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TRANSFORMATIONS OF GRAPEVINE PATHOGENS *EUTYPA LATA* AND *PHAEOMONIELLA CHLAMYDOSPORA*

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ABSTRACT

A transformation system has been developed for the grapevine pathogenic fungi *Eutypa lata* and *Phaeomoniella chlamydospora* using a positive selection system based on the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*). The system developed could give large, stable transformants at frequencies between 0.7 and 6.5 transformants per µg of DNA. A second type of colony also grew on the selective media. These were believed to be abortive transformants. The first type of transformants were characterized using classical molecular biological technologies such as PCR and Southern hybridization, and the transformation was shown to be successful.

Plasmids (pBCH-*gfp* and pCT74) containing a *gfp* reporter gene were also transformed into these two fungal species. Expression of the *gfp* gene was checked using a fluorescence microscope and *gfp*-expressing *E. lata* transformants were inoculated onto the host plants blackcurrant and grapevine. Confocal observation of the movement of fungal mycelia in wood tissues was performed but its interaction with host plant was not established in the time available. Purified *gfp*-expressing *P. chlamydospora* transformants were also obtained. A vector containing a fragment of the *P. chlamydospora* putative toxin gene *moxY* was constructed and transformed into *P. chlamydospora*. Putative *moxY* gene disruption transformants were screened with PCR followed by Southern hybridization. The putative *moxY* gene disruption transformants were spore purified and further confirmed with Southern hybridization. Whilst both PCR and Southern hybridization confirmed disruption of the *moxY* gene, clear evidence for the presence of an additional wild type *moxY* was also seen in the same transformants. This led to the suggestions that either *P. chlamydospora* is a natural diploid, or that *moxY* is essential for growth and that selective pressures led to the formation of a wild type: *moxY-hph* diploid.

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CHAPTER ONE INTRODUCTION

1.1 WINE INDUSTRY--A PROMISING INDUSTRY IN NEW ZEALAND

1.1.1 THE HISTORY OF WINE

According to the recorded wine history, it was the early inhabitants of Asia Minor (more than seven thousand years ago) who first discovered wine. Since that time, wine has been widely used as a safe and healthy beverage to provide calories and essential vitamins required by the body and to get stress relief in daily life (Amerine *et al.*, 1980).

Generally speaking, there are two categories of wine. White wines which result from rapid separation of the juice from the skins and seeds, and do not allow materials such as tannins to remain in the finished wine (Ough *et al.*, 1992), and red wines where the colors and flavors from the skins are allowed to be extracted into the juice (Surico *et al.*, 2000). White and red wines are also made from different grape varieties.

1.1.2 WINE INDUSTRY IN NEW ZEALAND

1.1.2.1 Wine history in New Zealand

Grapevines were first planted at Kerikeri in New Zealand in 1819. During the second half of the 19th century, only large quantities of cheap and low quality wine were produced (Cooper, 1984). With the encouragement of the removal of the Muller-Thurgau vineyard, and planting of varieties such as Chardonnay, Pinot noir and Merlot etc, New Zealand's reputation as a producer of premium wines has been gradually established (Cooper, 1984).

1.1.2.2 Recent development of vine industry in New Zealand

Recently, New Zealand has improved its wine technology greatly, and obtained a high reputation in the international wine industry, which has led to New Zealand wine exports in excess of NZD$100 million (Spense *et al.*, 1998) per annum. Recently, the vineyard

area rose to 8,716 hectares in 2000 (an increase of over 40% since 1993), to over 10,000 hectares in 2002 (Spense *et al.*, 1998), and to 15,479 hectares in 2003 with 42%, 26% and 13% of this area situated in Marlborough, Hawkes Bay and Gisborne, respectively (Anonymous *et al.*, 2003). Half the 55 million litres produced in 2003 were exported at a value of NZD$281 million. Sauvignon Blanc and Chardonnay are the two most commonly grown varieties (4344 ha and 3513 ha, respectively). As for red varieties, they are estimated to be planted in 2549 hectares. It is expected that by 2005, a further 2768 hectares will have been planted (Anonymous *et al.*, 2003).

New Zealand is relatively isolated from the world, but a variety of pathogens have been unconsciously introduced into New Zealand, which has a huge impact on this country in which agriculture and horticulture are of great importance. New strategies must be developed to maintain New Zealand's isolation from further grapevine pests and to research better management of existing pathogens such as *Eutypa lata* and *Phaeomoniella chlamydospora.*

1.2 REASONS WHY NEW ZEALAND CAN PRODUCE PREMIUM WINES WITH HIGH QUALITY

1.2.1 GEOGRAPHICAL REASON

New Zealand is a South Pacific island country with parts of the land uneven and hilly, and with alluvial clay loams in some areas. Thus soil types are generally quite fertile and very suitable for large scale growth of grass and grapevines (Jackson and Schuster, 1994).

1.2.2 CLIMATIC REASON

The average temperature of the main areas of wine-grape production in New Zealand is relatively low (less than 1390 degree days) and it is classed as a cool-climate region. The mechanism by which cool climates generally produce the best quality table wines still

remains elusive, but it is thought that the lower temperatures in autumn are of special significance (Jackson and Schuster, 1994). As warm climates shorten the time of ripening of grapes, and cause rapid development of sugars, rapid loss of acids, and high pHs, the direct consequence is that the juice is often unbalanced with respect to sugar, acid, and pH, and the time for accumulation of essential chemical compounds which add distinction to the wine is insufficient. However, a cool autumn can slow down this development process, thus the juice can be well balanced, and more aroma and flavor constituents are accumulated (Jackson and Schuster, 1994). A very good rainfall in these areas (650-1050 mm per annum) is another advantage for wine grape production (Jackson and Schuster, 1994).

1.3 GRAPEVINE PATHOGEN *E. LATA*

The vine pathogen *E. lata*, the fungus responsible for dying-arm disease in grapevines, and *P. chlamydospora*, the fungus responsible for Petri grapevine decline can both produce toxins. However toxin genes or genes involved in the toxin production have not yet been characterized. Previous studies on *E. lata* have demonstrated that eutypine, a phytotoxin produced by *E. lata*, can only be detected by GC-MS and MS-MS analyses in the crude sap and the inflorescences of diseased plants and can't be detected in healthy material (Tey-Rulh *et al.*, 1991), thus it has been deduced that eutypine is the toxin responsible for the symptoms of dying-arm disease. Further evidence supporting the above conclusion is from Deswarte, *et al.* (1994). Ultrastructural alterations induced by eutypine in leaf cells and protoplasts isolated from plantlets were observed by transmission electron microscopy. The eutypine-induced alterations of the cellular ultrastructure are similar to those previously described *in vivo* in the leaves of diseased grapevines (Deswarte *et al.*, 1996). However a more recently published paper expanded those investigations. A differential production of acetylenic phenol metabolites *in vitro* by three strains of *E. lata* was observed. This evidence suggests that eutypine is not solely responsible for phytotoxicity in grapevines but that dying-arm disease may result from a suite of compounds elaborated by the fungus (Molyneux *et al.*, 2002). With the development of a transformation system, it will be possible to make targeted gene

replacement mutants to determine if the genes are involved in toxin production and if the toxins are involved in the development of disease symptoms.

1.3.1 ROLE OF EUTYPINE

In order to gain a better understanding of the internal mechanism of *E. lata* infection on grapevines, Fallot *et al.,* (1989) successfully isolated an aldehyde compound, i.e. 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl aldehyde, a weak acid, later known as "eutypine", in the tissues of vines infected by *E. lata*. No such product has been detected in healthy vine wood. After colonization, *E. lata* interacts with host wood, and leads to the production of eutypine, which induces ultra-structural alterations in leaf cells and protoplasts. At the same time, foliage symptoms are often observed (Tey-Rulh *et al.*, 1991). Convincing evidence for a role of eutypine in the disease is the speed of symptom appearance. In addition their intensity was proportional to the eutypine concentration, which further confirmed the functions of eutypine on the disease development (Philippe *et al.*, 1992).

A further study performed *in vitro* indicated an accumulation of eutypine in the cytoplasm through passive diffusion due to an ion trapping mechanism, which lead to proton leakage and uncoupling of mitochondrial oxidative phosphorylation (Deswarte *et al.*, 1996). The direct consequence of this is the modification of the rate of respiration and the energy balance of the grapevine cells. Some genotypes of grapevines have the ability to detoxify eutypine, and therefore show some resistance to its infection (Fallot *et al.*, 1997). Recently, more study focused on derivatives of eutypine, such as eutypinol (4-hydroxy-3-(methyl-3-butene-1-ynyl), but only limited effects have been detected (Amborabe *et al.*, 2001). A gene encoding an aldehyde reductase, which is able to convert the toxic eutypine to its biologically inactive form (eutypinol), have been introduced into *Vitis vinifera* cells, and conferred resistance to the eutypine toxin after expression (Guillen *et al.*, 1998). It is quite possible that new varieties of grapevines could be tested and planted in the near future somewhere in the world.

1.3.2 FORMATION OF ASEXUAL AND SEXUAL STAGES

It usually takes several years for *E. lata* growing on the vines to develop perithecial stromata (Figure 1.1). These are black in colour, and stromatic tissues can be seen on the surface of dead wood. It is perithecial stromata from which ascospores are discharged (Jones, 2001). The anamorph of *E. lata* produces conidiomata, which often exude characteristic single-celled conidia. Based on a previous study performed by Munkvold *et al.*, (1993), it is unlikely that spread of *Eutypa* dieback is mainly caused by conidia as vine-to-vine spread of this disease in vineyards without perithecia was not detected. At present, information about the role of the asexual stage of *E. lata* in the disease cycle is still limited (Jones, 2001).

1.5 EPIDEMIOLOGY OF *E. LATA*

1.5.1 DISSEMINATION OF THE PATHOGEN

1.5.1.1 Production and local dispersal of ascospores

It is said that transfer of viable fragments of mycelium to healthy wood is unlikely to function successfully as inoculum unless they are incubated in a moist environment (Carter, 1991). Carter has demonstrated that when the stromata were dry, a minimum rainfall of about 2 mm sufficed to initiate the liberation of ascospores from the perithecia. Currently, rain is the only known means of release and dispersal of ascospores (Jones, 2001). As vineyards that contain perithecia of *E. lata* have consistently higher rates of disease incidence than those that do not (Hughes *et al.*, 1998; Munkvold *et al.*, 1993), this indicates that internal inoculum sources have a direct correlation with disease incidence. Carter (1965) also found that transport of inoculum of *E. lata* from perithecia to open vessels at pruning wounds during intermittent rainfall is a two-fold process, i.e. deposition from the air to the tree surfaces followed by redistribution of the deposited spores, during subsequent rain showers.

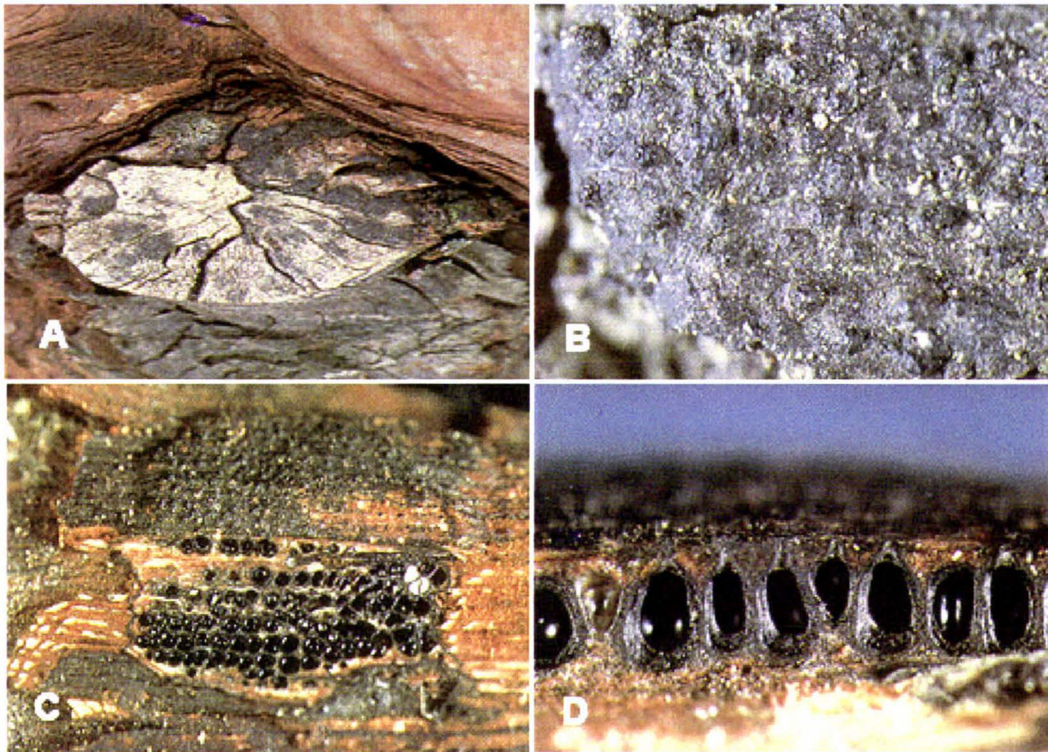

Figure 1.1 Perithecial stroma pictures adapted from Carter, (1991).
A: Perithecial stroma developing on the surface of an old pruning wound on the trunk of a grapevine; B: Close-up view of intact perithecial stroma; C: Stroma cut tangentially to reveal the locules of the perithecia; D: Vertical section through a stroma.

It seems that *E. lata* has evolved a very successful procedure for establishing its mycelium in the vessels of its hosts, and even a single ascospore is able to initiate infection (Ramos *et al.*, 1975).

1.5.1.2 Long range dispersal

Moller & Carter (1965) noted that winds that prevailed during times of rainfall would collect ascospores of *E. lata* liberated from stromata and carry them for long distances to reach the naturally arid areas, and this has been shown by Ramos *et al.*, (1975). This can partly explain the prevalence of *Eutypa* dieback disease in arid localities apparently devoid of perithecia.

1.5.2 ASCOSPORE SURVIVAL

Trese *et al.*, (1980) showed up to 65% germination after holding spores at –20°C for 14 days indicating that the ascospores of *E. lata* are able to survive prolonged periods of freezing after discharge from the perithecia (Carter, 1991). This is consistent with that performed in 1975 by Ramos, Moller & English. A seasonal pattern of ascospore production and release has been revealed in Australia (Moller and Carter, 1965) and in California (Ramos *et al.*, 1975).

1.5.3 PROCESS OF INFECTION

A very interesting phenomenon is the internal etiology of woody trunk diseases. The occurrence of the disease is sometimes attributed to one single or multiple causal agents. As Mugnai *et al.* (1999) noted "it is a complex disease whose symptoms arise from structural and physiological changes that cannot be reduced to a simple scheme of cause and effect". In terms of classic *Eutypa* dieback symptoms, a number of other fungi, including *Cephalosporium spp.* and *Botryosphaeria spp.* have been isolated in addition to the major causal agent, *E. lata* (Ferreira *et al.*, 1989).

As stated by Carter (1965), the transport of *E. lata* inoculum from the perithecia to the open ends of vessels exposed by pruning wounds during intermittent rainfall is a two-fold process. *E. lata* ascospores usually germinate within the vessels 2 mm or more beneath the wound surface (Moller and Kasimatis, 1978). Carter, (1991) determined the germination rate in vitro, i.e. 11-12 h at the optimal temperature of 20-25°C.

Newly made wounds are more likely to be infected, and with the extension of time, the opportunity for infection decreases gradually, and after four weeks the wounds are unlikely to become infected (Munkvold and Marois, 1995). The maximum duration of wound susceptibility is still not known, but wounds more than one year old are not likely to be infected (Moller and Kasimatis, 1980). In addition, the temperature is a strong factor affecting the susceptibility of pruning wounds. At a low temperature, infection of

the pruning wounds increased while the growth of other microorganisms is reduced (Munkvold and Marois, 1995; Carter, 1991; Chapuis *et al.*, 1998). The mechanism by which temperatures influence the susceptibility of wounds to infection is partly because it can exert an effect on the accumulation of suberin and lignin, which are common in a wound response reaction. Accumulation of suberin and ligin are linked to the decline in wound susceptibility during the first 28 days after pruning (Munkvold and Marois, 1995).

1.5.4 INTERNAL REASONS FOR INFECTION

When vines are "stressed" from factors such as grafting, water and nutrient deficiencies, severe frosting, overcropping etc, its defense system is unable to cope with the infection caused by a pathogen and unable to combat disease development (Scheck *et al.*, 1999). A typical example was from Ferreira *et al.*, (1999), who found that 70% of artificially inoculated vines exposed to water stress died, compared to 45% in unstressed vines.

1.5.5 SYMPTOM DEVELOPMENT OF *EUTYPA* DIEBACK

The progress of *E. lata* on vines is a chronic process, and usually lasts several years for mature vines until the complete collapse and death of the vines. The external symptoms on leaves and new season shoots are most evident. New shoots appear deformed and discolored. The young leaves are smaller than normal, cupped, chlorotic, and often develop small necrotic spots and tattered margins. Larger areas of necrosis could develop in time coupled with the dwarfing of the internodes (Jones, 2001). Philippe *et al.*, (1992) discovered internal physiological changes and structural alterations in leaf cells, using electron microscopy, they observed cytoplasmic lysis with plasma membrane detachment and complete chloroplast disorganization. On mildly affected shoots, leaves looks tattered on the first few nodes (sometimes with immature berries), and this is usually obscured from view by adjacent healthy growth (Jones, 2001).

The symptoms on foliage of diseased arms are increasingly extensive with the development of this disease, even the shoots could not be produced on the diseased arm

in the spring when normal shoots are usually developed. Trunks are developed with imbalance, i.e. one side of the vine dead while the other side appears healthy until the whole vine is completely dead (Jones, 2001).

Trunk cankers are often found inside the bark connecting to shoots bearing foliar symptoms, sometime with only a narrow strip of live wood observed through the canker. The cankers occurring in the trunk tend to penetrate towards the center of the arm or trunk with the presence of a wedge shaped zone of necrotic sapwood (Figure 1.2). Due to the chronic feature of the infection process, early stage treatment or prompt remedial surgery seems unlikely to be of value (Jones, 2001).

1.5.6 PATHOGENIC DIFFERENCES IN *E. LATA* ISOLATES

Cultivars of grapevines vary in susceptibility to *E. lata* infection, and some isolates of the pathogen do not cause the stem and foliar symptoms typical of the disease on grapevine cuttings (Munkvold and Marois, 1995). Peros *et al.*, 1997 demonstrated that 55 *E. lata* isolates, each collected from a different vine in a single vineyard showed a large variation in pathogenicity after testing in a greenhouse. In order to examine the internal differences, molecular markers such as isozymes and random amplified polymorphic DNA sequences (RAPDs) were tested. RAPDs appear to be more useful than isozymes to describe the genetic variation of the fungus as RAPD analyses identify more polymorphisms and the uniqueness of each isolate was confirmed (Peros *et al.*, 1999). However, the shortage of these markers limited the ability to group the isolates according to their pathogenicity (Jones, 2001). In addition, random mating between *E. lata* populations in two vineyards was also confirmed using RAPD (Peros and Larignon, 1997; Peros and Larignon, 1998). In 1999, DeScenzo *et al.* identified two clades of *Eutypa* species, i.e. *E. armeniacae and E. lata*, through Amplified Fragment Length Polymorphism (AFLP) analysis and sequence analysis of the Internal Transcribed Spacer

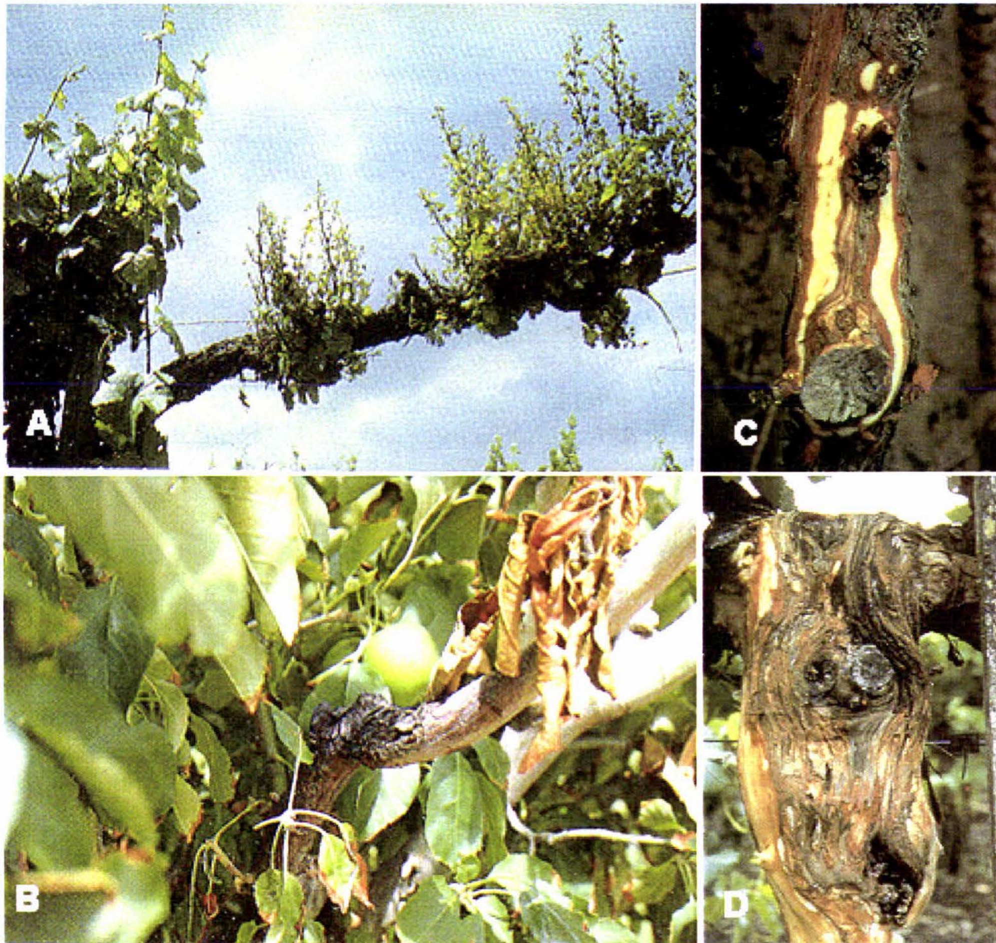

Figure 1.2 Symptoms of *Eutypa dieback* adapted from Carter, (1991).
A: acute foliage symptoms on grapevine; B: Typical symptoms of Eutypa dieback in apple; C: Longitudinal slice of an infected grapevine arm, showing invasion of sapwood below an old pruning wound; D: Grapevine trunk, showing an advanced canker centred on an old pruning wound.

(ITS) region of the ribosomal DNA, hence molecular markers have proven to be useful tools in evaluation the genetic systems and structures of *Eutypa* population (Jones, 2001). Serological identification of *E. lata* was investigated in the early 1970's, but the results of this method have not been promising (Jones, 2001).

1.5.7 SENSITIVITY OF GRAPE VARIETIES TO *E. LATA*

The issue of susceptibility of grape varieties to *E. lata* has been controversial, and there are no known grape cultivars immune to *E. lata* infection. However it is still possible that

the susceptibility of cultivars to *E. lata* could be variable depending on individual cultivars. Two years later, this point of view was challenged by the evidence observed in France, i.e. Cabernet Sauvignon was susceptible while Merlot was tolerant (Carter, 1991). This also was supported by a report from Hawkes Bay vineyards in New Zealand. The reason for argument is partly attributed to the word "susceptibility" itself, how serious the external or internal symptoms are defined as "infected" or "normal". There is no international standard for this at the moment. The conclusion made by Chapuis, *et al.*, 1998 is widely accepted at present: there are no differences in susceptibility to infection between Cabernet Sauvignon and Merlot cultivars, although their symptom expressions are markedly different. Some "infection symptoms", such as foliage, are just a kind of defense reaction of the plant cells toward fungal invasion, and it is inappropriate to put them into the category of infection.

1.6 EFFECT ON YIELD

Undoubtedly, *Eutypa* dieback has caused a significant reduction in the yield of infected grapevines, and has gradually become a major threat in the vine industry. The direct yield losses resulted from it have been estimated between 11% and 100% in Washington State, USA and Greece respectively according to the extent of severity (Johnson and Lunden, 1985). Yield reduction is primarily due to a diminished number of clusters per vine although its effect on mean cluster weight is not always significant because of the mechanism of compensation by producing more fruit on shoots that arise from the remaining healthy buds (Lider *et al.*, 1975). As the chronic characteristic of disease process, economic losses due to *Eutypa* dieback may be minor in early years, and its impact on yield thus increases with age. In addition to the direct losses, this disease can also exert an effect on vineyard longevity (Munkvold *et al.*, 1994), wine quality reduction, and the long-term loss of productivity (Jones, 2001).

Australian vineyards have gradually suffered more from *Eutypa* dieback, and this has seriously affected their sustainability (Pascoe and Cottral, 2000). In New Zealand, this kind of threat is increasing. Hence, it is urgent to have an in-depth study to gain a better understanding and to find an ideal method of treatment of this disease.

1.7 GRAPEVINE PATHOGEN *P. CHLAMYDOSPORA* AND PETRI DISEASE

1.7.1 THE HISTORICAL EVOLUTION OF THE NAME FOR *P. CHLAMYDOSPORA*

It was Lionello Petri who first discovered the characteristic symptom of Petri disease in 1912 (Chiarappa, 1999). For a long time, the causal agent for Petri disease remained elusive. *Cephalosporium spp* were initially regarded as the agent of Petri disease; however, this fungus has also been isolated from other trunk diseases such as esca (Chiarappa, 1959) and dieback (Ferreira *et al.*, 1989). Thus the direct relationship between Petri disease and its causal agent could not be established. Later, other fungi, similar but morphologically different that caused dieback symptoms, were isolated. From their morphology and genetic study, they were ascribed to a new genus *Phaeoacremonium*, and renamed as *P. chlamydosporum* and *P. aleophilum,* respectively (Crous *et al.*, 1996). *P. chlamydosporum* was unique in the *Phaeoacremonium* genus. For example, the mycelium is initially like yeast and darkens with age, it has chlamydospore-like cells and microsclerotia and unimorphic conidia. In addition, genetic analysis revealed it is unique in conservative gene sequences. Based on this, *P. chlamydosporum* was renamed as *Phaeomoniella chlamydospora*.

As the disease caused by *Phaeomoniella(Pm) chlamydospora* (Adalat *et al.*, 2000; Wallace *et al.*, 2003) has a wide range of names and different areas have their own names, it was resolved at the 2nd International Workshop on Grapevine Trunk Diseases, that the current name of "Petri disease" be used (Surico, 2001).

1.7.2 SYMPTOMS OF PETRI DISEASE

The effects of *P. chlamydospora* on the growth of vines are different according to their ages. For young vines, the external symptoms include slow establishment, poor growth and smaller rootstocks (Pascoe and Cottral, 2000).The parameters for growth also include above and below ground dry weight and the number of internodes and roots. After inoculation, these parameters are all reduced (Adalat *et al.*, 2000). A survey performed by

Pascoe and Cottral (2000) in Australia showed that nearly half of the plants showed a problem in establishment after *P. chlamydospora* infection.

As for older vines, the external effects include poor or late budburst, weak and stunted shoot growth, reduced internode length, reduced leaf size and leaves that are occasionally chlorotic and/or necrotic (Mugnai *et al.*, 1999).The mechanism underlying this is probably toxin production from these pathogenic fungi. Toxins and/or toxic secondary metabolites can cause damage to host cells, including damage to cell membranes, cellular transport systems, or enzymatic reactions involved in biological pathways (Tabacchi *et al.*, 2000). Some similar metabolites, such as phytotoxins have been extracted from *P. chlamydospora* and *P. aleophilum*, and their functions on host vines have been demonstrated by Sparapano *et al.*, 2000. After direct application, a 57% reduction in grapevine callus dry weight, chlorosis and necrosis in leaves and other typical Petri disease symptoms were observed.

The internal symptoms of vines with Petri disease include extensive browning of the vascular tissue, and excretion of a tar-like substance (Morton, 1995). This browning is attributed to the production of peroxidase by *P. chlamydospora,* which leads to the transformation of resveratrol and related compounds (Mugnai *et al.*, 1999). Some other symptoms, such as brown/black streaking, and formation of tyloses, were also observed on the cut section of infected vines (Pascoe and Cottral, 2000).

1.7.3 DISSEMINATION OF THE PATHOGEN

Some researchers have suggested that the main source of infection from *P. chlamydospora* is the use of material from diseased mother-vines (Larignon and Dubos, 1999; Mugnai *et al.*, 1999). This has been demonstrated by Chicau *et al.*, (2000) after isolation of *P. chlamydospora* pathogen from rootstock mother-vines, rootstock cuttings (Fourie and Halleen, 2001) and the rootstock section of young grafted vines (Bertelli *et al.*, 1998; Zanaotto *et al.*, 2001). In contrast, many researchers have consistently failed to isolate the pathogen from the scion part of grafted vines (Bertelli *et al.*, 1998; Zanzotto *et*

al., 2001). Pascoe and Cottral (2000) suggested that movement into the current season's growth occurs through movement of conidia in the vascular system. However this has been argued by Edwards *et al.* (2003), who suggested that movement may occur through active mycelial growth of *P. chlamydospora.*

Like the infection progress of *E. lata*, it is possible the infection by *P. chlamydospora* results from the aerially disseminated conidia that persist on the surface of canes, when rehydration of material, disbudding, making of grafting cuts and root forcing (Mugnai, 2000; Zanaotto *et al.*, 2001), it infects wounds, inoculation experiments from Scheck *et al.*, 1998 and Larignon and Dubos, (2000) have demonstrated this.

The effects of the presence of *P. chlamydospora* and *P. aleophilum* on grafting have been proved to be significant. In 1994, Ferreira *et al.* demonstrated that callus formation rate was reduced from 76% (controls) to 29% (grafted vines dipped in conidial suspensions of *P. parasitica,* which was later reclassified as *P. chlamydospora*). Recently, Adalat *et al.*, 2000; Wallace *et al.*, 2003 demonstrated that when *P. chlamydospora* was inoculated, the callus formation at the base of cutting had been dramatically reduced.

Another possibility of infection is from soil (Bertelli *et al.*, 1998; Surico *et al.*, 2000). This is consistent with the result from Adalat *et al.*, 2000, who reported that 7% of single bud cuttings of Chardonnay planted in sand artificially infested with *P. chlamydospora* were later found to be infected.

1.7.4 INTERNATIONAL DISTRIBUTION

Petri disease has been observed worldwide. In New Zealand, Jaspers *et al.*, 2000 reported that 84% of vines showing external symptoms of Petri disease had internal staining and *P. chlamydospora* was the fungus most commonly isolated.

While *P. chlamydospora* was first isolated on grapevines; this does not mean other plants are not susceptible to its infection and colonization. In 2000, Di Marco *et al.* reported that

P. chlamydospora as well as other similar fungi including *P. aleophilum* were also isolated from kiwifruit vines with a similar esca disease.

1.7.5 DISEASE MANAGEMENT

1.7.5.1 Cultural control

To control Petri disease, selection of resistant cultivars seems necessary. However the view of "no apparent difference in varietal susceptibility" (Pascoe and Cottral, 2000) has been widely accepted. Hence selecting high quality cuttings that are stress-free, disease-free and injury-free has been highly recommended (Morton, 1999). Other methods to prevent Petri disease infection include avoiding cultivar preparation during wet periods (Larignon and Dubos, 2000), reducing stress of cutting materials (Pascoe *et al.*, 2000), application of hot-water treatment (although negative and positive views are co-existing) (Edwards *et al.*, 2003; Fourie and Halleen, 2003), and use of fungicides (Jaspers, 2001).

From a variety of experiences obtained from controlling of other plant diseases, application of chemicals (fungicides) is likely to be a feasible method during the grafting process and the time when vines are getting infected. However no products are currently available specifically against *P. chlamydospora*. The near future of this seems to be bright, a number of systemic products have been tested *in vitro,* and shown to be very promising (Groenewald *et al.*, 2000; Jaspers, 2001), with a reduction in mycelial growth of the pathogen and conidial germination (Jaspers, 2001) have been observed *in vitro.* Some *in vivo* tests have been performed in the glasshouse with fungicides, such as thiabendazole (Fragoeiro pers. Comm.), Prochloraz, managanese (Laukart *et al.*, 2001), and various phosphorous based products (Di Marco *et al.*, 1999; Khan and Gubler, 2001; Laukart *et al.*, 2001). Large scale practical applications still need serious consideration although the potential as a treatment has been demonstrated. Further, as these chemicals usually are hazardous to the environment, some countries have banned their use.

1.7.5.2 Sanitation methods

It is believed that regular pruning of grapevines each year provides a multitude of entry points for the pathogen. Because of the slow growth of the pathogen, manifestation of the infection symptoms is usually delayed; this makes recognition of the disease difficult, and in time measures for controlling this infection seems unrealistic (Jones, 2001). Thus the time for pruning should avoid wet weather due to ascospore dispersal during this period (Chapuis *et al.*, 1998; Moller and Kasimatis, 1980).

1.7.5.3 Fungicide control

Although no ideal fungicides are available to control the disease at present, some chemicals, such as benomyl and flusilazole do provide barriers against the invasion of the pruning wood if these chemicals are flooded to the exposed vesicles at the surfaces of the pruning woods before the spores arrive (Moller and Kasimatis, 1980).

1.7.5.4 Biological control

Biological control seems to be a good idea for controlling this disease based on the mechanism that these biological organisms can serve as competitors. However the prerequisite is that the organisms must be able to grow under field conditions (Jones, 2001) and are not harmful to the environment. As for economic considerations, it is dependent on the biological agents themselves and the labor cost during application of these agents (Jones, 2001).

In order to control and or prevent infection, vineyard management, such as good vineyard sanitation to remove diseased material and maintain good soil structure is highly recommended to maintain a healthy environment for vines (Emmett and Magarey, 1994; Mugnai *et al.*, 1999).

1.8 DETECTION

1.8.1 DETECTION BY MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF PATHOGEN

It has been believed that *P. chlamydospora* and *P. aleophilum* are the two major causal agents of Petri disease. In term of the power to infect grapevines, *P. chlamydospora* shows higher aggressiveness than *P. aleophilum* or *P. inflatipes* (Adalat *et al.*, 2000). More recently, callus formation has been reduced in the *P. aleophilum* inoculated vines rather than in *P. inflatipes* ones (Wallace *et al.*, 2003).

The common feature of these pathogens is that they all are Petri disease causing agents; hence it is necessary to distinguish them from each other before application of treatments. The typical morphological features and characteristics of *P. chlamydospora* have been described by Crous *et al.*, (1996) and Pascoe, (1999), such as deep olive color with a white slimy margin (on potato dextrose agar and malt agar); grow slowly; green/brown mycelium; large number of chlamydospores; global brown pycnidia, etc.

1.8.2. DETECTION OF FUNGI USING THE ITS REGION

Based on the information on morphology of colonies described by Cater and Moller, 1977, culturing the pathogen from diseased wood and comparing with a reliable reference culture transferred at the same time may be another way for the diagnosis of *E. lata.* However due to the interference from other fungi that are co-existing on the diseased wood (infected vines are more vulnerable to other fungi), it may be difficult to confirm its presence through this method. Application of the ITS region by PCR using universal DNA primers specific for the conserved 18S and 28S elements (White *et al.*, 1990) followed by direct sequencing has been successfully used to detect fungal plant pathogens (Jones, 2001). Fungi detected using this method include *Armillaria* species Harrington and Wingfield, 1995) and *Phaeoacremonium* species (Tegli *et al.*, 2000).

Whiteman *et al.,* (2002) reported using species-specific PCR for the detection of *P. chlamydospora* in soil. Using a nested PCR, this method preamplified a 600 bp region of the ribosomal DNA, followed by amplification of a 360 bp species-specific region. This assay was able to detect 10^2 conidia/ml when a spore suspension was added to sterilized soil samples and 50 fg when genomic DNA was added directly to the reaction. Compared to traditional method (agar plate isolation), this method is faster and more precise.

1.9 *gfp* AS A REPORTER GENE

1.9.1 DISCOVERY OF THE *gfp* AS A REPORTER GENE

In 1978, Prendergast and Mann isolated the green fluorescent protein (*gfp*) from *Aequorea forskalea*. In the following year, Ward and Cormier, (1979), characterized the Renilla green-fluorescent protein, and found that it is an energy transfer protein in coelenterate bioluminescence. However, studies at the molecular level on this protein were not performed until 1992. Prasher *et al.* reported the cloning and sequencing of cDNA and genomic clones of *gfp* from the cnidarian, *Aequorea victoria*. The *gfp*10 cDNA encodes a 238-aa-residue polypeptide with a calculated Mr of 26,888. The great discovery from Chalife *et al.*, 1994 and Inouye and Tsuji, 1994 demonstrated that when the *gfp* gene was introduced into heterologous organisms, it could generate fluorescence upon UV or blue light excitation. This discovery made the widespread usage of *gfp* as a reporter gene possible and thus led to the worldwide application in biological studies at a molecular level.

Green-fluorescent proteins (*gfp*s) can be used as energy-transfer acceptors in bioluminescence. Upon receiving energy from either a luciferase-oxyluciferin excited-state complex or a Ca^{2+}-activated phosphoprotein, *gfp*s emit green fluorescence *in vivo*. These highly fluorescent proteins are unique due to the chemical nature of their chromophore, which is comprised of modified amino acid (aa) residues within the polypeptide (Prasher *et al.*, 1992).

This gene or derivatives have been successfully expressed and conferred fluorescence to a variety of organisms such as bacteria (Chalfie *et al.*, 1994); yeast (Flach *et al.*, 1994; Niedenthal *et al.*, 1996), filamentous fungi (Spellig et al., 1996); mammals (Pines, 1995), *Drosophila* (Wang and Hazelrigg, 1994), and plants (Haseloff and Amos, 1995; Sheen *et al.*, 1995).

This unique expression of *gfp* makes it very useful in detection of expression of specific tissue *in vivo* or monitoring the interactions between organisms, such as the interaction between pathogenic fungi and their host plants (this project is based on this consideration). *gfp* serves as a reporter mainly because of the unique qualities of this 238-amino-acid, 27-kDa protein which absorbs light at maxima of 395 and 475 nm and emits light at a maximum of 508 nm (Lorang *et al.*, 2001).

1.9.2 CHARACTERISTICS OF GFP

GFP is extremely stable *in vivo*, and is not affected by destructive sampling, cell permeablization, or leakage of products. GFP itself is not able to cross most membranes, with the exception of the nuclear membrane through the nuclear pores (Grebenok *et al.*, 1997). Furthermore, the fusion of the GFP to the C or N terminus of many cellular and extracellular proteins make it more stable and will not lead to a loss of activity, thereby permitting the tagging of proteins for gene regulation analysis, protein localization, or specific organelle labeling (Lorang *et al.*, 2001). The mature protein resists many proteases and is stable up to 65°C and at pH 5 to 11, in 1% sodium dodecyl sulfate or 6 M guanidinium chloride (Cubitt *et al.*, 1995; Ward, 1998), and in tissue if fixed with formaldehyde, methanol, or glutaraldehyde.

1.9.3 THE ADVANTAGES AND DISADVANTAGES OF USING *gfp* AS A REPORTER

The popularity of *gfp* as a biomarker in biological research has its reasons. As DNA sequences encoding specific secretory proteins or signals can be fused to *gfp*, usually without altering their targeting, it is a useful alternative to conventional dyes previously

used to investigate compartments *in vivo* (Brandizzi *et al.*, 2004). Compared with other traditional reporters such as 3 -glucuronidase, β -galacturonidase, chloramphenicol acetyltransferase, and firefly luciferase, which rely on cofactors or substrates for activity, the fluorescence of GFP requires only UV or blue light and oxygen, and therefore, *in vivo* observation of *gfp* expression is possible with individual cells, with cell populations, or in whole organisms interacting with symbionts or environments in real time (Lorang *et al.*, 2001).

On the other hand, GFP has its disadvantages, for example, pH values can affect GFP fluorescence, the optimal pH range for *gfp* expression is from pH 7.2 to 8.0 (Ward, 1998). GFP will not function as a biomarker in methanol-acetic acid (3:1) as a result of an absence of fluorescence, and it can be masked by autofluorescent aldehyde groups in tissue fixed with glutaraldehyde (Lorang *et al.*, 2001). Other limitations on *gfp* as a reporter for some applications include its low turnover rate, 2-h lag time for autoactivation of its chromophore, improper folding at high temperatures (37°C), which results in nonfluorescence and insoluble forms of the protein, and requirement for oxygen, which is not present in equal concentrations in all subcellular locations or cell types (reviewed by Cubitt *et al.*, 1995 and Ward, 1998).

1.9.4 RECENT DEVELOPMENT OF *gfp* AS A REPORTER MARKER

In order to overcome the problems posed by these limitations, a variety of *gfp* derivatives have been produced and demonstrated high promise. Most of these derivatives are mutant forms of *gfp*, which have a higher ability to resist the influences of high temperature and can still maintain a good conformation. In addition, these modifications can increase solubility and fluorescence, reduce photobleaching (Crameri *et al.*, 1996, Cubitt *et al.*, 1995, Siemering *et al.*, 1996), and reduce half-lives (Andersen *et al.*, 1998). Combined with fluorescence-activated cell sorting, confocal microscopy or quantitative image analysis techniques, *gfp* technology can be used to isolate transformed cells or specific cell types from populations of cells (Cormack *et al.*, 1996), and to quantify gene expression of individual cells within whole organisms (Brand, 1995).

1.9.5 *gfp* ULITIZATION IN FUNGAL STUDY

gfp as a reporter has been widely used in fungal studies, including yeast and filamentous fungi. *Ustilago maydis* was the first filamentous fungus for which successful expression of *gfp* was reported (Spellig *et al.*, 1996), followed by *Aspergillus nidulans* (Fernández-Ábalos *et al.*, 1998) and *Aureobasidium pullulans* (Vanden Wymelenberg *et al.*, 1997). In a review from Lorang *et al.*, (2001), *gfp* expression has been reported for 16 species comprising 12 genera of filamentous fungi, including *Colletotrichum* (Dumas *et al.*,1999), *Mycosphaerella* (Skinner *et al.*, 1998), *Magnaporthe* (Kershaw *et al.*, 1998), *Cochliobolus* (Maor *et al.*, 1998), *Trichoderma* (Bae and Knudson., 2000), *Podospora* (Berteaux-Lecellier *et al.*, 1998), *Sclerotinia* (Vautard-Mey *et al.*, 1999), *Schizophyllum* (Lugones *et al.*, 1999), *Aspergillus* (Du *et al.*, 1999) and *Phytophthora* (Bottin *et al.*, 1999).

Expression of *gfp* in filamentous fungi requires a well-developed transformation system, and a fungal promoter that can drive the expression of *gfp*. A new vector containing the designed *gfp* gene and a promoter (prefer a strong promoter) is required to be constructed prior to fungal transformation. A new fungal transformation vector that expresses *Sgfp* under the control of the *ToxA* gene promoter from *Pyrenophora tritici-repentis* was developed by Ciuffetti *et al.*, (1997) who demonstrated its use in plant pathogens belonging to eight different genera of filamentous fungi (*Fusarium, Botrytis, Pyrenophora, Alternaria, Cochliobolus, Sclerotinia, Colletotrichum*, and *Verticillium*).

1.9.6 WILD-TYPE AND ENGINEERED *gfp* GENES

The prerequisite of *gfp* utilization in fungal transformation is its successful expression. However the wild-type *A. victoria gfp* gene does not confer appreciable fluorescence in many transformed fungi due to the translation problem, hence the wild-type *gfp* gene is required to be modified with optimized codon usage. After modification, the *gfp* variants express proteins (SGFP, yEGFP, and EGFP1) that also contain a serine-to-threonine substitution at amino acid 65 (S65T), that causes a "red shift" from excitation maxima of 395 and 470 nm to a maximum of 488 nm (Lorang *et al.*, 2001).

After comparison of the expression of four *gfp* variants driven by a common promoter in *A. nidulans,* Fernández-Ábalos *et al.*, (1998) concluded that SGFP (Chiu *et al.*, 1996) conferred the highest GFP concentration and level of fluorescence to transformants. SGFP contains the S65T mutation as well as plant-optimized codon usage that also delete a cryptic intron splice site reported to reduce *gfp* expression in *Arabidopsis* (Haseloff *et al.*, 1997). *Sgfp* (Blue-*Sgfp-TYG*) has been the *gfp* gene most often used for transformation of filamentous fungi. *Egfp1* (Clonetech, Inc., Palo Alto, Calif.) is similar to *Sgfp* in that it contains the S65T mutation and 190 silent base mutations corresponding to human codon usage preferences (Yang *et al.*, 1996). Examples of *Egfp1* utilizations have been reported in *Aspergillus flavus, A. pullulans, Magnaporthe grisea,* and *Podospora anserina* (Berteaux-Lecellier *et al.*, 1998; Du *et al.*, 1999; Liu and Kolattukudy, 1999, van West *et al.*, 1999).

1.9.7 THE UTILITY OF *gfp* AS A REPORTER GENE

Unlike other reporters, usually with destructive sampling that limit observations to a moment in time, *gfp* affords detection of gene expression and protein localization that is continuous in time and development within a single living specimen. In addition, *gfp* does not require additional substrates or fixing of tissue (Lorang *et al.*, 2001). Furthermore, *gfp* gene expression is easily quantified in whole cultures via fluoroimetry (Fernández-Ábalos *et al.*, 1998) or in individual cells or subcellular compartments with confocal microscopy (Liu and Kolattukudy, 1999; Spear *et al.*, 1999).

1.9.8 INVESTIGATING CELL DYNAMICS WITH *gfp*

The emergence of *gfp* has created a milestone in the study of cellular biology of filamentous fungi *in vivo*. Filamentous fungi are regarded as an ideal organism for cell dynamics study due to their simple anatomy and rapid growth rates. Transformation of filamentous fungi with *gfp* expressing vector enables this study to be more delicate and more dynamic. One example is from *A. nidulans* transformed with *gfp* vector (Suelmann and Fischer, 2000), a model system for investigating the molecular basis of eukaryotic,

cellular morphogenesis. Nucleus-targeted GFP in *A. nidulans* allowed real-time visualization of nuclear migration and mitosis, and the behavior of specific nuclei at various developmental stages (Fernández-Ábalos *et al.*, 1998, Suelmann *et al.*, 1997).

1.9.9 VISUALIZING FUNGI IN THEIR ENVIRONMENTS: HOST-PATHOGEN INTERACTIONS, MYCOPARASITISM, AND THE PHYLLOSPHERE

Filamentous fungi that have been transformed with *gfp* so far are mainly plant pathogens or residents of plant surfaces, and genes of interest in these systems are those that play roles in host-fungus interactions. The merits of *gfp* in elucidating regulation of genes and the cellular location of their protein products are obvious. Tracking fungal strains that carry mutations for or that overexpress such genes in plants is also of great interest (Lorang *et al.*, 2001).

In order to detect *gfp* expression in fungi, track fungi in plants, monitor their distribution, and to estimate their biomass, laser scanning confocal microscopy and epifluorescence microscopy using a computer-controlled, z-stepper motor and filters in conjunction with a video camera were used by Spear *et al.*, (1999) for the fungus *A. pullulans*.. Data gathered by either method was analysed with image analysis software (Optimas v6.2 for PC; Media Cybernetics, Del Mar, Calif.). The advantages and disadvantages of Laser scanning confocal microscopy were compared with conventional epifluorescence microscopy. Compared with conventional epifluorescence microscopy, the optical sectioning capability of confocal microscopy affords clear visualization of GFP despite the autofluorescence and light-scattering properties of plant cell walls (Haseloff and Amos, 1995).

Labelling whole fungi with *gfp* generally results in a cytoplasmically located protein occurring in all fungal morphotypes (hyphae, spores, appressoria, etc.) with no obvious effects on fungal growth or pathogenicity (Maor *et al.*, 1998; Spellig *et al.*, 1996). Of *gfp* tagging of plant pathogenic fungi, S*gfp* was easily detected with epifluorescence microscopy (Leica DMRB and Endow *gfp* filter cube, exciter HQ470/40, and emitter HQ525/50 with beamsplitter Q495LP) (Lorang *et al.*, 2001).

1.9.10 VECTORS FOR *gfp* TAGGING OF FILAMENTOUS FUNGI

A variety of *gfp* or modified *gfp* expression vectors have been developed for all major classes of filamentous fungi. Each individual fungus has its own unique properties; hence selection of vector will depend on the fungus itself. In addition to modification of the *gfp* gene itself, modification of the gene promoter to get a strong promoter is an alternative way to enhance GFP fluorescence. Limited numbers of fungal promoters and the variability of the strength of promoters in heterologous fungi have posed a problem for *gfp* application in fungal studies. In addition, not only *gfp* but also selectable markers need to be taken into consideration prior to transformation. One commonly used *gfp* vectos is pCT74, which contains a *Sgfp* driven by *ToxA* promoter (appendix 3F).

1.9.11 *gfp* AND THE FUTURE OF FUNGAL BIOLOGY

Recently the complete sequences of the *S. cerevisiae* and *S. pombe* genomes were determined, and genome projects for several filamentous fungi, including *A. nidulans, Neurospora crassa, M. grisea,* and *Candida albicans* are well under way (Lo *et al.*, 2000, Lim *et al.*, 2004). The promoter sequences for every gene in all of these organisms will gradually be available. Coupled with the rapid developments in Microarray, digital imaging, and bioinformatics technologies, and with high-throughput systems that detect, store, and display complex fluorescent images, *gfp* technology is increasingly powerful (Lorang *et al.*, 2001).

