lesion appears. The mechanism, based on the formation of metabolites, is thought to involve peroxidase catalysed oxidation of dothistromin by hydrogen peroxide. Cells adjacent to those killed by dothistromin die and the lesions expand over 2-3 days. Since most of the dothistromin is degraded or metabolised, the cause of the expanding lesion is thought to be due to other toxic substances rather than dothistromin. It is found that the cells adjacent to those initially killed, synthesise and accumulated benzoic acid, which is toxic to *P. radiata* needle mesophyll cells and is highly fungistatic. The length of *D. pini* induced lesions were found to be proportional to the amount of benzoic acid injected into the needles and the necrotic tissue adjacent to the bands also accumulated benzoic acid which prevents colonisation of that tissue by the fungus. Benzoic acid is also bound tightly to lignin polymers which are found in disproportionately high amounts in dark green tissue adjacent to the dothistromin induced lesions. Benzoic acid therefore has been put forward as the phytoalexin of *P. radiata* needles (Franich *et al.*, 1986).

D. pini produces dothistromin as a major metabolic byproduct (Gallagher and Hodges, 1972). [¹³C]-labelling pattern shows that dothistromin is a difuroanthraquinone fused to a substituted tetrahydro-2-hydroxy-bisfuran ring system (Shaw, 1978). It is structurally related to the mycotoxins, sterigmatocystin and aflatoxin B1 from *Aspergillus flavus*. It is these structural features of aflatoxin B1 that is considered to be responsible for the toxicity

and potential human carcinogenicity associated with this toxin (Elliot *et al.*, 1989; Harvey *et al.*, 1976).

On the strength of the remarkable similarity between dothistromin and aflatoxin B1, mutagenicity studies were initiated with dothistromin. Harvey *et al.*, (1976) demonstrated that dothistromin inhibited RNA synthesis, as measured by [3H]-uridine incorporation in *Chlorella pyrenoidosa* and *Bacillus megaterium*. It was observed that dothistromin was 10 times more active in inhibiting the growth of *B. megaterium*. Various field samples, extensive environmental monitoring and human exposure studies were carried out by both the Department of Health and the FRI. Dothistromin was tested for mutagenicity in a wide variety of *in vitro* bioassays, most of which were positive. For example, chromosome damage in human peripheral blood lymphocyte cultures, accompanied by lysis of red blood corpuscles and for complete risk assessment a mouse *in vivo* mutagenicity assay. This work suggests that although it acts in a slightly different way, dothistromin may be just as hazardous as aflatoxin B1. However neither are much of health risk unless there is some underlying pathology present, for example smoking or the Hepatitis B Virus (Elliot *et al.*, 1989; Stoessl *et al.*, 1990).

1.6 Inactivation of the *Dothistroma* Toxin

Copper fungicide is only a control mechanism and resistant varieties are only successful as long as the pathogen retains its present virulence levels (Gallagher, 1971). So when the much shorter life-cycle of the plant pathogen is compared with its hosts, it is plausible that more virulent strains of *D. pini* could arise which would overcome the current resistant mechanisms. For this reason different possibilities of *Dothistroma* control such as monoclonal antibodies and development of a transformation system to produce *D. pini* isolates which are specifically blocked in dothistromin biosynthesis, must be investigated.

A team of research scientists at the Hort Research Immunology Group (led by Dr Paul Reynolds), situated in Palmerston North, have prepared monoclonal antibodies to dothistromin. They have cloned the gene encoding this dothistromin-specific antibody, and prepared a single-chain antibody epitope. They aim to produce transgenic plants which express this antibody, thus inactivating the dothistromin toxin if *D. pini* infects them and hence eliminating the need for spraying by copper fungicides. 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. The fundamental

assumption underlying this approach is that the dothistromin toxin is the primary cause of disease symptoms. This is suggested by experimental work in which purified dothistromin toxin was injected into pine needles (Franich *et al.*, 1986).

However, genes required for toxin-biosynthesis have recently been cloned from several plant pathogenic fungi which, when disrupted (leading to loss of toxin production), had no effect on fungal pathogenicity in two out of five cases (VanEtten *et al.*, 1994). These results lead us to question whether *D. pini* would also still be pathogenic to pine trees in the absence of dothistromin toxin: if so, the antibody approach to "immunising" pine trees may be ineffectual. We aim to answer this question by generating non-dothistromin producing isolates of *D. pini* so that their pathogenicity can be assessed. This will be achieved by disruption of dothistromin pathway genes.

The biosynthetic pathway of dothistromin has been investigated and intermediates have been identified which also occur in the biosynthesis of aflatoxin. Clones of some of the aflatoxin biosynthetic genes from *Aspergillus* are being used as hybridisation probes in the hope that they will be sufficiently homologous to *D. pini* biosynthetic genes to enable their detection. Once the dothistromin biosynthetic genes are cloned, a transformation system is required so the genomic copies can be disrupted or replaced to produce isolates of *D. pini* which are specifically blocked in dothistromin biosynthesis. The ploidy of *D. pini* is unknown. Consequently experimental work to determine the ploidy will have to be completed prior to gene disruption. Once disrupted the isolates will be checked for the production of toxic pathway intermediates, or other toxic products formed by branching pathways. These isolates can then be tested in the field to determine whether they have lost their pathogenicity.

1.7 Transformation of Fungal Plant Pathogens

The accessibility of a DNA-mediated transformation system is one of the first requirements to carrying out modern molecular biological research. Gene transformation of filamentous fungi is a relatively new area. The first description of such a system was in *Neurospora crassa* (Case *et al.*, 1979), followed soon after with gene transfer in another ascomycete, *Aspergillus nidulans* (Ballance *et al.*, 1983). Many other transformation systems have been developed for a range of commercially and agriculturally important fungal species (Hargreaves and Turner, 1992; Punt and van den Hondel, 1992).

To permit selection of transformed cells markers are used that are capable of complementing a mutation such as auxotrophic markers (negative selection) or that provide a new property to the host cell, for example antibiotic resistance (dominant or positive selection). An advantage of using a 'negative' selectable marker is that transformants for potential field release would be wild-type for the selectable marker rather than acquiring an additional characteristic. Dominant selection however has the advantage that mutant strains are not required so a genetically uncharacterised species can also be transformed (Punt and van den Hondel, 1992). Examples of genes used as markers in positive (dominant) or negative selection systems for transformation of plant pathogenic fungi are given in Table 1 (Fincham, 1989; Hargreaves and Turner, 1992).

In developing a transformation system positive selectable markers which confer resistance to hygromycin and phleomycin will be used. Hygromycin B (HmB) is an aminoglycosidic antibiotic which disturbs protein synthesis by interfering with peptidyl-tRNA translocation, causing misreading. Hygromycin B resistance genes encode the enzyme hygromycin B phosphotransferase which phosphorylates the antibiotic HmB. HmB resistant genes are isolated from *Streptomyces hygroscopicus* and from *Escherichia coli* and these genes have been used with many pathogenic fungi (see Table 1).

A less toxic and cheaper alternative to hygromycin is phleomycin. Phleomycin is a metalloglycopeptide antibiotic causing DNA strand scission. Phleomycin resistance genes isolated from *Streptoalloteichus hindustanus* and *E. coli* encode proteins which bind to and inactivate the antibiotic

1.8 Isolation of a *Dothistroma pini* β -tubulin Gene for use as an Endogenous Promoter

Due to a low success rate when transforming *D. pini* using promoters from *Aspergillus nidulans* to drive the expression of selectable markers, an alternative approach was investigated. One reason for this low transformation efficiency may be poor expression of the selectable markers which are under the control of the *Aspergillus nidulans* *gpdA* promoter, when placed in the *D. pini* genome. In the literature the use of endogenous promoters has been reported to both increase (Skatrud *et al.*, 1986) and decrease (Smith *et al.*, 1992) transformation efficiencies in *Acremonium chrysogenum*. In *Glomerella cingulata* an endogenous promoter showed no increase in transformation efficiency over an *A. nidulans* promoter (Bowen *et al.*, 1995) whereas in *Trichoderma reesei* a homologous

Table 1. Selectable Genes for Transforming Plant Pathogenic Fungi (Fincham, 1989)

Selectable gene	Species of origin	Phenotype(s) conferred	Genera in which marker was used
<i>hph</i>	<i>E. coli</i>	Hygromycin B resistance	<i>Cochliobolus</i> <i>Colletotrichum</i> <i>Fulvia</i> <i>Septoria</i>
<i>nptII</i>	<i>E. coli</i>	Kanaycin, G418 resistance	<i>Colletotrichum</i>
<i>tub-2</i>	<i>N. crassa</i>	Benomyl resistance	<i>Colletotrichum</i>
<i>argB</i>	<i>A. nidulans</i>	Arginine synthesis	<i>Magnaporthe</i>

promoter greatly increased the transformation efficiency (Gruber *et al.* 1990).

Due to the very low transformation efficiency in *D. pini* we decided to isolate a gene from *D. pini* with a view to using an endogenous promoter in the hope of increasing expression and transformation efficiency. The most widely used homologous promoter which has been used to increase transformation rates of dominant selection systems, is the constitutively-expressed glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene. This gene is highly conserved throughout the kingdoms of organisms (Jungehulsing *et al.*, 1994). Another gene which is also highly conserved (so the gene can easily be isolated using heterologous probes) and usually constitutively expressed is β -tubulin. The β -tubulin gene was chosen for use in our system, over the *gpd* gene, as it is slightly more variable and consequently could have other uses, for example, as a control probe for Northern blots and for phylogenetic studies.

Microtubules are distinct fibrous structures found in all eukaryotic cells. They play a role in a variety of intracellular processes including intracellular transport, mitosis and meiosis, and maintenance of cell shape. The constituent protein of microtubules is tubulin and it is composed of two similar sub-units α - and β -tubulin. A central question concerning the regulation of microtubule function is how microtubules can play a role in such a variety of cell processes. Two differing hypothesis have been suggested to explain this. The multi-tubulin hypothesis (Fulton and Simpson, 1976) states that microtubules with different functions are composed of different types of tubulins. The other hypothesis proposes that tubulin genes are functionally equivalent but different sets of microtubule-associated proteins are associated with microtubules having different functions (Weatherbee and Morris, 1984). Multiple β -tubulin genes are seen in many organisms, for example *A. nidulans* contains three (Weatherbee and Morris, 1984) whereas two have now been identified in *Colletotrichum gloeosporioides* (Buhr and Dickman, 1994).

Most mutations conferring resistance to the fungicide benomyl in fungi (such as *A. nidulans* and *Saccharomyces cerevisiae*, Orbach *et al.*, 1986) have been mapped in the β -tubulin structural genes. Consequently the mutational change in the β -tubulin genes responsible for benomyl resistance has been determined, and is then able to be used as a dominant selectable marker in transformation experiments. An example of cloning and characterising a gene for β -tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its subsequent use as a dominant selectable marker was performed by Orbach *et al.*, 1986.

Using a plasmid (pBT6) which contains the β -tubulin gene from *N. crassa* we aim to isolate the β -tubulin promoter from *D. pini*. We are hoping that the isolation of an endogenous promoter may facilitate the development of a more efficient transformation system.

1.9 Aims and Objectives

Transformation protocols vary between different fungi and different laboratories. Therefore, when developing a transformation system for *D. pini* it is necessary to try different protocols under many conditions to develop a method which is efficient, reliable and hopefully yields high numbers of transformants.

The simplest place to start developing a transformation system for *D. pini* is to try positive selection of transformants for phleomycin resistance. The first step requires the growing of *Dothistroma* in liquid culture or on cellophane discs without contamination and developing methods for optimising the isolation of protoplasts. There is a choice of enzymes for digesting the cell wall, including helicase and chitinase, but Novozyme234 prepared from the fungus *Trichoderma viride* and used at concentrations of 5-25mg/ml seems to be the one most commonly used. Young cultures are used because young hyphae are more susceptible to the cell wall digesting enzyme and the protoplasts are more easily separated. Protoplasts must be prepared and maintained in the presence of an osmotic stabiliser to prevent bursting. For this purpose a variety of osmotic stabilisers can be used, including sorbitol, mannitol, potassium chloride and magnesium sulphate, which are used at concentrations of 0.6-1.2M and pH 5-6 for an incubation time from 5 minutes to 3 hours. Again all these conditions need to be optimised (Fincham, 1989; Hargreaves and Turner, 1992).

After the protoplasts have been purified and numbers of $>10^7$ protoplasts/ml have been obtained, the protoplasts are induced to take up DNA molecules by treatment with calcium ions and poly ethylene glycol (PEG: causes the treated cells to clump which may facilitate in trapping of the DNA). For the regeneration of the protoplasts the osmotic stabiliser has to present in the growth media until the cell wall has been regenerated: this can be the same, or a different, osmotic stabiliser to that used for the protoplast isolation (Fincham, 1989).

For positive selection of transformed cells a concentration of the inhibitory substance (in this case phleomycin or hygromycin) that completely blocks growth of sensitive, non-transformed cells must be used. To test the resistant levels of *D. pini* to the antibiotic, macerated mycelium fragments will be plated on solidified *D. pini* media containing 0-

1000 μ g/ml hygromycin or 1-100 μ g/ml of phleomycin will be used. The composition of the growth media may influence resistant levels, for example, phleomycin has reduced antibiotic activity in acidic media (pH<5) and both phleomycin and hygromycin antibiotic activity is decreased in a hypertonic media (such as the one used for protoplast regeneration) (Punt and van den Hondel, 1992). The ploidy of *D. pini* is unknown but this will not interfere when selecting transformants as we will be using dominant selectable markers. Once the transformants are selected they will be analysed for stability by being taken through several rounds of subculturing. Analysis of location of the plasmid DNA, copy number and site or sites of integration of the plasmid in individual transformants will be performed by Southern blotting.

This project will establish the basic procedures of transformation for the first time in *D. pini*. The work is complementary to that of Ms C. Gilman and Ms B. Morgan who are currently isolating genes involved in the biosynthesis of dothistromin. The ultimate aim is to use the transformation system methodologies developed in this project to disrupt the wild-type copies of dothistromin biosynthetic genes to create a toxin-minus mutant. This will be useful for two reasons. The first reason being that although dothistromin is implicated as the primary cause of disease symptoms, the extent of the damage to the tree caused by the invasion of the fungus itself is unknown. The benzoic acid which is produced by the plant when it is challenged with the toxin, is a major source of tissue and needle damage: benzoic acid may still be produced by invasion of the fungal mycelium alone. Maybe the dothistromin is the elicitor which initiates the hypersensitive response of the plant and without it *D. pini* could become fully pathogenic. This information is very important to the Hort Research group who are developing monoclonal antibodies against dothistromin.

The second reason for disrupting the *D. pini* biosynthetic pathway is that, if *Dothistroma* mutants can be isolated which are found to be non-pathogenic, there is a possibility that they could be used in field trials to out-compete the naturally occurring toxin producing isolates. Clearly a field-release could not be contemplated without extensive studies on the *D. pini* - *P. radiata* interaction. The development of a transformation system for *D. pini* will provide a vital tool for further studies of this interaction.

Chapter 2. Materials and Methods

2.1 Fungi, Bacterial Strains and Plasmids

Fungal and bacterial strains, λ clones, and plasmids used in this study are listed in Table 2.

2.2 Media

2.2.1 Bacterial Media

2.2.1.1 Liquid Media

Luria Broth (LB)

(g/l): Tryptone, 10.0; NaCl, 5.0; Yeast Extract, 5.0. pH 7.5 (Miller, 1972).

SOC Medium

(g/l): Tryptone, 20.0; Yeast Extract, 5.0; NaCl, 0.6; KCL, 0.2; MgCl₂, 0.95; MgSO₄, 2.5; Glucose, 3.6 (Dower *et al.*, 1988).

NZCYM

(g/l): NZ Amine, 10.0; NaCl, 5.0; Casamino Acids, 1.0; Bacto Yeast Extract, 5.0; MgSO₄.7H₂O, 2.0. NaOH to pH 7.5.

2.2.1.2 Solid Media

Luria Agar

LB containing 15g/l agar.

Top Agarose

(g/l): Tryptone, 10.0; NaCl, 5.0; Agarose 15, 8.0. After cooling to 45-55°C it was supplemented with MgSO₄ to 10mM.

Table 2. Fungal and Bacterial Strains, λ Clones and Plasmids

Strain, λ Clone or Plasmid	Relevant Characteristics	Source/Reference
<u>FUNGAL STRAINS</u>		
<i>Dothistroma pini</i>		
Dp-1	wild type strain (forest isolate, 1993)	Philip Debnam FRI, Rotorua
AB1-AB8	wild type strain containing pAN7-1	This study
<u>BACTERIAL STRAINS</u>		
<i>Escherichia coli</i>		
LE392	<i>supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1</i>	Borck <i>et al.</i> 1976
XL-1	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F' [proAB⁺ lacI^q lacZΔM15 Tn 10 (tet^r)]</i>	Bullock <i>et al.</i> 1987
<u>λ CLONES</u>		
λ GEM-12	λ replacement library vector based on	Frischauf <i>et al.</i> 1983
λ AB1-5	λ GEM-12 clones containing genomic DNA from <i>Dothistroma pini</i> homologous to β -tubulin from <i>Neurospora crassa</i>	This study
<u>PLASMIDS</u>		
pAN7-1	6.5 kb HmB ^R Amp ^R	Punt <i>et al.</i> 1987
pAN8-1	5.9 kb Phleo ^R Amp ^R	Mattern and Punt 1988
pBT6	5.8 kb based on pBT3 containing the <i>N. crassa</i> Bml ^R β -tubulin gene	Marc Orbach University of Arizona
pUC118	3.2 kb Amp ^R	Messing 1983
pAB1	pUC118 containing a 1.3kb <i>Xho</i> I fragment from λ AB1	This study

2.2.1.3 Media Supplements

When required the antibiotic concentration used for selection was:

100µg/ml Ampicillin from a stock solution of 100mg/ml.

When required isopropylthio-β-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide (X-gal) were also supplemented:

30 µg/ml of IPTG

60µg/ml X-gal.

10mM MgSO₄ and 0.2% (w/v) maltose were supplemented to LB in which an overnight culture of LE392 (plating cells) for library screening were grown.

2.2.2 Fungal Media

2.2.2.1 Liquid Media

D. pini Liquid Media (DM Broth)

(g/l): Malt Extract, 50.0; Nutrient Broth, 20.0.

2.2.2.2 Solid Media

D. pini Media (DM)

(g/l): Malt Extract, 50.0; Nutrient Agar, 28.0.

D. pini Top Media (DM Top)

(g/l): Malt Extract, 50.0; Nutrient Agar, 11.2; Sucrose, 273.9 (0.8M).

Osmotically Stabilised DM (DM Suc)

(g/l): Malt Extract, 50.0; Nutrient Agar, 28.0; Sucrose, 273.9 (0.8M).

D. pini Sporulation Media (DSM)

(g/l): Malt Extract, 50.0; Yeast Extract, 20.0; Agar, 15.0.

2.2.2.3 Antibiotic Concentration

When required the antibiotic concentration used for selection was:

60-70 µg/ml Hygromycin B from a 50mg/ml H₂O stock

5-10 µg/ml Phleomycin from a 5mg/ml H₂O stock.

2.3 Growth of Cultures

2.3.1 Bacterial Cultures

E. coli strains were maintained on LB plates supplemented as required. The cultures were grown at 37°C, then stored at 4°C with regular subculturing.

Alternatively for plasmid DNA preparations, 2-5mls of LB supplemented appropriately, was inoculated with a single bacterial colony and incubated with shaking (300rpm) overnight at 37°C.

2.3.2 Fungal Cultures

A (8.0mm x 8.0mm) chunk (cut with scapple) of *D. pini* mycelia was ground in 1ml of sterile milliQ (MQ) water using a plastic grinder in an eppendorf tube. From this 200µl was spread onto DM plates (with or without cellophane discs) and incubated at 20°C for 6-14 days. The cultures were then stored at 4°C for up to 6 months before subculturing.

DM broth cultures were inoculated in the same way with 1ml of inoculum/100ml of DM broth in a 1 litre siliconised flask. These were grown at 20°C with gentle shaking (100rpm).

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

2.4 Common Solutions

2.4.1 10x TAE Buffer (Tris Acetate EDTA buffer)

400mM Tris, 11.4ml glacial acetic acid, 20mM EDTA (pH 8.5).

2.4.2 TE Buffer (Tris EDTA buffer)

10mM Tris-HCL/1mM EDTA (10:1.0 TE) or 10mM Tris-HCL/0.1mM EDTA (10:0.1 TE).

2.4.3 20x SSC (Standard Saline Citrate)

3M NaCl, 0.3M Na citrate.

2.4.4 10x Gel Loading Dye

50.0% (w/v) glycerol, 1x TAE, 12.0% (w/v) urea, 0.4% (w/v) bromophenol blue.

2.4.5 Phenol (Tris-equilibrated)

Phenol was liquified by heating at 50°C prior to hydroxyquinoline being added to a final concentration of 0.1% (w/v). The phenol was washed three times with an equal volume of 0.1M Tris-HCl (pH 8.0). The equilibrated phenol was stored under 0.1M Tris-HCl, at 4°C, in a dark bottle.

2.4.6 OM Buffer

1.6M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, with 10mM Na_2HPO_4 /100mM NaH_2PO_4 buffer (pH 5.8).

2.4.7 ST Buffer

1.0M sorbitol, 100mM Tris-HCl (pH 8.0).

2.4.8 STC Buffer

1.2M sorbitol, 50mM Tris-HCl (pH 8.0), 50mM CaCl_2 .

2.4.9 SM Buffer

100mM NaCl, 8mM MgSO_4 , 1M Tris.

2.5 DNA Preparations

2.5.1 Small Scale Alkaline Lysis Plasmid DNA Preparation

This method is based on that of Sambrook *et al.*, (1989). 2ml of LB + ampicillin was inoculated with a single bacterial colony and shaken overnight at 37°C. 1.5ml of the culture was pelleted by centrifugation for 1 minute (min) in a 1.5ml eppendorf tube. The supernatant was removed and the pellet resuspended in 100µl of TEG containing 50mM glucose, 25mM Tris (pH 8.0) and 10mM EDTA for 5 min at room temperature. 200µl of a solution containing 0.2M NaOH and 1.0% (w/v) SDS was added, mixed rapidly by inversion several times and stored for 5 min on ice. A 150µl ice cold solution of potassium acetate (60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml of MQ water) was added, mixed well and incubated a further 5 min on ice. After centrifugation for 5 min the supernatant was transferred to a fresh tube and a phenol/chloroform extraction (Section

2.6.1) followed by an ethanol precipitation (Section 2.7) were performed before resuspension of the pellet in TE. RNase was added to restriction digests at 0.5 μ g/ μ l.

2.5.2 Cesium Chloride-Ethidium Bromide Density Gradient Plasmid Preparation

E. coli cells were grown overnight with shaking at 37°C, in 250ml of LB + ampicillin (1/100 inoculum), and harvested at 10400g (8000rpm, GSA) for 10 min. The cells were washed by resuspending in 100ml of TE and then pelleted as above. Resuspension was achieved in 30ml of a solution containing 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA and 150mg lysozyme. After 10 min incubation at room temperature 60ml of a solution containing 0.2M NaOH and 1.0% (w/v) SDS was mixed by inversion with the suspension, incubated on ice for 10 min, then 45ml of a solution containing 3M potassium acetate and 11.5ml glacial acetic acid per 100ml was added, mixed by inversion then incubated for a further 10 min on ice. After a further 10 min centrifugation the supernatant was transferred to a fresh tube and the DNA was precipitated with isopropanol (Section 2.7). This method was based on that of Ish-Horowicz and Burke, (1981). The DNA was resuspended in 3.5ml TE and Cesium Chloride (CsCl) was added in a ratio of 1.05g/ml of DNA solution. Ethidium bromide was added in a ratio of 75 μ l/ml of DNA/CsCl solution (from 10mg/ml stock), mixed well and left at 4°C overnight. The solution was then spun at 17300g (12000rpm, SS34) for 10 minutes and the refractive index of the supernatant was checked to be between $n = 1.3860 - 1.3920$, and adjusted if necessary. The solution was then ultracentrifuged for 5 hours (hr) at 223000g (55000rpm, Sorvall combi TV865). The plasmid band was removed with a 18 gauge hypodermic needle and syringe and the ethidium bromide was subsequently removed by extraction with equal volumes of SSC saturated isopropanol (prepared by stirring equal volumes of 20x SSC and isopropanol for several hours). CsCl was then removed by dialysis against TES (10/1/100, 10mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 100mM NaCl) with 4 changes. Dialysis was performed with stirring at 4°C. After dialysis the DNA was quantitated as in Section 2.8.

2.5.3 Fungal DNA Extraction

DNA was extracted from *D. pini* and HmB resistant *D. pini* transformants using a method based on that of Raeder and Broda (1985). Mycelia were harvested, frozen (-20°C or snap-frozen by dipping in liquid N₂) and then freeze-dried overnight before being ground in liquid nitrogen with a mortar and pestle. Ground mycelia were placed in a corex tube, freshly made DNA extraction buffer (200mM Tris (pH 8.0), 250mM NaCl, 25mM EDTA, 0.5%

(w/v) SDS) was added (1ml/0.1g dry weight of mycelia) and vortexed thoroughly. Then 0.7 volumes of phenol (Section 2.4.5) and 0.3 volumes of chloroform were added, with thorough vortexing between each addition. Following centrifugation at 17300g (12000rpm, SS34) for 1 hr, the supernatant was again extracted with phenol/chloroform (Section 2.7.1) and centrifuged for 15 min. 250µg/ml of RNase was added to the supernatant and incubated for 30 min at 37°C, before a further phenol/chloroform extraction (with 15 min centrifugation) and a 1 volume chloroform extraction (20 min centrifugation). Following precipitation with isopropanol (Section 2.7) the pellet was resuspended fully in 1ml 1M NaCl and transferred to an eppendorf in which polysaccharides were precipitated by centrifugation for 5 min. The supernatant was transferred to a fresh tube and an isopropanol precipitation was performed (Section 2.7) before resuspending the DNA in TE (20-200µl) overnight.

