

Transformation of *Rhizoctonia solani*

By

Jiang Wu

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School of Biological Sciences and Biotechnology
Division of Science and Engineering
Murdoch University
Perth, Western Australia

Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institute.

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Jiang Wu

Abstract

The aim of this study was to develop a genetic transformation system for the plant pathogenic fungus *Rhizoctonia solani* (telomorph, *Thanatephrus cucumeris* Frank [Donk]). The availability of a transformation system would allow us to study gene exchange, epidemiology, and to use techniques such as gene disruption or gene silencing to investigate the role of fungal enzymes in pathogenesis.

The approach adopted was to use *Agrobacterium tumefaciens* to transform the fungus as reports in the literature suggested that this was the most efficient and easiest method to use. As a preliminary test, *Fusarium oxysporum* was transformed using a binary vector (pBINAN) containing a hygromycin resistance gene under control of an ascomycete promoter and terminator. Hygromycin resistant transformants were obtained after co-incubation of fungal conidia with the bacterium. The presence of the transgene was confirmed by analysis of DNA. The number of transformants depended on the genetic background of the *A. tumefaciens*. Strains AGLO or AGRO gave higher numbers of transformants compared to LBA4404. No transformants were obtained when the hygromycin gene was under control of a basidiomycete (pBINHL1), or a plant (CaMV35S) promoter. Since the basidiomycete promoter used in pBINHL1 originates from *Ustilago maydis*, the vector was tested by transformation of *Ustilago cynodontis*. Stable transformants of *U. cynodontis* were obtained with this vector.

A series of experiments were carried out on transformation of *R. solani* mycelium. Both the protoplast and the *Agrobacterium* transformation methods were tested. Parameters affecting protoplast production and regeneration were examined. Protoplast production varied with the age of the mycelium, with the osmotic stabilizer used, and with time of treatment with protoplasting enzymes. Regeneration of protoplasts was also affected by the osmotic stabilizer and the growth medium. Transformation of several isolates from different anastomosis groups (AG) was attempted by inducing protoplasts to take up DNA using polyethyleneglycol. Two plasmids were used; (1) pAN7-1 containing the

resistance gene under control of an ascomycete promoter, and (2) pHL-1 in which the resistance gene is under control of a basidiomycete promoter. No transformants were obtained.

Attempts were then made to transform mycelium and protoplasts using *A. tumefaciens*. The experiments used both mycelium and protoplasts as the recipient. A number of small resistant colonies were obtained using binary plasmid (pBINHL1) in which mycelium was transformed with the resistance gene was driven by the basidiomycete promoter. On transfer to fresh medium these colonies would grow to about 2cm diameter, and then stop growing. On a second transfer to fresh medium they failed to show any growth. No resistant colonies were obtained from *A. tumefaciens* transformation of protoplasts.

To improve transformation efficiency, a vector was constructed in which the hygromycin resistance gene was fused to an *R solani* laccase promoter sequence. No resistant colonies were obtained using this vector. Further experiments were carried out using a hygromycin resistance gene specially modified for expression in basidiomycetes by the insertion of artificial introns in the 5' and 3' untranslated regions, and a number of AT to CG conversions in the coding region. Most of the recipient isolates gave transformants with the unstable resistance phenotype. However, one AG 6 isolate gave transformants with a stable resistance phenotype. Of six transformants recovered from this isolate, five were shown by PCR and southern blotting to contain the transgene. In four of these transformants the resistance phenotype was stable in the absence of selection.

Table of Contents

TABLE OF CONTENTS	I
ACKNOWLEDGMENTS	IX
ABBREVIATIONS	X
CHAPTER 1 INTRODUCTION	1
1.1 Biology of <i>Rhizoctonia solani</i>	1
1.1.1 Characteristics of the fungus	1
1.1.2 Diseases caused by <i>R.solani</i>	3
1.2. Aims of This Thesis	6
1.3. Transformation of Fungi	8
1.3.1 Selection of transformants	9
1.3.2 Transformation vectors	11
1.3.3. Methods for Transformation of Fungi.	12
CHAPTER 2 MATERIALS AND METHODS	17
2.1 Growth and maintenance of <i>E. coli</i> cultures	17
2.1.1 Short-term storage of <i>E. coli</i> cultures	17
2.1.2 Long-term storage of bacterial cultures	17
2.1.3 Antibiotics and blue/white screening	17
2.1.4 <i>E. coli</i> strains genotypes	18
2.2 Growth and storage of <i>Agrobacterium tumefaciens</i>	18
2.2.2 Long-term storage of <i>A. tumefaciens</i>	19
2.3 Growth and storage of fungal material	19
2.3.1 <i>Rhizoctonia solani</i> isolates	19
2.3.2 <i>Fusarium</i> isolates	20
2.3.3 <i>Ustilago</i> isolates	20
2.4 General methods	20
2.4.1 Extraction of plasmid DNA from <i>E. coli</i> liquid cultures	20
2.4.2 Extraction of fungal DNA	22
2.4.3 Transformation of <i>E. coli</i>	24
2.4.4 Conjugal transfer of plasmid into <i>Agrobacterium</i>	26
2.4.5 DNA general Methods	27
2.4.6 Electrophoresis	31
2.4.7 Southern Blot analysis	32
2.4.8 DNA sequencing	34

CHAPTER 3. CONSTRUCTION OF VECTORS AND TRANSFORMATION OF <i>FUSARIUM</i>	37
3.1 Introduction	37
3.2 Materials and Methods	38
3.2.1 Construction of binary vectors	38
3.2.2 Introduction of vectors into <i>A. tumefaciens</i>	43
3.2.3 Transformation of <i>F. oxysporum</i>	43
3.2.4 Analysis of <i>F. oxysporum</i> transformants.	44
3.3 Results	44
3.3.1 Sensitivity to hygromycin	44
3.3.2 Transformation with <i>A. tumefaciens</i> strains	45
3.3.3 Growth curve of transformants	47
3.3.4 Confirmation of transformants by PCR	48
3.3.5 Analysis of transformants by Southern blotting	49
3.3.6 Stability of the transformants	50
3.3.7 Effect of pre-treatment with Acetosyringone	51
3.3.8 Transformation of germinated conidia	52
3.4 Discussion	53
CHAPTER 4 TRANSFORMATION OF <i>RHIZOCTONIA SOLANI</i>	55
4.1. Introduction	55
4.2 Material and Methods	55
4.2.1 Fungal isolates and cultivation	55
4.2.2 Preparation of mycelium for transformation	55
4.2.3 Preparation of protoplasts for transformation	56
4.2.4 Polyethyleneglycol transformation of protoplasts	57
4.2.5 <i>Agrobacterium</i> transformation of <i>R. solani</i> mycelium	57
4.2.6 <i>Agrobacterium</i> transformation of <i>R. solani</i> protoplasts	57
4.3 Results	58
4.3.1 Factors affecting protoplast production.	58
4.3.2 Effect of induction medium on protoplast regeneration	62
4.3.3 Transformation of <i>R. solani</i> protoplasts with pAN7-1 and pHL1	63
4.3.4 Transformation of <i>R. solani</i> with <i>A. tumefaciens</i> .	64
4.4 Discussion	67
CHAPTER 5. CONSTRUCTION OF A HYGROMYCIN RESISTANCE GENE WITH AN <i>R. SOLANI</i> PROMOTER.	71
5.1 Introduction	71
5.2 Materials and Methods	72
5.2.1 PCR amplifications: Isolation of a laccase promoter sequence from <i>R. solani</i>	72
5.2.2 DNA sequencing.	72
5.2.3 Cloning of PCR products	73

5.2.4 Plasmid extraction and enzyme digestion	73
5.2.5 Agrobacterium mediated transformation of <i>R. solani</i>	73
5.3 Results	73
5.3.1 Cloning the <i>R. solani</i> laccase promoter	73
5.3.2 Construction of the hygromycin resistance gene	78
5.3.3 Transformation of <i>R. solani</i> SCR122 and RH165 with pBINlcc	79
5.4 Discussion	80
CHAPTER 6 TRANSFORMATION OF <i>USTILAGO</i> AND <i>RHIZOCTONIA</i> WITH A MODIFIED HYGROMYCIN GENE	83
6.1 Introduction	83
6.2 Materials and Methods	84
6.2.1 Construction of the vector pJW2.2hyg	84
6.2.2 Transformation of <i>Ustilago cynodontis</i>	85
6.2.3 Agrobacterium mediated transformation of <i>R. solani</i> .	86
6.3 Results	86
6.3.1 Construction of a binary vector with the modified hph gene.	86
6.3.2 Transformation of <i>Ustilago cynodontis</i> .	90
6.3.3 Transformation of <i>R. solani</i>	93
6.4 Discussion	100
CHAPTER 7 GENERAL DISCUSSION	104
REFERENCES	108

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Abbreviations

AG	anastomosis group
amp	ampicillin
ARS	autonomous replicating sequence
AS	Acetosyringone
ATP	adenosine triphosphate
ATMT	<i>Agrobacterium</i> mediated transformation
bp	base pair
BSA	Bovine Serum Albumin
Bleo	bleomycin
cfu	colony forming unit
⁰ C	degree Centigrade
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
C-terminus	carboxyl terminus
cm	centimeter
cm ²	square centimeter
CIAP	calf intestine alkaline phosphatase
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DNAase	deoxyribonuclease
dsRNA	double strand RNA
EDTA	ethylene diamine tetra-acetic acid
EM	electron microscope
Fig.	figure
g	gram
GFP	green fluorescent protein
GUS	β-glucuronidase
h or hr	hour
HCl	hydrochloric acid
hyg	hygromycin
ha	hectare
<i>hph</i>	hygromycin phosphotransferase
IPTG	isopropyl β-D-thiogalactoside
IMA	induction medium agar
IMB	induction medium broth
IM	induction medium
kb	kilobase pair
KDa	kilodalton
Kg	kilogram
Km	kanamycin

L	litre
LB	T-DNA left boarder
M	moles per litre
mA	milliampere
mcs	multiple cloning site
MM	minimal medium
mRNA	messenger ribonucleic acid
min	minute
MW	molecular weight
N-terminus	amino terminus
NAA	α -naphthaline acetic acid
NaCl	sodium chloride
OD	optical density
O/N	over night
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PEG	polyethylene glycol
pH	a measure of hydrogen ion concentration
pmol	picomole
polyA	riboxynucleic acid with polyadenine tail
RB	T-DNA right boarder
RFLP	restriction fragment length polymorphism
Rep	replicase
RIP	ribosome inactivating protein
RNA	riboxynucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SA	South Australia
SDS	sodium dodecyl sulphate
Sec	second
SM	selection medium
STC	sorbitol Tris CaCl ₂ solution
SOBAG	SOB basal medium with ampicillin and glucose
Tas	Tasmania
T-DNA	transferred DNA
Ti	tumor inducing
Tr	transformant
TAE	Tris-HCl-EDTA buffer
TBE	Tris borate buffer
Taq	<i>Thermus aquaticus</i> DNA polymerase
TE	Tris-EDTA buffer
Tris	tris-hydroxymethylaminomethane

Tween-20	polyoxyethylenesorbitan monolaurate
U	unit
UTR	un-translated region
U/ml	unit per milliliter
UV	ultra violet
V	volt
Vic	Victoria,
Vir gene	virulence genes
W	watt
WA	Western Australia
X-gal	5-bromo-4-chloro-3-indolyl- β -galactopyranoside
ZG	pectic zymogram group
μ F	capacitance

Chapter 1 Introduction

1.1 Biology of Rhizoctonia solani

1.1.1 Characteristics of the fungus

Rhizoctonia solani is a very common soilborne pathogen with a great diversity of host plants (Agrios, 1988). The most widely recognized species of *Rhizoctonia* was originally described by Julius Kühn on potato in 1858. *R. solani* is the imperfect state of the basidiomycete fungus that does not produce any asexual spores (called conidia) and only occasionally produce sexual spores (basidiospores). In nature, *R. solani* exists primarily as vegetative mycelium and/or sclerotia. Unlike many basidiomycete fungi, the basidiospores are not enclosed in a fleshy, fruiting body. The sexual fruiting structures and basidiospores (i.e. teleomorph) were first observed by Prillieux and Delacroix in 1891. The sexual stage of *R. solani* is known as *Thanatephorus cucumeris* (Frank Donk).

The genus *Rhizoctonia* contains over 100 species (Parmeter and Whitney, 1970). There is a wide range of variation in mycelium types, sclerotia forms and perfect states (Tu and Kimbrough, 1975; Sneh *et al.*, 1996). The genus also contains a heterogeneous mixture of fungi including basidiomycetes, ascomycetes and deuteromycetes (Parmeter and Whitney, 1970; Sneh *et al.*, 1991). Although the basic characters of the genus were production of sclerotia of uniform texture with mycelium, the taxonomy changed in past decades. A more recent and generally accepted characteristics of *R. solani* was described by Ogoshi (1987) and Sneh (1996).

- (1) Pale to dark brown mycelium of larger diameter with branching near the distal septum of hyphal cell, often at nearly right angles to the hyphae;
- (2) Formation of a septum in the branch near the point of the origin;
- (3) Construction of the branch at the point of the origin;

- (4) Production of sclerotia of uniform texture varying in size and shape from 1mm to crusts several cm across;
- (5) Possession of a prominent septal pore apparatus;
- (6) Multinucleate cells in actively growing hyphae;
- (7) No clamp connection;
- (8) No conidia except moniloid cells often called barrel shaped cells or chlamydospores in chains or aggregates called sporodochia;
- (9) Sclerotia not differentiated into rind and medulla.

Because *R. solani* and other *Rhizoctonia* fungi do not produce conidia and only rarely produce basidiospores, the classification of these fungi has been difficult. Before the 1960's, researchers relied mostly on differences in morphology observed by culturing the fungus on a nutrient medium in the laboratory and/or pathogenicity on various plant species to classify *Rhizoctonia* (Atkins and Lewis, 1954; Bolkan and Butler, 1974). In 1969, Parmeter and his colleagues introduced the concept of "hyphal anastomosis" to characterize and identify *Rhizoctonia*. The concept implied that isolates of *Rhizoctonia* that have the ability to recognize and fuse (i.e. "anastomose") with each other are genetically related, whereas isolates of *Rhizoctonia* that do not have this ability are genetically unrelated. After that Ogoshi further developed this theory. If hyphal anastomosis occurs between the paired isolates, these isolates are considered to belong to the same anastomosis group (Ogoshi, 1987). Hyphal anastomosis criteria have been used to classify isolates of *Rhizoctonia* into taxonomically distinct groups called anastomosis groups. The commonly used method is to pair two isolates on a plate and allow them to grow together. The zone of merged hyphae is stained and examined under microscope for the results of hyphal interactions. Hyphal attraction (both perfect fusion and imperfect fusion) and the killing reaction (imperfect fusion) are regarded as "reliable clues" for detecting hyphal anastomosis (Ogoshi, 1987). Currently there are fourteen recognized AG (Cubeta and Vilgalys, 1997; Carling *et al.*, 2002).

Different AGs vary in host specificity and pathogenesis (Ogoshi, 1987; Sneh *et al.*, 1991). AG-1 isolates infect rice, sugar beet and buckwheat (Ogoshi, 1987). AG-2 can cause

sheath blight of mat rush and root rot of sugar beet (Ogoshi, 1987). AG-3 attacks *Solanaceae*, most notable potato, tomato and eggplant. AG-4 has a wide host range including *Chenopodiaceae*, *Leguminosae* and *Solanaceae*. AG-5 is a weak pathogen to potato, turf-grass and beans. AG-9 causes diseases on crucifer and potato. AG-6, AG-7 and AG-10 are regarded as non-pathogens. AG-8 is considered to be the most causal pathogen for cereal bare-patch disease (Neate and Warcup, 1985; Rovira, 1986; Sweetingham, 1991; Sneh *et al.*, 1991).

1.1.2 Diseases caused by *R.solani*

Rhizoctonia solani has a wide range of hosts and cause various types of disease (Domsch *et al.*, 1980). There is a correlation between AG and host specificity. Sometimes, isolates from different AG have some overlaps (Roberts and Sivasithamparam, 1986; MacNish *et al.*, 1993). Generally, the pathogen causes following diseases.

1.1.2.1 Damping off

Damping-off diseases of seedlings are found worldwide and can be caused by *R. solani* under various weather conditions (Gutierrez *et al.*, 1997). The name damping-off usually refers to the disintegration of stem and root tissues at and below the soil line. The plant tissues become water-soaked and mushy, and the seedling wilts and falls over. The fungi that cause these diseases can attack the seed or the seedling before it emerges above the soil surface. It also causes a seed rot or pre-emergent rot. When this happens, the result is a poor stand that may be mistakenly ascribed to poor seed quality or seed maggots rather than to the presence of a disease. The death of seedlings after emergence or transplanting is called post-emergent damping-off and is the condition most often identified as damping-off.

R. solani is found in most agricultural soils and remains on plant residues and as mycelium or microsclerotia. This pathogen usually attacks seedlings at or near the soil surface. Initial symptoms are stem lesions that are brick red to brown and sunken. When the disease progresses, the stem may become girdled. Stem canker, soreshin, wirestem,

and damping-off are names associated with seedling and postemergent diseases caused by *R. solani*.

The environmental conditions that produce damping-off vary according to the pathogens. *R. solani* often causes injury to beans during spring germination. Cole crops transplanted in the fall are often victims to wirestem caused by this pathogen.

1.1.2.2 Root Rot

Plant root and stem rot, caused by the fungus *Rhizoctonia solani*, is a common early season disease of soybeans. Infected roots are killed back to brown stumps causing plants stunted and spindly. The disease usually causes loss of seedlings (damping-off) in small patches called bare patch caused by AG-8, and is called purple batch in England (MacNish *et al.*, 1993 fig.1.1). It is more common when wet conditions prevail, but may be found in moderately wet soils where germination is slow or emergence is delayed.

One of the most serious diseases caused by the root rot fungus *R.solani* is bare patch disease. This patch is found in cereal and pasture crops. Patches are distinct with abrupt edges, forming circular or oval in the direction of sowing and 30cm-5m area. Both the tap and lateral roots are pinched off by dark brown spear topped lesions. Infection by *R. solani* occurs in juvenile and senescent tissues (MacNish and Neate 1996). As the fungus attacks the roots, the plants can no longer absorb and transport nutrients, stunting growth. The result of the infection seems to cut roots heavily.

Bare patch disease caused by *R.solani* leads to great loss in Australia. In severe cases 25-39% of crops may be affected, and the pathogen survives for several years causing reinfections in both cereal and legume crops (MacNish and Neate, 1996). In some areas the fungus was so severe that it had limited the production of the crops. There are no resistance crops and no effective fungicides to control this disease. Crop rotation is ineffective as the pathogen has a wide host range and it can infect the break crops. Although cultural measures such as tillage and chemical fallow are used to reduce symptoms, they do not eliminate the pathogen. This measure increases the cost, and an

additional cost of the disease is that the need to use cultivation has lead to soil erosion problem for many years (Table1.1). It can be seen that South Australia suffered the biggest lost from bare patch disease, and other states were also affected by *R.solani* and whole Australia cost \$113,998,190 dollars for this disease every year.

Table1.1 Economic cost of bare patch disease (Source: O'Brien, ABARE1995/1996)

	Vic	SA	Tas	WA	Australia
\$/ha	\$2	\$35	\$0.3	\$1	\$38
No ha	1782000	2984000	27000	6207000	11,000,000
Cost of bare patch	\$3,082,860	\$104,141,600	\$8,100	\$6,765,630	\$113,998,190



Figure 1.1 *R. solani* bare patch disease in wheat. The infected region is clearly demonstrated by the circular patch of stunted growth. (photo courtesy of G. MacNish)

1.1.2.3. Seed Decay

The pathogen may infect the seed while on the fruit, only causing symptoms after germination in the soil. Alternatively the fungus may infect seed in the soil. Either way the fungus takes the seeds as a food to spread to other seedlings nearby.

Generally, the disease cycle of *R. solani* is summarized in Fig.1.2

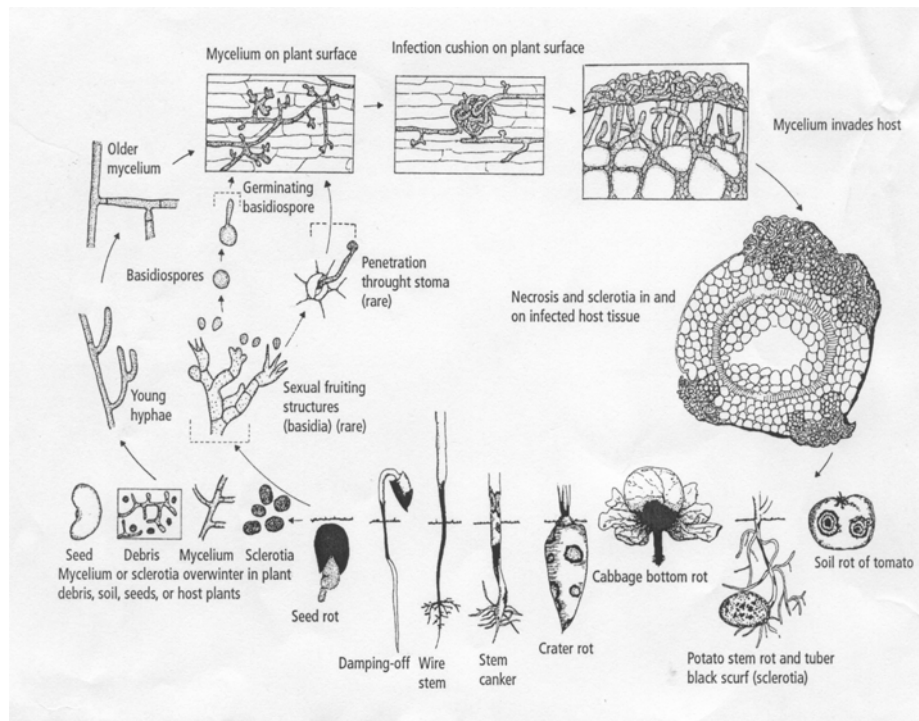


Figure 1.2 Disease cycle of *R. solani* (Source: Agrios, G.N.1997.Plant pathology, 4th edition. San Diego: Academic Press.)

1.2. Aims of This Thesis

Although *R. solani* causes extensive damage of the crops, very little is known about the behavior of these fungi in soil and plant debris. We also know little about the mechanisms by which these fungi cause diseases. So far some researchers have reported the virulent fungi produced cell wall degrading enzymes which included laccases, cutinases, pectinases and cellulases (Isshiki *et al.*, 2001). Of the enzymes produced, the pectic degrading enzymes are the ones mainly concerned with pathogenicity. However,

the exact role of each enzyme in disease is not clear. If we can determine different enzyme function during the symptom development and know which is the key enzyme we can engineer the plant to inhibit the activity of the enzyme. This issue can be addressed by developing a transformation system for *R. solani*. A transformation system will allow us to disrupt specific genes so that their role in pathogenicity can be assessed.

The extranuclear nucleic acid molecules in *R. solani* were investigated to have relationship with pathogenicity. They are in the form of plasmids or double stranded RNA (dsRNA). The plasmids exist in the nucleus or the mitochondrion while dsRNA exist in the cytoplasm (Bharathan and Tavantzis, 1991; Yang *et al.*, 1994b). Plasmids extracted from the same AG/subgroup but from different location isolates in Japan showed homology to each other, but not to plasmids from different AG/subgroup (Miyasaka *et al.*, 1990). Analysis of dsRNA elements in isolates from AGs 1 to 5 from different locations with cross-hybridization between dsRNA's indicated that the sequence homology of dsRNA's from same AG isolates, but different locations ranged from 32-80% (Bharathan and Tavantzis, 1991). Although these accessory genetic elements can offer some characters for analysis of the pathogenesis and the classification of *R. solani* isolates, they are not present in all the isolates and not consistent for some isolates (Sneh *et al.*, 1996). The question whether dsRNAs affect the pathogenicity (virulence) has been argued for a long time. An early report showed dsRNA linked with hypovirulence (Castano and Butler 1978), however subsequent study did not support this observation. Finkler (1985) reported that dsRNA produced virulence while Zanzinger' group (1984) demonstrated that there was no apparent relationship between the presence of dsRNA and degree of pathogenicity in *R. solani*. The role of dsRNA in pathogenicity could be addressed by developing transformation system. We could transform fungi with these dsRNA and compare with the parents which do not contain the elements.

In addition, as there is not a marker which can identify isolates of *R. solani*, it is very difficult to study genetics. The availability of marked isolates can also allow us to study the epidemiology of this pathogen.

The objective of this thesis is to develop a transformation system for *R. solani*. This includes the following aspects.

(1). To construct binary vectors for *Agrobacterium tumefaciens* mediated transformation system (ATMT).

(2). Preparation of protoplast of *R. solani* for both ATMT and conventional protocol.

(3). To optimize the transformation parameters which include:

Agrobacterium tumefaciens strain selection

Treatment of the bacterium

Age of fungal mycelium and cultural conditions

Regeneration of mycelium and selection of putative transformants

(4) Confirmation of transformants and its stability

1.3. Transformation of Fungi

Transformation describes the introduction of foreign DNA molecule into an organism.

The first report of transformation of a fungus was demonstrated in *Neurospora crassa* by Mishra and Tatum (1973). Further success came when a routine procedure was reported for the transformation of *Saccharomyces cereviae* protoplasts (Hinnen *et al.*, 1978).

Hinnen found protoplasts prepared from a *leu2* mutant could be readily transformed to leucine independence by treatment with wild-type DNA in the presence of calcium chloride. The first generally accepted filamentous fungal transformation was with protoplasts of *N. crassa* (Case *et al.*, 1979).

Generally, the conventional fungal transformation procedure includes following steps:

1. Choice of gene as selectable markers and construction of vector

2. Preparation of protoplast of fungal cell
3. Procedure for fungal cell to take up DNA
4. Selection of transformed protoplasts
5. Regeneration into mycelium

1.3.1 Selection of transformants

Table 1.2 lists selectable marker genes used for transformation of plant pathogens and different methods of transformation.