Using *gfp*-tagged fungi for tracking fungal distributions in natural systems seems to be a good idea. However, a big concern is that a large number of easily dispersible spores produced by many filamentous fungi tend to accumulate to very high concentrations in the air, hence contained environments for working with these engineered fungi should be used, and the ecological implications of introducing these organisms into the environment must be seriously considered (Lorang *et al.*, 2001).

The utilization of *gfp* and its spectral variants have changed the approach to studying the dynamics of the plant secretory pathway. *gfp* technology has shed new light on secretory

events by allowing bioimaging *in vivo* right to the heart of a plant cell (Brandizzi *et al.*, 2003).

Although *gfp* has become an invaluable tool for *in vivo* investigation, *gfp* technology has to be used wisely to avoid artefacts and data misinterpretations. In certain cases, the expression of *gfp* may involve protein overexpression, and this overexpression could exert effects on internal systems in the organisms, such as on the plant endomembrane system. There, the value of controls on transformation levels and ensuring that the labelling with GFP fusions is due to correct targeting should not be underestimated. In addition, GFP tagging of proteins may result in loss of a specific function or, worse, acquisition of new function (Brandizzi *et al.*, 2003).

Further malfolding of proteins and malfunction caused by *gfp*, and sensitivity to pH values should be taken into serious consideration. GFP may accumulate but its fluoresence may be easily quenched by the acidic pH and specific experimental procedures may need to be adopted to visualize GFP fluorescence. In addition, reporter systems could have an influence on *gfp* expression (Tamura *et al.*, 2003).

Further, the methods through which fungi grow and spread in their host plant still remain unknown. Currently, the use of the green fluorescent protein (*gfp*) as a biomarker for observation of fungal spreading in plants has been widely used, such as for *sapstain* fungi (Lee *et al.*, 2002). *gfp* is introduced into a vector containing an *hph* gene, which will subsequently be introduced into a fungal protoplast, therefore, after incubation; any movement of the transfected fungus in the plant can be monitored through tracking the expression of *gfp* by confocal microscopy. In order to achieve these it is necessary to develop a transformation system for these fungi.

In conclusion, recent advances in *gfp* molecule engineering, fluorescence detection, and imaging analysis are opening the door for us to gain considerable information about fungal genomes. A clever utilization of these technologies and information is likely to lead to a bright future for fungal biology.

1.10 TARGETED GENE REPLACEMENT

Gene targeting ('knock-out') technology is now widely used in the basic science of all disciplines of pathology and is the wilful introduction of precise mutations into the genome of organisms, affecting the function of a single gene or genes (Riminton, 2002).

1.10.1 OVERVIEW OF GENE TARGETING TECHNIQUE

To successfully disrupt a gene *in vivo* a number of tasks need to be completed. A targeting vector needs to be designed using knowledge of the genomic sequence containing the genes of interest. Design is governed by three principles. First, to disrupt one or more exons of the target gene by the interruption/excision of sequence, or by the introduction of a stop codon. Second, to incorporate a selectable marker, that enables the identification of transfected cells in culture. Third, to provide adequate sequence homology in flanking regions enabling homologous recombination (Riminton, 2002). After transfection by protoplasts/PEG method or electroporation, the vector sequence may be incorporated into the host genome randomly, or by the homologous replacement of endogenous sequence. Thus the normal function of the genes can be determined through observation of any alterations in comparison with wild-type organisms.

1.10.2 ADVANCES IN GENE TARGETING TECHNIQUES

Recent advances in gene targeting technology enable the specific inactivation of genes restricted both in time and anatomic location. The characterization of tumor necrosis factor (TNF), the ubiquitously expressed and most studied of cytokines, provides an ideal illustration of the effectiveness of gene targeting experiments in the investigation of inflammatory biology. The complexities of TNF have halted definitive progress in TNF biology. Gene targeting enabled precise inactivation of TNF and definition of its roles (Riminton, 2002).

This technique developed includes the use of the recombinase protein Cre, and the Cre-specific recognition sequence *LoxP*. The targeting construct is designed with the targeted allele flanked by two *LoxP* sites ('floxed'), with site-specific excision of the intervening sequences achieved by Cre expression. The Cre-*LoxP* system is used in 'knock-ins' where the neomycin resistance cassette is floxed and deleted from the replaced gene.

Gene targeting is now established as a key experimental technology for the identification of participating genes in the complex disease processes, which can be introduced into the current project for identifying those genes and factors involved in the grapevine disease black goo and Petri grapevine decline.

As this project was designed for a one year MSc, the convenient "one step" gene disruption method through homologous recombination was chosen for disruption of the *P. chlamydospora* putative toxin gene *moxY* (cloned by Hayley Ridgway, Lincoln University). A vector containing part of the *moxY* gene and selective marker *hph* will be constructed. After transformation of this vector into *P. chlamydospora* protoplasts, transformants selected based on hygromycin resistance are screened through PCR for targeted integration and concurrent disruption of the toxin genes or genes involved in toxin production. Therefore these genes' functions can be determined by comparing with wild-type organisms. However the prerequisite of this is the successful development of a highly efficient transformation system.

Fungal genomics and the remarkable improvements in transformation technology, together with the achievements in higher homologous recombination efficiencies (Garcia-Maceira *et al.*, 2000), and possibly targeted PCR (Wach *et al.*, 1997), make development of genomic approaches a reality for filamentous fungi. These developments would allow elucidation of fungal gene (particularly for those putative toxin genes) functions on a massive scale (Lorang *et al.*, 2001).

1.11 TRANSFORMATION OF FUNGAL PLANT PATHOGENS

At present, *E. lata* and *P. chlamydospora* are still poorly understood, partly due to a lack of molecular analyses that could characterize pathogenicity and virulence factors of these fungi. The need for a transformation protocol was emphasized in several studies as a necessary instrument for the investigation of disease-causing mechanisms. This transformation system is essential to the genetic analysis of gene regulation and biochemical features of these fungal species and the expression of recombinant proteins in them. Gene transformation of filamentous fungi is a relatively new area. With the rapid development of molecular biology this area has undergone unbelievable improvements.

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. The first description of such a system was in *N. crassa* (Case *et al.*, 1979), followed soon after with gene transfer in another ascomycete, *A. nidulans* (Ballance *et al.*, 1983). Many other transformation systems have been developed for a range of commercially and agriculturally important fungal species (Punt and van den Hondel, 1992).

Currently, the mainly existing methods that could be used for transformation are protoplast/PEG method, electroporation, biolistic transformation, and *Agrobacterium tumefaciens*-mediated transformation; the most popular one is the classical protoplast/PEG method using hygromycin B resistance gene (*hph*) as a dominant selectable marker.

1.11.1 FUNGAL PROTOPLASTS

Bacteria, fungi, and plants have rigid, mainly polysaccharide cell walls that give a characteristic morphology to the cells and provide support and protection to the enclosed protoplast (Peberdy & Ferenczy, 1985). They described 'protoplast' as a "naked cell completely devoid of cell wall residues". They still represent the organized entities of the

living components of cells, which are able to carry out active metabolism and energy transfer. Fungal protoplasts have been used and still are used extensively in cell wall synthesis studies and are providing a means of studying a wide range of cellular, biochemical, and genetic processes.

1.11.2 SELECTABLE MARKERS

The markers that have been selected for transformation include not only genes encoding antibiotic resistance enzyme but also those encoding special enzymes, even unique fluorescent proteins that can be easily detected. Most recently developed transformation systems are mainly focused on marker selection and vector construction. Both markers that are capable of complementing a mutation such as auxotrophic markers (negative selection), and those that can confer a new property to the host cell, like antibiotic resistance (positive selection), are useful. Each has its own advantages and disadvantages, such as a wild-type transformant (thus harmless to the environment) would be obtained after using a negative selectable marker, rather than acquiring a mutant with an additional characteristic. However the advantage of using positive selectable marker is that transformation does not need a mutant.

However the transformation frequencies based on the protoplast/PEG method are not always high. As obtaining a large number of transformants is essential to the eventual aim (i.e. to determine genes' function through gene targeting), any factors beside the selective markers involved in this process are all required to be seriously considered. To optimize those conditions and find a specific optimal protocol for the establishment of the transformation system is crucial for making successful targeted gene replacement.

Environmental consideration for these genetic modified organisms is essential. Techniques for eliminating selectable markers from transformed organisms have been developed (Komari *et al.*, 1996), but research that addresses the fitness, epidemiology, and possible ecological implications of releasing transformed fungi into ecosystems is lacking.

1.12 AIMS AND OBJECTIVES

As there is no single all-encompassing method to transform fungal cells, many factors including protoplast isolation, shuttle vector, selection system etc, can affect transformation success. In addition, transformation protocols will vary between different fungi and different laboratories. Therefore, transformation systems for *E. lata* and *P. chlamydospora* will be developed to find a method which is reliable to obtain expected transformants.

Aim 1: *Development of transformation systems for E. lata and P. chlamydospora.*

To develop transformation systems for *E. lata* and *P. chlamydospora*. The transformation method will be based on classic PEG/protoplast. Fungal protoplasts will be transformed with plasmid pAN7-1 and *gfp*- containing plasmids pBCH-*gfp* and pCT74.

Aim 2: *Molecular characterization of transformants.*

To characterize transformants with PCR and Southern hybridization. The characteristic genes (*hph, gfp* and *moxY*) in each plasmid will be amplified with PCR and confirmed with Southern hybridization. Check of the *gfp* expression will also be performed with a UV microscope.

Aim 3: *Establishment of a relationship between E. lata and host blackcurrants.*

To inoculate a purified *E. lata* transformant containing an expressed *gfp* gene onto blackcurrants, one of its host plants, followed by observation of its colonization and penetration in the wood tissue through detection of GFP fluorescence. Therefore, an internal interaction between *E. lata* and blackcurrants will be established.

Aim 4: *Obtaining a putative toxin gene moxY disrupted P. chlamydospora transformant.*

To construct a plasmid containing a *moxY* gene homologous fragment, and transform *P. chlamydospora* based on the previously developed transformation system. The expected

moxY gene disrupted transformants through homologous recombination will be screened through PCR, followed by Southern hybridization.

CHAPTER TWO MATERIALS AND METHODS

2.1 PLASMIDS, BACTERIA, FUNGI AND PRIMERS

Details of all organisms, plasmids and primers used in this study are given in Tables 2.1-2.4

Table 2.1 Plasmids used in this study

Plasmid	Relevant characteristics	Source/Reference
pAN7-1	6.5 kb HmB^R, Amp^R	Punt *et al.* 1987
pBC-hygro	$Chl^{R,}$ HmB^R , lacZ (6.8 kb)	Orbach 1994 Silar, 1995
pBCH-GFP	pBC-hyg containing fragment from pFAT-3 gfp (gfp gene, Anp-gpd) (9.1 kb)	West, 2004
pCT74	SGFP, Tox A promoter, $hygB^R$, Amp^R (~6 kb)	Lorang *et al*, 2001
pGEMT-MOXY	T7 promoter, SP6 promoter, MCS, pGEM-T with the insert of part of the *moxY* gene fragment from *P. chlamydospora.*	This study
pBCH-MOXY	pBC-hygro containing fragment of part of *P. chlamydospora moxY* gene (7.4 kb)	This study

Table 2.2 Bacterial strains used in this study

Bacterial strains	Relevant characteristics	Source/Reference
Escherichia coli XL-1	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lacF [proAB lacq*Δ*(lacZ) M15 Tn10(tet*^{r}*)]*	Bullock *et al*, 1987
XL-1 (pAN7-1)	XL-1 containing pAN7-1	This study
XL-1 (pGEMT-MOXY)	XL-1 containing PGEMT-MOXY	This study
XL-1 (pBCH-MOXY)	XL-1 containing PBCH-MOXY	This study

Table 2.3 Fungal isolates used in this study

Fungus	Relevant characteristics	Source/Reference
Eutypa lata (*E10-10*)	wild type strain	This study, collected from Cabernet Sauvignon grapevines at Erindale Vineyard, Hawkes Bay.
Eutypa lata Transformants	*E 10-10* containing plasmid pAN7-1, pBCH-gfp, or pCT74	This study
Phaeomoniella chlamydospora	wild type strain	This study, from Hayley Ridgway, Lincoln University.
Phaeomoniella chlamydospora transformants	*P. chlamydospora* containing plasmid pAN7-1, pCT74, or pBCH-MOXY	This study

Table 2.4 PCR and sequencing primers.

Name	Length (nt)	Tm (°C)	Sequence (5'-3')	Source
5'*hph*2672	18	56	ATCTTAGCCAGACGAGCG	This study
3'*hph*3032	20	62	GTCTGCTGCTCCATACAAGC	This study
GFP1	20	64	GGAACTGTTCACTGGCGTGG	This study
GFP2	20	64	CAGCTGCACGGATCCATCCT	This study
MOXY1	21	60	CAGTTCATAAAGTTCCAGCAC	This study
MOXY2	19	60	ACGGCGACAACCGACAGAA	This study
MOXY3	21	60	TGGTATCAATGTTGCTCGCAA	This study
MOXY4	20	60	ACCAGAGCTTCCAGATAACC	This study
pBCH-MOXY1	20	60	TATAGGGCGAATTGGGTACC	This study
pBCH-MOXY2	20	60	TGACCATGATTACGCCAAGC	This study

2.2 MEDIA

All media were prepared with Milli-Q purified water and were sterilized by autoclaving at 15 p.s.i. (121°C) for 15 min. Liquid media were cooled to room temperature before supplements were added. Solid media were cooled to ~55°C before the addition of supplements and pouring.

2.2.1 BACTERIAL MEDIA

Liquid Luria Broth (LB)

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

Solid Luria Agar

LB containing 15 g/l Bacteriological agar

Media Supplements

When required the antibiotic concentration used for selection was: 100 μg/ml ampicillin from a stock solution of 100 mg/ml. or 170 μg/ml chloramphenicol from a stock of 30 mg/ml.

For blue-white selection. LB was supplemented with 30 μg/ml isopropylthio-β-D-galactoside (IPTG) and 60 μg/ml 3-indolyl-β-D-galactoside (X-gal).

2.2.2 FUNGAL MEDIA

For intact fungi:

(g/l): Potato Dextrose Agar (PDA). 39.0

(g/l): Potato Dextrose Broth (PDB). 24.0

For protoplasts (Osmotically Stabilised Media):

(g/l): PDA. 39; Sucrose. 273.9 (0.8 M).

Media Supplements

When required the antibiotic concentration used for selection was: 100 μg/ml Hygromycin B from a stock solution of 48 mg/ml or 52 mg/ml.

2.3 GROWTH AND MAINTENANCE OF CULTURES

2.3.1 BACTERIAL CULTURES

E. coli strains containing pAN7-1, pBCH-gfp, or pBCH-MOXY were maintained on LB agar plates supplemented with antibiotics. Plates were sealed with parafilm and stored at 4°C with regular subculturing after growing at 37 °C for one day. For long-term storage, glycerol stocks of selected *E. coli* cultures were prepared by pelleting cells from an overnight liquid culture by a 1 min centrifugation, then resuspended in 1 ml of 25% (v/v) glycerol and stored at -80°C.

For plasmid DNA preparation, about 5 ml of LB appropriately supplemented with antibiotics was inoculated with a loop after touching a single bacterial colony and incubated with shaking (300 rpm) overnight at 37 °C. A large scale preparation (500 ml) was performed when the plasmid DNA preparation was required for transformation.

2.3.2 FUNGAL CULTURES

Pieces of *Eutypa lata* or *Phaeomoniella chlamydospora* colonies were cut with a cork borer and subcultured onto PDA plates (with or without cellophane discs), and incubated at 20°C for 3-10 days depending on specific requirements. The cultures were then stored on PDA plates sealed with parafilm in the cold room at 4°C until required for further sub-culturing or every 3 months. For spore purification, a culture was grown in 100 ml of potato dextrose broth in a flask, supplemented with appropriate antibiotics. The flasks were incubated at 22°C for four days with constant shaking at 100 rpm. For long-term storage, cultures (mycelium) were kept in 25% glycerol at −80°C. This method was modified from Manavathu *et al*, (1999).

2.4 PREPARATION OF FUNGAL PROTOPLASTS

Protoplasts of *Eutypa lata* and *Phaeomoniella chlamydospora* were prepared based on the methods described by Punt and van den Hondel, (1992). Wild type mycelia (inoculated with mycelium) grown on cellophane for 5 days for *E10-10* or 3 weeks for *P. chlamydospora* were collected by peeling them directly from PDA plates. The whole cellophane with mycelia was transferred to a fresh plate into which 10-20 ml of the 5 mg/ml wall-digestion enzyme glucanex (Novozymes Switzerland), dissolved in OM buffer was added. After 3 h shaking at 37°C, protoplast formation was checked under a microscope, and harvested when many free protoplasts were observed but the mycelia had not completely broken down. The digestion mixture was filtered through a sterile nappy liner and harvested in a 15 ml corex tube, five milliliter of protoplast solution was overlaid very carefully with 2 ml of ST buffer followed by centrifugation for 5 min at 5000 rpm on a SS34 (Sorvall RC-5B). A white band containing protoplasts that formed at the interface of the two solutions was removed to a fresh tube, and washed twice in 5 ml of STC buffer. The protoplasts were pelleted between washes by centrifuging as above. Finally, the pellet was resuspended in STC buffer and the concentration of the protoplasts was estimated using a haemocytometer. A series of dilutions were needed when the original concentration was extremely high. Extra protoplasts were stored at –80 °C after the addition of 40% PEG at the ratio of 4:1 (protoplasts: PEG).

2.5 DETERMINATION OF PROTOPLAST REGENERATION RATE

2.5.1 *E. LATA* PROTOPLAST REGENERATION RATE

First, it was necessary to determine that the protoplasts had the ability to regenerate after isolation and could still survive after treatment with 40% PEG. The fresh protoplast suspension was diluted 100-fold, 1000-fold, and 10000-fold in STC buffers. Aliquots of 100 μl from each suspension were plated onto PDA plates and incubated for 10-14 days. After counting the colonies in each plate the regeneration rates were calculated.

2.5.2 CHECK *P. CHLAMYDOSPORA* PROTOPLASTS

The fresh protoplast suspension was diluted in STC buffer and water, at 1000-fold, and 10,000 fold. An aliquot of 100 μl from each suspension was plated onto PDA plates (for protoplasts diluted in water) or PDA plates supplemented with appropriate sucrose (for protoplasts diluted in STC) and incubated for 14-21 days. After counting the colonies in each plate the regeneration rates were calculated.

2.6 TRANSFORMATION OF *E. LATA* AND *P. CHLAMYDOSPORA* USING PROTOPLASTS/PEG METHOD

Transformations were performed based on the method described by Murray *et al*, (1992) and Bradshaw *et al*, (1997). Plasmid DNA (5 μg of pAN7-1 or pBCH-GFP) was added to 150 μl of a protoplast suspension in STC buffer (concentration ranging from 8.6 x 10^6 to 2.3 x 10^7 protoplasts/ml for *E . lata,* and from 1.9 x 10^7 to 6.3 x 10^8 protoplasts/ml for *P. chlamydospora*) and incubated at 20°C for 20 min. In three steps 250, 250 and 850 μl of sterile 40% poly-ethylene glycol (PEG) 6000 solution (in 50 mM $CaCl_2$, 1 M sorbitol, 50 mM Tris-HCl pH 8.0) were added to the DNA/protoplast suspension with thorough mixing between each addition. Then the suspension was incubated for a further 20 min at 20°C followed by a dilution in 5 ml STC buffer. The protoplasts were collected by centrifugation (1085 *g*, 20°C, 10 min) and resuspended in 500 μl STC buffer. Aliquots (100 μl) of this suspension were spread onto 20 ml plates of osmotically stabilized PDA. After incubation for 24 h at 20°C, the plates were overlaid with 5 ml of PDA top agar containing sufficient hygromycin B to give a final concentration of 100 μg/ml. Negative controls without pAN7-1 and hygromycin B were included. Dilutions for determination of the regeneration rate were also performed.

2.7 SUBCULTURING AND PURIFICATION OF TRANSFORMANTS

Transformants were subcultured by cutting a small piece of the colony from the original

plate and growing in a fresh plate without hygromycin for 6 days followed by growing them on selective media.

For purification, one single hypha observed under the microscope was picked out and transferred onto fresh plates with or without hygromycin (the same as subculturing), the successful colony was chosen for subculturing onto a fresh plate with cellophane.

2.8 PCR CHARACTERIZATION OF PRESUMED *E. LATA* TRANSFORMANTS

2.8.1 OPTIMIZATION OF PCR CONDITIONS

The genomic DNA used for this optimization was *E10-10* pAN7-1 transformant P3, which previously demonstrated growth in selective medium. Three different concentrations of [Mg^{2+}], i.e. 1.5 mM, 2 mM, and 3 mM, in 50 μl of final PCR reaction mixture were tested. In addition, two different amounts of DNA (25 ng and 50 ng) used for template were tested in each of the above three [Mg^{2+}] concentrations. The PCR product was separated by electrophoresis on 1% gel for the determination of the optimal conditions. The optimal combination of the amount of template DNA and the concentration of [Mg^{2+}] was thus used for subsequent PCR characterization.

2.8.2 PCR CHARACTERIZATION

After extraction of presumed transformants' genomic DNA and wild-type genomic DNA, PCR experiments were performed using *hph* primers and *GFP* primers (Table 2.4) for amplification of the *hph* or *gfp* gene, respectively. At the same time, positive controls using plasmid PAN7-1or pCT74 as template DNA (1.4 ng/50μl mixture solution) and a negative control with wild-type genomic DNA were also included. The cycling conditions for *hph* gene amplification are listed below:

94°C	2 min	
94°C	**30 sec**	
58°C	**30 sec**	**repeated 35 cycles**
72°C	**1 min**	
72°C	7 min	
4°C	hold	

The condition for *GFP* amplification was the same except that an annealing temperature of 64°C was used instead of 58°C.

2.9 SEQUENCING OF PCR PRODUCTS

The PCR products were cleaned after flowing through a PCR clean-up kit (Qiagen, Germany). Before sending to sequence, the concentration of the PCR product was determined through comparison with concentration standards following agarose gel electrophoresis. Ten micro litre of 15 ng/µl of PCR product as well as 10 µl 3.2 pmol/µl primers were sent for sequencing. Sequencing reactions were performed using the ABI BigDye™ Terminator 3.1 Sequencing Ready Reaction Kit (Applied Biosystems). The automated sequencer was an ABI 3730 DNA Analyzer. The primers MOXY1 and MOXY2 (Table 2.4) were used for sequencing the PCR product from the constructed plasmid pBCH-MOXY.

2.10 SEQUENCE ALIGNMENT

DNA sequences were aligned using the Gene-Jockey II® sequence handling program (Cambridge, UK). Protein sequences from translated DNA sequence were also aligned in this program for identification of conserved domains. Translation between nucleotide and corresponding peptide was performed using the Emboss sixpack (www.hgmp.mrc.ac.uk/software/EMBOSS/Apps/sixpack.html).

2.11 CHECKING THE EXPRESSION OF *GFP* IN *E. LATA* AND *P. CHLAMYDOSPORA* TRANSFORMANTS

Transformants of *E. lata* and *P. chlamydospora* containing vector pBCH-GFP or pCT74, were purified (Section 2.23) before *gfp* expression was determined. The materials screened for *gfp* expression with a fluorescence microscope at wavelengths of 450-490 nm were mycelium in 75% glycerol on slides, or whole colonies. Wild type samples under the same corresponding conditions were included as a control. Pictures were taken and stored in a fluorescence microscope Leica MPS 30 (Germany).

2.12 *E. LATA* GFP TRANSFORMANT INOCULATION

Woody blackcurrant or grapevines were cut into 4-5 cm length blocks with a saw (sized to fit the universal bottle). A piece of agar made with a cork borer with a *GFP*-expressing *E. lata* transformant was immediately inoculated upside down onto the top of a newly cut end of the blackcurrant or grapevine wood, allowing a direct physical contact between the fungus and the freshly-cut surface of the wood. Half of the blackcurrant or grapevine blocks were sterilized by autoclaving in universal bottles containing 3 ml Milli-Q water each. The other half was surface-sterilized with 75% ethanol before placing into autoclaved universal bottles containing the same amount of autoclaved Milli-Q water. The bottle caps were left loose to allow gas exchange. Half of the bottles containing the samples from each group (autoclaved or 75% ethanol sterilized) were incubated at a constant temperature of 22°C, while the others were incubated at room temperature. Regular checks of the water in the universal bottles were done to prevent the blackcurrant or grapevine samples drying out. Wild type *E. lata* was also included as a control under the same conditions.

2.13 CONFOCAL MICROSCOPY

Blackcurrant or grapevine samples were prepared every one and half months after

inoculation. The outside section of the wood was removed and the inside section of the wood block was used for sample preparation. The infected wood blocks were cut longitudinally and transversely into 20-35 μm thick sections using a Leica RM 2145. Slices were mounted in 75% glycerol on slides or in Citifluor mounting fluid, followed by a coverslip. These samples then were observed using confocal microscopy (Lee *et al*, 2002).

A pre-check with a UV fluorescence microscope (Olympus BX51, Japan) at the excitation wavelengths of 460-490 nm (exciter filter) and emission wavelengths of 510-550 nm (barrier filter) for wide band ultra violet (WUV) was performed, and positive samples were forwarded to confocal microscopy for further examination.

The samples were examined under a Leica TCS 40 (Germany) confocal laser scanning microscope using filters with exciter wavelengths of 488 nm and imaging wavelengths between 515 and 545 nm. Confocal images were produced by depth scanning to create optical sections taken 5 μm apart and combined to produce the final 3D image.

2.14 DNA PREPARATIONS

2.14.1 SMALL SCALE PLASMID DNA PREPARATION

Commercial kits: QIAprep Spin Miniprep Kit Protocol.

This protocol is for preparation of up to 20 μg of high-copy plasmid DNA using a QIAprep column (Germany). Five ml of LB supplemented with ampicillin was inoculated with a single bacterial colony and shaken overnight at 37°C. The culture was pelleted by centrifugation (9000 rpm) for 1 minute in several 1.5 ml eppendorf tubes. The pellets were used for plasmid extraction according to manufacturer's instructions.

2.14.2 LARGE SCALE PLASMID DNA PREPARATION

The QIAGEN Plasmid Mega Kit Protocol is the same as the QIAprep Spin Miniprep Kit Protocol except that the initial volume of the culture solution growing in selective LB

medium was 500 ml. Centrifugations (11,000 rpm in Sorvall GSA rotor) with suitable tubes were required.

2.14.3 WILD TYPE AND TRANSFORMANT GENOMIC DNA EXTRACTION

2.14.3.1. CTAB Method

Fungal (*E. lata* and *P. chlamydospora*) genomic DNA were extracted based on a modification of the method described by Raeder and Broda (1985). After purification, mycelium was subcultured onto PDA plates with cellophane on the top of the agar. Wild type *E. lata* and its transformants were grown for 10 days before harvesting of the mycelia from cellophane with a sterilized scalpel. For *P. chlamydospora*, incubation time needed to be extended to three weeks. Mycelia were frozen at -80°C and then freeze-dried overnight before being ground in liquid nitrogen with a mortar and pestle. CTAB buffer (600 µl) together with 2 µl RNAse (20 mg/ml) were added into the tubes containing mycelial powder, mixed well and incubated at 65 °C for 30-45 minutes. Chloroform (600 µl) was added to the mixtures to allow separation of aqueous and organic phases. The centrifugation speed was increased to 10000 rpm instead of 6000 rpm (rotor SS-34). The upper (aqueous) phase was transferred to a clean tube after a brief centrifugation. Isopropanol (600 µl) was added for precipitation of DNA. Then 600 µl of 70% ethanol was used to clean the DNA after drawing off some of the liquid and leaving only about 300 µl liquid plus the DNA in the tube. After washing twice more with 70% ethanol, the ethanol was decanted and the tubes air dried until all ethanol had evaporated. The DNA was re-suspended in 100 µl TE buffer.

2.14.3.2. Genomic DNA Extraction Method (AL-Samarrai and Schmid, 2000)

Small scale DNA extractions of wild type and transformants of *E. lata* or *P. chlamydospora* were extracted based on the method described by Al-Samarrai and Schmid (2000). After purification and subculturing, mycelia were harvested, frozen at -80°C and then freeze-dried overnight. Around 30 mg freeze-dried mycelia were ground to a fine power in liquid nitrogen using a pre-cooled pestle, followed by mixing with about 500 µl of freshly prepared lysis buffer (appendix 2.5.2). The suspension was

pipetted vigorously with a Gilson P1000 pipetman (about 50 times) until lots of froth formed, indicating the DNA was free from polysaccharides. Then, 165 µl 5 M NaCl was added to this solution. After thorough mixing (about 5 inversions), the suspension was centrifuged for 15 minutes at 4°C at 13000 rpm (1.5 ml eppendorf tubes), this stage had precipitated cellular debris, protein and polysaccharides. The supernatant was recovered in a fresh tube, followed with an immediate addition of 1 volume chloroform. A good mixing was achieved with gentle inversion until the solution became milky (about 50 inversions). The mixture was spun at 13000 rpm for 10-20 minutes before slowly sucking off the aqueous phase to a fresh tube. To precipitate DNA, 2 volumes of ice cold 95% ethanol was added and immediately spun for 5 minutes at 13000 rpm. The DNA pellets were washed with 70% ethanol 5-6 times before resuspending in 200-300 µl sterile water or diluted TE (10/0.1). To increase the efficiency of precipitation, 4 M LiCl was added (at the ratio of 1: 40) (v/v) to the solution before precipitation.

2.15 DETERMINATION OF DNA CONCENTRATION AND PURITY

2.15.1 DETERMINATION OF DNA USING A FLUOROMETER

A Hoefer Dynaquant 200 fluorometer was used according to manufacturer's instructions. Before the estimation of the concentration of DNA, the fluorometer was standardised by using 2 µl calf thymus (1mg/ml). The results on the machine reflected the actual concentration in the glass cuvette. A series of dilutions were required when the original DNA concentration was extremely high.

2.15.2 DETERMINATION OF DNA USING CONCENTRATION STANDARDS

The concentration of DNA was estimated visually by comparing with uncut lambda DNA standards of 25 ng, 50 ng, and 100 ng which were run on TBE agarose minigels beside an aliquot of the DNA (diluted if necessary).

2.16 DNA MANIPULATIONS

2.16.1 RESTRICTION ENZYME DIGESTION OF DNA

Plasmid DNA (200 ng) was digested with 0.5 µl of 10 u/µl restriction enzyme at 37 °C in a volume of 15 µl for at least 1 h or overnight depending on how well the digestion worked.

Between 2 and 5 µg of fungal genomic DNA was digested in a total volume of 35 µl. Appropriate digestion buffers were used and restriction enzymes added at 3U/µg DNA. Genomic DNA was digested with *Hin*dIII (Roche, Germany), *Cla*I (Roche, Germany) and *Eco*RI (Roche, Germany) at 37°C overnight. The following day, a sample (usually 1-2 µl of the total reaction mix) was checked for complete digestion on a 1% mini-gel. A low concentration of gel (0.75%) was used when the 1% agarose gel did not separate the bands of digested genomic DNA, particularly those bands that were bigger than 10,000 bp. When smears were achieved, indicating a complete digestion, the digestion solutions were run on a gel for Southern blot.

2.16.2 AGAROSE-GEL ELECTROPHORESIS

Unless otherwise stated, DNA samples were fractionated through a 1.0% (w/v) agarose gel in 1xTBE buffer. Gel loading dye (1/10 volume of total digestion mixture) was added to each sample of DNA before loading in the wells. Gels were run at 80 V at room temperature for 30-60 min followed by staining the gel in ethidium bromide solution (1 µl ethidium bromide : 10 ml H_2O) for 15-20 min. After a brief destaining in MQ water, the bands were visualized under short wave UV light and photographed. The sizes of the DNA fragments were estimated by comparing with a 1kb plus ladder (Invitrogen, USA) run alongside the DNA samples.

2.17 DNA PURIFICATION

2.17.1 PCR PRODUCT PURIFICATION

2.17.1.1 Commercial kits: QIAquick PCR Purification Kit (50)

This protocol (p18, QIAquick Spin Handbook) is designed for fragments ranging from 100 bp to 10 kb. DNA products were purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge, according to the manufacturer's instructions.

2.17.1.2 DNA extraction from seaplaque agarose

DNA was run on a 1% SeaKem LE agarose gel (Cambrex, USA) in 1x TAE at 4°C for 1-2 h at 70-80 V. After DNA staining and a brief destaining in water. The gel was viewed under long wave UV light and a very small portion of the gel containing DNA of interest was excised with a scalpel and placed into pre-weighed 1.5 ml eppendorf tubes. The DNA was then extracted from the melted agarose using a commercial kit, i.e. QIA quick Gel Extraction Kit (Qiagen). The resulting DNA concentration was determined by checking on a minigel as in Section 2.15.2.

2.17.2 FUNGAL GENOMIC DNA PURIFICATION

2.17.2.1 Phenol/chloroform extraction and ethanol precipitation

An equal volume of phenol and chloroform were added to the sample of genomic DNA. After 3 min centrifugation, the aqueous (top) phase was transferred to a clean tube and an equal volume of $CHCl_3$ was added. The components were mixed thoroughly before centrifugation for 1 min. The aqueous phase was removed, followed by addition of 1/10 volume of 3 M Na acetate and incubated at 4 °C for 5 min. Subsequently 2.5 volumes of 95% ethanol or 0.6 volumes of isopropanol were added prior to 15 min centrifugation at 13000 rpm (1.5 ml eppendorf tubes) at 4°C. The precipitated DNA was washed with 1 ml

of 70% cold ethanol to remove excess salt before drying for 30 min at room temperature. Finally, DNA was resuspended in sterile water or diluted TE buffer (10/0.1) overnight before storage at 4°C.

2.17.2.2 Al-Samarrai and Schmid (2000) method purification

This purification method was based on that described by Al-Samarrai and Schmid (2000). DNA samples (about 500 µl) were added to 165 µl 5 M NaCl. After several inversions for mixing, the suspension was centrifuged for 15 min at 4 °C at 13000 rpm (1.5 ml eppendorf tubes). This stage further precipitated the remaining cellular debris, protein and polysaccharides. The supernatant was recovered in a fresh tube, followed with an immediate addition of 1 volume chloroform.

2.18 CONCENTRATING FUNGAL GENOMIC DNA

When necessary such as for loading of DNA onto a gel for a Southern Blot, fungal genomic DNA was concentrated on the Speed Vacuum Concentrator before loading. When the volume of DNA solution was more than 500 µl, using the Speed Vacuum Concentrator was inappropriate, and reprecipitation and resuspension were performed using the ethanol or isoproponal precipitation method described in Section 2.17.2.1.

2.19 LIGATION

The ligation method was based on a modification of the method of by Dugaiczyk *et al* (1975). Ligations were carried out using 1 µl of 10 x ligation buffer (Roche, Germany), 20 ng of DNA insert, 60 ng of vector, 1 µl of T4 DNA ligase (Ro`che, Germany), and MQ water to 10 µl. Incubation was at room temperature for 2 h, followed by 4°C overnight.

2.20 TRANSFORMATION OF *E.COLI* BY ELECTROPORATION

Plasmids were transfected into *E. coli* XL-1 cells by the method of Dower *et al*., (1988) using a Biorad Gene Pulser Transfection Apparatus set to 25 μF and 2.5 kV and Pulse Controller set to 200 Ω. After electroporation, the cells were immediately resuspended in LB medium and incubated for 30 min at 37°C with vigorous shaking. Positive controls with uncut circular vector and negative controls with water were also included. The cells were then plated on selective (ampicillin or chloramphenicol, Section 2.2.1) LB plates. X-gal and IPTG (Section 2.2.1) were also used to allow for screening of the white recombinant transformants

2.21 SOUTHERN BLOTTING AND HYBRIDIZATION

2.21.1 GEL PREPARATION

The DNA samples for Southern blotting were digested by the method described in Section 2.16.1. After complete digestion, all DNA, including wild type and transformants' genomic DNA, and 100 pg linearized plasmid DNA were separated by electrophoresis (Section 2.16.2) at 4°C with a voltage of 30 V for about 8 h. The gel was stained with ethidium bromide (Appendix 2.8) solution for 15 min and photographed with a ruler adjacent to it to determine the size of the hybridising bands.

2.21.2 SOUTHERN BLOTTING

The method used for Southern blotting was based on that of Southern (1975). The gel was depurinated in 250 mM HCl for 10 min with constant agitation. After a brief rinse with Milli-Q water, the gel was denatured in denaturing solution (appendix 2.15.2) for 2x 15 min, followed by directly transferring into neutralization solution (appendix 2.15.3) for at least 30 min. All procedures were performed with constant gentle agitation. The gel was finally washed with 2x SSC before blotting.

During gel treatment, a nylon membrane and blotting wicks were prepared. The membrane was prepared with Hybond-NX (Amersham, UK), and the long wicks were prepared from Whatman 3 MM (Whatman, UK) chromatography paper.

Wicks were wetted one by one in 20x SSC and lay across a glass bridge on top of each other. Bubbles from between the glass plates and each of the wicks were removed. A sheet of gladwrap was laid over the wicks, and a "window" approximately 1-2 mm smaller than the size of the gel to be blotted was removed. A few ml of 20 x SSC was pipetted over the "window" of the top wick, then the gel was gently placed upside down on top of the wicks, followed by a flood with 20 x SSC and the nylon membrane placed over the gel. Then three small filter papers were placed on the top of the nylon. All these procedures were devoid of bubbles. Finally, paper towels were laid over the filter paper plus a weight on top of them. This assembly was left to blot overnight. The exact length of time required depended on the thickness of the gel.

2.21.3. PROBE PREPARATION

Probe DNA was from PCR products. Three kinds of PCR products were obtained, i.e. *hph* gene, *GFP* gene, and part of the *P. chlamydospora* MOXY gene were amplified with the corresponding pair of primers. Templates for PCR were three plasmids containing each gene, respectively (General PCR conditions refer to Section 2.8.2, each annealing temperature refers to Table 2.4). PCR products were checked through electrophoresis, followed by purification with a commercial QIAquick PCR purification kit (Qiagen) and gel extraction (QIAquick Gel Extraction kit, Qiagen) before quantification with a fluorometer (Hoefer DyNA Quant 200) (Section 2.15.1).

After PCR products were purified and quantified, they were used for probe labeling with a DIG high prime DNA labeling and detection starter Kit II (Roche, Germany). After labeling, these successfully labeled probes were quantified again with a fluorometer, and labeled before stored at –20°C until use.

Hybridization (including prehybridization) and probe detection were according to the instruction manual of Roche DIG Kit (Roche, Germany). After film development (~30

min), the probed membrane was washed for stripping the hybridized probe according to the instruction manual before other hybridizations with different probes.

2.22 SCREENING FOR *P. CHLAMYDOSPORA MOXY* GENE DISRUPTED TRANSFORMANTS

2.22.1 FIRST ROUND SCREENING

2.22.1.1. Sample preparation

After subculturing and purification of *P. chlamydospora* transformants containing plasmid pBCH-MOXY, groups of 5 different transformants were subcultured as a pool onto a PDA plate with cellophane on the top of the agar until around 100 transformants were subcultured. Then these plates were incubated at a temperature of 22°C for 20-28 days before being harvested for genomic DNA extraction (Section 2.14.3).

2.22.1.2 Transformant genomic DNA extraction

After incubation, the cellophanes with grown *P. chlamydospora* transformants were peeled off from the agar, carefully harvested, and each pooled mycelium placed into an eppendorf tube with sterilized scalpels for freeze-drying. Finally genomic DNA extraction was performed using the method described in Section 2.14.3.

2.22.1.3 PCR screening for the P. *chlamydospora moxY* gene knock outs based on the pooled genomic DNA.

The pooled transformant genomic DNA was quantified using a fluorometer before PCR preparation. Around 20-60 ng DNA was used for each PCR template. One primer (i.e. pBCH-MOXY1) was designed from the region just upstream of the cloned MOXY gene fragment, and the other primer (i.e. MOXY3) was designed from plasmid pBC-hyg which is downstream of the cloned MOXY gene fragment (refer to chapter four). The direction of the DNA sequence for primers design was based on the successful integration of the constructed plasmid (pBCH-MOXY) through homologous recombination. Another group of PCR with primer MOXY2 (one primer used to amplify

the cloned MOXY fragment) and MOXY3 was also included on the same PCR conditions, and served as a control. The concentrations of PCR reagents were (these concentrations were same for all PCR in this thesis): dNTPs: 0.2 mM; Taq polymerase (Invitrogen, USA): 0.04 u/µl; $MgCl_2$: 2 mM; Buffer: 1-fold; Primer: 0.2 µM. The conditions for this PCR were as follows:

94°C 2 min

94°C 30 sec

60°C 30 sec } **repeated 35 cycles**

72°C 1 min 30 sec

72°C 7min

4°C hold

Finally, PCR products were visualized by gel electrophoresis and UV transillumination as described in Section 2.16.2. The DNA pools that contained MOXY gene knock outs were determined by the appearance of a positive band with a size of ~1kb.

2.22.2 SECOND ROUND OF PCR SCREENING

The individual colonies in the pools that contain the *moxY* gene knock-outs were

determined, and each was inoculated onto single PDA plates with cellophane on the top. After incubation at 22°C for three weeks, the mycelia were harvested with a sterilized scalpel for genomic DNA extraction as described in Section 2.14.3, followed by a fluoremeter quantification (Section 2.15.1). Using the same conditions described as in the first round screening for PCR, the second round PCR was performed using each genomic DNA as a template, and the specific MOXY gene knock-outs were determined by the appearance of a positive band with the same size as in the first round screening, i.e. around 1kb.

2.23 PURIFICATION OF *P. CHLAMYDOSPORA* TRANSFORMANTS

2.23.1 MICROSCOPE PURIFICATION

The method for purification of all kinds of *P. chlamydospora* transformants was similar to that described in Section 2.7 except that mycelium at the very edge of the colony was taken instead of a section of single hyphae as a single hypha could not be distinguished and picked out even under a microscope.

2.23.2 SPORE PURIFICATION OF *P. CHLAMYDOSPORA* TRANSFORMANT

Plasmid pBCH-MOXY *P. chlamydospora* transformants were further purified through spore suspension and plating. To allow rapid production of conidial suspensions, a liquid culture was grown for this isolate in 100 ml of potato dextrose broth, which had been prepared in a 250 ml conical flask sealed with cotton wool and tin foil. Each flask was inoculated with five actively growing culture blocks of 3 mm diameter made with a cork borer. The flasks then were incubated at 22°C for four days with constant shaking at 100 rpm. Subsequently, the spore suspension was quantitated with a cytometer as that described in Section 2.4. When necessary, dilutions were performed before plating onto selective PDA plates supplemented with Hygromycin (100 μg/ml). Then these plates were incubated at 22°C for 7-10 days for single spore isolates. Finally, a single spore was picked up and subcultured for a repeat of the above process.

2.24 COMPARE THE GROWTH RATE OF MOXY GENE KNOCK-OUTS AND WILD TYPE *P. CHLAMYDOSPORA*

After purification, MOXY gene knock-out transformants PC82 and PC87, ectopic transformant PC86, pAN7-1 transformant, and pCT74 transformant, were inoculated at the same time to fresh PDA plates with wild type colonies in the middle and other colonies around the wild type *P. chlamydospora* colony. The size of agar plugs

containing each sample was exactly the same (prepared with 3 mm cork borer). Then all these plates were incubated at 22ºC for two weeks. Finally the plates were photographed and the sizes of colonies in the plates were measured and recorded.

2.25 COMPARISON OF THE TRANSFORMANTS METABOLITES

2.25.1 CULTURING SAMPLES

Pure *P. chlamydospora* cultures, including two MOXY gene knock-out transformants PC87 and PC82, one ectopic transformant PC86, and wild type *P. chlamydospora*, were inoculated onto PDA plates after purification. After 2-3 weeks incubation, five agar plugs from each strain and pure agar (uninoculated PDA plate) made with a 3 mm cork borer were inoculated into a 125 ml flask containing autoclaved 50 ml PDB, covered with cotton wools or foam bungs, followed by a constant shaking (at least 100 rpm) at 22ºC for ten days. Four replicates from each strain and from uninoculated PDA were performed. During the incubation period, marked Whatman filter circles that fit the Buchner funnel were dried individually in an 80ºC oven for four hours. Then the papers were cooled in a dessicator with silica gel and weighed to the nearest mg.

2.25.2 COLLECTION OF FILTRATE AND MYCELIUM

A clean Buchner funnel and flask with a vacuum arm was used for each strain. The

content of each flask was poured into the Buchner funnel with one of the filter papers in place, followed by collections of the filter papers (each containing the mycelium from one flask). The filter papers were dried in an oven at 80 ºC for four hours before cooling in a dessicator. Then they were reweighed on a fine balance to the nearest mg. Approximately 10 ml of the filtrate collected in the flask from each sample was placed into an autoclaved glass universal bottle for subsequent spectrophotometer assays.

CHAPTER THREE TRANSFORMATION OF *E. LATA*

3.1 INTRODUCTION

Due to a lack of molecular analyses that can characterize pathogenicity and virulence factors of this fungus, *E. lata* is still poorly understood. Recent studies on *E. lata* are mainly focused on putative toxic secondary metabolites such as eutypine (Smith *et al.*, 2003). However the roles of these metabolites in disease have not yet been determined. This is partly due to the lack of naturally occurring mutants and partly to the lack of a well-developed transformation system that would allow toxin genes or genes involved in toxin production to be characterized by transformation-mediated gene disruption. Thus, it is desirable to develop a transformation system and use it as a tool to investigate mechanisms involved in grapevine disease.

Furthermore, the mechanism by which *E. lata* colonizes and infects its host grapevine, i.e. the internal interaction between the fungus and its host has not yet been discovered. We are unsure how this fungus interacts with its host tissues and moves inside these tissues. Recent developments in the use of the reporter gene *gfp* to study the interactions between pathogens and host plants inspired us to use *gfp* as a tool for tracking and monitoring the movement of *E. lata* inside grapevine wood. A transformation system was required to introduce the *gfp* gene into *E. lata*.

Gene transformation of filamentous fungi is a relatively new area although great advances have been achieved. The literature illustrates a diverse range of strategies and procedures which work for specific fungal species, but unfortunately none of the approaches can be universally applied to all fungi. Currently, the most popular method for filamentous fungi is the classical protoplast/PEG method using hygromycin B resistance gene (*hph*) as a dominant selectable marker.

Overall, a well developed transformation system for *E. lata* is an important tool for a thorough study on this fungus at the molecular level.

3.2 OVERVIEW OF TRANSFORMATION RESULTS

Subculturing of wild type *E. lata* for harvesting fresh protoplasts was performed before each transformation. The method used for fungal protoplast preparation was described in Section 2.4 and the method used for *E. lata* transformation was described in section 2.6. In order to have statistical significance, four independent transformations with newly prepared protoplasts were performed on separate occasions with the same conditions. Transformations with plasmid pAN7-1 as well as plasmid pBCH-*gfp* were performed simultaneously. Plasmid pAN7-1 (appendix 3A) is widely used for fungal transformation as it contains a selective marker, an antibiotic resistance gene *hph*, which confers hygromycin B resistance to transformants. Plasmid pBCH-*gfp* (appendix 3C) was constructed based on the plasmid pBC-hygro (appendix 3B) into which a *S-gfp* gene from plasmid pFAT-3*gfp* (appendix 4A) was introduced. Hence it not only contained an *hph* gene, but also an *S-gfp* gene. Transformants containing this *S-gfp* gene were examined for *gfp* expression with a fluorescence microscope. If expressed, inoculations with this *gfp*-expressing transformant were performed for monitoring the interaction between *E. lata* and a host plant, blackcurrant. In addition, transformation with plasmid pCT74 (appendix 3F) (Lorang *at al*, 2001) was also performed for obtaining transformants containing an *S-gfp* gene driven by a stronger promoter (*ToxA* promoter) than that (p-*gpd*) in plasmid pBCH-*gfp*. Controls without plasmid DNA were also included.

In general, transformations with plasmids pAN7-1 and pBCH-*gfp* were successful. The pBCH-*gfp* transformants G8, G21, and G22 were examined for *gfp* expression under a fluorescence microscope (Section 2.11), but the *S-gfp* was not expressed. Transformation with plasmid pCT74 was also successful. Transformants S1, S4, and S6 were examined with the same method. S-*gfp* was expressed in transformants S1 and S6; however it was not expressed in transformant S4. These results are included in Section 3.7.

3.3 PROTOPLAST REGENERATION AND TRANSFORMATION

The transformation results and *E. lata* protoplast regeneration rate are summarized in Table 3.1.

NUMBER OF TRANSFORMANTS			*REGENERATION RATES*
Replicates	*pAN7-1 (4 μg)*	*pBCH-gfp(4μg)*	
1	*1*	*0 (19)*	*NT*
2	*7*	*5 (25)*	*2.29×10^{-3}*
3	*1*	*1 (25)*	*3.22×10^{-3}*
4	*6*	*5 (39)*	*4.95×10^{-3}*
Average rate	*Average rate: 1 per μg DNA*	*Average rate: 0.7 per μg DNA*	*3.49×10^{-3}*

Table 3.1 ***E10-10*** **Transformation results with plasmid pAN7-1 and pBCH-*gfp*, and the protoplast regeneration rate.**

NT: **Not tested. In pBCH-*gfp* transformations, the numbers in the brackets indicate total numbers of presumed transformants, including vigorously growing big colonies indicated in the table and non-vigorously growing colonies (abortive transformants). The regeneration rate was calculated as the ratio of regenerated protoplasts (colonies) : actual counted protoplasts.**

Most transformants obtained with plasmid pAN7-1 were large colonies, and could grow vigorously on selective media. However, around 90% of the presumed transformants obtained with plasmid pBCH-*gfp* was relatively small and most of them could not re-grow after subculturing onto selective media from non-selective media. Therefore, these presumed transformants were not real transformants, but were probably abortive transformants or leaky transformants (i.e. colonies escaped from hygromycin inhibition as a result of a large number of protoplasts in the plate).