2.5.4 Lambda Phage DNA Preparation

The method used was a modification of the Liquid Lysate method of phage preparation based on Current Protocols in Molecular Biology, (1994). 100µl of an overnight culture (Section 2.3.1) of LE392 in LB supplemented with maltose and MgSO₄ (Section 2.2.1.3) was combined with 100µl of titred eluted phage (10⁶-10⁷) and incubated at 37°C for 30 min. The phage mixture was added to 50ml of NZCYM, shaken vigorously at 37°C until lysis occurred (6-8 hr) and then harvested immediately. The solution was centrifuged for 10 min at 16300g (10000rpm, GSA) and the lysate removed and stored overnight at 4°C. RNase and DNase were added to 10µg/ml and the solution was incubated for 1 hr at 37°C, then 0.5M NaCl and 10% (w/v) PEG 6000 were added and dissolved using a magnetic stirrer prior to precipitating on ice for 2 hr. The phage were pelleted by centrifugation at 4920g (5500rpm, GSA) for 10 min and the supernatant drained completely. Resuspension of the pellet was in 1ml SM buffer and the solution was then transferred to an eppendorf before centrifugation for 10 min at 12000rpm to pellet remaining bacterial debris. 0.1mg/ml of Proteinase K was added to the phage suspension followed by an incubation at 37°C for 30 min. The phage suspension was then extracted twice with an equal volume of phenol/chloroform (each with 20 min of vortexing), followed by a chloroform extraction (Section 2.6.1) with a 5 min vortex. An ethanol precipitation was then undertaken (Section 7) followed by a resuspension in TE 10/0.1 for 20 min at 65°C. After a final 10 min centrifugation to remove debris the supernatant was transferred to a fresh eppendorf.

2.6 Purification of DNA

2.6.1 Phenol/Chloroform Extraction

DNA samples were extracted when an equal volume of Tris equilibrated phenol (Section 2.4.5) and chloroform were added, the sample was vortexed and then centrifuged for 5 min at 12000rpm in an eppendorf centrifuge, or for larger samples, 15 min at 17300g (12000rpm, SS34). The aqueous phase was removed and re-extracted. Any residual traces of phenol were then removed with a final extraction of 1 volume of chloroform. The DNA was then ethanol precipitated as described in Section 2.7.

2.6.2 Commercial Kits

The usage of commercial kits was confined to extraction of DNA from Seaplaque agarose (Section 2.9.3). The kits used were:

The GENECLEAN Kit; manufactured by BIO 101 Inc.

The GLASSMAX DNA Isolation Spin Cartridge System; manufactured by GibcoBRL Life Technologies, Inc.

The kits were used as per the kit manufacturers instructions.

2.7 Ethanol or Isopropanol Precipitation of DNA

To precipitate DNA 0.1 volume of 3M Na acetate and either 2.5 volumes of 95% ethanol or 0.6 volumes of isopropanol were added to the DNA, mixed by inversion and incubated at room temperature for 5 min. The DNA was then pelleted via centrifugation for 15-30 mins at 12000rpm in an eppendorf centrifuge, or SS34 or GSA rotors. The pelleted DNA was washed once with cold (-20°C) 70% ethanol to remove excess salt and the pellet was then dried in the vacuum desiccator, before resuspension in water or TE.

2.8 Determination of DNA Concentration and Purity

2.8.1 Spectrophotometric Determination of DNA Concentration

DNA concentration was estimated by measuring the light absorption of diluted DNA at 260nm and 280nm. The reading at 260nm allowed quantitation of DNA in the sample as an optical density (OD) of 1.0 unit is equivalent to 50µg/ml of double stranded DNA. Protein

absorbs maximally at 280nm, so the purity of the DNA is estimated by the ratio of OD_{260}/OD_{280} . Pure DNA has the ratio of approximately 1.8.

2.8.2 Determination of DNA Concentration using Concentration Standards

For plasmids, linearised pBR322 concentration standards of 2.5ng, 5ng, 10ng and 20ng/5 μ l were run on 1% TAE agarose minigels beside an aliquot of the DNA (diluted if necessary) to be quantitated. The concentration of the DNA of interest was then estimated by visual comparison with the standards.

Genomic DNA concentration was estimated visually in comparison with uncut lambda standards of 10ng, 20ng, 50ng, 100ng and 200ng/5 μ l which were used instead of the pBR322 standards.

2.9 DNA Manipulations

2.9.1 Restriction Enzyme Digests of DNA

2.9.1.1 Digests of Lambda and Plasmid DNA

0.5-5.0 μ g of DNA was digested in a commercially prepared buffer specifically matching the appropriate enzyme, which was added in excess (eg. 3-4 fold), to a 10-50 μ l reaction mix. The digestion was carried out at 37°C for 1-5 hr. Where necessary RNase was added at a concentration of 0.5 μ g/ μ l at the completion of the digestion, and the reaction was continued for another 15 min. The digests were then checked for completion on an agarose gel (Section 2.9.2).

2.9.1.2 Digests of Genomic DNA

5.0 μ g of *D. pini* genomic DNA was digested in a 30 μ l total reaction mix with the appropriate restriction enzyme added at 3U/ μ g of DNA. Bovine serum albumin was added to a final concentration of 1mg/ml and the digestion was performed overnight at 37°C. Digestion was then checked for completion on an agarose minigel (Section 2.9.2).

2.9.2 Agarose-gel Electrophoresis

DNA samples were size fractionated through a 1.0-1.2% (w/v) agarose gel in 1x TAE buffer, immersed in 1x TAE buffer. 1/10 volume of 10x Gel Loading Dye (Section 2.4.4) was added to each sample of DNA before loading in the wells. Life Technologies Horizon minigel boxes were run at 80-95V at room temperature for 30-60 min whereas medium horizon gel boxes (110 x 140mm) or Biorad DNA Sub-Cell boxes (150 x 200mm) were run at 34V at 4°C overnight. The gels were stained in 5µg/ml ethidium bromide solution for 10-20 min, before briefly destaining in water. Bands were visualised under short wave UV light and photographed on Polaroid type 667 film.

DNA fragment sizes were determined by running a Lambda *EcoRI/HindIII* ladder alongside the DNA sample. Large gels intended for hybridisation experiments were photographed beside a ruler so the mobility of the DNA could be measured accurately. The molecular weights of the DNA samples were then determined using a graph plotted on semi-log paper of relative mobility versus molecular weight markers or by a computer program such as Cricket graph or Gel Frag Sizer (Gilbert, 1989 - Version 1.4 on HyperCard).

2.9.3 DNA Extraction From SeaPlaque Agarose

A 1% SeaPlaque agarose gel, in 1x TAE, was poured in the medium horizon gel box (Section 2.9.2) was run at 4°C for 2-4 hr at 60-90V. After staining the DNA, the fragment(s) of interest were viewed under a long wave UV light, excised with a scapel and placed into pre-weighed 1.5ml eppendorf tubes. The DNA was then extracted from the melted agarose using a commercial kit (Section 2.6.2). The resulting DNA concentration was determined by checking on a minigel (Section 2.8.2).

2.10 Cloning Procedures

2.10.1 Preparation of Insert DNA

The DNA was digested with the appropriate restriction enzyme(s) (Section 2.9.1.1) to release the fragment of interest. The DNA was then electrophoresed through a SeaPlaque agarose gel (Sections 2.9.2 and 2.9.3), the fragment of interest excised and then purified (Section 2.6.2).

2.10.2 Linearisation and CAP-Treatment of Vector DNA

Approximately 5.0µg of vector DNA was linearised with the appropriate enzyme (Section 2.9.1.1), then dephosphorylated by the addition of 0.5 units of calf intestinal alkaline phosphatase (CAP, Boehringer 1U/µl) in a 30 min incubation at 37°C. 5mM EDTA, 0.5% (w/v) of SDS and 50µg/ml Proteinase K were added to the reaction mix, mixed by inversion and incubated for 30 min at 56°C. A phenol/chloroform extraction and ethanol precipitation (Sections 2.6.1 and 2.7) were then performed and the resulting DNA was resuspended in TE (10/1) at a concentration of 20ng/µl. This method was based on Sambrook *et al.* (1989).

2.10.3 Ligation

Ligations were carried out using 2µl of (New England Biolabs) 5x ligation buffer, 20ng of DNA insert, 20ng of vector, 1µl of 1/10 (40 units) T4 DNA ligase (NEB) and MQ water to 10µl, overnight at 4°C. This was based on a modification of the method proposed by Dugaicznyk *et al* (1975).

To check whether ligation had occurred a 1µl aliquot of the ligation mix was removed before and after adding the T4 DNA ligase and checked on an agarose gel (Section 2.9.2) alongside a vector only, before and after T4 DNA ligase, control.

2.10.4 Transformation of *E. coli* by Electroporation

Plasmids were transferred into *E. coli* XL-1 cells by the method of Dower *et al.*, (1988) using a Biorad Gene Pulser Transfection Apparatus set to 25µF and 2.5kV and Pulse Controller set to 200Ω.

Following electroporation the cells, including positive (with uncut circular vector) and negative (with water) controls, were immediately resuspended in either SOC or LB medium (Section 2.2.1.1). After incubation for 1 hr at 37°C, appropriate dilutions of the cells were plated on selective (Ampicillin) LB plates. X-gal and IPTG were also used if pUC118 was used as the vector for subcloning, to allow for screening of the white recombinant transformants (Section 2.2.1.3).

Transformants were screened via informative restriction digests (Section 2.9.1.1) of plasmid DNA isolated by the alkaline lysis method (Section 2.5.1) followed by gel electrophoresis (Section 2.9.2).

2.11 Preparation of *Dothistoma pini* Protoplasts

2.11.1 Protoplast Protocol 1 (most successful)

Protoplasts of *D. pini* were prepared using a modification of the methods described by Punt and van den Hondel, (1992) and Yelton *et al.*, (1984). Young fungal mycelia (grown as in Section 2.3.2) were collected after 6 days growth by filtration through a sterile nappy liner, (standard brand) rinsed and suspended in osmotic medium (OM buffer: 1.6M MgSO₄, pH 5.8) at 1g wet weight mycelium/20ml. Filter-sterilised Novozyme 234 (Interspex, 5mg/ml OM buffer) were added, and protoplasts were prepared by incubation at 37°C with gentle shaking (50-100rpm). Protoplast formation was checked under a microscope, and harvested after 2.5-3.0 hrs when many free protoplasts were observed but the mycelia had not completely broken down. Protoplasts were separated from most of the mycelium by filtration through a sterile nappy liner and harvested in a 15ml corex tube by overlaying 5ml of the protoplast solution with 1ml of ST buffer (Section 2.4.7) and then centrifuging for 5 min at 1085g (3000rpm, SS34). The protoplasts formed a white band at the interface of the two solutions and were removed and washed twice in 5ml of STC buffer (Section 2.4.8), being pelleted in between washes by centrifuging as above. Finally, the pellet was resuspended in STC buffer and the concentration of the protoplasts (typically 10⁷-10⁸/ml STC buffer) estimated using a haemocytometer.

2.11.2 Protoplast Protocol 2

This method is based on protocol 1 (Section 2.11.1) with the differences being: 4 mycelial covered cellophane discs were placed, face down in the Novozyme/OM buffer solution and were harvested directly via the flotation method with no prior separation of mycelial fragments by filtering through gauze.

2.11.3 Protoplast Protocol 3

The main difference between protoplast protocol 3 and protocol 1 is that harvesting of mycelia and protoplasts is achieved by centrifugation alone, without the use of nappy liner

filters. 240ml of DM broth (Section 2.2.2.2) inoculated with *D. pini* and grown as in section 2.3.2, was harvested by centrifugation for 10 min in 8x 30ml sterile Naglene tubes at 27000g (15000rpm, SS34). Mycelia were resuspended in approximately 15ml sterile MQ water and combined into 4 tubes before further centrifugation as above. 5ml of Novozyme solution (5mg/ml in OM buffer) was added to the mycelial pellet in each tube, vortexed gently to resuspend the pellet and gently shaken (80-100rpm) for 2.5 hr (or until protoplasts were formed) at 30°C. The solution was distributed into 15ml falcon tubes (5ml in each) and overlaid with 1.0M ST buffer. A protoplast band at the buffer interface was formed by centrifugation in the Heraeus centrifuge 2500rpm (1085g) for 5 min. The protoplasts were pooled after removal from the interface, washed in STC buffer and resuspended and counted as in Section 2.11.1.

2.12 Transformation of *Dothistroma pini*

2.12.1 Transformation Protocol A

Transformations were performed based on the method described by Murray *et al.*, (1992). To 80µl of protoplasts in STC buffer (1.25×10^8 protoplasts/ml) 20µl of a 40% poly-ethylene glycol (PEG) 4000 solution (in 50mM CaCl₂, 1M sorbitol, 50mM Tris-HCl pH 8.0), 2µl of spermidine (50mM) and 5µg of DNA were added. The solution was mixed and incubated on ice for 30 min. Then, 900µl of 40% PEG solution was added to the protoplast solution, mixed and incubated at room temperature for 15-20 min. Aliquots (100µl of a 1/10 dilution in STC buffer) of this mixture were spread onto DM plates before overlaying with *D. pini* top media (containing the appropriate antibiotic) immediately or 24 hr later.

2.12.2 Transformation Protocol B

This transformation method was almost identical to protocol A (Section 2.12.1). The changes were concerned with the method of mycelia growth (on DM plates covered with cellophane discs) and harvesting (Section 2.11.3). Other factors were also investigated such as the protoplast regeneration media and subculturing techniques.

2.12.3 Transformation Protocol C (most successful)

Transformations were performed in 1.5ml eppendorf tubes based on the method of Punt and van den Hondel (1992). 150µl of protoplast suspension (7.5×10^6 - 1.8×10^7 protoplasts) was

mixed with 5 µg of plasmid DNA at 22°C for 20 min. In 3 steps, 250, 250, and 850µl of 40% polyethylene glycol (PEG) 4000 solution in STC buffer were carefully mixed with the DNA-protoplast mixture and the final suspension was incubated for a further 20 min at 22°C. This suspension was then diluted in 5ml of STC buffer and the protoplasts were collected by centrifugation (10 min, 3000rpm, SS34) and resuspended in 500µl of STC. 100µl of this suspension was spread onto plates containing 20ml osmotically stabilised DM (Section 2.2.2.2). 24 hr later the plates were overlaid with DM Top agar (Section 2.2.2.2) to which a selecting antibiotic had been added (Section 2.2.2.3). The plates were incubated at 20°C and were examined for transformants 12-16 days later. Protoplasts were also plated out for viability by plating out serial dilutions (10^{-3} to 10^{-6}) of the protoplast suspension, both before and after PEG treatment. A negative control (no DNA) was also always included.

2.13 Subculturing of Transformants

Transformants were subcultured by cutting a small piece of the colony from the original plate and after placing it onto a fresh plate containing selective media, grown for 7 days. This was followed by 2 rounds of growth on non-selective media, followed by growth on selective media again.

2.14 Southern Blotting and Hybridisation

2.14.1 Southern Blotting

Restriction enzyme digests were electrophoresed overnight through a 1% TAE agarose gel, stained and photographed as described in Section 2.9.2, then transferred to a nylon membrane based upon the method of Southern (1975).

The gel was depurinated by placing in a dish and covering with 250mM HCl for 15 mins. It was then denatured in 500mM NaOH, 500mM NaCl for 30 min and neutralised with 500mM Tris (pH 7.4), 2.0M NaCl for 30 min. The gel was finally washed in 2x SSC. All steps were performed with the gel immersed in the solution with gentle shaking, and between each step the gel was rinsed with MQ water.

The gel was then inverted and placed on a blotting stand which had been prepared whilst the gel was treated. The stand was a narrow plastic container with two wells at either end and was prepared by placing two sheets of Whatman 3MM chromatography paper soaked in 20x

SSC (Section 2.4.3) over the top (taking care to trap no air bubbles underneath) so the ends sat in the wells which were then filled with 20x SSC. Gladwrap was placed over the stand and pressed flat. A grid 2mm less than the gel size was then marked and removed from the centre of the gladwrap over which the inverted, treated gel was placed. A Hybond nylon membrane (Amersham) cut 2mm larger than the gel and presoaked in 2x SSC was placed on the gel, and then covered with two sheets of 3MM Whatman paper cut 2mm less than the gel size soaked in 2x SSC, followed by two identically sized sheets of unsoaked 3MM paper. This was followed by a stack of towels approximately 10cm deep on which a light weight was placed. The apparatus was then left to blot by capillary action at room temperature overnight after which it was dismantled and the membrane washed in 2x SSC for 5 min, then baked in a vacuum oven at 80°C for 2 hr.

2.14.2 Radioactive [α - 32 P]dCTP Labelling of the DNA Probe

DNA was labelled using the High Prime DNA Labelling Kit (Boehringer Mannheim). 25-50ng of template DNA was added to sterile MQ water so the final volume was 8 μ l and this was denatured in a boiling water bath for 2 min then placed on ice to cool. The denatured DNA was then mixed with 4 μ l of High prime reaction mixture, 3 μ l of dATP, dGTP, dTTP mix and 5 μ l of [α - 32 P]dCTP. The reaction mix was then incubated at 37°C for 1 hr.

Incorporation of the radiolabel was checked by thin layer chromatography on phosphoethyleneimine paper developed in 2N HCL. Unincorporated nucleotides were separated from labelled DNA on a Sephadex G-50 minispin column after the addition of TES and by centrifugation for the time required for the individual column. The three types of columns used at different times in this work were:

- Columns were made by filling a 1ml syringe with 3mm of siliconised glass wool, which was then filled with Sephadex G-50 in TE/NaCl and centrifuged for 3 min at 1600g (3000rpm, MSE bench centrifuge). This process was repeated until the volume of the resin in the syringe was unchanged at approximately 1ml. 100ml of TES was added to the probe, which was then added to the column, centrifuged for 2 min and collected in a clean eppendorf.
- Quick SpinTM Columns (Boehringer Mannheim) were used as per manufacturers instructions. 80 μ l of TES was added and the sample eluted in 4 min at 1100g.

- ProbeQuantTM G-50 Micro Columns (Pharmacia Biotech) were used as per manufacturers instructions. 30µl of TES was added and the sample eluted at 735g for 2 min.

Incorporation of the probe was again estimated by comparing the radioactive counts remaining in the column to those in the eppendorf containing eluted probe. The probe was heat denatured in a boiling water bath for 2 mins before being cooled on ice and added to the hybridisation buffer (Section 2.14.3).

2.14.3 Hybridisation of Probe DNA to Southern Blots

The nylon membrane was prehybridised at 65°C for 2 hrs in approximately 30mls of hybridisation buffer (3x SSC, 0.02% (w/v) Denhardt's solution, 0.5% (w/v) SDS and 50µg/ml sonicated salmon sperm DNA). After prehybridisation, all but approximately 5mls of the hybridisation buffer was poured off and the denatured probe was added. The hybridisation tube was left rotating at 65°C overnight. After hybridisation, the filter was removed and washed 3 times with 0.1 to 3x SSC and 0.1% SDS as outlined in the text.

2.14.4 Autoradiography of Southern Blots

The filter was wrapped in Gladwrap, and exposed, with the use of an intensifying screen, to a sheet of Kodak Scientific Imaging or Fuji Medical X-Ray film in a X-ray cassette. After a period of time suitable for the appropriate band intensity the film was developed and fixed using the appropriate solutions in the dark room, washed with water and air dried.

2.14.5 Stripping the Hybridised Probe from Southern Blots

Removal of the probe was performed by soaking the filter in boiling 0.1% (w/v) SDS and 1mM EDTA with gentle shaking until cooled. This was repeated two times and stripping of the filter was checked by autoradiography.

2.15 Library Screening

2.15.1 Determination of Library Titre and Primary Round of Library Screening

To determine the concentration of viable phage serial dilutions of the phage lysate were assayed. The phage suspension was diluted in SM buffer (Section 2.4.9) and 10^{-3} to 10^{-8}

dilutions were plated out in duplicate. LE392 was used as a host for phage λ : 100 μ l of an overnight culture (supplemented as in Section 2.2.1.3) was added to 100 μ l of diluted phage and incubated for 30 min at 37°C. The phage/LE392 mixtures were added to a 3ml aliquot of top agarose (Section 2.2.1.2) at 50°C, vortexed gently to mix, and poured onto LB plates. The top agarose was allowed to set and the plates were incubated overnight at 37°C. Plaques were counted and the titre determined to be 4.6×10^5 plaque forming units/ml (pfu/ml). Eight plates, each containing 2.5×10^3 pfu/plate, giving a total of 2.0×10^4 pfu were then prepared for lifts.

Note: A 99.9% probability of having a representative library with mean insert size of 16kb and genome size of 4×10^4 kb, requires that 1.7×10^4 clones had to be plated.

2.15.2 Plaque Lifts

The diluted phage were plated out as above, the plates were incubated for about 6 hr at 37°C (or until small even plaques had formed) and then placed at 4°C. Colony /plaque screen discs (Biotechnology Systems) were laid on the plate and marked so they could be orientated in reference to the plate later. Two lifts were taken from each plate, to guard against false positives, with the first membrane being left in contact with the plaques for 1 min after completely wetting and the second membrane being left for two min. Filters were then placed DNA side up on 3 layers of 3MM paper soaked in 0.5M NaOH, 0.5M NaCl for 2 min, 0.5M Tris (pH 7.4), 2.0M NaCl for 5 min and 2x SSC for 2 min before being air dried and vacuum baked for 2 hr at 80°C.

2.15.3 Hybridisation of a [α - 32 P]dCTP Labelled Probe to Phage λ DNA

Labelling of the DNA to be used as a probe, hybridisation of the probe to the filters and autoradiography of the film were all performed as previously specified in Sections 2.14.2 to 2.14.4. The only difference in techniques was that the lifts were placed in small, round plastic pots rather than round tubes for hybridisation and these were incubated at 65°C in a shaking water bath. The positions of the positive plaques on the film were lined up with the membranes and to the positive plaques on the plates. Plaques corresponding to the signals on the autoradiographs were removed with the end of a pasteur pipette, and were placed in an eppendorf with 1ml of SM buffer and 20 μ l of chloroform.

2.15.4 Second and Third Round of Library Screening

The phage from the plugs of positive plaques were purified twice more by screening as above, the only difference being the number of plaques on each plate. Dilutions of the plaques were made in SM buffer (10^{-2} to 10^{-5}) and only the plates with 30-300 plaques on them were screened with the hybridisation probe, as a pure positive plaque was required. Only one lift was necessary on the third screen as all of the plaques should by then be positive. DNA was then extracted from the positive plaques as detailed in Section 2.5.4.

2.16 DNA Sequencing

DNA for sequencing was prepared by subcloning a λ DNA fragment into pUC118 and transforming it into *E. coli* XL-1 cells (Sections 2.10) from which DNA was extracted by an alkaline lysis preparation (Section 2.5.1).

2.16.1 Sequencing Reactions

Sequencing reactions were carried out using the AmpliCycle Sequencing Kit by Perkin Elmer. This kit uses a modification of the dideoxy-mediated chain termination method of Sanger (1977). It uses the AmpliTaq DNA Polymerase, a modified form of Taq DNA polymerase and uses thermal cycling for the sequencing reactions in a PCR machine.

Eight different primers were used to sequence one template, these are listed in Table 3. For each different primer/template mix a cocktail containing 1 μ l (20 μ M) primer, 1 μ l [α - 33 P]-dCTP, 4 μ l 10x cycling mix, 1 μ l (20ng) template, 23 μ l H₂O was made of which 6 μ l was dispensed into 2 μ l of each of the G, A, T, and C termination mixes, in 0.2ml thin wall PCR tubes. The tubes were then placed in the preheated (95 $^{\circ}$ C) PCR machine for 25 cycles of 95 $^{\circ}$ C 30 sec denaturation, 60 $^{\circ}$ C 30 sec annealing, and 72 $^{\circ}$ C 1 min extension. After thermal cycling 4 μ l of stop solution was added to each of the tubes. They were then stored for up to a week at -20 $^{\circ}$ C.

2.16.2 Polyacrylamide Electrophoresis

The sequencing reactions were analysed through a 6% (w/v) polyacrylamide (34:2 acrylamide:bis-acrylamide) gel containing 8M urea in TBE sequencing buffer (134mM Tris, 2.5mM EDTA, 45mM Boric acid). Following heat denaturation, 3 μ l aliquots of each

reaction were loaded in the order of GATC after the gel had pre-run for 15 min with constant power (65W). These reactions were run for 2 hr and then the same samples were loaded for another 2 hr hence generating long and short runs. Extra long runs (6 hr) or very short runs (1 hr) were run if necessary. The gel was then disassembled and fixed in a solution containing 10% acetic acid, 10% ethanol for 15 min, dried for 30 min under vacuum at 80°C and then autoradiographed overnight.

Table 3. Primers used in Sequencing Reactions

Primer	Size	Tm ^{oC} ^a	Sequence ^b	Source
Foward M13 (<i>lacZ</i>)	22mer	70	GCCAGGGTTTTCC CAGTCACGA	Perkin Elmer
Reverse M13 (<i>lacZ</i>)	24mer	70	GAGCGGATAACA ATTCACACAGG	Perkin Elmer
AB TUB1	22mer	70	GGGCAAGGGTCA CTACACTGAG	This Study ^c
AB TUB2	24mer	70	CGAAGGTAGACG ACATCTTGAGAC	This Study ^c
AB TUB3	23mer	68	TCCAGGTCAGCT CAACAGTGATC	This Study ^d
AB TUB4	23mer	70	TTGCGGAGATCA CTGTTGAGCTG	This Study ^d
AB TUB 5	22mer	68	ACAACGTGCAGA CTGCCCTTTG	This Study ^d
AB TUB 6	23mer	70	TGATCTGGAATC CCTGAAGGCAG	This Study ^c

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

^b 5' to 3' sequence.

^c Pharmacia custom-made.

^d Life technologies custom-made.

3. Optimisation of Protoplast Isolation from *Dothistroma pini*

Isolation of pure, undamaged protoplasts in sufficient numbers ($>10^7$) is vital for a successful transformation and as it has never been attempted in *D. pini* before, it is a crucial first step in developing an efficient transformation system. Young hyphae are used as starting material as the walls of these cells are more vulnerable to enzymic degradation and the resulting protoplasts are easier to separate from the mycelia and cell debris.

Many factors affect the successful isolation of sufficient numbers of protoplasts, which are competent to take up DNA, and consequently become transformed mycelia. Peberdy (1976) demonstrated that protoplast yields were affected by the type and molarity of the osmotic stabiliser used, the pH of the lytic medium, duration of lytic digestion along with the amount and age of the mycelium used in the digestion mix. These various factors were examined and the results are presented.