Table 1.2 Selectable genes for transforming plant pathogenic fungi

Pathogen	Method	Selection ²	Reference
<i>Botryotinia squamosa</i>	Protoplast	hygB	Huang <i>et al.</i> , 1989
<i>Aspergillus nidulans</i>	Protoplast	bleo	Punt <i>et al.</i> , 1991
<i>Cochliobolus heterostrophus</i>	Protoplast	amdS ⁺	Turgeon <i>et al.</i> , 1985
<i>Colletotrichum capsici</i>	Protoplast	hygB	Soliday <i>et al.</i> , 1989
<i>Fulvia fulva</i>	Protoplast	hygB	Oliver <i>et al.</i> , 1987
<i>Fusarium moniliforme</i>	Protoplast	hygB	Leslie <i>et al.</i> , 1991
<i>Glomerella cingulata</i>	Protoplast	amdS ⁺	Rodriguez and Yoder 1987
<i>Leptosphaeria maculans</i>	Protoplast	hygB	Farman and Oliver 1988
<i>Magnaporthe grisea</i>	Protoplast	argB	Parsons <i>et al.</i> , 1987
<i>Nectria haematococca</i>	Protoplast	niaD	Daboussi <i>et al.</i> , 1989
<i>Ophiostoma ulmi</i>	Protoplast	ben	Royer <i>et al.</i> , 1991
<i>Septoria nodorum</i>	Protoplast	hygB	Cooley <i>et al.</i> , 1988
<i>Ustilago maydis</i>	Protoplast	hygB	Wang <i>et al.</i> , 1988
<i>U. violacea</i>	Protoplast	hygB	Bej <i>et al.</i> , 1989
<i>Aspergillus niger</i>	Electroporation	hyg B	Ozeki <i>et al.</i> , 1994
<i>Metarhizium anisopliae</i>	Electroporation	benomyl	St.Leger <i>et al.</i> , 1995
<i>Colletotrichum gloeosporioides</i>	Electroporation	GFP	Robinson and Sharon, 1999
<i>Scedosporium prolificans</i>	Electroporation	hygB	Ruiz-Diez and Martinez-Suarez, 1999
<i>Trichoderma harzianum</i>	Biolistic method	hygB	Lorito <i>et al.</i> , 1993
<i>Erysiphe graminis</i>	Biolistic method	GUS	Christiansen <i>et al.</i> ,

			1995
<i>Neocallinastix frontalis</i>	Biolistic method	GUS	Durand <i>et al.</i> , 1997
<i>Paecilomyces fumosporoseus</i>	Biolistic method	hygB	Barreto <i>et al.</i> , 1997
<i>Coccidioides immitis</i>	Biolistic method	hygB	Yu <i>et al.</i> , 1998
<i>Puccinia graminis</i>	Biolistic method	GUS	Schillberg <i>et al.</i> , 2000
<i>Cerocospira caricis</i>	Biolistic method	GUS	Aly <i>et al.</i> , 2001
<i>Aspergillus awamori</i>	ATMT ¹	hygB	de Groot <i>et al.</i> , 1998
<i>A.niger</i>	ATMT	hygB	de Groot <i>et al.</i> , 1998
<i>Fusarium venenatum</i>	ATMT	hygB	de Groot <i>et al.</i> , 1998
<i>Trichoderma reesei</i>	ATMT	hygB	de Groot <i>et al.</i> , 1998
<i>Colletotrichum gloeosporioides</i>	ATMT	hygB	de Groot <i>et al.</i> , 1998
<i>Neurospora crassa</i>	ATMT	hygB	de Groot <i>et al.</i> , 1998
<i>Agaricus bisporus</i>	ATMT	hygB	de Groot <i>et al.</i> , 1998
<i>A. awamori</i>	ATMT	Cutinase	Gouka <i>et al.</i> , 1999
<i>Coccidioides immitis</i>	ATMT	hygB	Abuodeh <i>et al.</i> , 2000
<i>Magnaporthe grisea</i>	ATMT	hygB	Rho <i>et al.</i> , 2001
<i>Penicillium chrysogenum</i>	ATMT	GUS	Sun <i>et al.</i> , 2002

ATMT¹, *Agrobacterium tumefaciens* mediated transformation

Selection², hygB, hygromycin resistance; bleo, bleomycin resistance; amdS⁺, acetamide resistance.

Transformation of filamentous fungi with eletroporation was reported by Marek *et al.*, (1987) and Ward *et al.*, (1989). Some selection systems in fungi are based on complementation of a nutritional mutant, but this method requires that appropriate mutant strains are available and limits transformation to these strains, and for this reason there have been few attempts to transform plant pathogens in this way.

Dominant selectable markers are often advantageous since they can be used to transform the wild-type cell. They can be bacterial antibiotic resistance markers such as Tn5 encoded neomycin phosphotransferase (Banks, 1983) bleomycin, and the related antibiotic phleomycin are derived from three different sources: the transposon Tn5, a *Staphylococcal* plasmid (pUB110) and *Streptoalloteichus hindustanus*. The most widely used selectable marker is the hygromycin phosphotransferase gene from *E. coli*. These antibiotic resistance genes must be placed under the control of a suitable fungal promoter. Other positive selection systems involve unusual catabolic enzymes (acetamidase) or mutant alleles of wild-type genes that confer antibiotic or fungicide resistance. Certain

mutant of alleles ATP synthetase subunit 9 and β -tubulin confer resistance to oligomycin and beomyl respectively (Ward *et al.*, 1986; Vollmer and Yanofsky. 1986).

1.3.2 Transformation vectors

For fungi there are two kinds of vectors, a) integrating vectors, and b) autonomously replicating vectors. The first yeast transformation vector used by Hinnen *et al.*, (1978) was based on the integrating vector pYEleu10 (Ratzkin and Carbon. 1977). Functional expression of cloned yeast DNA containing the LEU2 gene to transform a yeast leu23-112 mutant forms prototrophy. More than 100 putative transformants were analyzed by Southern blot analysis. Three types of transformants were identified. In type I transformants, the plasmid DNA was integrated adjacent to the leu2 locus. In the type II transformants, the plasmid integrated at other locations in the yeast genome. Type III transformants took place a precise replacement of the mutant allele with the wild-type copy from the transforming vectors. This required a double crossover at homologous sites on either side of the mutant chromosomal locus.

For the filamentous fungi, ectopic integrations seem to be homology-independent (Yelton *et al.*, 1984; Balance *et al.*, 1983) and this has been proved in *Podospora anserina* transformants by sequence analysis at the junctions of the integration site (Razanmparany and Begueret, 1988). Most transformants produced by integration were mitotically stable (Tilburn *et al.*, 1983; Kelly and Hynes, 1985; Kim and Marzluf, 1988). However, there have been several reports of mitotic instability. Transformants of *Neurospora crassa* are often reported as unstable mitotically (Case, 1986). There was a recent report about transient transformation of *R. solani* which was not stable either (Robinson or Deacon, 2001). Instability of some transformants has been attributed to the excision of plasmid from the genome (Rossier *et al.*, 1985; Razanmparany and Begueret, 1986).

An autonomously replicating vector for yeast was first reported by Beggs (1978). He constructed a shuttle vector that was replicated in *E. coli* and *S. cerevisiae*. Incorporation

of the yeast 2 μ m plasmid into the transforming vector conferred the capacity to replicate in the yeast cells. Chromosomal replication origins (autonomously replicating sequence ARS sequences) were also inserted into vectors and conferred autonomous replication. The efficiency of transformation was increased to $0.5-2 \times 10^4$ transformants/ μ g DNA (Gietz and Woods, 2001).

Because the early reports of transformation of filamentous fungi showed low frequency of transformation, researches hoped to improve transformation efficiency by constructing plasmids containing genomic sequences. Autonomously Replicating Sequences (ARS) from filamentous fungi have been reported to work in yeast, but most of these failed to work in the original species (Banks, 1983; Ballance and Turner, 1985) due to their integration into the genome. A few successful examples for high efficiency transformation of filamentous fungi for which functional ARS sequences have been cloned are *Mucor circinelloides* (Van Heeswijck, 1986) and *Ustilago maydis* (Tsukuda *et al.*, 1988).

1.3.3. Methods for Transformation of Fungi.

1.3.3.1. Transformation of protoplasts by CaCl₂/PEG.

The protoplast preparation is a crucial step to the success of a transformation. The general procedure for protoplast preparation of filamentous fungus is based on the method of Hinnen *et al.*, (1979). The young hyphae or germinating spores were harvested, and the cell wall was removed by digestion using different lytic enzymes. During the enzyme reaction the protoplasts are stabilised in a buffer containing an osmoticum. After digestion the cell wall and other debris are removed. The protoplasts collected by centrifugation or filtration are suspended in buffer for transformation.

Protoplasts are induced to take up DNA molecules by treatment with CaCl₂ for a short time before polyethylene glycol (PEG) is added to cause the protoplasts to clump and to become permeable to DNA. The normal protocol is to plate protoplast suspension and

DNA buffer onto the non-selection medium for an appropriate time to initiate regeneration of protoplasts, and then selection medium containing a suitable concentration of the drug is overlaid onto the plates and put into the incubator. The duration of recovery time varies with the fungus and with the drug.

1.3.3.2 Electroporation

Other methods of transformation include electroporation, glass bead and biolistic transformation (Gietz and Woods 2001). Electroporation was initially developed by Neumann *et al.*, (1982) to induce DNA uptake and transformation in mouse cell. It has been used for transformation of plant (Bates 1995) and yeast (Karube *et al.*, 1985; Hashimoto *et al.*, 1985). Transformation of filamentous fungi with electroporation was also applied by Marek *et al.*, (1987) and Ward *et al.*, (1989). The transformation frequencies were similar to the protoplast method. Generally, electroporation protocols require the delivery of an electric pulse to cell, either in an electroporation cuvette or between electrodes in a petri dish. The electroporation parameters: field strength (kV/cm), capacitance (uF), and resistance (Ω), are different in each protocol, as are the specifics of preparation of the cells, and it is clear that there is big variation in the yield of the transformants. Electroporation takes less time than either LiAc/PEG or spheroplast transformation, but the initial cost is high.

1.3.3.3. Biolistic Transformation

The biolistic transformation firstly developed for plant cells (Klein *et al.*, 1987). It has been used for yeast (Johnstone *et al.*, 1988) and filamentous fungi (Lorito *et al.*, 1993; Christiansen *et al.*, 1995). Lorito *et al.*, (1993) have compared the biolistic and protoplast methods for transforming *Gliocladium virrens* and *Trichoderma harzianum*. Conidia were bombarded using a helium-driven biolistic device to accelerate M5 tungsten particles coated with plasmid or genomic DNA. DNA from either source contained a bacterial hygromycin B resistance gene (*hph*) as a dominant selectable marker. The same DNA was also used to transform protoplasts using traditional PEG/CaCl₂ protocol.

Hygromycin resistance transformants were obtained using both methods. The biolistic procedure was relatively simple and the transformation efficiency was relatively higher as compared with the protoplast mediated transformation. The recent transformation of *Erysiphe graminis*, an obligate fungus that can only grow on hosts, demonstrated the utility of biolistic transformation as a tool for studying this and other obligate fungi (Chaure *et al.*, 2000).

1.3.3.4. *Agrobacterium tumefaciens* mediated transformation.

Agrobacterium tumefaciens mediated transformation was initially used for transformation of plants. This gram-negative soil bacterium contains a tumor inducing plasmid (the Ti plasmid) and causes crown gall tumor at wound sites of infected plants (Citovsky *et al.*; 1992; Cooley and Kado 1991). During the process of infection, *A. tumefaciens* transfers part of the Ti plasmid, T-DNA (transfer DNA), which is flanked by left and right 25 base pair border sequences, to plant cells. Expression of the genes on the integrated T-DNA causes the formation of the crown gall tumor. The only parts of the T-DNA required for transfer are the 25bp borders. For construction of transgenic plants the sequences between the borders can be replaced with recombinant DNA. The transfer of T-DNA also relies on the induction of a set of virulence (*vir*) genes, which are located on the Ti plasmid outside the T-DNA region. The *vir* genes encode proteins, which carry out the transfer of the T-DNA to the plant cell (Sheng and Citovsky 1996). This complex helps T-DNA get into host cell wall and integrate into host genome. The *vir* genes are induced by compounds secreted by wounded plant cells, such as acetosyringone (AS). There are now several reports in the literature that *A. tumefaciens* will also transfer T-DNA to filamentous fungi and yeasts (de Groot *et al.*, 1998; Bundock *et al.*, 1995; Bundock *et al.*, 1996).

The advantage of ATMT is that the procedure is simple compared with conventional methods. Tissues such as conidia, hyphae, protoplasts and even a slice of fruit body tissue can be used for transformation (de Groot *et al.*, 1998; Chen *et al.*, 2000).

R. solani is considered as a collective species with a character of no production of conidia. There is only one report of transformation of *R. solani* demonstrated by Robinson and Deacon (2001). They conducted transformation of protoplasts with PEG method by using plasmid pES2000 containing *hph* gene driven by a *trpC* promoter from *Aspergillus nidulans*. The transient transformants were obtained with slow growth resistant colonies on selection medium (25-35 mm diameters after 14 d) then ceased growth. Southern blots indicated that the transgene was not integrated into the host genome, therefore, the transformants are not stable.

In this thesis we firstly use both mycelium and protoplast as start materials to develop the conventional transformation system. Secondly, we focused on establishing ATMT for *R. solani* because recent research indicated ATMT could be more efficient method for transformation of filamentous fungi (de Groot *et al.*, 1998; Dunn-Coleman and Wang 1998; Chen *et al.*, 2000).

The advantages and disadvantages of transformation methods are summarized in Table 1.3

Table 1.3 Advantage and disadvantage of transformation methods

Technique	Advantages	Disadvantages
Protoplast + DNA/CaCl ₂ /PEG	<ol style="list-style-type: none"> 1. Efficient transformation procedure 2. No special equipment needed 3. Low cost 	<ol style="list-style-type: none"> 1. Difficult to optimize 2. Cell must be placed in osmotically neutral medium 3. Strains specific variation in transformation 4. Preparation of cells necessary
Electroporation	<ol style="list-style-type: none"> 1. Efficient procedure 2. Short protocol 3. Easily optimized 4. Frozen cells can be used 	<ol style="list-style-type: none"> 1. Equipment setup cost 2. Strain specific variation in transformation efficiencies
Biolistic transformation	<ol style="list-style-type: none"> 1. Relatively high efficient transformation 2. Wide species can be applied 	<ol style="list-style-type: none"> 1. High setup cost 2. Difficult to measure efficiency 3. Preparation of projectiles necessary
ATMT	<ol style="list-style-type: none"> 1. Efficient transformation 2. Easy to operate 3. Low cost 	<ol style="list-style-type: none"> 1. Preparation of bacterial cells 2. longer protocol

Chapter 2 Materials and Methods

2.1 Growth and maintenance of *E. coli* cultures

2.1.1 Short-term storage of *E. coli* cultures

For short-term storage of *E. coli* up to 3 months at 4⁰C, single colonies were streaked onto LB agar (15g/L agar, 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl). Recombinant bacterial strains were positively selected for by the appropriate antibiotic (Table 2.1). Non-recombinant strains were plated without selection.

2.1.2 Long-term storage of bacterial cultures

Single colonies were isolated by standard techniques. After overnight growth in LB medium, 0.8 volume of the bacteria cultures were mixed with 0.2 volume of glycerol and stored at -80⁰C.

2.1.3 Antibiotics and blue/white screening

E. coli strains containing plasmid were plated on selective media. All antibiotics were added to molten agar at approximately 55⁰C to a final concentration indicated in Table 2.1. X-GAL and IPTG were added at the same time to the molten agar to a final concentration of 40µg/ml and 0.1mM respectively when needed.

Table 2.1 Liquid stock solutions of antibiotics and working concentration

Compound	Dissolved in	Stock concentration	Working concentration
Kanamycin (Progen Industries)	Water	50mg/ml	50µg/ml
Ampicillin (Progen Industries)	Water	100mg/ml	100µg/ml
Chloramphenicol (Sigma)	Ethanol	25mg/ml	170µg/ml
Hygromycin B (Sigma)	Water	50mg/ml	50,100 and150µg/ml
Timentin (Smithkline Beecham.)	Water	150mg/ml	150µg/ml

2.1.4 *E. coli* strains genotypes

- 1 JM83 (Yanisch-Perron, 1985) *ara*[□] (*lac-proAB*), *rpsL*, Φ80*lacZ*ΔM15
- 2 JM103 (Yanisch-Perron, 1985) *endA1*, *hsdR*, *supE*, *sbcB15*, *thi1*, *strA*, Δ (*lac-proAB*), [F', *traD36*, *proAB*, *lavI*^qZΔM15]
- 3 JM109 (Yanisch-Perron, 1985) *endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (*r_K*⁻, *m_K*⁺), *relA1*, *supE44*, Δ(*lac-proAB*), [F', *traD36*, *proAB*, *lavI*^qZΔM15]
- 4 TOP10 (Invitrogen) F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74* *deoR* *recA1* *araD139* Δ(*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*
- 5 XL-10 Blue (Stratagene) *Tet*^rΔ (*mcrA*) 183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* Hte [F' *proAB* *lacI*^qZΔM15 Tn10 (Tet^r) Amp^r Cam^r]

2.2 Growth and storage of *Agrobacterium tumefaciens*

2.2.1 Short-term storage of *A. tumefaciens*

A. tumefaciens strains AGRO, AGLI (Lazo *et al.*, 1991), and LBA 4404 (Hoekema *et al.*, 1983) were obtained from S. Wylie, Murdoch University. For short-term storage up to 4

weeks at 4⁰C, cultures were streaked on Minimal Medium agar plates and grown for two days at 28⁰C followed by storage at 4⁰C

The composition of minimal medium for *A. tumefaciens*:

K-salts: 10ml; M-salts: 20ml; NH₄NO₃ (20%): 2.5ml; CaCl₂(1%): 1ml; Glucose (20%): 10; Agar: 18g; Total In 1 L medium.

K-salts composition: 20.5% K₂HPO₄; 14.5% KH₂PO₄

M-salts composition: 3%MgSO₄; 15%NaCl; 2.5% (NH₄)₂SO₄

2.2.2 Long-term storage of *A. tumefaciens*

Long-term storage of both recombinant and non-recombinant strains was with 50% glycerol, 50% overnight MM broth culture and stored at -80⁰C.

2.3 Growth and storage of fungal material

2.3.1 Rhizoctonia solani isolates

The isolates of Rhizoctonia solani used in this study and their characteristics are given in Table 2.2. Isolates were stored on Potato Dextrose Agar (PDA) medium at 4⁰C.

Table 2.2: Characteristics of the *R. solani* isolates used in this study.

Isolate	AG	ZG ¹	Origin	Host	Plasmid (kbp) ³	dsRNA (kbp) ³	Source
ST-11-6	3	7	Japan	NR ²	6	7.8	A Ogoshi
1342	4	8	SA	NR	NT ⁴	NT	S Neate
RH 165	4HGII	8	Japan	Sugarbeet	2.5	-	A Ogoshi
SCR122	6		SA	NR	NT	NT	J Harris
R829	8	1.3	WA	NR	NT	NT	M Sweetingham
1461	8	1.3	SA	Barleygrass	NT	NT	S Neate
1026	11	3	Wa	Wheat root	-	-	M Sweetingham

¹ZG, pectic zymogram grouping according to Sweetingham et al., (1986)

²NR, not recorded.

³unpublished, O'Brien

⁴Not tested

2.3.2 *Fusarium* isolates

Fusarium oxysporum and *F. solani* were obtained from Max Dawson, Murdoch University and were cultured onto PDA plates for 5-7 days at 26 °C until the conidia formed. The plates were stored at 4 °C for storage.

2.3.3 *Ustilago* isolates

Ustilago cynodontis (Henn.) Henn was obtained from the Culture Collection, Dept. Primary Industries Queensland (Cat No 28040). The isolates were stored on PDA at 4°C.

2.4 General methods

2.4.1 Extraction of plasmid DNA from *E. coli* liquid cultures

2.4.1.1 Isolation of plasmid DNA from *E. coli*

Plasmid DNA isolation from *E. coli* was conducted based on the Birnboim and Doly (1979) alkaline lysis method. 3-10 ml of LB Broth containing appropriate selective antibiotics was inoculated and incubated at 37⁰C and shaken at approximately 225 rpm overnight. The cultures were incubated on ice for 10min at 4⁰C until needed (up to 24 hr). 1.5 ml was centrifuged for 5 min at 14,000rpm in a bench top centrifuge. The supernatant was discarded. (If high yields of DNA were required, more chilled culture was added to the microcentrifuge tube and recentrifuged and again the supernatant removed). The bacterial pellet was resuspended in 200 µl of Solution 1 (25 mM Tris-HCl pH8.0, 10 mM EDTA, 50mM glucose) then incubated on ice for 10min. 200 µl of fresh Solution 2 (200 mM NaOH, 1% SDS) was added, followed by thorough mixing and another 10min incubation on ice. 150 µl of Solution 3 (3M Sodium Acetate, adjusted to pH 4.8 with glacial acetic acid) was added, mixed and then incubated for 10min on ice. The mixture was centrifuged at 14,000 rpm for 10 min and then the supernatant transferred to another 1.5ml microcentrifuge tube. The DNA was concentrated by ethanol precipitation. The pellet was resuspended in 90 µl of water and 10 µl of RNaseA (20 mg/ml) (Sigma) and incubated at 37⁰C for 15 min. Proteins were removed by Phenol:Chloroform extraction followed by an ethanol precipitation to concentrate the DNA. The DNA was then resuspended in 50 µl TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA Na₂ pH 8.0) or water.

For large scale preparations of plasmid DNA, the method was scaled up so that the cells from overnight cultures of 80-250 ml were resuspended in 2-5 ml of solution 1. Solutions 2 and 3 were scaled proportionally. The mixture incubated for 10 min on ice and the solution was centrifuged at 14000 rpm for 10 min and the supernatant transferred a fresh 1.5 ml tube. DNA was precipitated by the addition of 0.6 volumes of room temperature isopropanol and mixing well. The precipitate was collected by centrifugation at 14000rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 90µl of water and 10µl of RNaseA (20 mg/ml) (Sigma) and incubated at 37⁰C for 15 min. Proteins were then removed by Phenol: Chloroform extraction. 2 volumes of ethanol

were added to the solution to precipitate the DNA. The DNA pellet was then re-suspended in 50 μ l TE buffer or water.

2.4.1.2 Isolation of plasmid DNA using QIAprep Spin Miniprep Kit

Using the QIAprep Spin Miniprep Kit (QIAGEN), plasmid DNA was isolated from 1-5 ml overnight *E. coli* cultures. The plasmid DNA isolation was performed as directed by the manufacturer. The DNA was eluted in TE buffer.

2.4.1.3 Extraction of plasmid from *A. tumefaciens* using QIAprep Spin Miniprep Kit

A. tumefaciens strains were cultured in LB with Km liquid for 16-20 hours at 28⁰C. 10-20 ml culture were centrifuged at 5000 rpm for 5 min. Plasmid DNA was extracted by using QIAprep Spin Miniprep Kit. The DNA was eluted in 50 μ l of pH7.5 injection water.

2.4.2 Extraction of fungal DNA

2.4.2.1 Extraction of fungal DNA from agar culture.

A 1x2 cm piece of agar from growing edge was cut and placed in a sterile 1.5 ml tube and mixed with 400 μ l extraction buffer. The mixture was ground and incubated at 65 ⁰C for 60 min. The tube was centrifuged at 14,000 rpm for 15 min and the supernatant transferred to a new tube and mixed with 600 μ l Bresa-salt and 5 μ l of glass milk. The mixture was incubated at room temperature for 15 min with occasional shaking. The solution was centrifuged for 10 sec and the supernatant discarded. The pellet was mixed with 600 μ l of Bresa-wash and centrifuged for 10 sec. This step was repeated twice. The pellet was re-suspended with 500-600 μ l 100% cold ethanol and centrifuged for 1 min to collect the precipitate. Excess liquid was removed by inverting over tissue paper for 30

min and the pellet dissolved in 40 μ l of TE. To aid dissolution the tube was incubated at 55 $^{\circ}$ C for 10 min and then centrifuged for 1-2 min to collect evaporated liquid at the bottom of the tube. The supernatant containing DNA was transferred to a new tube and stored at -20° .

The composition of Extraction buffer:

200 mM Tris.HCl pH 8.5; 250 mM NaCl;

25 mM EDTANa₂ ; 0.5% SDS

Breasa-Wash:

100 mM NaCl; 10 mM Tris pH7.5;

1 mM EDTA; 50% EtOH

Breasa-salt:

100g of NaI put in 100ml of Delta water. Add small amount of Na Sulphite to dissolve NaI. Filter and store in light protected bottle in the fridge.

2.4.2.2 Extraction of fungal DNA from liquid culture

DNA was extracted from mycelium by a modification of the method of Raeder and Broda (1985). 50 ml of PD broth was inoculated with a plug of fungal mycelium in 250 ml flask. The flask was shaken at 100 rpm at 26 $^{\circ}$ C for 3-5 days. The mycelium was harvested, washed with distilled water and pressed between sheets of paper towels to dry. The mycelium was transferred to a small vial. Liquid nitrogen was added and the mixture was ground. The powder was poured into a 1.5 ml eppendorf tube. 400 μ l of extraction buffer was added and the mixture was shaken thoroughly and incubated at 65 $^{\circ}$ C for 1 hr. 350 μ l of phenol and 150 μ l chloroform were added and mixed thoroughly. The mixture was centrifuged at 14000 rpm for 1 hr. The upper aqueous phase was withdrawn to a new tube (it is better to leave some of the upper phase behind than to disturb the inter-phase material). 25 μ l of RNAase (20mg/ml) solution was added in the tube that was incubated

at 37⁰C for 10 min. An equal volume of chloroform was mixed with above solution and the mixture was centrifuged at 14000 rpm for 3 min. The upper aqueous phase was transferred to a fresh tube and 0.54 volumes of isopropanol were added to precipitate the DNA. The mixture was centrifuged at 14000 rpm for 20 seconds and the supernatant was decanted off. The pellet in the tube was dried in vacuum for 1 hour and then dissolved in 50 µl of TE buffer.