Table 3.1 revealed that the frequencies of large transformants were consistent between the transformation experiments with the two different plasmids. The transformation

frequency in experiments 2 & 4 was higher than those in experiments 1 & 3. One possible reason for this was the difference in fresh protoplast concentration. The protoplast concentrations in experiments 2 (1.4×10^7 protoplast/ml) & 4 (2.1×10^7 protoplast/ml) were higher than those in experiments 1 (8.6×10^6 protoplast/ml) & 3 (5.75×10^6 protoplast/ml). Protoplast regeneration rate was generally poor and varied between $2.29 - 4.95 \times 10^{-3}$ (mean ± standard deviation of $3.49 \pm 1.35 \times 10^{-3}$) over three independent experiments although it was relatively consistent.

Two transformations with *gfp* plasmid pCT74 were also performed with the same method, and nine transformants were obtained in total. No abortive transformants were obtained. The transformation frequency was 0.9 transformants per μg DNA, which was consistent with previous transformations with pAN7-1 and pBCH-*gfp*. After subculturing for purification (Section 2.7), three of the vigorously growing transformants numbered S1, S4, and S6 were selected for further investigation along with the pAN7-1 and pBCH-*gfp* transformants.

3.4 SUBCULTURING AND PURIFICATION OF PRESUMED TRANSFORMANTS

The method for subculturing pAN7-1 and pBCH-*gfp* *E10-10* transformants was described in Section 2.7. Twenty-two transformants taken at random were numbered 1 to 22 (the letter "P" or "G" before the number indicates pAN7-1 or pBCH-*gfp* transformants, respectively), subcultured onto non-selective media, then back on to selective media again. In comparison with colonies on non-selective media, which all grew vigorously, growth of some colonies on selective media was totally inhibited, while others still grew vigorously (Table 3.2).

P1(V)	P2(V)	P3(V)	P4(V)	P5(V)	P6(V)	P7(V)	G8(V)	G9(V)	G10(V)	G11(V)
G12	G13	G14	G15	G16	P17(V)	P18(V)	G19	G20(V)	G21(V)	G22(V)

Table 3.2 Selection and Purification of *E10-10* transforamants.
Letter "P" indicates plasmid pAN7-1 transformant; Letter "G" indicates plasmid pBCH-*gfp* transformant; Letter "V" indicates the colony grew vigorously. Samples in shade are those that could grow after being resubcultured onto selective media.

The results above demonstrated that less vigorously growing colonies could not re-grow after purification. In contrast, more than half of the vigorously growing colonies could re-grow after purification. Based on this result, six of the well-growing colonies, i.e. P1(V), P2(V), G8(V), P18(V), G21(V), and G22(V) were selected for further purification on selective media and further characterization.

After transformants had been harvested and purified (method described in Section 2.7), each transformant was subcultured onto plates containing selective and non-selective media for checking their growth, with wild type *E10-10* included as a control (Figure 3.1).

Colonies like those numbered P1; P2; P3; G22, S1, etc, could still grow on selective medium after non-selective medium purification. In contrast, the wild type colonies in the centre (refers to Figure.3.1) were totally inhibited by hygromycin in the selective media.

3.5 CHARACTERIZATION OF *E10-10* TRANSFORMANTS WITH PCR AMPLIFICATION.

3.5.1. OPTIMIZATION OF PCR CONDITIONS

The method for PCR condition optimization was described in Section 2.8.1. The genomic DNA used for this optimization was from pAN7-1 transformant P3. The primers used for amplification were *hph* primers (Table 2.4), and the selective marker gene *hph* was amplified. The optimal amount of genomic DNA and the optimal concentration of [Mg^{2+}] required for PCR were determined as shown in Figure 3.2.

The optimization result revealed that in a total volume of 50 µl PCR reaction, 2 mM [Mg^{2+}] was optimal for this PCR reaction (PCR products with this concentration are most effective), while there was not a big difference between 25 ng and 50 ng of genomic DNA. Lane 8 without Mg^{2+} served as a negative control.

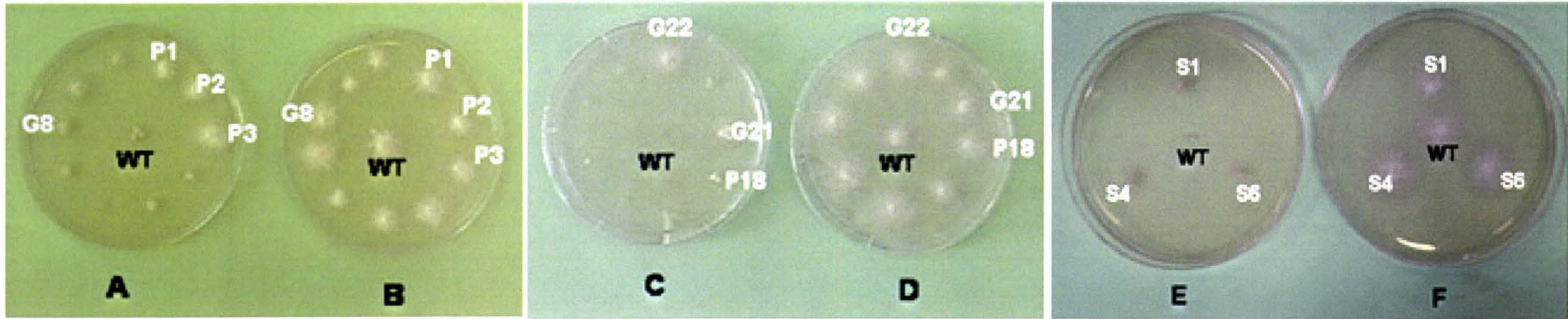

Figure 3.1 Some transformants grow on selective medium after subculturing from purified colonies on non-selective media.

P: pAN7-1 transformants; G: pBCH-*GFP* transformants; S: pCT74 transformants.
The right three plates (B, D, & F) contain non-selective media; the left three plates (A, C, & E) contain selective media. The colonies subcultured into the middle of the plates are wild type *E. lata.* Presumed transformants were subcultured at the edges of the plates; those chosen for further characterization are labeled with their names.

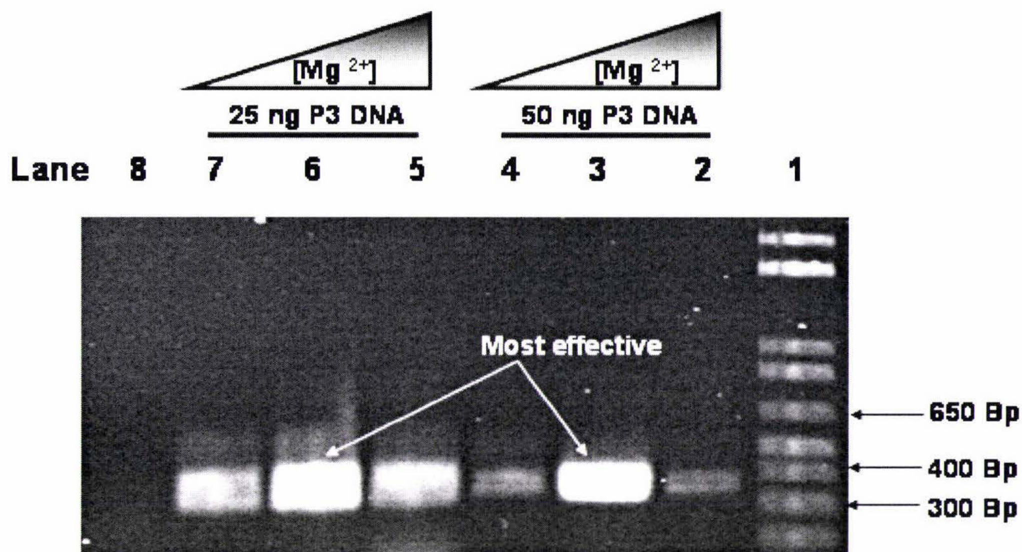

Figure3.2 Optimization of the amount of template DNA and the concentration of Mg^{2+} for PCR characterization.
Lane 1: 1kb^{+} ladder (Invitrogen, USA); Lane 2-4: PCR with 50 ng of P3 genomic DNA each; Lane 5-7: PCR with 25 ng of P3 genomic DNA each; Lane 8: PCR with 50 ng of P3 genomic DNA without Mg^{2+}; From Lanes 2, 3, 4 and lanes 5, 6, 7, the concentration of [Mg^{2+}] used for PCR decreased from 3 mM, 2 mM, to 1.5 mM, respectively.

3.5.2 CHARACTERIZATION OF PAN7-1 AND PBCH-*gfp* TRANSFORMANTS WITH *HPH* PRIMERS

Irrespective of the type of plasmid, a common component of plasmids used in this study is that they all contain an antibiotic resistance gene (*hph*) encoding hygromycin B which is a selection marker for transformation. Hence PCR amplification of the *hph* gene can be used to verify that transformants contain the plasmid.

Transformants were grown separately on PDA media for genomic DNA extraction using the method described in Section 2.14.3. Figure 3.3 shows that most of the transformants contain the *hph* gene, as the sizes (around 360 bp) of the PCR products in lanes 2, 3, 4, 5, 6, 9, 10, and 11 are identical with that in lane 12, which served as a positive control. In contrast, there is no PCR product in lane 13, which was a negative control with wild type *E10-10* genomic DNA. However, lanes 7 and 8 (transformants G10 and G11) do not contain this band although they are hygromycin resistant. Thus, the presence of the *hph* gene in these transformants was not shown with this method. Repeating this PCR still did not give positive results for these two assumed

transformants. The reason for this discrepancy is most likely due to a loss of the plasmid during the purification period.

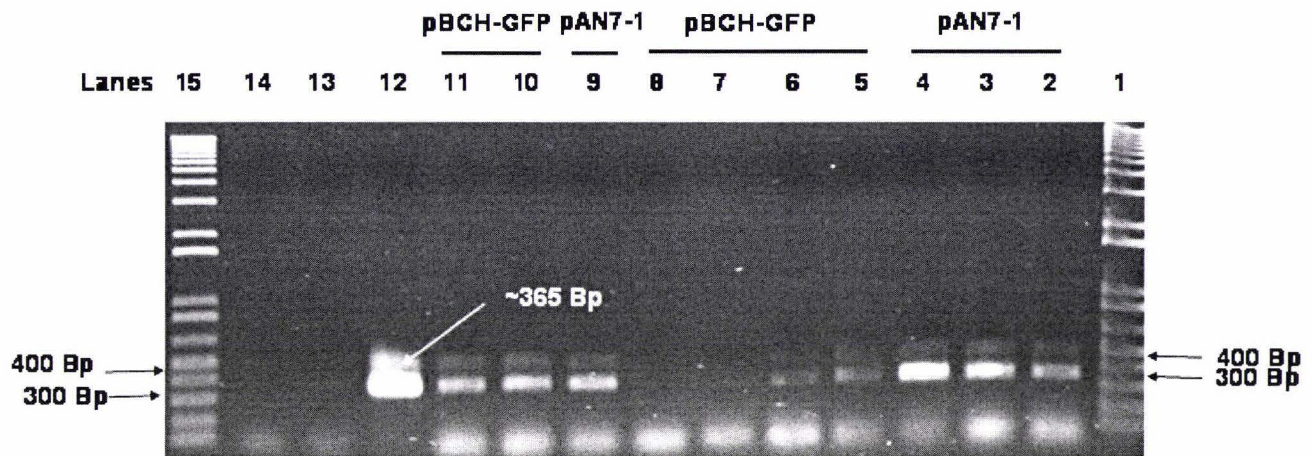

Figure 3.3 PCR characterizations of presumed *E.lata* transformants using genomic DNA and pAN7-1 DNA templates with *hph* primers.
Lane 1 & 15: 1kb⁺ ladder; Lanes 2-11: PCR products obtained using transformants genomic DNA as template. Lanes 2, 3, 4, and 9 are pAN7-1 transformants numbered P1, P2, P3, and P18 respectively; Lane 5, 6, 7, 8, 10, 11 are pBCH-*gfp* transformants numbered G8, G9, G10, G11, G21, and G22, respectively; Lane 12: PCR with 2 ng plasmid pAN7-1 DNA; Lane 13: PCR with wild type *E10-10* genomic DNA; Lane14: PCR without DNA template.

Based on this result, pAN7-1 transformants numbered P1, P2, and P18 (positive samples were chosen at random); pBCH-*gfp* transformants numbered G8, G21, and G22 were prepared for further analysis with Southern Blot and hybridization.

3.5.3 CHARACTERIZATION OF PCT74 TRANSFORMANTS WITH *HPH* PRIMERS

Since the pCT74 transformants were obtained after the pAN7-1 and pBCH-*gfp* transformants, they were characterized separately. PCR characterization with *hph* primers was performed for presumed pCT74 transformants, as shown in Figure 3.4 below.

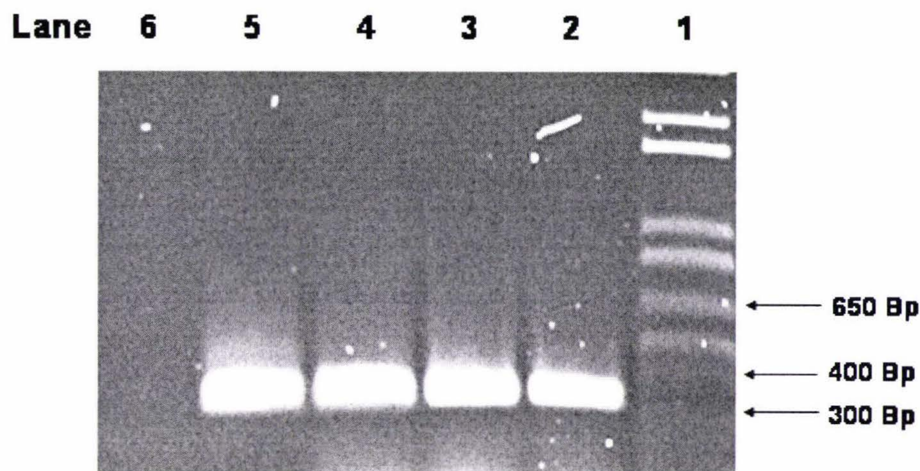

Figure 3.4 PCR characterizations of presumed pCT74 *E10-10* transformants.

Lane 1: 1kb+ ladder; Lane 2-4: PCR using template of transformants genomic DNA numbered S1, S4, and S6, respectively; Lane 5: PCR with plasmid pCT74 DNA; Lane 6: PCR with wild type *E10-10* genomic DNA;

This result revealed that a *hph* PCR product at ~365 bp was present in transformants S1, S4, and S6, respectively. As expected, this size is equivalent with that from the positive control (lane 5). The negative control with WT DNA (lane 6) lacks the PCR product.

3.5.4 CHARACTERIZATION OF pBCH-*gfp* TRANSFORMANTS WITH *gfp* PRIMERS.

As well as the *hph* gene, plasmids pBCH-*gfp* and pCT74 also contain the reporter gene *gfp*. Therefore, identification of this reporter *gfp* gene is an additional way to characterize these transformants. Primers *gfp*1 and *gfp*2 (Figure 3.5) were designed to amplify a 520 bp region of the *gfp* gene. The full sequence of plasmid pFAT-3*gfp* is shown in appendix 4A. The region containing the *gfp* ORF is shown below:

```
2951  ACTCCCGTCC TCGATCCTCA TCCTCATCTT CTCATCTCCT GCCTCTCCCC
                                                GFP ORF ——►
3001  GTCCTCTTCC TCCTCAGGTC TCTCGCTCCG CCTCTTGTCT GTCAAGATGA
                 ————GFP1 ————►
3051  GCAAGGGCGA GGAACTGTTC ACTGGCGTGG TCCCAATTCT CGTGGAACTG

3101  GATGGCGATG TGAATGGGCA CAAATTTTCT GTCAGCGGAG AGGGTGAAGG

3151  TGATGCCACA TACGGAAAGC TCACCCTGAA ATTCATCTGC ACCACTGGAA

3201  AGCTCCCTGT GCCATGGCCA ACACTGGTCA CTACCTTCAC CTATGGCGTG

3251  CAGTGCTTTT CCAGATACCC AGACCATATG AAGCAGCATG ACTTTTTCAA

3301  GAGCGCCATG CCCGAGGGCT ATGTGCAGGA GAGAACCATC TTTTTCAAAG

3351  ATGACGGGAA CTACAAGACC CGCGCTGAAG TCAAGTTCGA AGGTGACACC

3401  CTGGTGAATA GAATCGAGCT GAAGGGCATT GACTTTAAGG AGGATGGAAA

3451  CATTCTCGGC CACAAGCTGG AATACAACTA TAACTCCCAC AATGTGTACA

3501  TCATGGCCGA CAAGCAAAAG AATGGCATCA AGGTCAACTT CAAGATCAGA
                 ◄————GFP2————
3551  CACAACATTG AGGATGGATC CGTGCAGCTG GCCGACCATT ATCAACAGAA

3601  CACTCCAATC GGCGACGGCC CTGTGCTCCT CCCAGACAAC CATTACCTGT

3651  CCACCCAGTC TGCCCTGTCT AAAGATCCCA ACGAAAAGAG AGACCACATG

3701  GTCCTGCTGG AGTTTGTGAC CGCTGCTGGG ATCACACATG GCATGGACGA

3751  GCTGTACAAG TGAGATCCAT GTGCTGAGCA TTGGAGGATG TTGATGAGAA
```

Figure 3.5 Sequence analysis of *gfp* gene for primer design.
DNA sequence in red which is from 5' to 3' is *gfp* ORF DNA sequence. Two DNA sequences in green are the regions to which two primers were designed. The arrows are indicating primer direction from 5' to 3'.

Figure 3.6 shows the results of the PCR of the *gfp* gene. This result indicates that plasmid pBCH-*gfp E10-10* transformants G8 & G21 did contain the *gfp* gene, which was introduced by plasmid pBCH-*gfp*. However, presumed transformants G9 & G22 did not contain it.

3.5.5 CHARACTERIZATION OF PCT74 *E10-10* TRANSFORMANTS WITH *gfp* PRIMERS

The *gfp* gene located in plasmid pBCH-*gfp* was cloned from plasmid pFAT-3*gfp* based on which *gfp* primers were designed, whilst the *gfp* gene in pCT74 was from pBlue-S*gfp*-TYG-nos-KS (from Jen Sheen, Department of Molecular Biology,

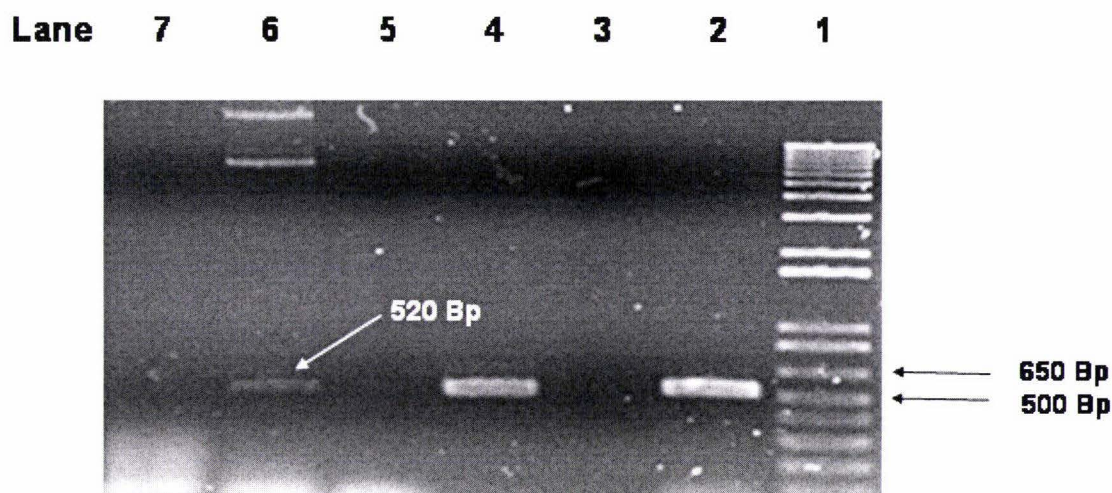

Figure 3.6 PCR characterizations of presumed *E. lata* transformants using genomic DNA and pBCH-*gfp* plasmid DNA as templates and *gfp* primers.
Lane 1: 1kb$^+$ ladder; lanes 2-5: PCR products based on different transformants (numbered G8, G9, G21, and G22, respectively) genomic DNAs; lane 6: PCR product based on pBCH-*gfp* DNA; lane 7: PCR with wild type *E10-10* genomic DNA.

Massachusetts General Hospital, Harvard University), which contains S*gfp*-TYG (for brighter fluorescent signals, the serine at position 65 in the chromophore is replaced with a threonine, i.e. a shift from SYG to TYG) (Chiu *et al*, 1996) on an *Nco*I/*Not*I DNA fragment (Lorang *et al*, 2001). Therefore it was necessary to test these primers were still functional for amplification of the *gfp* gene in plasmid pCT74. This test was performed simultaneously with PCR characterization of pCT74 *E10-10* transformants. These results are shown in Figure 3.7 below.

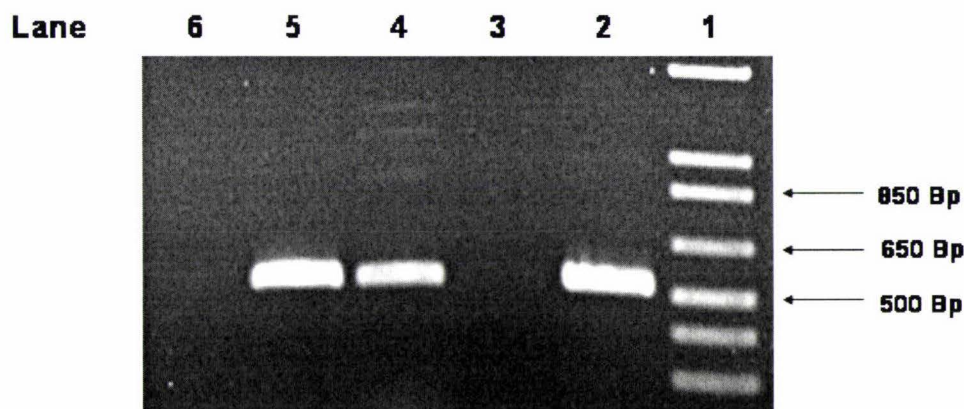

Figure 3.7 Characterization of presumed pCT74 transformants with *gfp* primers
Lane 1: 1kb$^+$ ladder; lane 2: sample S1; lane 3: sample S4; lane 4: sample S6; lane 5: PCR based on plasmid pCT74 DNA; lane 6: PCR with Wild type *E10-10* genomic DNA as template.

Compared with the negative control of wild type *E10-10* genomic DNA as a template in lane 6, lanes 2, 4, and 5 (positive control) have positive bands with the same size of around 520 bp. This result revealed that the *gfp* primers with a design based on plasmid pFAT-3*gfp* for amplification of the *gfp* gene in pBCH-*gfp* were also suitable for amplification of the *gfp* gene located in plasmid pCT74. Positive results from samples S1and S6 demonstrated that these presumed pCT74 *E10-10* transformants do contain *gfp* gene(s). As for how many copies of this *gfp* gene, this needs to be confirmed by Southern blotting and hybridization. The transformant S4 (lane 3) did not give a positive band, indicating an absence of the *gfp* gene in the genome. This also is consistent with the later results from fluorescence microscope check for fluorescence and Southern hybridization (for more details, see discussion in this chapter).

3.6 CHARACTERIZATION OF *E10-10* TRANSFORMANTS WITH SOUTHERN HYBRIDIZATION

3.6.1 DIGESTION AND GEL ELECTROPHORESIS FOR SOUTHERN BLOTTING

The transformants including plasmids pAN7-1, pBCH-*gfp*, and pCT74 that gave a positive *hph* PCR result were chosen for further characterization through Southern hybridization. The method used for genomic DNA digestion is described in Section 2.16.1, and method for Southern blotting is in Section 2.21.

Lane18 17 1615 1413 1211 10 9 8 7 6 5 4 3 2 1

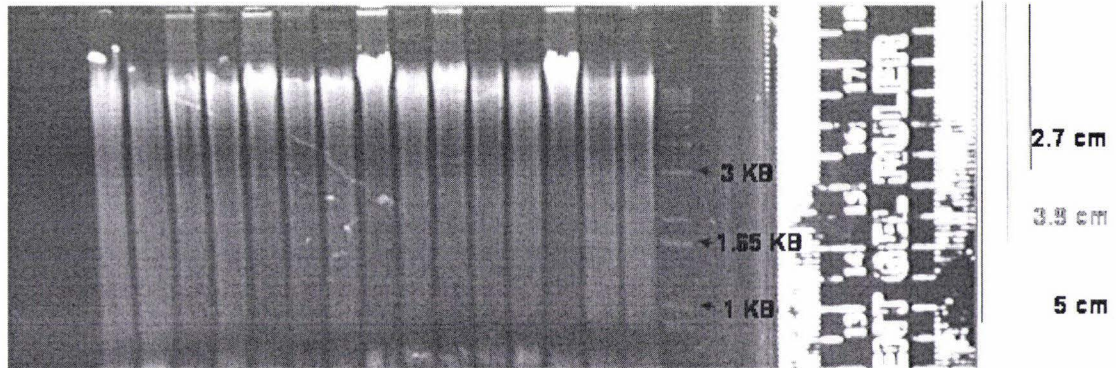

Figure 3.8 Digested transformants' genomic DNA in the gel before Southern Blot

Lane 1: 1kb$^+$ ladder; Lane 2, 3, and 5: pAN7-1 transformants (P1, P2, and P18) with *Hin*dIII; Lane 4 & 6: pBCH-*gfp* transformants (G8 and G21) with *Hin*dIII; Lane 7, 8, and 10: pAN7-1 transformants (P1, P2, and P18) with *Eco*RI; Lane 9 & 11: pBCH-*gfp* transformants (G8 and G21) with *Eco*RI; Lane 12-14: pCT74 transformants (S1, S4, and S6) with *Eco*RI; Lane 15: Wild type *E10-10* with *Hin*dIII; Lane 16: Wild type *E10-10* with *Eco*RI; Lane 17: 100 pg plasmid pAN7-1 with *Eco*RI; Lane 18: 100 pg plasmid pCT74 with *Eco*RI;

3.6.2 HYBRIDIZATION WITH LABELLED *hph* PROBE.

The gel shown in Figure 3.8 was transferred to a nylon membrane through Southern blotting as described in Section 2.21.2, followed by *hph* probe hybridization (Section 2.21.3). The *hph* probe and *gfp* probe were prepared with the method described in Section 2.21.3 and were based on PCR products from each corresponding gene. Figure 3.9 below is the result of hybridization with labeled *hph* probe.

From the Figure 3.9, positive bands appeared in each lane except Lanes 15 & 16 (wild type *E10-10* genomic DNA), which served as negative controls. Lanes 17 & 18 were two digested plasmids pAN7-1 & pCT74, which served as two positive controls. From this, it was confirmed that the labeled *hph* probe was capable of hybridizing to *hph* DNA sequence in these plasmids. According to the plasmid maps in appendix 3A, there is only one *Hin*dIII site but two *Eco*RI restriction sites in plasmid pAN7-1. Therefore, when digested with restriction enzyme *Eco*RI, two bands of 2.5 kb & 4.1 kb were formed. From the positive control in lane 17, it was determined that the labeled *hph* probe hybridized to the larger band with 4.1 kb as expected. For plasmid pCT74, there is only one *Eco*RI and one *Hin*dIII restriction site, thus when digested with one of these enzymes, it would result in just one band with the whole plasmid size ~6 kb, which was consistent with the result shown in lane 18.

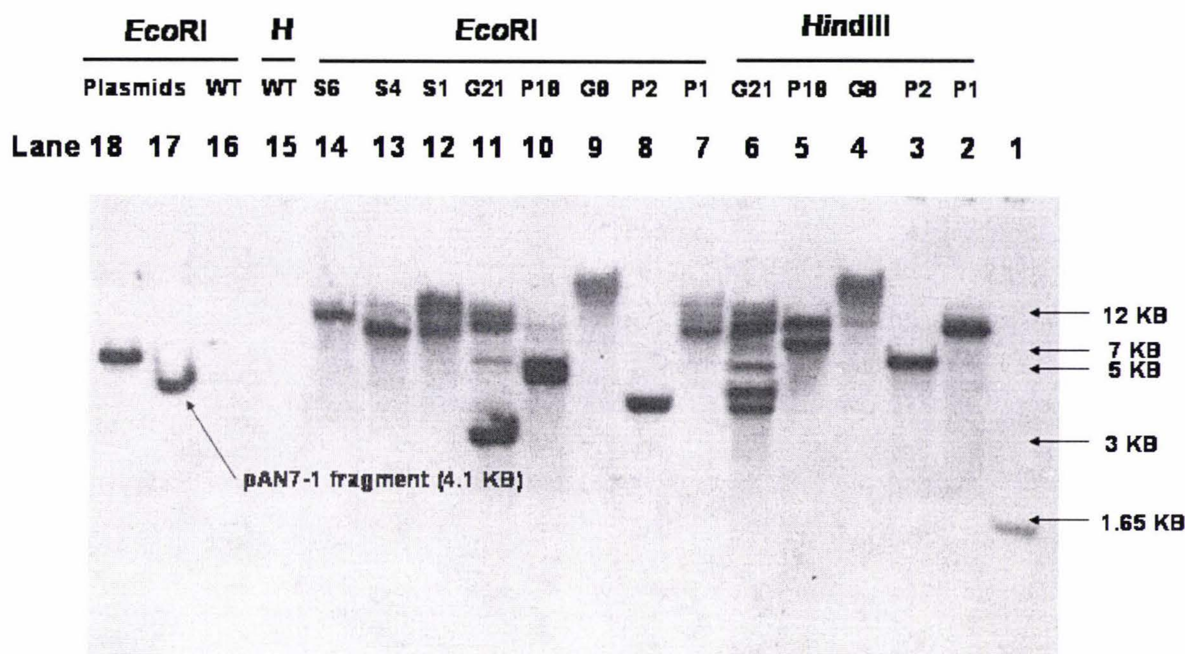

Figure 3.9 Hybridization of *E10-10* transformants' genomic DNA with labeled *hph* probe. For details see Figure 3.8.

From Lane 2 to Lane 14, *hph* probes also successfully hybridized to each *E10-10* transformants' genomic DNA, indicating that those transformants' genomic DNA contained the hybridization target, i.e. the *hph* gene DNA, which in turn supported this point that plasmids pAN7-1 & pCT74 had successfully integrated into fungal genomic DNA after transformation. In addition, lanes 5, 6, 10, 11, and 12, i.e. transformants P18, G21, S1 had multiple bands, indicating that multiple plasmid integration had occurred. For lanes 4 and 9 (sample G8), a clear hybridization band was not present, this was probably due to incomplete digestion of genomic DNA.

The hybridization bands varied in size depending on the site that plasmids integrated. The variability of the band size in this hybridization picture suggested that for plasmids pAN7-1 or pCT74, integration into the fungal genome was random. Size of fragments depends on the distance of restriction sites from the site of integration. For example, in transformant G21 with multiple plasmid pBCH-*gfp* integration in lane 11, an *Eco*RI site is close to one integrated plasmid, which resulted in a short hybridization band at ~ 3.1 kb, while another *Eco*RI restriction is further from the second integrated plasmid, which resulted in a big hybridization band with the size of ~10 kb.

3.6.3 HYBRIDIZATION WITH LABELLED *gfp* PROBE.

The DNA blot was stripped (described in Section 2.21.3 and re-probed with labeled *gfp* probe. The hybridization result is shown in Figure 3.10.

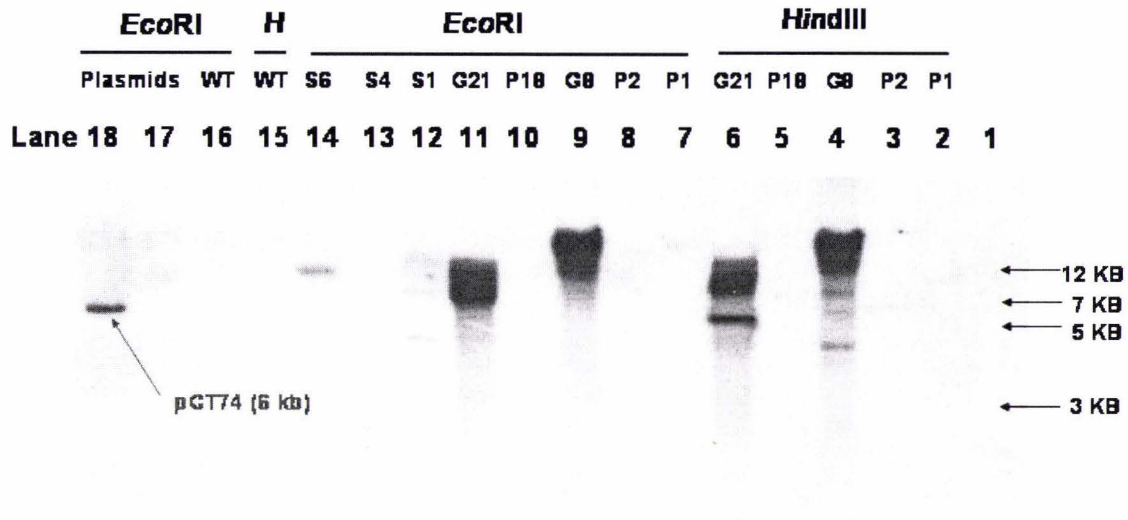

Figure 3.10 Hybridization of *E10-10* transformants' genomic DNA blot with labeled *gfp* probe. Legend is the same as that in Figure 3.8.

Using the labeled *gfp* DNA sequence as a probe, if positive bands appeared, then it could be concluded that plasmids pBCH-*gfp* or pCT74 had successfully integrated into the fungal *E10-10* genomic DNA. This result above revealed that the labeled *gfp* probe was hybridized to positive control plasmid pCT74 in lane 18 rather than plasmid pAN7-1 in lane 17, indicating that the *gfp* probe prepared from plasmid pBCH-*gfp* was capable of hybridizing to the *gfp* gene in plasmid pCT74. Lane 15 and 16 were wild type *E10-10* DNA, which served as two negative controls, no band(s) were present in these lanes. Lane 17 was plasmid pAN7-1 without a *gfp* gene, therefore no positive hybridization appeared.

Positive bands also appeared in lanes 4 & 6 and lanes 9 & 11, which were samples of pBCH-*gfp* transformants G8 & G21. Lanes 12-14 contained pCT74 transformed DNA samples. Positive band(s) also appeared in lane 12 & 14, which suggested that plasmid pCT74 had successfully integrated into *E10-10* genomes of transformants S1 & S6. Transformant S4 in lane 13 had no *gfp* hybridization band, indicating the absence of the *gfp* gene in the genome of transformant S4. However, from the hybridization result with

the *hph* probe (Figure 3.9), the presence of the *hph* gene was demonstrated. This suggested that pCT74 transformant S4 did not fully contain plasmid pCT74. Either pCT74 had not fully integrated into the fungal genome during transformation (i.e. only the *hph* gene and not the *gfp* gene were integrated), or that part containing the *gfp* gene had subsequently been lost during the selection or purification period. On the other hand, a further potential reason for the lack of *gfp* hybridization was because of the positions of restriction sites in the genomic DNA that might exert influences on Southern blotting. When the size of the restriction fragment containing the *gfp* gene was large, such as more than 12 kb, a successful blotting for this large fragment would not be achieved. This could lead to the failure of subsequent hybridization.

By referring to the PCR characterization result for transformant S4 shown in Figure 3.7, a *gfp* PCR product was not obtained, this was consistent with the result of *gfp* hybridization. However *hph* PCR characterization (Figure 3.4) indicated an integration of *hph* gene in the genome of S4. This suggested that only part of plasmid (containing *hph*) integrated, which conferred S4 hygromycin resistance, while leaving the *gfp* part outside of the genome.

It was also noticed that hybridizations of pCT74 transformants were fainter than that for pBCH-*gfp* transformants. From the first hybridization with probe *hph*, any gel loading problem was ruled out. As the probe was made based on plasmid pBCH-*gfp*, it was likely that *gfp* genes in pBCH-*gfp* transformants were hybridized more efficiently with the *gfp* probe. This indicated that these two *gfp* DNA sequences were slightly different, and were competitive against each other for binding *gfp* probe. However, this difference did not affect *gfp* primer annealing during PCR characterization (Figure 3.7).

In addition, two copies of pCT74 had entered into sample S1, which showed two separate bands in lane 12.

3.6.4 CONFIRMATION OF HYBRIDIZATIONS OF pCT74 TRANSFORMANTS WITH LABELED *hph* and *gfp* PROBES.

As the hybridization of pCT74 transformants was weak and because of an incomplete digestion of S4 indicated in Figure 3.8, Southern Blot and hybridization was repeated for pCT74 transformants digested with a different restriction enzyme, *Cla*I, rather

than *Eco*RI. According to the plasmid pCT74 map in appendix 3F, there is only one *Cla*I restriction site, and this site is not located in the *Sgfp* fragment, thus when digested, would result in one linear fragment with intact *Sgfp*. The digestion results for the Southern blot are shown in Figure 3.11 below.

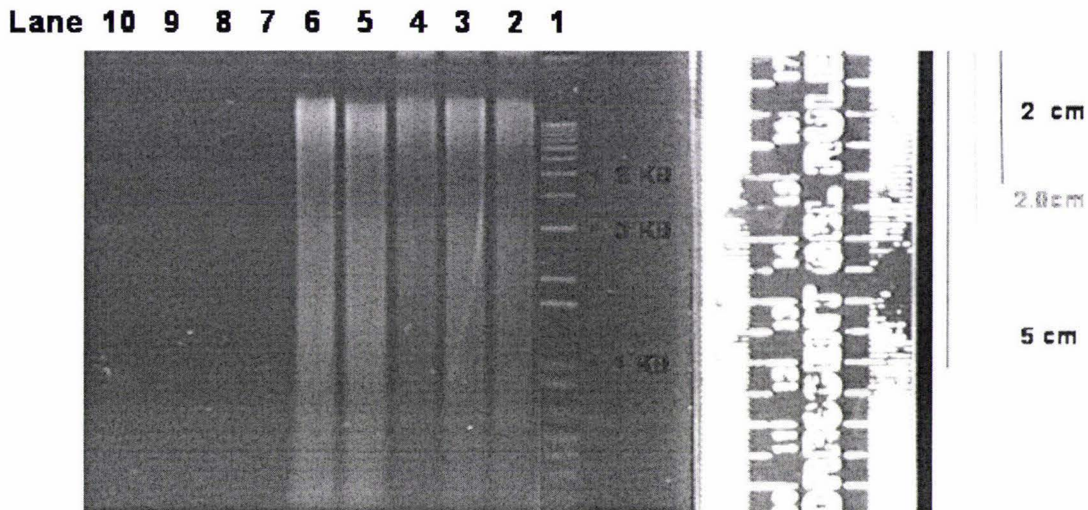

Figure 3.11 Digestion of pCT74 transformants' genomic DNA for Southern blot.

Lane 1: 1kb$^+$ ladder; Lane 2-4: pCT74 transformants (S1, S4, and S6, respectively) with *ClaI*; Lane 5: Wild type *E10-10* with *Hin*dIII; Lane 6: Wild type *E10-10* with *ClaI*; Lane 7: 10 pg plasmid pAN7-1 linearized with *Hin*dIII; Lane 8: 100 pg plasmid pAN7-1 linearized with *Hin*dIII; Lane 9: 10 pg plasmid pCT74 linearized with *ClaI*; Lane 10: 100 pg plasmid pCT74 linearized with *ClaI*;

The results shown in Figure 3.11 indicated that digestion of pCT74 transformants' genomic DNA was complete. Therefore Southern hybridization was performed with labeled *hph* and *gfp* probes. Hybridization results are shown in Figure 3.12.

In Figure 3.12, Lanes 7 & 8 and Lanes 9 & 10 served as controls for the *hph* probe and *gfp* probe, respectively. Lanes 4 & 5 was wild type DNA, which served as negative controls. Figure A indicated that the *hph* gene which is located in plasmid pCT74 has integrated into the genomes of all transformants S1, S4, and S6. Two copies were integrated into tranformant S1 (two bands appeared). From Figure B, it can be concluded that the *gfp* gene has integrated into the genome of transformants S1 and S6. However Lane 3 has no appropriate hybridization band and this is not consistent with the results probed with the *hph* probe, indicating that the *gfp* gene is not present in this transformant and confirming the results of the previous Southern blot.

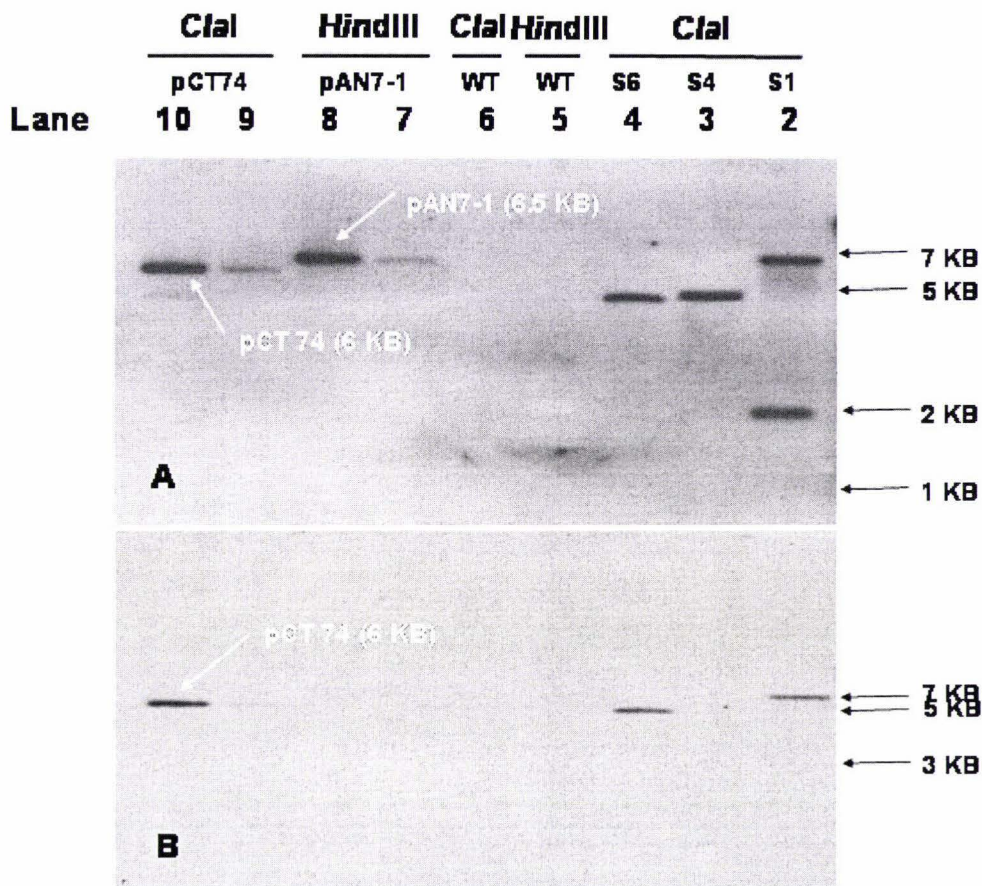

Figure 3.12 Confirmation of *hph* and *gfp* hybridizations to pCT74 transformants' DNA.

Legend refers to Figure 3.11. A: The DNA blot was probed with *hph* probe; B: The same DNA blot was probed with *gfp* probe prepared based on plasmid pBCH-*gfp*

3.7 DETECTION OF *gfp* GENE EXPRESSION IN *gfp* TRANSFORMANTS

3.7.1 DETECTION OF PBCH-*gfp* *E10-10* TRANSFORMANTS

Colonies of pBCH-*gfp* *E10-10* transformants (G8, G21, and G22) and wild type *E10-10* were checked for *gfp* expression with a fluorescence microscope. The method used for *gfp* expression detection was described in Section 2.11, and the results are shown in Figure 3.13.

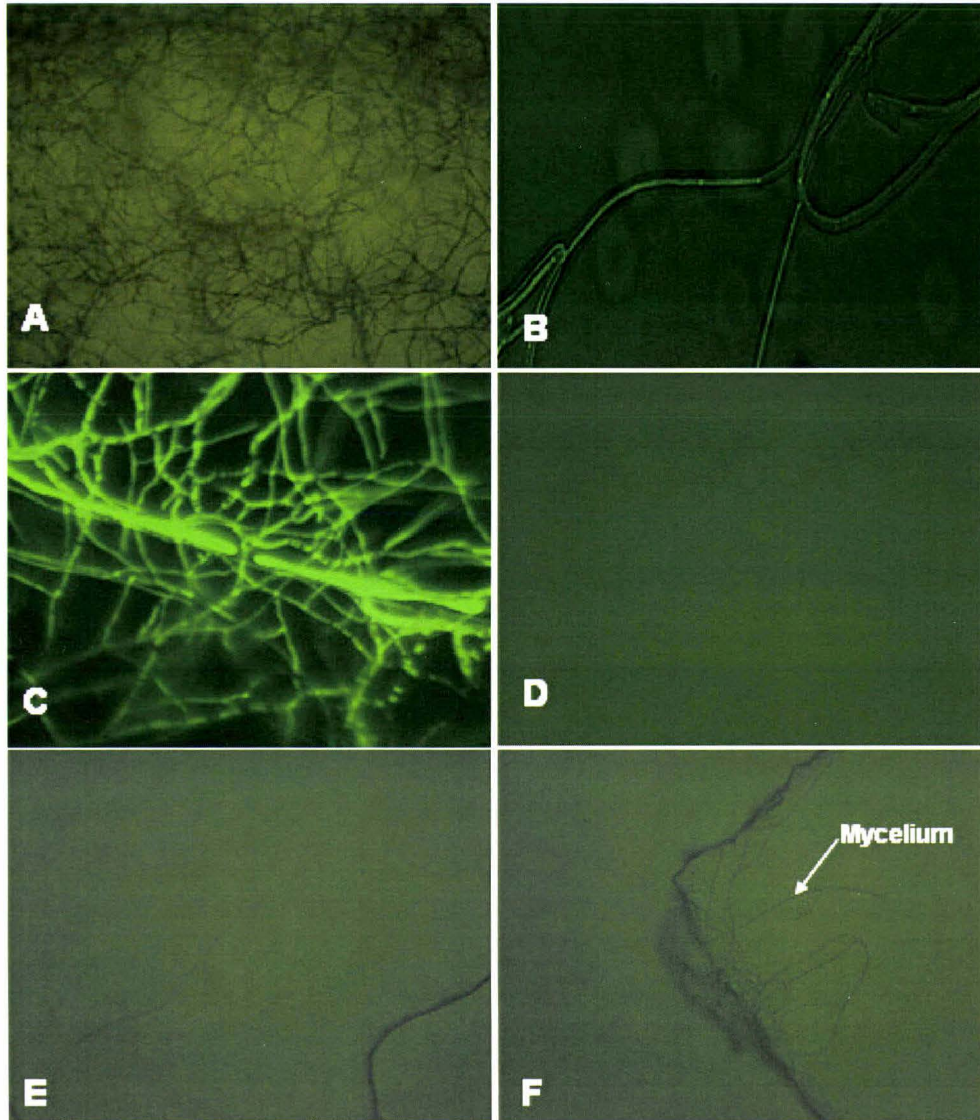

Figure 3.13 Fluorescence microscope check of *gfp* expression in pBCH-*gfp* transformants.

A & B: WT *E10-10* mycelia under UV light, mycelia in "A" were from an intact colony and checked directly in the plate; mycelia in "B" were checked on a slide. C: *gfp*-expressing pCT74 transformant S1, served as a positive control. D, E, and F: pBCH-*gfp* transformants G22, G21, and G8. Scale refers to Figure 3.16.

Compared with the controls, the Figures 3.13 above indicated that although the *gfp* gene in plasmid pBCH-*gfp* had successfully integrated into transformants G8, G21, and G22, it was not successfully expressed. These were repeated along with a positive control of pCT74 transformants, and the same results were obtained.