3.1 Investigation into the Parameters which Affect Protoplast Isolation

A point to note is that only one variable was altered in each separate experiment with remaining conditions kept standard unless a change is specified. A quarter of a cellophane disc, on which mycelium of *D. pini* had been grown for 6 days, was placed mycelium side down in 10ml of 5mg/ml Novozyme 234 in 1.6M $MgSO_4$ osmotic stabiliser, pH 5.8. This was incubated at 37°C for 3 hr. An aliquot was removed and placed on a haemocytometer (0.0025mm²) and counted using a phase contrast microscope. A further point to note is that although the results are presented in a quantitative form, there were unavoidable differences in the amount of mycelium used in each case (approximately 100mg \pm 15% wet weight per quarter of cellophane disc). The overall aim was to determine, on a qualitative level, which conditions would yield sufficient protoplasts for transforming, hence rigorous statistical tests were not carried out.

3.1.1 Analysis of Various Osmotic Stabilisers

During their isolation and subsequent regeneration, protoplasts have to be stabilised with an osmoticum due to the lack of a cell wall. It is widely known that the type and concentration

of osmotic stabiliser can influence both the yield and stability of the protoplasts. These factors also depend on the individual fungi, and subsequently there is no one 'universal' osmoticum for all fungi. Many different osmotic stabilisers have been used in the past including; sorbitol, mannitol, NaCl, MgSO₄ and KCl at concentrations ranging from 0.6-1.2M (Hargreaves and Turner, 1992).

In initial experiments with *D. pini*, protoplasts were obtained using 0.7M NaCl, 1.2M Sorbitol, 1.2M MgSO₄ and 0.6M KCl as osmotic stabilisers (OM buffers). Protoplasts were obtained with all of these but further experimentation was continued with MgSO₄ and NaCl only, as larger protoplasts were evident with these stabilisers, which would hopefully prove beneficial for the flotation method of purification (Section 2.11).

3.1.2 Investigation into Varying Concentrations of MgSO₄, NaCl and Novozyme per ml of Osmotic Buffer

Experiments were performed using 1.0, 1.2 and 1.4M MgSO₄ along with 0.5, 0.7 and 0.9M NaCl as osmotic stabilisers. Results (data not shown) indicated increased protoplast numbers with increased molar concentrations for both of these salts. Further investigation of 1.4, 1.6M MgSO₄ and 0.9, 1.1M NaCl was conducted concurrently with testing total protoplast numbers yielded with 5 and 10mg/ml Novozyme solutions (Table 4). The results indicate higher numbers of *D. pini* protoplasts form when generated in 5mg/ml Novozyme, and they also show quite definitively that MgSO₄ yields about 10-fold higher protoplast numbers than NaCl. Differences between the buffer concentrations (for example, comparing 1.4M with 1.6M MgSO₄) were not substantial.

Another consideration was that protoplasts which formed in NaCl buffer were of two types, large and small, whereas only large protoplasts formed in MgSO₄ buffer. Peberdy (1979) showed that these large protoplasts contained large vacuoles (whereas smaller ones did not) and they formed when *A. nidulans* was digested with lytic enzymes in the presence of MgSO₄. Qualitative observation by Skatrud *et al.* (1987), of different sized regenerating protoplasts under a light microscope, revealed that large protoplasts of *Cephalosporium acremonium* produced new hyphae whereas the small ones remained

Table 4. Protoplast Numbers^a with Differing Concentrations of MgSO₄, NaCl and Novozyme

OM buffer	Novozyme concn		Mean protoplasts/ OM buffer
	5mg/ml	10mg/ml	
1.4M MgSO ₄	130	7.8	68.9
1.6M MgSO ₄	150	5.8	77.9
0.9M NaCl	8.5	5.3	6.9
1.1M NaCl	5.1	4.9	5.0
Mean protoplasts/ Novozyme concn	73.4	6.0	

^a All protoplast numbers are given $\times 10^4$ /ml of osmotic stabiliser and results given are the average from duplicate experiments.

inert. These large vacuolated protoplasts were found to have more buoyancy and hence they were able to be harvested by flotation after centrifugation, which was first demonstrated successfully by Tilburn *et al.* (1983). Subsequently, in the hope of harvesting by flotation, further experiments were performed to compare 1.4 and 1.6M MgSO₄ until it was shown that more protoplasts were usually generated with 1.6M MgSO₄ under optimal conditions (Table 5).

3.1.3 Assessing Different pHs of OM Buffer

Generally the effect of pH is associated with the activity of the lytic enzyme and osmotic stabiliser (Davis, 1985). The optimum pH for Novozyme activity is between 5 and 6 (Hargreaves and Turner, 1992). With 1.2M MgSO₄ the most common pH used is 5.8 (Tilburn *et al.* 1983 and Yelton *et al.* 1984). As I had previously determined the benefits of using a buffer with a higher molarity with *D. pini*, I thought it essential to optimise the pH. 1.4 and 1.6M MgSO₄ were made at pH 5.6, 5.8, and 6.0 and used for protoplast isolation. Results (Table 6) indicate the optimal pH for protoplast generation in *D. pini* is as previously observed in other fungi, pH 5.8.

3.1.4 Influence of Mycelium Age on Protoplast Numbers

As mentioned earlier young mycelium, in the exponential phase of growth, is generally chosen for generating protoplasts. *D. pini* is a relatively slow growing fungus. After 6 days incubation a thin layer of mycelium is seen covering a cellophane disc (Section 2.3.2), hence it was considered to be in exponential growth phase at this time. To assess whether this mycelium would lead to optimal protoplast numbers, protoplast yields were determined from 5, 6 and 7 day old mycelium (Table 7), establishing that optimal culture incubation time was 6 days.

3.1.5 Analysis of Exposure Time of Mycelium to the Lytic Enzyme

Various reports in the literature advise to keep the digestion time of the enzyme with the mycelium to a minimum, as prolonged incubation may cause early-formed protoplasts to degenerate (Davis, 1985), or reduce transformability (Hargreaves and Turner, 1992). Punt *et al.*, (1992) suggest that protoplast formation be checked at 30 min intervals and harvested when many free protoplasts are observed but the mycelium has not completely broken down. Protoplast formation was calculated every 30 min in a lytic digestion using

Table 5. Protoplast Numbers^a from 1.4 versus 1.6M MgSO₄

MgSO ₄ concn	Mean protoplasts
1.4M	97 ± 47
1.6M	120 ± 43

^a All protoplast numbers are given x10⁴/ml of osmotic stabiliser and results given are the average from duplicate experiments.

Table 6. Protoplasts Numbers^a Using Differing pHs of OM Buffer

Concn of OM buffer	pH 5.6	pH 5.8	pH 6.0	Mean protoplasts/OM buffer
1.4M MgSO ₄	54	97	10	54
1.6M MgSO ₄	85	120	44	83
Mean proto-plasts/pH	70	109	27	

^a All protoplast numbers are given x10⁴/ml of osmotic stabiliser and results given are the average from duplicate experiments.

Table 7. Protoplast Numbers^a Obtained from Varying Aged Mycelia

Concn of OM buffer	Age of <i>D. pini</i> cultures			Mean protoplasts/ OM buffer
	5 Days	6 Days	7 Days	
1.4M MgSO ₄	16	97	30	48
1.6M MgSO ₄	16	120	15	50
Mean proto- plasts/mycelia age	16	109	23	

^a All protoplast numbers are given $\times 10^4$ /ml of osmotic stabiliser and results given are the average from duplicate experiments.

mycelium on a whole cellophane disc, incubated at 37°C. The results (Table 8) indicate that protoplasts should be harvested after 2.5 hr.

3.1.6 Protoplast Isolation from Liquid versus Agar Cultured Mycelium

Fungal material used to generate protoplasts can be grown on cellophane discs laid on the surface of agar plates (Penttila *et al.* 1987) as was convenient for optimisation experiments, or alternatively in liquid shake-flask cultures.

In order to ascertain whether liquid-grown cultures give superior protoplast yields, mycelium was grown in liquid cultures as in Section 2.3.2 but on a smaller scale (25ml DM broth in 250ml siliconised flask), harvested by filtering through a Buchner funnel, weighed and then digested with the Novozyme/MgSO₄ mix. The mean number of protoplasts generated from duplicate experiments using liquid cultured mycelium was 2.8×10^4 /mg wet weight of mycelium compared to the mean of 1.2×10^4 protoplasts/mg wet weight mycelium generated with cellophane/agar cultures.

Although protoplast production numbers were increased 2-fold using mycelium from liquid cultures over mycelium grown on cellophane discs, I continued to use the latter at this stage as they were found to be more convenient with fewer contamination problems (an important consideration when dealing with a slow growing fungus).

3.1.7 Analysis of Results of Optimising Protoplast Isolation

Success was shown when optimising protoplast isolation with initial yields of 5.0×10^4 /ml increasing to an average of 130×10^4 protoplasts/ml as conditions were optimised. After a moderately long culture incubation time (6 days), protoplast isolation was deemed optimal using a high concentration of MgSO₄ (1.6M) at pH 5.8, with Novozyme at 5mg/ml for 2.5 hr. Potentially, protoplast numbers could still be increased, for example another lytic enzyme may work better with *D. pini* mycelia, also slightly better yields were seen when using mycelia from liquid cultures (Section 3.1.6). However since mycelium from just one quarter of a cellophane disc was used in all these initial experiments, with no harvesting to concentrate the numbers, optimisation seemed successful. The next step was to regenerate the harvested protoplasts.

Table 8. Protoplast Numbers^a Formed at 30 Min Intervals

Time (min)	Protoplast numbers		
	Sample 1	Sample 2	Mean of Samples
30	2	3	3
60	45	76	61
90	129	189	159
120	357	440	399
150	386	575	481
180	370	580	475

^a All protoplast numbers are given $\times 10^4$ /ml of osmotic stabiliser.

3.2 Regeneration of Protoplasts

Following transformation the protoplasts are regenerated and exposed to appropriate selection pressure. The regeneration frequency can vary greatly (between 0.1 to 70%) and failure to regenerate is likely to be caused by damage or stress during protoplast isolation, lack of nuclei, the lethal effects of PEG treatment during the transformation, and/or protoplast origin in relation to hyphal organisation (Hargreaves and Turner, 1992 and Perberdy, 1979).

To obtain growing colonies from protoplasts it is vital to maintain osmotically stabilised conditions until the cell wall is regenerated. Sorbitol and sucrose are both commonly used for this purpose. Protoplasts are plated either directly on to osmotically stabilised media or in an agar overlay. It is important for both methods of protoplast plating to be tried as regeneration frequencies may be affected. Ballance and Turner (1985) found increasing the overlay agar concentration from 0.9 to 2.0% enhanced the recovery of transformants of *A. nidulans*, whereas, Yelton *et al.* (1984) found plating protoplasts in agar overlays to have no effect compared to spreading over pre-poured plates.

Four mycelial covered cellophane discs were placed face down in the Novozyme/OM buffer solution, and the protoplasts were harvested by flotation in a sorbitol buffer (0.6 or 1.0M) as described in Section 2.11.2. Dilutions were made (so as to expect 10 or 100 colonies per plate after regeneration) and the diluted protoplast solution either spread directly on to osmotically stabilised DM (0.8 or 1.2M sucrose) plates or added to a 5ml agar overlay.

The two main areas which were targeted for optimisation of protoplast regeneration were the concentration of the ST (sorbitol) buffer used for harvesting and the concentration of osmotic stabiliser in the DM regeneration media. In previous papers the ST buffer contained 0.6M sorbitol, but that was for protoplasts isolated using 1.2M MgSO_4 in the protoplast isolation (OM) buffer (Tilburn *et al.*, 1983 and Yelton *et al.*, 1984). In contrast, the maximum number of protoplasts yielded from *D. pini* mycelium was with 1.6M MgSO_4 , therefore it was considered necessary to try raising the concentration of other osmotic stabilisers as well eg. 1.0M sorbitol in ST buffer and 1.2M sucrose in the regeneration medium (compared with the 1.0M sucrose norm).

Many regeneration experiments were performed using different combinations of buffers and stabilisers (Table 9). A large difference in regeneration frequencies was not observed

between the different conditions, although protoplast regeneration after using 1.0M ST buffer with 0.8M sucrose was consistently more efficient than with any of the other buffer combinations. 1.0M ST buffer with 0.8M sucrose was also believed to be optimal, for protoplast regeneration as it produced a higher percentage of the large over the small colony types. This was important as I believed only the large colonies were regenerated protoplasts (Section 3.4). Overall the percentage of protoplasts regenerating ranged from 2-14%.

Experimentation with the two opposing means of plating protoplasts on the osmotically stabilised medium (spreading and adding to an agar overlay) had no effect on the regeneration frequency (data not shown). It was decided that since the transformation selection procedure requires cell wall regeneration time before addition of an antibiotic, it would be simpler to spread the protoplast suspension on to the osmotically stabilised DM medium before overlaying with DM top agar containing the antibiotic (Section 2.2.2.2). Results shown in table 9 only include regeneration frequencies from plates on which protoplasts were spread.

3.3 Examination of Different Colony Morphologies of Regenerated Protoplasts

During the time that I have been growing different *D. pini* cultures (Section 2.3.2) it has become obvious that *D. pini* shows morphological instability, with almost a slightly different appearance each time it is subcultured. A similar occurrence appeared to be happening on the regenerated protoplast plates, where two different colony sizes were observed (large and small). Various explanations could account for this, such as mycelial fragments being present, different nuclei numbers (with protoplasts forming at the apex of the hyphae containing more) or prolonged incubation in the lytic enzyme which can cause the early-formed protoplasts to regenerate aberrant hyphae Davis (1985).

Common features were seen between the large and small colonies. Both appeared to produce the toxin dothistromin (seen by brown staining of the media) and both had the same morphology when observed under 100 and 400x magnification. In order to discern whether one of the colony types originated from mycelial fragments, rather than

Table 9. Regeneration (%) of *D. pini* Protoplasts^a

	Mean ^b % protoplast regeneration			
	0.8 M	0.8 M	1.2 M	1.2 M
[Sucrose] in DM regeneration medium	0.8 M	0.8 M	1.2 M	1.2 M
[Sorbitol] in ST flotation buffer	0.6 M	1.0 M	0.6 M	1.0 M
Colony morphology ^c				
Large	6 ± 2.4	14 ± 5.7	2.0 ± 0	2 ± 0.6
Small	17 ± 17.8	9 ± 4.2	20 ± 20.5	9 ± 5.7

^a Before dilution the average number of protoplasts generated was $3.5 \times 10^6 \pm 2.9 \times 10^6$ /ml.

^b Mean of at least 2 experiments in which protoplasts were spread onto plates.

^c Two different morphologies were observed; only the larger were considered regenerated protoplasts (see Section 3.3).

regenerated protoplasts, 1:10 and 1:100 dilutions of the protoplasts were made with water prior to plating on non-osmotically stabilised DM. Growth seen on the agar medium after a regeneration period would come from mycelium, as protoplasts would burst in these conditions and be unable to regenerate. In this experiment only small colonies grew on the DM, leading to the theory that the larger colonies seen were in fact regenerated protoplasts (Section 3.3) and the smaller colonies originated from the remaining mycelial fragments. Why the difference in growth size was observed was not apparent.

3.4 Further Attempts to Improve Protoplast Isolation and Harvesting/Purity

Isolation of protoplasts followed by purification had so far yielded an average of 1.3×10^6 protoplasts/ml of osmotic stabiliser whereas most transformation protocols require at least 10^7 protoplasts/ml. Consequently I tried various methods to enhance protoplast numbers and purity.

3.4.1 Filtration of the Hyphal Debris Through a Sintered Glass Filter

Negligible alteration in protoplast numbers were observed using a sintered glass filter (porosity 1) and the filtered protoplasts were still largely contaminated by hyphal debris. Hence the ST flotation method of harvesting was deemed superior.

3.4.2 Increasing Mycelia Concentration in the Preparations

Previously (Section 3.3) I had been using total mycelium from four cellophane discs in each protoplast preparation. Now I tried using a total of 4, 5, 6 and 12 mycelia covered cellophane discs in 10ml of Novozyme solution, along with mycelia harvested from a liquid culture of 300ml of DM broth (Section 2.3.2). By comparing protoplast yields between disc numbers (Table 10), a trend of increasing protoplasts with increasing mycelium was seen up to 5 discs. A vital point to note here is that these results are from only one experiment and I was unable to repeat the success shown here (including the high protoplast numbers with 5 discs) for a long time.

In retrospect and with increased knowledge I feel the main reason for the lack of reproducibility in my results, and the lower protoplast numbers evident when using 6 and 12 discs, or mycelium grown in liquid cultures, is the fact that I failed to take the

Table 10. Harvested Protoplast Numbers Obtained using Different Amounts of Mycelium

Amount of Mycelium	Protoplast numbers ^a
4 Discs	3.5
5 Discs	160
6 Discs	50
12 Discs	58
300ml DM broth	73

^a All protoplast numbers are given $\times 10^6$ /ml of osmotic stabiliser using 10ml of 5mg/ml Novozyme.

mycelium weight into consideration and adjust the volume of the Novozyme solution accordingly, to 50mg wet weight of mycelium per ml of Novozyme. Hargreaves and Turner, (1992) mention that the use of too much starting material results in poor recovery of protoplasts contaminated with large amounts of debris. Realisation of this, and the use of modified harvesting methods (Chapter 4), contributed to my eventual success in developing a transformation system.

3.4.3 Trying Another Centrifuge for Harvesting

Whilst harvesting protoplasts by flotation using the Sorvall centrifuge (with a fixed-angle rotor) some mycelial debris seemed to be very buoyant and collected near the buffer interface, leading to mycelial contamination of the isolated protoplasts. With the use of a Heraeus Sepatech Megafuge (with a swinging bucket rotor) far fewer mycelial fragments were seen in the protoplast preparations. The swinging bucket rotor ensures all of the mycelia is spun down to the bottom of the tube, this enabled a pure protoplast band to be collected from the buffer interface.

4. Developing a Transformation System

There is no single all encompassing method to transform fungal cells. The literature available on the subject illustrates a diverse range of strategies and procedures which work for specific fungal species but unfortunately not one of the approaches can be universally applied to all fungi. Since it is impossible to perform all of the methods when attempting to develop a transformation system for a previously untransformed fungus, the approaches must be limited to the ones which have the greatest chance of success. Bearing the above in mind I optimised protoplast isolation as successfully as I could (Chapter 3) and then set out to actually transform some *D. pini* protoplasts with the plasmids pAN7-1 or pAN8-1. During the transformation attempts further optimisation of protoplast isolation was achieved.

4.1 Preparatory Work

4.1.1 Investigating the Antibiotic Concentrations which Inhibit *D. pini*

In order to use either of the antibiotics hygromycin B (HmB) or phleomycin (Phleo) it is first necessary to determine what concentrations inhibit the growth of *D. pini*. Concentrations ranging from 50 to 200 μ g/ml of HmB and 20 to 50 μ g/ml of Phleo are generally sufficient to inhibit fungal growth (Hargreaves and Turner, 1992). Stock solutions of the antibiotics were made up in sterile MQ water (50mg/ml HmB and 5mg/ml Phleo). Growth of *D. pini* mycelium spread onto DM plates containing the antibiotics, was completely inhibited by HmB at 50 μ g/ml and Phleo at 6 μ g/ml.

4.1.2 Preparation and Molecular Analysis of pAN7-1 and pAN8-1

Plasmids pAN7-1 (Appendix 1) and pAN8-1 (Appendix 2) contain the bacterial hygromycin B resistance and the phleomycin resistance genes, respectively, under the control of the *Aspergillus nidulans* *gpdA* promoter and *trpC* terminator. When these plasmids are transformed into a fungal cell they convey resistance to the antibiotics HmB or Phleo.

The plasmids pAN7-1 and pAN8-1 were used with the permission of Dr. C.A.M.J.J. van den Hondel (Punt *et al.*, 1987 and Mattern and Punt, 1992). Plasmid DNA was prepared by transforming it into *E. coli* cells (Section 2.10.4) followed by a cesium chloride plasmid

preparation (Section 2.5.2). Both the plasmids were checked by informative restriction enzyme digests and electrophoresed on an agarose gel (Sections 2.9.1 and 2.9.2).

4.2 Analysis of Different Transformation Methods

4.2.1 Attempts at Transforming *D. pini* with Transformation Protocol A

Protoplasts were isolated using the optimised methods developed in chapter 3 (see protoplast protocol 2, Section 2.11.3). Transformations were performed using Transformation Protocol A (Section 2.12.1), based on the method of Murray *et al.* (1992), except that heparin was omitted as it was found to have no effect on transformation efficiency in *A. nidulans* (D. Bird, unpublished). The key features of this transformation method were: the addition of 20 μ l of a 40% PEG 4000 solution (containing 1M sorbitol as osmoticum) to the protoplast solution with 2 μ l of spermidine, followed by a further addition of 900 μ l of the PEG solution 30 min later.

Growth on non-selective plates suggested that the protoplasts were viable after PEG treatment. The mean protoplast regeneration for transformations 1, 2 and 3 was 2.0%. Both small and large colonies were still seen on the non-selective plates of the untransformed negative controls, except for results from transformation 3 in which only large colonies were seen (see Table 11). As explained in Section 3.4 the small colonies were found to be due to mycelial fragments in the protoplast preparation. The lack of small colonies with transformation 3 was due to the use of the swinging bucket Heraeus Sepatech Megafuge for protoplast isolation rather than the fixed rotor Sorvall centrifuge (Section 3.4.3). Mycelial fragments should not be a problem since when performing a transformation only protoplasts can be transformed and only transformed protoplasts are able to grow on selective media. The different centrifuge, along with the use of liquid grown mycelium, also contributed to the increase in protoplast numbers available for transformation 3 (7.3×10^7 protoplasts/ml), compared with 1.0×10^6 and 2.47×10^6 for transformation attempts 1 and 2 respectively (Table 11, second column).

The lack of transformants from transformations 1 and 2 was reinforced by the inability of any of the colonies which had grown on the selective medium to grow upon sub-culturing on selective medium. Sub-culturing techniques were developed over this period: it was necessary to cut off small pieces of the colonies with a scapel before placing them on fresh

Table 11. Transformation of Fungal Protoplasts with pAN7-1 or pAN8-1 using Transformation Protocol A

Transformation no.	DNA added ^a	Protoplasts used/transformation (x10 ⁶)	Colonies/plate ^b		Transformation freq/ μ g DNA
			Large	Small	
1	0	0.08	21	u/c ^c	-
	pAN7-1	"	0	4	0
	pAN8-1	"	0	6	0
2	0	0.2	46	u/c	-
	pAN7-1	"	1	11	1
	pAN8-1	"	22	u/c	22
3	0	5.8	65	0	-
	pAN7-1	"	1	10	1
	pAN8-1	"	0	24	0
4		4.7	All plates contaminated		

^a 0 μ g of DNA added to protoplasts for the negative control or 5 μ g added of the appropriate plasmid.

^b Results are shown for the non-selective plates for the negative control (0 plasmid) and selective plates for the transformations (70 μ g/ml HmB for pAN7-1 or 7 μ g/ml for Phleo pAN8-1. The large colonies with 0 μ g of plasmid equate to regenerated protoplasts and the small to mycelial fragments. The large colonies generated when selecting for the plasmid are stable transformants whereas the small are abortive transformants.

^c Too many to count.

media (Section 2.13). The one large HmB resistant colony seen from transformation 3, continued to grow when subcultured onto selective medium (DM + HmB) as in section 2.13 and was thus considered to be a genuine transformant (transformant AB1). The HmB resistance of this positive transformant compared to untransformed (control) *D. pini* is seen by growth of the transformed mycelia on a DM + HmB plate in Figure 1.

Although transformation Protocol A was not very successful overall, it proved to be useful in so far as it provided one positive transformant and from this the essential features causing success could be identified, and the method improved. Some of the points essential to the success of transformation 3 are the increased protoplast numbers, the use of liquid grown mycelium and the use of a different centrifuge (Heraeus Sepatech Megafuge, compared with a Sorvall) which probably led in turn to the lack of mycelial contamination.

4.2.2 Attempts at Transforming *D. pini* with Transformation Protocol B

The method employed for the ensuing transformations is very similar to Protocol A. The crucial differences were the methods of mycelia growth and harvesting (protoplast protocol 3, Section 2.11.3): mycelia grown in DM broth was harvested by centrifugation instead of by filtration, washed in water before being resuspended in Novozyme solution and incubated, with gentle shaking at 30°C (or 37°C, experiment 6).

The viability of protoplasts before the addition of PEG (expressed as a percentage of the total protoplasts), was determined to be approximately 6% for transformation 5 and 2% for transformation 6 (Table 12). Although these values are quite low, they are within the range (0.1 to 70%) depicted by Hargreaves and Turner (1992). These relatively low transformation regeneration frequencies further emphasise the need for higher protoplast isolation numbers with *D. pini*, to increase the chance of a viable protoplast being transformed. Unfortunately the protoplast isolation method trialed in this protocol seems to be a step backward in protoplast numbers (mean of 0.65×10^7 /ml with protocol B compared to 6.6×10^7 /ml with the last two transformations using protocol A. However, transformation attempt 6 did benefit from increased protoplast numbers compared to transformation 5 which may reflect the fact that protoplast incubation in experiment 6 was at 37°C, not 30°C as with experiment 5 which is the temperature Yelton *et al.* (1984) use.

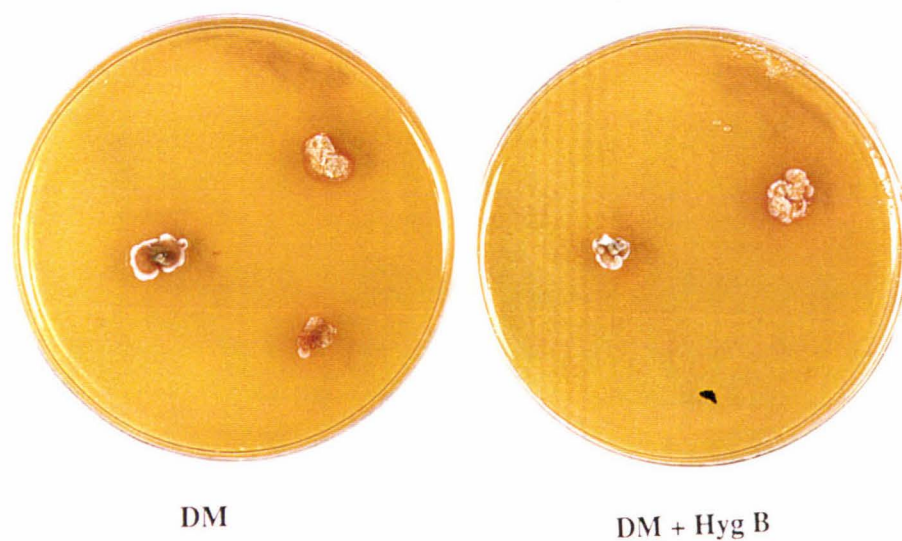


Figure 1. Transformants AB1 and AB2 plated on selective and non-selective DM

Non-selective ($0\mu\text{g/ml}$ HmB) and selective ($70\mu\text{g/ml}$ HmB) DM plates, left to right respectively, showing growth of transformed and untransformed *D. pini*. Colonies on the plates clockwise from the top are; transformant AB1, untransformed *D. pini* and transformant AB2.