Extraction buffer is the same as the above.

2.4.3 Transformation of *E. coli*

2.4.3.1 Growth of *E. coli* cells

To prepare competent cells to take up plasmid DNA, two different methods were used. Both of these started with a 5 ml overnight culture of the desired strain of *E. coli* grown in LB broth shaking (225 rpm) at 37⁰C. This culture was inoculated with a single colony from an overnight LB agar plate culture. The 5ml overnight culture was diluted 1:20 with LB broth and incubated while shaking at 37⁰C. This culture was ready to harvest after 2-3.5 h of growth (dependent on the *E. coli* strain) when the O.D.₆₀₀ was between 0.3-0.6 (Sambrook *et al.*, 1989). The cells were chilled on ice for 10-20min. The cells were added to a cooled, opaque 40ml Oakridge tube, and centrifuged at 5000rpm for 8min, at 4⁰C in a Sorvall SS-34 rotor. The supernatant was then discarded. Following this, two different methods were used to prepare the cells for transformation as indicated in sections 2.4.3.2a and 2.4.3.2b.

2.4.3.2.1 Method 1 for the preparation of competent *E. coli* cells

A modified method based on the Hanahan (1983) method was used for the preparation of competent cells (Sambrook *et al.*, 1989).

To the prepared cells, 8 ml of ice cold TFB (100 mM KCl, 10 mM CaCl₂, 45 mM MnCl₂, 3 mM Hexamine CoCl₂, 10 mM MES) were added. The cells were gently resuspended by pipetting up and down, and then incubated on ice for 15 min. They were then centrifuged at 5000rpm for 8 min, at 4⁰C in a Sorvall SS-34 rotor and the supernatant discarded. The cells were gently resuspended in 2 ml of ice cold TFB, and then 140 µl of dimethylformamide (DMF) was mixed in by gentle pipetting. The suspension was incubated on ice for 5 min, 140 µl of β-mercaptoethanol was added and the cells were iced for a further 10 min. Another aliquot of DMF was added followed by 5 min incubation on ice. The cells were used immediately or stored on ice for up to 2 h before they were transformed

2.4.3.2.2 Method 2 for the preparation of competent *E. coli* cells

This method was based on the method by Dagert and Ehrlich (1979). To the pellet of cells, cold 0.1M MgCl₂ was added to a final volume of 1/20th the original culture volume. The cells were gently resuspended by pipetting up and down. The resuspended cells were kept on ice for 20min. The cell suspension was centrifuged at 4000 rpm for 8 min, at 4⁰C in a Sorvall SS-34 rotor. The pellet was resuspended in 0.1M CaCl₂ to 1/50th the initial culture volume. The cells were used immediately or stored on ice until they were needed. They were stored at 4⁰C overnight and then transformed the next day.

2.4.3.3 Transformation of competent *E. coli* cells

The procedure outlined below for the transformation of competent *E. coli* cells, is a modification of the method by Cohen *et al.* (1972).

100-200 µl of competent *E. coli* cells was pipetted into pre-cooled 1.5 ml microcentrifuge tubes. Up to 20 µl of plasmid DNA solution or ligation reaction was added to each of the tubes. The DNA-cell solution was stored on ice for 20 min. The cells were heat-shocked

by placing them in a 42⁰C water-bath for 60 sec and then quickly back into the ice without shaking. The cells were then iced for 2min. Cool or room temperature SOB (2% tryptone, 0.5% yeast extract, 10 mM NaCl₂, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂) or SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl₂, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂, 20 mM glucose) broth was added to the cells to a final volume of 1ml. The mixture was then incubated at 37⁰C with shaking at 200 rpm for 1 hr. After that 100 µl of the mixture was spread onto selective agar plates with a sterile glass hockey stick. The plates were incubated overnight at 37⁰C, and examined for antibiotic resistant colonies.

If a low number of colonies were expected then the cells were centrifuged at 3000 rpm for 5 min in a bench-top microcentrifuge. 800-900 µl of the supernatant was removed and discarded. The cells were then resuspended in the remaining supernatant, and 100 µl was then spread on selective agar plates.

2.4.4 Conjugal transfer of plasmid into *Agrobacterium*

2.4.4.1 Introduction of plasmid into *A. tumefaciens* via conjugation

Binary vectors were mobilized from *E. coli* into *A. tumefaciens* by the tri-parental conjugation method described by Hooykaas (1988). The donor *E. coli* containing the binary vector, the *E. coli* strain containing the conjugative plasmid pRK2013 (km^r), and the recipient *A. tumefaciens* were inoculated in LB medium (5-10 ml in 100 ml erlenmeyer flasks and grown overnight with shaking (for *A. tumefaciens* at 29⁰C and *E. coli* at 37⁰C). The overnight cultures were diluted to OD (666 nm) 0.05-0.1 in the same medium and incubated for another 4 hours with shaking. Equal volumes of each donor cell and the recipient cell were mixed and 50-100 µl of the mixture was spread onto a sterile membrane filter that was placed on the top of the agar layer in the petri dishes. The plates were incubated overnight at 29⁰C. The filter was suspended in the 0.9% NaCl solution by vigorous shaking. Transconjugants were selected by plating on minimal medium with kanamycin. The transconjugants were purified by single colony isolation.

2.4.4.2.1 Introduction of plasmid into *A. tumefaciens* via transformation

The bacterium was grown in LB liquid medium at 29⁰C overnight in the shaker. The overnight culture was diluted to OD (666nm) of 0.02 by adding the same medium and incubated for another 4 hours at 29⁰C with shaking. The cell was centrifuged at 5000 rpm for 5 min and the pellet washed with 10 mM Tris-HCl (pH7.4-7.6). The cell was concentrated 10 fold by re-suspending in 1/10 volume of LB medium. 20 µl of the bacterial suspension was mixed with 10 µl of plasmid DNA (1-5µg). The mixture was incubated at -70⁰C for 5 min and then brought to 37⁰C for 25 min. The suspension was diluted with 70 µl fresh medium and incubated at 29⁰C for 1 hour and then plated onto the selective medium for further isolation.

2.4.5 DNA general Methods

2.4.5.1 Restriction enzyme digestions

Restriction enzyme digestions were performed using reaction conditions as suggested by the manufacturer unless stated otherwise. The restriction enzymes used were *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Sac*I, (Biotech International) and *Kpn*I, *Stu*I, *Not*I, *Pst*I and *Sal*I (Promega). For analytical purposes, digestions were run for 1.5-3 h. Digestions that were to be used as preparative digestions for further cloning were performed for 3h to overnight. To decrease the amount of undigested DNA, further aliquots of enzyme were added part way through the digestion. Preparative digestions contained higher concentrations of enzyme. The units of enzyme to ng of DNA ratio was always less than what the manufacturer had suggested to prevent star activity. Analytical digestions were performed with final reaction volumes of 20 to 50 µl. Preparative digestions were performed in 100 to 400 µl reactions. To determine if the digestion had proceeded through to completion, samples were electrophoresed on agarose gels and compared against undigested DNA.

2.4.5.2 Cloning PCR products into pCRII[®]-TOPO

Cloning into pCRII[®]-TOPO (Invitrogen) was performed using PCR products that were ethanol precipitated following thermocycling. The reactions were set up with 400-500 ng of PCR product DNA, 1 µl salt solution, 0.5µl pCRII[®]-TOPO vector and water to 6 µl. The reactions were incubated at room temperature for 30 min and then kept on ice. 3 µl of the reaction was then used to transform *E. coli* Top10 cells provided. Transformed cells were selected on ampicillin (100 µg/ml) LB agar.

2.4.5.3 Polymerase Chain Reaction (PCR)

PCR was performed in 0.5 ml microcentrifuge tubes or 0.2 ml PCR reaction tubes. Reactions (10µl) were set up with 1 unit of *Tth* Plus DNA polymerase, and 1X PCR Polymerisation Buffer, 1 mM MgCl₂ (Biotech International), 25 pmol of each primer and 5-50 ng of template DNA. Water was added to 10 µl. dNTP's required for the reaction and all other cofactors were supplied in the PCR Polymerisation Buffer supplied as a 5 fold concentrated stock solution.

- (1) The hygromycin resistant gene fragment was amplified with *hph* primers (Table 2.3). A PCR was conducted by the following protocol: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30sec, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec, and additional extension at 72 °C for 7 min.
- (2) M13 primers were universal primers that amplify inserts in the MCS of pBIN19, pUC plasmids, and phage M13 cloning vectors. The modified M13 PCR amplification was conducted by the following protocol: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 3 min, and additional extension at 72 °C for 7 min.
- (3) The *vir* primers amplify a region of the virulence operon of the Ti plasmid (Sawada *et al.*, 1995). The *vir* gene fragment was amplified with *vir* primers (Table2.2). A PCR

reaction was conducted as follows: initial denaturation at 95 °C for 2.5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and additional extension at 72 °C for 7 min.

- (4) ITS1 and ITS4 primers were designed for amplifying 5.8S rDNA region of fungal genomic DNA (Lanfranco *et al.*, 1998). PCR was conducted as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 45 sec, extension at 72 °C for 1 min, and additional extension at 72 °C for 7 min
- (5) Hf primers were used for amplifying the hygromycin resistant gene from pCAMBIA1300 plasmid. The PCR was conducted as following: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 2 min, and additional extension at 72 °C for 7 min.
- (6) Lcc primers were designed from laccase promoter sequence (Wahleithner *et al.*, 1996). The PCR reaction was conducted as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 48 °C for 30 sec, extension at 72 °C for 30 sec, and additional extension at 72 °C for 7 min.
- (7) Hsp70 primers were designed for amplifying promoter of hygromycin resistant gene from pHL1 plasmid. PRC was conducted as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55°C for 30 sec, extension at 72 °C for 30 sec, and additional extension at 72 °C for 7 min.

2.4.5.3 Screening recombinant colonies for inserts via PCR

PCR was used to screen for the presence of inserts in all recombinant plasmids. The primers and PCR thermocycling conditions were the same as for the original amplification of the inserts. The reactions were set up with the same reaction conditions as in section 2.2.4.8. Rather than the template DNA being in solution, a small scrape of a

bacterial colony was pipetted up and down in the PCR reaction mix. An appropriate amount of water was added to make the solution up to 10 μ l to replace the template DNA solution.

Table 2.2 PCR Primers used in this project.

A Set of Primers	Tm	Sequence	Anneals to
hph	47°C	5'CTGAACTCACCGCGACG 3'	<i>hph</i> gene fragment
	42°C	5'ACGGACGCACTGACGTG3'	
vir	42°C	5'-ATCATTTGTAGCGACT-3'	<i>vir</i> gene
	43°C	5'AGCTCAAACCTGCTTC-3'	
M13	63°C	5'CGCCAGGGTTTTCCCAGTCACGAC 3'	Insert of MCS
	55°C	5'TCACACAGGAAACAGCTATGAC 3'	
ITS	55°C	5' TCCGATGGTGAACCTGCG 3'	5.8S rDNA
	50°C	5' TCCTCCGCTTATTGATAT 3'	
Hf	59 °C	5'TTAACGAAGCTTTAATTCGGGGGA3'	<i>hph</i> gene
	63 °C	5'TGACCATGGAAAAGCCTGAACTCACC 3'	
Lcc	64 °C	5'GAGCTCGGAATTCATTCCTCGATCA 3'	Laccase promoter
	72 °C	5'AAGTGGATCCCCATGGCAGAGGCAACCTG 3'	
hsp70	55°C	5' CCGTACCCTGTGAATAGAG 3'	<i>hsp</i> promoter
	46°C	5' AGAATTCCTGGTCGTTCC 3'	

2.4.5.4 Measuring DNA concentrations

DNA concentrations were measured using a DyNA Quant 200 Fluorometer (Hofer) as directed by the manufacturer.

2.4.6 Electrophoresis

2.4.6.1 Agarose gel electrophoresis

Agarose gels were prepared as 0.8 or 1.0% w/v solution with TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 20 mM EDTA Na₂). The solution was heated in a microwave to dissolve the agarose and then cooled to approximately 60⁰C and poured into a gel mould. Samples were loaded onto the gel with gel loading buffer (Biotech International) or 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) to a final concentration of 1X. Electrophoresis was carried out in TAE at 6.7–10 volts/cm. The electrophoresis was stopped when the bromophenol blue had traveled two-thirds of the distance from the wells to the end of the gel.

The gel was stained for 10-20min in an ethidium bromide (1 µg/ml) TAE solution and washed three times with tap water. The DNA was visualized on either a Foto/PhoresisI (Fotodyne) or TFX-35M (Life Technologies) ultraviolet (UV) light box. When viewed on the Foto/PhoresisI light box a photograph was taken using Polaroid 667 film. Using the TFX-35M light box a digital photo was taken using a Kodak DC120 Zoom Digital Camera. The image was processed using Kodak Digital Science 1D (3.0.2). Image analysis was also performed using the Kodak Digital Science 1D (3.0.2) software program.

2.4.6.2 DNA molecular weight markers

MW1 - FN-1 – 2686, 1563, 1116, 859, 692, 501/489, 401, 331, 342, 190 147, 111/110, 67, 34, 26bp (Biotech International).

MW2 - Lambda/*Hind*III – 2310, 9416, 6557, 4361, 2322, 2027, 564, 125bp (Biotech International).

MW3 - Lambda/*Eco*RI+*Hind*III – 21227, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125bp (Biotech International).

2.4.6.3 Isolation of DNA from agarose gels

DNA fragments were cut from agarose gels following electrophoresis. Exposure to UV light was kept to a minimum to reduce damage to the DNA.

2.4.6.3.1 Isolation of DNA from agarose gels using Wizard[®] PCR Preps DNA purification system

Extraction of DNA from agarose using Wizard[®] PCR Preps DNA Purification System (Promega) was performed as directed by the manufacturer for high melting temperature agarose. The procedure was performed using a vacuum system.

2.4.6.3.2 Isolation of DNA from agarose gels using Bresa-Clean

Isolation of DNA from agarose gels using Bresa-Clean (BresaGen) was performed as directed by the manufacturer for TAE gels.

2.4.7 Southern Blot analysis

2.4.7.1 DNA transferred to membrane

3-5 µg fungal DNA was digested with restriction enzyme (*Hind*III) overnight. The digested DNA was loaded on agarose gel and electrophoresed at 3.5 V overnight. The gel was stained and marked with the lambda fragments. The gel was soaked in 0.25 M HCl for 5 min and repeated once and then rinsed with distilled water. The gel was soaked in 0.5 M NaOH 1.5 M NaCl for 2 x 20 min and then washed in water. The gel was soaked in transfer buffer (1 M ammonium acetate + 20 mM NaOH) for 2 x 30 min. Hybond N membrane was cut to the same size as the gel and then wet properly. DNA transfer from the gel to the membrane was carried out by capillary action overnight (Maniatis *et al.*, 1982). DNA was fixed onto the membrane either by baking at 80°C for 1-2 hour, or using the UV crosslinker (Bio Rad).

2.4.7.2 Labelling of DNA probes

The DNA was diluted to a concentration of 2.5-25 ng in 45 µl of TE buffer (10 mM Tris HCl at pH8.0, 1 mM EDTA). The DNA was denatured by heating to 95-100°C for 5 min in a boiling water bath and then cooled down by placing on ice for 5 min. The denatured DNA was transferred to the reaction tube and 5 µl of Redivue [³²P] dCTP (Amersham sp.act. 5000 Ci/mmol) was added to the tube and mixed by pipetting up and down about 12 times. The mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 5 µl of 0.2 M EDTA. For use in hybridization, the labeled DNA was heated at 95-100°C for 5 min and then cooled on ice for 5 min before adding to the bag containing the filter.

2.4.7.3 Hybridization of the membrane and post hybridization washes

The membrane was wetted with hybridization buffer and sealed in a plastic bag which was placed into a water bath (65°C) for 1 hour with shaking. The denatured probe DNA solution was added into the bag and mixed with rest of the solution. The bag was resealed and put into water bath (65°C) overnight.

The membrane was removed from the plastic and transferred into a lunchbox and washed with 2xSSC+ 1% SDS for 20 min. The buffer was poured off.

Then the filter was washed in the box:

2xSSC, 0.1%SDS for 30 min at 65°C

0.1xSSC, 0.1%SDS for 30 min at 65°C

Repeat the last wash

The filter was placed on Whatman paper and wrapped in clingfilm.

2.4.7.4 Autoradiography

The filter was taped to the bottom of a film cassette. An X-ray film was placed on the filter in a dark room and the cassette was closed and placed in the -80°C freezer for exposure. The exposure time was 1-3 days.

2.4.8 DNA sequencing

2.4.8.1 Plasmid DNA template preparation and reaction protocol

Plasmid DNA was purified by using the Qiagen QIAprep kit. The DNA was further cleaned with ethanol precipitation. The DNA was re-suspended in injection water and the concentration was measured with the fluorometer (manufacturer). The DNA concentration was recommended to be over 100 µg/ml. The sequencing reaction was set up in 0.2 ml tube. Both standard half reaction and half term half reaction were used as below.

Standard half reaction

4 µl Dye terminator mix
1.6 pmoles primer
400-800 ng of double-stranded plasmid DNA template
make up to 10 µl final volume with de-ionised water.

Half term half reaction

2 µl Dye terminator mix
2 µl Half term (Half term is a new product that can be used as a partial substitute for dye terminator mix to lower the cost of sequencing reactions without affecting the accuracy)
1.6 pmoles primer
400-800 ng of double-stranded plasmid DNA
make up to 10 µl final volume with de-ionised water

2.4.8.2 PCR DNA template preparation and sequencing reaction protocol

If the PCR reaction produces a single product (only one band on an agarose gel), use a Promega DNA wizard or Qiagen PCR cleanup kit. Ethanol precipitation can be used to concentrate the sample but should not be required.

If the PCR reaction produces more than one product, then run the reaction on the gel and cut the desired band out of the gel. The DNA was eluted by using Qiagen Qiaquick gel

elution kit (which also removes primers and salts). The DNA concentration was measured for sequencing reaction.

Standard half reaction

4 μ l Dye terminator mix

1.6 pmoles primer

60-180 ng PCR product DNA template

Make up to 10 μ l final volume with de-ionised water

Half term half reaction

2 μ l Dye terminator mix

2 μ l Half term

1.6 pmoles primer

60-180 ng of PCR DNA as a template

make up to 10 μ l final volume with de-ionised water

2.4.8.3 Thermal cycling

The samples were vortexed and centrifuged briefly to ensure the reactions were mixed and at the base of the tubes. For 10 μ l reaction volumes it is necessary to use a thermal cycler with a heated lid. The tubes were kept on ice until the thermal cycler was heated to 96°C. The program was as follows:

Hold on 96°C for 2 minutes

25 cycles of

96°C for 10 seconds (denaturation)

50-60°C for 5seconds (annealing)

60°C for 44 minutes (extension)

Hold on 14°C for 99.99 (ie infinity)

2.4.8.4 Post-reaction purification

After thermal cycling, the reaction mixture was purified to remove salts and unincorporated dye terminators. Two methods were used.

2.4.8.4.1 Centrisep spin columns.

These products are produced by ABI. The procedure follows the directions on the pamphlet in the kit. It is noted that it takes a long time to dry the samples in the Speedvac (1-1.5 hours) because they end up in water.

2.4.8.4.2 Ethanol precipitation

For each half reaction, a 0.5 ml tube with the following 25 μ l of 95% ethanol 1 μ l of 3 M sodium acetate (pH4.6) was prepared. The 10 μ l contents were transferred into the 0.5 ml tube containing the ethanol and sodium acetate. The tubes were vortexed and left on ice for 10 minutes and then centrifuged for 30 minutes. The supernatant was discarded and the pellet was rinsed by adding 125 μ l of 70% ethanol. The tubes were spin at 14000 rpm for 5 minutes and the supernatant was removed as much as possible. The samples were dried in the Speedvac for at least 15 minutes and submitted for sequencing.

Chapter 3. Construction of Vectors and Transformation of *Fusarium*

3.1 Introduction

Fusarium are necrotrophic pathogens which cause severe disease in the host plants. The pathogen infects plant root tissues of susceptible plant species resulting in vascular wilt disease (Kacprzak *et al.*, 2001). In solid media culture, such as potato dextrose agar (PDA), different species of *Fusarium* can have varying appearances. *F. oxysporum* produces three types of asexual spores: microconidia, macroconidia, and chlamydospores (Agrios, 1988). Microconidia are one or two celled, and are the type of spore most abundantly and frequently produced by the fungus under all conditions. It is also the type of spore most frequently produced within the vessels of infected plants. Macroconidia are three to five celled, gradually pointed and curved toward the ends. These spores are commonly found on the surface of plants killed by this pathogen as well as in sporodochia like groups. Chlamydospores are round, thick-walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. These spores are either one or two celled (Agrios, 1988).

It has been reported that transformation of *Fusarium* was successfully conducted by two methods- *Agrobacterium* mediated transformation and naked DNA transfer (de Groot *et al.*, 1998; Mullins *et al.*, 2001)

In this chapter we investigated transformation of the ascomycete fungus *Fusarium oxysporum* with the aims: a) determining which of our strains of *A. tumefaciens* were the most efficient at transfer of DNA to the fungal cells; b) testing our vectors to see whether they are functional; c) testing our experimental protocols to see if we could pick up transformants.

3.2 Materials and Methods

3.2.1 Construction of binary vectors

3.2.1.1. pBINAN

The pBINAN vector was constructed on the backbone of the plant binary vector pBIN19 (Fig 3.1) (Bevan, 1984). The *hph* gene in pAN&-1 is contained on a 3.7 kb fragment of pAN7-1 (Fig 3.2). The coding region is flanked by an ascomycete *gpd* promoter and *trp* terminator (Punt *et al.*, 1987). The *Stu*1 site in pAN7-1 was converted to a *Kpn*1 site by cutting with *Stu*1 followed by treatment with DNA polymerase I in presence of dNTPs to create blunt ends. *Kpn*1 linkers were ligated onto the ends of the insert. After digestion with *Kpn*1 and *Hind*III the insert was inserted into *Hind*III/*Kpn*1 digested pBIN19 to create pBINAN.

3.2.1.2. pBINHL1 construction

The second vector pBINHL was derived from pHL1 (Fig 3.3) (Wang *et al.*, 1988) which contains the *hph* gene under control of the *Ustilago maydis hsp70* promoter. The 3.3 kb *hph* gene was isolated by cutting pHL1 with *Hind*III, and ligating the digest to *Hind*III digested pBIN19. Colonies containing the *hph* gene were identified by PCR screening using the *hph*F/*hph*R primer pair.

3.2.1.3. pCAMBIA1300

The third vector used for transformation in this study was pCAMBIA1300 (CAMBIA, Canberra, Australia) which contains the *hph* gene under control of Cauliflower mosaic virus 35S (CaMV35S) promoter and polyA. This gene was in T-DNA region between left and right borders (Fig.3.4).

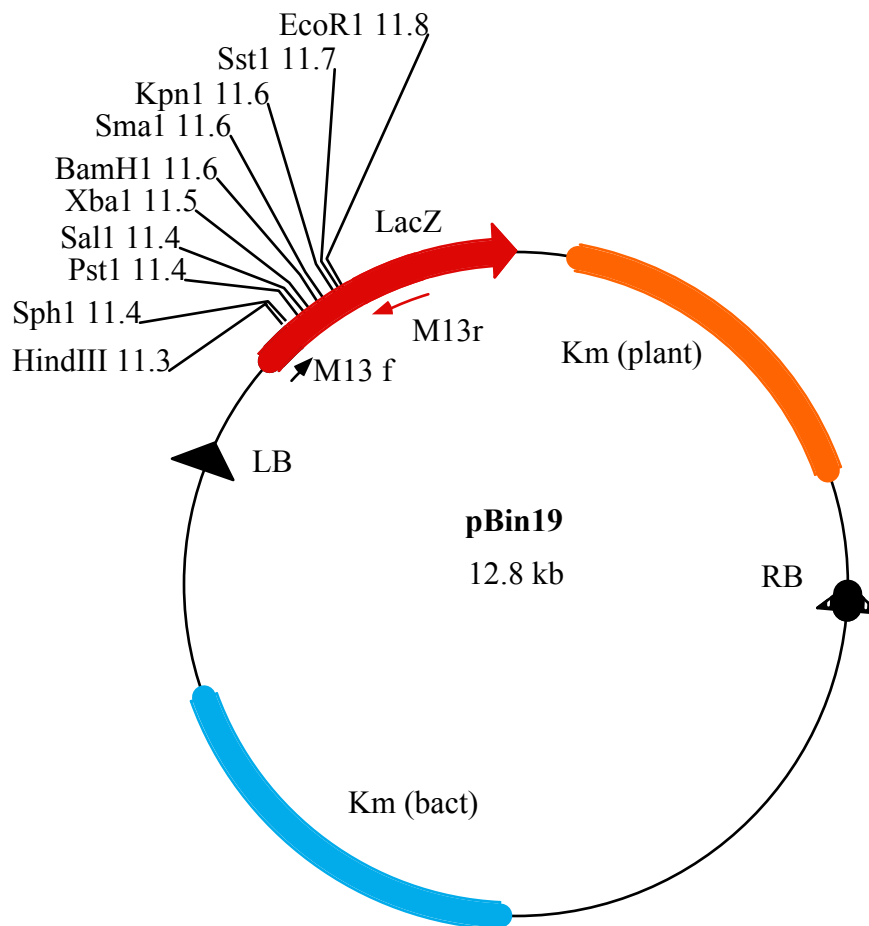


Figure 3.1. The plant binary vector pBin19 (Bevan, 1984). The vector contains the lac operon insert from bacteriophage M13 with the multipurpose cloning site and has blue/white selection for identification of recombinant colonies in *E. coli*. The vector has kanamycin resistance genes for selection in plants and bacteria. The *A tumefaciens* T-DNA left border (LB) and right border (RB) sequences define the sequence transferred to the plant/fungus. M13f and M13r denote the positions of the M13 primers used for analysis of transformants.