3.7.2 DETECTION OF pCT74 *E10-10* TRANSFORMANTS

Colonies of pCT74 transformants (S1 and S6) and wild type *E10-10* were checked under the fluorescence microscope on plates with the same method as for pBCH-*gfp* transformants. The detection result is pictured in Figure 3.14 below:

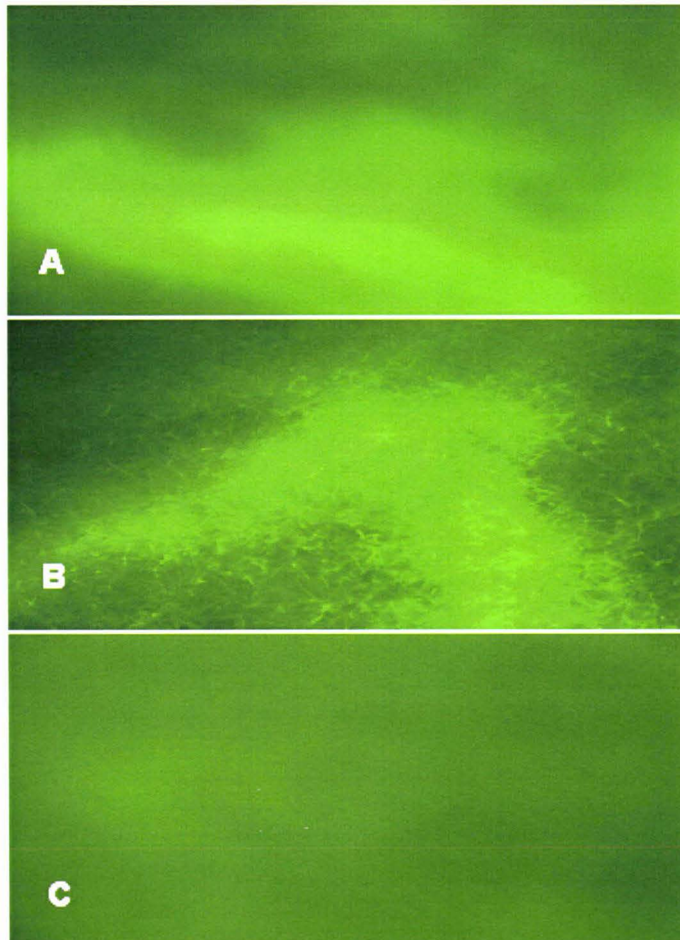

Figure 3.14 fluorescence microscope check of *gfp* expression in plasmid pCT74 *E10-10* transformant.

A: pCT74 *E10-10* transformant S1; B: pCT74 *E10-10* transformant S6; C: wild type *E10-10*

The results indicated that compared with the wild type image taken under the same conditions as that for pCT74 transformants, the *gfp* gene located in plasmid pCT74 was successfully expressed when integrated into the fungal genome after transformation.

gfp expression detection was studied in more detail through checking of single or clustered hyphae on slides under the fluorescence microscope (method described in Section 2.11). The results are shown in Figure 3.15 and 3.16 below:

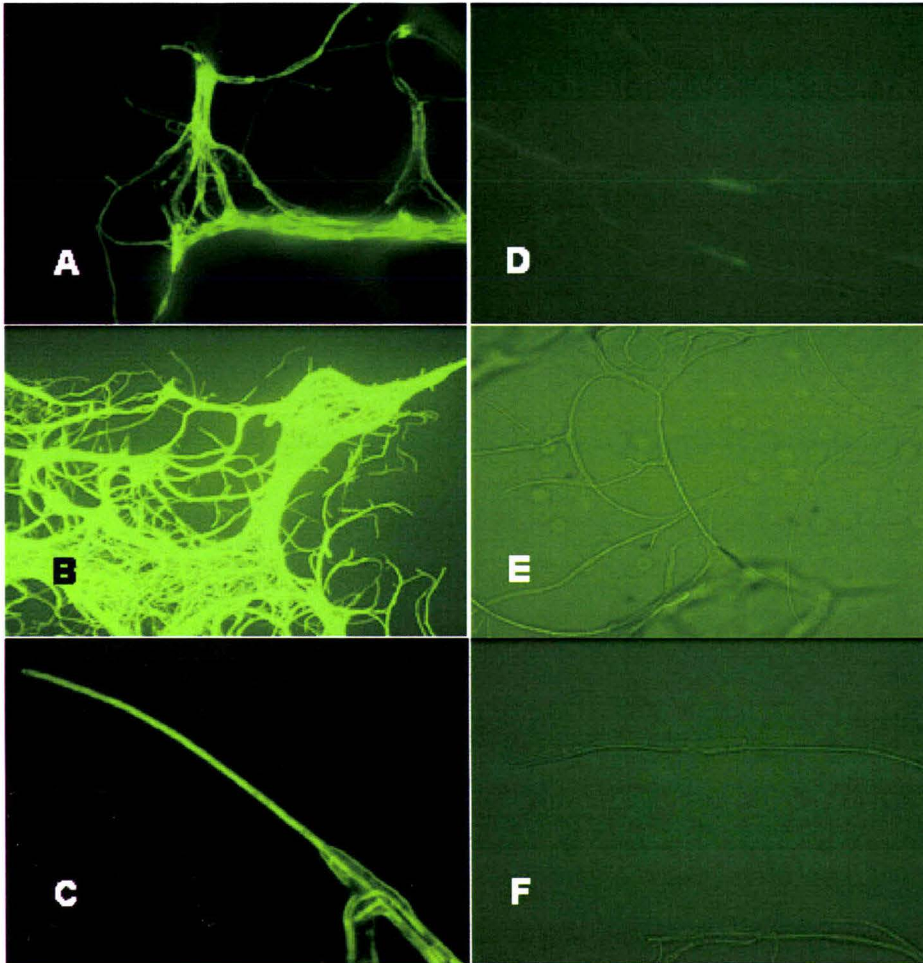

Figure 3.15 ***gfp*** **has been expressed in pCT74** ***E10-10*** **transformants.**

A, B, D, and E are clusters of hyphae. A and B are pCT74 transformants S6 and S1, and D and E are wild type ***E10-10*** **controls. C and F are single hyphae under the fluorescence microscope, and C is pCT74 transformant S1, while F is wild type hyphae. A, D, C, and F were under the same light intensity background conditions, while the brightness for B and E were increased for clearer hyphae image. Illumination with blue light at 460-490 nm wavelengths.**

3.8 CONFOCAL MICROSCOPE OBSERVATION OF *gfp* EXPRESSION AND THE INTERACTION BETWEEN *gfp E10-10* TRANSFORMANT AND ITS HOST BLACKCURRANT

With the achievement of a *gfp* expressing *E10-10* transformant, it is feasible to use this as a reporter to study interactions between the fungus and its host plants, including blackcurrant and grapevine. Thus any growth and movement of the fungus during the period of infection can be monitored and tracked in its host plant through a fluorescence microscope or confocal microscopy. As *E. lata* can colonize and grow in blackcurrant most vigorously, inoculations onto blackcurrants as well as grapevines were performed with the method described in Section 2.12.

The expressed *gfp* pCT74 *E10-10* transformant S1 as well as wild type *E10-10* were inoculated onto host blackcurrants, respectively. After three months incubation, pictures were taken for each sample as in Figure 3.17.

From these results, it can be concluded that fungus *E10-10* (both WT and transformant) could colonize and grow on both non-autoclaved and on autoclaved blackcurrant stems. However, growth on autoclaved samples (section A) was more active than that on nonautoclaved ones (Section B), suggesting that live blackcurrants may be resistant to the pathogenic fungus *E10-10* colonization and infection. In contrast, dead tissues had lost the ability to prevent such infection. An alternative suggestion is that *E. lata* is more capable of a saprophytic than a parasitic mode of growth.

After incubation (Section 2.12), blackcurrant blocks were harvested (as pictured in Figure 3.17) for sample preparation (method described in section 2.13) for confocal microscope observation (Section 2.13). Confocal pictures are summarized in Figure 3.18.

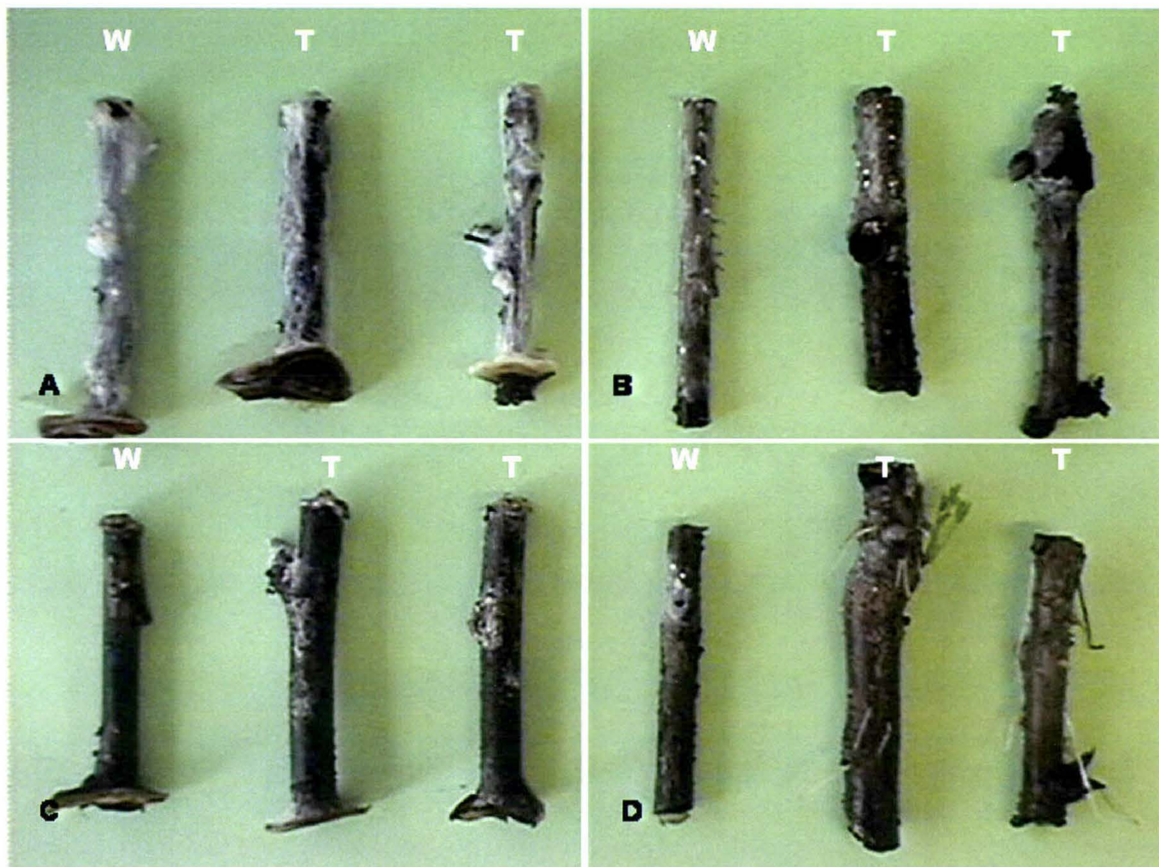

Figure 3.17 *gfp* expressing transformant and wild type *E10-10* growing on blackcurrant stems (three months since inoculation).

A & B: Inoculated blackcurrant incubated at room temperature; C & D: Inoculated blackcurrant incubated at constant 22°C; A & C: Blackcurrants were autoclaved before inoculation; B & D: Blackcurrants were not autoclaved, but cleaned with 75% ethanol before inoculation; W: Inoculated with wild type *E10-10;* T: Inoculated with *gfp* expressing pCT74 *E10-10* transformant S1; The top end of each blackcurrant stem was inoculated.

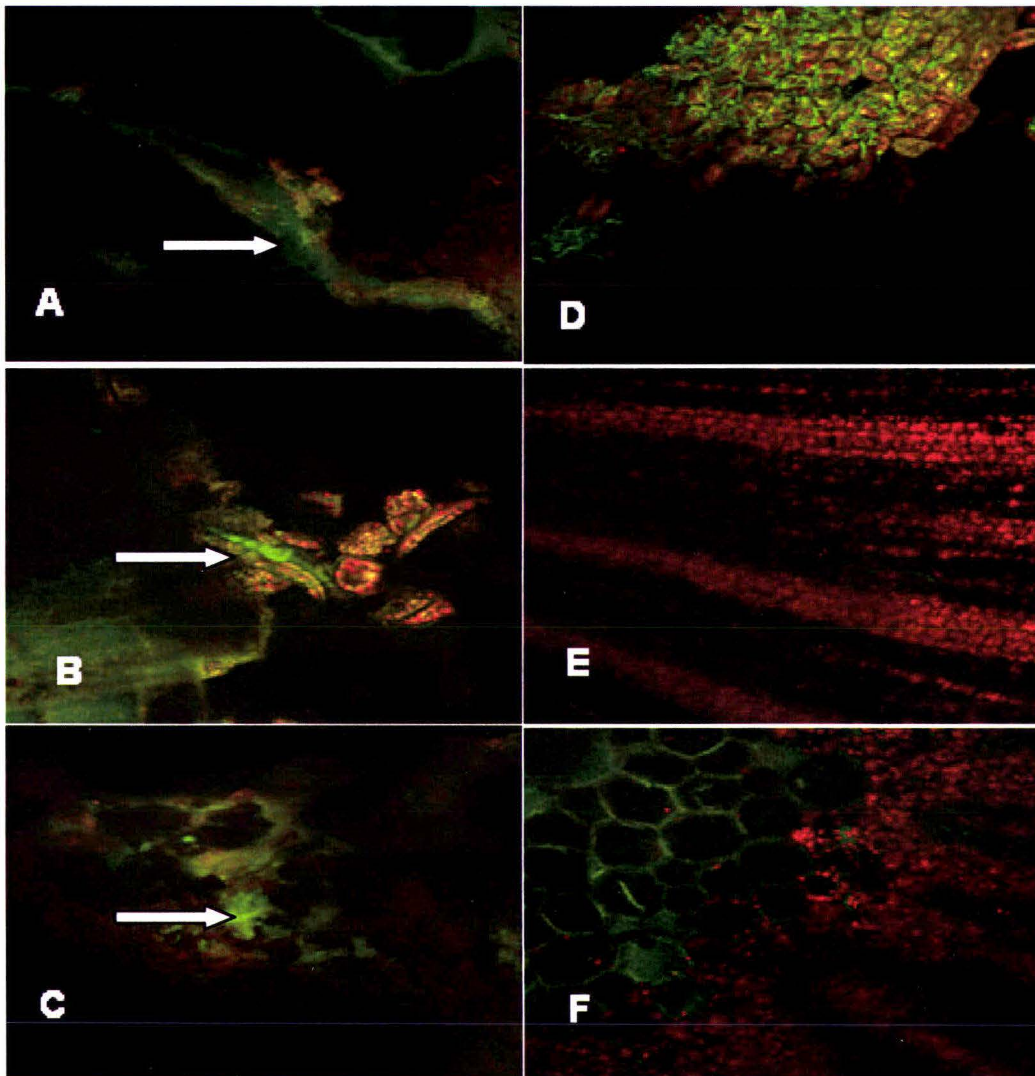

Figure 3.18 Confocal microscope observation of *gfp* expression in *E10-10* transformant growing outside and inside of inoculated blackcurrant stems (three months since inoculation).

A, B, and C: Woody tissue observations from pCT74 transformant inoculated blackcurrant; D: Bark picture taken from outside; E and F: Woody tissue observations inoculated with wild type *E10-10*; Arrows are pointing to the detected *gfp* expressing *E. lata*. All samples were prepared as transverse sections.

The confocal observation results suggested that *E10-10* did colonize and actively grow on bark of the grapevine blackcurrant block (Figure D). However the penetration of *E. lata* in its host plant seems very weak. *gfp* fluorescence could only be detected at the interface between the bark and wood tissues. In contrast and as expected, no fluorescence detections were observed in samples shown in Figure E and F, which were inoculated with wild type *E. lata* controls. Specific interactions

between plant cells and the fungus, or penetration inside the lignified wood tissues were not observed. This indicated that the penetration of *E. lata* in the plant tissues was a relatively slow progress. A two to three months incubation period (the incubation time at which these pictures were taken) was not sufficient for establishment of widespread colonization of the woody tissues.

When a large number of samples were screened, detection of *gfp* expression in host plants was carried out with a fluorescence microscope (Section 2.11). Examples of these results are summarized in Figure 3.19.

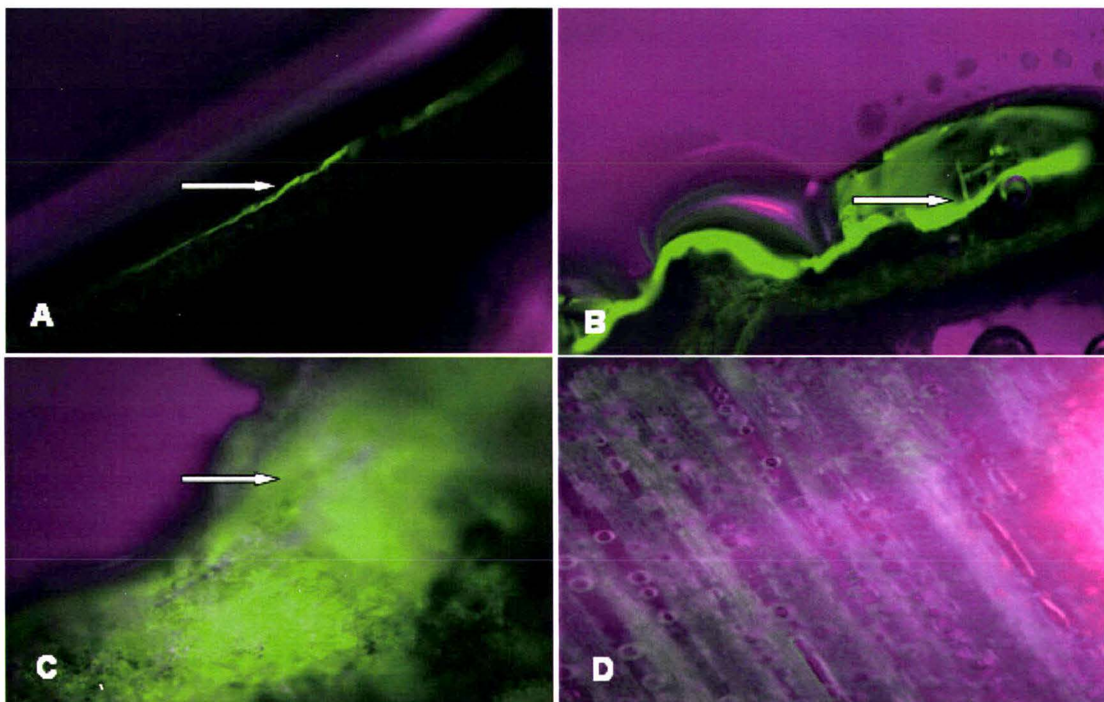

Figure 3.19 fluorescence microscope observation of *gfp* expressing *E10-10* in inoculated grapevine shoot pieces (three months since inoculation).

A, B, and C: Pictures from slides of pCT74 *E10-10* transformant inoculated onto grapevine wood tissues; D: Picture from slide of wild type *E10-10* inoculated wood tissues; Arrows are pointing to the detected *gfp* expressing *E. lata*. All samples were prepared as transverse sections.

The results from the above are consistent with those obtained with confocal observation.

3.9 DISCUSSION

3.9.1 PCR AND SOUTHERN CHARACTERIZATIONS

In Figure 3.3 and Figure 3.6, which are for characterization of *E10-10* transformants, including plasmid pAN7-1 and pBCH-*gfp* transformants, some presumed transformants, such as G10 and G11 in Figure 3.3, G9 and G22 in Figure 3.6 were not confirmed through PCR amplification. The reason for this discrepancy is most likely due to a loss of the plasmid during purification (the plasmid was lost when facing non-selective pressure in PDA plates without hygromycin supplement). Further confirmation of pCT74 transformants with Southern hybridization indicated that:

1: When digested with *Cla*I, transformant S1 contains two *hph* hybridization bands while transformants S4 contain one *hph* band (Figure 3.12 A).

2: When digested with *Cla*I, S1 contain just one *gfp* band, and S4 does not have any *gfp* bands (Figure 3.12 B).

3: When digested with *Eco*RI, S1 *gfp* hybridization has two bands (Figure 3.9).

4: When digested with *Eco*RI and *Cla*I, transformant S6 contains one *hph* band and one *gfp* band (Figure 3.9, 3.10, and 3.12).

The difference (2 & 3) in *gfp* bands in S1 is attributed to the difference in restriction sites in the genome. When digested with *Cla*I, S1 contains two *hph* bands while one *gfp* band. Could there be a second copy of *gfp* fragment the same size as the first one? This is not likely to be the case as the intensity of the band was no greater than that in S6. Possibly there is a second copy that is too big to transfer. Obviously two copies of plasmid pCT74 have integrated into transformant S1 while only one integrated into transformant S4. However, for transformant S4 it is possible that just the part of the plasmid containing the *hph* gene was integrated into the fungal genome while the part containing the *gfp* gene was not integrated. Only one copy of pCT74 was integrated into transformant S6, and this was consistent between *hph* and *gfp* hybridizations. In summary, plasmid integration was a complicated process; plasmid integration was sometimes non-stable, occurred fully in most cases, or partly in rare cases. Sometimes the integrated part might pop out again when under several rounds of subculturing or purifications.

3.9.2 *gfp* EXPRESSION IN TRANSFORMANTS

With the availability of two *gfp* vectors pBCH-*gfp* and pCT74, using *gfp* as a reporter gene for establishment of a pathogen/host relationship was proposed in this project. Both are S-*gfp*, which was modified from the original *gfp* through a Ser65-Thr mutation. The reason for using S-*gfp* instead of the original *gfp* was that it has increased fluorescence and solubility as well as decreased photobleaching (Cubitt *et al*, 1995; Crameri *et al*, 1996; Siemering *et al*, 1996). In addition, successful use of S-*gfp* as a reporter has been reported in a number of filamentous fungi (Dumas *et al*, 1999; Horowitz *et al*, 2002).

The results in this project indicated that the *gfp* gene located in plasmid pBCH-*gfp* was not successfully expressed in its transformants (Figure 3.13) while the *gfp* gene in plasmid pCT74 was successfully expressed (Figure 3.14, 3.15, and 3.16). The *gfp* gene located in plasmid pBCH-*gfp* was cloned from plasmid pFAT-3*gfp*, whilst the *gfp* gene in pCT74 was from pBlue-S*gfp*-TYG-nos-KS (from Jen Sheen, Department of Molecular Biology, Massachusetts General Hospital, Harvard University), The difference in expression may be attributed to the difference in the two promoters, i.e. pBCH-*gfp* has the promoter from the *gpd*A gene from *Aspergillus nidulans* whilst pCT74 has the *ToxA* promoter from *Pyrenophora tritici-repentis* (Ciuffetti *et al,* 1997). Therefore, it could be the difference in promoters that determines the different expression in the host cells.

Confocal examination of the movement and distribution of *E. lata* in host plants was performed. This type of microscopy captures images from within a sample, allowing visualization of undisturbed *E. lata* cells in intact vessels. It is convenient as fixation, washing, and staining are not needed, and sample dissection is minimal, eliminating the potential for artifacts (Newman *et al.*, 2003).

3.9.3 LIMITATIONS OF THE BLACKCURRANT INOCULATION EXPERIMENTS

As this project was designed for a one year thesis, obtaining good woody blackcurrants was difficult during winter when the *gfp* expressing pCT74 transformants (S1 and S4) were achieved. Very few blackcurrants with woody tissues were inoculated with purified transformant S1. Grapevines were also inoculated, although growth of *E. lata* was not optimal on this substrate. These season and material reasons have posed limitations on this experimental design. If time is not a limit, re-inoculation with transformant S1 designed to fit these requirements should be performed. Another limitation is the slow infection progress and the slow disease development caused by *E. lata* as dieback disease needs a couple of years for development. Apparently, a one year design was not the most appropriate for this purpose.

CHAPTER FOUR TRANSFORMATION OF *P. CHLAMYDOSPORA*

4.1 INTRODUCTION

The filamentous fungus *Phaeomoniella chlamydospora* is a grapevine pathogen. The disease caused by *P. chlamydospora* is usually known as Petri grapevine decline, and information about this fungus and the disease it causes are in the general introduction in chapter one.

Scientists at Lincoln University have identified a gene *moxY* from *P. chlamydospora* that is similar to a monooxygenase gene required for aflatoxin biosynthesis. On this basis it is possible that *moxY* is a putative toxin biosynthetic gene. Part of the DNA sequence of *P. chlamydospora* putative toxin gene *moxY* was obtained from Dr Hayley Ridgway (National Centre for Advanced Bio-Protection Technologies, Lincoln University) (Ridgway *et al*, 2005). The next step is to characterize this gene through gene disruption based on an efficient transformation. Gene disruption technology is now widely used in the basic science of all disciplines of pathology and it is the wilful introduction of precise mutations into the genome of organisms, affecting the function of a single gene or genes (Riminton, 2002). However, an efficient gene disruption needs an efficient transformation; literature available shows that until the present no single *P. chlamydospora* transformant has been obtained. Thus, development of an efficient transformation system is a prerequisite for gene disruption in this fungus.

The aim of this project was to develop a transformation system for *P. chlamydospora*, and to use this transformation system to disrupt this putative toxin *moxY* gene. Based on previous experience with *E. lata*, transformation of *P. chlamydospora* with the highly effective PEG/protoplast method was performed with plasmid pAN7-1 and the

constructed vector pBCH-MOXY containing part of a *moxY* gene for homologous recombination.

4.2 PRIMER DESIGN FOR PCR CLONING OF PART OF THE *moxY* GENE AND FOR VECTOR CONSTRUCTION

In order to disrupt the *P. chlamydospora* putative toxin gene *moxY* through homologous recombination, a vector containing part of the *moxY* gene was required to be constructed prior to transformation. The method used for this cloning was PCR amplification of an internal fragment; therefore primer design was essential before cloning was performed.

In order to achieve disruption of the gene function, it is ideal to disrupt a conserved domain or to separate two or more conserved domains as shown in Figure 4.1. Sequence similarity alignment was carried out to identify the conserved domains (Figure 4.2).

4.2.1 ANALYSIS OF THE *moxY* GENE SEQUENCE

A corresponding peptide sequence similarity comparison of the putative toxin gene *moxY* from filamentous fungi (*Aspergillus oryzae, Aspergillus parasiticus, and Emericella (Aspergillus) nidulans*) on the NCBI gene database was performed using the software "Gene Jockey" (Cambridge, UK). The results are shown in Figure 4.2.

From this result, several possible conserved domains in this gene were found based on the high level of amino acid identity between the genes from the four species. In order to disrupt this gene, an internal fragment between the two domains shaded in Fig 4.2 was designed for PCR cloning and vector construction. A summarized flow diagram in Figure 4.3 indicates how plasmid pBCH-*moxY* was prepared.

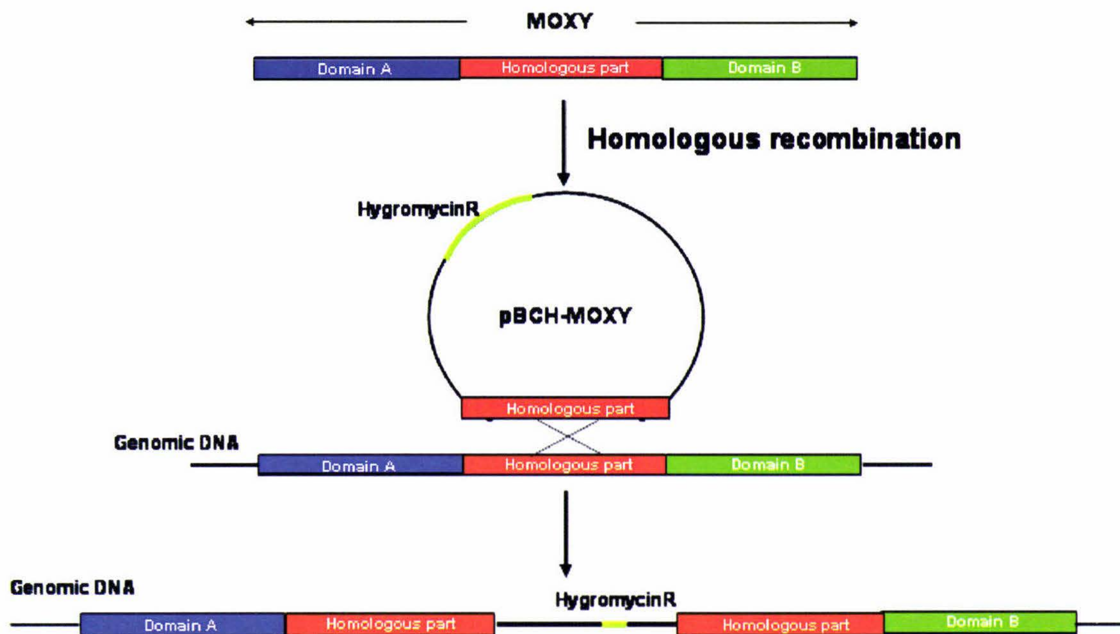

Figure 4.1 Disruption of *moxY* gene through homologous recombination

```
                                  10        20        30        40        50
                                   |         |         |         |         |
                                                                            .
Phaeomoniella moxY         A----------------------------------------------------PG
Aspergillus oryzae         MDPANRPLRVVTIGTGISGILMAYQIQKQCPNVEHVLYEKNADVGGTWLENRYPM
Aspergillus parasiticus    MDPANRPLRVVTIGTGISGILMAYQIQKQCPNVEHVLYEKNADVGGTWLENRYPM
Emericella nidulans        MDPNNRRLRVITIGAGFSGILMAYQIQKQCANIEHVVYEKNHDIGGTWLTNRYPN

                                60        70        80        90       100       110
Phaeomoniella moxY         CGCDIPSHNYQYSWAPNPRWNQYYSSQGEILTYFQDAAEKSGLIQFIKFQHKVVE
Aspergillus oryzae         AGCDVPSHAYTYPFAPNPDWPRYFSYASDIWNYLDRVCKFFDLRRYMVFHTEVVG
Aspergillus parasiticus    AGCDVPSHAYTYPFAPNPDWPRYFSYAPDIWNYLDRVCKVFDLRRYMVFHTEVVG
Emericella nidulans        AGCDVPSHAYTYRFALYPDWPRYFSYASDIWEYLDKVCAAFKLRQYMQFRTEVIK

                                  120       130       140       150       160
Phaeomoniella moxY         AVWANESRGVWQFKIENLATGET---FQDYAHFFINASGYLNNWKWPDIAGLQD-
Aspergillus oryzae         CYW-NEDRGEWTVRLRQHASGSEPREFEDHCHVLVHASGVFNNPQWPQIPGLHDR
Aspergillus parasiticus    CYW-NEDRGEWTVRLRQHVGGSEPRDFEDHCHILVHASGVFNNPQWPQIPGLHDR
Emericella nidulans        ACW-NEEEGQWKVRLRRQRPGQEPEEFDDHCHILLNACG------WPDTPGLHDR
```

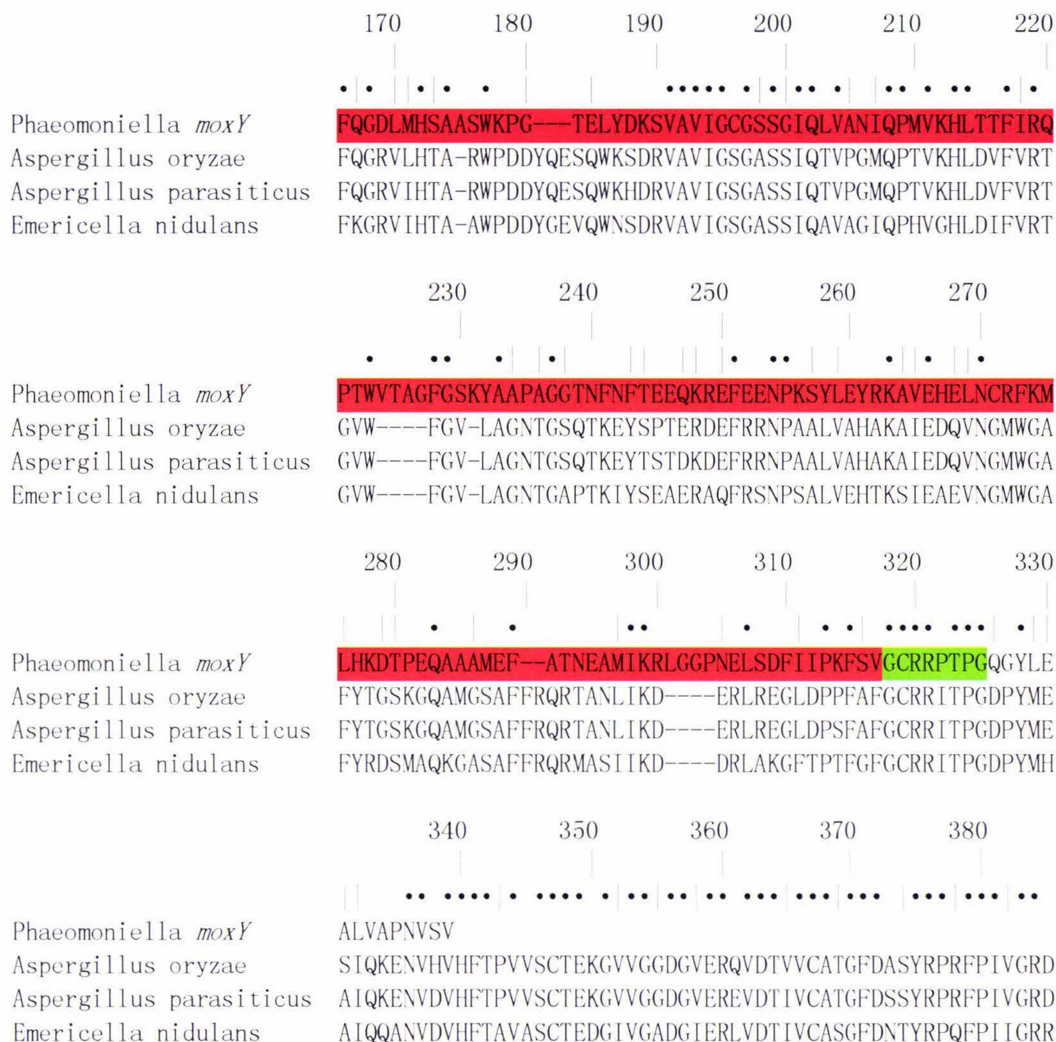

Figure 4.2 Multiple peptide sequence alignment between query sequence *Phaeomoniella chlamydospora moxY* (accession number: AY921607) and *Aspergillus oryzae moxY* (accession number: BAC20334), *Aspergillus parasiticus moxY* (accession number: AAF26281), and *Emericella nidulans steW* (accession number: Q00730) (Partial gene only). Green shaded areas are possible conserved domains that have been found; red shaded region is the internal fragment for PCR amplification.

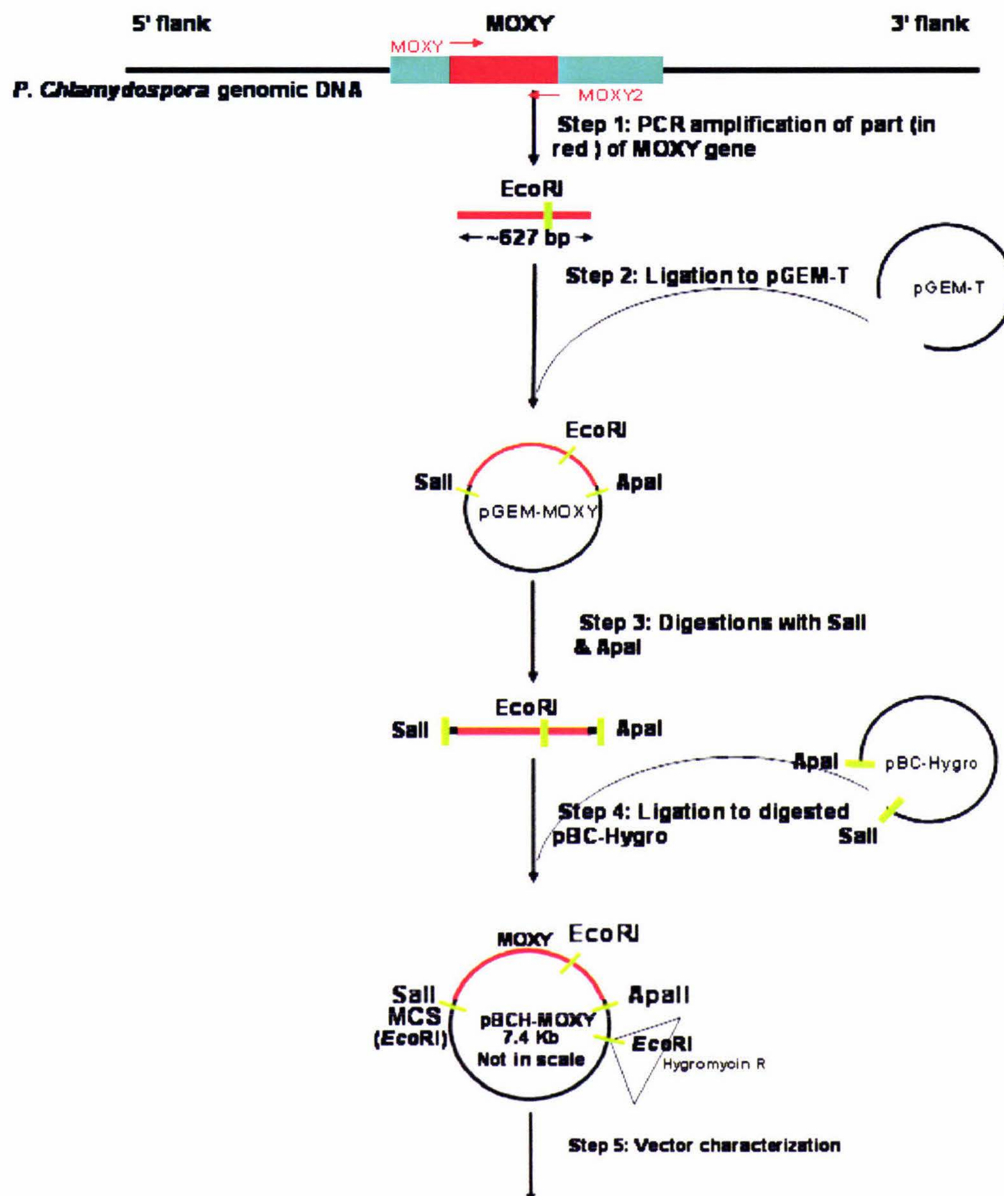

Figure 4.3 Flow diagram of vector pBCH-MOXY preparation.
***Sal*I and *Apa*I are restriction sites in the multiple cloning site of pGEM-T**

4.2.2 PRIMER DESIGN FOR PCR CLONING

Using the software EMBOSS GU1V1.12: sixpack (refers to Section 2.1), the peptide and its corresponding reading frame was determined. This DNA sequence with the direction from 5' to 3' has been translated into a peptide sequence (Figure 4.4).

```
    Q  G  E  I  L  T  Y  F  Q  D  A  A  E  K  S  G  L  I  Q  F

241 TCAAGGAGAGATATTAACTTACTTCCAAGATGCTGCGGAAAAGAGTGGTTTGATACAGTT 300
  5'      PC moxY1
    ------------------->
    I  K  F  Q  H  K  V  V  E  A  V  W  N  E  S  R  G  V  W  Q

301 CATAAAGTTCCAGCACAAAGTGGTGGAGGCAGTATGGAACGAGAGCAGGGGTGTGTGGCA 360

    F  K  I  E  N  L  A  T  G  E  T  F  Q  D  Y  A  H  F  F  I

361 ATTTAAGATTGAAAACCTCGCTACGGGAGAGACATTTCAAGATTATGCACATTTCTTCAT 420

    N  A  S  G  Y  L  N  N  W  K  W  P  D  I  A  G  L  Q  D  F

421 CAACGCTTCCGGTTACCTTAACAACTGGAAATGGCCAGATATTGCTGGACTTCAAGACTT 480

    Q  G  D  L  M  H  S  A  S  W  K  P  G  T  E  L  Y  D  K  S

481 CCAAGGCGATTTAATGCACAGCGCATCTTGGAAACCGGGAACGGAATTGTACGACAAGTC 540

    V  A  V  I  G  C  G  S  S  G  I  Q  L  V  A  N  I  Q  P  M

541 GGTTGCAGTCATTGGTTGTGGGTCCAGTGGTATTCAATTAGTCGCAAACATTCAACCTAT 600

    V  K  H  L  T  T  F  I  R  Q  P  T  W  V  T  A  G  F  G  S

601 GGTCAAGCATCTGACGACCTTTATCCGACAGCCTACATGGGTGACGGCAGGGTTTGGAAG 660

    K  Y  A  A  P  G  G  T  N  F  N  F  T  E  E  Q  K  R
                                                          EcoRI
661 CAAGTATGCAGCACCGGGAGGCACTAACTTCAATTTCACGGAAGAGCAAAAGCGAGAATT 720

    E  E  N  P  K  S  Y  L  E  Y  R  K  A  V  E  H  E  L  N  C

721 CGAGGAAAATCCAAAGTCCTATCTTGAATACCGCAAGGCTGTTGAACATGAACTCAACTG 780

    R  F  K  M  L  H  K  D  T  P  E  Q  A  A  A  M  E  F  A  T

781 CCGATTCAAAATGCTACACAAGGATACACCCGAACAAGCAGCTGCTATGGAGTTTGCAAC 840

    N  E  M  I  K  R  L  G  G  P  N  E  L  S  D  F  I  I  P  K

841 AAATGAAATGATCAAACGCCTTGGTGGTCCCAACGAACTCTCTGATTTTATTATCCCCAA 900
    3'      PC moxY2       5'
      <------------------
    F  S  V  G  C  R  R  P  T  P  G  Q  G  Y  L  E  A  L  V  A

901 ATTTTCTGTCGGTTGTCGCCGTCCGACACCTGGCCAGGGTTATCTGGAAGCTCTGGTGGC 960

    P  N  V  S
```

Figure 4.4 ***P. chlamydospora moxY*** **DNA sequence and its corresponding peptide reading frame (Courtesy of Hayley Ridgway, Lincoln University).**

The DNA sequence is from 5' to 3'. Based on the two DNA sequences in green, two primers were designed. The arrows indicate primer direction from 5' to 3'.

Based on the above information, two primers were designed as shown for disruption of the putative toxin *moxY* gene at the two conserved domains.

4.3 CONSTRUCTON OF THE VECTOR CONTAINING THE HOMOLOGOUS *moxY* GENE SEQUENCE

4.3.1 PCR AMPLIFICATION OF PART OF THE *moxY* GENE

Using the two primers designed above and *P. chlamydospora* genomic DNA as a template, PCR was performed to amplify the internal fragments between the two conserved domains for vector construction. The expected PCR product was 650 bp in size (Figure 4.5), which is consistent with the size expected.

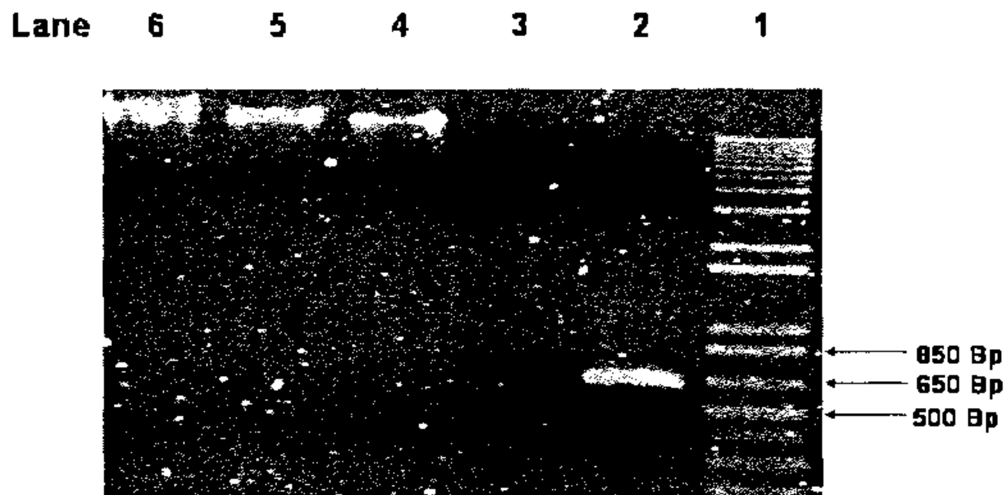

Figure 4.5 PCR cloning of a part of the putative toxin gene in *P. chlamydospora*.
Lane 1: 1kb[+] ladder; lane 2: PCR product using *P. chlamydospora* genomic DNA as a template; lane 3: PCR without *P. chlamydospora* genomic DNA; lane 4, 5, and 6: DNA standards corresponding to 25 ng, 50 ng, and 100 ng, respectively.

4.3.2 LIGATION OF THE AMPLIFIED FRAGMENT INTO pGEM-T VECTOR

In order to have convenient enzyme restriction sites, the PCR fragment was cloned into the pGEM-T vector (appendix 3G) first before being cut out and re-cloned into final vector pBC-Hyg (appendix 3B). *Apa*I and *Sal*I restriction sites which were available in pGEM-T multiple cloning site (MCS) were not found in the amplified DNA sequence, thus they were chosen for subsequent digestion after successful cloning.

The PCR fragment was purified (Section 2.17.1) and quantitated with DNA standards (Section 2.15.2) (data not shown), followed by ligation (Section 2.19) to vector pGEM-T. Subsequently, electroporation of *E. coli* was performed with the ligation mixture as described in Section 2.20).White colonies were selected on plates containing X-gal and IPTG. After purification with streaking, the purified single colony was grown and prepared for plasmid pGEM-*moxY* extraction (Section 2.14.1).

4.3.3 DIGESTION OF THE PLASMID pGEM-*moxY* WITH RESTRICTION ENZYMES *Apa* I and *Sal* I

Since the final vector into which the cloned *moxY* fragment would be placed was plasmid pBC-Hygro, digestions of plasmid pBC-Hygro with enzymes *Apa*I and *Sal*I were essential. The manufacturer's instruction (Roche, Germany) showed that the optimal digestion buffers for the two enzymes were different, so the plasmid was digested twice with one enzyme at each step. Two rounds of digestion of the plasmid pGEM-*moxY* were performed based on the method described in Section 2.16.1.

The result in lane 2 of Figure 4.6 A indicates that the cloned *moxY* fragment contained an *Eco*RI restriction site because one small band (~300 bp) was obtained in addition to the 3.5 kb band. This further confirmed that this plasmid contained the *moxY* fragment of interest. This was consistent with the *moxY* gene sequence analysis result in Figure 4.4.

DNA in lane 2 was discarded and DNA in lane 3 was recovered through isopropanol precipitation and digested with second enzyme *Apa*I (data not shown). The double digestion product was run on a gel, and the expected band size of ~620 bp was recovered

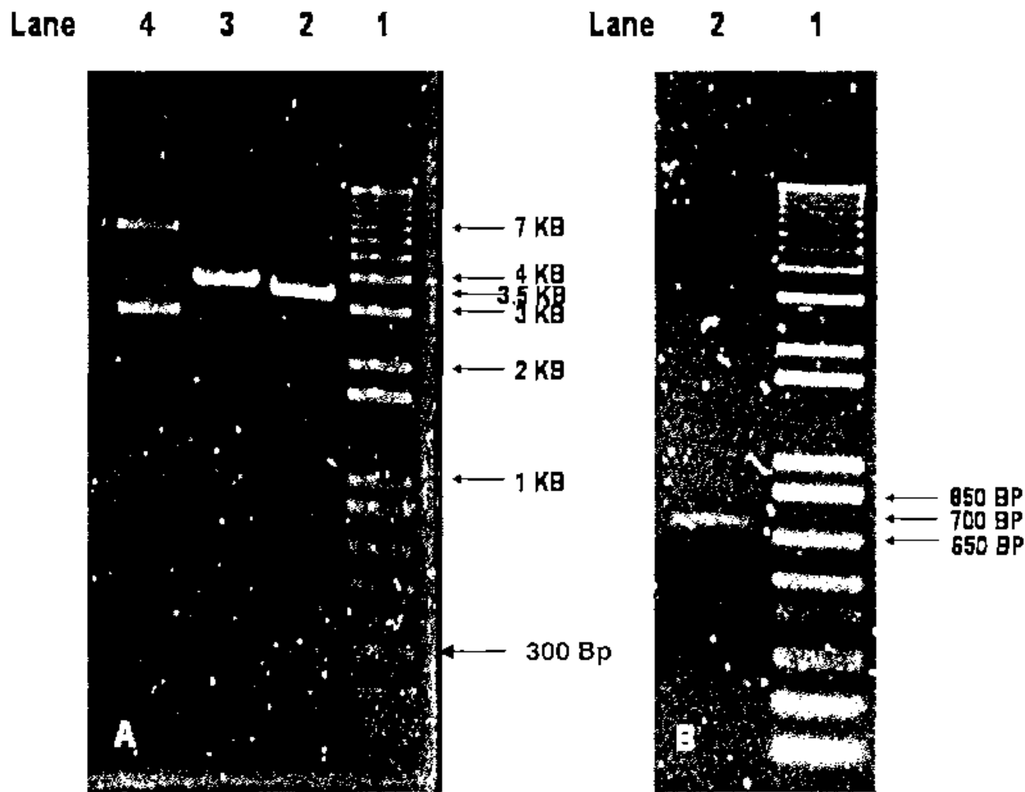

Figure 4.6 Digestions of plasmid pGEM-*moxY* for preparation of the insertion fragment for digested plasmid pBC-Hygro
A: First round digestion; Lane 1: 1kb^{+} ladder; lane 2: Plasmid digested with restriction enzyme *Apa*I and *Eco*RI; Lane 3: Plasmid digested with restriction enzyme *Sal*I; Lane 4: original plasmid pGEM-*moxY* without digestion. B: Recovered and purified insertion fragment containing *moxY* gene after double digestions; Lane 1: 1kb^{+} ladder; lane 2: pure DNA fragment containing *moxY* gene with *Apa*I and *SalI* sticky ends on each side.

and purified through gel extraction (Section 2.17.1.2). Finally the recovered DNA containing the cloned *moxY* gene was re-checked on the gel (Figure 4.6 B).