Table 12 Transformation of Fungal Protoplasts with pAN7-1 or pAN8-1 using Transformation Protocol B

Transformation no.	DNA added ^a	Protoplasts used/transformation (x10 ⁶)	Viable protoplasts (%) - PEG ^b	Transformants/plate ^c		Transformation freq/ μ g DNA
				Large	Small	
5	pAN7-1	4.5	4.8	1	2	1
	pAN8-1	"	"	con ^d	-	-
6	pAN7-1	5.9	1.7	0	0	0
	pAN8-1	"	"	1	8	1

^a 5 μ g added of the appropriate plasmid.

^b The percent viable protoplasts refers to the large colonies (on non-selective media) only which had been shown to be genuine regenerated protoplasts before PEG treatment.

^c Selected on 70 μ g/ml HmB (pAN7-1) or 7 μ g/ml Phleo (pAN8-1). The large colonies are stable transformants whereas the small are abortive transformants.

^d Unable to count *D. pini* colonies due to contamination.

Overall this protocol was not very successful for generating transformants. Only two transformants were identified which were stable after successive rounds of sub culturing: a HmB resistant transformant AB2 from protoplasts transformed with pAN7-1 (transformation 5; Table 12), and a Phleo resistant transformant (transformation 6; Table 12). The resistance of transformant AB2 to HmB is shown in Fig. 1. All of the stable transformants isolated so far (AB1, AB2 and one Phleo resistant transformant) have come from large colonies. The small colonies are probably abortive transformants, (see Section 4.3) as they do not remain resistant to the appropriate antibiotic after sub-culturing.

A potential reason for the low number of transformants generated is the richness of the DM Suc medium which was being used to regenerate the protoplasts. This hypothesis was tested in transformation 6 by the use of DM Suc with one half of the standard amounts of malt extract (25g/l) and nutrient agar (14g/l). Protoplasts were harvested and transformed as in Protocol B. Protoplasts were plated onto the less nutrient-rich DM Suc as well as standard DM Suc. The results indicated no significant change in the viability of protoplasts plated on the less nutrient rich mediums (1.1% on DM + half malt extract and 1.4% on DM + half nutrient agar, compared to 1.7% protoplast viability when generated before addition of PEG on standard DM + Suc) An increase in the frequency of transformed mycelia was also not observed. One Phleo resistant transformant was seen on a DM + half nutrient agar plate and no transformants were counted on the half malt extract plates.

The results of the two transformation protocols trialed were carefully considered. Although the liquid method of culturing mycelia seems effective I was still having problems with mycelial debris contaminating the protoplasts. This contamination practically invalidates the protoplast viability results as it is difficult to ensure only large (genuine) regenerated protoplasts are counted. Other problems also occurred such as contamination of many of the plates and low protoplast numbers. Further trouble was experienced when protoplast yields for one protoplast isolation experiment, using Protocol B, generated extremely low numbers of protoplasts (5×10^4 /ml) which, subsequently, were not used for transforming. A possible cause of these low protoplast numbers was detergent residue in the Nalgene tubes used for centrifugation, but variable protoplast yields are common with this method when applied to other fungi such as *Acremonium loli* (K. Saunders and C. Berkahn, unpublished).

4.2.3 Transforming *D. pini* with Transformation Protocol C

As previously stated there were continuous problems in the form of mycelial debris contamination, in my protoplast preparations. Although improvement was made using a swinging bucket centrifuge (compared to a fixed rotor) some contamination by mycelial fragments was still seen (two colony sizes evident on the non-selective plates). The protoplast isolation method performed by Punt *et al.*, (1992) offered an alternative method for separating the protoplasts from mycelia debris by filtering through myra cloth (protoplast protocol 1, Section 2.11.1) More easily available than myra cloth are nappy liners, which were being used successfully for first filtering the mycelium from the growing medium and then harvesting the protoplasts from the mycelial debris in *A. nidulans* (T. McGowan, unpublished). In protocol C I adopted this filtration technique in addition to altering the PEG treatment conditions.

For fungal, bacterial and animal cells, the universal components of the transformation mixtures are DNA and calcium ions (Fincham, 1989). After an initial period of exposure to the DNA, protoplasts are induced to take up the DNA molecules by treatment with PEG and calcium ions which causes the protoplasts to aggregate and become temporarily permeable to the DNA molecules. PEG (molecular weight 3350 to 6000) is used at concentrations ranging from 25 to 66% and CaCl_2 is present at concentrations from 10mM to 100mM (Hargreaves and Turner, 1992). In the two previous transformation protocols 40% PEG 4000 has been used with 50mM CaCl_2 , 50mM Tris-HCl and 1.0M sorbitol, whereas in this protocol (C) 60% PEG 6000 was used with 50mM CaCl_2 , 10mM Tris-HCl but with no osmoticum.

In the previous transformation protocols A and B, 20 μ l of PEG solution was added initially to the protoplast/DNA mixture and after a 30 min incubation a further 900 μ l of the PEG solution was added. Both Fincham (1989) and Hargreaves and Turner (1992), suggest an initial period without any PEG solution, followed by slowly adding PEG in increasing aliquot volumes, with gentle mixing between each aliquot. Step-wise addition of PEG during transformation, compared to the addition of one large aliquot, is another significant change in transformation protocol C. PEG can be toxic to some fungi and the length of time the protoplasts are exposed to it should be kept to a minimum.

Initially (transformation experiment 7), *D. pini* protoplasts were prepared as in Section 2.11.2 but using as an OM buffer 0.27M CaCl_2 , 0.6M NaCl. The wet weight of the

mycelium was 6.85g, and Novozyme was added at 5mg/ml to 140ml of the OM buffer. After incubation at 30°C with slow agitation (50-100rpm) for 2.5 hrs, protoplasts were separated from the mycelium by filtration through a sterile nappy liner. The protoplast suspension was diluted (1:1) in 1.2M sorbitol, 10mM Tris.HCl (pH 7.5), 50mM CaCl₂, 35mM NaCl (STC1700) and incubated on ice for 5-10 min. Protoplasts were collected by centrifugation for 10 min at 1085g (3000rpm, SS34) and washed twice in STC1700. Finally the protoplasts were resuspended (10^7 - 10^8 /ml) in STC1700. Unfortunately the final solution yielded using this method of protoplast isolation with *D. pini*, was very brown and dirty, and protoplasts were unable to be seen in it. The protoplasts were salvaged by gently resuspending in 1.6M MgSO₄ and over laying with 1ml ST buffer (see Section 2.4.7), before collecting at the buffer interface and washing as in Section 2.11.

The protoplast yield, shown in Table 13, was 1.5-fold higher than with transformation protocol B, but almost 7-fold less than the last two protoplast isolations performed with protocol A. The fact that protoplast numbers decreased using the latter two protocols, may reflect mycelium/Novozyme incubation at 30°C, rather than 37°C as in protocol A. Alternatively the diminished numbers could be due to less efficient centrifugation of the protoplast solution as the Heraeus Sepatach centrifuge was found to be faulty. Both these conditions were altered when protoplasts were isolated in transformation 8. In transformation 8 the protoplasts were isolated as in Section 2.11 and the transformation performed as in Section 2.12. 1.6M MgSO₄ (Section 2.4.6) was used as the osmotic stabiliser, and immediately after some of the mycelial debris was filtered from the protoplast solution with a nappy liner, the protoplast solution was overlaid with ST buffer (2.4.7) and the protoplasts were collected at the buffer interface.

The alteration of the incubation temperature during protoplast isolation, along with using both the nappy liner and the protoplast-flotation method of purifying the protoplasts, led to increased protoplast yields (Table 13. transformations 8 to 11 row 3) which were uncontaminated with mycelia fragments (seen by the absence of the small, mycelial fragment colonies on the non-selective plates).

Transformation 7 was performed as in Section 2.12.3, but using 60% PEG 6000 in 10mM Tris-HCl and 50mM CaCl₂, rather than osmotically stabilised 40% PEG 4000 with 1M sorbitol. The transformation frequency was 0 protoplasts/μg of DNA. The protoplast viability results, show a low percentage (0.8%) of viable protoplasts after PEG treatment

Table 13 Transformation of Fungal Protoplasts with pAN8-1 and Various forms of pAN7-1 using Transformation Protocol C

Transformation no.	DNA added ^a	Protoplast used/transformation	Viable protoplasts (%) ^b		Transformants per plate ^c		Transformation freq per μ g DNA
			- PEG	+ PEG	Large	Small	
7	pAN7-1	1.8	11.9	0.1 ^d	0	40	0
	pAN8-1	"	"	"	0	6	0
8	pAN7-1 ²	9.3	11.1	1.8; 4.2 ^e	48	u/c ^f	48
	pAN8-1	"	"	"	1	48	1
9	pAN7-1 ²	1.9	2.0	0.6	4	150	4
	L pAN7-1 ²	"	"	"	1	1	1
	pAN7-1	"	"	"	0	19	0
10	pAN7-1 ²	5.3	6.0	2.2	9	u/c	9
	LpAN7-1 ²	"	"	"	6	u/c	6
	CpAN7-1 ²	"	"	"	5	u/c	3
	pAN7-1	"	"	"	21	u/c	21
	LpAN7-1	"	"	"	28	u/c	28
11	pAN7-1 ²	18	3.4	0.8	10	u/c	10
	LpAN7-1 ²	"	"	"	62	u/c	62
	CpAN7-1 ²	"	"	"	21	u/c	11
	pAN7-1	"	"	"	32	u/c	32
	LpAN7-1	"	"	"	26	20	26

^a 5 μ g added of the appropriate plasmid. Prefix L denotes linearised vector, prefix C denotes 10 μ g DNA used, superscript² denotes an alternative batch of pAN7-1 used.

^b The percent viable protoplasts refers to the large colonies, on non-selective media only.

^c Selected on 70 μ g/ml HmB (pAN7-1) or 7 μ g/ml Phleo (pAN8-1). The large colonies are stable transformants whereas the small are abortive transformants.

^d Using PEG with no osmotic stabiliser.

^e First figure obtained using PEG with no osmotic stabiliser and second with osmotically stabilised PEG.

^f Unable to count *D. pini* colonies due to contamination.

compared to before PEG treatment which I think may be the cause of the lack of transformants (Table 13). The literature indicates that the absence of an osmoticum in the PEG solution may lead to a decreased transformation frequency due to bursting of some of the protoplasts. This theory was examined in transformation 8 when both non-osmotically stabilised (60% PEG 6000) and osmotically stabilised (40% PEG 4000) were added to the protoplasts. Although protoplast viability was increased when non-osmotically stabilised PEG was used in transformation 8, compared to transformation 7, a 2-fold increase in viability was observed using PEG stabilised with 1M sorbitol. Osmotically stabilised PEG was used with both pAN8-1 and pAN7-1 and transformants were observed with both plasmids. pAN7-1 does seem to integrate and/or express more successfully in *D. pini* than pAN8-1, so subsequently further transformation experiments were performed using pAN7-1 only. This also made experimentation with different forms and concentrations of pAN7-1 possible.

With a successful transformation system now developed, it was important to investigate other factors which could influence the transformation frequency, such as the state of the DNA. In the literature, several groups have made investigations into transformation frequencies obtained when using either covalently closed circular or linear DNA, with various results. Yelton *et al.* (1984) found no variation in the transformation frequency when using a linearised plasmid encoding the *trpC* gene in *A. nidulans*. Conversely, Kelly *et al.* (1994) found transformation frequencies to be improved 4- to 6-fold when using linear over circular DNA to transform the fungus *Zalerion arboricola*.

Linearisation of pAN7-1 was performed with the enzyme *Hind*III (see Section 2.9.1.1) which cuts the plasmid just after the 3' coding region of the *trpC* gene (Appendix 1). The effects of linearisation are summarised in Table 14. No change is seen when pAN7-1 from preparation 1 is linearised. Though an increase in transformation frequency is seen with the average of two experiments using pAN7-1 from preparation 2. However such a large standard deviation somewhat invalidates any positive effect of the linearisation.

Garnand and Nelson (1995) found linearising transforming DNA in *Neurospora crassa* did not significantly increase the generation of stable transformants, but it was shown to significantly reduce the number of abortive (Section 4.3) transformants. This reduction in the number of unstable transformants, could be beneficial when determining the transformation frequency, as it would ensure only stable transformants were counted. A reduction in abortive transformants was seen only twice in five separate transformations with

Table 14. Transformation of Fungal Protoplasts with Linear and Circular pAN7-1 from Two Separate CsCl-density Preparations and with Two Concentrations of pAN7-1 from CsCl Preparation 2

DNA added ^a	Transformation frequency/ μg DNA ^b
<u>Preparation 1</u>	
pAN7-1 (circular)	27 ± 7.8
pAN7-1 (linear)	27 ± 1.4
<u>Preparation 2</u>	
pAN7-1 (circular)	10 ± 0.7
pAN7-1 (linear)	34 ± 39.5
pAN7-1 (10μg circular)	8 ± 4.2

^a 5 or 10μg added of the appropriate plasmid.

^b Number of transformants per 10⁶ protoplasts using the average of transformation experiments 10 and 11.

linear pAN7-1 (transformation experiments 9 to 11). Table 13 shows a reduction in small colonies when linearised pAN7-1 (preparation 2) was used in transformation 9 and when linearised pAN7-1 (preparation 1) was used in transformation 11.

Effects of linearisation were investigated in transformation 9 using pAN7-1 from preparation number 2 and in transformation 10 and 11 using pAN7-1 from preparations 1 and 2 (Table 13 and 14). Transformation frequencies were obtained when transforming *D. pini* with pAN7-1 prepared at two different times, as it was necessary to determine whether the increased transformation frequencies seen in transformations 8 to 11 were due to the use of pAN7-1 prepared later/separately (pAN7-1 preparation 2) rather than the pAN7-1 (preparation 1) which was used for transformation experiments 1 to 6. If the transformation frequency was different between the two plasmid preparations it would indicate a difference in the purity of the DNA.

The purity of the vector DNA used for the transformation has been shown to influence the transformation frequency. An increase in the number of transformants was observed by Wendt *et al.* (1990) when using pAN7-1 which was purified twice by CsCl-density gradient, over pAN7-1 which had been purified once, to transform *A. giganteus*. All the plasmids used during the transformation experiments 1 to 11 were purified by CsCl-density gradient (Section 2.5.2) but the DNA purity does vary between different preparations. The results are summarised in Table 14 and indicate pAN7-1 preparation 1 leads to almost 3-fold higher transformation frequencies than pAN7-1 preparation 2 (when using circular DNA) indicating that it was the changes in transformation protocol C that were effective to generate transformants and not the DNA change. The fact that transformation 7 was unsuccessful, see Table 13, even though pAN7-1 from preparation 2 was used, also indicates that it was the change in protocol, not the plasmid which caused the later increased transformation frequencies (transformations 8 to 9).

The optimal amount of DNA to include in the transformation mixture has been studied by only a few groups. One of the more extensive studies was performed by Wnendt *et al.* (1990) who discovered a non-linear, hyperbolic increase in the number of transformants seen when using 1 to 10 μ g of DNA. Higher amounts of DNA led to a decrease in the total yield of transformants. The highest transformation efficiency obtained was 30 per μ g of DNA per 3×10^3 protoplasts when using 1 μ g of DNA. The results were supported by Yelton *et al.* (1984) who found when using less than 0.05 μ g of DNA 300 transformants per μ g of DNA were obtained, compared to 20 transformants per μ g when 10 μ g of DNA was used.

Preliminary experiments using 10 μ g of pAN7-1 as vector DNA in *D. pini* transformations indicate a decrease in the number of transformants generated (see Table 13 transformations 10 and 11) and a decrease in the transformation frequency (Table 14). More experimentation has to be done in this area using varying amounts of pAN7-1 such as 1,5,10,15 and 20 μ g of DNA.

4.3 Abortive Transformants

In the transformation systems developed for filamentous fungi, the presence of 'abortive' transformants are commonly observed. Abortive transformants are defined as those which begin to grow and form small colonies on the selection plates, but stop growth prior to becoming a full sized colony. These transformants fail to grow upon subculturing and are thought to arise when DNA enters the cell but fails to become stably integrated into the genome (Garnand *et al.*, 1995). Tilburn *et al.* (1983) observed that at least some of the abortive transformants still contain the transforming DNA. After prolonged incubation sectors of vigorous growth could develop from the colony where stabilisation had occurred by integration of the DNA.

Small colonies were visible in all eleven transformation attempts (Tables 11, 12 and 13). Although these colonies still produced the toxin dothistromin (dark brown pigment Fig. 2) they were not viable after subculturing and so were termed abortive transformants.

4.4 Suggestions to Further Improve Transformation Frequencies

With the initial crucial step of developing a transformation system which consistently yields transformants completed successfully, I feel that there are still parameters which require investigation and which may lead to increased numbers of transformants. Transformation numbers have increased steadily over the last three transformations (Fig. 3), and even though the literature suggests that transformation frequencies vary greatly between individual transformations I feel that the transformants obtained from *D. pini* are only just getting high enough now (Fig. 3; transformation 11) and more parameters need optimising to obtain consistently higher transformation frequencies. One of the factors which should be investigated when trying to optimise the transformation procedure is the addition of PEG. PEG, although a necessary part of the transformation system as it causes aggregation of the protoplasts, is also seen to be toxic to the protoplasts.

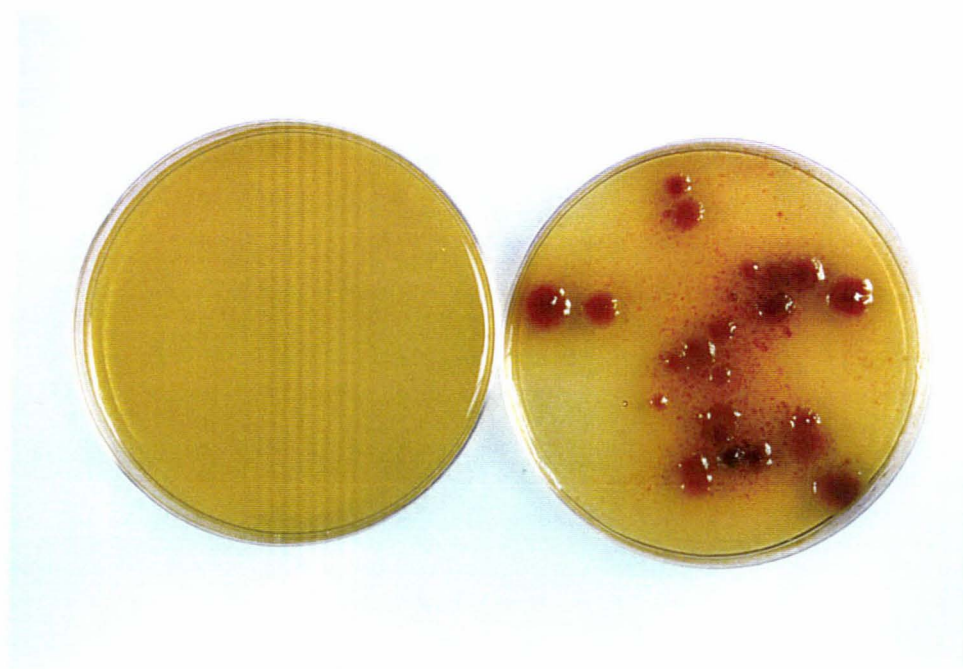


Figure 2. Stable and abortive transformants

10^5 untransformed *D. pini* protoplasts (left plate) and protoplasts transformed with pAN7-1 (right plate) were spread on to DM + Suc plates. After 24 hr HmB was overlaid in 3ml of DM top agar to a final concentration of $70\mu\text{g/ml}$. The abortive transformants are clearly differentiated amongst the large stable transformants, although it should be noted that their size and lack of clarity make it difficult for them to be counted individually. The toxin dothistromin is clearly visible as the red/brown pigment seen colouring the colonies and the surrounding agar.

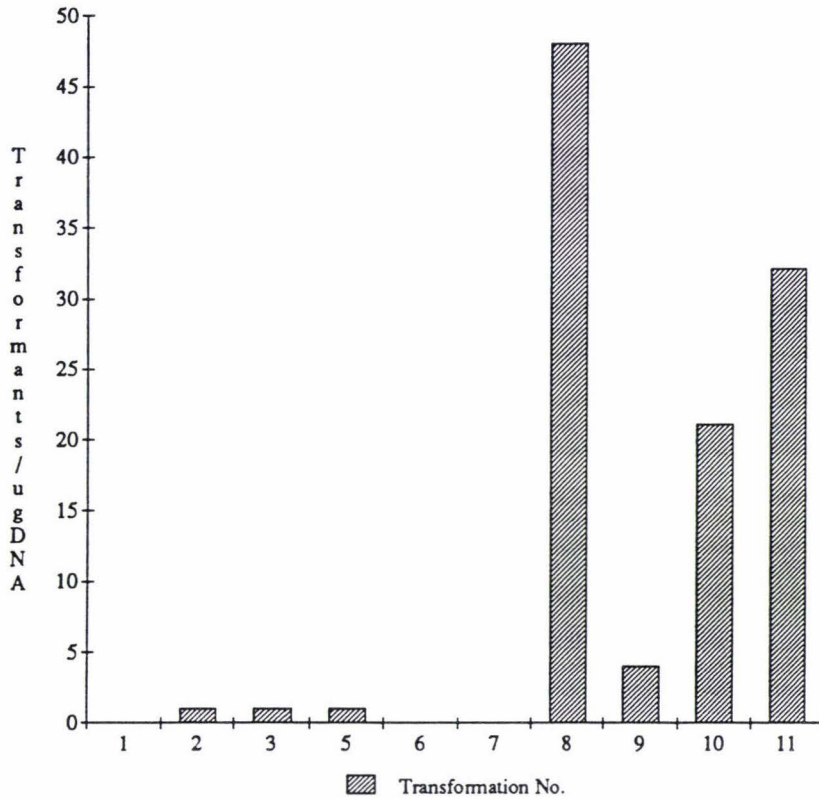


Figure 3. The number of transformants/ μg of DNA generated during various transformations

The numbers of stable transformants obtained per μg of DNA are shown. Results from transformation experiments 8 and 9 are from preparation 2 of vector DNA pAN7-1 while the remaining results are generated using pAN7-1 from preparation 1.

This toxicity can be demonstrated by the average of a 4-fold decrease in protoplast viability after exposure to PEG (Table 13). Kelly *et al.* (1994) became concerned as they noted that protoplast regeneration was reduced about 55-fold after the transformation steps. By minimising the time that the protoplasts were incubated with PEG from 30 min (Mathison *et al.* 1993) to 15 min they noted a decrease of only 12-fold in the protoplast regeneration frequency subsequent to transformation. The modified procedure also resulted in a 10-fold improvement in transformation frequency. A decreased incubation time (from 20 to maybe 15 or 10 min) for *D. pini* protoplasts with PEG may also lead to similar results.

The effect on the transformation frequency, of using varying concentrations of sorbitol as the osmotic stabiliser in the transformation procedure, has also been investigated. Tilburn *et al.* (1983) found increased transformants when using 1.0M over 1.2M sorbitol. Whereas Crowhurst *et al.* (1992) found that the incorporation of either 0.6, 0.8, or 1.0M of sorbitol in the buffer used for transformation (compared to no sorbitol), increased transformation frequencies 23-, 39-, or 34-fold. The use of 1.0M sorbitol instead of no osmoticum, has already been shown to increase protoplast viability by 2-fold with *D. pini* (compare experiments 7 and 8 Section 4.2.3) during transformation. I suggest that a further increase to 1.2M sorbitol should also be trialed, based on the fact that to generate the maximum amount of protoplasts from *D. pini* requires a higher than usual concentration of $MgSO_4$ in the OM buffer (1.6M; Section 3.1.2).

Another parameter concerning the usage of PEG which should be experimented with *D. pini* is the concentration at which PEG is used. Tilburn *et al.* (1983) found that transformation frequencies increased up to 24.8 transformants/ μg of DNA when using 60% PEG compared to 3.2 transformants/ μg of DNA when using 25%. Crowhurst *et al.* (1992) obtained similar results, observing a 4- and 7-fold increase in transformation frequency when increasing the PEG concentration from 20 to either 40 or 60% respectively.

All of the other variables which have been reported to have been altered in the literature to increase the efficiency of transformations involve the vector DNA. The purity of the DNA has already been noted in Section 4.2.3, but more extensive investigation would be beneficial. To know whether increased purity of the DNA leads to a subsequent increase in the transformation frequency as it did with Wnendt *et al.* (1990), or has no impact at all as Crowhurst *et al.* (1992) discovered using both CsCl gradient purified and non-CsCl gradient purified DNA, would have an impact on the preparation of the transforming DNA. To purify twice through a CsCl gradient in order to obtain an increased transformation

frequency would be advantageous, as would doing a simple plasmid mini-prep method if the purity of the DNA has no effect on transformation efficiency.

As mentioned earlier, although preliminary experiments have been performed with increasing the concentration of the vector DNA more investigation needs to be done in this area with *D. pini*. Results so far (Table 15) indicate linearisation of the plasmid pAN7-1 to have no effect when used in my system of transforming *D. pini*. Linearisation of the transforming DNA may be expected to increase the transformation frequency as the DNA ends recombine more readily. This increase would be expected to be greatest where homology exists between the ends of the transforming DNA fragment and the recipient genome (Crowhurst *et al.*, 1992). The impression that linearisation has no effect with *D. pini* therefore is not unexpected, because of the lack of homology between the vector and the genome of *D. pini*.

Another aspect which was found to influence transformation efficiency by Wnendt *et al.* (1990) was the protoplast concentrations. They found that the optimal transformation efficiency was obtained with protoplast concentrations ranging between 5×10^7 /ml and 1×10^8 /ml. A wide variation in protoplast numbers was used in each of my transformation attempts, ranging from 1.6×10^7 to 1.5×10^8 in the last three experiments alone. It seems as if protoplast numbers do have a transformation efficiency effect with *D. pini*. Transformation 11 had 3-fold more protoplasts in the transformation mix than transformation 10, and has an average of 2-fold more transformants. Hopefully with a standardised protoplast isolation and transformation procedure, and with using a more consistent weight of mycelium to digest for protoplasts, protoplast numbers will become more uniform.