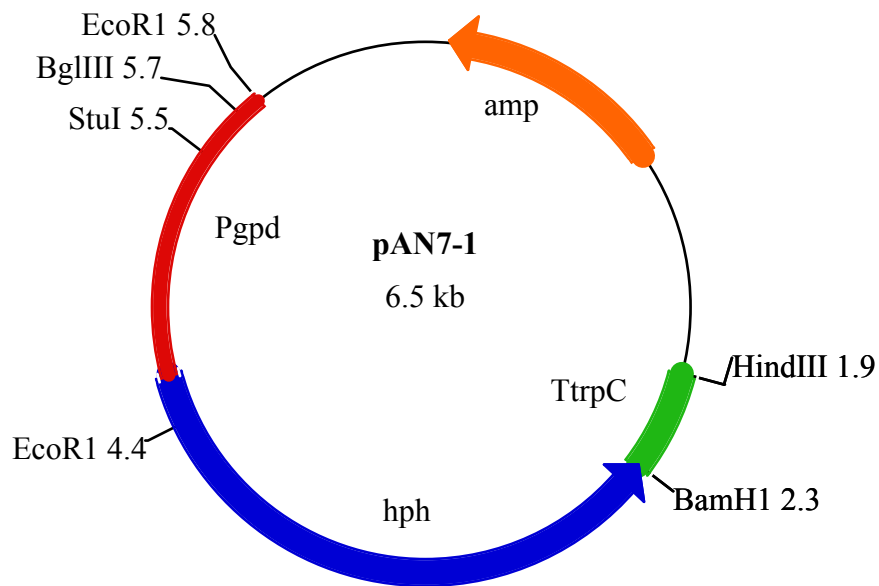


Figure 3.2 The fungal cloning vector pAN7-1. The vector contains the *hph* gene under control of the ascomycete *Pgpd* promoter and the *TrpC* terminator (Punt *et al.*, 1987).

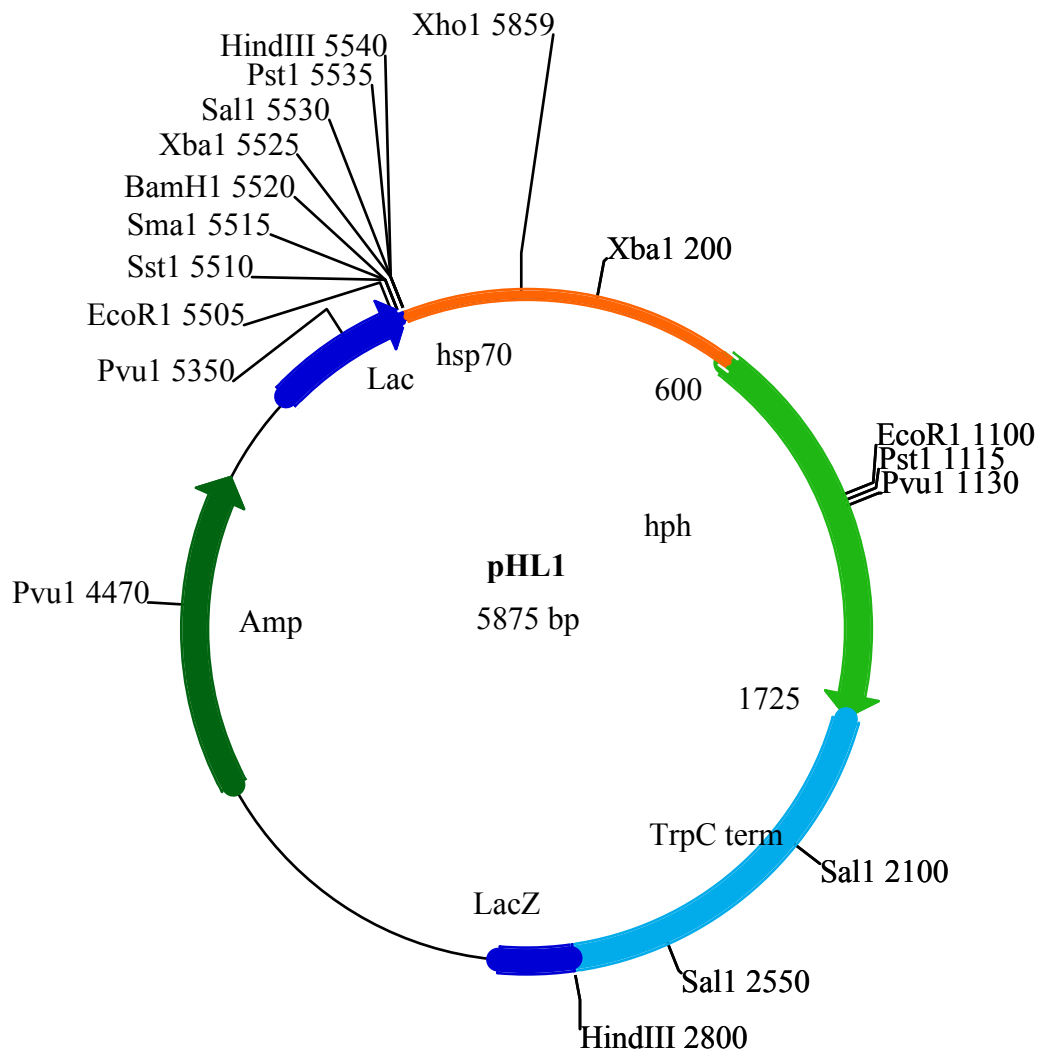


Figure 3.3 The fungal vector pHL1. This contains the hygromycin resistance gene under control of the *Ustilago maydis* *hsp70* promoter and the *TrpC* terminator (Wang *et al.*, 1988). The *hph* gene was transferred to pBin19 as a HindIII fragment.

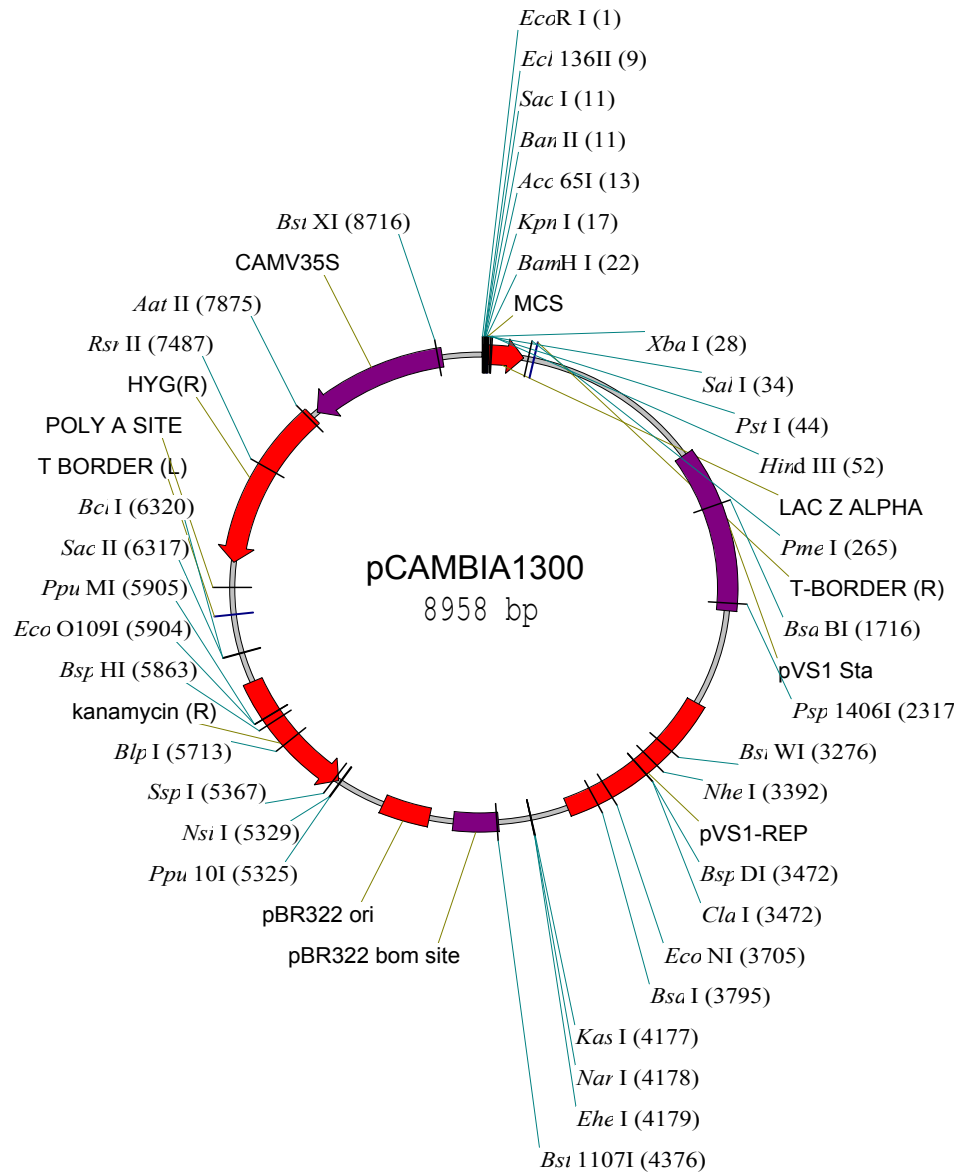


Figure 3.4 Map of Binary Vector pCambia1300

3.2.2 Introduction of vectors into *A. tumefaciens*

These vectors were introduced into the *A. tumefaciens* strains AGLO, AGL1, and LBA4404 by conjugation as described in section 2.4.4.1

3.2.3 Transformation of *F. oxysporum*

3.2.3.1 *F. oxysporum* conidia preparation

F.oxysporum-C3316 was cultured on Potato Dextrose Agar (PDA) at 25°-26°C for 7-8 days until the mycelium turned purple. 5ml 0.9% NaCl sterile solution were added to the plate and conidia suspended by rubbing with a glass spreader. The liquid was recovered from the plate and filtered through two layers of sterile gauze to remove mycelial fragments. The concentration of the conidia was counted with a haemocytometer. The concentration of conidia was adjusted to 10⁶ per ml.

3.2.3.2 Preparation of germinated conidia

Conidia collected from a PDA plate were washed with 10 ml of distilled water. The conidia suspension were inoculated into 40 ml induction medium at 28°C with a rotary shaker (200 rpm) for 0, 4 and 8 h. Conidia recovered by centrifugation were resuspended in 40 ml of ice-cold sterile water, centrifuged again, resuspended in 4ml of ice-cold water and kept on ice.

3.2.3.3 Transformation of conidia

A. tumefaciens strains containing the vectors were grown at 28°C for 24 hours in minimal medium (MM) (Hooykass *et al.*, 1980) with kanamycin (50 µg/ml). 1ml was transferred into 5 ml fresh induction medium broth (IMB) (Bundock *et al.*, 1995) with and without acetosyringone (AS 200 µM) and incubated until the cell concentration reached 10⁸ cfu/ml. Equal volumes of bacteria and conidia suspension were mixed and poured onto induction medium agar (IMA) with or without AS. Small pieces of sterile

filter papers (0.5 x 0.5cm) were placed onto the plates. The plates were incubated at 26°C for 1-3 days. The filter papers were transferred to selective medium (SM) containing hygromycin 50 µg/ml and timentin 150 µg/ml and the plates incubated at 26 °C for 3-5 days until the resistant colonies grew. Individual resistant colonies were purified and sub-cultured on selective agar for further testing.

3.2.4 Analysis of *F. oxysporum* transformants.

3.2.4.1 Test of transformants stability

To determine the stability of transformants, transformants were chosen to grow on PDA without hygromycin B. When the mycelia grow to the edge of the plate, a small plug of culture was transferred to fresh PDA, and this procedure repeated 5 times. After that, the transformant conidia were placed onto PDA with hygromycin B again (150 µg/ml).

3.2.4.2 Analysis of transformants by Southern blotting

DNA was extracted from transformants as described in section 2.4.2. The DNA was digested with restriction enzyme, and analyzed by Southern blotting as described in section 2.4.7

3.2.4.3 PCR Analysis of transformants.

The presence of the transgene was confirmed by PCR as described in section 2.4.5.

3.3 Results

3.3.1 Sensitivity to hygromycin

In order to determine hygromycin concentration for selection of transformants, *F. oxysporum* was grown on PDA with different concentrations of hygromycin. The results are shown in Figure 3.5.

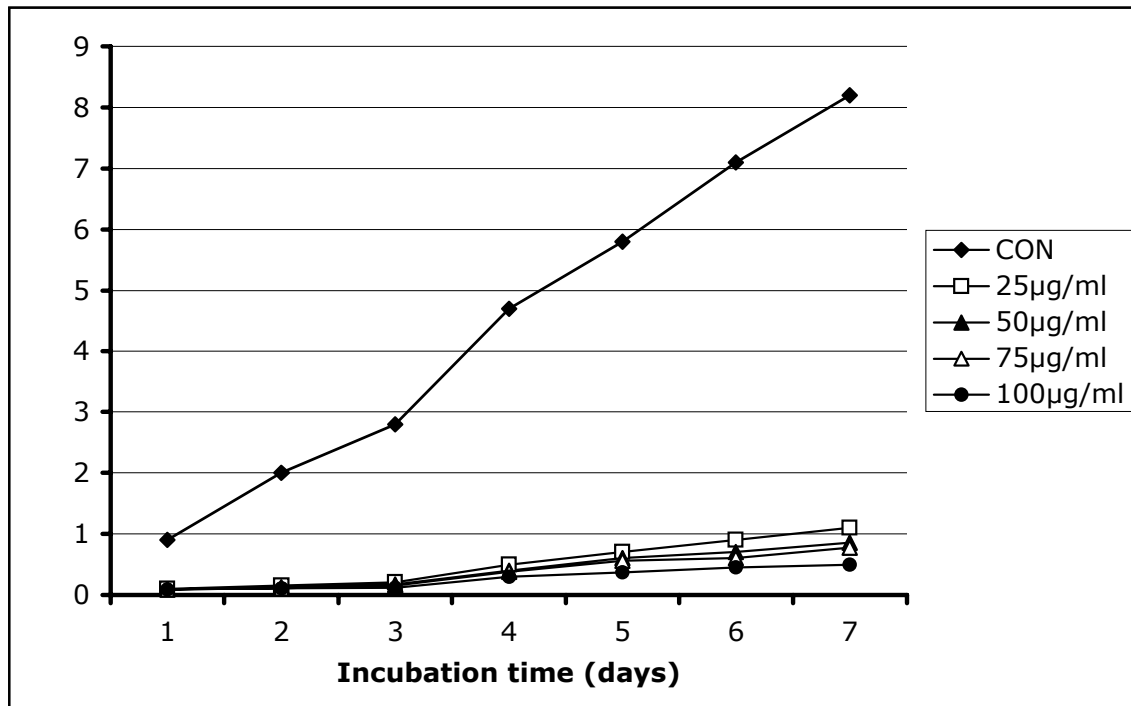


Figure 3.5 Growth curve of *F. oxysporum* on PDA at different concentrations of hygromycin.

The results show that *F. oxysporum* was sensitive to hygromycin at 25 µg/ml, and completely inhibited at 50 µg/ml. In this study we used hygromycin at 50 µg/ml for selection of transformants.

3.3.2 Transformation with *A. tumefaciens* strains

In plant transformation the efficiency of transformation depends partly on the strain of *A. tumefaciens* (Hansen and Wright 1999). We compared three strains of *A. tumefaciens* as donors and three vectors which contain the *hph* gene driven by different promoters. The results are given in Table 3.1.

Table 3.1 Transformation of *F. oxysporum* with different strains of *A.tumefaciens* and different vectors. (figures are the average of three replicates).

Bacterial Strains	Vector	No. of Transformants Per 10 ⁶ conidia
AGLO	pBINAN	13
	PCAMBIA1300	2
	pBINHL	0
AGL1	pBINAN	35
	PCAMBIA1300	7
	pBINHL	0
LBA4404	pBINAN	0
	PCAMBIA1300	0
	pBINHL	0

The results show that different strains have different transformation efficiency. Transformants were consistently obtained when strain AGLO and AGL1 were used as a host. In contrast no transformants were obtained when *A. tumefaciens* LBA4404 was used as the host.

One of the main requirements for obtaining transformants of *R. solani* will be the degree of expression of the *hph* gene. The higher the level of expression the easier it will be to obtain transformants. For this reason we tested vectors in which the *hph* gene is driven by different promoters. The *hph* construct in pBINAN is known to function in *Fusarium*, and it has also been reported that the CaMV35S promoter in pCAMBIA1300 also functions in *Fusarium* (Mullins *et al.*, 2001) but it is not known if it functions in basidiomycetes. It is not known if the *U. maydis hsp70* promoter used in pBINHL functions in *Fusarium*.

Higher numbers of transformants were obtained with the pBINAN construct containing the *hph* gene under control of the ascomycete Pgpd promoter (Punt *et al.*, 1987). The

CaMV35S promoter in pCAMBIA1300 also worked but at lower efficiency. No transformants were obtained with the pBINHL vector.

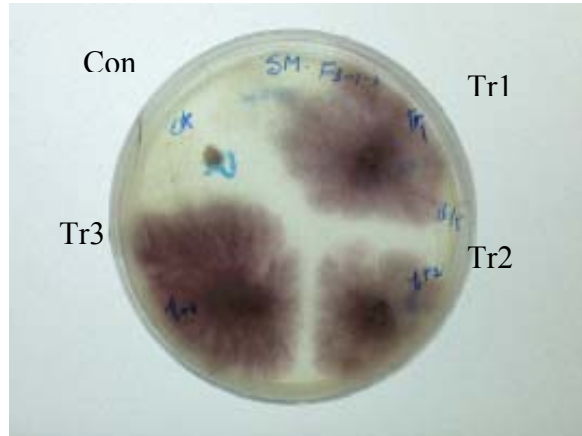


Figure 3.6 *F.oxysporum* transformants 1.2 and 3 with non-transformant control (Con)

3.3.3 Growth curve of transformants

Resistant colonies were transferred to fresh selection medium with hygromycin at 50 $\mu\text{g}/\text{ml}$ to test growth. The morphology of the resistant colonies is shown in Fig. 3.6 Transformants 1, 2 and 3 grew on hygromycin agar whilst the untransformed control did not grow.

Growth of one of the transformants on hygromycin agar is shown in Fig. 3.7. Growth was observed at both 50 and 150 $\mu\text{g}/\text{ml}$ hygromycin. Growth was not significantly different at the higher concentration of hygromycin. The growth rate was similar to that observed on non-hygromycin agar (compare with Fig 3.5).

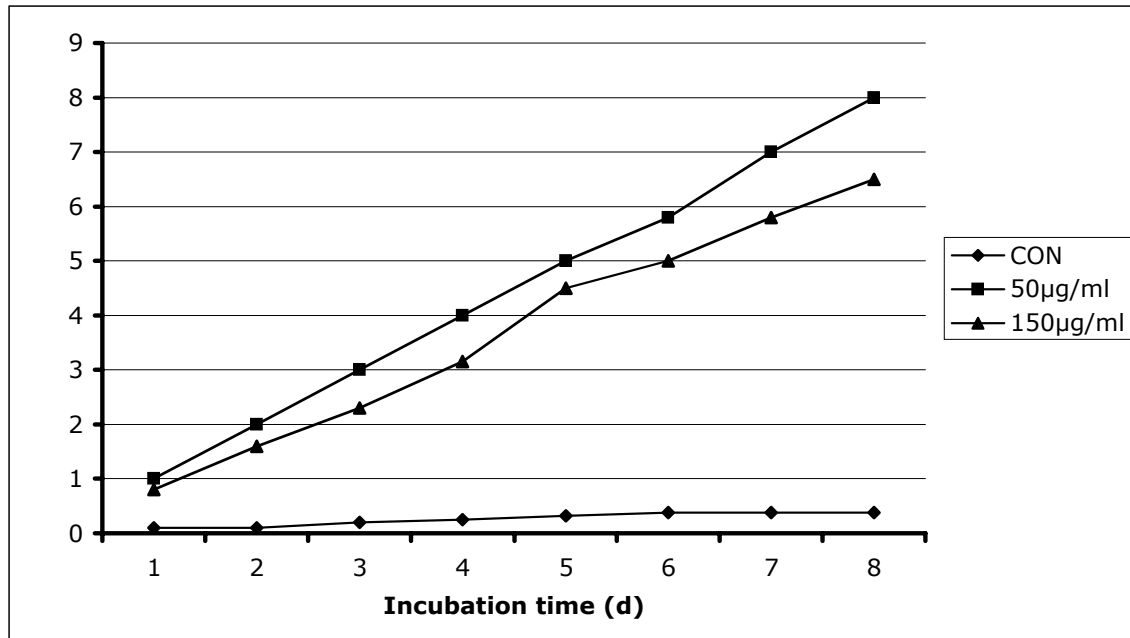


Figure 3.7 Growth of transformants in the presence of hygromycin. The colonies were grown on PDA in the presence of the indicated concentrations of hygromycin.

3.3.4 Confirmation of transformants by PCR

Resistant colonies were analyzed by PCR to establish whether they contain *hph* gene. The results (Fig.3.8A) show that the expected 527 bp *hph* gene fragment was obtained by PCR with specific *hph* gene primers. The same size band is obtained from pAN7-1. The second pair of primers that anneal to M13 sequences flanking the *Hind*III-*Kpn*I sites of pBIN19 were used to confirm that the entire insert was transferred to the fungus. The results also confirm the presence of the 3.7 kb band in each transformant (Fig 3.8B). No product was obtained from the untransformed control.

The results in Fig 3.8A and Fig3.8B could be explained by the presence of a contaminating *A. tumefaciens* cell being carried over during purification of the transformant. To exclude this possibility we carried out an amplification with primers to the *vir* region of the *A. tumefaciens* Ti plasmid. The *vir* region of the plasmid is the part of plasmid that is not included in the transfer to the fungus. The presence of *vir* sequences would therefore indicate the presence of contaminating bacteria (Sawada *et al.*, 1995).

DNA from the putative transformants was amplified with the VF1/VR1 primer pair specific for the *vir* region. No *vir* PCR products were obtained from selected transformants (Fig.3.8C) confirming that the positive transformation results were not due to a contaminating bacterial cell

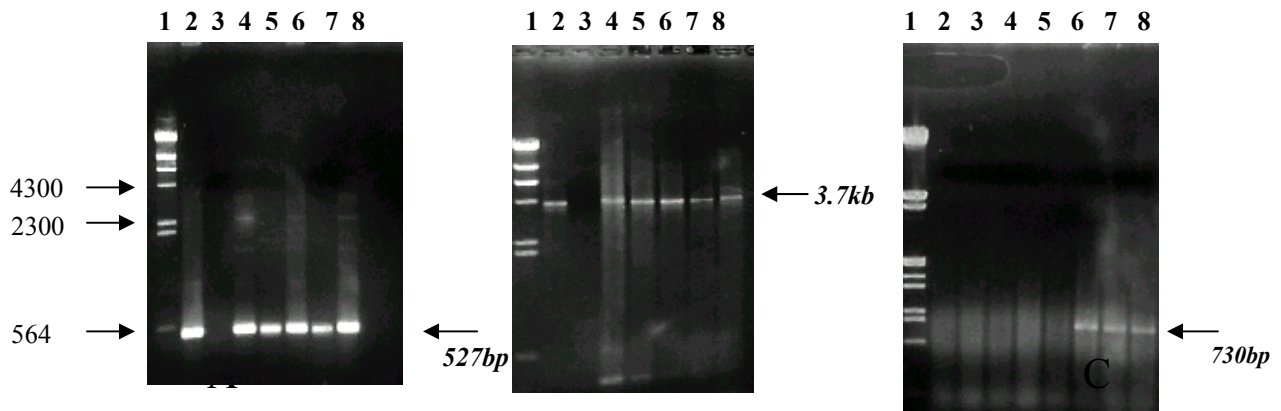


Figure 3.8 (A). Analysis of DNA from transformants with *hph* primers (A), M13 primers (B) and *vir* primers (C). Lane contents,: A and B, lane 2, positive controls (pAN-7 and pBINAN in A and B respectively), lane 3, negative control; lanes 4-8, putative transformants. (C), lanes 2-5, putative transformants; lanes 6-8: *A tumefaciens* strains AGLO, AGR1 and LBA4404 respectively. Product sizes are indicated in bold italics on the right of each gel.

3.3.5 Analysis of transformants by Southern blotting

To verify that the transformants were independently generated, the site of insertion of the Ti DNA was analysed by Southern blotting. Restriction enzyme *Hind*III digested DNA from putative transformants was electrophoresed on an agarose gel, and blotted onto a Hybond membrane. The blot was hybridized with a probe specific for the *hph* gene. The results show that transformant 1 is different from transformants 2 and 3 (Fig 3.9).

Transformants 2 and 3 show a major band of the same size indicating that the site of insertion was the same in both transformants. However, the probe hybridizes to a less intense higher molecular weight band in transformant 3, and to a lower molecular weight band in transformant 2, indicating that there may be secondary sites of insertion in these two transformants, and that the transformants are not the same.

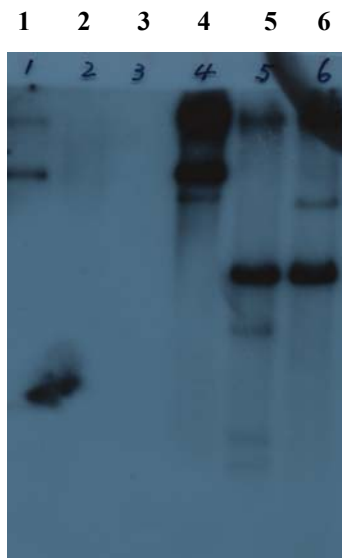


Figure 3.9 Southern blot analysis of the putative transformants. The genomic DNA was digested with *Hind*III. Lane 1: Undigested plasmid pBINAN as a control; Lanes 2-3: Non-transformants; Lanes 4-6: Putative transformants 1, 2 and 3. The blot was probed with pBINAN.