After two rounds of digestion, plasmid pGEM-*moxY* was cut with two enzymes, and an *moxY* gene fragment with one *Apa*I cutting end and one *Sal*I end was ready for cloning into the destination vector pBC-hygro (Section 4.3.4).

4.3.4 DIGESTION OF PLASMID pBC-Hygro for *moxY* FRAGMENT INSERTION

The digestion method was described in Section 2.16.1. The first round enzyme digestion result indicated a complete digestion of the plasmid (Figure 4.7 A). The digested plasmid DNA was precipitated using isopropanol as described in Section (2.17.2.1), the second round digestion was done with the other enzyme (Figure 4.7 B).

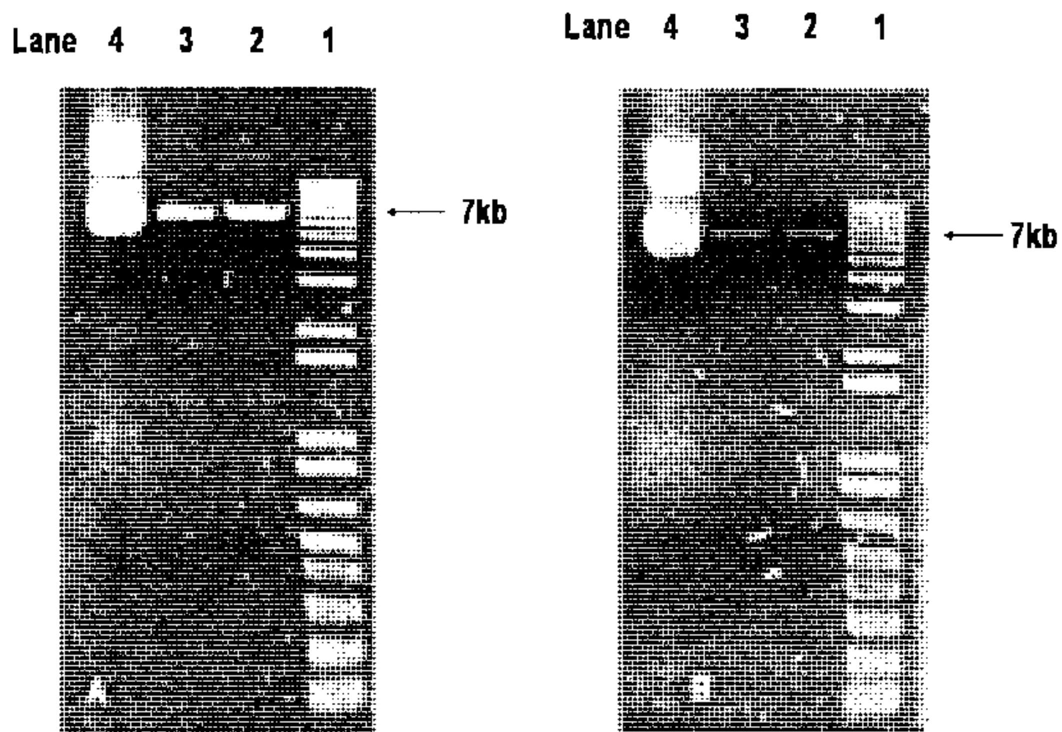

Figure 4.7 Plasmid pBC-Hygro digestion with restriction enzyme *Apa*I and *Sal*I
A: First round digestion; Lane 1: 1kb+ ladder; lane 2: Plasmid digested with restriction enzyme *Apa*I; Lane 3: Plasmid digested with restriction enzyme *Sal*I; Lane 4: original plasmid without digestion.
B: Second round digestion; Lane 1: 1kb+ ladder; lane 2: Plasmid re-digested with restriction enzyme *Sal*I; Lane 3: Plasmid re-digested with restriction enzyme *Apa*I; Lane 4: original plasmid without digestion.

After the two rounds of digestions, plasmid pBC-hygro (~7 kb) with one *Apa*I cutting end and one *Sal*I cutting end was ready for insertion of the purified PCR *moxY* fragment. Although the *Apa*I and *Sal*I cutting sites are very close together and a double digest cannot be distinguished from single digest by gel electrophoresis, the efficient cutting of

both enzymes separately in the first round suggested that double digestion would have been achieved.

4.3.5 ELECTROPORATION, TRANSFORMANT SELECTION, AND LARGE SCALE PLASMID EXTRACTION

Electroporation of XL-1 cells (Section 2.20) with the ligation mixture was performed, followed by plating and transformant selection. The white colonies that grew on plates with 170 μg/ml chloramphenicol were selected and purified through streaking. After a small scale plasmid checking, a purified single transformant was inoculated into LB broth for large scale plasmid preparation (Section 2.14.2).

4.3.6 CHARACTERIZATION OF CONSTRUCTURED PLASMID pBCH-*moxY*, pAN7-1, AND pCT74 FOR TRANSFORMATION

The constructed vector pBCH-*moxY* and other two plasmids were quantified with a fluorometer (Section 2.15.1) before characterization with enzyme digestion and PCR.

4.3.6.1 Characterization of vectors with restriction enzyme digestion

The map of plasmid pAN7-1 in appendix 3A shows it is about 6.5 kb, and it contains two *Eco*RI restriction sites, thus when digested with *Eco*RI, two bands (4 kb and 2.5 kb) should appear in the gel, as in Lane 3. Similarly, the map of plasmid pBC-Hygro and constructed vector pBCH-*moxY* (appendix 3B & 3E) shows pBCH-*moxY* is ~7.4 kb, and contains three *Eco*RI restriction sites: one in the cloned *moxY* fragment, one in the *hph* gene, and one in the multiple cloning site (MCS) (refer to flow graph Figure 4.3) and Figure 4.12. As shown in figure 4.12, pBCH-MOXY should give three bands (about 4 kb, 3 kb, and 550 bp) when cut with restriction enzyme *Eco*RI. This is consistent with the digestion result shown in Lane 5 of Figure 4.8. A further comparison of plasmid pBCH-*moxY* with its original plasmid pBC-Hygro was performed with a restriction enzyme *Eco*RI digestion (Figure 4.9).

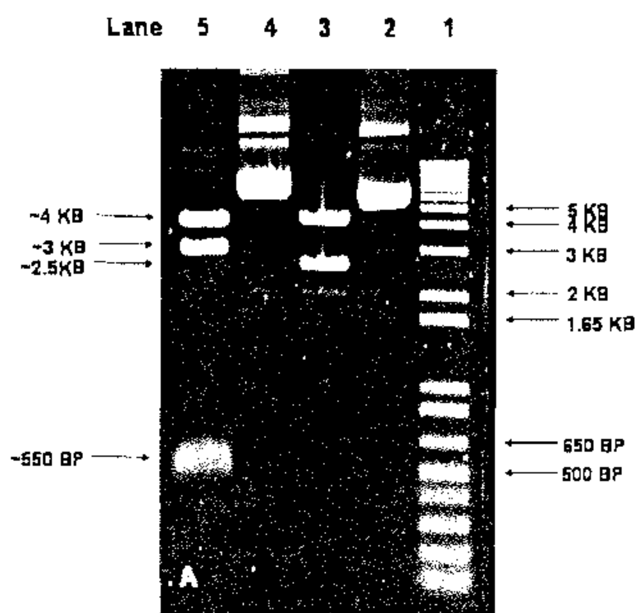

Figure 4.8 Restriction enzyme digestion of plasmids pAN7-1 and pBCH-*moxY*
Lane 1: 1kb$^+$ ladder; lane 2: plasmid pAN7-1 without enzyme digestion; Lane 3: Plasmid pAN7-1 digested with restriction enzyme *Eco*RI; Lane 4: original plasmid pGEM-*moxY* without digestion; Lane 5: Plasmid pGEM-*moxY* digested with restriction enzyme *Eco*RI.

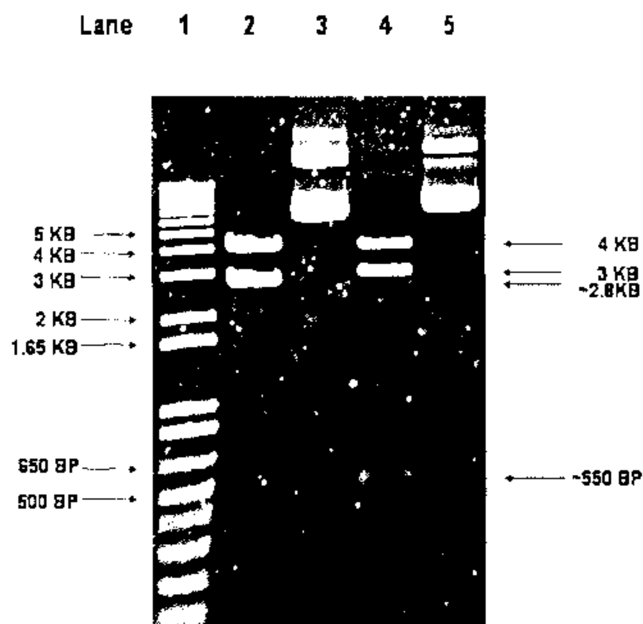

Figure 4.9 Comparison of plasmid pBCH-*moxY* with its original plasmid pBC-Hygro through restriction enzyme digestion *Eco*RI.
Lane 1: 1kb$^+$ λ ladder; lane 2: Plasmid pBC-Hygro digested with restriction enzyme *Eco*RI; Lane 3: plasmid pBC-Hygro without enzyme digestion; Lane 4: Plasmid pBCH-*moxY* digested with restriction enzyme *Eco*RI; Lane 5: Original plasmid pBCH-*moxY* without digestion.

This result demonstrates that the original plasmid pBC-hygro contains two *Eco*RI restriction sites, while the constructed plasmid pBCH-*moxY* contains three sites, two from the original pBC-hygro plasmid, and the other from the cloned *P. chlamydospora moxY* gene fragment.

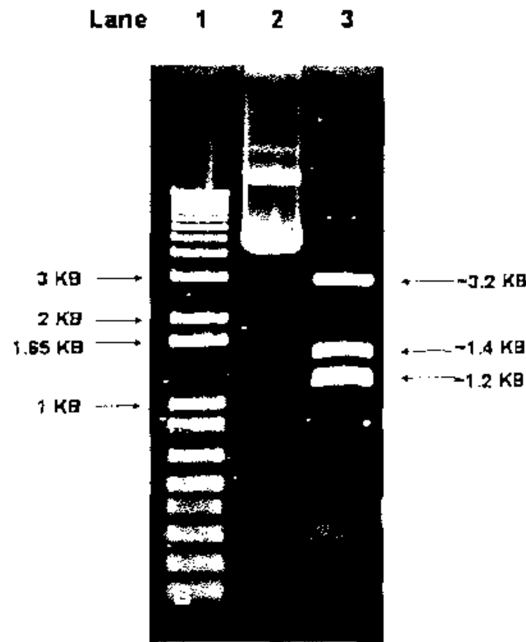

Figure 4.10 Restriction enzyme digestion of plasmid pCT74
Lane 1: 1kb⁺ λ ladder; lane 2: Original plasmid pCT74 without enzyme digestion; Lane 3: Plasmid pCT74 digested with restriction enzymes *Pst*I and *Sal*I.

The map of the *gfp*-containing plasmid pCT74 (appendix 3F) shows that the size is about 6 kb. It contains two *Pst*I restriction sites and one *Sal*I restriction site, hence when digested with these two enzymes, should result in three bands with sizes of 3.4 kb, 1.4 kb, and 1.2 kb. This is shown in Figure 4.10.

In general, the three plasmids, pAN7-1, pCT74, and pBCH-*moxY*, which were prepared for transformation of the fungus *P. chlamydospora* were characterized as correct by enzyme digestions.

4.3.6.2 Characterization of constructed plasmid pBCH-*moxy* through PCR amplification

Further characterizations with PCR and sequencing were required to confirm this characterization since the correct plasmids are critical for intended transformations. The presence of the *hph* gene in all three plasmids and of the cloned *moxY* gene in pBCH-MOXY were tested by PCR with the appropriate primer pairs (Figure 4.11).

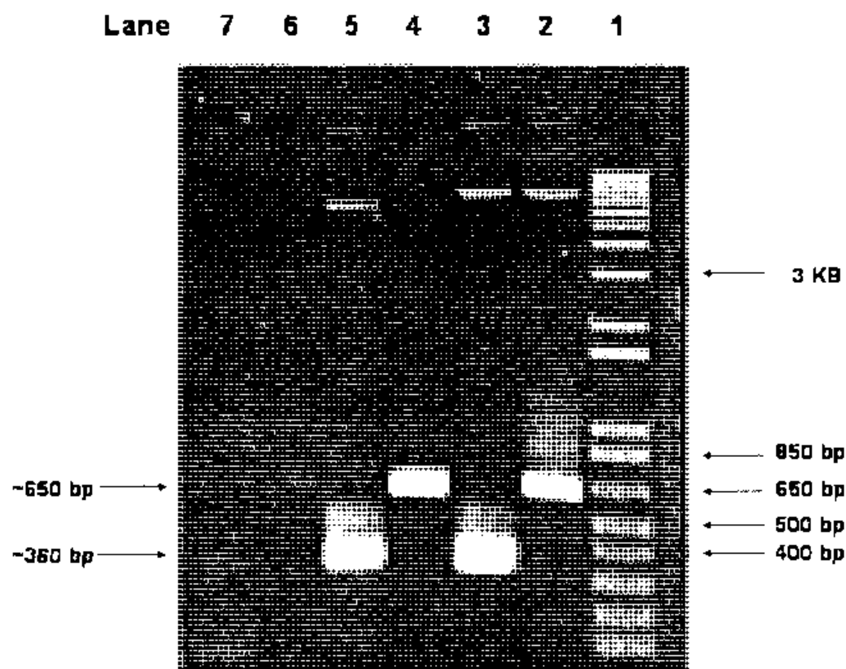

Figure 4.11 PCR characterizations of plasmids pAN7-1 and pBCH-*moxY*

Lane 1: 1kb⁺ ladder; Lane 2: Test for cloned *moxY* gene fragment using plasmid pBCH-*moxY* as a template and *moxY*1 and *moxY*2 as primers; Lane 3: Test for hygromycin resistance gene *hph* using plasmid pBCH-*moxY* as a template and 5'*hph* and 3'*hph* as primers; Lane 4: Positive control for the original *moxY* gene fragment using wild type *P. chlamydospora* genomic DNA as a template and *moxY*1 and *moxY*2 as primers; Lane 5: Positive control for the hygromycin resistance gene *hph* using plasmid pAN7-1 as a template and 5'*hph* and 3'*hph* as two primers; Lane 6: Negative control 1: wild type *E10-10* genomic DNA as a template and two *hph* primers. Lane 7: Negative control 2: *moxY* primers in the absence of template DNA.

Figure 4.11 above indicated that constructed plasmid pBCH-*moxY* contained the cloned *moxY* fragment which had been amplified with *moxY* primers (lane 2). Moreover its size was equivalent to the band in lane 4 that amplified from wild type *P. chlamydospora* genomic DNA, which served as a positive control. Lane 3 indicated that pBCH-*moxY* contained a *hph* gene as amplification of hygromycin gene with *hph* primers gave positive bands equivalent to the positive control in lane 5. Both lanes 6 and lane 7 served as negative controls.

In conclusion, based on PCR characterization, the constructed plasmid pBCH-*moxY* contains a *moxY* gene fragment and an *hph* gene.

4.3.6.3 Characterization of plasmids through sequencing of the amplified PCR product and sequence comparison with original *moxY* gene DNA sequence.

The above two characterization methods have demonstrated that the designed plasmids should be able to fulfill the requirements of selection and gene disruption after transformation. In order to ensure the cloned sequence was exactly the *moxY* gene fragment, and was not identified solely by fragment size, sequencing of the fragment was carried out after purification of the PCR product as described in Section 2.9. The sequencing results were compared with those of the original *P. chlamydospora moxY* gene sequence using "Gene Jockey" (Appendix 5).

This sequence similarity comparison has demonstrated that the PCR product is the *moxY* gene fragment, which in turn further indicates that the constructed plasmid pBCH-*moxY* authentically contains the cloned homologous *moxY* gene sequence for gene disruption.

There were two mismatches: nucleotide "G" in position 408 and "C" in 562 in the cloned PCR product. The mismatches could have occurred during initial PCR amplification of the *moxY* gene for cloning. However these minor "mistakes" will not interfere with the final aim of gene disruption as more than 98% of these two sequences are identical, and are sufficient for providing a homologous sequence for recombination. On the other hand, if the final aim was *moxY* gene expression rather than homologous recombination, any one of these mismatches could incur a big problem. So for *moxY* gene expression, cloning

of this gene through restriction digestion of the wild type genomic DNA should be performed rather than a PCR cloning.

4.3.7 DETERMINATION OF THE ORIENTATION OF THE CLONED *moxY* GENE FRAGMENT

The direction of the cloned *moxY* fragment in plasmid pBCH-*moxY* is very important and critical for subsequent screening of *moxY* gene disruption transformants as the primers used for PCR screening can be designed based on the DNA sequence of the inserted *moxY* gene. The result of restriction enzyme *Eco*RI digestion (Figure 4.9) shows a digestion fragment of ~550 bp rather than a fragment of ~200 bp, showing that the *Eco*RI restriction site in the *moxY* fragment is near the restriction enzyme *Apa*I end shown in Figure 4.12 A rather than the *Sal*I site shown in Figure 4.12 B.

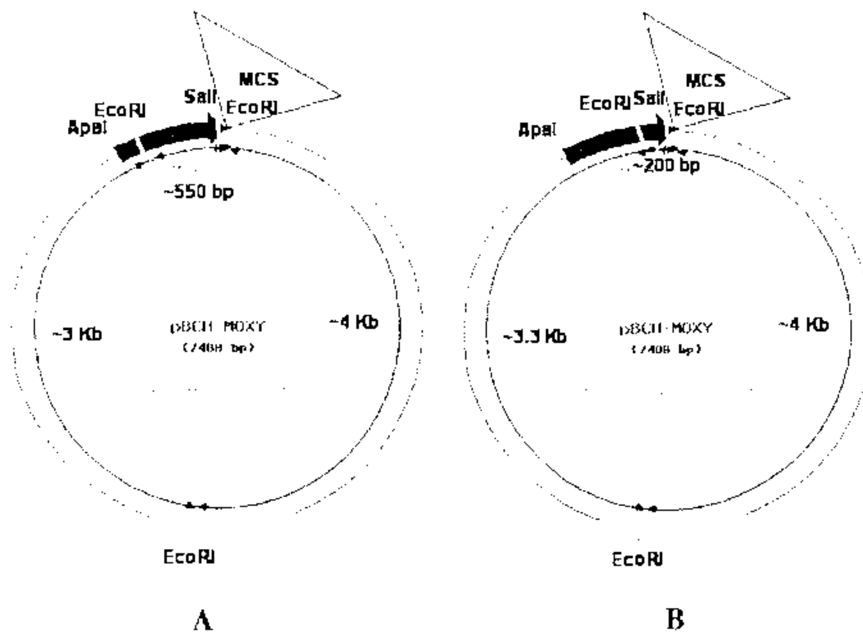

Figure 4.12 Possible orientations of the inserted *moxY* fragment in plasmid pBCH-*moxY*. A is the correct orientation.

4.4 TRANSFORMATION OF *P. CHLAMYDOSPORA* USING PROTOPLASTS/PEG METHOD.

The method that was used for *P. chlamydospora* protoplast isolation and transformation has been described in Section 2.6. At least three independent transformations with newly prepared protoplasts were performed on separate occasions with the same conditions. Transformations with plasmid pAN7-1 as well as plasmid pBCH-*moxY* were performed simultaneously. In addition, transformation with plasmid pCT74 was also performed for harvesting a transformant containing a *gfp* gene driven by a strong promoter (*toxA* promoter). Controls without plasmid pAN7-1 were also included. In general, transformations with plasmid pAN7-1 and pBCH-*moxY* were successful. Table 4.1 is a summary of transformation and protoplast regeneration for independent transformations of *P. chlamydospora*.

NUMBER OF TRANSFORMANTS (AMOUNT OF DNA)			*REGENERATION RATES*
REPLICATE	***pAN7-1***	***pBCH-moxY***	
1	*5(4 ug)*	*34(8 ug)*	1.28×10^{-2}
2	*30(4 ug)*	*44(8 ug)*	2.54×10^{-2}
3	*31(8 ug)*	*28(4 ug)*	3.68×10^{-2}
4	*Non-applicable*	*101(12 ug)*	3.39×10^{-1}
Average rate	*4.1 ± 3.1 per ug DNA*	*6.5 ± 1.8 per ug DNA*	$1.0 \pm 1.6 \times 10^{-1}$

Table 4.1 *P. chlamydospora* transformation results with plasmid pAN7-1 and pBCH-*moxY*, and protoplast regeneration rate.

The regeneration rate was calculated at the ratio of regenerated protoplasts (colonies) : actual protoplasts.

Table 4.1 revealed that the transformation frequencies for the two plasmids pAN7-1 and pBCH-*moxY* were similar, i.e. 4.1 ± 3.1 transformants per μg pAN7-1 DNA and 6.5 ± 1.8 for pBCH-MOXY DNA. In addition, this result is consistent with those developed for other filamentous fungi (refer to result for *E. lata* transformation Table 3.1). Most of these transformants grew vigorously on selective media supplemented with hygromycin, a few of them were small and grew non-vigorously. Subsequent subculturing and purification indicated that the small colonies were abortive transformants. Compared with that of *E. lata* (average 3.49 x 10^{-3}) for *E10-10*, the average protoplast regeneration frequency for *P. chlamydospora* is higher, i.e.1.0 ± 1.6 x 10^{-1}. This discrepancy between them may be attributed to the impurity of the protoplast suspension as newly made *P. chlamydospora* protoplast suspensions contain some conidiospores. This was shown through dilution in sterilized water or STC buffer and plating on non-osmotically stabilized medium (Table 4.2). Protoplasts would lyse in water and would be unable to regenerate on non-somatically stabilized media.

Experiment / Dilution	3	
1000-Fold	STC	H_2O
	520 colonies/plate	230 colonies/plate
10,000-Fold	STC	H_2O
	110 colonies/plate	71 colonies/plste

Table 4.2 *P. chlamydospora* protoplast check based on plating.

In experiment 3 in Figure 4.2, plating of the harvested assumed protoplasts was performed in order to check whether they are real protoplasts. Protoplasts diluted in STC buffer were plated onto plates containing PDA supplemented with sucrose. As a control, protoplasts diluted in water were plated onto plates containing PDA only.

To achieve successful transformation, it is essential to have a high concentration of protoplasts. From Table 4.1, it was noticed that there was a difference in transformation rate between experimental replicates, which was dependent on the concentration of the

protoplasts. Experiment 4 had a higher concentration of protoplasts as mycelium used for protoplast preparation was relatively young (15-19 days). Some of the protoplasts harvested were not real protoplasts, as indicated for experiment 3 in Figure 3, suggesting that assumed protoplast suspension contained some conidiospores and/or mycelial fragments since protoplasts would not be able to regenerate when diluted in water. However transformants were harvested in experiment 3, indicating that this presumed protoplast suspension contained real *P. chlamydospora* protoplasts. Those real *P. chlamydospora* protoplasts contributed to transformation while conidiospores played their role in regeneration after plating.

In addition, transformations with *gfp* plasmid pCT74 for harvesting *gfp* transformants were also performed, and eleven pCT74 transformants in total were obtained. After purification, only 8 still remained viable. These transformants were observed with a fluorescence microscope for *gfp* expression (Section 2.11).

4.5 CHARACTERIZATION OF PRESUMED *P. CHLAMYDOSPORA* TRANSFORMANTS

All transformants, including those with pAN7-1, pBCH-*moxY*, and pCT74, were harvested and purified by the method described in Section 2.7. Genomic DNA was extracted (Section 2.14.3), followed by fluorometer quantification (Section 2.15.1). Subsequently DNA was used for PCR characterization, Southern blot and hybridization confirmation.

4.5.1 MORPHOLOGY OF *P. CHLAMYDOSPORA* TRANSFORMANTS IN SELECTIVE AND NON-SELECTIVE MEDIA.

After transformants had been harvested and purified, each type of transformant was subcultured onto plates containing selective and non-selective media for checking their growth. Plasmid pAN7-1 transformants were labelled as pAN7-1-1, pAN7-1-2, etc; plasmid pCT74 transformants were labelled as pCT74-1, pCT74-1, etc; One hundred and two *P. chlamydospora* pBCH-MOXY transformants in total were harvested and they

were numbered from PC1 to PC102. Wild type *P. chlamydospora* was included as a control (Figure 4.13).

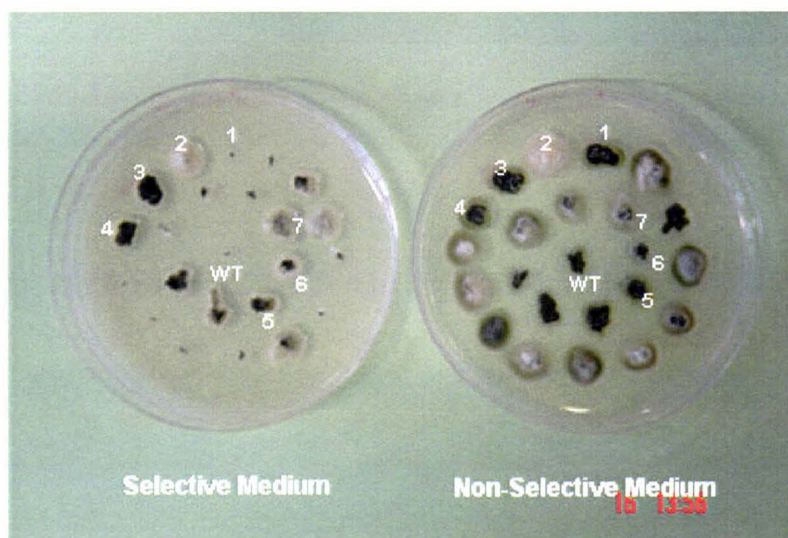

Figure 4.13 Some presumed transformants growing on selective medium with hygromycin after subculturing from purified colonies on non-selective medium.
Transformant 1: pAN7-1-1; Transformant 2: pAN7-1-2; Transformant 3: pCT74-1; Transformant 4: pCT74-7; Transformant 5: PC82; Transformant 6: PC86; Transformant 7: PC87
The colonies subcultured into the middle of the plates are wild type *P. chlamydospora*. Presumed transformants were subcultured at the edges of the plates. Each type of transformant taken at random and not all transformants were included.

The colonies such as those numbered 1, 2, 3, etc still could grow on selective medium after non-selective medium purification. In contrast, the wild type colonies in the centre, which served as a control, were totally inhibited by hygromycin in the selective medium. This morphological difference between wild type and transformed *P. chlamydospora* demonstrated hygromycin resistance ability in these transformants. The numbered colonies were subcultured for genomic DNA extractions and subsequent PCR characterization and Southern hybridization.

4.5.2 CHARACTERIZATION THROUGH PCR AMPLIFICATION OF THE *hph* GENE

The methods used for characterization were similar as for *E. lata* (Section 2.8.2). Before

characterization, the wild-type and transformants' genomic DNA were quantitated (method described at 2.15.1). Using *hph* primers, all the bands obtained by PCR were identical in size (~365 bp) with lane 1 containing DNA from WT *P. chlamydospora* as a negative control (Figure 4.14). Samples pAN7-1-1 and PC84 each have only a very faint band with the same size. This was possibly due to the impurity of these samples' DNA. This has demonstrated that except for presumed transformants pAN7-1-1 and PC 84, all other transformants contain the characteristic gene *hph,* confirming they are real transformants. The two presumed transformants pAN7-1-1 and PC 84 have not been supported with this PCR method. A possible reason of this is due to the loss of plasmid when facing non-selection conditions during purification. This conclusion has been supported by a further morphological check (Section 4.5.1 and Southern hybridization (Section 4.8).

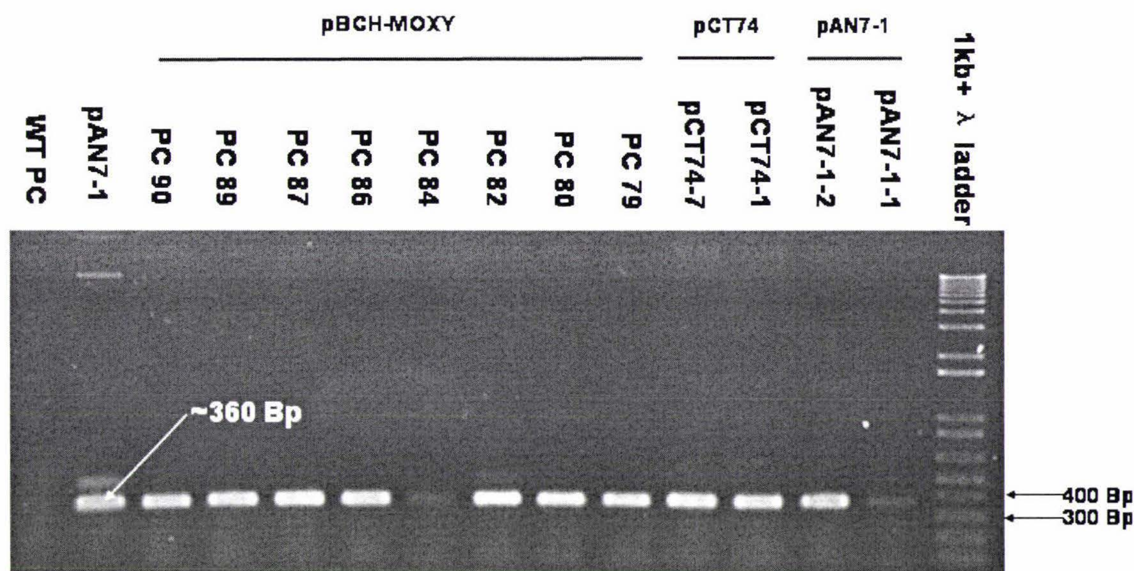

Figure 4.14 Characterization of *P. chlamydospora* transformants through PCR amplification
P. chlamydospora **transformants containing plasmid pAN7-1, pCT74, and pBCH-*moxY* were characterized through PCR amplification of the hygromycin resistance gene *hph*.**

4.5.3 CHARACTERIZATION OF *P. CHLAMYDOSPORA* pCT74 TRANSFORMANTS USING A fluorescence microscope

As with the *E10-10* transformants containing the *gfp* gene, *P. chlamydospora* pCT74 transformants were checked under a fluorescence microscope for *gfp* expression on the slides, and the results photographed (Figure 4.15 A).

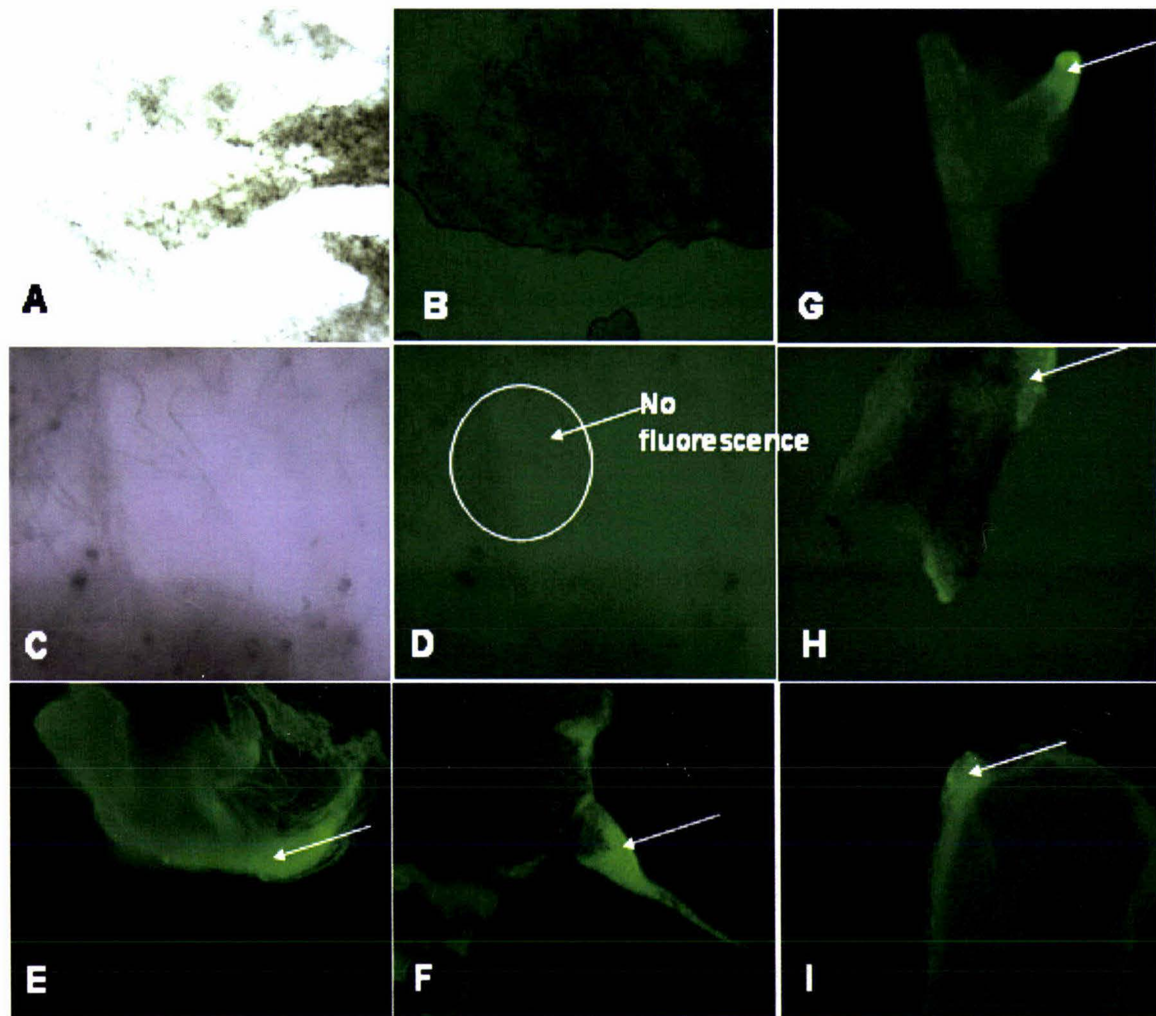

Figure 4.15A. fluorescence microscope check of *gfp* expression in pCT74 *P. chlamydospora* transformants.
A: Wild type *P. chlamydospora* colonies in white light; B: Wild type *P. chlamydospora* colonies in UV light; C: Wild type *P. chlamydospora* mycelium in white light; D: Wild type *P. chlamydospora* mycelium in UV light; E, F, G, H, and I: are pCT74 transformants pCT74-1, pCT74-3, pCT74-6, pCT74-7, and pCT74-8, respectively, in UV light. All pictures were taken under 40-fold magnification.

To obtain *gfp* fluorescence in single hyphae, pCT74-1 and pCT74-7 were further observed under higher magnification (100 fold) (Figure 4.15 B).

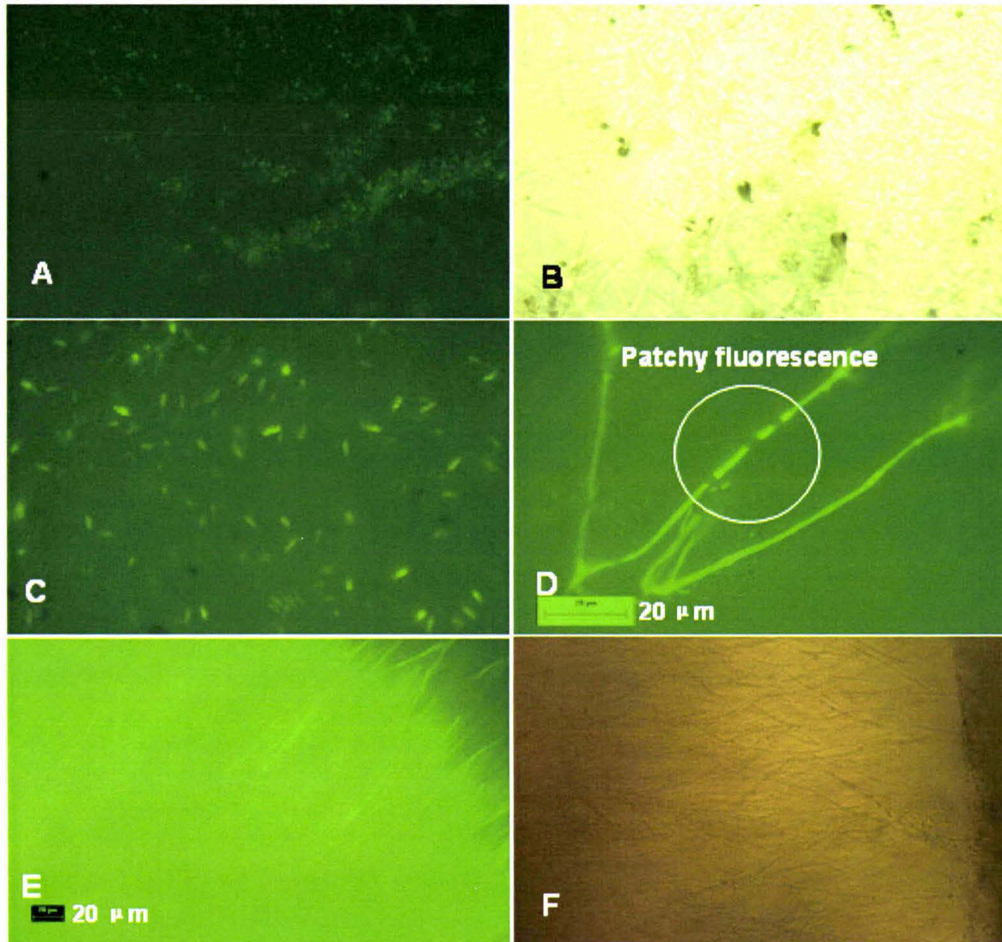

Figure 4.15B. fluorescence microscope check of *gfp* expression in pCT74-1 and pCT74-7.
A & C: pCT74-1 in UV light, A is under 40-fold magnification, C is under 100-fold magnification; B: pCT74-1 in white light; D & E: pCT74-7 in UV light, D is under 100-fold magnification, E is under 40-fold magnification; F: pCT74-7 in white light. The exposure times were 1.037s. Scale bars were included in D & E. Illumination with UV light at 460-490 nm wavelengths.

Figure 4.15 A showed that through comparison with wild types without UV light (A & C) and that were not fluorescent with UV light (B & D), pCT74 transformants pCT74-1, pCT74-3, pCT74-6, pCT74-7, and pCT74-8 were glowing under UV light (E, F, G, H, and I). Figure 4.15 B showed that through comparison with B & F, pCT74-1 spores (C) and pCT74-7 (D) hyphae were fluorescent. These observations indicated that the *gfp* in plasmid pCT74 which has integrated into *P. chlamydospora* genomic DNA (based on previous PCR and later Southern hybridization) was expressed.

4.6 PRIMER DESIGN FOR *moxY* GENE DISRUPTION TRANSFORMANTS SCREENING

After harvesting of pBCH-*moxY* transformants, screening of the *moxY* gene knock-outs was performed through PCR amplification (method described in Section 2.22). The two primers *moxY*3 and pBCH-*moxY*1 used for screening are indicated in Figure 4.16. The primer *moxY*3 was designed based on the *P. chlamydospora* genomic DNA just upstream of the cloned *moxY* gene fragment (red), while the primer pBCH-*moxY*1 was based on the plasmid pBCH-*moxY* DNA that was just downstream of the *moxY* gene fragment. The total size of the expected PCR product was around 1 kb. The specific DNA sequence was in Figure 4.17.

4.7 SCREENING OF *moxY* GENE DISRUPTED TRANSFORMANTS

4.7.1 RESULT OF THE FIRST ROUND OF SCREENING THROUGH PCR AMPLIFICATION

The method used for screening of *moxY* gene disruption transformants has been described in Section 2.22. The PCR amplified product contains *P. chlamydospora* genomic DNA as well as part of the integrated plasmid pBCH-*moxY* DNA. If the transformant is a *moxY* gene disruptant, a PCR product could appear with one primer from *P. chlamydospora* DNA and another one from original plasmid pBCH-*moxY* (not from the cloned MOXY). One hundred and two *P. chlamydospora* pBCH-MOXY transformants were harvested into 14 groups (refer to Figure 4.18 legend) for initial PCR screening (Section 2.22.1).

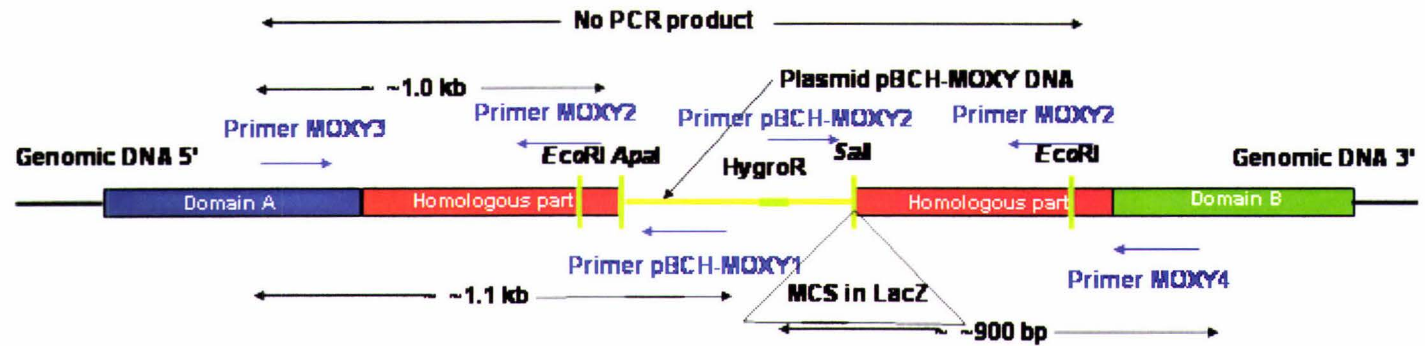

Figure 4.16 Graph of disruption of *moxY* gene after homologous recombination

Refer to Figure 4.1 The whole *moxY* gene comprising of Domain A, Domain B, and homologous part (cloned *moxY* fragment) was disrupted after homologous recombination. The plasmid pBCH-*moxY* DNA was integrated into the host fungal genomic DNA. The yellow fragment stands for original plasmid DNA (i.e. from plasmid pBC-Hygro). Positions of primers are shown.

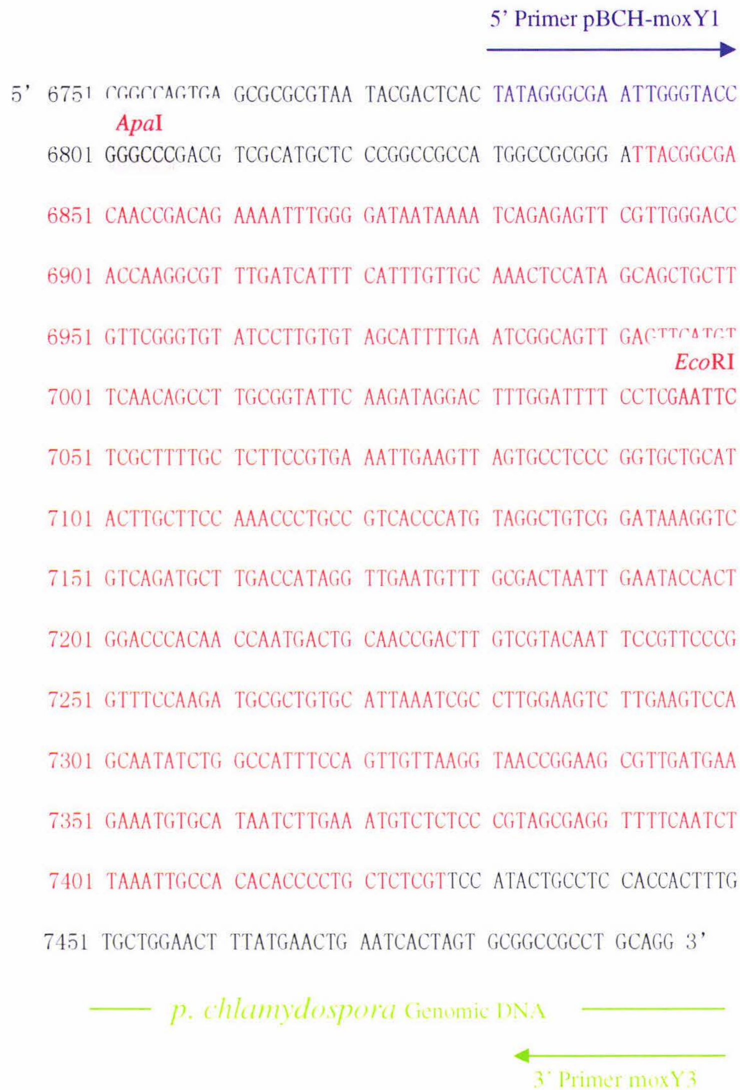

Figure 4.17 Recombination site DNA sequence of the MOXY disruption *P. chlamydospora* transformant.
The red part of the DNA sequence is cloned *moxY* fragment; the black part is plasmid pBCH-MOXY DNA. The blue sequence was used for primer pBCH-*moxY*1 design, the *P. chlamydospora* genomic DNA sequence used for primer *moxY*3 design was not shown and was included in Appendix 4B.

Controls with primers MOXY2 and MOXY3 (refer to Figure 4.16) were performed simultaneously. As these two primers were based on wild type *P. chlamydospora* genomic DNA, *P. chlamydospora* wild type and transformants would have positive PCR

results after amplification with these two primers. The first round of screening is shown in Figure 4.18 below;

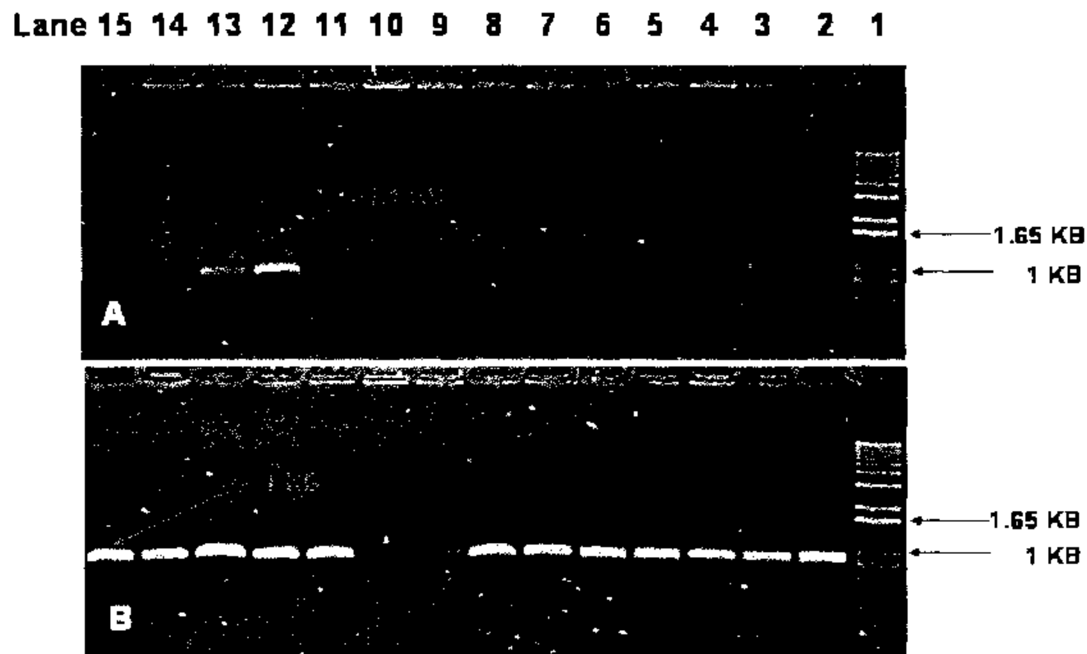

Figure 4.18 First round of PCR screening for *moxY* gene knock-outs in *P. chlamydospora* plasmid pBCH-*moxY* transformants.
A: PCR amplification of pools of pBCH-*moxY* transformants and wild type genomic DNA with primers *moxY*3 & pBCH-*moxY*1; B: PCR amplification of pools of pBCH-*moxY* transformants and wild type genomic DNA with control primers *moxY*3 & *moxY*2.
Lane1: 1kb⁺ ladder; 2:Combined DNA from transformants numbered from PC1 to PC6; 3: Numbered from PC7 to PC12; 4: from PC13 to PC18; 5: from PC19 to PC24; 6: from PC25 to PC30; 7: from PC31 to PC36; 8: from PC37 to PC42 plus number PC44 and PC46; 9: from PC48 to PC53; 10: from PC55 to PC61; 11: from PC62 to PC68; 12: from PC69 to PC73 plus number PC77; 13: from PC78 to PC83; 14: number PC84, PC86, PC87, PC88, PC89, and PC90; 15: number PC91, PC96, PC98, PC101, and PC102.
PC: *P. chlamydospora* pBCH-MOXY transformant.

The first screening result indicated that lanes 12 & 13 contained expected *moxY* gene disruption transformants as a band in the expected size (~1.1 kb) appeared in these lanes (Figure 4.18 A). Figure 4.18 B demonstrated that PCR amplification was successful for each pool apart from those in lanes 9 and 10.