A factor which should be more precisely timed is the incorporation of the antibiotic (in the form of an agar overlay) in the regeneration medium. I determined a length of time (about 24 hrs) before the addition of the antibiotic was necessary, otherwise low transformation numbers resulted (Section 3.2). Upchurch *et al.*, (1994) also found it essential to allow the protoplasts to incubate in the regeneration medium for 12 to 18 hr prior to the addition of the selective overlay. They found this timing important as they also noticed virtually no transformants were recovered when the selective agent was incorporated directly into the agar overlay. In addition they discovered that if protoplasts were incubated for more than 24hr prior to the addition of the selective agent, a high background level of abortive growth occurred, making identification of true transformants difficult. A shorter regeneration time before addition of selection is well worth investigating with *D. pini*, as a high level of background abortives did in fact occur, especially in transformations 10 and 11.

A final condition which may influence the regeneration of the transformants is the choice of osmotic stabiliser used in the selective media plates. I have been using 0.8M sucrose in DM for protoplast regeneration although 1.2M sucrose was also trialed initially in Section 3.2. Another osmotic stabiliser should be tested as it may yield a higher regeneration frequency. This was found when 1.0 to 1.2M sorbitol gave two to four times higher transformation frequencies than 1.2M sucrose (Penttila *et al.*, 1987).

4.5 Molecular analysis of transformants

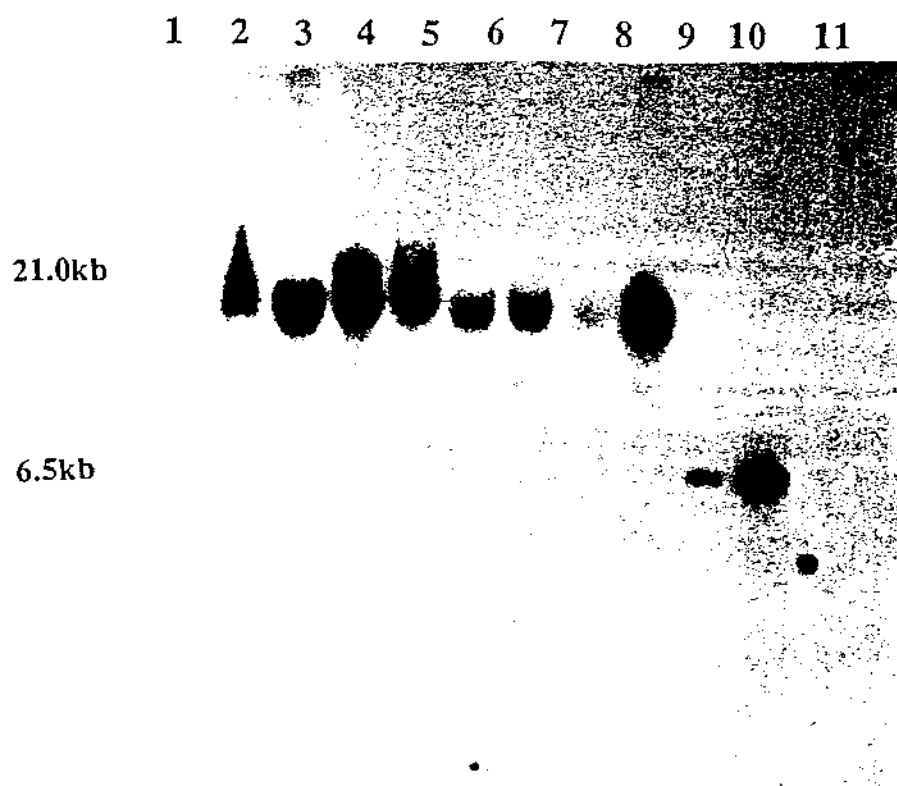
To ensure that the putative hygromycin resistant transformants contain plasmid DNA, genomic DNA was isolated (Section 2.5.3) from eight of them (AB1-8) as well as from untransformed *D. pini*. All of the isolates had undergone several rounds of subculturing (Section 2.13) before being grown on DM without antibiotic selection for DNA isolation. Genomic DNA from the transformants and untransformed control were all digested (Section 2.9.1.2) with *Hind*III which cuts once in pAN7-1 and *Eco*RV for which there are no recognition sites in pAN7-1 (Appendix 1), or left undigested. Southern blots of these digests and the undigested DNA were performed and probed with [α - 32 P]dCTP labelled pAN7-1 4.2kb *Eco*RI fragment (Section 2.14 and Appendix I). Hybridisation of these blots revealed differences in both the size and number of fragments hybridising as well as the intensity of the signal (Figs. 4A, 4B and 4C).

No hybridisation was detected between the pAN7-1 probe and the untransformed *D. pini* (Fig. 4A, B and C, lane 1) indicating there is no substantial homology between the vector and genomic sequences. The high stability of the HmB resistance phenotype seen during subculturing of the transformants suggested that the transforming vector was integrated into the genomic DNA (Wnendt *et al.* 1990). Further support of this was given by Southern analysis of the undigested transformant DNA, as the probe hybridised only to high molecular weight DNA (>23kb), suggesting that the vector had integrated into the genome and did not replicate autonomously (Fig. 4A). The intensity of the major band differed between transformants, even though approximately the same amount of DNA was loaded in each case.

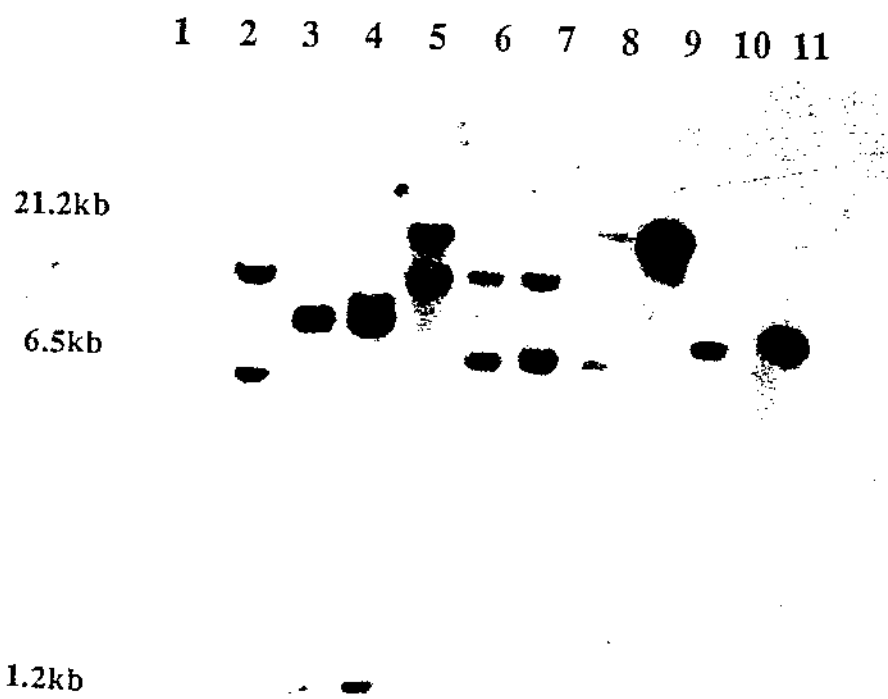
Figure 4. Hybridisation of pAN7-1 to transformants AB1 to AB8

Genomic DNA (lane 1), Transformants AB1 to AB8 (lanes 2-9), 10pg of pAN7-1 (lane 10), and 100pg of pAN7-1 (lane 11). **A:** Undigested DNA; **B:** *HindIII* digested; **C:** *EcoRV* digested.

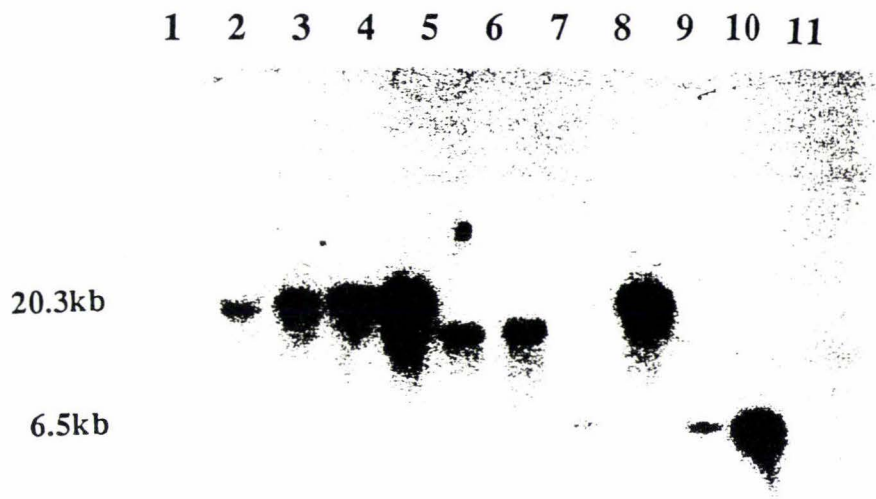
A



B



C



When using pAN7-1 to transform phytopathogenic fungi, people have found that the vector often integrates more than once, to form tandem repeats and/or multiple integration sites. Oliver *et al.* (1987) found when transforming *Fulvia fulva* (a fungal pathogen of tomato) to HmB resistance that copy numbers ranged from 1 to 10, integrating at either one or several sites per transformant. More recently, Hamada *et al.* (1994) found pAN7-1 integrated mainly in tandem repeats, either once or several times in the phytopathogen *Botrytis cinerea*.

The restriction enzyme *Hind*III cuts within pAN7-1 once and thus a single integration event will generate two fragments of homology to pAN7-1, the sizes of which will vary according to the position of plasmid integration (Fig. 4B). An additional band the size of the vector (6.5kb) indicates the presence of tandem repeats at site(s) of insertion. In the *D. pini* transformants tested no 6.5kb bands were seen in the *Hind*III digest (Fig. 4B), which indicates that the vector had integrated as a single copy rather than a tandem array in all transformants analysed.

Most of the transformants analysed showed a single integration site, for example transformants AB1, 4, 5, and 6 (Fig. 4B, lanes 2, 5, 6, and 7) with two bands being seen. Transformants AB5 and 6 have the same sized bands (14 and 6.4kb) which suggests the possibility of the same integration site. Transformant AB6 has slightly more intense bands than transformant AB5 which probably indicates a slight difference in the concentration loaded onto the gel. Transformants AB2 and 8 are seen as only one migrating band in lanes 3 and 9 (Fig. 4B). This may be due to the co-migration of the two bands or to integration of pAN7-1 at a site very close to the *Hind*III site so the border sequences, i.e., those regions at either end of the integrated plasmid DNA which contain both plasmid and chromosomal DNA sequences, are too small to be seen. Transformant AB7 also appears to have a single integration site when digested with *Hind*III as two migrating bands are seen (Fig. 4B, lane 8), but when digested with *Eco*RV two bands of about 18 and 6.9kb are seen (Fig. 4C, lane 8). This is probably due to a partial digest with *Eco*RV as more intense bands would be expected if it was due to co-migration of two or more of the bands. Another point to be noted about transformant AB7 is that although roughly the same amount of DNA was loaded from each transformant on the gel, and the intensity of the DNA looked the same on the gel (photo not shown), the bands hybridising from this transformant look a lot lighter. An explanation for the less intense hybridisation seen with transformant AB7 is that it may be a heterokaryon, with not all the nuclei being transformed.

Transformants AB7 and 8 are both from linearised pAN7-1. Linearisation has been shown to decrease the number of plasmid copies being transformed into the plasmid. As only single copies of the integrated DNA were seen in all the transformants analysed, linearising pAN7-1 before transformation in *D. pini* had no observable effect. Two integration sites are clearly visible with transformant AB3 (Fig. 4C, lane 4), but only one high molecular weight, >19kb band is observed with the *EcoRV* digest. This single signal however is very intense, suggesting that more than one fragment is migrating at this position in the gel.

As the transformants show different hybridisation patterns no targeting of the vector into a specific locus has occurred. This type of integration, when the DNA is introduced at random sites in the genome, was designated Type II integration by Hinnen *et al.* (1978) (Fig. 5B). This ectopic integration of tandem repeats and/or single copies of pAN7-1 has been observed in the transformation of many other filamentous fungi including *A. nidulans* and *Septoria nodorum* (Punt *et al.*, 1987 and Cooley *et al.*, 1988).

4.6 Further Transformant Analysis

Untransformed *D. pini* and transformants AB1 to 8 were plated on DM containing increasing concentrations of HmB. All of the inoculated mycelium grew on 0µg of HmB (Fig. 6, top left-hand plate), but different transformants were resistant to different concentrations of the antibiotic (Table 15 and Fig. 6). All of the transformants were resistant to at least 100µg of HmB/ml, a concentration which completely inhibited growth of the wild type. Transformant AB7 proved to be the most resistant to HmB still growing on the plate containing 800µg/ml. Southern blots (Figs. 4A-C) indicated a single copy of pAN7-1 has integrated in all the transformants analysed (except in transformant AB3 which had two integration sites) yet a range of HmB resistance was seen (200 to 800µg/ml). And although transformant AB3 had two copy numbers it had the lowest level of resistance to HmB (Table 16). Thus it appears that the number of copies of the plasmid does not influence resistance levels. This observation is supported by Mellon and Casselton, (1988) who found that increasing the gene copy number in *Coprinus cinereus* did not necessarily lead to increased enzyme activity. They discovered that the site of integration of the gene was important in determining whether or not it is expressed and to what level it is expressed.

Figure 5. Diagram of integration events as described by Hinnen *et al.* (1978)

A Type I: integration of plasmid into the fungal genome at a sequence complementary to the β -tubulin sequence carried by the plasmid. B Type II: integration of the DNA at a random site in the genome. C Type III: integration of the fungus DNA sequences of the plasmid into the genome by a double crossover event.

Key: Genes A, B, C.

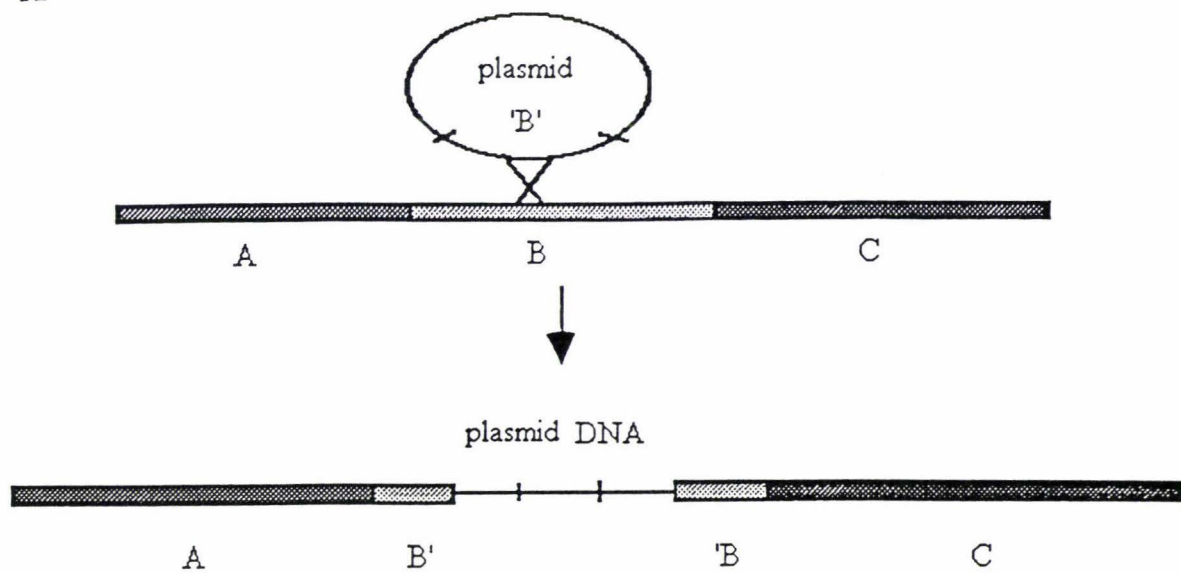
'B truncated at the 5' end.

B' truncated at the 3' end.

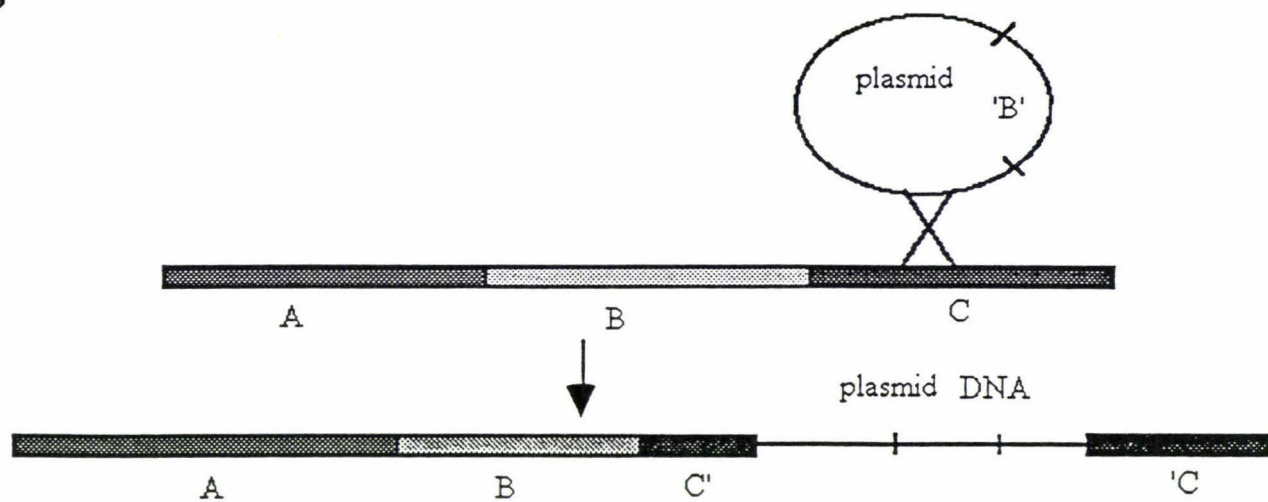
'B' truncated at both ends.

B mutant.

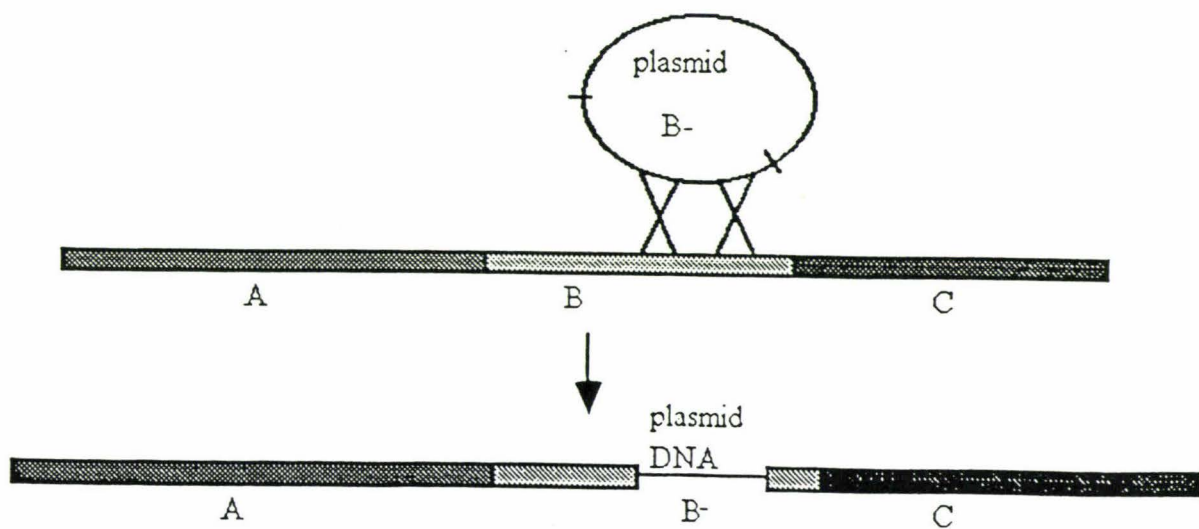
A



B



C



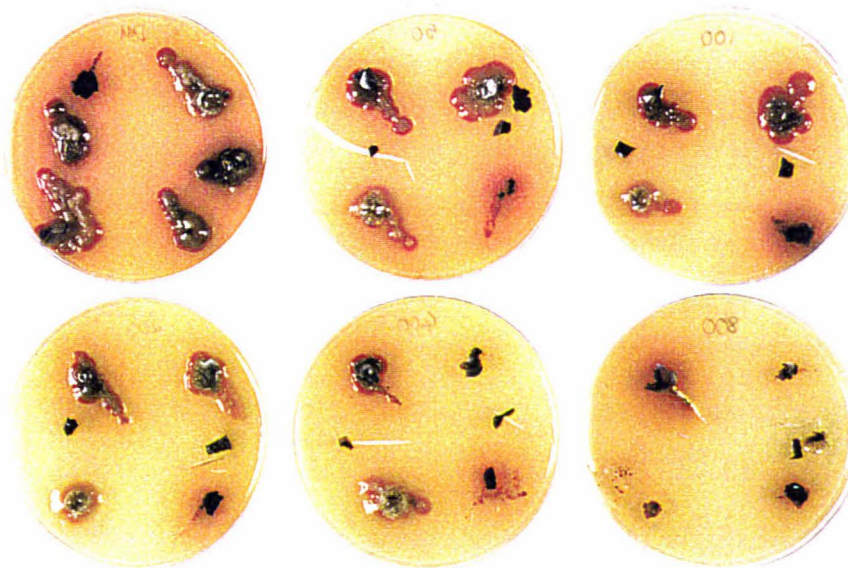


Figure 6. Sensitivity of wild-type *D. pini* and four independent transformants to various concentrations of hygromycin B.

Clockwise from the top left-hand colony: transformants AB2 and AB1, wild-type, transformant AB3 and AB4, and wild-type (see key). The concentration of HmB in $\mu\text{g/ml}$ in each plate clockwise from the top left is 0, 50, 100, 800, 400, and 200. Wild type *D. pini* was sensitive to all concentrations of the drug, whereas the transformants were all able to grow on 100 μg of HmB per ml. The transformants all showed different resistance levels, though all showed significant retardation at 800 μg HmB per ml.

Note: Only transformants AB1 to AB4 are shown. Transformants AB5 to AB8 showed similar patterns (see Table 16).

Table 15. Resistance levels shown by transformants AB1 to 8 when plated on increasing concentrations of hygromycin B

Trans- formant ^a	Hygromycin B concentration ($\mu\text{g/ml}$)					
	0	50	100	200	400	800
Control ^b	+	-	-	-	-	-
AB1	+	+	+	+	-	-
AB2	+	+	+	+	+	(+)
AB3	+	+	+	(+)	(+)	-
AB4	+	+	+	+	+	-
AB5	+	+	+	+	(+)	(+)
AB6	+	+	+	+	(+)	(+)
AB7	+	+	+	+	+	+
AB8	+	+	+	+	+	-

a. *D. pini* transformed with pAN7-1.

b. Untransformed *D. pini* (Dp-1).

Key: + growth and toxin formation
 (+) reduced growth and toxin formation
 - no growth or toxin formation

The PEG treatment used in transformation protocols causes fusion of protoplasts, with the consequence that transformants could be heterokaryons, containing a mixture of transformed and untransformed nuclei. This was investigated by harvesting *D. pini* spores from two of the transformants (AB4 and AB5) by overlaying mycelium grown on *D. pini* sporulation media (DSM) with about 500 μ l of water, and scraping gently with a glass rod before collecting and counting the spores. After plating 100 spores on DM they were subcultured onto DM and DM + HmB. All the spores grew on both non-selective media and selective media indicating that they were all resistant transformants. This suggests that the transformants contain predominantly transformed nuclei, although all transformants including AB7 were not tested this way.

5. Developing a Homologous Transformation System for *Dothistroma pini*

Transformation of filamentous fungi with positively selectable vectors, which lack homology with the recipient genome, tends to yield rather low numbers of transformants, around 1-30 per microgram of DNA (Cooley *et al.* 1987; Wnendt *et al.* 1990; Hamada *et al.* 1994). Transformation frequencies may be limited by the ability of the vector DNA to integrate into the genome. This was suggested by Farman and Oliver (1992) when they discovered that transformation frequencies are enhanced and vector DNA is targeted during retransformation of *Leptosphaeria maculans*. They transformed *L. maculans* with pAN8-1, and then transformed protoplasts from a vigorous Phleo resistant transformant using the partially homologous vector, pAN7-1 which encodes HmB resistance. They discovered that transformation consistently occurred at frequencies 2-fold higher than with the wild-type. Molecular analysis of five transformants generated with pAN7-1 indicated that in four cases the pAN7-1 vector had integrated into pAN8-1 sequences.

Another factor that limits transformation frequencies may be poor expression of the selectable marker. The *A. nidulans* *gpdA* promoter, which is present in pAN7-1, may not function as effectively in other fungi. Recently many groups have described transformation systems based on the use of homologous promoters. Ridder and Osiewacz (1992) have increased the transformation frequency of *Podospora anserina* by using vectors which contain the HmB selectable marker under the regulation of the homologous *P. anserina* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene. Likewise Skatrud *et al.* (1986) reported improved transformation of *Cephalosporium acremonium* when using the homologous isopenicillin N synthetase (IPNS) gene as the promoter for a plasmid encoding for HmB resistance. It was thus decided that the β -tubulin gene, would be isolated from a *D. pini* genomic library, by heterologous hybridisation with the corresponding gene from *Neurospora crassa*. The *D. pini* β -tubulin gene would then be used as a homologous promoter to drive the expression of HmB resistance in the hope of increasing the transformation frequency of *D pini*.

With the use of homologous DNA in the transforming vector, three classes of transformants can be defined, according to Hinnen *et al.* (1978) and their work with with the *leu2* mutant strain of *S. cerevisiae*. Type II integration events, occur when DNA is introduced at random

sites in the genome as previously described in Section 4.5 (Fig. 5B). Type I integration involves the introduced sequence integrating at the resident site, along with the homologous sequence in the host genome. This type of integration has been attributed to homologous crossing over (Fig. 5A). The third type of integration, Type III or homologous replacement integration, is when the introduced sequence completely replaces the homologous resident sequence, without the integration of additional plasmid sequence. This type of integration has been attributed to a double crossover event or gene conversion (Fig. 5C).