3.3.6 Stability of the transformants

Loss of transgenes from transgenic fungi during growth in nonselective conditions has been described by a number of investigators for a variety of fungal species (Bailey *et al.*, 1993; deGroot *et al.*, 1998; Fincham, 1989; Leslie and Dickman, 1991; Renno *et al.*, 1992) To determine the stability of transformants, we tested transformants for several generations of growth on nonselective media. After that, the transformant conidia were placed onto PDA with hygromycin B again (150 μ g/ml). The transformant growth rate was the same as before (Fig 3.7). The test result (Fig 3.10) indicated these transformants were still resistant to hygromycin. Furthermore, the transformants were again tested by PCR with *hph* gene primers and M13 primers, the results were all positive.

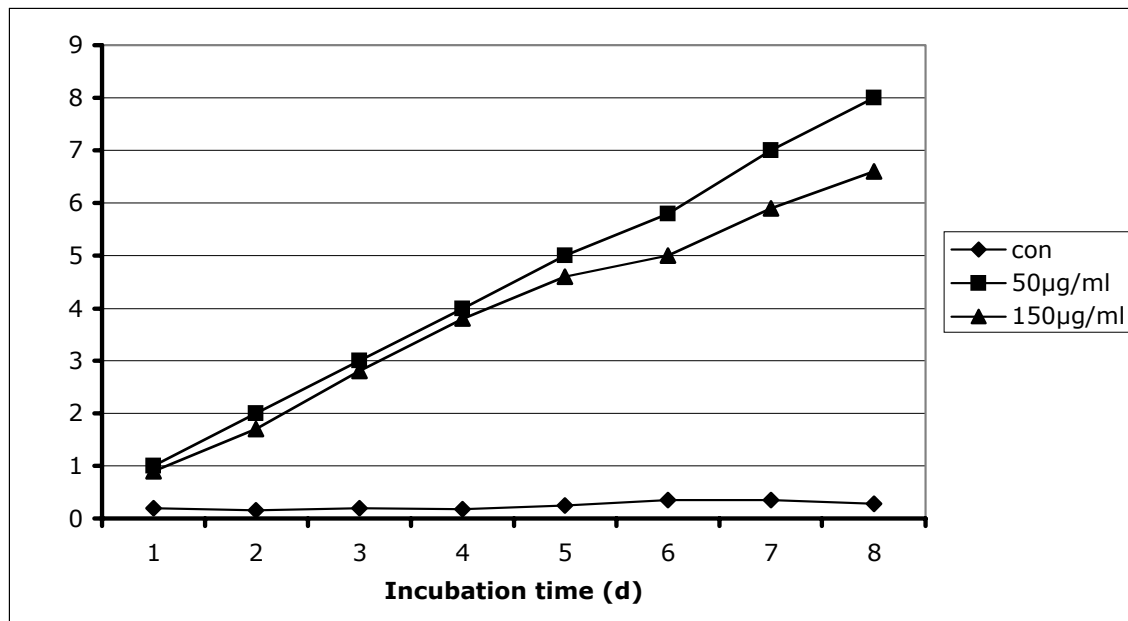


Figure 3.10. Stability of transformed phenotype. Colony growth was measured on PDA supplemented with 50, or 150µg/ml hygromycin.

3.3.7 Effect of pre-treatment with Acetosyringone

Acetosyringone (AS) is a crucial signaling compound during T-DNA transformation of plant and fungus (Pier *et al.*, 1996). In this study, we first tested transformation during co-cultivation with and without AS. No transformants were obtained when AS was omitted from the co-cultivation medium.

In order to improve the transformation, the effect of pretreating *A. tumefaciens* with AS prior to mixing with conidia was tested as it has previously been shown to enhance transformation (Mullins *et al.*, 2001). *A. tumefaciens* was grown in MM medium for 24 hours, then AS was added to the medium for 10 hours prior to co-cultivation with *F. oxysporum* conidia.

The results (Table 3.2) show that pretreatment with AS resulted in greater numbers of transformants. This effect was observed after 24 hr co-cultivation, and was even more enhanced after 48 hr co-cultivation.

Table 3.2. Effect of pre-treating *A. tumefaciens* with AS and effect of co-cultivation time on transformation frequency

Vector	AS Pretreatment	Co-cultivation time (h)	No. of transformants per 10 ⁶ conidia
AGL1(pBINAN)	+AS	24	9
	-AS	24	2
	+AS	48	31
	-AS	48	8
AGL1(pCAMBIA1300)	+AS	24	5
	-AS	24	0
	+AS	48	9
	-AS	48	3

3.3.8 Transformation of germinated conidia

Sanchez and Aguirre (1996) and Dantas-Barbosa *et al.*, (1998) reported that higher transformation efficiencies could be obtained by using germinated conidia as the starting material for transformation by lithium acetate or electroporation respectively. In these experiments we have compared the efficiency of transformation with germinated conidia to that obtained with ungerminated conidia (Table 3.3).

Table 3.3. Effect of germination time on transformation efficiency with AGL1(pBINAN)

Germination time (hour)	No. Transformants/ml
0	18
2	31
4	30
8	8

The results show that pregermination of the conidia did not significantly increase the transformation efficiency.

3.4 Discussion

In this chapter we demonstrated that the strains of *A. tumefaciens* are competent in transferring DNA to fungal cells, and that at least one of the vectors we constructed pBINAN was expressed and able to confer hygromycin resistance.

In this study, I used three different promoters for driving expression of the hygromycin resistance gene. The first vector contained the *E. coli hph* gene fused to the 5' expression signals of the *A. nidulans gpd* gene (Punt *et al.*, 1987) and the transcription-termination region of the *A. nidulans trpC* gene. The second vector contains a *hph* gene fused to the *U. maydis hsp70* promoter (Wang *et al.*, 1988). In the third vector the *hph* gene is fused to the Cauliflower mosaic virus-35S promoter. The results show that ascomycete *gpd* promoter and CaMV35S promoter can both function in this fungus. While pBINHL vector did not result in any transformants, it could be because the *hps70* promoter may not work in *Fusarium*. The species from which this promoter is derived (*U maydis*), is a Basidiomycete and may not function in ascomycetes..

Compared with the efficiency of transformation by using pBINAN and pCAMBIA1300, more transformants (5 times) were obtained from using pBINAN. An obvious explanation is that the *gpd* promoter gene is from *Aspergillus nidulans* which like *Fusarium* belongs to the ascomycetes. Consequently we would expect it to function more efficiently in an ascomycete host compared to the plant virus CaMV35S promoter. The CaMV35S promoter is a widely used promoter for driving a selection gene in plant transformation (Mushegian and Shepherd, 1995) and the binary vector carrying the hygromycin B resistance gene under control of the CaMV35S promoter is widely available.

It has been reported with plant transformation systems that the efficiency of transformation varies with the strain of *A tumefaciens* used (Lazo *et al.*, 1991; Tzfira *et al.*, 2001). Lazo (1991) found that in plant transformations the AGLO strain consistently gave a 10 fold higher transformation frequency than the LBA4404 strain. Piers *et al.*, (1996) reported a similar effect of strain background on AMT of yeast. They reported up

to 100 fold difference in transformation efficiency with different strains. AGL1 is a recombinant deficient strain derived from AGL0 (C58 pTiBo542) (Lazo *et al.*, 1991). The higher transformation efficiency of strain AGLO compared to LBA4404 in plant transformation experiments is ascribed to genes in the *vir* region of the Ti plasmid. Bacterial proteins such as the VirD2 are required for transfer of the T-DNA to the plant, and presumably the fungus (Gelvin, 2000). The Ti plasmid of LBA4404 is of a different lineage and possibly its VirD2 protein is less effective. There is evidence that the VirD2 protein of the bacterium interacts with histone type proteins in the host to achieve integration of the T-DNA (Gelvin, 2000).

In this study, conidia and germinated conidia were compared for transformation efficiency. Sanchez (1996) and Dantas-Barbosa *et al* (1998) reported 5 and 10 fold higher transformation efficiencies when germinated conidia were used for transformation by lithium acetate and electroporation respectively. In this study, pregermination of the conidia did not have a significant effect on transformation efficiency. In the AMT process bacteria and conidia are mixed and co-cultivated prior to selection of transformants. The results reported by other researchers suggests that the transformation event does not occur immediately, but occurs over a 48 or 72 hr period (Chen *et al.*, 2000; de Groot *et al.*, 1998; Mullins *et al.*, 2001). This gives ample time for the conidia to germinate.

Chapter 4 Transformation of *Rhizoctonia solani*

4.1. Introduction

In chapter 3 we have conducted *Agrobacterium* mediated transformation of *Fusarium*, developed transformation system for *Fusarium* and successfully obtained the transformants. In this chapter we developed a transformation system for *Rhizoctonia solani*. Because *R. solani* does not form conidia, we are restricted to using mycelium for transformation. It has been reported that *Agrobacterium* can not only transform fungal conidia but also it can transform fungal hyphal tissue (de Groot *et al.*, 1998).

A potential barrier to transformation is the fungal cell wall. Traditional fungal transformation protocols use protoplasts created by enzymatically removing the wall (Fincham, 1989). The wall is essentially a layer of polysaccharide, chitins and glucans covering the cell. The exact structure varies widely from species to species. Older cells have thicker walls than younger cells at the hypha tip. One way of getting around this problem is to treat the cells with cell wall degrading enzymes which would wholly or partially remove the wall leading to more efficient transformation.

4.2 Material and Methods

4.2.1 Fungal isolates and cultivation

Isolates RH165 (AG 8) and SCR122 (AG 6) were used to determine the conditions for protoplast formation. The characteristics of these isolates are described in Ch2.

4.2.2 Preparation of mycelium for transformation

To prepare mycelium for protoplast production, a plug of fungal culture was inoculated in 250 ml flask containing 50 ml of PD medium at 25 °C for two days with shaking at 150

rpm. The mycelium was harvested and washed with 20 ml of sterile water. The mycelium was placed into blender with 50 ml water and blended for two min. The suspension was centrifuged at 1500 rpm for 5 min, the supernatant was discarded and the blended mycelium inoculated into 50ml of fresh PD medium at 25⁰ C for indicated time.

4.2.3 Preparation of protoplasts for transformation

Mycelium prepared as in section 4.2.2 was harvested by centrifugation and the fresh wt was determined. For protoplasting 300mg mycelium was suspended in 5 ml of a solution of lytic enzyme (Novozyme 234 Sigma L-2265; or lysing enzyme Sigma L-1412) 10 mg/ml in 0.1 M potassium hydrogen phthalate-NaOH buffer (pH5.7) with osmotic stabilizer. The solution was sterilized by filtration using a 0.45 µm membrane (Millipore). Protoplasting treatment was carried out at 30⁰C.

Protoplasts were harvested by filtering the solution through a double layer of Miracloth (Calbiochem, 475855). The filtrate was centrifuged at 3000 rpm for 5 minutes, the supernatant was discarded and protoplasts were washed twice with osmotically stabilized buffer to remove the residual enzymes. Protoplasts were re-suspended in osmotically stabilized buffer and the concentration of protoplast was measured with a haemocytometer. The concentration of protoplasts was adjusted to about 10⁵-10⁶/ml for transformation.

4.2.3.1 Regeneration of protoplasts in NaCl, sucrose and sorbitol at different concentrations

To determine optimal conditions for protoplast regeneration, the osmotic stabilizers NaCl, sucrose and sorbitol were incorporated into PD or minimal medium and 0.5% agar, autoclaved at 121⁰ C for 20min. The mixture was cooled down to 55⁰ C, 0.2 ml protoplast solution was added to the molten agar which was poured into Petri dishes, mixed and spread evenly. The plates were incubated at 25⁰ C, regeneration frequency was observed after 48 hours (Hashiba and Yamada, 1982)

4.2.4 Polyethyleneglycol transformation of protoplasts

100 µl of the protoplast in STC (0.8 M sorbitol, 10 mM Tris-Cl pH7.5, 50 mM CaCl₂) was mixed with 5 µg of plasmid DNA suspended in 20 µl of distilled water and incubated for 30 min on ice.

200 µl of 60% polyethylene glycol 4000 (PEG) was added to the protoplast suspension with gentle shaking. This step was repeated and an additional 800 µl of 60% PEG was then added with gentle mixing. The mixture was incubated at 10⁰ C for 20 min, 12 ml of STC was added. Transformed protoplasts were centrifuged at 3000 rpm for 10 min at 4⁰ C. Protoplasts were resuspended in 200 µl STC. This suspension was mixed with 5ml of 0.5% osmotically stabilized molten PDA and poured onto PDA agar (1.5%) plates containing 50 µg/ml hygromycin. The plates were incubated at 26⁰ C and monitored for the appearance of colonies.

4.2.5 *Agrobacterium* transformation of *R. solani* mycelium

Mycelium prepared as in section 4.2.2 was used for transformation directly after blending, or was incubated for a period between blending and transformation. Where the mycelium was used directly after blending, the mycelium was resuspended in 2 ml of liquid induction medium. 0.5 ml of this suspension was mixed with equal volume of *Agrobacterium* culture. The subsequent steps were as described for transformation of *Fusarium* in section 3.2.3.

4.2.6 *Agrobacterium* transformation of *R. solani* protoplasts

1. 300 µl of the protoplast at a concentration of 10⁶ was mixed with 50 µl of *Agrobacterium* culture.

2. The mixture was gently shaken and added to 2 ml of induction medium (IM) top agar (0.5% agar) with osmotic stabilizer.
3. The agar was poured onto IM plates and incubated at 26⁰ C for 2 days.
4. 2 ml of sterile water was added to the IM plate and the top agar scraped off into a centrifuge tube. The agar was allowed to settle and a dilution series was made from this mixture. Aliquots of dilutions were plated onto selection medium and incubated at 26⁰ C. Incubation was continued until it was apparent that no more colonies would appear on the plates. As resistant colonies appeared they were transferred to fresh selection plates.

4.3 Results

4.3.1 Factors affecting protoplast production.

4.3.1.1 Age of mycelium.

Previous studies with other species of fungi have shown that mycelium age has a significant effect on the yield of protoplasts and hence transformation efficiency (Hashiba *et al.*, 2001; Robinson and Deacon, 2001; Yang *et al.*, 1992). As the mycelium ages the cell wall thickens due to continued deposition of chitin and glucan polysaccharides. In this experiment we investigated the effect of mycelium age on protoplast yield. Blended mycelium was resuspended in growth medium and incubated for various times at 26⁰ before treatment with protoplasting enzymes.

For isolate SCR122, 24-36 hour mycelium was found to be most suitable for protoplast production (Fig 4.1). With incubation time of 48h the mycelium began to clump and the yield of protoplasts dropped dramatically. For isolate RH165, the maximum yield of protoplasts was observed with 36-48 hr mycelium. Neither of the isolates gave appreciable yields of protoplasts at 12h or at incubation times longer than 48 hr. Since

both isolates gave good yields with 36h mycelium this was used in subsequent experiments to prepare protoplasts.

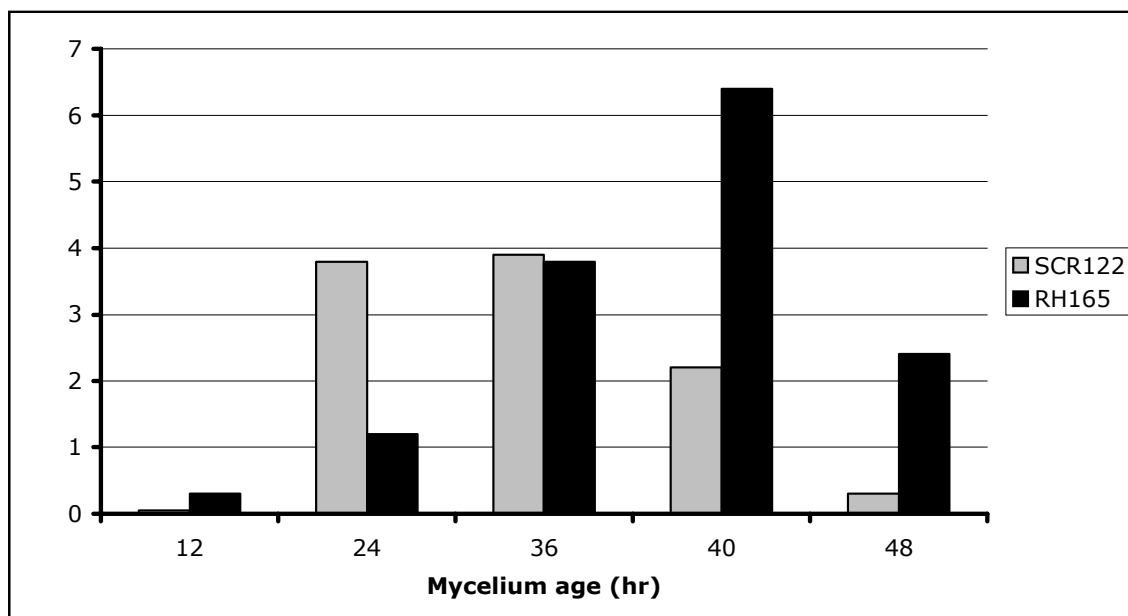


Figure 4.1 Effect of the age of mycelium on yields of protoplasts from *R solani* SCR122 and RH165. Blended mycelium was resuspended in growth medium for the indicated period before being harvested and treated with protoplasting enzymes for 3 h.

4.3.1.2 Effect of stabilizer on protoplast releasing

Osmotic stabilizers are required to prevent lysis of the protoplasts during the transformation process. Usually sugars, sugar alcohols and inorganic salts are used as osmotic stabilizers at concentrations in the range 0.6 – 1.2M. However, although required they have an adverse effect on growth (O’Brien unpublished; Peberdy, 1991). Different species respond differently to different stabilizers and the most appropriate stabilizer to be used for any given species must be determined by experiment.

Three stabilizers were compared for their effect on the yield of protoplasts from isolate SCR122. The results show that as the concentration increased from 0.4 – 1.0M the yield

of protoplasts increased (Fig 4.2). Among the three stabilizers compared, 0.9M magnesium sulphate produced the highest yield of 6.5×10^6 protoplasts/g mycelium. When magnesium sulphate concentration reaches 1.0M, it becomes insoluble, therefore I used 0.9 M MgSO_4 , 8.4mM Na_2HPO_4 , 1.6mM NaH_2PO_4 as stabilizer together with lysing enzyme to prepare protoplasts. NaCl was also a very effective stabilizer resulting in comparable numbers of protoplasts at 0.8M; however the number decreased slightly at 1M. The yields obtained with the organic stabilizer sorbitol were much lower.

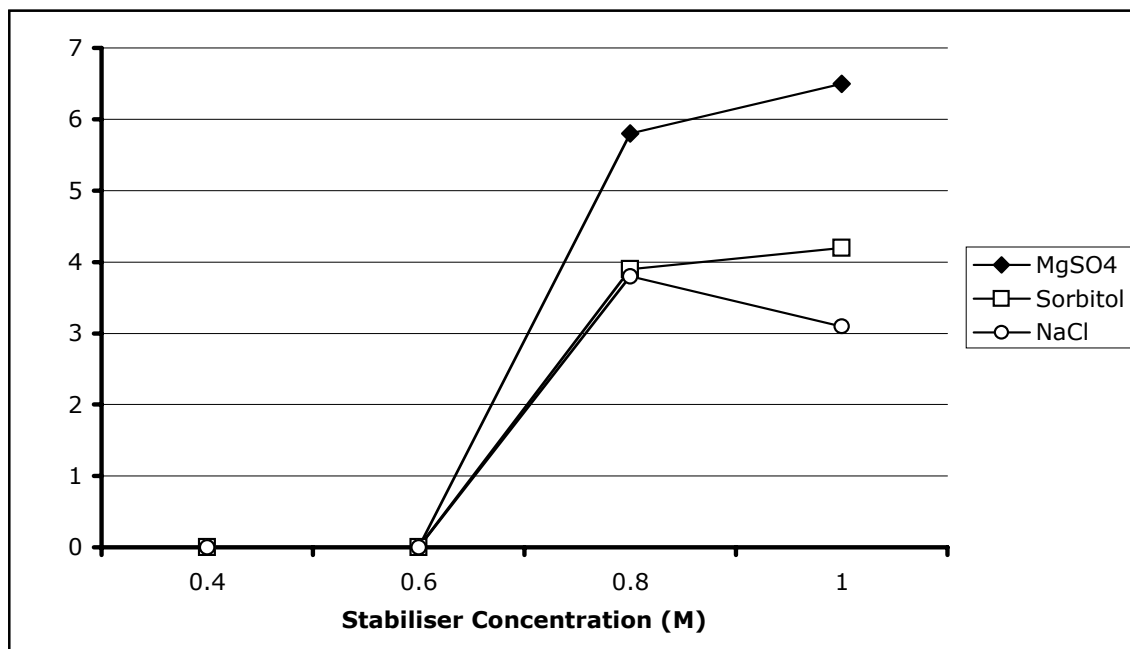


Fig.4.2 Effect of osmotic stabilizers on protoplast production of SCR122. Blended mycelium was resuspended in growth medium and incubated for 36h before treatment with lytic enzymes for 3h.

4.3.1.3 Effect of osmotic stabilizer on protoplast regeneration

The effects of the stabilizers on the regeneration of protoplasts was also tested. Protoplasts were mixed with molten top agar containing different stabilizers and plated onto PDA supplemented with the same stabilizer. Since MgSO_4 was relative lower for protoplast regeneration, MgSO_4 was omitted from this experiment, and sucrose was included.

The protoplast regeneration frequency was affected by the nature of the osmotic stabilizer used (Fig 4.3). Although NaCl was good stabilizer for protoplast release but it was not good for protoplast regeneration. Of the three stabilizers tested it gave the lowest level of regeneration. Sorbitol proved to be the best stabilizer for protoplast regeneration, whilst sucrose was only slightly better than NaCl. The concentration of stabilizer was also crucial in regeneration. Max regeneration occurred at a concentration of 0.8M.

Regeneration frequency declined in the range 0.8 to 1M. Therefore regeneration medium was prepared with PDA containing 0.8 M sorbitol.

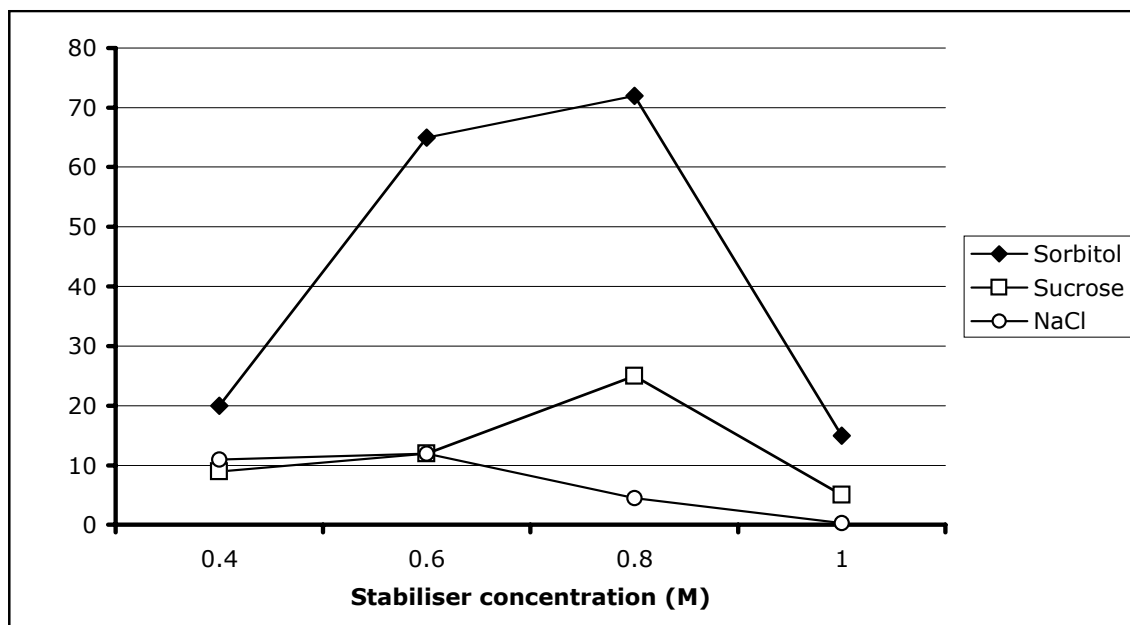


Figure 4. 3 Protoplast reversion percentage following 4 day incubation at 26⁰ C on PDA containing different concentration of stabilizers.

4.3.1.4 Enzyme reaction time.

The time of treatment with lytic enzymes is another factor which significantly affects protoplast yield. If insufficient time is allowed for digestion protoplasts will not be formed. In contrast treating cells for longer times can result in lysis of the protoplasts. The mechanisms is not known. The optimal treatment has to be determined for each

species and set of conditions. In this experiment, samples were treated with Novozyme 234 for different times and the yield of protoplasts were determined. The results show that treatment for 3 hours resulted in the maximum yield of protoplasts (Fig 4.4).