4.7.2 RESULT OF THE SECOND ROUND OF SCREENING THROUGH PCR AMPLIFICATION

The second round of screening was similar to the first except that it used individual transformant's genomic DNA from the two pools that tested positive with primers MOXY3 and pBCH-MOXY1 instead of the combined DNA. Therefore, positive bands identified the specific tranformants that are potentially *moxY* gene disrupted transformants (Figure 4.19).

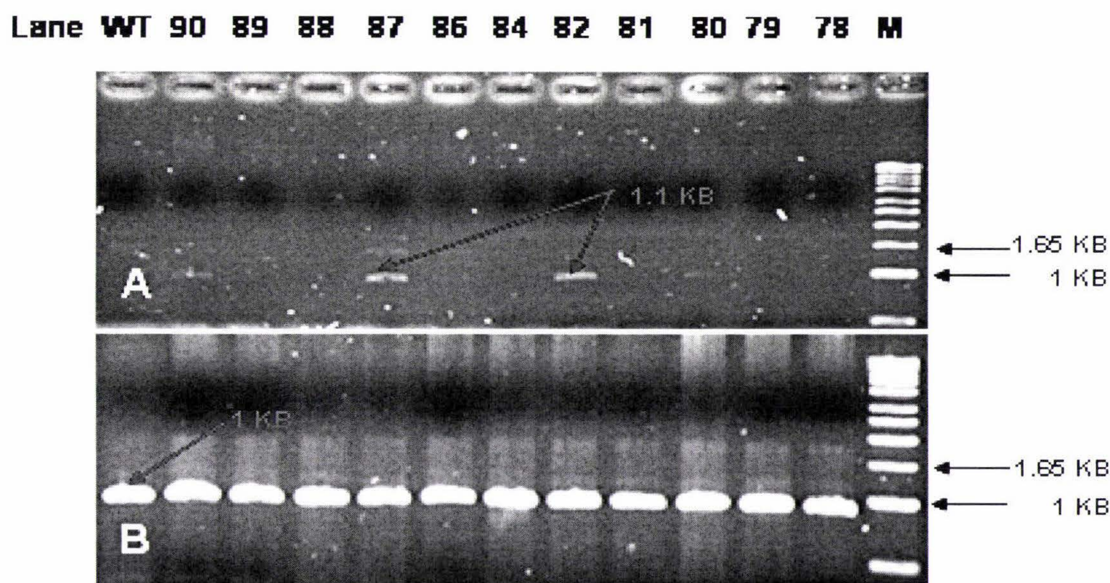

Figure 4.19 Second round of PCR screening of *moxY* gene disrupted transformants from transformants identified in pools 12 & 13.
The numbers above each lane indicates the individual plasmid pBCH-*moxY* *P. chlamydospora* transformant, WT served as a negative control in part A. A: PCR amplification with primers MOXY3 & pBCH-MOXY1; B: PCR amplification with control primers MOXY3 & MOXY2.

From part "A" in Figure 4.19, transformants numbered PC82 and PC87 are demonstrated as putative *moxY* gene disrupted transformants compared with wild type and other pBCH-*moxY* transformants (there is a faint band from PC90) in which no positive bands are present. Part "B" has the same meaning as in Figure 4.18 for the first round of screening. However these results need to be confirmed by Southern blot and hybridization.

4.8 CHARACTERIZATION OF *P. CHLAMYDOSPORA* TRANSFORMANTS AND CONFIRMATION OF *moxY* GENE DISRUPTION WITH SOUTHERN HYBRIDIZATION

4.8.1 GEL CHECK OF COMPLETE DIGESTION OF THE GENOMIC DNA

The method used for genomic DNA digestion has been described in Section 2.16.1, and the method for Southern blots is in Section 2.21. A pre-digestion check with 1-2 μg of each total digestion solution in a mini gel was performed to check for completed digestion (Figure 4.20) before Southern blotting.

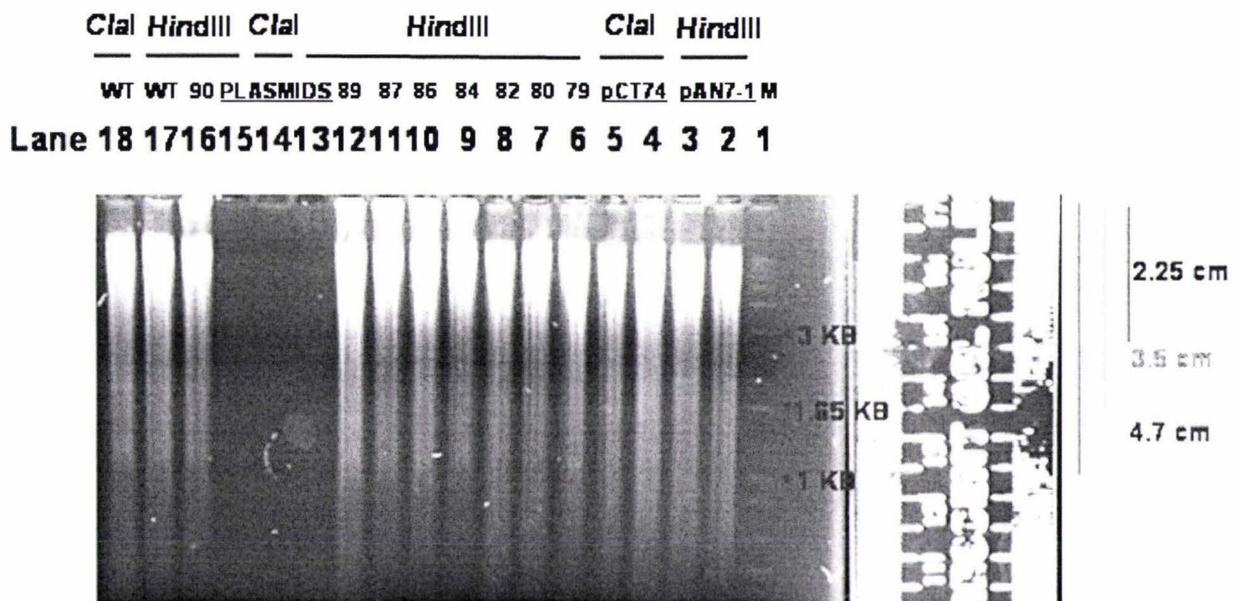

Figure 4.20 Gel of digested transformants' genomic DNA as well as plasmids DNA in the gel before Southern blotting.

The distances between the well and each band are shown alongside the gel.

Lane 1: 1kb[+] ladder; Lane 2: pAN7-1-1 digested with restriction enzyme *Hind*III; Lane 3: pAN7-1-2 digested with restriction enzyme *Hind*III; Lane 4: pCT74-1 digested with restriction enzyme *Cla*I; Lane 5: pCT74-7 digested with restriction enzyme *Cla*I; Lane 6-12: pBCH-*moxY* transformants numbered PC 79, 80, 82, 84, 86, 87, and 89 digested with restriction enzyme *Hind*III; Lane 13: 100 pg plasmid pAN7-1 linearized with restriction enzyme *Hind*III; Lane 14: 100 pg plasmid pCT74 linearized with restriction enzyme *Cla*I; Lane 15: 100 pg pBCH-*moxY* linearized with restriction enzyme *Hind*III; Lane 16: pBCH-*moxY* transformants numbered PC 90 digested with restriction enzyme *Hind*III; Lane 17: Wild type *P. chlamydospora* digested with restriction enzyme *Hind*III; Lane 18: Wild type *P. chlamydospora* digested with restriction enzyme *Cla*I.

4.8.2 HYBRIDIZATION WITH LABELLED *hph* AND *gfp* PROBE.

The gel containing those samples DNA was transferred for Southern blotting with the method described in Section 2.21.2, followed by *hph* and *gfp* probe hybridization (Section 2.21.3). Figure 4.21 below is the result of hybridization.

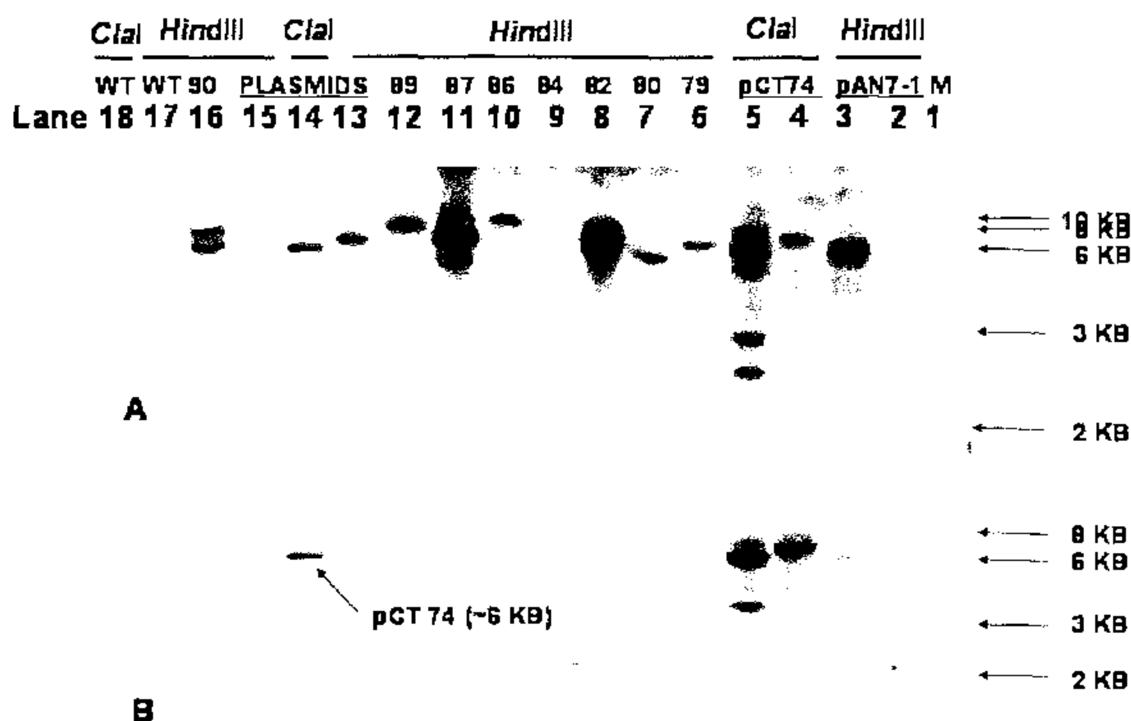

Figure 4.21 Hybridization of transformants' genomic DNA blot with labelled *hph* and *gfp* probe.
A: Membrane hybridized with *hph* probe; B: The same membrane reprobed with labelled *gfp* probe. Legend refers to Figure 4.20.

From part "A" in Figure 4.21, positive bands for the *hph* gene appeared in each lane except Lane 1 (which is 1kb$^+$ ladder), lane 2 (sample pAN7-1-1), lane 9 (PC 84), and Lane 17 & 18 (wild type *P. chlamydospora* genomic DNA), which served as negative controls. Lanes 13, 14, and 15 are three linearized plasmids i.e. pAN7-1, pCT74, and pBCH-*moxY*, which served as three positive controls. From this result, it was determined that labelled *hph* probe was capable of hybridizing to *hph* DNA sequence in these

plasmids. Based on the positive band(s) in each presumed *P. chlamydospora* transformants except presumed pAN7-1 transformant pAN7-1-1 and pBCH-*moxY* transformant PC84, we can concluded that the *hph* probes successfully hybridized to presumed *P. chlamydospora* transformants' genomic DNA. These results indicated that the transformants' genomic DNA contained the hybridization targets, i.e. *hph* gene DNA. This in turn confirms that plasmids pAN7-1, pCT74, and constructed plasmid pBCH-*moxY* have successfully integrated into fungal genomic DNA after transformation. In addition, lanes 5, 11, and 16 have multiple bands, indicating that multiple plasmid integration has occurred.

Part "B" confirms that gene *gfp* hybridization has occurred in the transformants in lane 14 but not those in lanes 13 & 15 which are plasmid pAN7-1 & pBCH-*moxY*, respectively. This further confirms that the designed *gfp* probe was able to hybridize to the *gfp* gene in pCT74, and pCT74 transformants (lane 4 & 5) contain the *gfp* gene.

As with the results obtained with each kind of *E10-10* transformant, the band size depends on the site at which the plasmids integrated. This variability suggests random plasmid integration into the fungal genome. Multiple copy integration of plasmid pCT74 in sample pCT74-7 in lane 5 has been demonstrated with the second *gfp* probe hybridization in part "B". These results are consistent except in lane 3 which most likely contains a non-specific *gfp* hybridization.

4.8.3 IDENTIFICATION OF *moxY* GENE DISRUPTION TRANSFORMANTS THROUGH HYBRIDIZATION WITH LABELLED *moxY* GENE FRAGMENT

In order to confirm *moxY* gene disruption, transformant(s) PC82 and PC87 (as well as PC90), which were identified through PCR screening, were re-hybridized with labeled MOXY probe with the same method as that for *hph* and *gfp* (Figure 4.22).

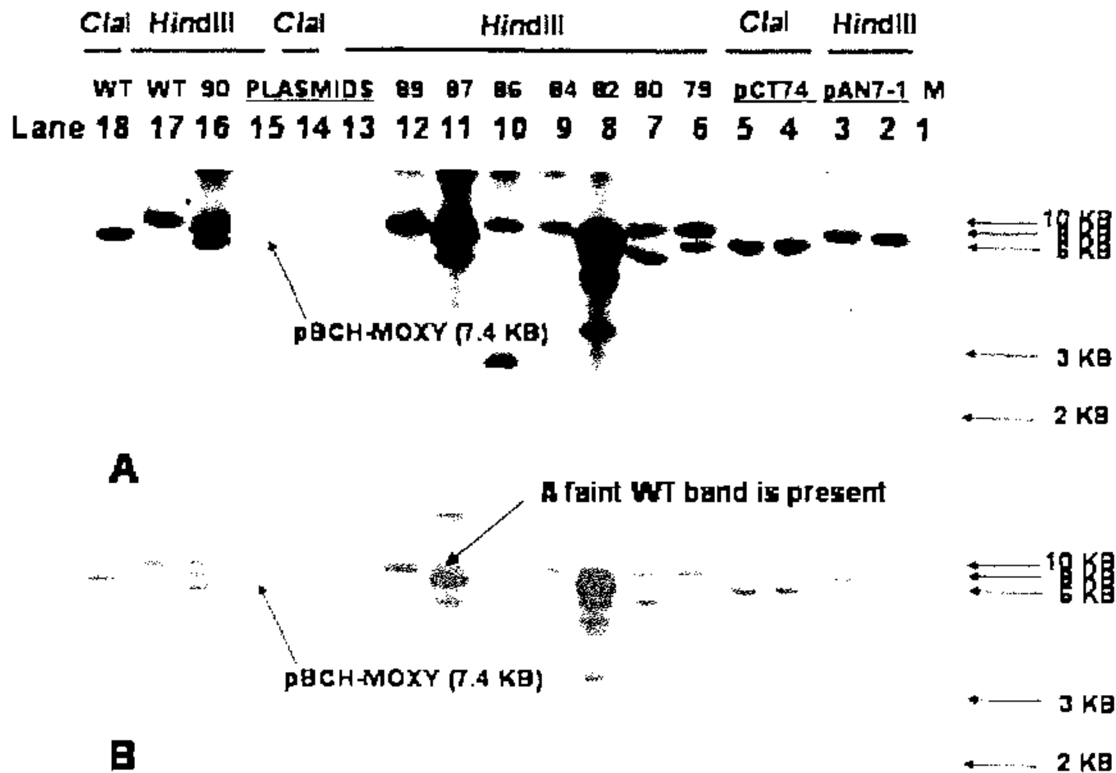

Figure 4.22 Hybridization of *P. chlamydospora* pBCH-*moxY* transformants' genomic DNA blot with labelled *moxY* gene fragment probe.
Legend is the same as that in Figure 4.20. "A" and "B" are the same MOXY hybridized membrane; "B" was adjusted with light brightness and light contrast.

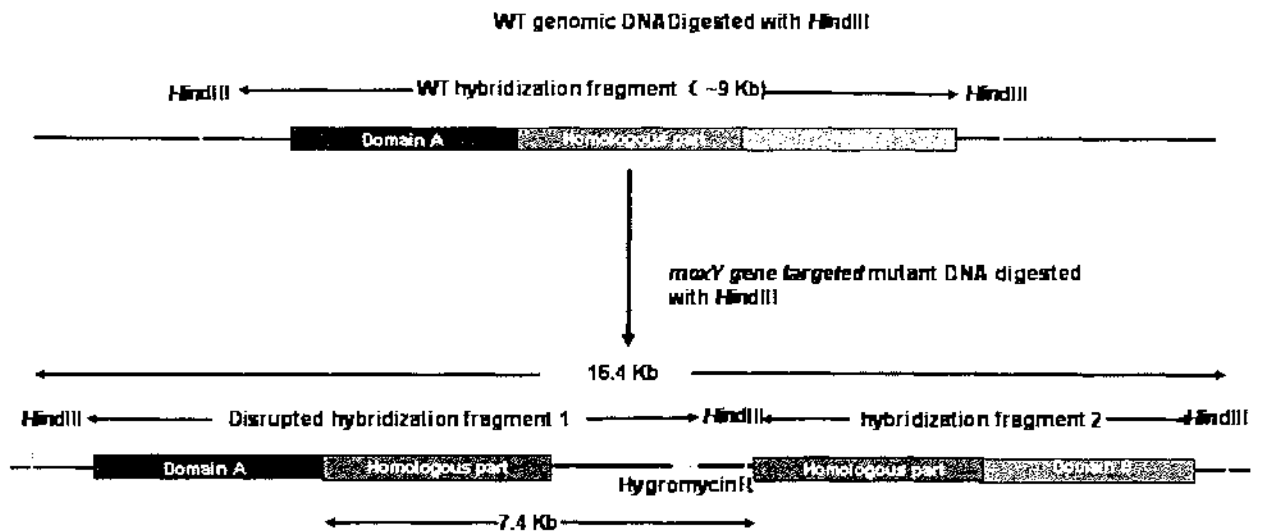

Figure 4.23 Comparison of *Hin*dIII digested hybridization fragment(s) between *P. chlamydospora* WT strain and *moxY* gene disrupted transformant.

There was a band in lane 15 which contained plasmid pBCH-*moxY*, which served as a positive control and indicated that the labelled *moxY* gene fragment had hybridized to target gene *moxY*. However the band in the positive control was very faint and by comparison with previous hybridization with probe *hph* (for pBCH-MOXY), which was also faint, while bands from other positive controls were strong, we concluded that the loaded DNA was insufficient. Since the wild type as well as *P. chlamydospora* transformants contained the original *moxY* gene in their genome, all positive band(s) were present in lanes 17 & 18 and showed that the *moxY* gene is present on an 9 kb *Hin*dIII fragment in the wild type genome.

moxY transformants PC79, PC80, and PC86 (lanes 6, 7, and 10) each had two bands, indicating that beside its original *moxY* gene, these pBCH-*moxY* transformants contained another ectopic copy of the *moxY* gene fragment which was introduced through plasmid pBCH-*moxY*. Lane 16 contained three copies of *moxY* gene fragments, hence two copies of plasmid pBCH-*moxY* had been integrated into the genome of transformant PC90. In addition, a clear WT band with ~8 kb was present; this excluded it as a *moxY* gene disruptant. Multiple copy integration was also found in lane 8 (PC82). Presumed pBCH-*moxY* transformant PC84 just contained one band, i.e. from its original *moxY* gene, thus it was concluded that this previously presumed pBCH-*moxY* transformant was not a real transformant, this was consistent with the hybridization result from probe *hph* and *gfp* (Figure 4.21). Size variation in the second copy of the *moxY* gene band in lanes 6, 7, and 10 indicated a random integration. Lane 11 (PC87) contained three copies of *moxY* gene fragment; only a faint wild-type size band was present besides two smaller bands. The probable reason for this was a mixed culture with wild type strain or non-disrupted transformant(s). Due to incomplete digestion and gel overloading, the presence or absence of a wild type band in lane 8 (PC82) was not clearly demonstrated. Hence both PC87 and PC82 were candidate *moxY* gene disruption transformants based on PCR and Southern hybridization results but the presence of a faint wild type band suggested they were both mixed cultures, further purification with spore purification method thus was required.

4.9 CHARACTERIZATION OF SPORE PURIFIED *P. CHLAMYDOSPORA* TRANSFORMANTS

The putative *P. chlamydospora moxY* gene disrupted transformants PC82 and PC87 were purified through the spore purification method described in Section 2.23.2. Again, genomic DNA of these two strains was extracted as before, followed by characterization through PCR amplification and Southern hybridization.

4.9.1 PCR CHARACTERIZATION

Three independently purified PC82 colonies numbered PC82.1, PC82.2, and PC82.3 and five PC87 colonies numbered PC87.1, PC87.2, etc were characterized through PCR with different combinations of primers (refer to Figure 4.16). In addition to primers MOXY3 and pBCH-MOXY1 that indicated gene disruption at the 5' end of the gene, new primers MOXY4 and pBCH-MOXY2 were used to indicate gene disruption at the 3' end (refer to Figure 4.16 showing positions of primers). MOXY2 and MOXY3 positive control primers were expected to give a 0.9 Kb with both wild type and *moxY* disruption, and could also give a bigger fragment (~8.5 Kb) with a *moxY* disruption. Similarly primers MOXY3 and MOXY4 should give ~1 kb in wild type and just ~8.6 Kb in a *moxY* disruption. PCR results are summarized in Figure 4.24.

Figure 4.24 A (checking 5' gene disruption) indicated that through comparison with WT strain (negative control), plasmid pBCH-*MOXY* was targeted to the *moxY* gene in all PC82 and PC87 clones; Figure 4.24 B (positive control) demonstrated that the PCR was working correctly; Figure 4.24 C (checking 3' gene disruption) further indicated that plasmid pBCH-*MOXY* was integrated into the target *moxY* gene through downstream amplification; Figure 4.24 D indicated that WT *moxY* gene(s) were still present in these transformants' genomes. Due to the PCR conditions not being suitable for long range PCR, no larger band(s) were observed in gel B and D, which would have been expected for disruption transformants (disruption transformants expected ~8.5--~8.6 Kb). Taken together, these results suggested that *P. chlamydospora* contained at least two *moxY* genes in the genome, a wild type copy and a disrupted copy. However at this stage, we can not distinguish whether the multiple copies of the *moxY* gene were from different loci

or as a result of diploid. In order to test these, Southern hybridizations were performed for confirmation of the presence of WT *moxY* gene(s).

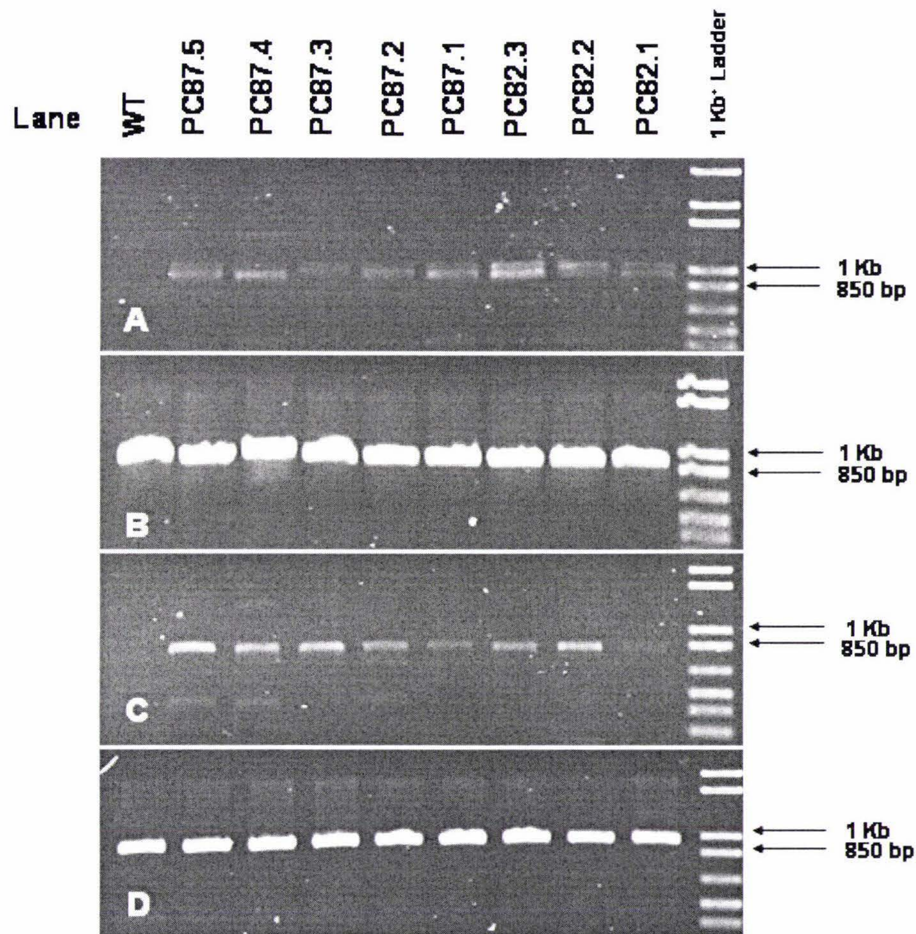

Figure 4.24 PCR characterization of spore purified *P. chlamydospora moxY* gene disruption transformants PC82 & PC87
A: PCR with primers *MOXY*3 & pBCH-*MOXY*1; B: PCR with primers *MOXY*2 & *MOXY3*; C: PCR with primers *MOXY*4 & pBCH-*MOXY*2; D: PCR with primers *MOXY*3 & *MOXY*4.

4.9.2 CHARACTERIZATION THROUGH SOUTHERN BLOTTING AND HYBRIDIZATION

The transformants and WT genomic DNA were digested with *Hin*dIII as before; the digestions are shown in Figure 4.25. This gel was blotted overnight and the membrane was hybridized with labeled probe *moxY* (Figure 4.26) and *hph* (Figure 4.27).

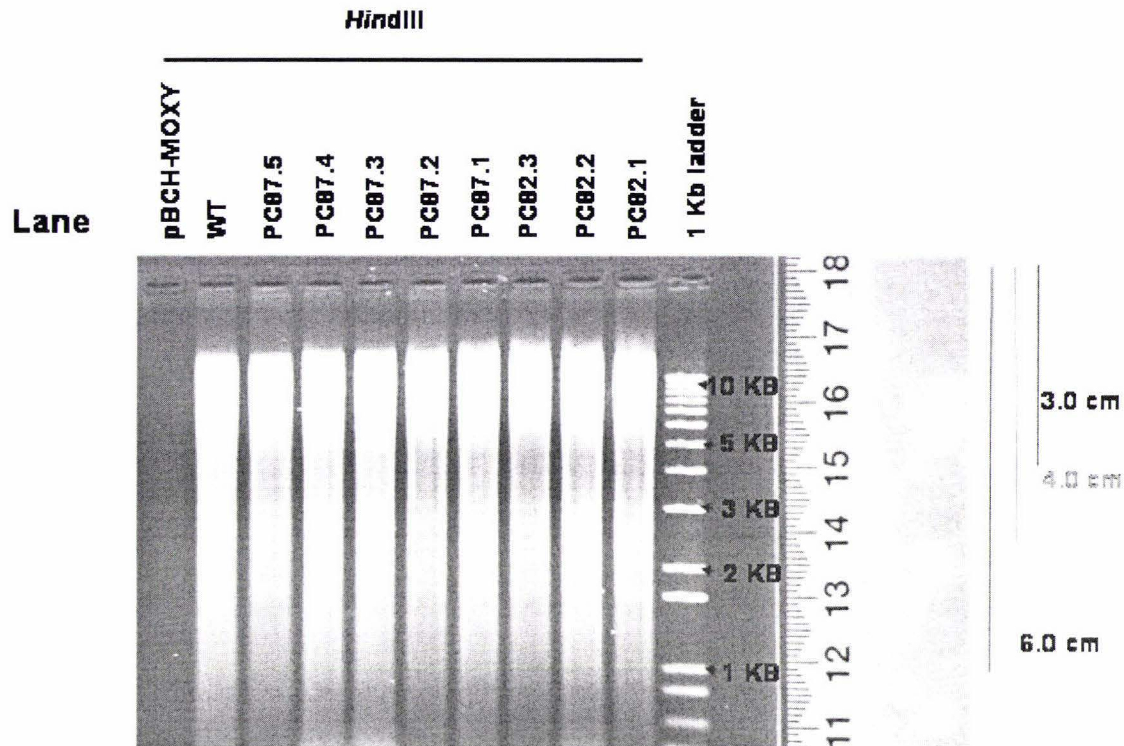

Figure 4.25 ***Hin*****dIII digestions of genomic DNA of** ***P. chlamydospora*** **PC82, PC87, WT, and digestion of plasmid pBCH-MOXY.**

Figure 4.26 indicated that all three PC82 colonies and all five PC87 colonies were genetically identical, but that PC82 and PC87 were different from each other. Linearized plasmid pBCH-MOXY and digested WT DNA (positive controls) indicated that the *moxY* probe successfully hybridized to its target gene. All samples have a band approximately the same as the wild type (band 1) and all have band 2, the same size as linearized pBCH-MOXY, suggesting multiple tandem copies of the disruption plasmid integrated (this was confirmed by Figure 4.27). The presence of the WT *moxY* gene in PC87 & PC82 from Southern hybridization affirmed the results from PCR with MOXY3 and MOXY4 primers (Figure 4.24 D) that suggested the presence of a wild type copy of *moxY* in addition to a disrupted copy. There are several possible reasons for this:

1: *P. chlamydospora* contains multiple copies of the *moxY* gene, either from different loci or because it is a genetic diploid. However as only one *moxY*-hybridizing band (~9 Kb)

occurred in WT DNA, this suggested that the multiple copies of *moxY* gene in the genome were more likely to be due to diploidy rather than to different loci.

2: The PCR screening results were false positives as a result of ectopic integrations.

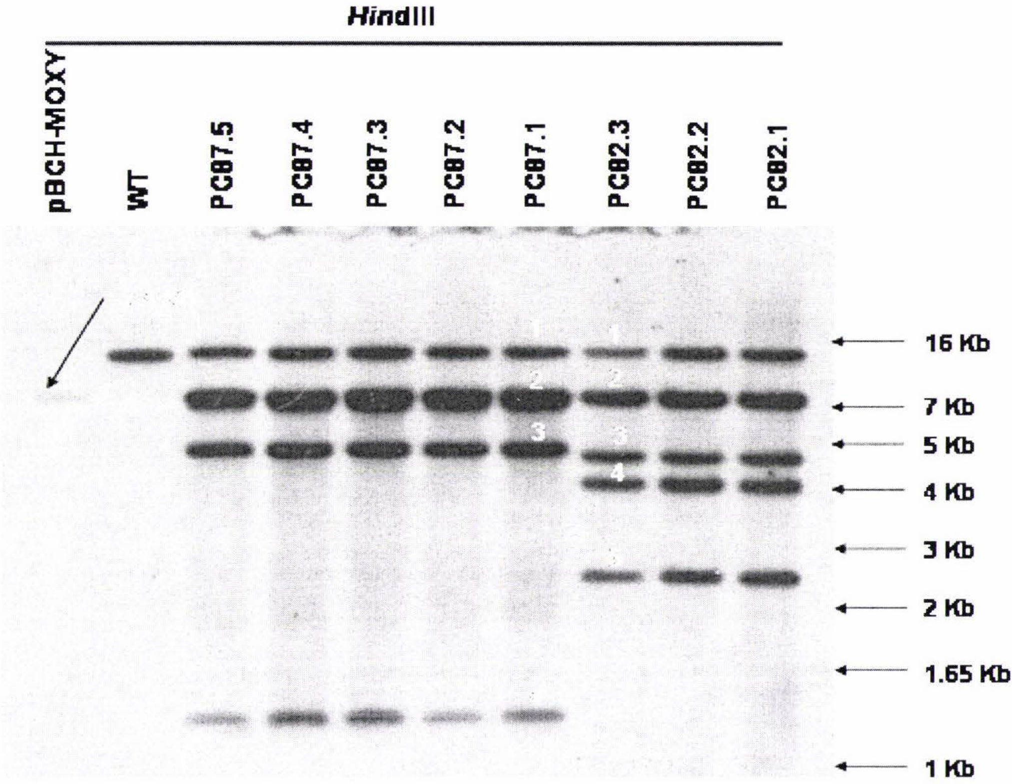

Figure 4.26 Hybridization with labeled probe *moxY*.

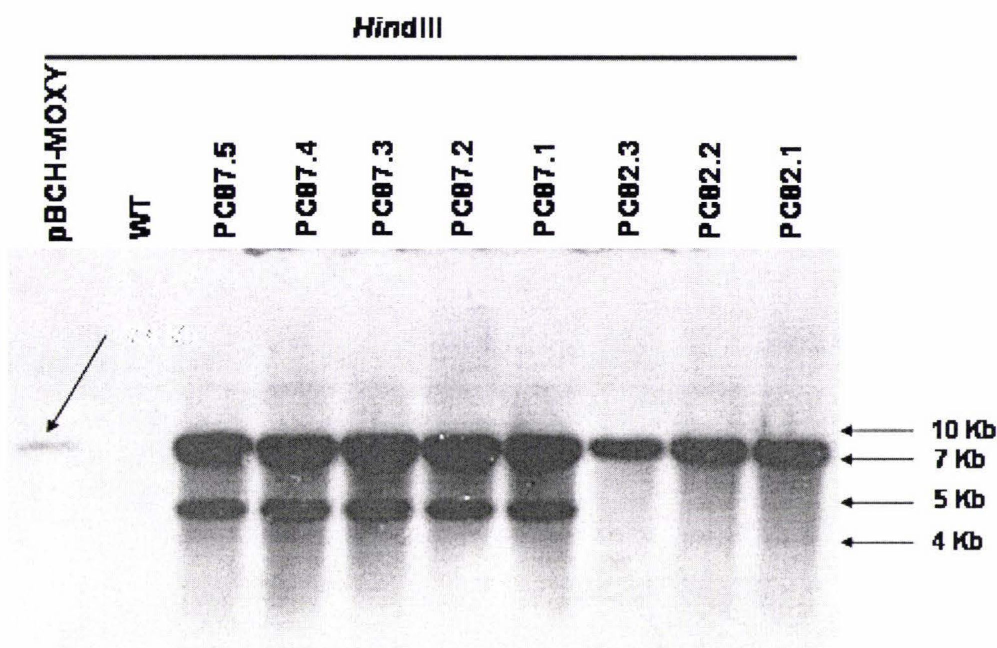

Figure 4.27 Hybridization with labeled probe *hph*.

If there was a *moxY* disruption, two hybridizing bands with a combined size of 16.4 Kb, would be expected (see Figure 4.23). Excluding bands 1 and 2 in Figure 4.26 (wild type and multicopy) transformant PC82 has bands much smaller than this. However, band 2 of PC87 has a higher intensity than any other bands, suggesting the possibility of two bands with a similar size, i.e. two bands with ~8 Kb and ~5 Kb (resulted from disruption) would be the hybridization bands in this case.

There is a further complication with both PC87 and PC82 in that the band 1 shows a slight difference in mobility with WT band, such that the presumed WT band 1 could be one of the disruption fragments. In this case, the disruption fragments would be ~9 kb and ~ 5 Kb (PC87) or ~9 Kb and ~4.5 Kb (PC82). The smallest band 4 of PC87 could have resulted from an ectopic integration. Taken together, a further checking with different enzymes thus was advisable.

Figure 4.27 indicated that labeled probe *hph* was capable of hybridizating to pBCH-MOXY (positive control), but did not hybridize to WT DNA (negative control). The intensity of bigger band (7.4 Kb, i.e. plasmid size) from PC87 is much higher than that of

the smaller one (4.8 Kb), suggesting an occurrence of tandem pBCH-MOXY integration or coincidence of two similar fragments. Only one band with ~7.4 Kb was present from PC82, this doesn't mean this transformant has only one copy of pBCH-MOXY as this is contradictory with the result from Figure 4.26. This can be attributed to the restriction fragments detected by *hph* being similar in size.

In order to exclude the possibility of multiple *moxY* genes in the genome, and to determine if a wild type copy remained in the transformants, Southern hybridization was repeated for 4 of the clones after digestion with different restriction enzymes (*Eco*RI & *Cla*I). The digestion results are shown in Figure 4.28.

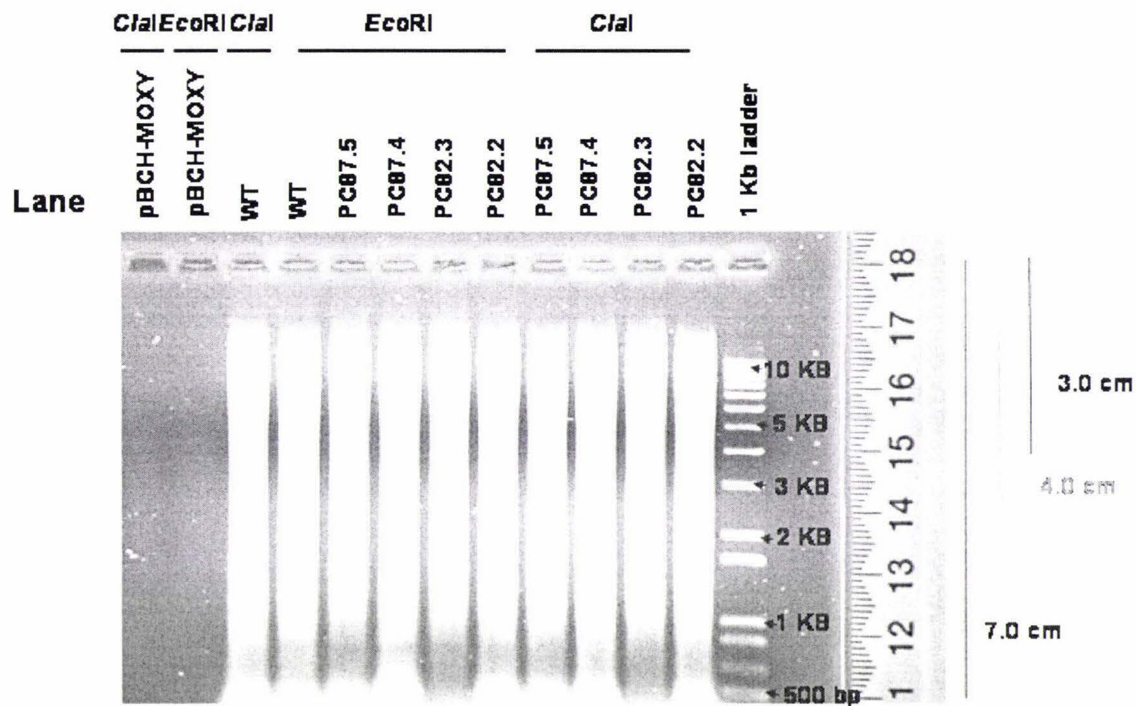

Figure 4.28 Restriction digestions of PC82, PC87, WT *P. chlamydospora*, and pBCH-MOXY with enzymes *Eco*RI and *Cla*I.

WT genomic DNA Digested with *Cla*I

*Cla*I ← WT hybridization fragment (~7.4 Kb) → *Cla*I

Domain A | Homologous part | Domain B

moxY gene targeted mutant DNA digested with *Cla*I

← 14.8 Kb →

*Cla*I ← Disrupted hybridization fragment 1 → *Cla*I ← hybridization fragment 2 → *Cla*I

Domain A | Homologous part | HygromycinR | Homologous part | Domain B

← 7.4 Kb →

← >7.4 Kb →

← <7.4 Kb →

Figure 4.29 Expected hybridization fragments when digested with *Cla*I.
WT has ~7.4 Kb fragment whilst *moxY* disruptants have one fragment bigger than 7.4 Kb, the other smaller than 7.4 Kb while the total size of the two hybridization fragments is 14.8 kb.

WT genomic DNA Digested with *Eco*RI

*Eco*RI ← ~2 Kb → *Eco*RI ← ~850 bp → *Eco*RI

Domain A | Homologous part | Domain B

moxY gene targeted mutant DNA digested with *Eco*RI

← 10.25 Kb →

*Eco*RI ← ~2 Kb → ← ~3 Kb → ~4 Kb ← ~550 bp → ← ~850 bp → *Eco*RI

Domain A | Homologous part | HygromycinR | Homologous part | Domain B

← 7.4 Kb →

Figure 4.30 Expected hybridization fragments when digested with *Eco*RI.

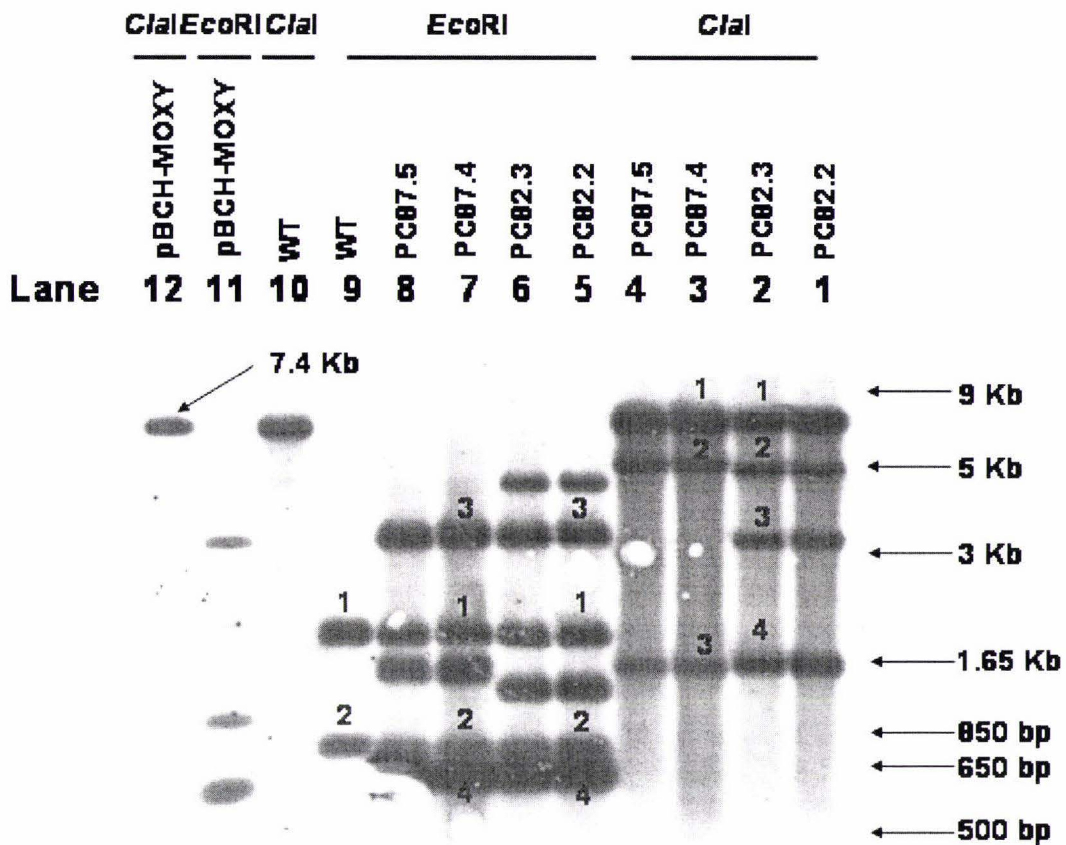

Figure 4.31 Hybridization with labeled probe *moxY*.

Figure 4.31 indicated that *moxY* probe hybridized as expected to *Cla*I linearized pBCH-MOXY (7.4 Kb in lane 12) and *Eco*RI digested pBCH-MOXY (~3 Kb band and ~550 bp in lane 11). In addition, a band with 900 bp and a faint band with 2 Kb also appeared in lane 11, this is probably due to non-specific hybridizations or incomplete digestion. WT genomic DNA was digested with the same two enzymes (lanes 10 & 9): these are controls for the determination of the presence and size of WT *moxY* genes in the transformants. By comparing *Cla*I digests, the WT *moxY* bands (lane 10) were also present in transformants PC82 and PC87 (lanes 1, 2, 3, and 4). However, it was noticed that these assumed WT bands had a slight difference in mobility with control WT band in lane 10. Since the exact sizes of disruption fragments expected are not known, it is still possible that these fragments (band 1 in lanes 1-4) are one of the expected disruption

fragments. Band 3 in lane 3 and Bands 3 & 4 in lane2 could be consequences of ectopic integration.

When digested with *Eco*RI, *moxY* disruptions were expected to have four *moxY*-hybridizing fragments as shown in Figure 4.30. Two of these bands would be the same size as the wild type (2 Kb and 850 bp, i.e. bands 1 & 2 in lane 9) whilst two bands would only be seen in a *moxY* disruption (~3 Kb and 550 bp, i.e. bands 3 & 4 in lanes 5-8). Both PC82 and PC87 have the wild type bands (1 & 2 in Figure 4.31) as well as the disruption bands (3 & 4 in Figure 4.31). PC82 contains another two fragments and PC87 contains another one: these might have resulted from ectopic integration.

All these results suggested that the WT *moxY* gene was present in the two putative *moxY* gene disruption transformants PC82 & PC87 whilst they also have a *moxY* disruption copy. In conclusion, two putative *moxY* gene disruption transformants PC82 & PC87 contain one disrupted as well as a WT *moxY* gene and an ectopic plasmid pBCH-MOXY.

4.10 COMPARISON OF GROWTH RATE BETWEEN WILD TYPE *P. CHLAMYDOSPORA* AND ITS TRANSFORMANTS

P. chlamydospora pBCH-MOXY transformants PC82, PC87, and PC86 (ectopic) were compared in terms of their respective growth rate. Subculturing of each transformant was described in Section 2.7 and results were measured after ten days incubation (Figure 4.32) and tabularized as in Table 4.3.

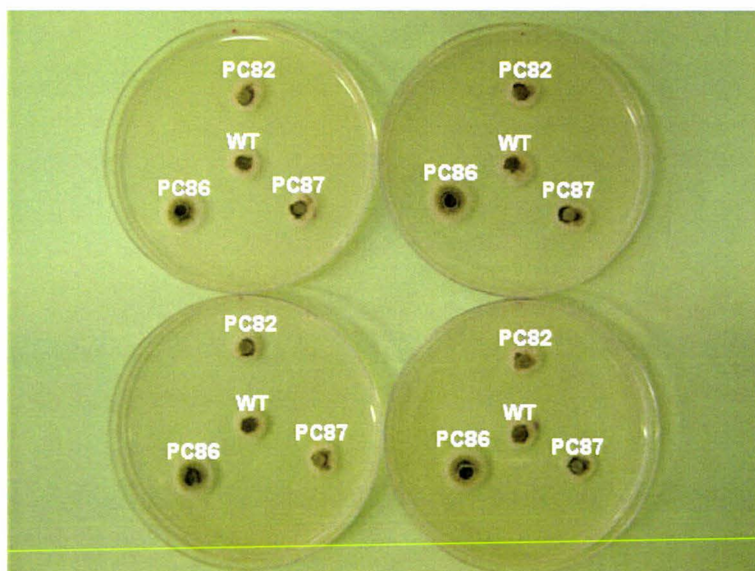

Figure 4.32 Morphological observation and comparison of growth rate between transformants and wild type *P. chlamydospora*. (Incubation at 22°C for 12 days).

Characteristic \ Strains	Wild type	PC82	PC86	PC87
Colony radius size (mm)	5.5	5.5	7.5	5.5
	6.5	6.0	7.5	6.0
	5.5	5.5	7.5	5.5
	5.5	6.0	7.5	5.5

Table 4.3 Comparison of growth rate between wild type *P. chlamydospora* and its transformants. WT and transformants colonies were grown at 22°C for ten days. The colony sizes were based on their radii. Statistical significance is studied through *t* test program (www.graphpad.com). Unpaired *t* test results show that all p values for each pair comparison are above 0.05 except comparison with PC86. P values: 0.0004 (WT & PC86); 1.0 (WT & PC82); 0.67 (WT & PC87); 0.0001 (PC86 & PC82); 0.0001 (PC86 & PC87); 0.54 (PC82 & PC87).

Result in Figure 4.32 and Table 4.3 demonstrated that the putative *moxY* gene disrupted transformants PC87 & PC82 didn't grow more vigorously than the WT. However, the ectopic strain PC86 grew more vigorously than the two transformants and its WT. This suggested that the site at which pBCH-MOXY integrated into the DNA of transformant PC86 played a negative role in colony growth.

4.11 CHECK MYCELIUM GROWTH OF WILD TYPE *P. CHLAMYDOSPORA* AND ITS *moxY* GENE KNOCK-OUT AND ECTOPIC TRANSFORMANTS

The *P. chlamydospora* mycelia including wild type and *moxY* gene disruption as well as ectopic transformants were grown in liquid PDB for ten days (refer to details given in Section 2.25.1, and were harvested after filtering through a membrane Section 2.25.2, followed by drying and weighing (Table 4.4).

Replicates / Strain	1	2	3	4	Average
Wild type	239	196	177	152	191
PC86	202	210	206	210	207
PC82	273	215	202	233	230
PC87	177	204	120	210	178

Table 4.4 Comparison of growth rate (mg dry weight) of wild type *P. chlamydospora* and its transformants grown in PDB for ten days at 22°C on a flask shaker rotating at 150 rpm. Unpaired *t* test results show that all p values for each pair comparison are above 0.05. P values: 0.42 (WT & PC86); 0.15 (WT & PC82); 0.65 (WT & PC87); 0.18 (PC86 & PC82); 0.21 (PC86 & PC87).