In addition to its potential use in enhancing transformation frequencies, the β -tubulin gene will be used for other aspects of the overall *Dothistroma* program:

- a) as a constitutively expressed internal control for regulatory studies of toxin biosynthesis genes
- b) as the sequence data will be used in a molecular phylogenetic study to assess how closely related *D. pini* is to other ascomycete fungi.

Moreover, since the β -tubulin gene is the first gene to be cloned in *D. pini*, it will be of general interest to look at the codon usage and other features.

5.1 Analysis of Unsuccessful Clones λ BT1, BT2 and BT3

Three positive β -tubulin λ clones (BT1, BT2 and BT3) were previously isolated from a *D. pini* genomic library and DNA was isolated from them (S. Campbell, 1995). The library was prepared by C. Gillman (1994) using the lambda GEMTM-12 *Xho*I half-Site Arms Cloning System, in which genomic DNA (from Dp-1) was partially digested with *Mbo*I and ligated into the Lambda GEM-12 arms at the *Xho*I site.

5.1.1 Restriction Digestion of λ Clones and Southern Hybridisation

DNA from the 3 positive λ clones, along with *D. pini* genomic DNA, was digested (Section 2.9.1.1) with the restriction enzymes, *Bam*HI and *Xho*I, which release the insert from the lambda vector and cut the clone into several fragments. The fragments were then separated by gel electrophoresis on a 1% agarose gel (Section 2.9.2, Fig. 7). Both digests gave a range of fragment sizes (from less than 1 to 15kb). A southern blot of this gel was performed, and it was hybridised at 60°C to an [α -³²P]dCTP-labelled (Section 2.14) 3.1kb pBT6 *Hind*III fragment (containing part of the *N. crassa* β -tubulin gene; Appendix I). After hybridisation, the filter was washed 3 times with 3x SSC, 0.2% SDS at 60°C, and exposed to X-ray film for 48 hrs. This blot indicated that all three clones contained fragments which

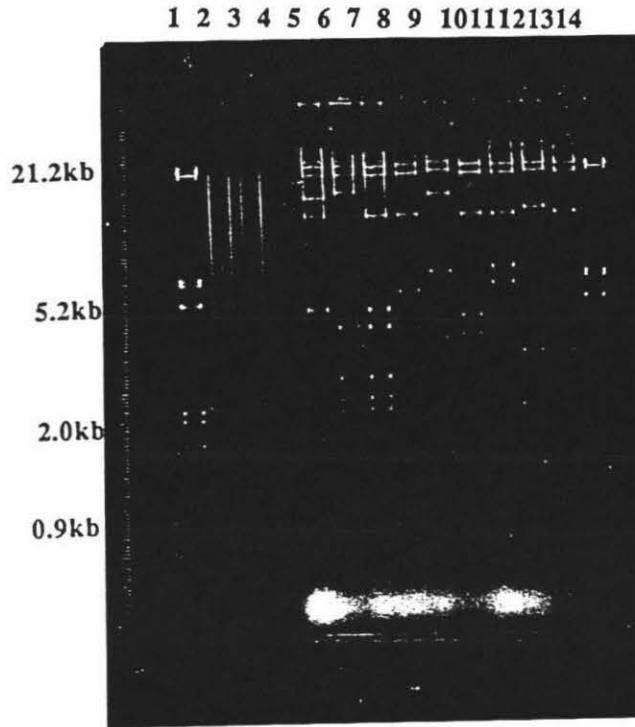


Figure 7. Restriction enzyme analysis of λ BT1, λ BT2 and λ BT3.

D. pini genomic DNA (lanes 2-4), λ BT1 (lanes 5-7), λ BT2 (lanes 8-10) and λ BT3 (lanes 11-13) digested with *Bam*HI, *Xho*I and *Bam*HI + *Xho*I flanked by λ *Eco*RI/*Hind*III ladder (lanes 1 and 14).

hybridised to pBT6. The *Bam*HI digest produced a commonly hybridising 2.9kb band which implied that all 3 λ clones are from the same genetic region (Fig.8A). This sized band was also seen in the genomic *Bam*HI digest. Further, more stringent, washing of the blot (60°C with 0.1x SSC and 0.2% SDS) caused basically all bands except the common 2.9kb, to wash off in the lanes containing the λ clones (Fig. 8B, lanes 5-13). Unfortunately this common 2.9kb hybridising band was not seen on the gel pictures of the *Bam*HI and *Bam*HI + *Xho*I digests of the λ clones with the exception of the λ BT3 *Bam*HI + *Xho*I digest (Fig. 7).

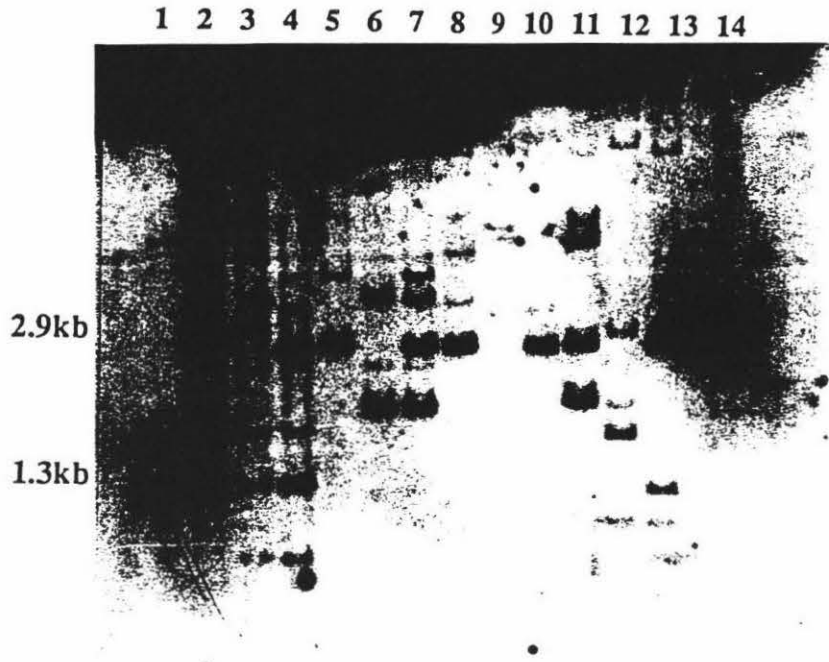
Due to the washing off of all the hybridising bands which did in fact correspond to bands from the digests, and the remaining stable band not corresponding to almost all bands obtained from the digests, the validity of the three λ clones was compromised. Also I had hoped to find a small fragment in the *D. pini* genomic DNA which corresponded to an adjacent fragment in the β -tubulin clones and which would be a convenient size for subcloning and sequencing. With this blot although small fragments were seen, 0.8 and 1.0kb on the *Bam*HI + *Xho*I lane of λ clone BT3, (Fig. 8A, lane 13), no corresponding band was observed in the genomic lanes. As a similar banding pattern was seen in all 3 clones, it was suspected that they were all from overlapping regions of the genome and so subsequent analysis involved only λ BT3.

Bearing the above points in mind another set of digestions on λ clone BT3 and a Southern blot were performed. The insert in λ BT3 and *D. pini* genomic DNA were digested with *Xba*I, *Kpn*I, *Hind*III and *Sma*I. These enzymes were chosen as they do not cut within the LambdaGEM-12 arms (see Appendix 1 for map). The digests were then electrophoresed through a 1% gel (Fig. 9) and information from them is summarised in Table 18?????. A Southern blot was hybridised to the 3.1kb *Hind*III fragment of pBT6. The filter was washed three times with 3x SSC and 0.2% SDS at 60°C and exposed to film, before being washed again with 0.1x SSC, 0.2% SDS at 60°C (Fig.10A and B). The results indicate a repeat of the first λ clones BT1-3 digests. Bands which were visible at a lower stringency wash (Fig. 10A) hybridised to bands seen on the digested DNA gel picture (Fig. 9), and were washed completely away when the stringency was increased (Fig. 10B). But bands which remained when the stringency of the washes was increased (Fig. 10B, lanes 2, 3, 4 and 5) did not correspond to the sizes of of λ BT3 bands seen on the gel photograph.

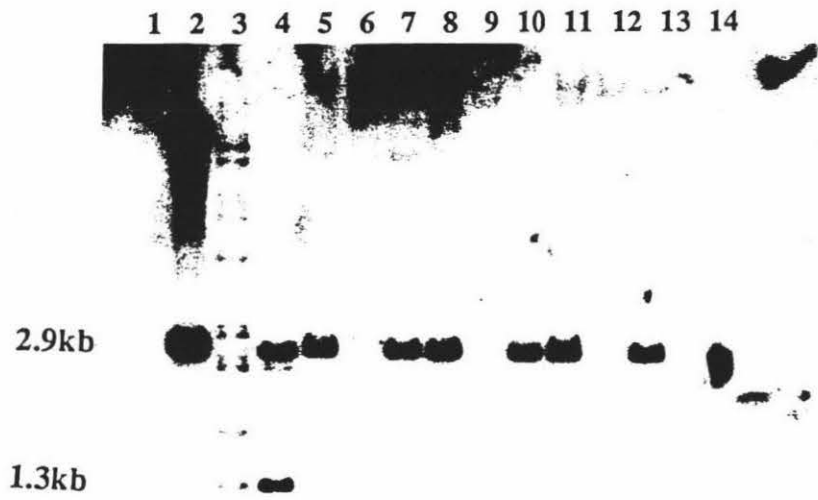
Figure 8. Hybridisation results of Southern blot of λ BT1, λ BT2 and λ BT3 washed at two different stringencies

Autoradiograph of the southern blot of the gel shown in Fig.7. *D. pini* genomic DNA (lanes 2-4), λ BT1 (lanes 5-7), λ BT2 (lanes 8-10) and λ BT3 (lanes 11-13) digested with *Bam*HI, *Xho*I and *Bam*HI + *Xho*I flanked by λ /*Eco*RI/*Hind*III ladder (lanes 1 and 14). A washed 3x SSC and 0.2% SDS at 60°C; B washed 0.1x SSC and 0.2% SDS at 60°C.

A



B



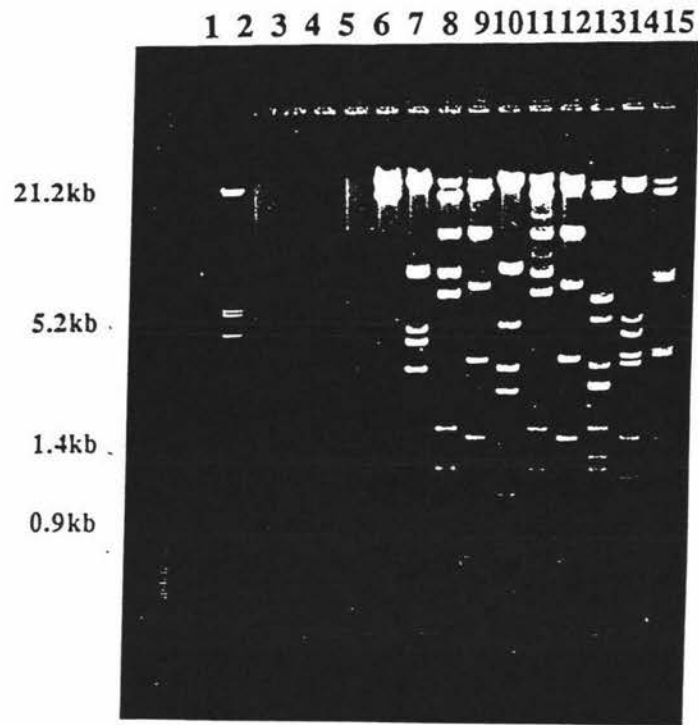


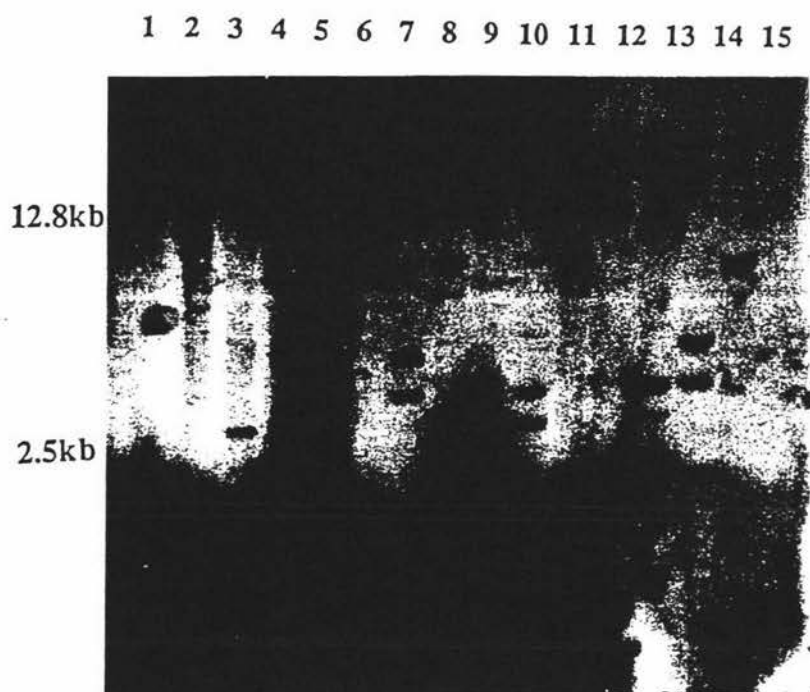
Figure 9. Restriction enzyme analysis of λ BT3.

λ EcoRI/HindIII ladder (lane 1). *D. pini* genomic DNA was digested with the enzymes *Xba*I (lane 2), *Hind*III (lane 3), *Kpn*I (lane 4) and *Sma*I (lane 5). λ clone BT3 was digested with the enzymes *Xba*I (lane 6), *Hind*III (lane 7), *Kpn*I (lane 8), *Sma*I (lane 9), *Xba*I + *Hind*III (lane 10), *Xba*I + *Kpn*I (lane 11), *Xba*I + *Sma*I (lane 12), *Hind*III + *Kpn*I (lane 13), *Hind*III + *Sma*I (lane 14) and *Kpn*I + *Sma*I (lane 15).

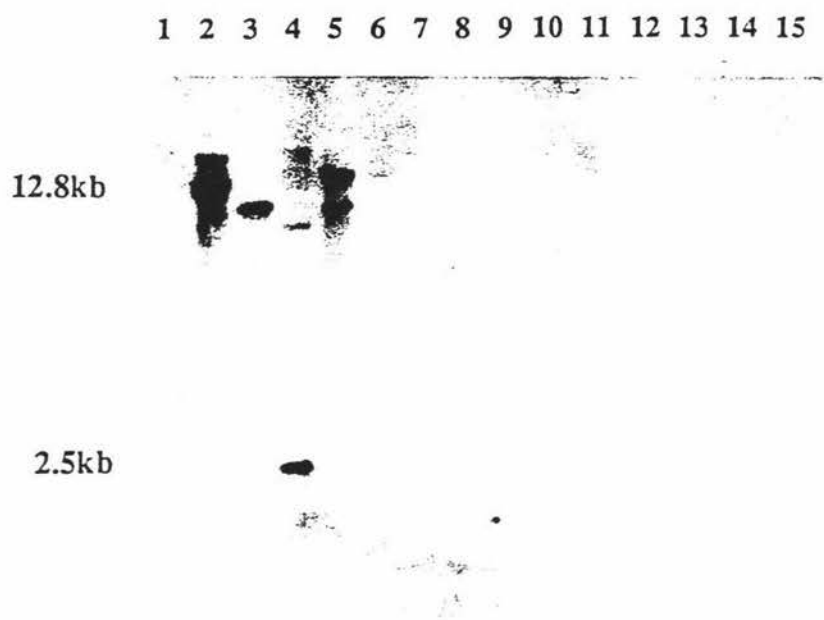
Figure 10. Hybridisation results from Southern blot of λ clone BT3 washed at two different stringencies.

Autoradiograph of the Southern blot of the gel shown in Fig.9. λ /EcoRI/HindIII ladder (lane 1). *D. pini* genomic DNA was digested with the enzymes *Xba*I (lane 2), *Hind*III (lane 3), *Kpn*I (lane 4) and *Sma*I (lane 5). λ clone BT3 was digested with the enzymes *Xba*I (lane 6), *Hind*III (lane 7), *Kpn*I (lane 8), *Sma*I (lane 9), *Xba*I + *Hind*III (lane 10), *Xba*I + *Kpn*I (lane 11), *Xba*I + *Sma*I (lane 12), *Hind*III + *Kpn*I (lane 13), *Hind*III + *Sma*I (lane 14) and *Kpn*I + *Sma*I (lane 15). **A** washed 3x SSC and 0.2% SDS at 60°C; **B** washed 0.1x SSC and 0.2% SDS at 60°C.

A



B



5.1.2 Computer Analysis of the λ Clones

Investigation was made into the results gained from probing these "positive" β -tubulin clones. My hypothesis to account for the high stringency bands (2.9kb *Bam*HI digest) was that the clones had been contaminated with some other DNA. *D. pini* genomic DNA was a possible source of contamination. This was implicated by the common occurrence of a 2.9kb band in genomic DNA and in all the λ clones digested with *Bam*HI, but no aligning band in the electrophoresed DNA gel picture. Alternatively contamination by the 3.1kb *N. crassa* probe was a possibility, as indicated by the closely matching sized (2.9kb) strongly hybridising band.

The hybridisation that was seen at a lower stringency may have been due to some cross-hybridisation between the lambda vector pGEM12 which the library was cloned into, and the plasmid pBT6, which was used in screening the library. Both the pUC12 vector and the *N. crassa* β -tubulin gene (making up pBT6) may cross-hybridise with lambda. The potential for cross-hybridisation between the lambda library and the plasmid pBT6 was investigated by comparing the various sequences using the BESTFIT program of the VAX GCG package.

Both strands of the *N. crassa* β -tubulin sequence (accession number M13630) were compared to the lambda EMBL3 left and right arms. The lambda EMBL3 vector sequence was used as lambdaGEM-12 vector is not on Genebank or other databases and is a derivative of EMBL3. The BESTFIT program indicated a high region of homology (73-81% over 23-40 nucleotides) between these two sequences, located within the first 750bp of the β -tubulin gene present in the pBT6 plasmid. From a map of the *N. crassa* β -tubulin sequence it was determined that by excising the β -tubulin gene from pBT6 at the *Eco*RI site (Appendix I) this region of high homology between lambda EMBL3 and the β -tubulin sequence could be avoided when probing the library and further Southern blots. By examining a comparison of β -tubulin sequences from six different organisms (Orbach *et al.*, 1986) it was determined that probing with the 1.6kb *Eco*RI only cuts off the region of the β -tubulin gene which is less highly conserved between the different organisms. Therefore by using the reduced 1.6kb β -tubulin gene any cross-hybridisation problems should be excluded and only true positive β -tubulin clones should be picked during library screening, while the reduced length of the probe should not effect the detection of positive signals.

To rule out the possibility of cross-hybridisation occurring between lambdaGEM-12 and pUC12, the BESTFIT program was run on these 2 sequences. pUC19 (accession number

M77789) was used in the place of pUC12, again due to the sequence not being held in any databases. A range of 65-76% identity over 28-61 nucleotides was detected between the two sequences, indicating a potential source of false positives when the *D. pini* genomic library was first screened. A gel picture of the 3.1kb *Hind*III pBT6 fragment which was used to probe the above Southern blots, indicated a faint residual 2.7kb pUC12 band. Any hybridisation between pUC12 and lambdaGEM-12 is likely to be weak, and therefore possibly could be the cause of the probe hybridising at a lower stringency and then washing straight off at a higher stringency. By using the 1.6kb *Eco*RI band of pBT6, any problem of pUC12 cross-hybridisation should also be avoided as the two bands (1.6 and 4.2kb) separate quite distinctly on a sea-plaque gel (Section 2.9.3) and the band of interest can easily be cut out and cleaned up with no contamination carry over from the other band (Section 2.6).

5.2 Analysis of Positive λ Clones AB1 to AB5

5.2.1 Library Screening

The *D. pini* genomic library was plated and plaque filters containing approximately 20000 pfu were screened with a [α - 32 P]dCTP labelled 1.6kb pBT6 *Eco*RI fragment (Section 2.15.3) at 65°C, 3x SSC and 0.2% SDS. The filters were washed (three washes at 20 mins each) in 1x SSC and 0.2% SDS at 65°C. After a more stringent wash (0.1x SSC, 0.2% SDS and 65°C) two hundred and eighty plaques were still hybridising to the probe, which is a higher number than expected with a first round of library screening (even with an amplified library). A BLAST search through the National Centre for Biological Information yielded no other sequences which hybridised to *N. crassa* β -tubulin with such high homology to cause so many intense signals on the autoradiograph. The filters were then stripped of the hybridising probe (Section 2.14.5) and were reprobed with the adjacent and more variant 1kb *Eco*RI, *Sal*I fragment of pBT6 at the previous conditions. After washing with 0.1x SSC and 0.2% SDS at 60°C and exposing after 24 hrs (Section 2.14.4), most of the positive signals, although fainter were still visible. The hybridising plaques were then all considered genuine positively hybridising plaques which potentially contained the β -tubulin gene.

Five positive plaques were picked and subjected to a second and third round of screening using the labelled 1.6kb pBT6 *Eco*RI fragment (Section 2.15.4). Washes on these screens were performed at 65°C using 0.1x SSC and 0.2% SDS. DNA was then isolated from the five positive plaques (Section 2.5.4).

5.2.2 Restriction Enzyme Analysis of λ Clones and Southern Hybridisation

DNA from the 5 positive λ clones was cut with the restriction enzymes *Bam*HI, *Eco*RI and *Xho*I which released the insert from the lambda vector. These enzymes were chosen as they do not cut within the lambda arms, therefore leaving them intact. They also cut the insert into a range of sizes (0.9 to 13kb) with plenty of smaller sizes suitable for subcloning and sequencing. The digested DNA was separated by agarose-gel electrophoresis on a 1.0% gel (Section 2.9.2, Fig. 11A-C). The results of these digests indicated that clones AB1, AB2 and AB3 were all from the same area of the genome or very closely overlapping as they had many common fragment sizes (Fig. 11A lanes 4-7; 11B lanes 5-8 and 11C lanes 2 and 4; Table 16). This result, along with the high proportion of positive signals in the primary screen, indicates that these clones were preferentially amplified in the library. Due to this common clone, Southern blots of these gels containing DNA from the λ clones AB1, AB2, AB3 and AB4 only, were hybridised to the 1.6kb pBT6 *Eco*RI (Section 2.14). These blots (washed at 65°C, 1.0x SSC and 0.2% SDS) confirmed that all four of these lambdaclones contained fragments which hybridised to this β -tubulin probe. Equally important was the fact that after a further more stringent wash (65°C, 0.1x SSC and 0.2% SDS), all the hybridising bands remained (Fig. 12A-C; Table 16). The blots also revealed that all four of the lambda clones are from overlapping regions of the genome as was indicated by the repeating hybridising pattern seen (Fig. 12A-C).

5.2.3 Mapping of λ Clone AB1

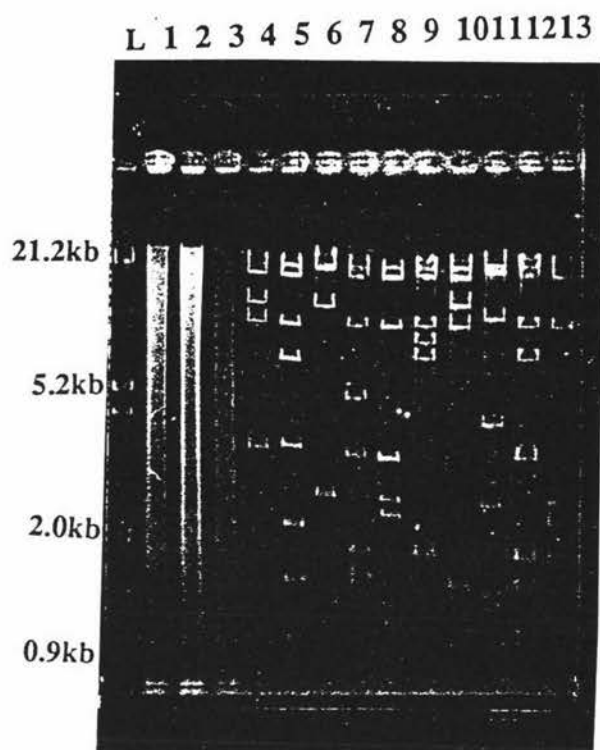
Due to all the lambda clones being from overlapping regions of the genome a restriction map was established for λ clone AB1 only. This was done using the results from the Southern blot of clone AB1 (Fig. 12A; Table 16) and a further Southern performed by Mr B. Monahan. This later Southern was a repeat of the λ clone AB1 digest with *Bam*HI, *Eco*RI and *Xho*I, and was executed because it was suspected that bands had migrated off the end of the previous agarose gel (Figs. 11A and 11C). Additional, smaller bands were seen on the agarose gel when repeated (not shown). And one extra 0.25kb band did hybridise in the *Eco*RI + *Xho*I lane when the Southern blot was probed with the 1.6kb *Eco*RI fragment of pBT6.

Both the Southern blots were stripped (Section 2.14.5) and reprobed with the hybridising 1.4kb *Eco*RI, 2.8kb *Xho*I, and the 5.8kb *Eco*RI/*Bam*HI fragments, by B. Monahan, giving

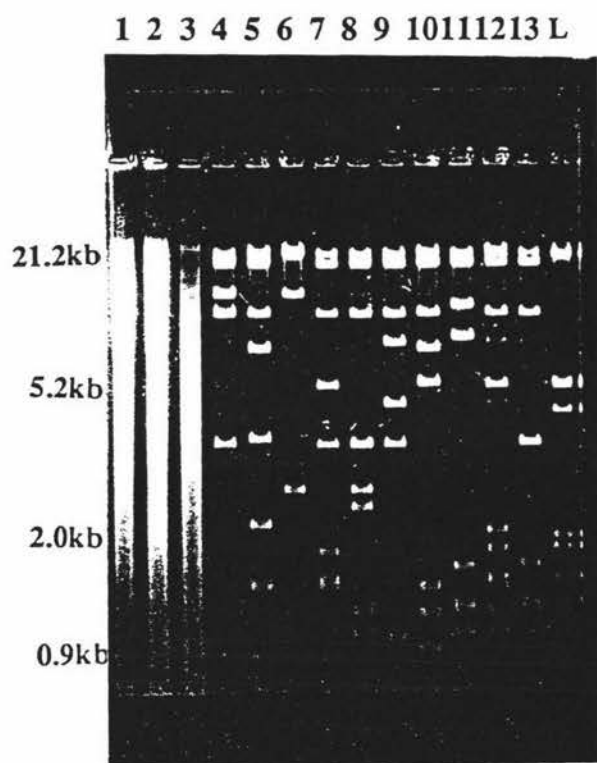
Figure 11. Restriction enzyme analysis of λ clones AB1, AB2, AB3 and AB4

A AB1 (lanes 4-8), AB2 (lanes 9-13) B AB3 (lanes 5-9), AB4 (lanes 10-14) digested left to right with enzymes: *Bam*HI, *Eco*RI, *Xho*I, *Bam*HI + *Eco*RI, *Bam*HI + *Xho*I. A (lanes 1-3) and B (lanes 2-4) *D. pini* genomic DNA digested with *Bam*HI, *Eco*RI and *Xho*I C AB1, AB2, AB3 and AB4 digested with *Eco*RI + *Xho*I. All digests are flanked by λ /*Eco*RI/*Hind*III ladder (L).