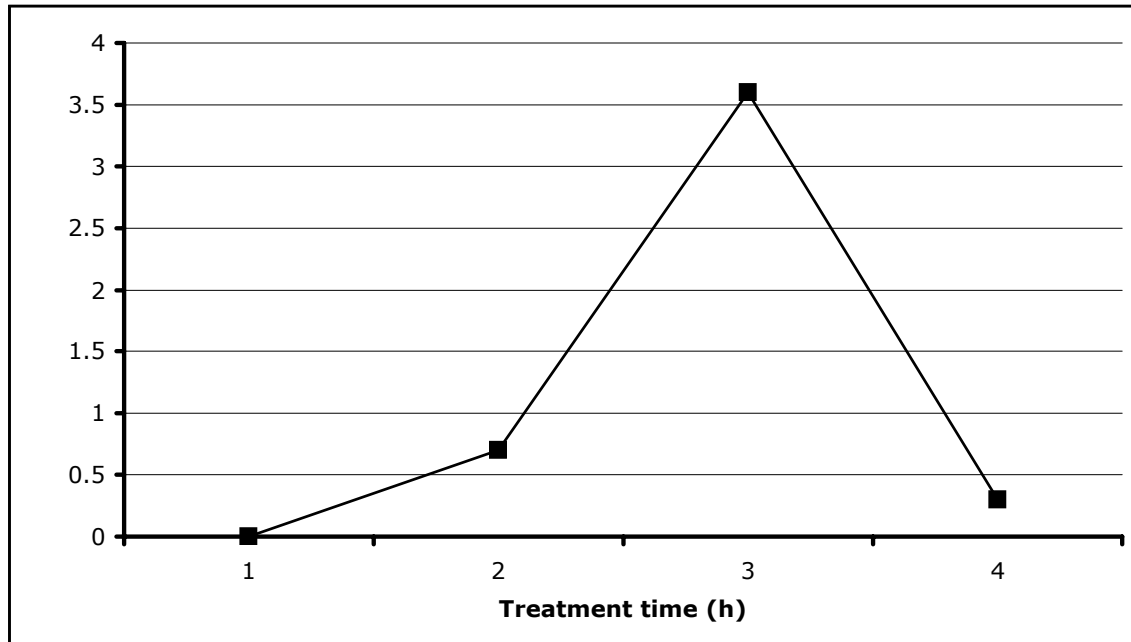


Figure 4.4 Time course of protoplasts yield from *R. solani* SCR122.

4.3.2 Effect of induction medium on protoplast regeneration

In the initial experiments with *A. tumefaciens* mediated transformation it was decided to try transformation of protoplasts. This should be highly efficient due to removal of the cell wall. However, we needed to test whether the induction medium would allow regeneration of protoplasts. The results of this show that IM supplemented with sorbitol did support regeneration of protoplasts although at a lower rate compared to PDA sorbitol medium (Fig. 4.5).

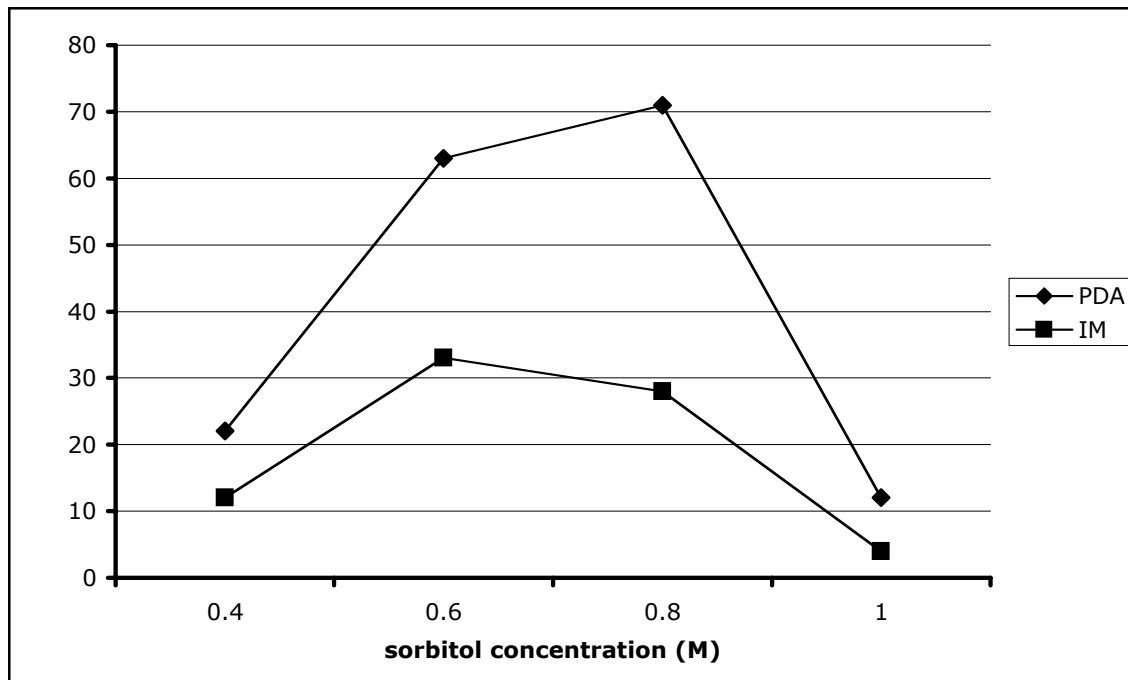


Figure 4.5 Effect of medium on protoplast regeneration. Protoplasts of *R solani* SCR122 were prepared and plated on PDA and induction medium (IM).

4.3.3 Transformation of *R. solani* protoplasts with pAN7-1 and pHL1

Plasmids pAN7-1 and pHL1 were initially developed for transformation of *Aspergillus* and *Ustilago* with bacteria hygromycin resistant gene respectively (Punt *et al.*, 1987; Wang *et al.*, 1988). The experiments were successful for transformation with 5-20 and 50-1000 transformants per μg vector DNA.

We attempted transformation of *R. solani* protoplasts from isolates SCR122 and RH165 but failed to obtain any resistant colonies that might indicate transformation had occurred. Varying the incubation time (0, 5, 10, 20 and 30 min), or the PEG concentration at 25, 40 and 60% did not result in the appearance of resistant colonies.

4.3.4 Transformation of *R. solani* with *A. tumefaciens*.

Both mycelium and protoplasts of *R. solani* were transformed with *A. tumefaciens*. In this experiment we used protoplasts recovered after both 2 and 3hr treatment with protoplasting enzymes and tested isolates from 4 different AG. After several days incubation a number of small resistant colonies appeared on the selective agar plates (Fig 4.6; Table 4.1). Of the isolates tested, ST-11-6 was the least susceptible to transformation. There was very little difference between the other three isolates. With SCR122 and RH165, higher transformation levels were obtained with protoplasts, whilst with 1342 protoplasting did not have a significant effect. Selections of the colonies from the different isolates were transferred to fresh selection medium plates where they grew to a size of about 20 mm. No further increase in size was observed even after prolonged incubation. These colonies did not show any growth when inoculum was taken from the edge of these colonies and transferred to fresh selection plates. PCR analysis of DNA extracted from these colonies using primers specific for the *hph* showed no clear bands, suggesting that they do not contain the transgene.

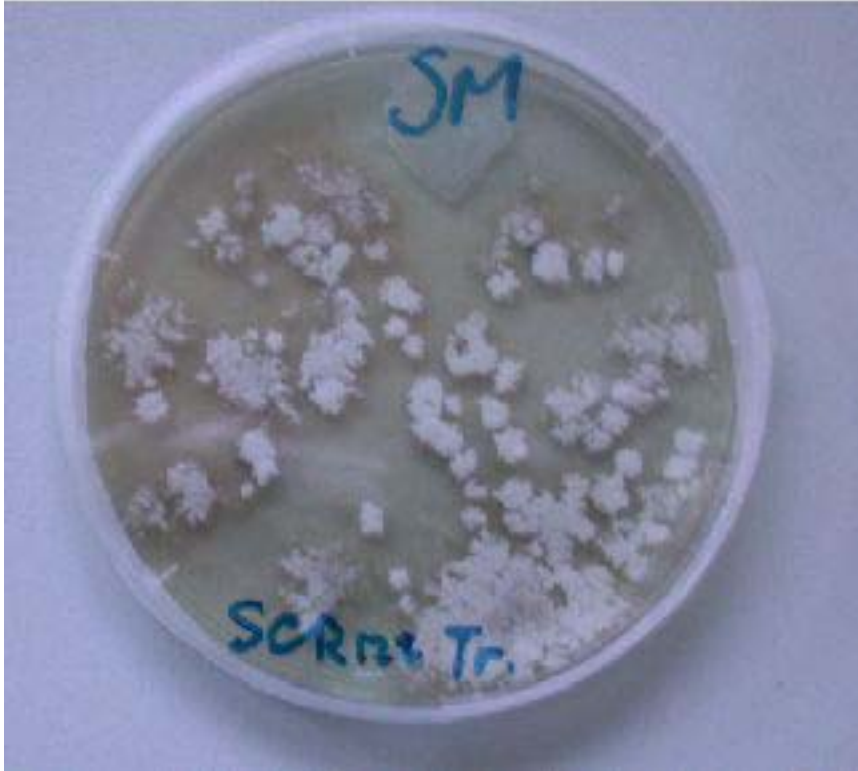


Figure 4.6 Small resistant colonies obtained from *Agrobacterium* transformation of *R. solani* isolate SCR122 which grew on selection medium.

Table 4.1 The number of hygromycin resistance colonies obtained from *Agrobacterium* transformation of *R. solani*. The number is averaged from two experiments

AG	Isolate	Protoplast Treatment (hour)	Transformants/10 ⁶ protoplasts	
			+AS	-AS
3	ST-11-6	0	2	0
		2	0	0
		3	0	0
4	1342	0	32	0
		2	0	0
		3	50	0
6	SCR122	0	6	0
		2	10	0
		3	32	0
8	RH165	0	5	0
		2	30	0
		3	17	0

4.4 Discussion

Initial protoplasting experiments had focused on conditions that produced large numbers of protoplasts. Further experiments to identify parameters that affected protoplast release returned to choice of osmoticum in the protoplasting buffer, NaCl, MgSO₄ and sorbitol were tested. MgSO₄ was effective in protoplasting release, and 0.9M MgSO₄ was found to be suitable concentration. The protoplast yield decreased rapidly at MgSO₄ concentrations below 0.8M. For this reason, the mycelium was washed in 0.9M MgSO₄ prior to addition of the buffer containing the protoplasting enzymes. Above 1.0M MgSO₄, the protoplasting release was very slow.

In the early experiments, the enzyme used in protoplasting formation was Novozyme 234 which was commercially produced from the fungus *Trichoderma harzianum*. Its main components are α -1,3-glucanase, β -1,3-glucanase, laminarinase, xylanase, chitinase and protease. For *R. solani* the cell wall components are chitin and β -1,3-glucan, therefore the Novozyme 234 was effective in protoplast production in buffered pH ranged from 5-7. Later on Novozyme 234 was not commercially available, so the lysing enzymes (Sigma No: L-1412, lot 69H1557) were used for protoplast preparation instead of Novozyme 234. Lysing enzymes were also produced from *Trichoderma harzianum* and contain cellulase, protease and chitinase activities. Glucanase and chitinase are important components in preparing fungal protoplasts (Peberdy, 1991). By comparing the two products, we found they were all effective in making protoplasts, while protoplasts yield by Novozyme234 was slightly higher than that of lysing enzymes.

Protoplast production was highly dependent on culture age. Mycelium that was older than 48 hour gave significantly lower yields of protoplasts. This result was quite different from the report of Robinson and Deacon (2001). They reported that when mycelium age was 60-70 hour, the protoplast yield reached the highest peak. This could be explained that different isolates have different growth rate, and different methods of cell culture were used. In our optimal experiment, the plug of culture was inoculated into PD broth and grown for 48 hours, and the mycelium was blended into very small piece of

fragments, and then these small fragments were re-inoculated into fresh PD broth for 24 hours. The absolute age varied from one inoculated mycelium to another. It is impossible to reach the same stage of growth in a set time from one experiment to the next. Therefore, microscopic examination of growing mycelium during 24 hour was used to check suitable size tips and whether they formed clumps.

An improved regeneration medium was developed by using induction medium for protoplast reversion and *Agrobacterium* mediated transformation. Composition of the medium was important to protoplast regeneration frequency. PDA was effective in protoplast reversion. In a comparison of the two media, the defined IM was significantly less effective than the complex PDA medium. The more complex nutritional environment of PDA seems to stimulate the regeneration process, or alternatively provides a greater degree of osmotic stability that aids the regeneration process.

Polyethyleneglycol transformation of *R. solani* with pAN7-1 and pHL-1 failed to generate any transformants. It is reported that DNAase's secreted by *R. solani* could be harmful to transformation by causing degradation of the transforming DNA (Robinson and Deacon, 2001). To overcome this the protoplasts were washed with STC solution twice to remove DNase activity and treated with different concentrations of CaCl₂. (Ruiz-Diez, 2002). Variables such as plasmid DNA concentration, plasmid form (linear vs circular) (Raymond *et al.*, 1999), PEG with different concentrations (Gietz *et al.*, 1995), time and temperature of protoplast mixed with the plasmids (Fincham, 1989; Raymond *et al.*, 1999), these factors were all tested for transformation. However, transformation was still not successful.

Agrobacterium mediated transformation of protoplast resulted in slow growing resistant colonies. Most of these failed to grow when transferred to fresh selection plates. Some did grow to a colony size of about 20 mm, but then stopped growing and failed to show any further growth. Similar results were reported by Robinson and Deacon (2001) who ascribed the results as a lack of integration of the transforming DNA into the endogenome despite the fact that the DNA was transported into the nucleus. de Groot *et*

al., (1998) also reported the appearance of unstable transformants of *Aspergillus niger* and *Colletotrichum gloeosporoides* after transformation by *A. tumefaciens*.

Work on *A. tumefaciens* transformation of plant cells has shown that the T-DNA is transferred to the plant cell as a single stranded linear segment associated with the bacterial *virD2* protein attached to the 5' end of the DNA (Gelvin, 2000; Tzfira *et al.*, 2001; Zupan *et al.*, 2000). Within the plant cell the DNA is coated with the *virE2* protein which is transported from the bacterium independently. Both proteins then assist the entry of the DNA into the nucleus. The *virD2* protein may also play a role in integrating the T-DNA into the endogenome (Gelvin, 2000; Zupan *et al.*, 2000). A comparison of transient vs stable expression of transgenes in plants has revealed that most of the DNA transferred to plant cells is not integrated into the genome and is lost through successive cell divisions (Gelvin, 2000). For many plant species T-DNA integration is the limiting step for transformation. It has been estimated that the stable transformation of apple decreased approx 10,000 fold from the initial transformation event (Maximova *et al.*, 1998). That T-DNA does not integrate efficiently in yeast cells has been shown by a 500 fold higher transformation frequency obtained with a replicative vector compared to a non replicative version of the same vector (Piers *et al.*, 1996).

In comparison with *R. solani*, it proved relatively easy to obtain transformants of *U. cynodontis* with pBINHL and *F. oxysporum* with pBINAN (see chapter 6). This suggests that DNA is transferred to the recipient and integrates at sufficiently high efficiency to obtain stable transformants. Why therefore do we not obtain stable transformants with *R. solani*? It has long been known in plant transformation, that varieties of a species show differences in their susceptibility to *A. tumefaciens* transformation. This may be due to the host proteins. Mysore *et al.*, (2000) isolated a mutant of *Arabidopsis thaliana* resistant to transformation by *A. tumefaciens* and identified a histone protein that is affected by the mutation. This suggests that the conformation of chromatin affects integration of the T-DNA.

R. solani also differs from *Fusarium* and *Ustilago* in that the vegetative cells are multinucleate (Adams, 1996; Flentje and Stretton, 1970). It can be speculated that nuclei containing the transgene are somehow selected against and lost through successive cell divisions. Robinson and Deacon (2001) have proposed similar explanations. They suggested that the resistant microcolonies arise through translocation of hygromycin phosphotransferase from transformed cells through to the growing hyphal tip. Although *R. solani* has been shown to have an efficient translocation system (Sherwood, 1970) the efficiency of translocation would be likely to decrease as the colony grows. At the same time there would be an increasing need for more enzyme at the growing tip.

Chapter 5. Construction of a hygromycin resistance gene with an *R. solani* promoter.

5.1 Introduction

In ascomycetes a construct containing a hygromycin phosphotransferase gene under control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and *trpC* terminator of *Aspergillus nidulans* has been successfully used for transformation of some species (Hanegraaf *et al.*, 1991). However, attempts to express this construct in basidiomycetes have not been successful (Gold and Alic 1993; Saito *et al.*, 2001). Only in *Phanerochaete chrysosporium* (Randall *et al.*, 1991), *Pleurotus ostreatus* (Peng *et al.*, 1992), *Lentinus edodes* (Hirano *et al.*, 2000) and *Schizophyllum commune* (Scholtmeijer *et al.*, 2001) has this construct been reported to be active, but transformation either occurred at lower frequency or involved a recombined autonomously replicating plasmid. Sometimes the transformation system was not reproducible. One possible reason why such constructs do not function efficiently in basidiomycete is that regulatory sequences from ascomycete genes are not recognized by transcriptional machinery (Frank *et al.*, 1998).

We have used three different promoters to construct vectors for transformation of *R. solani*. The first one was *gpd* promoter from *Aspergillus nidulans* (Punt *et al.*, 1987). The vector pUR5750 containing promoter *gpd* has been successfully used for transformation of several fungi such as *Aspergillus awamoni*, *A. niger*, *Fusarium venenatum*, *Trichoderma reesei*, *Colletotrichum gloeosporioides*, *Neurospora crassa* and *Agaricus bisporus* (de Groot *et al.*, 1998). The second is the heat shock promoter from the basidiomycete *Ustilago maydis* (Wang *et al.*, 1988), which has been used for transformation of *Pseudozyma flocculosa* (Cheng *et al.*, 2001), *Ustilago maydis* (Wang *et al.*, 1988), *Cryphonectria parasitica* (Churchill *et al.*, 1990). The third promoter 35S promoter is from plant virus, the cauliflower mosaic virus which has been used for constructing vectors for transformation of lots of plants (Yoshida and Shinmyo, 2000)

and fungi such as *Fusarium oxysporum* (Kim *et al.*, 1996a). We have used the above three promoters constructed in three different vectors and transformed *R. solani*, but only transient transformants were obtained with the *U maydis hsp70* promoter in the pBINHL1 vector (chapter 4). Punt *et al.*, (1991) suggested that it is best to choose the transcriptional control sequences either from the host organism itself or from a related species to drive selection marker gene for fungal transformation, because the promoter driving its expression can strongly affect the efficiency of transformation. In this chapter, we try to isolate a novel promoter from *R. solani* genome and construct a novel binary vector for transformation of the same isolate of *R. solani*.

A search of Genebank revealed that in addition to rRNA sequences, the only *R solani* sequences available are the sequences of four laccase genes from an isolate R122 (Wahleithner *et al.*, 1996). We used these sequences to design primers to amplify the laccase promoter sequence from SCR122. This promoter was fused in front of the hygromycin resistance gene and the composite gene inserted in pBIN19 to create a novel binary vector (pBINLcc). With this vector *Agrobacterium* mediated transformation was conducted.

5.2 Materials and Methods

5.2.1 PCR amplifications: Isolation of a laccase promoter sequence from *R. solani*

The laccase promoter sequence was amplified with the primer pair LacF/LacR, and the *hph* coding region from pCAMBIA1300 was amplified with the primer pair HpF/HpR (Table 2.2) as described in section 2.4.5.3.

5.2.2 DNA sequencing.

This was carried out as described in section 2.4.8

5.2.3 Cloning of PCR products

PCR amplicons were purified from agarose gels using the Wizard reagents (section 2.4.6.3) and cloned into pCRII TOPO (section 2.4.5). The ligated products were transformed into *E. coli*.

5.2.4 Plasmid extraction and enzyme digestion

Plasmid DNA extractions, restriction enzyme digestions, and ligations were as described in section 2.4.5.

5.2.5 Agrobacterium mediated transformation of *R. solani*

Transformation of *R. solani* was conducted by using young mycelium and protoplast respectively (section 4.2.5; 4.2.6).

5.3 Results

5.3.1 Cloning the *R. solani* laccase promoter

A search of the Genbank DNA sequence database for *R. solani* sequences revealed only laccase gene sequences. Four sequences were retrieved corresponding to different genes from a single isolate (Wahleithner *et al.*, 1996). Three of the sequences had an ORF close to the 5' end of the sequence and therefore presumably minimal promoter sequence if any. In the fourth sequence (Accession No Z54276) the first ATG occurs at position 266. It is presumably not functional since there is an in-frame stop codon four codons downstream. The second start codon ATG is located at 393, it is preceded by the sequence $^{-5}$ TCTCT $^{-1}$ and is followed by an ORF. This is considered a functional initiation triplet. Therefore the whole 329bp was used as a source of a promoter.

Because the isolate RS22 (AG 6) is not available from the author or Centre for Agriculture and Bioscience in UK, we have selected four different isolates SCR122 (AG 6), RH165 (AG 8), 1342 (AG 4) and ST-11-6 (AG 3) for isolating promoter products by

PCR. The PCR was conducted in section 2.4.5.3 using the touchdown PCR procedure which is designed to prevent spurious priming during PCR (Don *et al.*, 1991).

The primers designed for amplification of the promoter sequence are shown in Fig. 5.1. The forward primer has a site for the restriction enzyme *SacI* at its 5' end whilst the reverse primer has sites for the enzymes *BamHI*, and *NcoI*. The *NcoI* site contains the putative ATG start codon for the laccase.

SacI

GAGCTCGAGTGATCCG CCAGAGTTCA
 GAGTGATCCG CCAGAGTTCA GGCGGATAAG TTCCTAAATA GTCATTTCGCC
 TATTCGTGTA CCTCAGCATA CTGACGACAT ACCGCCAGAT CGCCCTCGGT
 TCGGGCGTGG CATACTGTCG CAAGGGCACC TCACGGAGCA AACTCTAAAA
 AGCTTCGGCA TGGATTGCAT TTTGTATTGT AAACAAGTTA CGAGAAAAAC
 AATAGATCAG TTTTGGCCGA ATCGGATGGC TTGAAACGGA AGTACCGATG
 GCCGATCCGA GTCGAATGAA TTAACGCATC TGAAACGGGA CCCTGAGTCG
 AGGCACCCGC CGGCCTTGGC CGTATAAGTC ACTTGTCGCC AACTAGCACT
 TTTTCATTCC CCCTTTTCTT CTTCCCTCGTC TTCTTCTTCT CTATGGCTCG
AG AAGAAGAAGA GGTACCCCTA
NcoI

LacF prime: 5'GAGCTCGAGTGATCCGCCAGAGTTCA 3'
SacI

LacR prime: 5'GTCGGGATCCCATGGAAGAAGAAGA 3'
BamHI NcoI

Figure 5.1 Laccase promoter primers design. The primer sequences are underlined in red with restriction enzyme sites.

5.3.1.1 Laccase promoter PCR optimisation

To optimise the amplification different initial annealing temperatures were tried. The first annealing temperature was tested at 55-60°C, did not result in a product (Fig.5.2A).

Decreasing the annealing temperature to 50 or 52 degrees resulted in a product (Fig.5.2B). Although all isolates gave amplicons at both temperatures, only one of the four isolates SCR122 gave a strong band. An annealing temperature of 52°C was chosen because there were less bands occurred at this temperature.

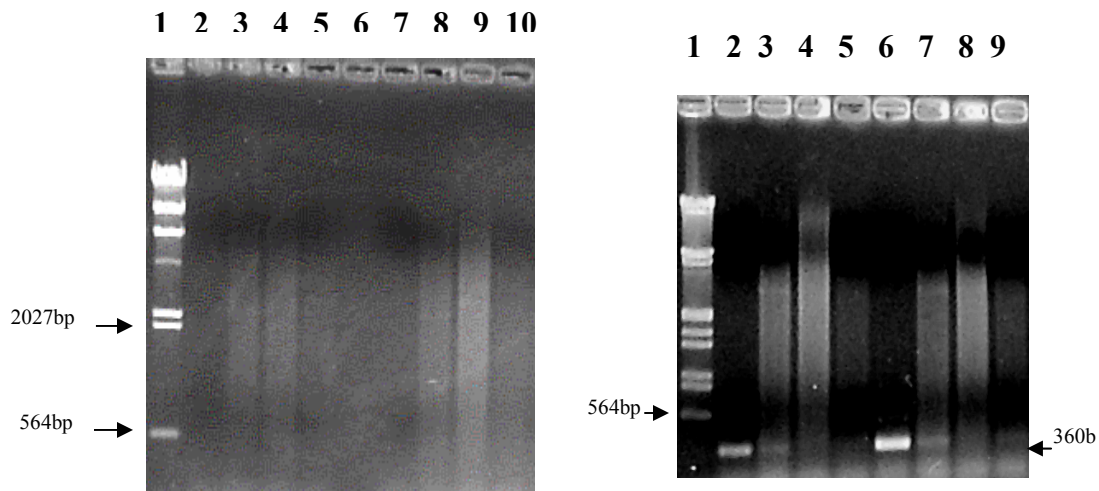


Figure 5.2A. Laccase promoter PCR optimization with different annealing temperature and isolates. Lane 1:Marker, λ /HindIII; Lane 2-5: Annealing temperature 60°C with isolates SCR122, RH165, 1342 and ST116; Lane 6: Blank; Lane 7-10: Annealing temperature 55°C with isolates same as lane 2-5.

Figure 5.2-B Laccase promoter PCR optimization: Lane 1: Marker λ /HindIII+EcoRI; Lane 2-5: Annealing temperature 52°C for four isolates; Lane 6-9: Annealing temperature 50°C.

From Fig 5.2B multiple bands can be seen on the gel. In order to decrease the amplification of non-specific products with primer pairs that produced distinct product, lower than normal primer concentration were tried. Initially 0.2 μ M primers were used, after optimisation 0.1 μ M primers were chosen for routine use.

For $MgCl_2$ optimisation, several concentration of $MgCl_2$ from 1.0, 2.0 and 3.0 mM were tested. From all test results, 2.0 mM $MgCl_2$ achieved the best amplification (Fig.5.3).

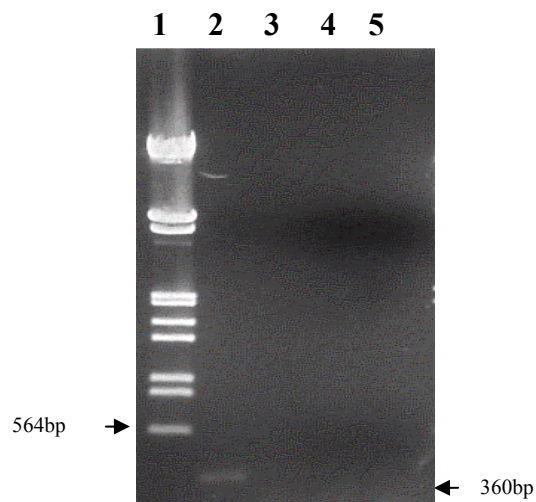


Figure 5.3 Laccase promoter PCR optimization with $MgCl_2$ concentration. Lane 1: Marker λ /HindIII+EcoRI; Lane 2-5: Different isolates: SCR122, RH165, 1342 and ST116.