Result in Table 4.4 demonstrated that the putative one *moxY* gene disruption transformants PC87 and PC82 did not grow more vigorously than any other kind of transformants and wild type strain. This is consistent with the result in Table 4.3 while is contradictory with the PC86 result drawn from Table 4.3. A possible reason for this can be attributed to the media difference.

4.12 DISCUSSION:

4.12.1 A TRANSFORMATION SYSTEM FOR *P. CHLAMYDOSPORA* WAS ESTABLISHED

From the transformation results in Table 4.1, it was concluded that the classic protoplast/PEG transformation method is suitable for this fungus and the transformation frequency (~4 per μg DNA) was higher than that for *E. lata* (~1 per μg DNA) and 1.6-2.5 transformants for *Crinipellis perniciosa* (Lima *et al.*, 2003), although it is still low compared with most of those developed for other fungi.

The aims of transformation are usually to integrate foreign DNA (such as for homologous recombination) or foreign gene(s) for expression (such as *gfp* gene). Targeted integration of foreign DNA into host genomes is essential for the former whilst random integration of foreign gene(s) still meets the requirements for expression. Southern hybridization of *E. lata* pCT74 transformants S1 and S4 and *P. chlamydospora* pCT74 transformants pCT74-1 and pCT74-7 indicated a pattern of random integration and this pattern did not affect the *gfp* expression. In the aim of achieving *moxY* gene disrupted transformants through homologous recombination with the constructed vector pBCH-MOXY, targeted and ectopic integration occurred simultaneously, but around 98% of transformants were ectopic. Thus, the rate of targeted integration was relatively low.

4.12.2 STUDY OF BIOLOGICAL QUESTIONS THROUGH GENETIC TRANSFORMATION

The introduction of foreign DNA into cells through transformation does not necessarily imply that it will be generally useful as a research tool unless the fate of the transformed DNA can be predicted (Ruiz-Diez, 2002). If the DNA integration is mainly through homologous recombination to give a relatively low frequency of transformation such as transformation of *S. cerevisiae,* this is predictable and most of the transformants can be used for genetic study. However, in general, filamentous fungi differ from *S. cerevisiae*

in that high frequency transformation often results from non-homologous (ectopic) integration of DNA into the genome, as well as homologous integration (Ruiz-Diez, 2002). In this project, transformation of *P. chlamydospora* with vector pBCH-MOXY for disruption of the *moxY* gene only resulted in two possible *moxY* gene disrupted transformants from 102 transformants screened. This feature of unpredictability emphasizes the need for a more efficient targeted transformant screening system.

Gardiner and Howlett, (2004) recently reported a method that using thymidine kinase as a negative selection was able to increase the efficiency of recovery of transformants with targeted genes in the filamentous fungus *Leptosphaeria maculans* although this method was originally developed for mammalian systems (Muller, 1999) and was recently modified for rice (Terada *et al,* 2002). The system uses the thymidine kinase gene (*hv-tk*) from the herpes simplex virus as a negative selectable marker. When thymidine kinase is expressed in the presence of particular thymidine analogues, these analogues are converted to toxic compounds which subsequently kill the cells that contains vector incorporated by illegitimate recombination events into the genome. When the desired homologous recombination events occur, the toxic gene is lost and the cell survives (Figure 4.33). Therefore, transformants that contain full-length ectopic copies of the transforming DNA are eliminated, enriching the pool of transformants for those carrying the desired modification (Gardiner and Howlett, 2004). This will largely increase targeted integration and reduce the colony screening.

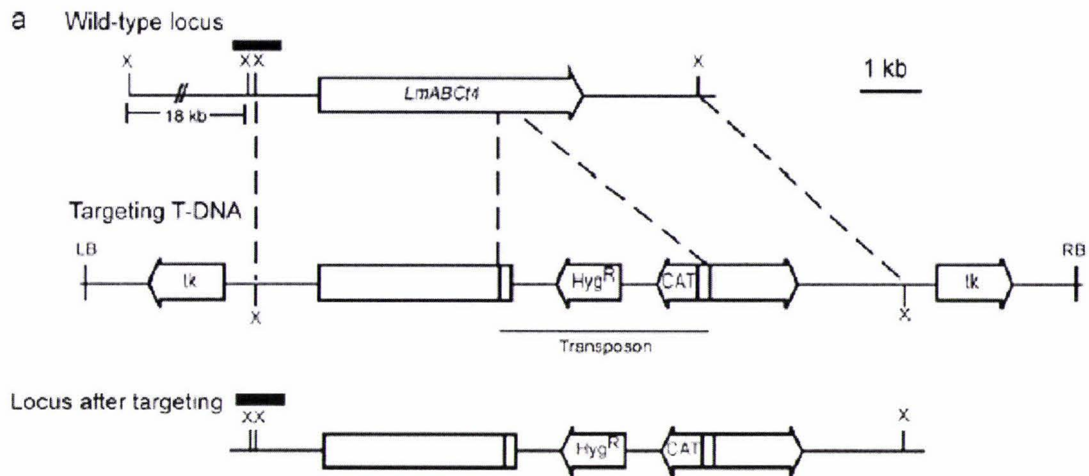

Figure 4.33 Strategy for disruption of *Leptosphaeria maculans* ATP-binding cassette (ABC) transporter 4 (*LmABCt4*). The targeting DNA shown contains the *hv-tk* cassettes. Regions of homology between the targeted gene and targeting construct are indicated by dashed lines. Double crossover events in the regions of homology between the T-DNA and wild-type locus result in incorporation of the disrupting fragment (transposon) and loss of the *hv-tk* cassettes. Transformants having undergone these events are resistant to thymidine analogues. Adapted from Gardiner and Howlett, (2004).

4.12.3 FIND AN EFFICIENT TRANSFORMANT SCREENING SYSTEM

In this project, a two round screening method with PCR followed by Southern hybridization was used. This method needs a large number of DNA extractions, and is laborious and time consuming. Finding a more efficient way to screen out the *moxY* mutant transformants is desirable. Colony hybridization procedures that have been developed for large scale transformant screening (Arganoza and Akins, 1995; Van den Brink *et al*., 1995), were not optimal as (van Zeijl *et al*., 1998) have said "the major disadvantages of this type of method are that nonspecific binding during hybridization may readily occur, screening for specific integration is, in many cases, not feasible and these procedures always take a few days". A direct PCR method on conidiospores developed for *Aspergillus* (Aufauvre-Brown *et al*., 1993) is only suitable for fragments

smaller than 1 kb (amplified fragment is ~1.1 kb in this case), and is therefore inappropriate for this type of screening.

A successful screening with a colony-PCR based method has been reported for filamentous fungi. In this method, PCR is done directly on mycelial pellets or colonies treated with NOVOzym 234 (an enzyme that removes the cell walls), and allows rapid screening of large numbers of transformants of both sporulating and non-sporulating fungi (van Zeijl *et al.*, 1998). Fragments more than 3 kb in size can be amplified by this method, thus is appropriate for *moxY* disrupted transformants screening. The NOVOzym 234 has been replaced by glucanex as NOVOzym 234 is no longer available. This modified screening was tried in this project and was unsuccessful. Were the mycelia used for screening too old and resistant to the removal of the wall by the enzyme glucanex? This was not the case as further tests on younger mycelia were still unsuccessful (data not shown). Perhaps the first step of incubation of the mycelia (60 min at 37°C followed by 95°C for 3 min) in the glucanex solution for removal of the cell wall was insufficient. Extending of the incubation time should be tested.

4.12.4 RECENT STUDY ON *P. CHLAMYDOSPORA*

P. chlamydospora is the main microorganism associated with the decline of young grapevines known as Petri grapevine decline, black goo or slow dieback (Mugnai *et al*, 1999; (Alves *et al.*, 2004). Previous studies on *P. chlamydospora* mainly focused on strain identification and characterization. *P. chlamydospora* has been reported in different regions of New Zealand (Ridgway *et al.*, 2002), and has also been reported in the United States (Stewart *et al.*, 2003), Australia (Edwards and Pascoe, 2004), and Chile (Auger *et al.*, 2004). However, investigations on this fungus at a molecular level were not widely implemented except those aimed at characterization through PCR amplification (Groenewald *et al.*, 2000);(Ridgway *et al.*, 2002). A PCR-based method for the detection of *P. chlamydospora* in grapevines was established using two specific primers, PCL1 and PCL2, synthesized from the variable internal transcribed spacers ITS1 and ITS2 of the ribosomal DNA(Groenewald *et al.*, 2000).

The presence of *P. chlamydospora* in New Zealand has been confirmed by analysis of the ribosomal gene region amplified from morphologically identified isolates as sequencing of six isolates revealed that their nucleotide sequences were 99.9-100% similar to those reported in GenBank (Ridgway *et al.*, 2002).

Papers that correlated this fungus with grapevine disease (Petri disease) were frequently found, whereas the underlying mechanisms of the disease caused by this fungus still remain elusive. Does Petri disease result from toxins produced by *P. chlamydospora* similar to the eutypine produced by *E. lata*? A recent study by Sparapano *et al*, (2000) on chemicals (such as pullulan) from *Phaeoacremonium* culture filtrates has given insights into the role of these chemicals in Petri disease development.

Pullulan, a mixture of α-glucans with varying molecular weight, is produced by species of *Phaeoacremonium,* including *Phaeoacremonium chlamydosporum* (i.e. renamed *Phaeomoniella chlamydospora*), *P. aleophilum* and *Fomitiporia punctata*, as well as by another plant pathogenic fungus, *Cryphonectria parasitica* (Murrill) Barr. It has ben shown to be toxic to grapevines (Corsaro *et al*, 1998; Sparapano *et al*, 2000). Phytotoxic metabolite extraction and purification followed by re-injection into plant grapevine woody tissue of shoots and branches demonstrated that these metabolites were able to produce foliar symptoms similar to those shown by the esca-affected vines. This provided new information on the production of phytotoxic metabolites with esca and related diseases of grapevine (Sparapano *et al*, 2000).

Through bioinformatics, a putative toxin gene, *moxY,* has been identified. The next step was to characterize this gene through the traditional genetic method of "gene disruption". This project has established a transformation system and obtained putative *moxY* gene disrupted transformants. However in each case there was evidence for an additional intact WT *moxY* gene, this suggested that either *P. chlamydospora* maybe a diploid with two sets of homologous genes, or that *moxY* mutation is lethal, and can only survive if wild type copies are presented (i.e. in a forced diploid). Therefore we still are not able to determine the function of *moxY* gene at this stage because of the presence of a WT *moxY*

gene. However it may be possible to separate *moxY*-disrupted and wild type nuclei or to disrupt the remaining WT *moxY* gene through homologous recombination. Thus identification of the potential role of the *moxY* gene in metabolite biosynthesis and therefore of the possible role in the Petri disease would be facilitated. In addition, chemical differences between *moxY* mutants and wild type/ectopic mutants can be determined using spectrophotometer or high performance liquid chromatography (HPLC). Further, with the achievement of a *gfp* gene expressing transformant, more detailed investigations of the relationship between this fungus and its host plant are possible, particularly of the initial stages on infection.

5.1 ESTABLISHMENT OF A TRANSFORMATION SYSTEM FOR *E. LATA* AND *P. CHLAMYDOSPORA*

The ability to transform *S. cerevisiae* using auxotrophic markers, and the development of *E. coli* shuttle vectors for this organism, represented the beginning of fungal molecular genetics (Hinnen *et al.*, 1978). Subsequently, transformation systems were developed for model filamentous fungi including *Neurospora. crassa* (Case *et al.*, 1979) and *Aspergillus nidulans* (Ballance *et al.*, 1983; Tilburn *et al.*, 1983). Transformation is of great importance and is an essential part of modern fungal research.

Currently, the main existing methods that can be used for transformation are protoplast/PEG method, electroporation, biolistic transformation, and *Agrobacterium tumefaciens*-mediated transformation. As none of these transformation methods can be universally applied to all fungi, individual species must be considered independently, and appropriate systems need to be chosen and optimized for each organism. The most popular one for filamentous fungal transformation at present is the classical protoplast/PEG method using hygromycin B resistance gene (*hph*) as a dominant selectable marker since it can be used directly in the wild-type strain, without need for a recipient mutant strain.

Meyer *et al.*, (2003) tested and compared these four different transformation methods in an attempt to facilitate the genetic transformation of *Aspergillus giganteus*, they found that the protoplast/PEG method worked well so it was chosen for these fungal transformations.

Based on the PEG/protoplast method, a transformation system was established for *E. lata* and *P. chlamydospora.* The transformation frequencies for *E. lata* transformation ranged from 0.7 (for plasmid pBCH-*gfp*) to 1 (for plasmid pAN7-1) transformants and for *P. chlamydospora* ranged from 4.1 (for pAN7-1) and to 6.5 (for pBCH-MOXY)

transformants per μg plasmid DNA. All transformants were characterized with PCR, followed by confirmation through Southern hybridization with labeled *hph* and *gfp* probes. In addition, transformants plated on selective media containing 100 μl/ml hygromycin demonstrated vigorous growth compared with non-growth of the wild type strain, indicating that the expression of antibiotic resistance gene *hph* conferred hygromycin resistance to these transformants.

Compared with those developed for other fungi, such as the 5-46 transformants per μg plasmid pAN7-1 for *Pleurotus ostreatus* (Peng *et al.*, 1992), 3-5 transformants per μg plasmid pAN7-2 for *Verticillium dahliae* (Dobinson, 1995), 7/μg for *Dothistroma pini* (Bradshaw *et al.*, 1997), and 1.6-2.5/μg for *Crinipellis perniciosa* (Lima *et al.*, 2003) this transformation frequency developed for *E. lata* is relatively low, but is still comparable as transformation efficiencies for filamentous fungi are generally low.

5.2 IMPROVEMENT OF THE TRANSFORMATION EFFICIENCY

Protoplast preparation from fungal cells using cell-wall degrading enzymes remains the most common method for preparation of cells for transformation.

Protoplast concentration is a critical factor for successful transformation. Usually, protoplasts are used at a density of about 10^8-10^9/ml. In this project, the concentrations of protoplast ranged from 5.8 x 10^6 to 2.3 x 10^8 for *E. lata*, and from 2.5 x 10^7 to 6.3 x 10^8 for *P. chlamydospora.* Obviously, improvement of the concentration of protoplasts is a potentially feasible way for the improvement of the transformation efficiencies. Factors such as cell type, growth phase, growth conditions, lytic enzymes, DNA forms (either linear or circular double-stranded), and regeneration medium all can exert an influence on protoplast generation or DNA integration, and therefore play a critical role in the transformation efficiencies (Ruiz-Diez, 2002). The regeneration rate for fresh *E. lata* protoplast was tested and the results ranged from 2.29 x 10^{-3} to 4.95 x 10^{-3}. In contrast, the regeneration rate for protoplasts stored at -80 °C was around 1 x 10^{-5} (Duan, 2003), hence the use of fresh rather than frozen protoplast is advisable.

Scientists have employed several alternative ways for filamentous fungal transformation. As early as 1989, electroporations of the filamentous fungi *Fusarium solani* and *A. nidulans* with DNA was investigated by (Richey *et al.*, 1989) based on fungal protoplasts. This method brought foreign vector DNA to fungal cells through a high-amplitude electric pulse delivered from a Gene Pulser apparatus. It provides an alternative method of introducing foreign DNA into fungi that cannot be transformed by traditional methods (Richey *et al.*, 1989). Electroporation of β-glucuronidase-treated germinating conidia of filamentous fungi *N. crassa* and *Penicillium urticae* with vectors containing an *hph* gene yielded transformation frequencies ranging from 1,100 to 1,750 transformants and even 2.8 x 10^3 transformants per μg plasmid DNA, respectively (Chakraborty and Kapoor, 1990). However, not all fungi show efficient uptake of foreign DNA with this method. Also, production of protoplasts has been problematic for some fungi.

A method called biolistic transformation for incorporation of plasmid DNA into intact, thick-walled fungal cells was introduced by Klein *et al.*, (1987). This method directly brings particles coated with DNA into fungal spores or hyphae by using the particle-inflow gun, and has been reported for fungal transformation such as *Trichoderma-harzianum* and *Gliocladium-virens* (Lorito *et al.*, 1993), *Neocallimastix frontalis* (Durand *et al.*, 1997), and more recently, *T. reesei* (Hazell *et al.*, 2000). However, due mostly to low efficiency, application of this method is still limited.

In order to improve the transformation frequency, *Agrobacterium tumefaciens* was investigated to transfer part of its Ti plasmid, the T-DNA, to filamentous fungal cells as was initially used for transformation of plant cells (de Groot *et al.*, 1998). It was reported that *A. tumefaciens* could not only transfer its T-DNA efficiently to the protoplasts but also to the conidia of *Aspergillus awamori* with transformation frequencies up to 600-fold higher than conventional techniques for transformation of *A. awamori* protoplasts. In addition, transformations of a variety of other filamentous fungi, including *Aspergillus niger, Fusarium venenatum, Trichoderma reesei, Colletotrichum gloeosporioides, Neurospora crassa,* and the mushroom *Agaricus bisporus*, were also successful, demonstrating that transformation using *A. tumefaciens* is generally applicable to filamentous fungi (de Groot *et al.*, 1998).

Meyer *et al.*, (2003) tested and compared these four different transformation methods in the study of genetic transformation of *Aspergillus giganteus*. They found that electroporation and biolistic transformation were inappropriate for transforming *A. giganteus*, due to a low transformation yield. The conventional transformation technique based on protoplasts/PEG yielded up to 55 transformants in 10^8 protoplasts/μg DNA and was enhanced to 140-fold by *A. tumefaciens*-mediated transfer. Due to the limited time and fulfillment of the aim to obtain *gfp*-expressing transformants, transformations with *A. tumefaciens*-mediated method were not performed in this study. However this remains the potential way for a dramatic improvement of transformation efficiency for *E. lata*.

5.3 MODIFICATION OF PROTOPLAST/PEG TRANSFORMATION

In order to obtain various mutants which can be subsequently used in the cloning of the genes affecting a particular characteristic, transformation can also be performed with protoplast/PEG method through restriction enzyme-mediated integration (REMI) (Riggle and Kumamoto, 1998). Genetic transformation of the basidiomycete *Ganoderma lucidum* with this method achieved a transformation yield of 4-17 transformants per μg plasmid DNA (Kim *et al.*, 2004) and of *Coprinus congregatus* up to 550 transformants per μg of DNA (Leem *et al.*, 1999). Further study on toxins or toxin production genes such a those required for eutypine biosythesis can use this method for specific gene cloning.

5.4 SELECTABLE MARKER

Most recently developed transformation systems are focused on the new discoveries on marker selection and vector construction, such as construction of the vector pSCFUM by introducing the sC gene encoding ATP sulfurylase into the pUC18 vector (De Lucas *et al.*, 2001). The sC gene conveys strong selenate resistance as a selective marker instead of the traditional *hph* gene. Another advantage of introducing the sC gene into pUC18 is for targeting specific genetic constructs to the *A. fumigatus* sC locus, thus facilitating studies of gene regulation and function (De Lucas *et al.*, 2001).

Many dominant selectable markers are now available for use in transformation. Auxotrophic markers have also been widely used, such as using the gene *pyrG* (*A. nidulans*) or *pyr*-4 (*N. crassa*) for complementation of uracil auxotrophs in these strains. However, these have the disadvantage of the requirement for generating the appropriate genotypes in desired recipient strains, which is difficult in species without good genetic systems (Ruiz-Diez 2002). Thus this kind of selective marker is inappropriate for the two fungi in this project.

Drug resistance markers have proved to be of great utility in studies with filamentous fungi. Examples of drug resistance genes used for transformation include the oligomycin resistance gene from *A. nidulans* (Ward *et al.*, 1986), bacterial resistance gene to bleomycin and phleomycin (Austin *et al.*, 1990; Drocourt *et al.*, 1990), and to hygromycin B. Particularly for hygromycin B resistance gene *hph*, this gene has been used very extensively with a variety of fungi (Kwon-Chung *et al.*, 1998) although some species like *A. nidulans* are relatively resistant to hygromycin. Successful fungal transformations with hygromycin B selection include those developed *Dothistroma pini* (Bradshaw, *et al.*, 1997), *Candida oleophila* (Yehuda *et al.*, 2001) and *Crinipellis perniciosa* (Lima *et al.*, 2003).

In this project, the positive selective marker used for selection is the antibiotic resistance gene *hph*. However not all fungi are suitable for this selective marker. There is no transformant which shows stable resistance against the antibiotic in *C. congregatus* (Leem *et al.*, 1999) as a result of the DNA methylation inside the transformed strains, which leads to an unstable expression of this gene (Mooibroek *et al.*, 1990). In this case, alternative ways (other antibiotics such as geneticin and phosphinothricin may not be methylated) for selection may be tried. Successful transformation of *C. congregatus* (Leem *et al.*, 1999) with the phosphinothricin resistance gene *bar* which inhibited glutamine synthetase was reported. In this project, the transformants of *E. lata* and *P. chlamydospora* show vigorous growth in selective media, indicating that the *hph* gene was stably expressed.

5.5 *gfp* EXPRESSION IN TRANSFORMANTS

The green fluorescent protein (*gfp*) was originally isolated from the bioluminescent jellyfish, *Aequorea victoria*. It is a very good biomarker and has been used widely as a reporter gene in variety of organisms. The advantages of using *gfp* gene as a reporter gene include that *gfp* expression is stable; it only requires oxygen for fluorescing and rarely causes adverse effects after expression in transformants (Chalfie *et al.*, 1994). Successful utilizations of *gfp* as a reporter gene in different phytopathogens including species of *Fusarium, Colletotrichum, Aspergillus, Cercospora, Alternaria* (Fernandez-Abalos *et al.*, 1998; Lorang *et al.*, 2001; Tavoularis *et al.*, 2001; Chung *et al.*, 2002; Horowitz *et al.*, 2002; Chen *et al.*, 2003; Oren *et al.*, 2003).

Recently, using gene *gfp* as a reporter gene for establishment of a relationship between pathogens and their host plants has been popular. In a study done by (Balint-Kurti *et al.*, 2001), a genetic transformation system was developed for three Mycosphaerella pathogens (*Mycosphaerella fijiensis, M. musicola, and M. eumusae*) of banana and plantain (*Musa* spp.). They were transformed with a construct carrying a synthetic gene encoding green fluorescent protein (*gfp*). *gfp* expression by transformants allowed them to observe extensive fungal growth within leaf tissue that eventually turned necrotic.

The recent studies on *E. lata* are mainly focused on the toxins (eutypine) or similar chemicals that are produced by this fungus. However the size, distribution, and behavior of *E. lata* colonies within the host tissue may be important factors in the generation of *Eutypa* dieback symptoms. Understanding the pattern of *E. lata* colonization and relating this pattern to symptom expression will further our progress in understanding *Eutypa* dieback disease as well as the life-style of the pathogen. Studies on this pathogen/host interaction might be achieved with traditional methods such as in a study of grapevine Pierce's disease caused by the bacterium *Xylella fastidiosa* with light microscopy (Hopkins, 1981), scanning electron microscopy (Tyson *et al.*, 1985), transmission electron microscopy (Mollenha and Hopkins, 1974), and immunofluorescence microscopy (Brlansky *et al.*, 1982). However these

methods need extensive preparation of the sample prior to microscopy. In addition, dissection and preparation of samples is fraught with procedural issues, leading to uncertainty about the original spatial distribution of the pathogen (Newman *et al.*, 2003). A report demonstrated that a better understanding of the pattern of the host colonization and its relationship to the Pierce's disease was achieved with a *gfp*-expressing *X. fastidiosa* strain using confocal microscopy (Newman *et al.*, 2003).

With the achievement of *gfp*-expressing *E. lata* and *P. chlamydospora* transformants, further studies on pathogen/host interaction will be facilitated.

5.6 GENE DISRUPTION

Classic approaches for identifying candidate virulence genes start with random mutagenesis and the selection of mutants based on a desired phenotype. Since the advent of reverse genetic approaches based on homologous recombination with transforming DNA, the introduction of defined, mutations specifically into the genes of interest and reintroduction of intact genes of interest into the mutants has been widely used (Kwon-Chung, 1998). Gene disruption is a powerful tool that can define pathogenic or virulence factors. In the past a few years gene disruption approaches have been widely used to identify fungal virulence genes, such as the capsule genes and certain kinases of *Cryptococcus neoformans* (Alspaugh *et al.*, 1997) and genes involved in hyphal formation (Braun and Johnson, 1997; Gale *et al.*, 1998; Lo *et al.*, 1997).

For filamentous fungi, disruption of genes in pathogenic *Aspergillus* spp. encoding various degradative enzymes, toxins or hyphal wall components failed to provide unambiguous evidence for their importance in pathogenicity (AufauvreBrown *et al.*, 1997; Chang *et al.*, 1998; Holden *et al.*, 1994; Jatonogay *et al.*, 1994; Kwon-Chung, 1998). However Tsai *et al.*, (1998) found that disruption of *alb1*, a gene regulating condial pigment synthesis, resulted in a measurable loss of virulence.

Another function of gene disruption is to determine whether a gene is essential for viability. A typical example is the disruption of the yeast actin gene through

integration by a single homologous recombinational event between the chromosomal actin gene and the internal homologous fragment in a constructed plasmid pRB111 (Figure 5.1) (Shortle *et al.*, 1982). In a diploid strain of yeast, they disrupted the actin gene on one chromosome; this resulted in a mutation that segregated as a recessive lethal tightly linked to a selectable genetic marker on the integrated plasmid pBC111. They therefore determined that the actin gene must encode an essential function for yeast cell growth. Similarly, for determination of the role of the *cycA* gene (cytochrome *c*) in the oxidative respiratory pathway in *Aspergillus nidulans*, replacement of one homologue of the *cycA* gene was carried out in a diploid strain. Segregation of the diploid showed that in this case, the gene-disrupted mutants were viable, although their growth was impaired (Bradshaw *et al.*, 2001).

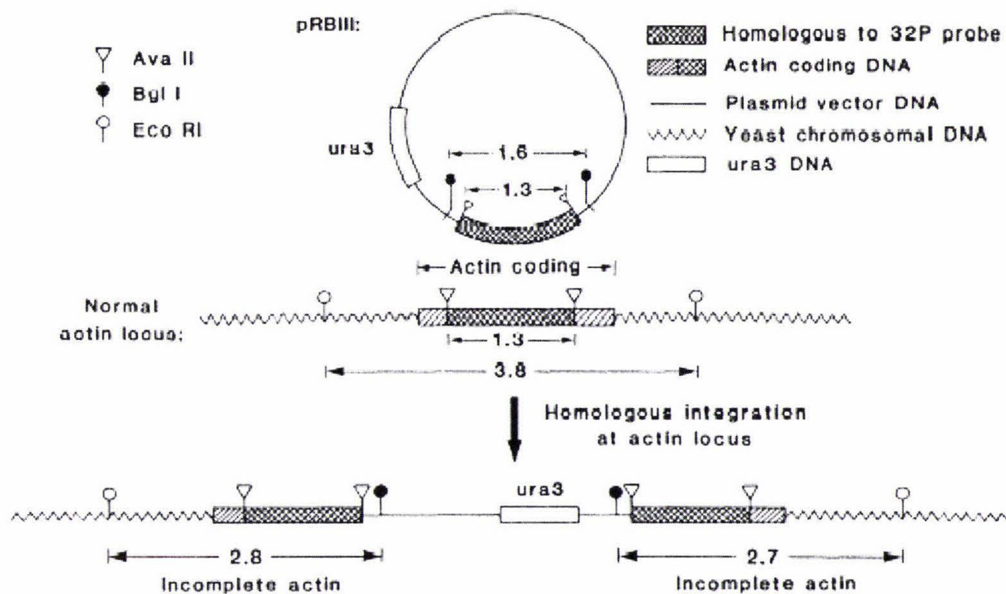

Figure 5.1 Structure of the yeast actin locus before and after recombination with the hybrid plasmid.pRB111. (adapted from Shortle *et al.*, 1982).

Recently, targeted gene disruption by *in vitro* transposon mutagenesis has been used to identify the genes required for biosynthesis of the *Haemophilus influenzae* Rd cell wall under standard cultivation conditions. Of the 28 genes known to be associated with the cell wall biosynthetic pathway, 14 were determined to be essential (Trepod and Mott, 2005).

All these demonstrated that gene disruption through homologous recombination is the most powerful and effective method so far for determination of a gene's function.

In this project,attempts were made to determine the role of the putative toxin gene *moxY* by *moxY* disruption. Transformants were obtained in which the *moxY* gene was disrupted but a wild type copy of *moxY* was retained despite extensive purification of the transformants. It is possible that *P. chlamydospora* is naturally a diploid, and has two sets of identical genes. This would make simultaneous disruption of two identical *moxY* genes extremely difficult. To determine whether the *moxY* gene is essential for viability of *P. chlamydospora*, Benomyl-induced haploidisation of this diploid could be performed.

Another possibility is spores themselves may be heterokaryotic (2 nuclei), i.e. one is wild type and the other is *moxY* disruption or *gfp* containing nucleus. This is supported by the patchy *gfp* expression seen in *P. chlamydospora gfp* transformants pCT74-7 and spores of pCT74-1 (D & C in Figure 4.15 B). However that whether *P. chlamydospora* is heterokaryotic or diploidy is still not known for sure, despite that *gfp* is still useful for the establishment of interactions between *P. chlamydospora* and its hosts, further experiments for distinguishing this are required.

CHAPTER SIX CONCLUSION AND FUTURE WORK

6.1 GENERAL CONCLUSION OF TRANSFORMATION OF FILAMENTOUS FUNGI *E. LATA* AND *P. CHLAMYDOSPORA*

Development of an efficient plasmid-mediated transformation system is the first step for molecular genetic manipulation of micro-organisms. As Ruiz-Diez, (2002) said in a review of strategies for the transformation of filamentous fungi: "transformation includes i) infusion of exogenous DNA into recipient cells; (ii) expression of genes present on the incoming DNA; and (iii) stable maintenance and replication of the inserted DNA, leading to expression of the desired phenotypic trait".

In this project, I developed a transformation system for two fungi *E. lata* and *P. chlamydospora* with the classic protoplast/PEG method, and the transformation frequencies for them are around 0.9 transformants (Table 3.1 in chapter three) and 5.3 transformants (Table 4.12 in chapter four) per μg plasmid DNA, respectively. Based on this method, the reporter genes *gfp* located in plasmid pBCH-gfp and pCT74 were transferred into *E. lata* protoplasts, and expression of the *gfp* gene in this transformants was checked using a UV microscope. However the *gfp* gene in pBCH-gfp was not expressed in *E. lata* transformants (G8, G21, G22) whilst the *gfp* gene in pCT74 was expressed (refer to Figure 3.13, 3.14, 3.15, and 3.16). The purified *gfp*-expressing pCT74 *E. lata* transformant S1 was inoculated onto blackcurrants as well as grapevines for tracking the internal movement of this fungus in host plants after colonization and for establishment of an internal interaction or relationship between the fungus *E. lata* and its host plants. However an internal interaction between them was not established as confocal examination showed that penetration of the mycelium in the wood tissues was not successful. Fluorescent mycelium was only observed outside the bark and the interface between the bark and wood tissues. The main reason for this is the limited incubation time as *E. lata* die-back disease development usually needs a few years (refer to chapter one introduction). Another possibility is that the transformed fungus had lost the ability to colonize this substrate. All kinds of

transformants were characterized with PCR amplification of characteristic genes (*hph* and *gfp*), followed by confirmation with Southern hybridization.

In addition, plasmid pCT74 containing a *gfp* reporter gene was also transformed into cells of *P. chlamydospora*. The *gfp* reporter gene was expressed in transformants of *P. chlamydospora* (pCT74-1, pCT74-3, pCT74-6, pCT74-7, and pCT74-8) and checked with a UV microscope.

Based on the publication database, *P. chlamydospora* was transformed for the first time and transformants were harvested. Due to the limitation of time for this project, inoculation of the *P. chlamydospora gfp*-expressing pCT74 transformants onto host plants was not performed. Spore purified transformants with one disrupted as well as a WT putative toxin gene *moxY* were obtained through homologous recombination after transformation with the constructed vector pBCH-MOXY containing part of the *moxY* DNA sequence. These *moxY*-containing disrupted transformants were screened with PCR and confirmed with Southern hybridization. The growth rates of these transformants were compared with ectopic and wild type strains. Additionally, chemical differences between them were examined through a spectrophotometer. The *moxY* work has led to speculation *P. chlamydospora* may be diploid, but is unlikely to be a heterokaryon due to the spore purification method used (Section 2.23.2).

6.2 POTENTIAL BENEFITS OF THIS WORK

All this work is based on the established transformation system. All transformants, including *gfp*-expressing pCT74 transformants of *E. lata* and *P. chlamydospora*, one *moxY* gene disrupted transformant and ectopic transformants, which have been genetically transformed, open the door to more detailed molecular analysis. Any aim of genetic analysis of specific genes in these two fungi could potentially benefit from having a transformation system available. The *P. chlamydospora* one *moxY* gene disrupted transformant will facilitate future infection studies with this pathogen on grapevine. This work leads to the suggestions that either *P. chlamydospora* is a natural diploid and that only one of the *moxY* copies was disrupted, or that it is usually a haploid but was forced into a balance lethal (wild type:*moxY-hph*) because of

selection on hygromycin and non viability of the *moxY* mutation. Further experiments for determination of heterokaryons or diploids include:

1: Spore plating on selective media, spores may have 50% viability if they are heterokaryons.

2: Compare spore DNA content to WT strain, if the *moxY* transformants is a forced diploid, the DNA content will be twice that of the wild type spores.

3: Attempt to haploidize the putative *moxY*-disrupted diploid by plating on an agent such as benomyl that causes non-disjunction in mitosis.

4: Attempt to disrupt a different gene in *P. chlamydospora*, and determine if a wild type copy is still present in this case also.

With the availability of the *E. lata* and *P. chlamydospora* *gfp*-expressing transformants, inoculation of the transformants onto host plant grapevine/blackcurrant for establishment of a pathogen/host plant relationship through confocal observation is thus facilitated. However as the transformation frequencies for these two fungi are relatively low, any potential method used for the improvement of the transformation frequency, such as *Agrobacterium tumefaciens*-mediated transformation still should be tried.

With the gradual releases of DNA sequences of fungal genomes and the development of functional genomics, more and more genes involved in toxin production such as those encoding *E. lata* eutypine, eutypinol, eulatachromene, siccayne, and eulatinol or *moxY* genes in *P. chlamydospora* will be identified through bioinformatics tools. The subsequent characterization of these genes will be facilitated with the transformation system developed in this project.

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APPENDICES:

APPENDIX 1: CHECK LIST OF GRAPE DISEASES

Adapted from Balasubramaniam, (1993).

1.1 FRUIT AND FOLIAR DISEASES

Common name	Cause	Importance (+-+ + + +)	Distribution	Control
Botrytis bunch rot *Botrytis* rot Grey mould	Fungus: *Botryotinia fuckeliana* (sexual stage RARE); *Botrytis cinerea* (asexual stage)	+ + + +	NZ; world wide	Canopy management; hygiene; fungicides
Powdery mildew Oidium	Fungus: *Uncinula necator* (sexual stage); *Oidium tuckeri* (asexual stage)	+ + + +	NZ; world wide	Canopy management; fungicides
Downy mildew	Fungus: *Plasmopara viticola*	+ + + +	NZ; world wide	Canopy management fungicides
Bunch rot Sour rot	Fungi: *Penicillium; Aspergillus; Cladosporium*; *Rhizopus*; Others	+ + +	(NZ*); world wide	Canopy management; fungicides
Phomopsis cane & leaf spot Excoriose "Dead arm"	Fungus: *Cryptosporella viticola* (sexual stage RARE); *Phomopsis viticola* (asexual stage)	+ + +	NZ; world wide	Pruning; hygiene; fungicides
Black rot	Fungus: *Guignardia bidwellii* (sexual stage); *Phyllosticta ampelicida* (asexual stage)	+ + + +	N. Amer. Europe;S.Amer	Hygiene; fungicides
Anthracnose Black spot Birds eye rot	Fungus: *Elsinoe ampelina* (sexual stage); *Sphaceloma ampelinum* (asexual stage)	+ + +	NZ; world wide	Fungicides
Ripe rot	Fungus: *Glomerella cingulata* (sexual stage); *Colletotrichum gloeosporioides* (asexual stage)	+ +	NZ; world wide	Hygiene; fungicides
Bitter rot	Fungus: *Greenaria uvicola* (syn.	+ +	(NZ*); N.Amer.;	Fungicides

	Melanconium fuliginuem)		Australia; Asia	
Rotbrenner	Fungus: *Pseudopezicula tracheiphila* (sexual stage); *Phialophora tracheiphila* (asexual stage)	+ + (+)	Europe	Fungicides
White rot "Hail disease"	Fungus: *Coniella diplodiella* (syn. *Coniothyrium diplodiella*)	+ +	Europe	Bunch damage reduction; fungicides
Rust	Fungus: *Physopella ampelopsidis*	+ +	Americas; Asia	Fungicides
Leaf blight Leaf spot	Fungus: *Mycosphaerella personata* (sexual stage); *Cercospora vitis* (asexual stage) (syn. *Isariopsis clavispora, Pseudocercospora vitis*, and others)	+	NZ; N.Amer.; S. Africa; Asia	Nil

- **Known to occur or probably occurring in NZ, but not officially recorded.**
- **Importance: Severity of disease caused by each fungus.**

1.2 WOOD AND ROOT DISEASES

Common name	Cause	Importance (+-+ + + +)	Distribution	Control
Eutypa dieback Dying dead arm Dead arm	Fungus: *E. lata* (sexual stage); *Libertella blepharis* (syn. *Cytosporina sp.*) (asexual stage)	+ + +	**NZ**; World wide	Hygiene; Old wood wound protection
Black dead arm "Botryosphaeria die back"	Fungus: *Botryosphaeria stevensii* (sexual stage); *Sphaeropsis malorum* (asexual stage)	+ + +	(**NZ***); Italy; Canada	As for *Eutypa*
Esca Black measles	(?) Fungi: *Cephalosporium, Chondrostereum, Phellinus.*	+ +	Europe; Nth Amer.	Hygiene; sodium arsenite at dormancy
Armillaria root rot Shoestring root rot	Fungus: *Armillaria mellea*	+ + (+)	**NZ**; Europe; Nth Amer. and elsewhere	Vine removal; soil fumigation; biological

				control(?)
Verticillium wilt	Fungus: *Verticillium dahliae*	+ (+)	(**NZ***); Nth Amer.; Germany	Vine removal
Phytophthora crown & root rot	Fungi: *Phytophthora cinnamomi, P. citricola, P. megasperma*	+	**NZ**; Sth Africa; Nth Amer.; Australia; Asia	Avoid excessive irrigation; vine removal
Crown gall	Bacterium: *Agrobacterium tumefaciens*	+ (+ +)	(**NZ***); Europe; Nth Amer.	Avoidance of freezing injury; biological control; nursery hygiene
Bacterial blight	Bacterium: *Xanthomonas ampelina*	+ + +	Europe; Argentina	Clean planting stock; hygiene; copper sprays
Pierce's disease	Bacterium: *Xylella fastidiosa*	+ + +	Nth, Central Sth Amer.	Quarantine (?); resistant cultivars

- **Known to occur or probably occurring in NZ, but not officially recorded.**

APPENDIX 2: COMMON SOLUTIONS

2.1 TE Buffer (Tris EDTA buffer)

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

2.2 OM Buffer

1.6 M $MgSO_4.7H_2O$, with 10 mM Na_2HPO_4/100 mM NaH_2PO_4 buffer (pH 5.8).

2.3 ST Buffer

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

2.4 STC Buffer

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

2.5 DNA extraction buffer

2.5.1. Hexadecyl Trimethyl-Ammonium Bromide buffer (CTAB)

0.8 g of 2% CTAB; 0.4 g of 1% PVP40; 11.2 ml 5M NaCl; 8.0 ml 100 mM EDTA; 4 ml 1 M Tris HCl (pH 8.0); dH_2O 16 ml.

2.5.2 Lysis buffer (AL-Samarrai and Schmid, 2000)

40 mM Tris Acetate buffer pH 7.8; 20 mM Sodium Acetate; 1 mM EDTA; 1% SDS.

2.6 10 x TBE Buffer

0.89 M Tris-HCl (pH 8.2); 0.01 M EDTA (Na Salt); 0.89 M Boric Acid.

2.7 Fluorometer working solution

Hoechst H 33258 stock solution (Section 2.10) 5 μl; 10x TNE (Section 2.11) 5 μl; Milli-Q water 45 ml.

2.8 Ethidium Bromide Staining Solution

1 mg/ml ethidium bromide

2.9 Gel Loading Dye

20% (w/v) Sucrose (BDH); 5 mM EDTA $Na_2.H_2O$ (BDH); 1% (w/v) SDS (BDH); 0.2% (w/v) Bromophenol blue (J. T. Baker Chemical Co); 0.2%(w/v) Xylene cyanol (Sigma).

2.10 10 x Hoechst dye stock solution

10 mg of Hoechst H 33258 (Amersham Biosciences) dissolved in 10 ml of milli-Q water, stored at 4°C for up to 6 months without light.

2.11 10 x TNE

12.11 g/l Tris; 3.72 g/l EDTA $Na_2.H_2O$ (BDH); 116.89 g/l NaCl (Merck). Dissolved in roughly 800 ml of Milli-Q water, followed by pH adjust to 7.4 with concentrated HCl (BDH) and top up to 1 Liter.

2.12 Fluorometer DNA standards

100 ng/μl Calf thymus DNA (Amersham Biosciences) in 1x TNE (Section 2.11)

2.13 Phenol (Tris-equilibrated)

The equilibrated phenol was stored under 0.1 M Tris-HCl at 4°C (avoiding light).

2.14 Protoplast preparation solution

0.05 g Glucanex was dissolved in 10 ml of 1.4 M $MgSO_4$ (Section 2.2).

2.15 Southern Blotting Solutions

2.15.1 Dupurination Solution

0.25 M HCl

2.15.2 Denature Solution

0.5 M NaOH; 1.5 M NaCl

2.15.3 Neutralization Solution

1.5 M NaCl; 0.5 M Tris (pH 7.2)

2.15.4 20x SSC (Standard Saline Citrate)

3 M NaCl; 0.2M Trisodium citrate. Adjust pH to 7.0

2.15.5 Hybridization Detection Solution

Maleic Acid Buffer

0.1 M Maleic Acid; 0.15 M NaCl; Adjust with NaOH (Solid) to pH 7.5

Washing Buffer

0.1 M Maleic Acid; 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20.

Detection Buffer

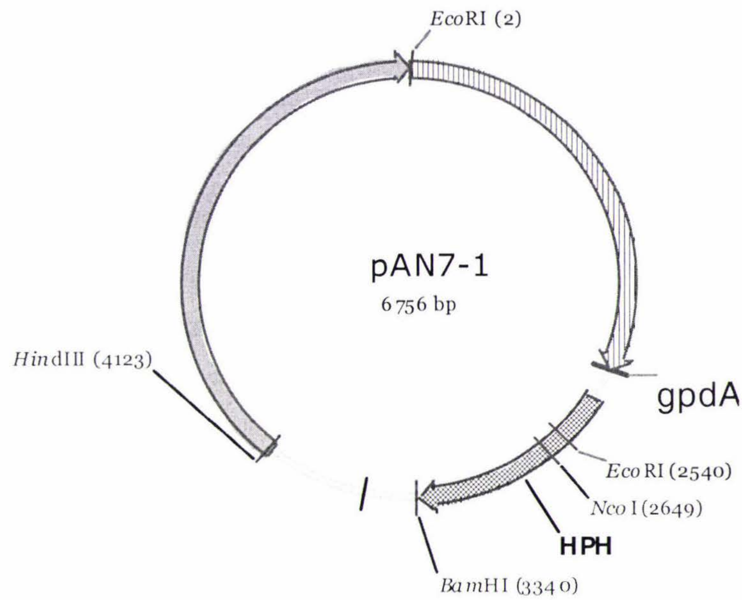
0.1 M Tris-HCl; 0.1 M NaCl; pH 9.5.