A



B



C

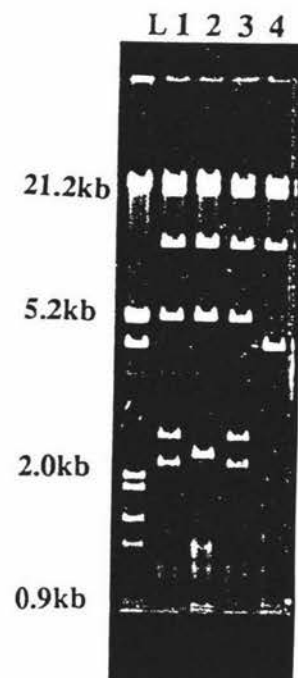
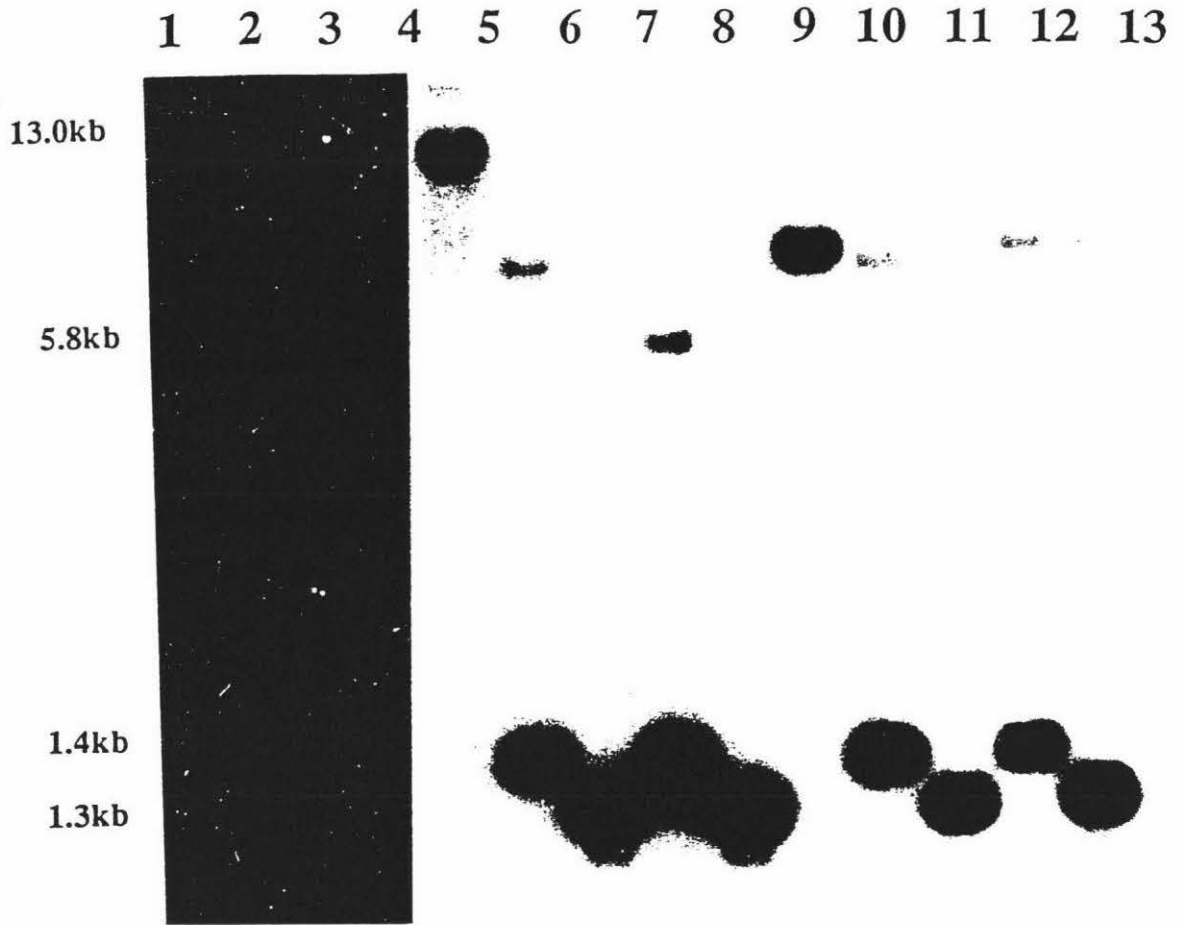
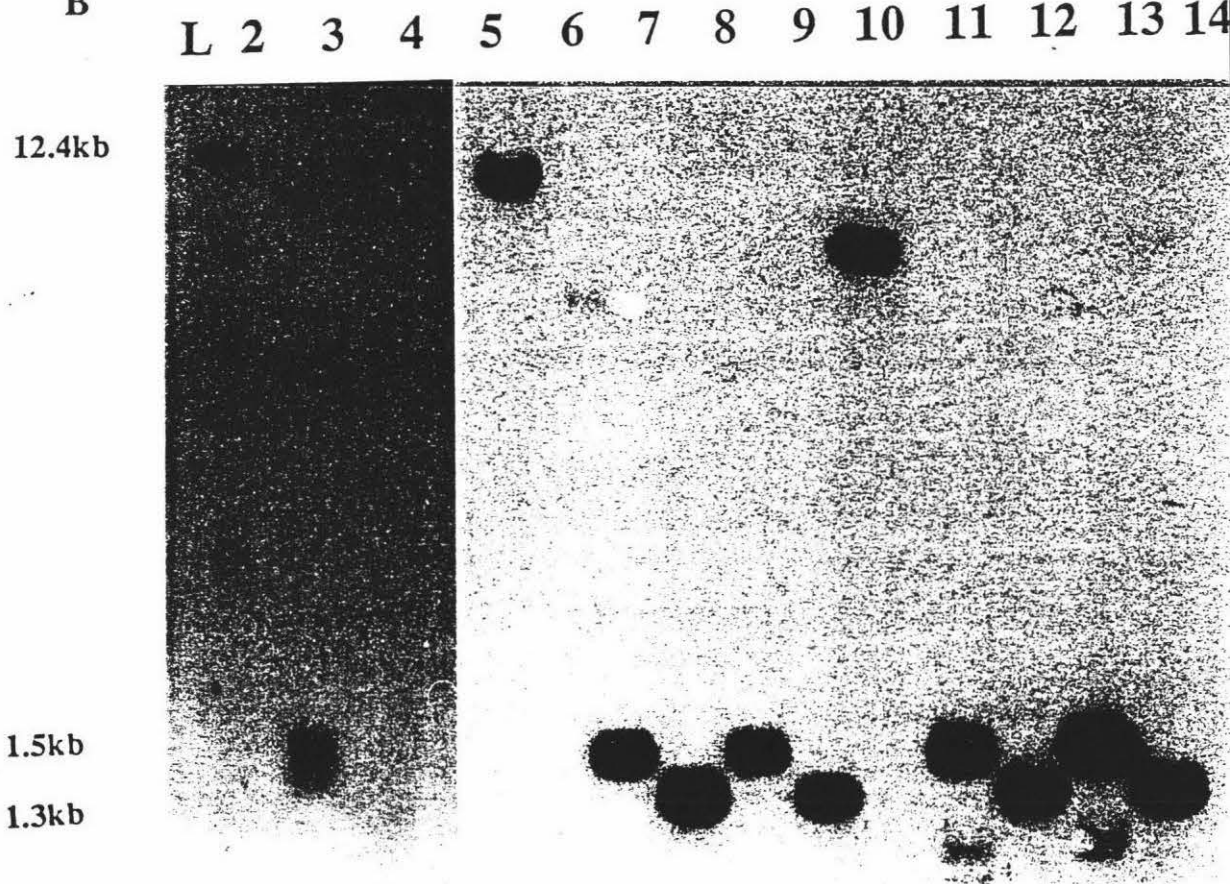


Figure 12. Autoradiograph of hybridisation results from blot of λ clones AB1, AB2, AB3 and AB4

A AB1 (lanes 4-8), AB2 (lanes 9-13) **B** AB3 (lanes 5-9), AB4 (lanes 10-14) digested left to right with enzymes: *Bam*HI, *Eco*RI, *Xho*I, *Bam*HI + *Eco*RI, *Bam*HI + *Xho*I. *D. pini* genomic DNA digested with *Bam*HI, *Eco*RI and *Xho*I (**A** lanes 1-3 and **B** lanes 2-4). **C** AB1, AB2, AB3 and AB4 digested with *Eco*RI + *Xho*I. All digests are flanked by λ *Eco*RI/*Hind*III ladder (L).



B



C

L 1 2 3 4

1.2kb



Table 16. Data from Restriction Enzyme Analysis and Southern Hybridisation of λ Clones from Figs. 11 and 12

λ Clone	Restriction enzyme	Fragment size (kb)
λ AB1	<i>Bam</i> HI	21.2, 19.0, 13.0*, 10.1, 3.4
	<i>Eco</i> RI	21.2, 19.0, 10.1, 6.9*, 3.5, 2.1, 1.4*, 0.9
	<i>Xho</i> I	21.2, 13.0, 2.7, 1.3*, 1.1
	<i>Bam</i> HI + <i>Eco</i> RI	21.2, 19.0, 10.1, 5.8*, 3.4, 1.8, 1.5, 1.4*, 0.9
	<i>Bam</i> HI + <i>Xho</i> I	19.0, 10.1*, 3.4, 2.5, 1.3*, 1.1
	<i>Eco</i> RI + <i>Xho</i> I	22.8, 18.6, 10.0, 5.2, 2.4, 2.2, 1.3, 1.2*, 1.1, 1.0
λ AB2	<i>Bam</i> HI	21.2, 19.0, 10.1, 7.5*, 4.4, 3.4, 1.1, ??
	<i>Eco</i> RI	21.2, 10.1, 6.9*, 5.0, 1.5*, 1.3, 1.0
	<i>Xho</i> I	21.2, 19.0, 11.1, 7.9, 1.6, 1.4, 1.3*, 1.1, 0.9
	<i>Bam</i> HI + <i>Eco</i> RI	21.2, 19.0, 10.1, 7.2*, 5.0*, 4.5, 3.4, 2.1, 1.8, 1.6, 1.5*, 1.3, 1.1, 1.0
	<i>Bam</i> HI + <i>Xho</i> I	21.2, 19.0, 10.1, 7.2*, 4.4, 3.4, 1.6, 1.4, 1.3*, 1.1, 0.9
	<i>Eco</i> RI + <i>Xho</i> I	18.6, 10.4, 5.2, 2.3, 1.4, 1.3, 1.2*, 1.0, 0.9
λ AB3	<i>Bam</i> HI	18.0, 12.4*, 10.0, 3.3
	<i>Eco</i> RI	17.1, 10.1, 6.6*, 3.4, 2.1, 1.5*, 0.9, 0.8
	<i>Xho</i> I	20.1, 18.0, 11.7, 2.4, 1.2*, 1.0
	<i>Bam</i> HI + <i>Eco</i> RI	16.2, 9.1, 4.6*, 3.0, 1.8, 1.4, 0.9
	<i>Bam</i> HI + <i>Xho</i> I	19.0, 16.2, 4.6*, 3.0, 1.8, 1.4*, 0.9
	<i>Eco</i> RI + <i>Xho</i> I	26.5, 22.8, 12.0, 5.7, 2.6, 2.5, 1.3, 1.2*, 1.1, 1.0
λ AB4	<i>Bam</i> HI	20.1, 18.0, 9.6, 7.9*, 6.6, 1.7
	<i>Eco</i> RI	19.0, 16.2, 11.7, 9.1, 1.5*, 1.0*, 0.9, 0.7
	<i>Xho</i> I	16.2, 9.6, 6.3, 3.9, 2.2, 1.3*, 1.2, 0.9
	<i>Bam</i> HI + <i>Eco</i> RI	20.1, 17.1, 9.1, 6.3, 3.0, 1.7, 1.5*, 1.0*, 0.9
	<i>Bam</i> HI + <i>Xho</i> I	16.2, 9.1, 2.4, 2.1, 1.7, 1.5, 1.4, 1.3, 1.0*, 0.9
	<i>Eco</i> RI + <i>Xho</i> I	26.5, 22.8, 11.5, 4.7, 1.5, 1.2*, 1.1, 1.0

* Indicates which bands hybridised to the 1.6kb pBT6 *Eco*RI probe in Fig. 12.

various hybridising bands. Analysis of these later Southern probings confirmed a restriction map of the λ clone AB1 (Fig. 13).

5.2.4 Subcloning and Sequence Analysis of λ Clone AB1

On the basis of the initial hybridisation results the 1.3kb *Xho*I fragment of λ Clone AB1 was chosen for subcloning and sequencing (Table 17). It was chosen because it hybridised to the *N. crassa* β -tubulin probe and was of a size suitable for sequencing.

The 1.3kb *Xho*I fragment was purified from a 1% sea-plaque agarose gel after electrophoresis (Section 2.9.3). It was then ligated into the *Sal*I site (compatible to *Xho*I ends) of pUC118 to create the plasmid pAB1 (Section 2.10). The identity of subclone pAB1 was confirmed by restriction enzyme digests (Section 2.9.1.1) followed by size fractionation by gel electrophoresis (Section 2.9.2).

DNA from pAB1 was extracted using the alkaline lysis method of preparation (Section 2.5.1). Initial sequencing was then carried out using the forward and reverse primers supplied with the AmpliCycle Sequencing Kit (Section 2.16; Table 3). From the sequence obtained more primers were designed (Table 3), until the entire 1316 nucleotide *Xho D. pini* fragment was sequenced (Fig. 14; Appendix 2) as outlined in Fig. 13B. By looking for intron consensus splice sites: 5' GTANGTY....YAG 3' (Ballance, 1986), the sequence was seen to contain two introns. Using the MAP program of the VAX GCG package a three frame translation of the nucleotide sequences was performed. One of these open reading frames showed very close similarity to the β -tubulin gene from *N. crassa*; out of 400 amino acids only 22 differed. The 1.3kb *Xho D. pini* sequence was directly compared with the β -tubulin nucleotide sequence from *N. crassa* using the GCG BESTFIT package (Appendix II). Even with two introns contained in the *D. pini* sequence a 84.6% similarity between the two sequences at the nucleotide level was obtained.

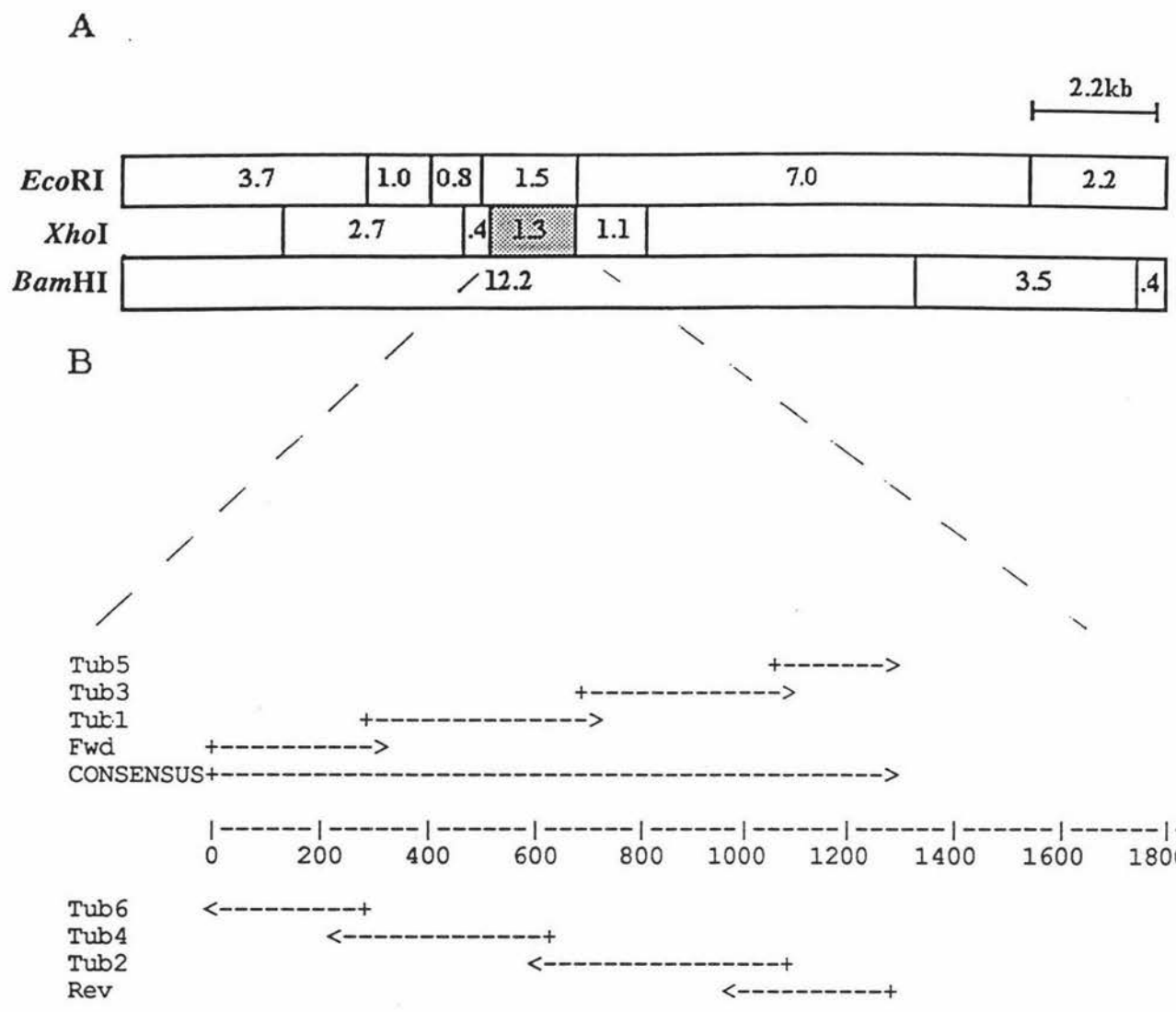


Fig. 13 Restriction map of the λ clone AB1 from a Dp-1 genomic library that hybridised to *Neurospora crassa* β -tublin gene.

A Restriction map of λ clone AB1. B Region of this clone which was sequenced showing the primer sites.

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1  C T C G A G C G C A T G A A T G T C T A C T T C A A C G A G g t a a t t c a c c g c c t g g c g c a a a a t c g c g c a   60
   L E R M N V Y F N E

61  t a a g g a c a g a a c a c t a a c a c c a c a c c c a g G C G T C C G G C A A C A A G T A T G T C C C T C G T G C C G   120
      A S G N K Y V P R A V

121 T C C T C G T C G A C T T G G A G C C A G G T A C C A T G G A T G C C G T C C G C G T G G T C C A T T C G G T C A G C   180
     L V D L E P G T M D A V R A G P F G Q L

181 T C T T C C G C C C A G A C A A C T T C G T C T T C G G T C A A T C C G G C G C C G G C A A C A A C T G G G C C A A G G   240
     F R P D N F V F G Q S G A G N N W A K G

241 G T C A C T A C A C T G A G G G T G C C G A G C T C G T C G A C C A G G T C C T C G A T G T C G T T C G C C G C G A A G   300
     H Y T E G A E L V D Q V L D V V R R E A

301 C T G A G G G C T G C G A C T G C C T T C A G G G A T T C C A G A T C A C C C A C T C C T T A G G G G T G G T A C T G   360
     E G C D C L Q G F Q I T H S L G G G T G

361 G T G C C G G T A T G G G T A C G C T C T T G A T T T C G A A G A T C C G T G A G G A G T T C C C A G A C C G C A T G A   420
     A G M G T L L I S K I R E E F P D R M M

421 T G G C C A C T T T C C C G T A T G C C A T C G C C A A A G G T C T C C G A C C C G T C G T T G A G C C A T A C A   480
     A T F S V M P S P K V S D T V V E P Y N

481 A T G C G A C C C T G T C C G T C C A C C A G C T T G T C G A G A A C T C G G A C G A G A C T T T C T G T A T C G A T A   540
     A T L S V H Q L V E N S D E T F C I D N

541 A C G A G G C G C T G T A C G A C A T T T G C A T G C G C A C C C T T A A G C T C A A C A C C C A T C A T A C G G C G   600
     E A L Y D I C M R T L K L N N P S Y G D

601 A C T T G A A C C A C C T C G T C C G C T G T G A T G T C C G G C G T C A C C A C T T G C C T G C G T T T C C C A G   660
     L N H L V S A V M S G V T T C L R F P G

661 G T C A G C T C A A C A G T G A T C T C C G C A A G T T G G C A G T C A A C A T G g t a a g t g a t t g a a a t c t a c   720
     Q L N S D L R K L A V N M

721 t t g g a t g c t t t t c g a a c a t c a t t a a c t t g t g a t a g G T G C C A T T T C C A C G T C T G C A C T T C T   780
      V P F P R L H F F

781 T C A T G G T C G G T T T C G C A C C A C T C A C C A G C C G T G G C G C A C A C T C C T T C C G T G C T G T C A C C G   840
     M V G F A P L T S R G A H S F R A V T V

841 T T C C C G A G C T C A C C C A G C A A A T C T T C G A C C C T A A G A A C A T G A T G G C C G T A G C G A C T T C C   900
     P E L T Q Q I F D P K N M M A A S D F R

901 G C A A C G G C C G T T A C C T C A C G T G C T C G G C T A T C T A T C G A G G A A A G G T C T C C A T G A A G G A G G   960
     N G R Y L T C S A I Y R G K V S M K E V

961 T C G A G G A C C A G A T C C G C A A C G T G C A G A A C A A G A A C A C T G C C T A T T T C G T C G A G T G G A T T C   1020
     E D Q I R N V Q N K N T A Y F V E W I P

1021 C A A A C A A C G T G C A G A C T G C C C T T T G C T C G A T C C C A C C A C G G G T C T C A A G A T G T C G T C T A   1080
     N N V Q T A L C S I P P R G L K M S S T

1081 C C T T C G T T G G C A A C A G C A C C T C C A T T C A G G A G C T G T T C A A G C G T G T C G G T G A C C A G T T C A   1140
     F V G N S T S I Q E L F K R V G D Q F T

1141 C T G C C A T G T T C A G G C G C A A G G C T T T C T T G C A T T G G T A C A C G G G C G A G G G T A T G G A C G A G A   1200
     A M F R R K A F L H W Y T G E G M D E M

1201 T G G A A T T C A C T G A G G C C G A G T C C A A C A T G A A C G A C C T T G T C T C G A G T A C C A G C A G T A C C   1260
     E F T E A E S N M N D L V S E Y Q Q Y Q

1261 A G G A G G C A T C C G T C T C C G A G G G T G A G G A G A G T A C G A C G A G G A G G C T C C A C T C G A G   1316
     E A S V S E G E E E Y D E E A P L E

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Figure 14. *Dothistroma pini* partial β -tubulin sequence (XhoI 1.3 kb fragment). Intron sequences are shown in lower case.

Chapter 6. Summary and Conclusions

The first step in developing an efficient transformation system for *D. pini*, the optimisation of protoplast isolation, has been successfully completed. Many factors affect protoplast isolation and most of these were investigated in this project. Experimentation to optimise the osmotic stabiliser indicated that 1.6M MgSO₄ gave the largest protoplasts in the highest numbers. Different pHs of 1.6M MgSO₄ were used until pH 5.8 was determined the most satisfactory. Two time factors were explored: the age of the mycelial culture before protoplast isolation along and the length of exposure time of the mycelium to the lytic enzyme. Young, rapidly growing six day old cultures of *D. pini* were found to give the maximum protoplast yields after 2.5 hrs incubation with the Novozyme solution at 37°C. With all of the above conditions optimised it seemed that, short of trying different lytic enzymes, developing the method to consistently obtain the maximum amount of protoplasts had been achieved.

After establishing the concentrations of the antibiotics HmB and Phleo which completely inhibit *D. pini* growth to be 50µg/ml and 6µg/ml respectively, the next task was to transform *D. pini* with the plasmids pAN7-1 and pAN8-1 which confer resistance to the two respective antibiotics. Various methods were trialed with the most successful involving harvesting the mycelia from DM broth cultures, and also separating the protoplasts from the majority of mycelial debris, through nappy liners. Protoplasts were subsequently harvested via the flotation method and incubated with 5µg of plasmid DNA for 20 min at 22°C before step-wise addition of PEG. 40% PEG 4000 which was osmotically stabilised (50mM CaCl₂, 50mM Tris-HCl and 1.0M sorbitol) caused the protoplasts to aggregate and become permeable to the DNA. Transformations were only performed using pAN7-1 after the first eight transformation attempts, as pAN8-1 showed an extremely poor transformation frequency. The DNA concentration, form (circular and linear), and purity all seemed to have little effect on the transformation efficiency of pAN7-1 in *D. pini*. The mean transformation frequency from the last three transformations using 8.4×10^6 protoplasts was 18 transformants/µg of DNA.

Molecular analysis of Southern blots of the uncut transformants indicated that they all contained stable copies of the *hph* gene as the vector had integrated into the genomic DNA. Examination of 8 transformants cut with *Hind*III (which has one site within pAN7-1), indicated that the plasmid had integrated in a single copy at different sites in all the

transformants. In one transformant (transformant AB3) two integration sites were seen while the remainder had only one site of integration. All 8 transformants analysed by molecular techniques were plated on DM containing increasing concentrations of HmB. All of the transformants were resistant to at least 100 μ g/ml of HmB with the untransformed control being sensitive to 50 μ g/ml of HmB. The fact that transformant AB3 (which had the highest copy number of pAN7-1) had the lowest resistance to HmB indicated that the number of copies of the plasmid does not affect resistance levels in *D. pini*. Propagation of two of the transformants through single spore analysis indicated that they were homokaryons. Though a low intensity signal on Southern blots of one of the transformants, transformant AB7, suggested heterozygosity.