5.3.1.2 Cloning and sequencing the potential laccase promoter gene

The amplification product from SCR122 was inserted into the pCRII-Topo vector to create the plasmid pTopoLcc and transformed into *E.coli* Top10. Four white colonies were screened by using PCR to confirm the transformants. Screening showed that all four colonies contained a recombinant plasmid with the correct sized insert (Fig. 5.4).

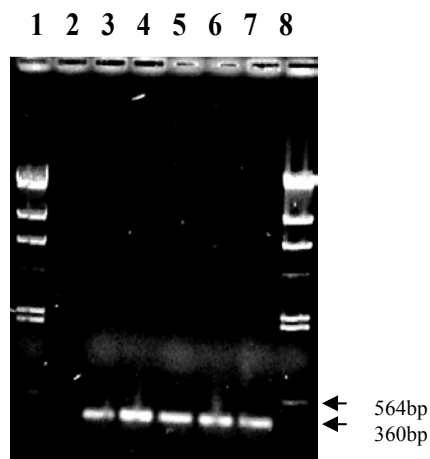


Figure 5.4 PCR analysis for screening the potential promoter gene from *E.coli* Top10 cell. Lane 1 and 8: Marker λ /HindIII; Lane 2: Negative control; Lane 3: Positive control; Lane 4-7: White colony No.1 to No.4 contained the gene

The plasmids from all four colonies were sequenced (Fig 5.5). The sequences were identical according to sequencing data analysis. This sequence was aligned with the sequence from the GenBank database. The results (Fig 5.6) show that the sequences are 91.2% similar over their entire length. The SCR122 PCR product lost some sequence insertions that are present in the Genebank sequence. Also from -108 to the ATG start codon both sequence share 97% homology. Therefore this PCR product was chosen for fusing to the *hph* gene.

```

1      GAGCTCGAGT GATCCGCCAG AGTTCAGGCG GATAAGTTCC TGAATAGTCT
51     GTTCGTGTAC CTCATACTGA CACATACGCC CTCGGTTCGG GCGTGGCATC
101    GCAAGGGCAC CTCAGAGCAA ACTGTAAAAA GCTTCATTGC ATTTAGTATT
151    GTAAACAAGT TACGAACAAT AGATCAGTTT TTGCCGAATC GGATGGCTTG
201    AAACGGAAGT ACCGAGCCGA TCCGAGTCGA ATGAATTCTG AAACGGGACC
251    CTGAGTCGAG GACCCGCCGG CCTTGGCCGT ATAAGTCACT TGTCGCCAAC
301    TAGCACTTTA TCATTCCCC TTTTCTTCTT CCTCGTCTTC TTCTTCTCCA
351    TGGGGATCCC GAC

```

Figure 5.5 Sequence of the cloned insert

```

Query: 71  cgccctcgggttcgggcgtggcat-----cgcaagggcacctca--gagcaaaactgtaaaa 123
          |||
Sbjct: 91  cgccctcgggttcgggcgtggcatacgttcgcaagggcacctcacggagcaaaactctaaaa 150

Query: 124 agcttc-----attgcatttagtattgtaaacaagttacga-----acaatagatcag 171
          |||
Sbjct: 151 agcttcggcatggattgcattttgtattgtaaacaagttacgagaaaaacaatagatcag 210

Query: 172 tttttgccgaatcggatggccttgaacggaagtaccga--gccgatccgagtcgaatgaa 229
          |||
Sbjct: 211 tttttgccgaatcggatggccttgaacggaagtaccgatggccgatccgagtcgaatgaa 270

Query: 230 tt-----ctgaaacgggaccctgagtcgagg-accgcggccttgccgtataagtc 281
          ||
Sbjct: 271 ttaacgcatctgaaacgggaccctgagtcgaggaccgcggccttgccgtataagtc 330

Query: 282 acttgtcgccaactagcacttttattcattcccccttttcttcttctcctcgtcttcttcttct 341
          |||
Sbjct: 331 acttgtcgccaactagcacttttattcattcccccttttcttcttctcctcgtcttcttcttct 390

Query: 342 ccatgg 347
          | |||
Sbjct: 391 ctatgg 396

```

Figure 5.6 Alignment of the SCR122 laccase amplicon (top) with the Genebank laccase sequence (Z54276) from *R. solani* RS22.

5.3.2 Construction of the hygromycin resistance gene

The source of the *hph* coding region was from the plasmid pCAMBIA1300. This coding region has been optimised for expression in plants which may make it more effective in fungi. In addition, this has removed the *Nco*I site from within the coding region making it easier for cloning. The *hph* coding region was amplified with the primer pair HpF/HpR (Fig5.7).

Hygromycin resistant gene forward primer HpF:

GAGCCATGGAAAAGCCTGAACTCACC
*Nco*I

Hygromycin resistant gene forward primer HpR:

TTAACGAAGCTTTAATTCGGGGGA
*Hind*III

Figure 5.7 Primers for amplification of the *hph* gene from pCAMBIA1300. The ATG in the *Nco*I site is the initiation codon for the *hph* gene.

The forward primer termed HpF was designed to contain the *hph* start codon region and contains an *Nco*I restriction enzyme site at the start codon. The reverse primer has a *Hind*III restriction site for cloning into pBIN19. The amplicon contains both the *hph* coding region and the terminator.

Amplicons were obtained at all annealing temperatures from 50-55°C (Fig.5.8) and annealing temperature at 52°C resulted in a better results. The both *hph* and laccase amplicons were digested with *Nco*I/*Hind*III and *Sac*I/*Nco*I respectively, and then ligated together and cloned into pBIN19 to create pBINlcc. After transformation into *E. coli*, the recombinant plasmid was extracted and the sequence of the recombinant gene confirmed by DNA sequencing. With ANGIS Mapping program the protein sequence from the ligation DNA is very close to hygromycin B transferase except the second amino acid is Glu instead of Lys.

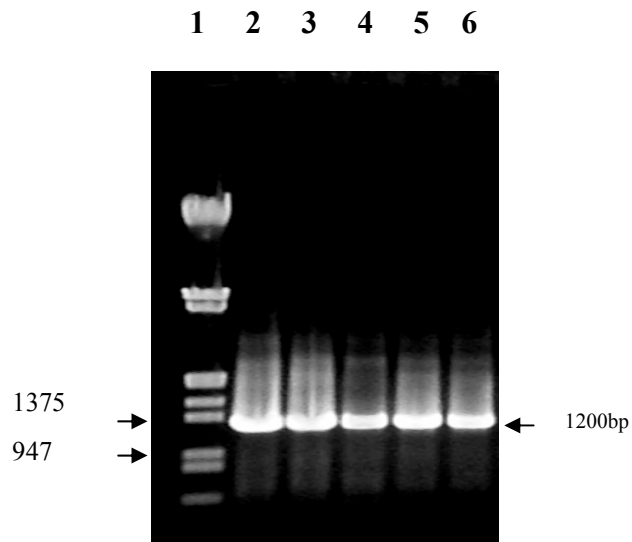


Figure 5.8 Effect of annealing temperature on amplification of the hph gene: Lane 1: Marker λ /HindIII+EcoRI; Lanes 2-6: annealing temperatures of 50, 51, 52, 54, 55°C respectively.

5.3.3 Transformation of *R. solani* SCR122 and RH165 with pBINlcc

The novel construct pBINlcc was transferred into *A. tumefaciens* AGL1 and AGL0 by conjugation as described in section 2.4. The presence of the plasmid was confirmed by screening *A. tumefaciens* cells with the hphF/hphR primer pair. All colonies tested were positive for the recombinant plasmid except AGL0 number 3 (Fig 5.9).

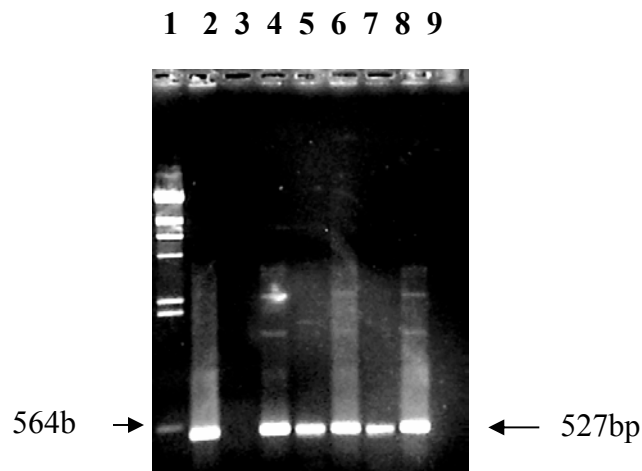


Figure 5.9 Screening *Agrobacterium* colonies for the presence of the *hph* gene. Colonies were screened for the presence of the gene using the *hphF/hphR* primer pair. Lane 1: Marker λ /HindIII; Lane 2: Positive control; Lane 3: Negative control; Lane 4-6: *A.tumefaciens* AGL1 colonies No.1, 2 and 3; Lane 7-9: AGL0 colonies No.1, 2 and 3.

Transformation of *R. solani* isolate SCR122 and RH165 was conducted by mycelium and protoplast. When prepared protoplast, take 0 hour enzyme reaction time as a control. 2 and 3 hour protoplasts were co-cultivated with *A. tumefaciens* AGL1 containing pBINlcc for two or three days on induction media. The mycelium was transferred to selection medium containing hygromycin at 50 μ g/ml. No resistant colonies were obtained. The isolates 1342 and ST-11-6 were also used as recipients, but without success.

5.4 Discussion

It is the first time to use a novel vector with *R. solani* laccase promoter to drive hygromycin resistant gene for *R. solani* transformation. Initially, we asked for the *R. solani* isolate RS22 from Mycelium Culture Centre in UK as this was the isolate from which the Genebank sequences were derived (Wahleithner *et al.*, 1996). However, this isolate was not available. Hence we used four the isolates from our fungal collection. The potential promoter was successfully isolated from one of the isolates named SCR122. Because isolate RS22 and our isolate SCR122 are all same in anastomosis

group 6 (AG 6), and are more likely to be similar in genomic background. From the promoter sequence analysis, the novel promoter obtained by PCR shared 91% homology with laccase promoter from GenBank (Wahleithner *et al.*, 1996). By an analysis of the sequence shows that there are conserved sequences- TATA box and GC box located around the -35 region (Appendix 1). These sequences are involved in the activation of transformation. In addition, it is reported that CT-rich stretch at and near the transcriptional initial area involved in a promoter function in some highly expressed genes of filamentous fungi (Noel *et al.*, 1995) In our promoter sequence, it was found that there are 8 CT repeats between -30 to -2 position.

Although the potential promoter shared 91% homology with the database laccase promoter from RS22, there are nearly 50 base pairs present in the RS22 sequence that are not present in the SCR122 original sequence. It is also noted that in the gene construct, there is an adenine base pair replaced by guanine at +4 site after start codon ATG, therefore, the second amino acid was changed from Lys to Glu, in this case it could affect enzyme activity.

It has been demonstrated that laccase production in *R. solani* is inducible (Bora, pers. comm.). Wahleithner *et al.*, (1996) reported that *R. solani* produced four laccases, two of which were produced at a low constitutive level whilst the remaining two were produced in response to the addition of inducers, eg., anisidine. The laccase promoter sequence used in these experiments may be appropriate for *R. solani* since it is derived from *R. solani*. However, we don't know the level of expression from the gene since that has not been measured. Analysis of promoter function would require measurement of RNA levels using techniques such as reverse transcriptase PCR (Egea *et al.*, 1999) or RNAase protection (Sambrook *et al.*, 1989).

Although gene expression depends on having a functional promoter, it is also affected by processes such as mRNA stability. Some species RNA are degraded rapidly whereas others are relatively stable. The factors that affect mRNA stability are not fully understood. Changing the promoter of a gene may decrease mRNA stability, and the effect may differ in different organisms. The hygromycin resistance gene has been used

for transformation of a wide range of fungal species (Ruiz-Diez, 2002). However, Sholtmeijer (2001) reported that the *hph* gene was not efficiently expressed in basidiomycetes due to instability of the mRNA. They found that the transformation efficiency could be improved by changing a number of the AT base pairs in the coding region to GC base pairs, and adding two artificial introns to the transcript. Apparently the presence of the introns stabilises the mRNA.

Chapter 6 Transformation of *Ustilago* and *Rhizoctonia* with a modified hygromycin Gene

6.1 Introduction

Almost all the genes of higher eukaryotes are interrupted by intervening sequences called introns. The biological role of introns has been questioned since their discovery. Recent data have implicated introns in qualitative changes in gene expression by mechanisms of alternative or *trans*-splicing (Chabot, 1996). Evidence that introns can also have quantitative effects on gene expression has been obtained since 1979, for example for the β -globin gene (Buchman and Berg 1988). Other evidence has been reported for a number of genes from higher eukaryotes whose introns contain transcriptional enhancer elements (Chung and Perry 1989; Xu *et al.* 1994 and Scholtmeijer *et al.* 2001).

Studies with transformed strains of *Schizophyllum commune* have demonstrated that introns are important for expression of transgenes (Lugones *et al.*, 1999). These authors found that the insertion of artificial introns in the 5' UTR or the 3' UTR were required for expression. Even better results were obtained when introns were placed in both regions simultaneously. Apparently, neither the nature nor the position of the intron is decisive in enhancing messenger steady-state levels. For the *SC6* mRNA, it was shown that the presence of introns only affected the accumulation of mRNA and not the rate of transcription. It has been suggested that for genes that lack a good polyA sequence, processing of the 3' end of the mRNA leads to degradation of the mRNA (Lugones *et al.*, 1999). Introns assist in stabilizing the mRNA leading to higher levels of expression. In contrast to ascomycetes whose genes contain an identifiable polyA sequence, those basidiomycete genes that have been studied lack such a sequence. It is believed that introns are a general feature of basidiomycete genes to counteract the lack of a polyA sequence and stabilize the mRNA Scholtmeijer *et al* (2001).

Scholtmeijer *et al* (2001) described the construction of a vector for the basidiomycete *Schizophyllum commune*. The vector contains the selectable marker *hph* gene but with

artificial introns inserted into the 5' and 3' UTR's. The *hph* coding region was further modified by changing a number of AT bp to CT bp at the 5' end of the coding sequence. It was established that these changes significantly elevated the level of expression relative to the unmodified gene. The unmodified gene was ineffective in selecting transformants, whereas transformants could be selected with the modified gene. In this chapter we describe experiments in which the modified gene was transferred to a plant binary vector and used for transformation of *Rhizoctonia solani*. As a positive control we included *Ustilago cynodontis* in the transformation experiments.

6.2 Materials and Methods

6.2.1 Construction of the vector pJW2.2hyg

The vector pHYM2.2 (Fig 6.1) was kindly donated by Dr K. Scholtmeijer.

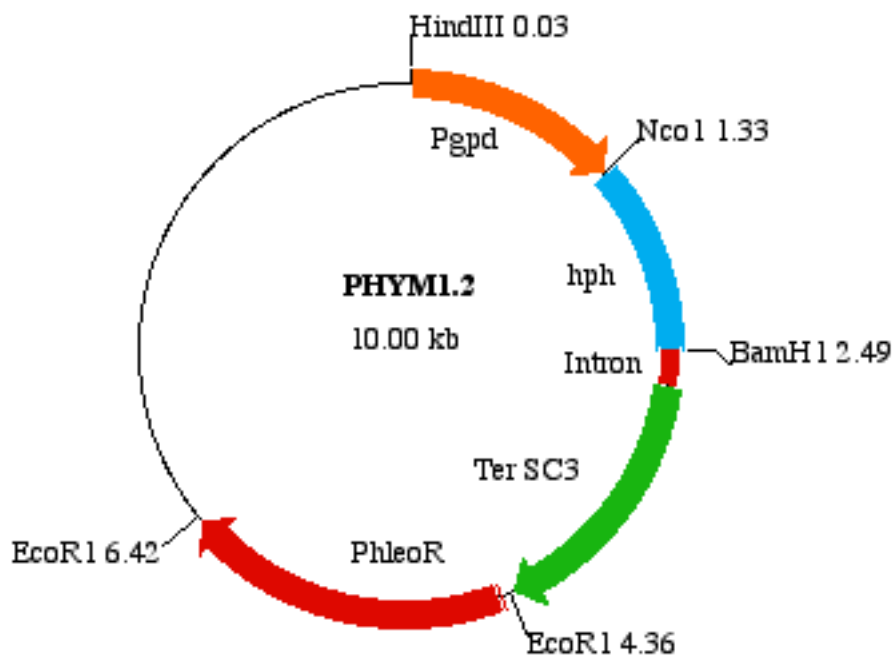


Fig 6.1. The vector pHYM2.2

The pJW2.2hyg vector was constructed with the backbone of the binary vector pBIN19 (Bevan *et al.*, 1984). The plasmid pHYM2.2 was digested with *HindIII* and *EcoRI*. The

3kb fragment contained the *hph* gene with the *gpd* promoter and *SC3* terminator from *Schizophyllum commune* which belongs to a basidiomycetes. This whole gene was extracted from an agarose gel (section 2.4.6.3) and inserted into *HindIII/EcoRI* digested pBin19 to create the binary vector pJW2.2hyg.

This vector was transformed into *E.coli* JM83 (section 2.4.3) and then introduced into *A. tumefaciens* AGL1 by conjugation (section 2.4.4). The vector was confirmed in *A. tumefaciens* by extracting the vector from the LB cell culture with QiaGen kit (section 2.4.2) and digesting it with *HindIII* and *EcoRI*

6.2.2 Transformation of *Ustilago cynodontis*

6.2.2.1. Preparation of *U. cynodontis* cells.

The *U. cynodontis* isolate-28040 was cultured in PD broth at 25⁰C for three days. 1 ml was diluted into 20 ml of PD or V8 medium in 100 ml flask and incubated at 25⁰C with shaking at 150 rpm for 24-48 hours. The cells were harvested by centrifuging at 5000 rpm for 5 min, washed twice with induction medium and then suspended in 5 ml induction medium. The cell concentration was determined by counting with a haemocytometer.

6.2.2.2. *Agrobacterium* mediated transformation of *U. cynodontis*

A. tumefaciens AGL1 containing pJW2.2hyg was grown at 28°C for 24 hours in minimal medium (MM) with kanamycin (50 µg/ml)(Hooykass, 1988). 1 ml was transferred into 5 ml fresh induction medium broth (IMB) (Bundock *et. al.*, 1995) with and without acetosyringone (AS 200 µM) and incubated until the cell concentration reached about 10⁸ cfu/ml. Equal volumes of bacteria and *U. cynodontis* suspension were mixed and poured onto induction medium agar (IMA) with or without AS. The plates were incubated at 26°C for 1-3 days. After co-cultivation the cells were transferred to selective medium (SM) containing hygromycin (50 µg/ml) and timentin (150 µg/ml) and the plates

incubated at 26 °C for 3-5 days until the resistant colonies grew. Individual resistant colonies were purified and sub-cultured on selective agar for further testing.

6.2.3 Agrobacterium mediated transformation of *R. solani*.

6.2.3.1 Preparation of protoplasts and mycelium.

Transformation of *R. solani* mycelium and protoplasts was conducted as described in chapter 4.

6.2.3.2 Stability of the transformants

The transformants were conducted with stability test by transferring the transformants from selection medium to a PDA plate. Once the colony had grown to the edge of the plate on PDA plates at 26⁰C for 4-5 days, the plugs of mycelium from the growing edge were transferred to fresh PDA and selection medium respectively. This procedure was repeated five times.

6.3 Results

6.3.1 Construction of a binary vector with the modified *hph* gene.

It can be seen that *Hind*III and *Eco*RI digestion of pHYM2.2 can produce three fragments (Fig.6.2). The three fragments represented *hph* resistant gene fragment, *phleo*^R gene fragment and the *amp*^R fragment. The three fragments sizes were 3, 2.2 and 1.2 kb respectively (Fig.6.2). The 3kb fragment was ligated to pBin19 to form pJW2.2hyg and transformants of *E. coli* colonies were screened by PCR with M13 primers. The result was seen in Fig 6.3. Four transformants were tested by PCR, three of them were showed right insert. Therefore, they were used as donor strains.

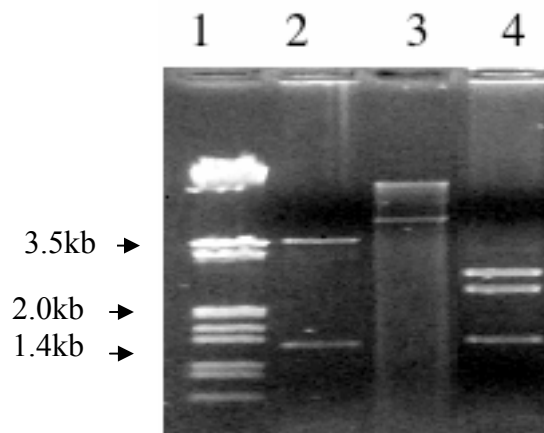


Figure 6.2. Restriction analysis of pHYM2.2. Lane 1: Marker λ *HindIII/EcoR1*; Lane 2: pHYM2.2 *EcoR1*; Lane 3: pBIN19 *EcoR1*; Lane 4: pHYM2.2 *HindIII/EcoR1*.

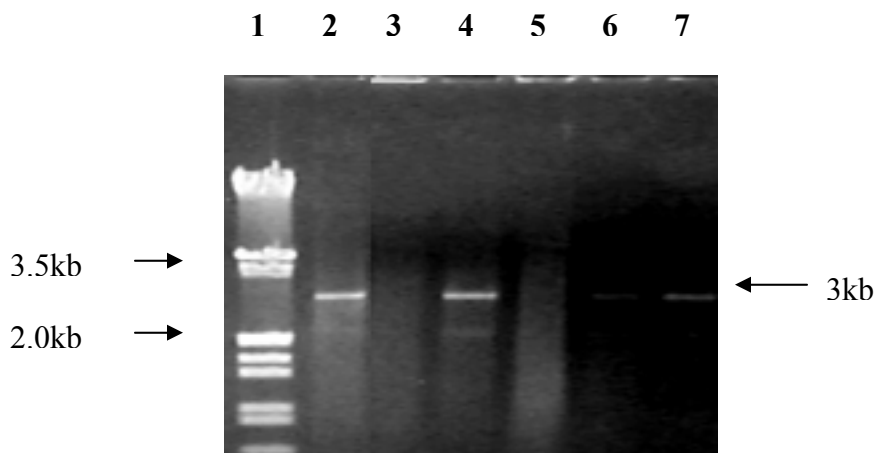


Figure 6.3. Screening *E. coli* colonies for the presence of pJW2.2 plasmid. Colonies were screened by amplification with M13 primers. Lane 1: λ DNA *HindIII/EcoR1* markers; Lane 2: positive control; Lane 3: Negative control; Lanes 4-7: *E. coli* transformants. The position of the correct 3kb band is indicated.

The pJW2.2hyg vector was mobilized into *A. tumefaciens* AGL1 by conjugation. Exconjugant *A. tumefaciens* colonies could be selected on minimal medium with kanamycin. Neither the donor nor recipient parents can grow on this medium. Colonies

were analyzed by PCR with the M13 and the *hph* primers to confirm the presence of pJW2.2hyg. For M13 PCR there were three colonies No.3, 4, and 5 showed the expected 3kb band, but the other two did not show any band (Fig 6.4). While for *hph* PCR four of the five colonies showed the right band 527bp, but only No.3 did not show any product on the gel (Fig 6.5). Interestingly colony No 3 which gave a positive result with the M13 primers, gave a negative result with the *hph* primers. The possibility that this may be a false negative was not further investigated in view of the fact that we have colonies showing the desired result.

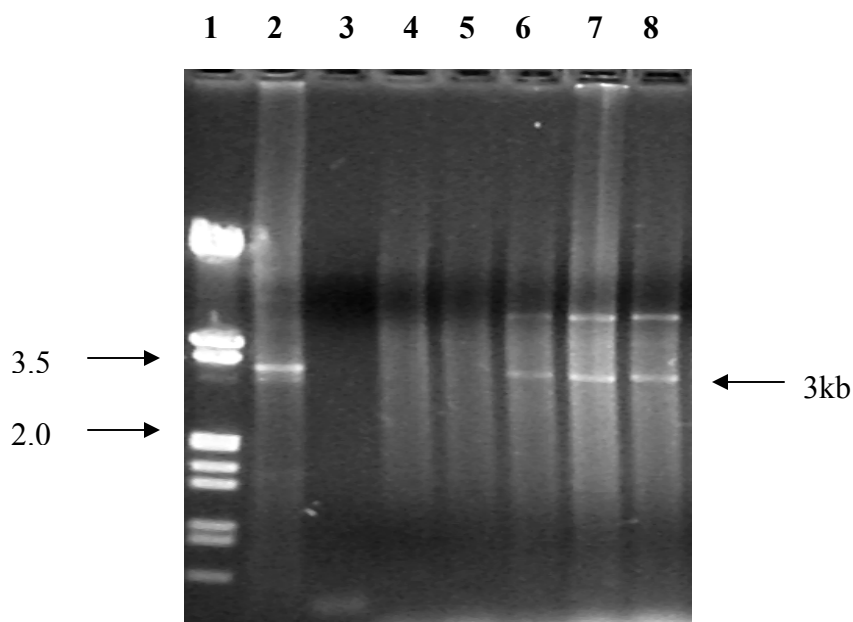


Figure 6.4. PCR analysis of exconjugant *A tumefaciens* colonies with M13 primers. Lane 1: Marker λ /*HindIII*+*EcoRI*; Lane 2: Positive control; Lane 3: Negative control; Lane 4-8: AGL1 colonies No.1-No.5. Position of the 3kb insert is indicated.