2.15.6 Solution for DNA blots Stripping

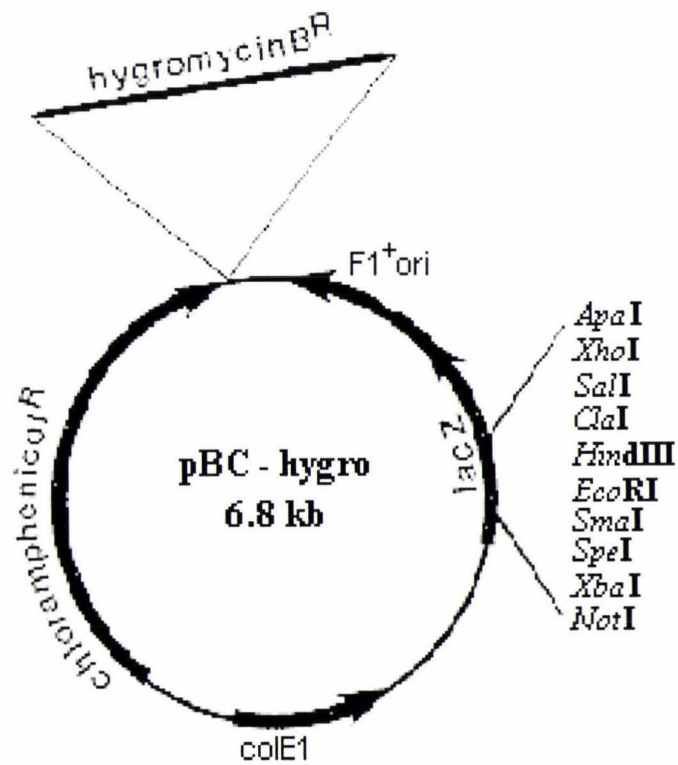
0.2 M NaOH; 0.1% SDS

APPENDIX 3: PLASMIDS

Appendix 3A: pAN7-1

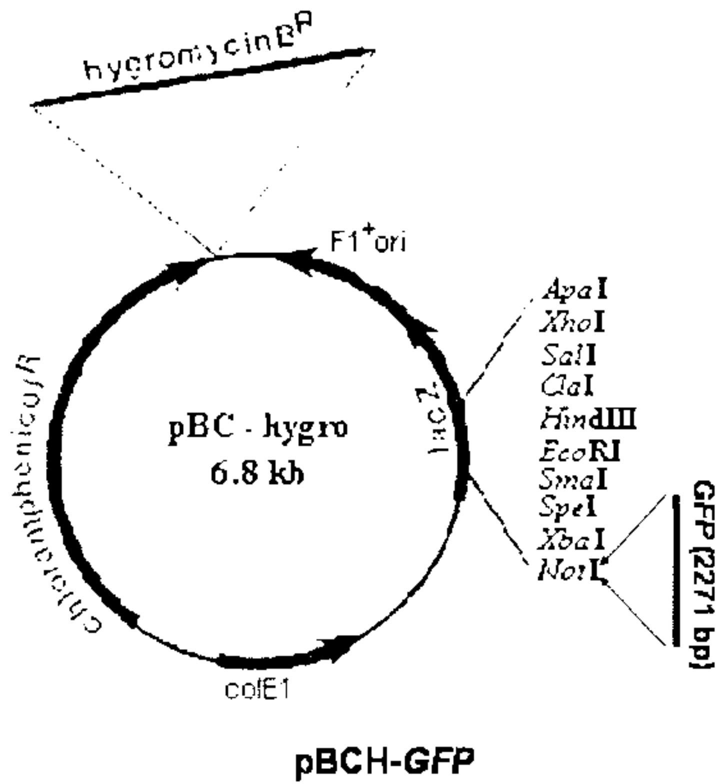


Appendix 3B: pBC-hygro



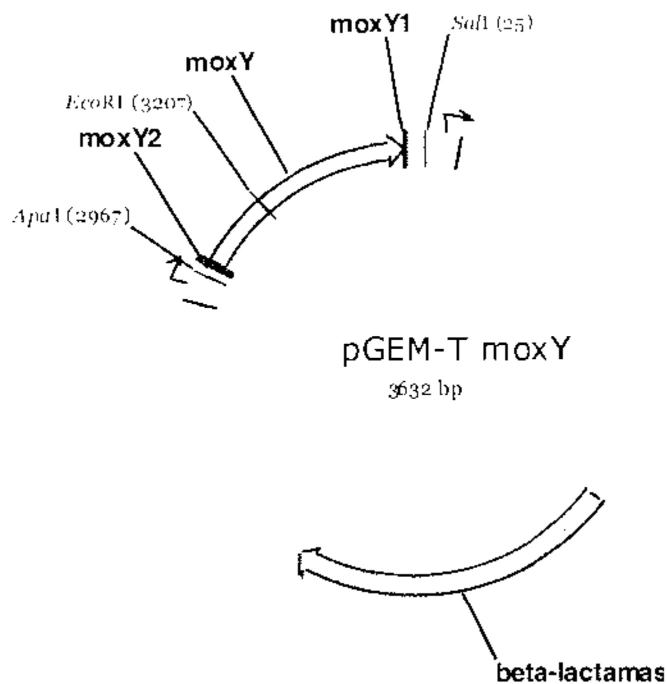
Adapted from www.fgsc.net/fgn42/silar.html

Appendix 3C: pBCH-*gfp*

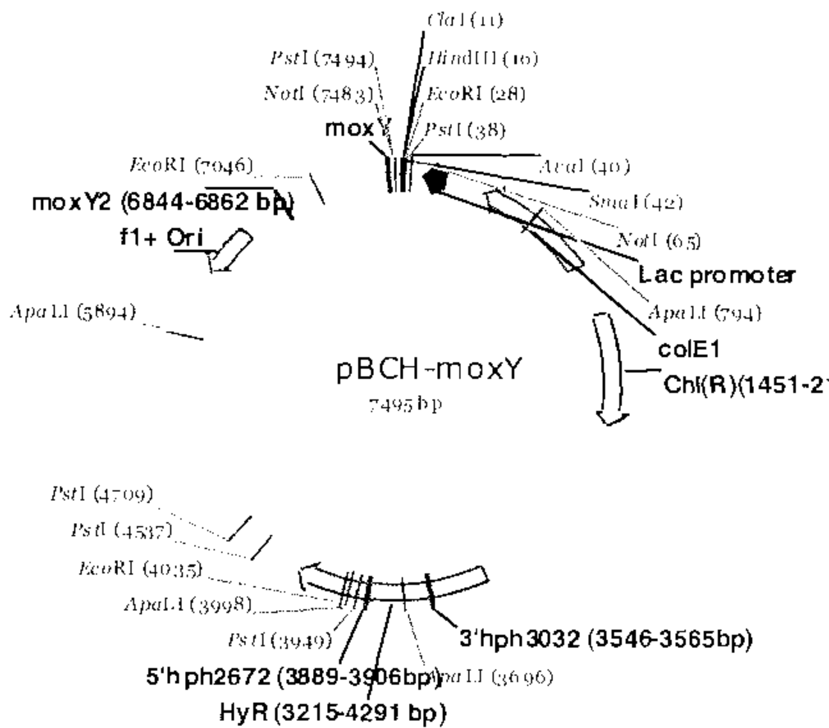


pBCH-*GFP*

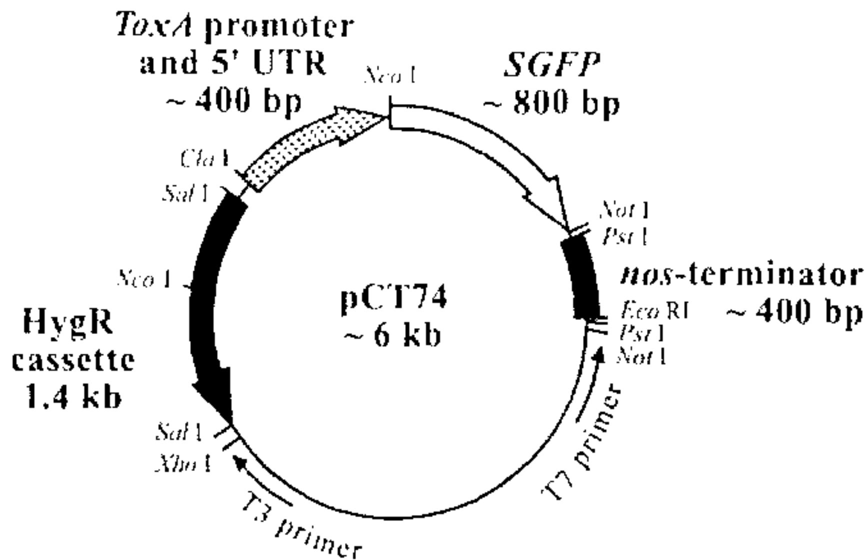
Appendix 3D: pGEM-TMOXY



Appendix 3E: pBCH-MOXY

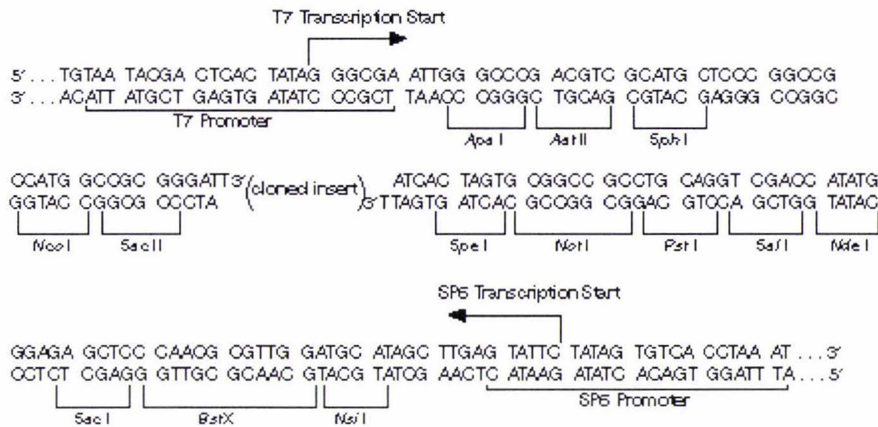


Appendix 3F: pCT74

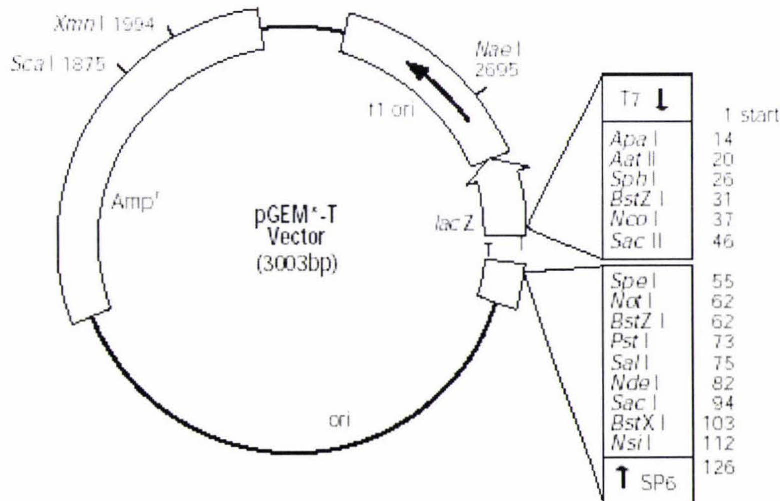


A *gfp* expression vector for filamentous fungi, pCT74. pCT74 is based on pBlue-S*gfp*-TYG-nos-KS (from Jen Sheen, Department of Molecular Biology, Massachusetts General Hospital, Harvard University), which contains S*gfp*-TYG on an *Nco*I/*Not*I DNA fragment inserted into the *Eco*RV site and the *nos* terminator on a *Pst*I/*Eco*RI DNA fragment inserted into the *Eco*RI site of pBluescript KS (Stratagene, La Jolla, Calif.). An *Nco*I site containing the start codon of *Sgfp* and a *Not*I site replaced *Eco*RV in this process (adapted from Lorang *et al*, 2001)

Appendix 3G: pGEM-T vector



pGEM-T vector promoter and multiple cloning site sequence
Adapted from Promega, website http://wheat.pw.usda.gov/~lazo/methods/pro/tb150.html



pGEM-T Vector circle map

Adapted from website:
http://www.tcd.ie/Genetics/staff/Noel.Murphy/recombinant%20dna%20ge4021/pgem.pdf

APPENDIX 4: SEQUENCES

Appendix 4A: Sequence of plasmid pFAT-3*gfp* (Length: 7001).

Genebank accession number: U25123. Arrows indicate two designed *gfp* primers (*gfp*1 & *gfp*2).

```
   1 GAATTCGCGC GATATGCAAT ACAACAAACG AAACACCAGT GACGAAAGAA
  51 GCCGTGCTGT GTATGGTGTT GTGTGTGATA CTGTGTGTGC CTGACCGACG
 101 ATGGGATCAG TGAAGAGGAA AGACGAACAT GGTCGGACTG ACTTCATGAC
 151 TCTTCGGATA CAAGTACCCC GAACGTCCAG AGCTGGGGCC CTGATTCAAG
 201 AGCAATGACC TCAGACCTCG GGTACGGCGG TCGGGATTTG GGGAAGAAGC
 251 GCAGAGTCTC GTCGAGTTCA GATGACAGCC GCAACCACCA CAATGATGAA
 301 GAACATCAAT AACCCAAAGA TGGAAAGTCA ACAAACAACA CCACCAAACA
 351 GGCACGGGCA ACGACCCAAG GAAGGCCCAC TCCGCTTTGC CGCGATGGGC
 401 AAACATCCCC AATGGTCTGC TCAGCGGTTG TGGGCCGCGA TAATCAATCG
 451 ACCAGGCAAT TACTTAACGG CGTCTACGGT ACGTAATTGA ATTTGAACCC
 501 CAAGGGTCAG GCACCTTACT GCGCCGTGAG GAAGCATTGG ATCAGGGGAG
 551 ACCCGTGTCG GGGGAGCTGA GAGAGCTCCG ACGGTGCAAT CTTAATTCAA
 601 TCATGCTTAT TCGGGGGGTT TCCGCGCCCA ACGGTTCTTA TCGCGCGGTC
 651 GGCAGAGAGG AGACTGACTG ACTCTGACTG AGGACCCGAC GAGAGAGTCT
 701 CTCGCCACTG TCTAGATTGC GACATCCAAT GGGACTGGCC TGACTGGACT
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 801 CTGATGAGCA CACGACTAGT ACCTTCTTGA GCCTTATCTT AAGAAAACAT
 851 GGAGACCGGC TGACTCGGCA GTCTTCTCGA AGAAACGCCC AGCACGATTG
 901 AGGAAGCTCA AACAAATCAC GATTCACCCC TCATCCGCAA AGTCAGTGAA
 951 CCGCCGGAGC GAAGAGATCA ATCGGGAAAG AGAGAAGGTC ACGAAGGTTT
1001 GTGAAATGTT CCGTCATGGA GTCGCGGGCA GAATATCAGC TCGTGCGAGC
1051 GATGGCACGA CATGGACGAA CTTTTGCAAA GGCAACCCAG CCCAGCTTCC
1101 CGAATGATGA ATCCCTCCTC CTCCAGCCCC AAATAATACG CGCGAAGAAG
1151 GTCCTTGTGT TGAGTTGAAT GCGACGGAGG GCAGGGTCCA GGCCGGTGCA
1201 GCCGCCAGCA CGTCATGAGA GCAGGGCAGC TCATGGCCAG ACGTATCGCC
1251 AAGATGGCAA CGACCGCCGA ACAATTCCGC TTCTTTGGGA TCCGGTGTTT
1301 GACGCAGAGA AGAGACGACG GAGACTTGGG TCCAGGCCCC CAGACATCTC
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1451 ATCTCGCCGT CTTCCATCAC CTGCACCACC ACCATCAGCA GAAGCACCCC

1501 AGTCCGCCTT GCCTTCCTTC CCGTCTTGGC CTCGATCTCG TGCCTGGGCG

1551 GCCCCAGACT TGGCGGAGGG CTGGCGGAGG GTTGGAGTTG CAATTCCAAG

1601 TCTTTGCTCG CATACGGGAC TGTGGGTGGG GACTTGGCGG TAAAGCTTGT

1651 TTCCATGTGC CGAGGCGAAT TGGCAAAGCG AAGTGAACGG GGGATCTGCC

1701 ATGCCAGTCT GCAGCTGTTG TGTTCAGTTA TTGGCGATTA ACAATGGGAA

1751 ACATGGTTGG GCCTGGTTCC AGCTCGGAGC CCCATGTGCG GTTTTGGGCT

1801 GGCGACTCCC GGAGCCATGT CCCGAGTAGG TAGGTGCTTT TATTTTCCTT

1851 CTTCTACGTA CTGCGTATCC GTACGAGTAC GTGTGAGCGA GCACTCAGAC

1901 TTGTCTGTTC GACTCGAACA CCGGTCCGGG GCTGTCGCGA GATGCGCTGG

1951 GAACATGGCA GAGACGATCA AGGATCTATC TGTCGAAGAG AGGAAAAGGG

2001 GTCCCGTCCG GCGTTGGCAT TGGCTGGTTG CGAGATGCAT GGCTGAGTGA

2051 ATCCATTCGT CTCCCCCGCC CTCGGCCCCA TCATCAGCTG CTCCAGTCGT

2101 GCGCTGTGCT TTTTTTTTCA TCTCATCTCC CGCCTTGGAC GAACGGAGAG

2151 CCGCGGTTTG GTCTGGCTGG GGAATTCGAC CTGCCTCTTT TTCATCTAGT

2201 TTCCCGCTGC TGGGGACTTG TGAAAAAGCT TAGGCCCAAG GTTGCGCCCA

2251 CCACCACCAC CACCATCTCG TTGTTCCGCT CTGCCCACCC TTGCACCACA

2301 TCACTCACCC ATGATCCATC CCACCCCTCC AACCCTCCTC CTCCTTGAGT

2351 CCTCCTCCTC CCATCCCACC CCTTCAGGAA GCCGGGAATA GGCTGGCTGT

2401 TGTTGCAATT GGCTCTTCGG GAGGAATGGA GCTACGTCAA GGTCCCTGGT

2451 TTGGCGTCCC CCGATGGACC ATGGCTAGTC AGTCCGACTT GGCTCGTTTA

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2651 ATGACACTAT GGGCACGATG CTCCCTCTTG GAAGGTGGTA TTCAACTCCC

2701 GGCTATCTTG TGCCGGTCAC CTTGAAAGCT TGACTTGACG ATCCCTTGTT

2751 GGCACCTCTT GGCGTTATCG AGCCTCGTGA ACGCTGGTCG ACTCCGAGTG

2801 ATATTGCGAT TTACCTATCG ACCGGAGTGG GTTCCTCCCA CAACAACACA

2851 CACGGCGGCG ATCTCTGTCG TGGAGTCGAA CATGAGGATA TAAGGCTCTG

2901 TTGATCACCC CGTTCATCTG GTCTTTTTTC CTCTTCATCC TCATCGGCTC

2951 ACTCCCGTCC TCGATCCTCA TCCTCATCTT CTCATCTCCT GCCTCTCCCC

GFP ORF →

3001 GTCCTCTTCC TCCTCAGGTC TCTCGCTCCG CCTCTTGTCT GTCAAGATGA

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3101 GATGGCGATG TGAATGGGCA CAAATTTTCT GTCAGCGGAG AGGGTGAAGG

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3201 AGCTCCCTGT GCCATGGCCA ACACTGGTCA CTACCTTCAC CTATGGCGTG

3251 CAGTGCTTTT CCAGATACCC AGACCATATG AAGCAGCATG ACTTTTTCAA

3301 GAGCGCCATG CCCGAGGGCT ATGTGCAGGA GAGAACCATC TTTTTCAAAG

3351 ATGACGGGAA CTACAAGACC CGCGCTGAAG TCAAGTTCGA AGGTGACACC

3401 CTGGTGAATA GAATCGAGCT GAAGGGCATT GACTTTAAGG AGGATGGAAA

3451 CATTCTCGGC CACAAGCTGG AATACAACTA TAACTCCCAC AATGTGTACA

3501 TCATGGCCGA CAAGCAAAAG AATGGCATCA AGGTCAACTT CAAGATCAGA

3551 CACAACATTG AGGATGGATC CGTGCAGCTG GCCGACCATT ATCAACAGAA

3601 CACTCCAATC GGCGACGGCC CTGTGCTCCT CCCAGACAAC CATTACCTGT

3651 CCACCCAGTC TGCCCTGTCT AAAGATCCCA ACGAAAAGAG AGACCACATG

3701 GTCCTGCTGG AGTTTGTGAC CGCTGCTGGG ATCACACATG GCATGGACGA

3751 GCTGTACAAG TGAGATCCAT GTGCTGAGCA TTGGAGGATG TTGATGAGAA

3801 GTACCAAGAC CGAAATGGTC TTTTAATGAA ATAAAGAAAC GACCAACAAA

3851 AGCAGTAAAA TCGGAAGATG GGCAGTCTTT CAGAAGGGGG GGCAACGATG

3901 ACGCAGAGTC TCGAGAGGCC GGGAAGGATT ATGGTTGTGT CTTTTCCTAA

3951 CAAAACTCCC AAACTACATG TCCTTATGAC GTCTATGAAA TAGATTATGC

4001 CTACCCATCA CTACAAAGTT CCTCCATCGC AAAGTGAAGT CTTGCTGCCT

4051 TTTTTCCTGC CAACGCGGGT GGGTTGTAGC GGCCGCCACC GCGGTGGAGC

4101 TCCAATTCGC CCTATAGTGA GTCGTATTAC GCGCGCTCAC TGGCCGTCGT

4151 TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA CTTAATCGCC

4201 TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA AGAGGCCCGC

4251 ACCGATCGCC CTTCCCAACA GTTGCGCAGC CTGAATGGCG AATGGGACGC

4301 GCCCTGTAGC GGCGCATTAA GCGCGGCGGG TGTGGTGGTT ACGCGCAGCG

4351 TGACCGCTAC ACTTGCCAGC GCCCTAGCGC CCGCTCCTTT CGCTTTCTTC

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4451  GGGGCTCCCT TTAGGGTTCC GATTTAGTGC TTTACGGCAC CTCGACCCCA
4501  AAAAACTTGA TTAGGGTGAT GGTTCACGTA GTGGGCCATC GCCCTGATAG
4551  ACGGTTTTTC GCCCTTTGAC GTTGGAGTCC ACGTTCTTTA ATAGTGGACT
4601  CTTGTTCCAA ACTGGAACAA CACTCAACCC TATCTCGGTC TATTCTTTTG
4651  ATTTATAAGG GATTTTGCCG ATTTCGGCCT ATTGGTTAAA AAATGAGCTG
4701  ATTTAACAAA AATTTAACGC GAATTTTAAC AAAATATTAA CGCTTACAAT
4751  TTAGGGCACT TTTCGGGGAA ATGTGCGCGG AACCCCTATT TGTTTATTTT
4801  TCTAAATACA TTCAAATATG TATCCGCTCA TGAGACAATA ACCCTGATAA
4851  ATGCTTCAAT AATATTGAAA AAGGAAGAGT ATGAGTATTC AACATTTCCG
4901  TGTCGCCCTT ATTCCCTTTT TTGCGGCATT TTGCCTTCCT GTTTTTGCTC
4951  ACCCAGAAAC GCTGGTGAAA GTAAAAGATG CTGAAGATCA GTTGGGTGCA
5001  CGAGTGGGTT ACATCGAACT GGATCTCAAC AGCGGTAAGA TCCTTGAGAG
5051  TTTTCGCCCC GAAGAACGTT TTCCAATGAT GAGCACTTTT AAAGTTCTGC
5101  TATGTGGCGC GGTATTATCC CGTATTGACG CCGGGCAAGA GCAACTCGGT
5151  CGCCGCATAC ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC
5201  AGAAAAGCAT CTTACGGATG GCATGACAGT AAGAGAATTA TGCAGTGCTG
5251  CCATAACCAT GAGTGATAAC ACTGCGGCCA ACTTACTTCT GACAACGATC
5301  GGAGGACCGA AGGAGCTAAC CGCTTTTTTG CACAACATGG GGGATCATGT
5351  AACTCGCCTT GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAAACG
5401  ACGAGCGTGA CACCACGATG CCTGTAGCAA TGGCAACAAC GTTGCGCAAA
5451  CTATTAACTG GCGAACTACT TACTCTAGCT TCCCGGCAAC AATTAATAGA
5501  CTGGATGGAG GCGGATAAAG TTGCAGGACC ACTTCTGCGC TCGGCCCTTC
5551  CGGCTGGCTG GTTTATTGCT GATAAATCTG GAGCCGGTGA GCGTGGGTCT
5601  CGCGGTATCA TTGCAGCACT GGGGCCAGAT GGTAAGCCCT CCCGTATCGT
5651  AGTTATCTAC ACGACGGGGA GTCAGGCAAC TATGGATGAA CGAAATAGAC
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5751  CAAGTTTACT CATATATACT TTAGATTGAT TTAAAACTTC ATTTTTAATT
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5851  CTTAACGTGA GTTTTCGTTC CACTGAGCGT CAGACCCCGT AGAAAAGATC
5901  AAAGGATCTT CTTGAGATCC TTTTTTTCTG CGCGTAATCT GCTGCTTGCA
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6351 CCGGTAAGCG GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG

6401 GGGAAACGCC TGGTATCTTT ATAGTCCTGT CGGGTTTCGC CACCTCTGAC

6451 TTGAGCGTCG ATTTTTGTGA TGCTCGTCAG GGGGGCGGAG CCTATGGAAA

6501 AACGCCAGCA ACGCGGCCTT TTTACGGTTC CTGGCCTTTT GCTGGCCTTT

6551 TGCTCACATG TTCTTTCCTG CGTTATCCCC TGATTCTGTG GATAACCGTA

6601 TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG AACGACCGAG

6651 CGCAGCGAGT CAGTGAGCGA GGAAGCGGAA GAGCGCCCAA TACGCAAACC

6701 GCCTCTCCCC GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT

6751 TTCCCGACTG GAAAGCGGGC AGTGAGCGCA ACGCAATTAA TGTGAGTTAG

6801 CTCACTCATT AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT

6851 GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG

6901 ACCATGATTA CGCCAAGCGC GCAATTAACC CTCACTAAAG GGAACAAAAG

6951 CTGGGTACCG GGCCCCCCCT CGAGGTCGAC GGTATCGATA AGCTTGATAT

7001 C

Appendix 4B: Part of the DNA sequence of *p. chlamydospora* putative toxin gene *moxY*

Adapted from Ridgway et al, (2005).

```
  1 TGGTATCAAT GTTGCTCGCA ACGTGAAGGA GCACATGAAG AACGTTGAGT
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101 CGATGTTAGT GATACTCATG CCACATGCCT ACTGCCTTGC TGACGATGCA
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201 CGTGGGCGCC AAATCCTCGA TGGAACCAAT ATTATTCGTC TCAAGGAGAG
251 ATATTAACTT ACTTCCAAGA TGCTGCGGAA AAGAGTGGTT TGATACAGTT
301 CATAAAGTTC CAGCACAAAG TGGTGGAGGC AGTATGGAAC GAGAGCAGGG
351 GTGTGTGGCA ATTTAAGATT GAAAACCTCG CTACGGGAGA GACATTTCAA
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601 GGTCAAGCAT CTGACGACCT TTATCCGACA GCCTACATGG GTGACGGCAG
651 GGTTTGGAAG CAAGTATGCA GCACCGGGAG GCACTAACTT CAATTTCACG
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751 CCGCAAGGCT GTTGAACATG AACTCAACTG CCGATTCAAA ATGCTACACA
801 AGGATACACC CGAACAAGCA GCTGCTATGG AGTTTGCAAC AAATGAAATG
851 ATCAAACGCC TTGGTGGTCC CAACGAACTC TCTGATTTTA TTATCCCCAA
901 ATTTTCTGTC GGTTGTCGCC GTCCGACACC TGGCCAGGGT TATCTGGAAG
951 CTCTGGTGGC GCCCAATGTA TCTGT
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Appendix 4C:pBCH-MOXY DNA sequence (Length: 7495)

Refer to Figure 4.17 for the location of *moxY* gene and the primers pBCH-MOXY1 and moxY3.

```
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  51 ACTAGTTCTA GAGCGGCCGC CACCGCGGTG GAGCTCCAGC TTTTGTTCCC
 101 TTTAGTGAGG GTTAATTGCG CGCTTGGCGT AATCATGGTC ATAGCTGTTT
 151 CCTGTGTGAA ATTGTTATCC GCTCACAATT CCACACAACA TACGAGCCGG
 201 AAGCATAAAG TGTAAAGCCT GGGGTGCCTA ATGAGTGAGC TAACTCACAT
 251 TAATTGCGTT GCGCTCACTG CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC
 301 CAGCTGCATT AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCGTAT
 351 TGGGCGCTCT TCCGCTTCCT CGCTCACTGA CTCGCTGCGC TCGGTCGTTC
 401 GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC
 451 ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA
 501 AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC
 551 TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG
 601 CGAAACCCGA CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC
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 701 CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCATAGCTC ACGCTGTAGG
 751 TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA
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 951 GTGGTGGCCT AACTACGGCT ACACTAGAAG GACAGTATTT GGTATCTGCG
1001 CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC
1051 GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA
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1151 CGGGGTCTGA CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC
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1401 CGGGCGTATT TTTTGAGTTG TCGAGATTTT CAGGAGCTAA GGAAGCTAAA

1451 ATGGAGAAAA AAATCACTGG ATATACCACC GTTGATATAT CCCAATGGCA

1501 TCGTAAAGAA CATTTTGAGG CATTTCAGTC AGTTGCTCAA TGTACCTATA

1551 ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC CGTAAAGAAA

1601 AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG CCCGCCTGAT

1651 GAATGCTCAT CCGGAATTAC GTATGGCAAT GAAAGACGGT GAGCTGGTGA

1701 TATGGGATAG TGTTCACCCT TGTTACACCG TTTTCCATGA GCAAACTGAA

1751 ACGTTTTCAT CGCTCTGGAG TGAATACCAC GACGATTTCC GGCAGTTTCT

1801 ACACATATAT TCGCAAGATG TGGCGTGTTA CGGTGAAAAC CTGGCCTATT

1851 TCCCTAAAGG GTTTATTGAG AATATGTTTT TCGTCTCAGC CAATCCCTGG

1901 GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG ACAACTTCTT

1951 CGCCCCCGTT TTCACCATGG GCAAATATTA TACGCAAGGC GACAAGGTGC

2001 TGATGCCGCT GGCGATTCAG GTTCATCATG CCGTTTGTGA TGGCTTCCAT

2051 GTCGGCAGAA TGCTTAATGA ATTACAACAG TACTGCGATG AGTGGCAGGG

2101 CGGGGCGTAA TTTTTTTAAG GCAGTTATTG GTGCCCTTAA ACGCCTGGTT

2151 GCTACGCCTG AATAAGTGAT AATAAGCGGA TGAATGGCAG AAATTCGAAA

2201 GCAAATTCGA CCCGGTCGTC GGTTCAGGGC AGGGTCGTTA AATAGCCGCT

2251 TATGTCTATT GCTGGTTTAC CGGTTTATTG ACTACCGGAA GCAGTGTGAC

2301 CGTGTGCTTC TCAAATGCCT GACAAGGAGA TGGCGCCCAA CAGTCCCCCG

2351 GCCACGGGGC CTGCCACCAT ACCCACGCCG AAACAAGCGC TCATGAGCCC

2401 GAAGTGGCGA GCCCGATCTT CCCCATCGGT GATGTCGGCG ATATAGGCGC

2451 CAGCAACCGC ACCTGTGGCG CCGGTGATGC CGGCCACGAT GCGTCCGGCG

2501 TAGAGGATCC TCTAGCTAGA AAGAAGGATT ACCTCTAAAC AAGTGTACCT

2551 GTGCATTCTG GGTAAACGAC TCATAGGAGA GTTGTAAAAA AGTTTCGGCC

2601 GGCGTATTGG GTGTTACGGA GCATTCACTA GGCAACCATG GTTACTATTG

2651 TATACCCATC TTAGTAGGAA TGATTTTCGA GGTTTATACC TACGATGAAT

2701 GTGTGTCCTG TAGGCTTGAG AGTTCAAGGA AGAAACAGTG CAATTATCTT

2751 TGCGAACCCA GGGGCTGGTG ACGGAATTTT CATAGTCAAG CTATCAGAGT

2801 AAAGAAGAGG AGCATGTCAA AGTACAATTA GAGACAAATA TATAGTCGCG

2851 TGGAGCCAAG AGCGGATTCC TCAGTCTCGT AGGTCTCTTG ACGACCGTTG

2901 ATCTGCTTGA TCTCGTCTCC CGAAAATGAA AATAGACTCT GCTAAGCTAT

2951 TCTTCTGCTT CGCCGGAGCC TGAAGGGCGT ACTAGGGTTG CGAGGTCCAA

3001 TGCATTAATG CATTGCAGAT GAGCTGTATC TGGAAGAGGT AAACCCGAAA

3051 CGCGTTTTAT TCTTGTTGAC ATGGAGCTAT TAAATCACTA GAAGGCACTC

3101 TTTGCTGCTT GGACAAATGA ACGTATCTTA TCGAGATCCT GAACACCATT

3151 TGTCTCAACT CCGGAGCTGA CATCGACACC AACGATCTTA TATCCAGATT

3201 CGTCAAGCTG TTTGATGATT TCAGTAACGT TAAGTGGATC CCGGTCGGCA

3251 TCTACTCTAT TCCTTTGCCC TCGGACGAGT GCTGGGGCGT CGGTTTCCAC

3301 TATCGGCGAG TACTTCTACA CAGCCATCGG TCCAGACGGC CGCGCTTCTG

3351 CGGGCGATTT GTGTACGCCC GACAGTCCCG GCTCCGGATC GGACGATTGC

3401 GTCGCATCGA CCCTGCGCCC AAGCTGCATC ATCGAAATTG CCGTCAACCA

3451 AGCTCTGATA GAGTTGGTCA AGACCAATGC GGAGCATATA CGCCCGGAGC

3501 CGCGGCGATC CTGCAAGCTC CGGATGCCTC CGCTCGAAGT AGCGCGTCTG

3551 CTGCTCCATA CAAGCCAACC ACGGCCTCCA GAAGAAGATG TTGGCGACCT

3601 CGTATTGGGA ATCCCCGAAC ATCGCCTCGC TCCAGTCAAT GACCGCTGTT

3651 ATGCGGCCAT TGTCCGTCAG GACATTGTTG GAGCCGAAAT CCGCGTGCAC

3701 GAGGTGCCGG ACTTCGGGGC AGTCCTCGGC CCAAAGCATC AGCTCATCGA

3751 GAGCCTGCGC GACGGACGCA CTGACGGTGT CGTCCATCAC AGTTTGCCAG

3801 TGATACACAT GGGGATCAGC AATCGCGCAT ATGAAATCAC GCCATGTAGT

3851 GTATTGACCG ATTCCTTGCG GTCCGAATGG GCCGAACCCG CTCGTCTGGC

3901 TAAGATCGGC CGCAGCGATC GCATCCATGG CCTCCGCGAC CGGCTGCAGA

3951 ACAGCGGGCA GTTCGGTTTC AGGCAGGTCT TGCAACGTGA CACCCTGTGC

4001 ACGGCGGGAG ATGCAATAGG TCAGGCTCTC GCTGAATTCC CCAATGTCAA

4051 GCACTTCCGG AATCGGGAGC GCGGCCGATG CAAAGTGCCG ATAAACATAA

4101 CGATCTTTGT AGAAACCATC GGCGCAGCTA TTTACCCGCA GGACATATCC

4151 ACGCCCTCCT ACATCGAAGC TGAAAGCACG AGATTCTTCG CCCTCCGAGA

4201 GCTGCATCAG GTCGGAGACG CTGTCGAACT TTTCGATCAG AAACTTCTCG

4251 ACAGACGTCG CGGTGAGTTC AGGCTTTTTC ATATGGGTAC CTGAGAACAT

4301 CTTGTTGCCC TGCTTTCCGT GCGAAATACT ACCGGTACTT TTGGGAAACA

4351 AGGGAACAGG AGGGCGCTGC TGTGCGCGGT TCTGAGTGTT CAGGATTGAA

4401 GCTGAAGAAG GTGCTGAGGA AGCGTAGAAC TGTTGCGGAC GCGAGTTCTG

4451 AGAAGAGCTG TACCGATTGG TGAAAGCCGA AGAAGTGAGT TGGTGCCCTG

4501 TTGCCTGGAT AATGTTTGCA ACTCGCTGGT TCTGCAGAGA CGGAGACAAA

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4601 TTTTGGTTTG ATAGGGTGGA TTTGGTTGCA GAGAAGAGAA AGGAAGGTCA

4651 AAGAGGGAAA ACTGGGCGGA GGGAAGGATT TTGTATCAGG CAGCAAACTG

4701 CCACTGCAGT GGCCCTGGCA GTGCCGGGCG AGGCACCCAC GCACGGCCGC

4751 GCAACCGGTT GGTCCTTGCC CACCACGAAA CCCTTCTGAA AGGTCAGATG

4801 GAAGTGTGCG ACAGTGCGCG TCCCCAAGCC AATGCAGGCG CCATGGATCC

4851 ACTCCCCACC CGCAAGATTT CACTGTGCGT TCTTATTGGT TGCCGCAAGG

4901 CCAGCCAAAG GGGGAAGTAT GAGTCACAGC ACCGATACAA GAAAATTGCA

4951 GAACTAACAT ATGGATGCGC GCGCTATTCT GTAGAGCTCT GGGCAAAGCA

5001 CCAATCCTGC GGGTCGGTAC ACACACTAGC ACTGCCCCAC CTGAGGCAGT

5051 CAGCCCCGCT GACCGAATTG CCAAGAGCCA ATGGAGACGG AAAGCCAACG

5101 CTGATGGAGC ACCATCTGAA TGGACCTCGC TCGCTTGCCT GGAAGGACA

5151 AGGGACACCG GAGACAGGGC CTCCAACCAA GGGCGCGGGA AAGACGATCC

5201 CCAAAGTCGC AACGGCCCAG AAAGAACGCA TCCATCACAT TCGGATGGGA

5251 TGGCTTGAAG CCAGCTTGCA GCAACTTCAA AGCTCGACGC GAGGGTGGAT

5301 GGCCTTCCCC ATTATGATTC TTCTCGCTTC CGGCGGCATC GGGATGCCCG

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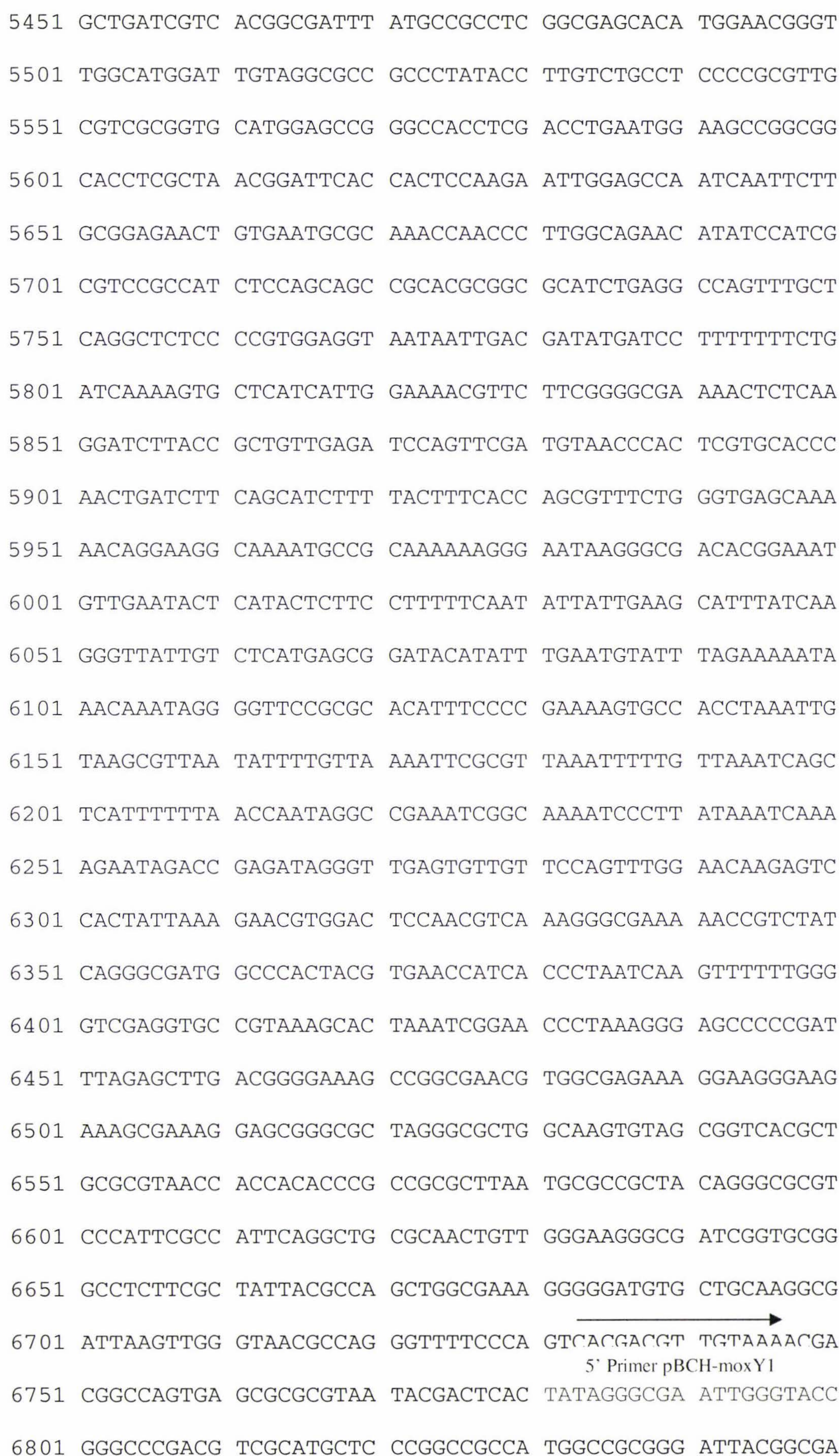

```
5451 GCTGATCGTC ACGGCGATTT ATGCCGCCTC GGCGAGCACA TGGAACGGGT
5501 TGGCATGGAT TGTAGGCGCC GCCCTATACC TTGTCTGCCT CCCCGCGTTG
5551 CGTCGCGGTG CATGGAGCCG GGCCACCTCG ACCTGAATGG AAGCCGGCGG
5601 CACCTCGCTA ACGGATTCAC CACTCCAAGA ATTGGAGCCA ATCAATTCTT
5651 GCGGAGAACT GTGAATGCGC AAACCAACCC TTGGCAGAAC ATATCCATCG
5701 CGTCCGCCAT CTCCAGCAGC CGCACGCGGC GCATCTGAGG CCAGTTTGCT
5751 CAGGCTCTCC CCGTGGAGGT AATAATTGAC GATATGATCC TTTTTTTCTG
5801 ATCAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA
5851 GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC
5901 AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
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6101 AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTAAATTG
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6301 CACTATTAAA GAACGTGGAC TCCAACGTCA AAGGGCGAAA AACCGTCTAT
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6451 TTAGAGCTTG ACGGGGAAAG CCGGCGAACG TGGCGAGAAA GGAAGGGAAG
6501 AAAGCGAAAG GAGCGGGCGC TAGGGCGCTG GCAAGTGTAG CGGTCACGCT
6551 GCGCGTAACC ACCACACCCG CCGCGCTTAA TGCGCCGCTA CAGGGCGCGT
6601 CCCATTCGCC ATTCAGGCTG CGCAACTGTT GGGAAGGGCG ATCGGTGCGG
6651 GCCTCTTCGC TATTACGCCA GCTGGCGAAA GGGGGATGTG CTGCAAGGCG
                                    ---------------------->
6701 ATTAAGTTGG GTAACGCCAG GGTTTTCCCA GTCACGACGT TGTAAAACGA
                                      5' Primer pBCH-moxY1
6751 CGGCCAGTGA GCGCGCGTAA TACGACTCAC TATAGGGCGA ATTGGGTACC
6801 GGGCCCGACG TCGCATGCTC CCGGCCGCCA TGGCCGCGGG ATTACGGCGA
```

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6851 CAACCGACAG AAAATTTGGG GATAATAAAA TCAGAGAGTT CGTTGGGACC
6901 ACCAAGGCGT TTGATCATTT CATTTGTTGC AAACTCCATA GCAGCTGCTT
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7001 TCAACAGCCT TGCGGTATTC AAGATAGGAC TTTGGATTTT CCTCGAATTC
7051 TCGCTTTTGC TCTTCCGTGA AATTGAAGTT AGTGCCTCCC GGTGCTGCAT
7101 ACTTGCTTCC AAACCCTGCC GTCACCCATG TAGGCTGTCG GATAAAGGTC
7151 GTCAGATGCT TGACCATAGG TTGAATGTTT GCGACTAATT GAATACCACT
7201 GGACCCACAA CCAATGACTG CAACCGACTT GTCGTACAAT TCCGTTCCCG
7251 GTTTCCAAGA TGCGCTGTGC ATTAAATCGC CTTGGAAGTC TTGAAGTCCA
7301 GCAATATCTG GCCATTTCCA GTTGTTAAGG TAACCGGAAG CGTTGATGAA
7351 GAAATGTGCA TAATCTTGAA ATGTCTCTCC CGTAGCGAGG TTTTCAATCT
7401 TAAATTGCCA CACACCCCTG CTCTCGTTCC ATACTGCCTC CACCACTTTG
7451 TGCTGGAACT TTATGAACTG AATCACTAGT GCGGCCGCCT GCAGG
```

Appendix 4D: Sequencing of MOXY primers (MOXY1 & MOXY2 in Table 2.4) amplified PCR product (Length: 600).

```
  1 GGGGAGCGTA TGGACGAGAG CAGGGGTGTG TGGCAATTTA AGATTGAAAA
 51 CCTCGCTACG GGAGAGACMT TTCAAGATTA TGCACATTTC TTCATCAACG
101 CTTCCGGTTA CCTTAACAAC TGGAAATGGC CAGATATTGC TGGACTTCAA
151 GACTTCCAAG GCGATTTAAT GCACAGCGCA TCTTGGAAAC CGGGAACGGA
201 ATTGTACGAC AAGTCGGTTG CAGTCATTGG TTGTGGGTCC AGTGGTATTC
251 AATTAGTCGC AAACATTCAA CCTATGGTCA AGCATCTGAC GACCTTTATC
301 CGACAGCCTA CATGGGTGAC GGCAGGGTTT GGAAGCAAGT ATGCAGCACC
351 GGGAGGCACT AACTTCAATT TCACGGAAGA GCAAAAGCGA GAATTCGAGG
401 AAAATCCGAA GTCCTATCTT GAATACCGCA AGGCTGTTGA ACATGAACTC
451 AACTGCCGAT TCAAAATGCT ACACAAGGAT ACACCCGAAC AAGCAGCTGC
501 TATGGAGTTT GCAACAAATG AAATGATCAA ACGCCTTGGT GGTCCCAACG
551 AACTCTCTGA TCTTATTATC CCCAAATTTT CTGTCGGTTG GTCGCCGTAA
```

APPENDIX 5: ALIGNMENT OF SEQUENCE 4D WITH THE ORIGINAL *moxY* SEQUENCE

Top: Sequence of MOXY1 & MOXY2 amplified PCR product (appendix 4D)
Bottom: The original *moxY* sequence from Dr. Hayley Ridgway, Lincoln University.

```
------------- GTATGGAACGAGAGCAGGGGTGTGTGGCAATTTAAGATTGAAAACCTCGCTACG
              ......................................................
TCCAGCACAAAGTGGTGGAGGCAGTATGGAACGAGAGCAGGGGTGTGTGGCAATTTAAGATTGAAAACCTCGCTACG
|         |         |         |         |         |         |         |
310       320       330       340       350       360       370       380
```

```
        70        80        90        100       110       120       130
        |         |         |         |         |         |         |
GGAGAGACATTTCAAGATTATGCACATTTCTTCATCAACGCTTCCGGTTACCTTAACAACTGGAAATGGCCAGATAT
....... .....................................................................
GGAGAGACATTTCAAGATTATGCACATTTCTTCATCAACGCTTCCGGTTACCTTAACAACTGGAAATGGCCAGATAT
  |         |         |         |         |         |         |         |
  390       400       410       420       430       440       450       460
```

```
140       150       160       170       180       190       200       210
|         |         |         |         |         |         |         |
TGCTGGACTTCAAGACTTCCAAGGCGATTTAATGCACAGCGCATCTTGGAAACCGGGAACGGAATTGTACGACAAGT
.............................................................................
TGCTGGACTTCAAGACTTCCAAGGCGATTTAATGCACAGCGCATCTTGGAAACCGGGAACGGAATTGTACGACAAGT
     |         |         |         |         |         |         |
     470       480       490       500       510       520       530
```

```
    220       230       240       250       260       270       280       290
    |         |         |         |         |         |         |         |
CGGTTGCAGTCATTGGTTGTGGGTCCAGTGGTATTCAATTAGTCGCAAACATTCAACCTATGGTCAAGCATCTGACG
.............................................................................
CGGTTGCAGTCATTGGTTGTGGGTCCAGTGGTATTCAATTAGTCGCAAACATTCAACCTATGGTCAAGCATCTGACG
|         |         |         |         |         |         |         |
540       550       560       570       580       590       600       610
```

```
    300       310       320       330       340       350       360
    |         |         |         |         |         |         |
ACCTTTATCCGACAGCCTACATGGGTGACGGCAGGGTTTGGAAGCAAGTATGCAGCACCGGGAGGCACTAACTTCAA
.............................................................................
ACCTTTATCCGACAGCCTACATGGGTGACGGCAGGGTTTGGAAGCAAGTATGCAGCACCGGGAGGCACTAACTTCAA
  |         |         |         |         |         |         |         |
  620       630       640       650       660       670       680       690
```

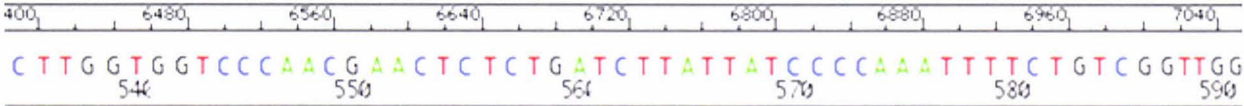
```
370       380       390       400       410       420       430       440
|         |         |         |         |         |         |         |
TTTCACGGAAGAGCAAAAGCGAGAATTCGAGGAAAATCCGAAGTCCTATCTTGAATACCGCAAGGCTGTTGAACATG
.................................... ........................................
```

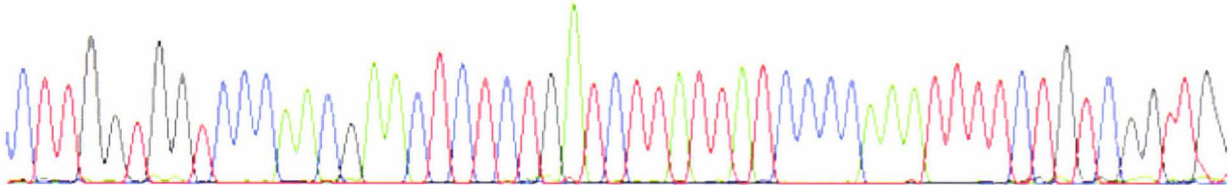
Sequence similarity comparison of the cloned *moxY* gene with the original *P. c* *moxY* gene DNA sequence. The top DNA sequence (in red) is the cloned PCR produ DNA sequence (in green) is the original *P. chlamydospora moxY* gene DNA s nucleotides in blue indicate non-matching sections in PCR product sequence.

APPENDIX 6: SEQUENCING OF *moxY* PCR PRODUCT

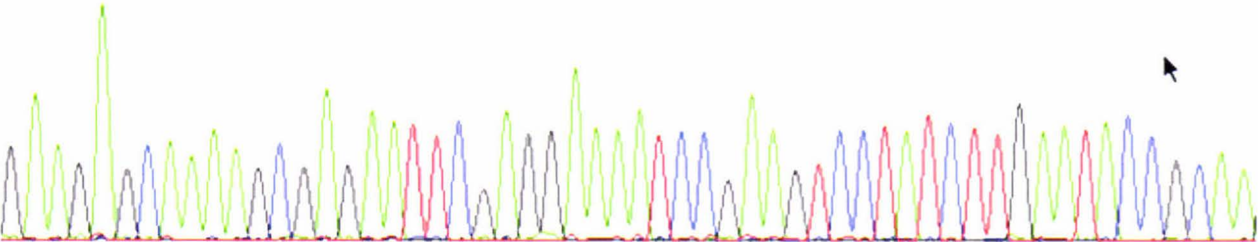
Sequence 1: with primer MOXY1; Sequence 2: with primer MOXY2 (Table 2.4). These two single strands are complementary sequences.

Sequence 1





Sequence 2



APPENDIX 7: DNA PREPARATION FOR PCR AND SOUTHERN HYBRIDIZATION

The methods for genomic DNA preparations were described in Section 2.14.3. The average yields of DNA of the fungi *E. lata* and *P. chlamydospora* were calculated, and the result was summarized in Table app1.

Method / Fungus	CTAB	Method from AL-Samarrai and Schmid, (2000)
E10-10	7 µg (ranging from 2.5 to 15µg)	6.1 µg (ranging from 3.3 to 10.2µg)
P. chlamydospora	7.5 µg (ranging from 2.5 to 13.8µg)	7 µg (ranging from 1.3 to 12µg)

Table app1. Comparison of DNA yielding between the method CTAB and the genomic DNA extraction method from AL-Samarrai and Schmid, (2000). The unit for the DNA yielding is per 30 mg of grounded dry mycelium, the displayed numbers are based on an average calculation.

This result indicated that both of the two methods were suitable for these fungal genomic DNA extractions. At the first glance, the CTAB method was more efficient than the second one although there was no big difference in DNA yield between these two methods. However when the quality of DNA was considered, the difference between these two methods was obvious. The CTAB method was easier whilst the purity of DNA was affected as cellular debris and polysaccharides were more likely to be simultaneously precipitated with the DNA. The second method was relatively laborious and time consuming, but the advantage was the DNA was more pure. This DNA was able to be used directly for enzyme digestion and Southern hybridization. As for PCR amplification, DNA from either of methods worked.

For obtaining cleaner DNA, the original CTAB method was modified through increasing the centrifugation speed up to 10000 rpm (1.5 ml eppendorf tubes), but DNA was still not clean enough for Southern blotting. The second method also was modified through addition of 4 M LiCl to increase the efficiency of precipitation; several subsequent washings washed the LiCl away from the DNA. This method did increase the precipitation efficiency (data not shown).