In order to obtain a homologous promoter to drive the expression of the hygromycin resistance gene three putative β -tubulin clones had previously been isolated from a *D. pini* genomic library (S. Campbell, unpublished). Southern blots of these digested clones were hybridised to a 3.1kb *Hind*III fragment of the *N. crassa* β -tubulin gene but there was evidence of contamination either by genomic DNA or the 3.1kb *N. crassa* probe. An additional series of bands were also seen which were easily washed off at a higher stringency of washing. By using the BESTFIT program of the VAX GCG package it was verified that a high region of homology existed between the lambda vector sequences and the pBT6 plasmid containing the *N. crassa* β -tubulin gene and determined that the cross-hybridisation problems should be eliminated by using the shorter 1.6kb *Eco*RI β -tubulin fragment as a probe.

The *D. pini* genomic library was re-screened with the 1.6kb *Eco*RI β -tubulin fragment and five β -tubulin clones were isolated. Restriction enzyme and Southern analysis indicated that all these clones were from the same area of the genome or very closely overlapping areas as they had many common fragment sizes. Consequently only one λ clone (AB1) was examined any further. A 1.3kb *Xho*I fragment of this clone was mapped and subcloned into pUC118 ready for sequencing. Sequencing confirmed that the fragment was 1316 nucleotides in length and contained two introns. By using the GCG MAP program a three frame translation of the nucleotide sequence was produced, one of which was very similar to the β -tubulin gene from *N. crassa*.

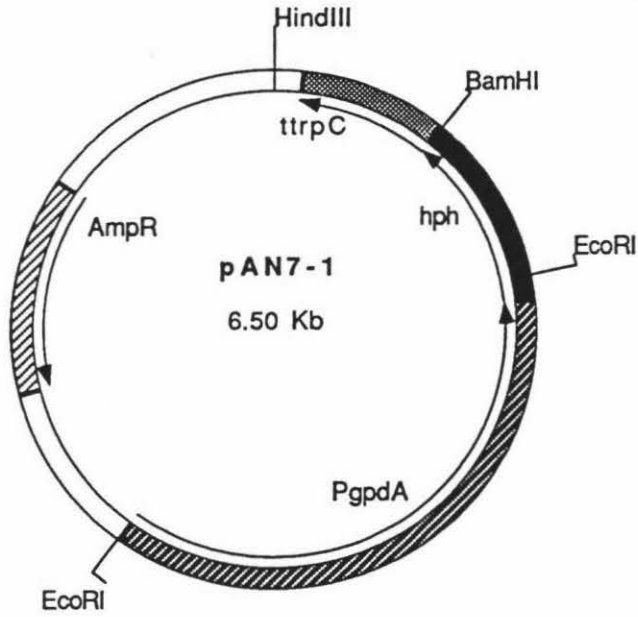
So to conclude, a transformation system was effectively developed in this thesis. Suggestions have been made as to how to increase the transformation frequency even more (Chapters 3 and 4). The *D. pini* β -tubulin gene has been cloned, sequenced and mapped so

to enable further work on determining the promoter region of this gene to proceed smoothly. Once the promoter region is determined it can be ligated with the hygromycin resistance gene to make a construct which will hopefully increase the expression of the selectable marker and subsequently increase the transformation of *D. pini*.

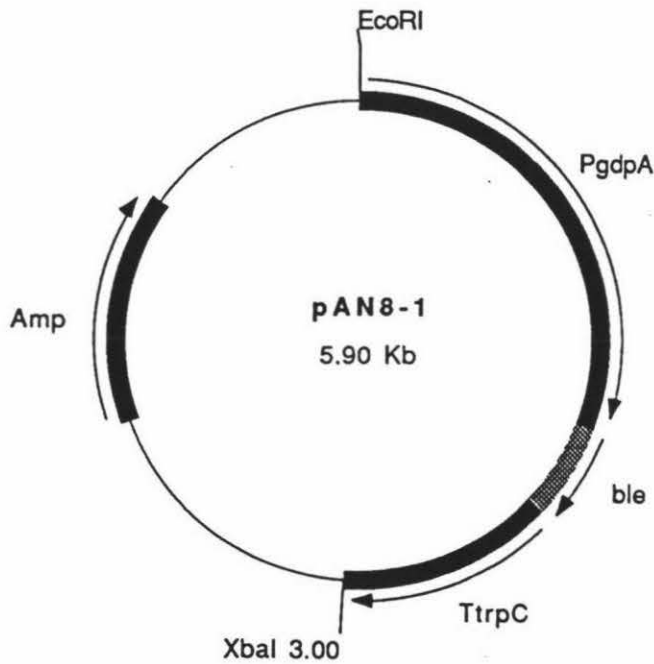
Appendices

Appendix I : Vector Maps

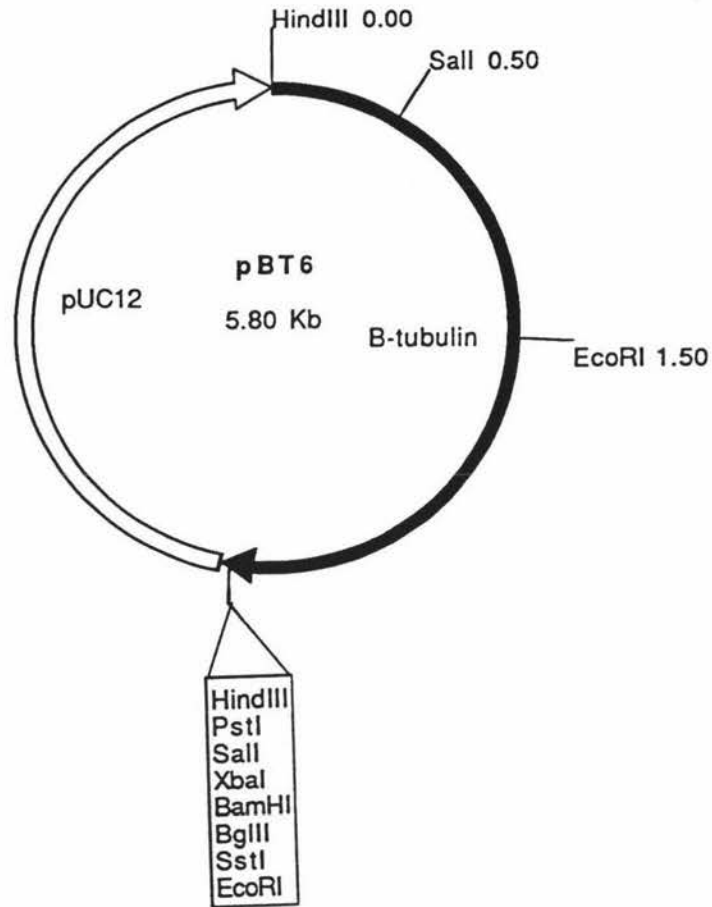
Restriction map of pAN7-1 showing *EcoRI*, *StuI*, *SalI*, *BamHI*, and *HindIII* restriction sites.



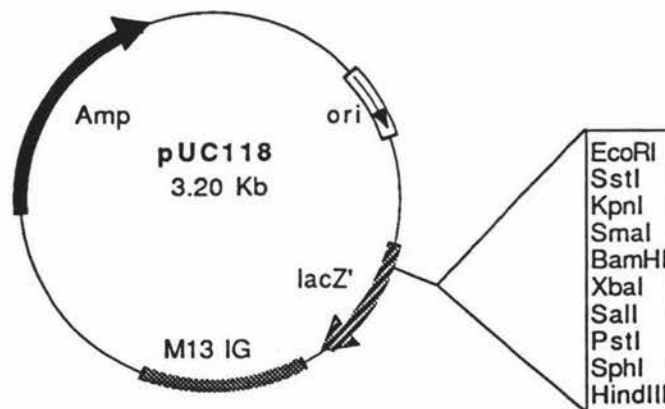
A restriction map of pAN8-1 showing *EcoRI*, *StuI*, *SalI*, and *XbaI* restriction sites.



A restriction map of pBT6 showing *Hind*III, *Sal*I, *Eco*RI, and the polycloning restriction sites.



A restriction map of pUC118 showing the polycloning restriction sites.



Appendix II : Sequence Data

PrettyOut of 1.3 *XhoI D. pini* β -tubulin Gene

From: 1 To: 1311

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Fwd      > GTCGAGCGCATGAATGTCTACTTCAACGAGGTAATTCACCGCCTGGCGCAAAATCGCGCA 60
Tub6     < GTCGAGCGCATGAATGTCTACTTCAACGAGGTAATTCACCGCCTGGCGCAAAATCGCGCA 60
CONSENSUS > GTCGAGCGCATGAATGTCTACTTCAACGAGGTAATTCACCGCCTGGCGCAAAATCGCGCA 60
          .....+.....+.....+.....+.....+.....+.....+

Fwd      > TAAGGACAGAACACTAACACCACACCCAGGCGTCCGGCAACAAGTATGTCCCTCGTGCCG 120
Tub6     < TAAGGACAGAACACTAACACCACACCCAGGCGTCCGGCAACAAGTATGTCCCTCGTGCCG 120
CONSENSUS > TAAGGACAGAACACTAACACCACACCCAGGCGTCCGGCAACAAGTATGTCCCTCGTGCCG 120
          .....+.....+.....+.....+.....+.....+.....+

Fwd      > TCCTCGTCGACTTGGAGCCAGGTACCATGGATGCCGTCCGCGCTGGTCCATTTCGGTCAGC 180
Tub6     < TCCTCGTCGACTTGGAGCCAGGTACCATGGATGCCGTCCGCGCTGGTCCATTTCGGTCAGC 180
CONSENSUS > TCCTCGTCGACTTGGAGCCAGGTACCATGGATGCCGTCCGCGCTGGTCCATTTCGGTCAGC 180
          .....+.....+.....+.....+.....+.....+.....+

Tub4     <                                     AAGG 4
Fwd      > TCTTCCGCCcAGACAACCTTCGTCTTCGGTCAATCCGGCGCCGGCAACAACCTGGGCCAAG 240
Tub6     < TCTTCCGCCcAGACAACCTTCGTCTTCGGTCAATCCGGCGCCGGCAACAACCTGGGCCAAG 240
CONSENSUS > TCTTCCGCCcAGACAACCTTCGTCTTCGGTCAATCCGGCGCCGGCAACAACCTGGGCCAAG 240
          .....+.....+.....+.....+.....+.....+.....+

Tub4     < GTCACTACACTGAGGGTGCCGAGCTCGTCGACCAGGTCCCTCGATGTCGTTTCGCCGCGAAG 64
Fwd      > GTCACTACACTGAGGGTGCCGAGCTCGTCGACCAGGTCCCTCGATGTCGTTTCGCCGCGAAG 300
Tub6     < GTCACTACACTGAGGGTGCCGAGCTCGTCGACCAGGTCCCTCGATGTCGTTTC          291
CONSENSUS > GTCACTACACTGAGGGTGCCGAGCTCGTCGACCAGGTCCCTCGATGTCGTTTCGCCGCGAAG 300
          .....+.....+.....+.....+.....+.....+.....+

Tub1     > TGAGGGCTGCGACTGCCTTCAGGGATTCCAGATCACCCACTCCTTAGGGGGTGGTACTG 59
Tub4     < CTGAGGGCTGCGACTGCCTTCAGGGATTCCAGATCACCCACTCCTTAGGGGGTGGTACTG 124
Fwd      > CTGAGGGCTGCGACTGCCTTCAGGGATTCCA          331
CONSENSUS > CTGAGGGCTGCGACTGCCTTCAGGGATTCCAGATCACCCACTCCTTAGGGGGTGGTACTG 360
          .....+.....+.....+.....+.....+.....+.....+

Tub1     > GTGCCGGTATGGGTACGCTCTTGATTTTCAAGATCCGTGAGGAGTTCCAGACCGCATGA 119
Tub4     < GTGCCGGTATGGGTACGCTCTTGATTTTCAAGATCCGTGAGGAGTTCCAGACCGCATGA 184
CONSENSUS > GTGCCGGTATGGGTACGCTCTTGATTTTCAAGATCCGTGAGGAGTTCCAGACCGCATGA 420
          .....+.....+.....+.....+.....+.....+.....+

Tub1     > TGGCCACTTTCTCCGTCATGCCATCGCCAAAGGTCTCCGACACCGTCGTTGAGCCATACA 179
Tub4     < TGGCCACTTTCTCCGTCATGCCATCGCCAAAGGTCTCCGACACCGTCGTTGAGCCATACA 244
CONSENSUS > TGGCCACTTTCTCCGTCATGCCATCGCCAAAGGTCTCCGACACCGTCGTTGAGCCATACA 480
          .....+.....+.....+.....+.....+.....+.....+

Tub1     > ATGCGACCCTGTCCGTCCACCAGCTTGTCGAGAAGTCCGACGAGACTTTCTGTATCGATA 239
Tub4     < ATGCGACCCTGTCCGTCCACCAGCTTGTCGAGAAGTCCGACGAGACTTTCTGTATCGATA 304
CONSENSUS > ATGCGACCCTGTCCGTCCACCAGCTTGTCGAGAAGTCCGACGAGACTTTCTGTATCGATA 540

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.....+......+......+......+......+......+.
Tub1      > ACGAGGCGCTGTACGACATTTGCATGCGCACCCCTTAAGCTCAACAACCCATCATAACGGCG 299
Tub4      < ACGAGGCGCTGTACGACATTTGCATGCGCACCCCTTAAGCTCAACAACCCATCATAACGGCG 364
CONSENSUS > ACGAGGCGCTGTACGACATTTGCATGCGCACCCCTTAAGCTCAACAACCCATCATAACGGCG 600

.....+......+......+......+......+......+.
Tub2      <                                     TGTGATGTCCGGCGTCACCACTTGCCTGCGTTTCCAG 38
Tub1      > ACTTGAACCACCTCGTCTCCGCTGTGATGTCCGGCGTCACCACTTGCCTGCGTTTCCAG 359
Tub4      < ACTTGAACCACCTCGTCTCCGCTGTGATGTCCGGCGTCACCACTT                       409
CONSENSUS > ACTTGAACCACCTCGTCTCCGCTGTGATGTCCGGCGTCACCACTTGCCTGCGTTTCCAG 660

.....+......+......+......+......+......+.
Tub3      >                                     AAGTGATTGAAATCTAC 17
Tub2      < GTCAGCTCAACAGTGATCTCCGCAAGTTGGCAGTCAACATGGTAAGTGATTGAAATCTAC 98
Tub1      > GTCAGCTCAACAGTGATCTCCGCAAGTTGGCAGTCAACATGGTAAGTGATTGAAATCTAC 419
CONSENSUS > GTCAGCTCAACAGTGATCTCCGCAAGTTGGCAGTCAACATGGTAAGTGATTGAAATCTAC 720

.....+......+......+......+......+......+.
Tub3      > TTGGATGCTTTTCGAACATCATTAACTTGTGATAGGTGCCATTTCCACGTCTGCACTTCT 77
Tub2      < TTGGATGCTTTTCGAACATCATTAACTTGTGATAGGTGCCATTTCCACGTCTGCACTTCT 158
Tub1      > T 420
CONSENSUS > TTGGATGCTTTTCGAACATCATTAACTTGTGATAGGTGCCATTTCCACGTCTGCACTTCT 780

.....+......+......+......+......+......+.
Tub3      > TCATGGTCGGTTTCGCACCACTCACCAGCCGTGGCGCACACTCCTTCCGTGCTGTCACCG 137
Tub2      < TCATGGTCGGTTTCGCACCACTCACCAGCCGTGGCGCACACTCCTTCCGTGCTGTCACCG 218
CONSENSUS > TCATGGTCGGTTTCGCACCACTCACCAGCCGTGGCGCACACTCCTTCCGTGCTGTCACCG 840

.....+......+......+......+......+......+.
Tub3      > TTCCCGAGCTCACCCAGCAAATCTTCGACCCTAAGAACATGATGGCCGCTAGCGACTTCC 197
Tub2      < TTCCCGAGCTCACCCAGCAAATCTTCGACCCTAAGAACATGATGGCCGCTAGCGACTTCC 278
CONSENSUS > TTCCCGAGCTCACCCAGCAAATCTTCGACCCTAAGAACATGATGGCCGCTAGCGACTTCC 900

.....+......+......+......+......+......+.
Tub3      > GCAACGGCCGTTACCTCACGTGCTCGGCTATCTATCGAGGAAAGGTCTCCATGAAGGAGG 257
Tub2      < GCAACGGCCGTTACCTCACGTGCTCGGCTATCTATCGAGGAAAGGTCTCCATGAAGGAGG 338
CONSENSUS > GCAACGGCCGTTACCTCACGTGCTCGGCTATCTATCGAGGAAAGGTCTCCATGAAGGAGG 960

.....+......+......+......+......+......+.
Rev       <                                     GAACACTGCCTATTTTCGTCGAGTGGATT 29
Tub3      > TCGAGGACCAGATCCGCAACGTGCAGAACAAGAACACTGCCTATTTTCGTCGAGTGGATT 317
Tub2      < TCGAGGACCAGATCCGCAACGTGCAGAACAAGAACACTGCCTATTTTCGTCGAGTGGATT 398
CONSENSUS > TCGAGGACCAGATCCGCAACGTGCAGAACAAGAACACTGCCTATTTTCGTCGAGTGGATT 1020

.....+......+......+......+......+......+.
Tub5      >                                     GTCGTCTA 8
Rev       < CAAACAACGTGCAGACTGCCCTTTGCTCGATCCCACCACGCGGTCTCAAGATGTCGTCTA 89
Tub3      > CAAACAACGTGCAGACTGCCCTTTGCTCGATCCCACCACGCGGTCTCAAGATGTCGTCTA 377

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Tub2      < CAAACAACGTGCAGACTGCCCTTTGCTCGATCCCACCACGCGGTCTCAAGATGTCGTCTA 458
CONSENSUS > CAAACAACGTGCAGACTGCCCTTTGCTCGATCCCACCACGCGGTCTCAAGATGTCGTCTA 1080
          .....+.....+.....+.....+.....+.....+.....+
Tub5      > CCTTCGTTGGCAACAGCACCTCCATT CAGGAGCTGTTCAAGCGTGTCCGGTGACCAGTTCA 68
Rev       < CCTTCGTTGGCAACAGCACCTCCATT CAGGAGCTGTTCAAGCGTGTCCGGTGACCAGTTCA 149
Tub3      > CCTTCG                                383
Tub2      < CCTTCG                                464
CONSENSUS > CCTTCGTTGGCAACAGCACCTCCATT CAGGAGCTGTTCAAGCGTGTCCGGTGACCAGTTCA 1140
          .....+.....+.....+.....+.....+.....+.....+
Tub5      > CTGCCATGTT CAGGCGCAAGGCTTTCTTGCATTGGTACACGGGCGAGGGTATGGACGAGA 128
Rev       < CTGCCATGTT CAGGCGCAAGGCTTTCTTGCATTGGTACACGGGCGAGGGTATGGACGAGA 209
CONSENSUS > CTGCCATGTT CAGGCGCAAGGCTTTCTTGCATTGGTACACGGGCGAGGGTATGGACGAGA 1200
          .....+.....+.....+.....+.....+.....+.....+
Tub5      > TGAATTCAC T GAGGCCGAGTCCAACATGAACGACCTTGTCTCCGAGTACCAGCAGTACC 188
Rev       < TGAATTCAC T GAGGCCGAGTCCAACATGAACGACCTTGTCTCCGAGTACCAGCAGTACC 269
CONSENSUS > TGAATTCAC T GAGGCCGAGTCCAACATGAACGACCTTGTCTCCGAGTACCAGCAGTACC 1260
          .....+.....+.....+.....+.....+.....+.....+
Tub5      > AGGAGGCATCCGTCTCCGAGGGTGAGGAGGAGTACGACGAGGAGGCTCCAC 239
Rev       < AGGAGGCATCCGTCTCCGAGGGTGAGGAGGAGTACGACGAGGAGGCTCCAC 320
CONSENSUS > AGGAGGCATCCGTCTCCGAGGGTGAGGAGGAGTACGACGAGGAGGCTCCAC 1311
          .....+.....+.....+.....+.....+.....+.....+

```

BESTFIT of 1.3 *XhoI* *D.pini* β -tubulin Gene with *N. crassa* β -tubulin Gene

Gap Weight: 5.000 Average Match: 1.000
 Length Weight: 0.300 Average Mismatch: -0.900

Quality: 825.2 Length: 1382
 Ratio: 0.630 Gaps: 5
 Percent Similarity: 84.622 Percent Identity: 84.622

Crassabtub.Seq x Tub.Con February 15, 1996 19:53 ..

```

936 tcgagcgcgatgaacgtctacttcaacgaggtgagcaacaaccggtctcga 985
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   2 TCGAGCGCATGAATGTCTACTTCAACGAGGTAATTCACCGCTGGCGCAA 51

986 cgatctccccctcctcgagaattcgctc.gctaactagcttcgcgcttga 1034
   ||| | || | || | || | || | || | || | || | || | || |
   52 .....AATCGCGCATAAGGACAGAACTAACCACAC...C 86

1035 caggcttccggcaacaagtatgtccctcgtgccgtcctcgatctcga 1084
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   87 CAGGCGTCCGGCAACAAGTATGTCCCTCGTGCCGTCCTCGTGC ACTTGA 136

1085 gcccggtagcatggacgccgttcgagccggtcccttcggccagctcttcc 1134
   ||| | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   137 GCCAGGTACCATGGATGCCGTCGCGCTGGTCCATTTCGGTCAGCTCTTCC 186

1135 gccccgataacttcgtcttcggccagtcggtgctggcaacaactgggcc 1184
   |||| | | ||||| ||||| || ||||| || ||||| || ||||| || |||||
   187 GCCCAGACA ACTTCGTCTTCGGTCAATCCGGCGCCGGCAACA ACTGGGCC 236

1185 aagggtcactacactgagggtgctgagcttgttgaccaggttctcgatgt 1234
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   237 AAGGGTCACTACTGAGGGTGCCGAGCTCGTCGACCAGGTCTCTCGATGT 286

1235 cgtccgtcgcgagggtgaggggtgcgactgcctccagggttccagatca 1284
   ||| | || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   287 CGTTCGCCGCGAAGCTGAGGGGTGCGACTGCCTTCAGGGATTCCAGATCA 336

1285 cccactccctcgggtggtggtaccggtgccggtatgggtaccctccttatc 1334
   ||||| || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   337 CCCACTCCTTAGGGGGTGGTACTGGTGCCGGTATGGGTACGCTCTTGATT 386

1335 tccaagattcgtgaggagtccccgaccgatgatgggtacctactccgt 1384
   || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
   387 TCGAAGATCCGTGAGGAGTTCAGACCGCATGATGGCCACTTTCTCCGT 436

1385 cgtgccctcccccaaggtctccgataccggttgtcgagccctacaacgcca 1434
   | |||| | | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
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1435 ccctctcgtccatcagctcgttgagaactccgacgagaccttctgcatt 1484
   |||| | ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
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1485 gacaacgagggcgtttacgacatttgcatgaggacctcaagctctccaa 1534
   || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||

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1535 cccctcttacggcgatcttaaccacctcgtctccgcccgtcatgtccgggtg 1584
||| || ||||| | ||||| ||||| ||||| ||||| ||||| |
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|||| | ||| ||||| || ||||| ||| ||||| |||||
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| || |||||
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1681 tcggggtcgtcccccttaccagccgcccggcggccaccatttccgtgccgtc 1730
|||| ||||| || || ||||| ||||| ||| ||||| |||||
787 TCGGTTTCGACCACCTCACCAGCCGTGGCGCACACTCCTTCCGTGCTGTC 836
1731 tcctgcccagagttgaccagcagatggttcgacccaagaacatgatggc 1780
|||| ||||| | ||||| || ||||| ||||| ||||| |||||
837 ACCGTTCCCGAGCTCACCCAGCAAATCTTCGACCCTAAGAACATGATGGC 886
1781 tgcttctgacttccgcaacgggctggttacctcacctgctctgccatcttgt 1830
||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
887 CGCTAGCGACTTCCGCAACGGCCGTTACCTCACGTGCTCGGCTATCT... 933
1881 aacagccgtggcaagggtctccatgaaggagggtgaggaccagatgcgcaa 1930
|| || ||||| ||||| ||||| ||||| ||||| ||||| |||||
934ATCGAGGAAAGGTCTCCATGAAGGAGGTCGAGGACCAGATCCGCAA 979
1931 cgttcagaacaagaactcttctacttctcgtcgagtggatccccaacaacg 1980
||| ||||| ||||| || ||||| ||||| ||||| ||||| |||||
980 CGTGCAGAACAAAGAACTGCCTATTTTCGTCGAGTGGATTCCAAACAACG 1029
1981 tccagactgccctctgctctatccctccccgcggtctcaagatgtcctcc 2030
| ||||| ||||| ||||| ||||| || ||||| ||||| ||||| |||||
1030 TGCAGACTGCCCTTTGCTCGATCCCACCACGGTCTCAAGATGTGCTCT 1079
2031 accttcgtcggtaactccaccgcatccaggagctcttcaagcgtatcgg 2080
|||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
1080 ACCTTCGTTGGCAACAGCACCTCCATTCAGGAGCTGTTCAAGCGTGTCGG 1129
2081 cgagcagttcactgccatggtcaagcgcgaaggcttcttgcattggtaca 2130
|| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
1130 TGACCAGTTCCTACTGCCATGTTCCAGGCGCAAGGCTTTCTTGCAATTGGTACA 1179
2131 ctggtgagggtatggacgagatggagttcactgaggctgagtccaacatg 2180
| || ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
1180 CGGGCGAGGGTATGGACGAGATGGAATTCCTGAGGCCGAGTCCAACATG 1229

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2181 aacgatctcgtctccgagtaccagcagtaccaggatgctggtggtgacga 2230
      ||||| || |||||||||||||||||||||||||||||||| || || |||
1230 AACGACCTTGCTCCGAGTACCAGCAGTACCAGGAGGCATCCGTCTCCGA 1279

      .
2231 ggaggaggaggagtacgaggaggaggccccc 2262
      || |||||||||||||| |||||||| || |
1280 GGGTGAGGAGGAGTACGACGAGGAGGCTCCAC 1311
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