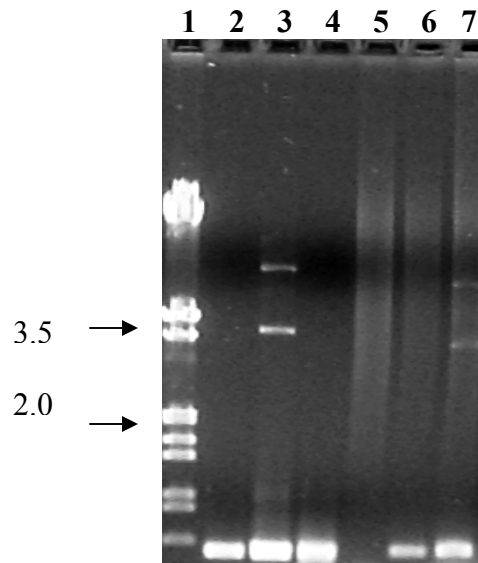


Figure 6.5 PCR analysis of exconjugant *A tumefaciens* colonies with *hph* primers
Lane 1: Marker λ /*Hind*III+*Eco*RI; Lane 2: Positive control; Lane 3-7: AGL1 colonies No.1-No.5

The presence of the plasmid was further confirmed by digestion of plasmid extracted from the AGL1 colonies with *Eco*R1 and *Hind*III (Fig 6.6). It can be seen that AGL1 No.5 contains the pJW2.2hyg with right insert (3kb of *hph* gene plus introns) in pBIN19. It is also interesting to find that AGL1 No.4 not only contains the right insert, but also it contains phleomycin resistant gene which is about 1.2 kb. Therefore this vector was named pJW2.2phl.

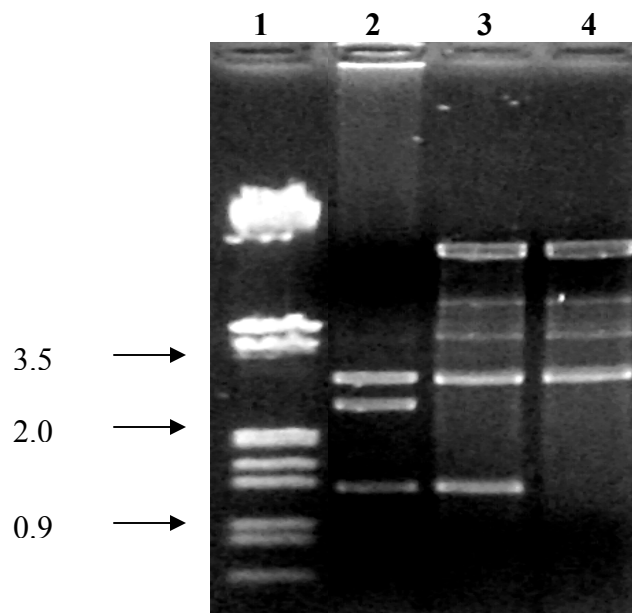


Figure 6.6 Restriction analysis of plasmid DNA extracted from exconjugant colonies of *A. tumefaciens*. The plasmid was extracted from colonies and digested with *Hind*III and *Eco*RI. Lane 1: Marker λ /*Hind*III+*Eco*RI; Lane 2: pHYM2.2/*Hind*III+*Eco*RI; Lane 3-4: AGL1 No.4 and No.5 plasmids cut with *Hind*III and *Eco*RI.

6.3.2 Transformation of *Ustilago cynodontis*.

Initially the sensitivity of *U. cynodontis* to hygromycin was tested. A culture was diluted to 100 cells per ml and aliquots spread onto PDA plates with 0, 30 or 50 μ g hygromycin per ml. *U. cynodontis* 28040 can grow very slowly on PDA with hygromycin B at 30 μ g/ml, but its growth was clearly inhibited by hygromycin B at a concentration of 50 μ g/ml (Fig 6.7). Therefore, in the following transformation test, hygromycin B was used for selection of transformants at a concentration of 50 μ g/ml. The efficiency with which different vectors transformed *U. cynodontis* was compared. The results show that the vector pJW2.2hyg was more efficient than pBINHL1, whilst no transformants were obtained with pBINAN or pBINlcc (Table 6.1).

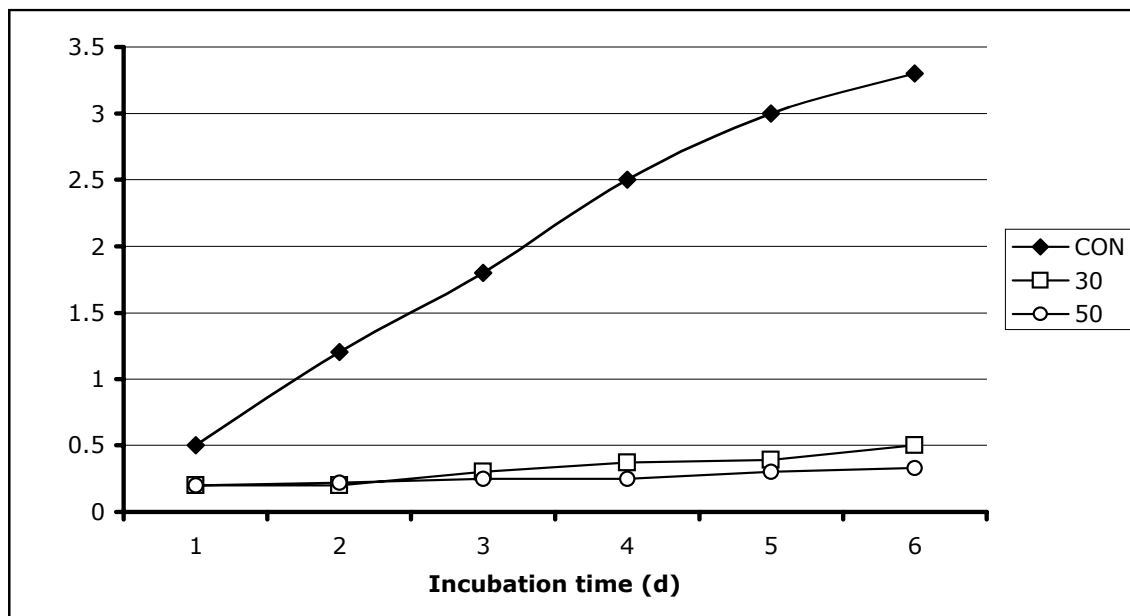


Figure 6.7 Growth rate of *Ustilago cynodontis* on PDA with different concentration of hygromycin B.

Table 6.1 Comparison of vectors for transformation of *U.cynodontis*

Vectors	No. of transformants/ 10^6 recipient cells.
pJW2.2hyg	5200
pBINHL	1000
pBINAN	0
pBINlcc	0

The growth response of the transformants on selective agar is shown in Fig 6.8. The transformants were capable of growth on this medium whilst the untransformed control could not grow.

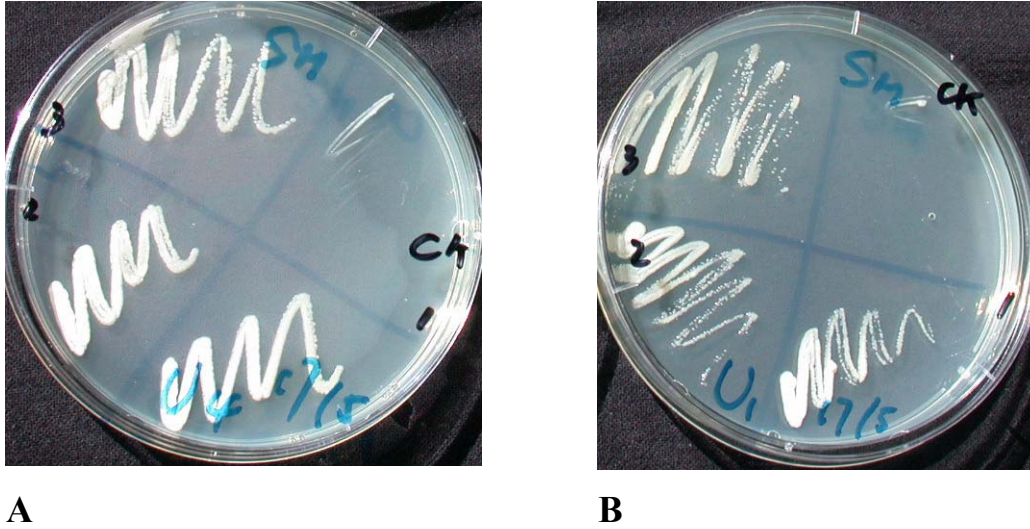


Figure 6.8 Growth of *U. cynodontis* transformants on selection medium.
(A): pJW2.2hyg transformants U4-1, U4-2 and U4-3, Labeled CK indicated non-transformant.
(B): pBINHL1 transformants U1-1, U1-2 and U1-3. The blank sector on each plate contains the untransformed control.

6.3.2.1 Analysis of transformants by PCR

The transformants were analyzed by PCR with the *hph* and M13 primers to confirm the presence of the transgene. All transformants except the U1-3 contained the expected *hph* gene fragment (Fig.6.9). However, with the M13 primers, only three of the six transformants gave a positive result.

The experiment shows that the pJW2.2hyg vector is an alternative vector to pBINHL for transformation of basidiomycetes.

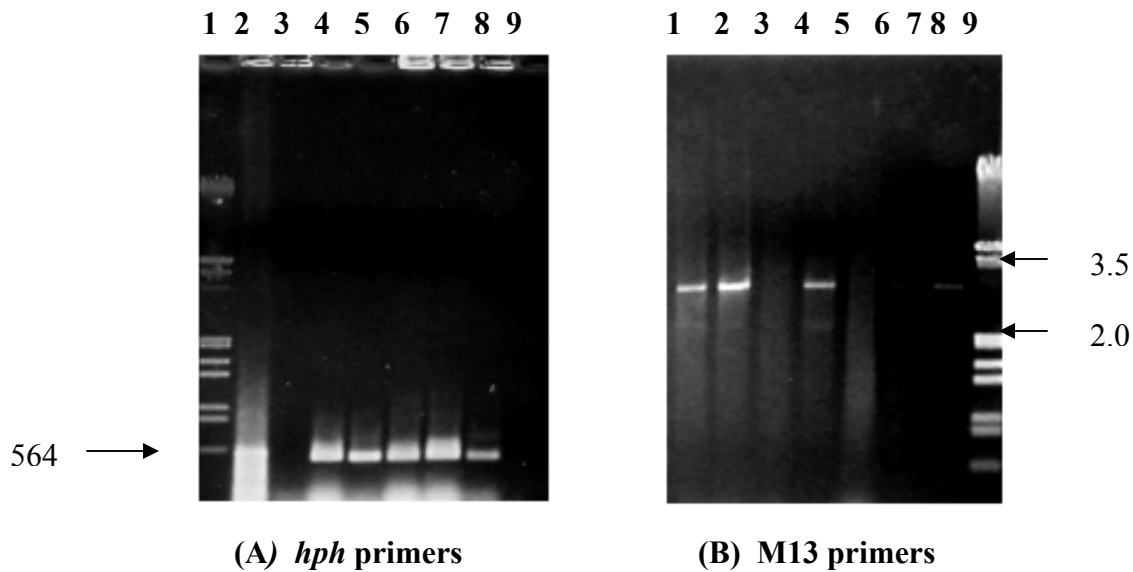


Figure 6.9A. PCR analysis of *U cynodontis* transformants; Lanes 4-6: Putative transformants U4-1,U4-2 and U4-3; Lane 7-9: Putative transformants U1-1, U1-2 and U1-3.

Figure 6.9B. Lane 9:Marker (λ /HindIII); Lane 8: positive control (faint line); Lane 7: Negative control(non-transformant); Lanes 1-3: putative transformants U4-1, U4-2 and U4-3; Lanes 4-6: putative transformants U1-1, U1-2 and U1-3.

6.3.3 Transformation of *R. solani*

For transformation of *R. solani* we used both mycelium and protoplasts generated after digestion of mycelium with Novozyme 234 for either two or three hours. In plant transformation systems different varieties within a plant species show significant differences in their susceptibility to transformation by *A. tumefaciens* (Gelvin, 2000). This is due to differences in chromatin structure. With this in mind we tried as recipients a number of isolates from different AG.

Except in the case of SCR122, treatment of mycelium with Novozyme 234 did not increase the number of resistant colonies observed (Table 6.2). Isolate 1342 appeared to be the most susceptible to transformation generating the highest number of

resistant colonies. RH165 was also susceptible, whilst SCR122 gave a lower number of resistant colonies. The isolate ST-11-6 gave intermediate levels.

The resistant colonies were transferred to fresh selective plates. As in previous experiments, most of the colonies grew for a short time and then ceased growing. However, the six transformants from SCR122 did continue to grow and four appeared to be stably transformed and are capable of giving rise to large colonies on selective agar Fig 6.10B.

Table 6.2 The number of resistance colonies obtained from *Agrobacterium* transformation of *R.solani*

Isolate	Protoplast treatment hr	Transformants /10 ⁶ protoplasts		
		No vector	pBINHL	pJW2.2hyg
1342	0	0	320	60
	2	0	na	na
	3	na	50	na
RH165	0	0	5	51
	2	0	30	20
	3	0	17	9
SCR122	0	0	6	6
	2	na	na	na
	3	0	34	9
ST-11-6	0	0	20	30
	2	0	0	0
	3	0	0	0

6.3.3.1 Growth of transformants on selective agar

The growth rate of the transformants on different concentrations of hygromycin plates was assessed by measuring colony diameter on the plates. At lower concentrations of hygromycin, 25 and 50µg/ml five of the transformants grew more rapidly than the control (Fig 6.10). Only transformant Tr6 appeared to be similar to the non-transformed control. The control continued to grow albeit at a much slower rate. The growth rate of the

transformants was considerably slower than on non-selective agar, so that although they are supposedly transformed with a resistance gene, they are affected negatively by the hygromycin. As the concentration of hygromycin increased, the differences between the transformants and the control were less obvious until at a concentration of 100 $\mu\text{g/ml}$, there was no significant difference between the transformants and the control (Fig.6.10). A photograph of one of the transformants growing on hygromycin agar is shown in Fig 6.11.

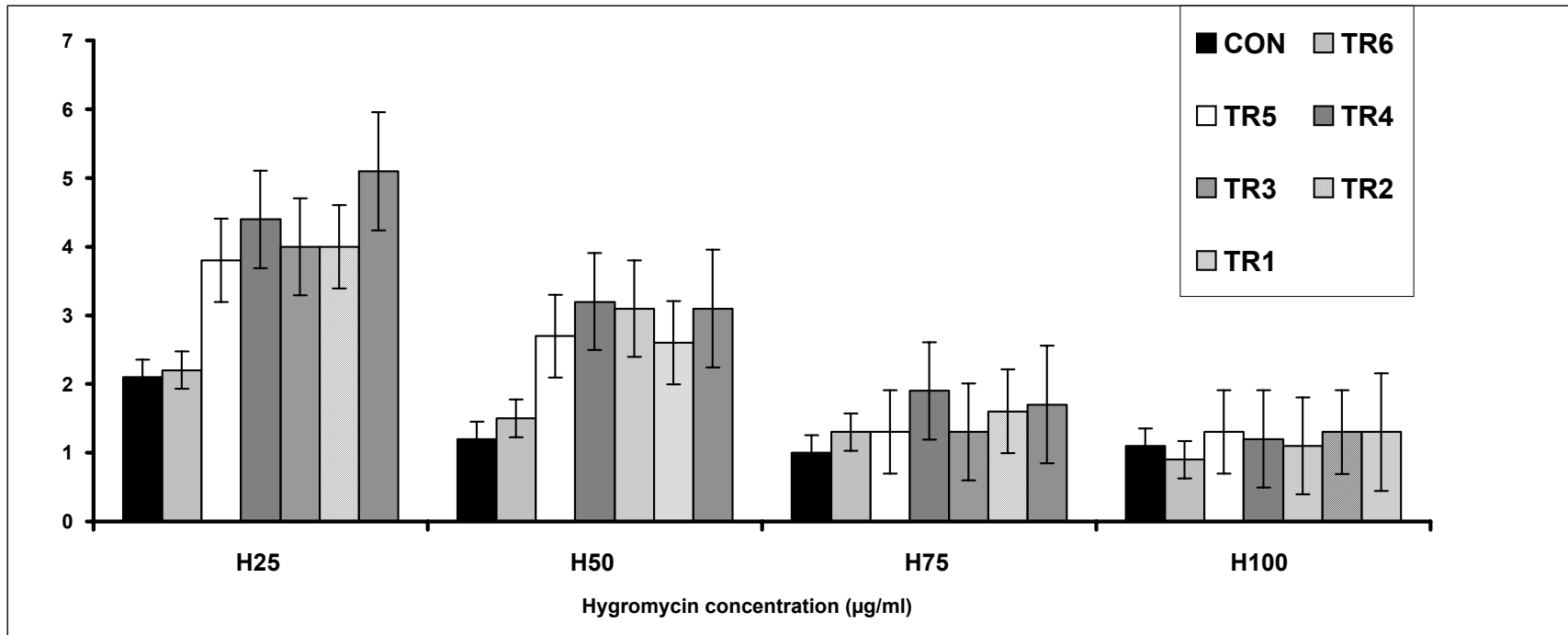


Figure 6.10. Growth of SCR122 transformants on PDA agar with different concentrations of hygromycin. The radial growth of colonies was measured at 11d incubation. Four replicates were measured for each isolate. Bars indicate std error.

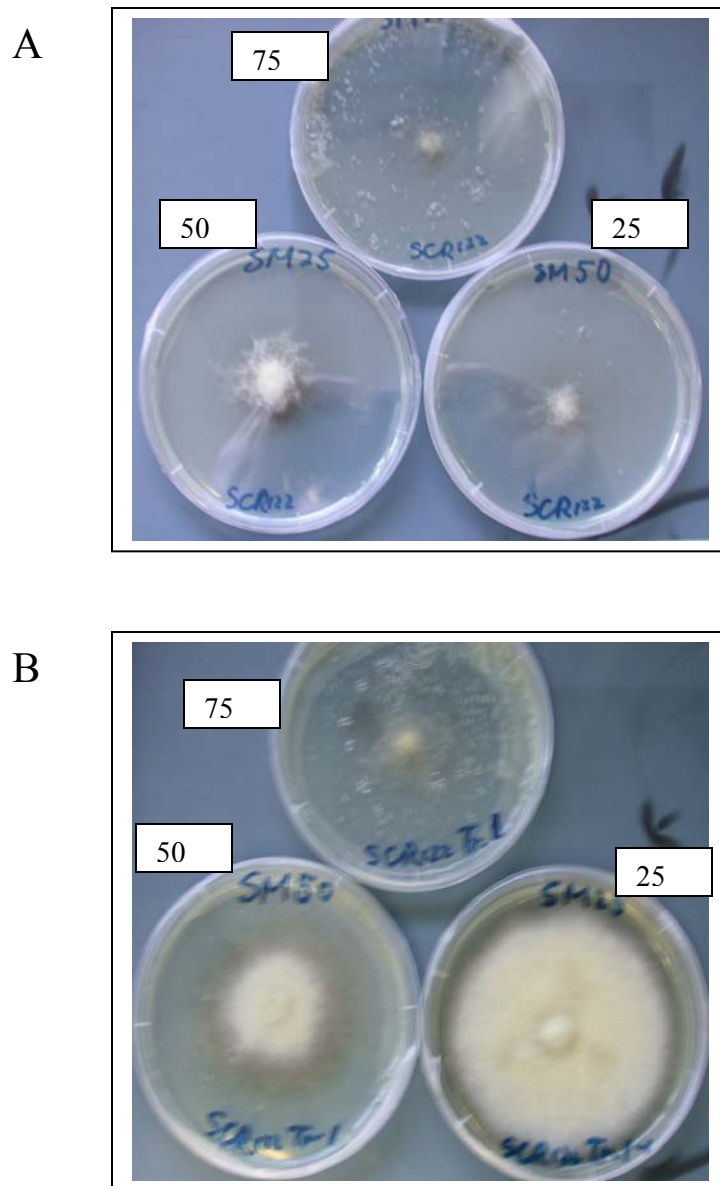


Figure 6.11 Growth of transformant Tr1 on hygromycin agar. (A) untransformed, (B) Transformant Tr1. The numbers beside the plates indicate the concentration of hygromycin in the agar (the top plate with hygromycin 75 $\mu\text{g/ml}$. (photograph of colony).

6.3.3.2 Analysis of transformants by PCR.

The six transformants were analyzed by PCR with both the M13 and *hph* primers to check whether they contain the *hph* gene. The result was shown in Fig.6.12.

Transformants Tr1, Tr2, Tr5 and Tr6 contain the *hph* gene, but transformants Tr3 and Tr4 did not produce the expected band. Only three of the transformants (2, 5 and 6) showed the expected band when the M13 primers were used (Fig 6.12B).

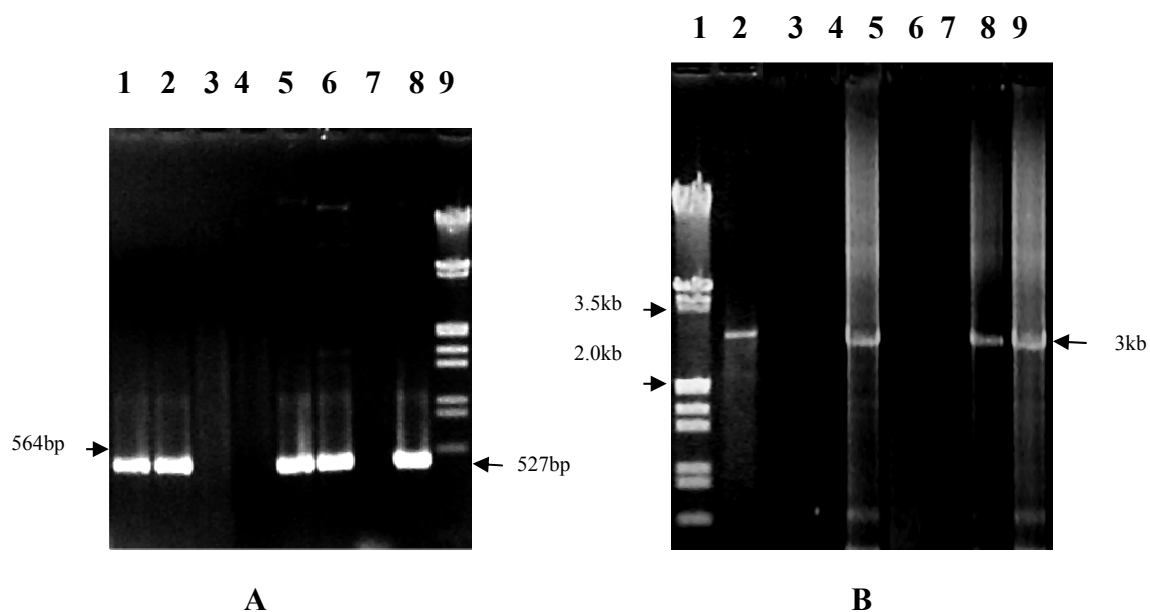


Figure 6.12. PCR analysis of transformants.

(A). Amplification of transformant DNA with *hph* primers. Lane 1: Marker (*HindIII*+*EcoRI*); Lane 2: positive control; Lane 3: negative control; Lane 4-9: transformants Tr1 to Tr6.

(B). Amplification of transformant DNA with M13 primers. Lane 1: Marker (*HindIII*+*EcoRI*); Lane 2: positive control; Lane 3: negative control; Lane 4-9: transformants Tr1 to Tr6.

To exclude the possibility that the PCR products observed were due to carryover of contaminating *A. tumefaciens* cells, we tested the DNA extracted from the transformants with primers for the *vir* region of the Ti plasmid. The *vir* region is not transferred to the recipient during transformation (Gelvin, 2000). None of the transformants showed a product with the *vir* primers. PCR is notorious for its sensitivity to components that

extract with DNA from tissue, especially tissue high in polysaccharides such as plant and fungal tissue. There is the possibility that the negative results obtained with some of the primers, ie., the *vir* primers and M13 primers may be due to inhibition of PCR. To exclude this possibility we tested all DNA extracts with primers for the rRNA-ITS region. All extracts gave a positive result

6.3.3.3 Analysis of transformants by Southern hybridization

Independent transformants, ie. resulting from independent integration events will contain the transgene at different sites. This is the expectation from studies on plant transformation which have shown that the site of T-DNA insertion is random (Gelvin, 2000). To determine whether the transformants are independent, the transformants were analysed by Southern hybridisation using the amplicon obtained with the *hph* primer pair as the probe. This showed the presence of the transgene in all transformants (Fig. 6.13). Transformant Tr1 and Tr6 were characterized by a high molecular weight band A. Transformants Tr2 and Tr5 were characterized by a low molecular weight band B. However, band A was very prominent in Tr6 and Tr1 but very faint in T2 and T5, whilst band B was more prominent in T2. These indicated they are different transformants.

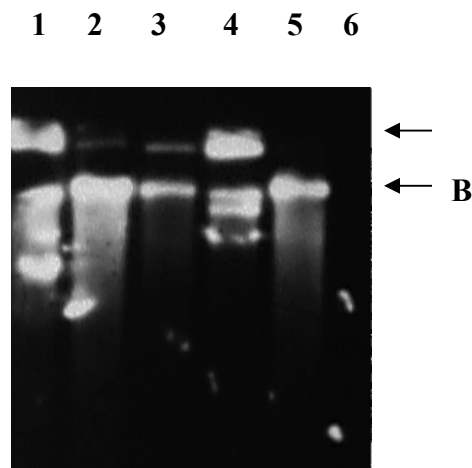


Fig 6.13 Southern blot analysis of transformants. The genomic DNA was cut with *Hind*III. The digest was electrophoresed on a 0.8% agarose gel, and a blot of the gel probed with ³²P labeled *hph* amplicon. Lane 1-4: Transformants 1. 2. 5 and 6; Lane 5: Positive control,pJW2.2hyg; Lane 6: negative control: non-transformant.

6.3.3.4 Stability of the transformed phenotype.

The six transformants were tested for stability of the hygromycin B resistant phenotype by growing the isolates in the absence of selection. The results were found in Table 6.3. Transformants Tr3 and Tr6 lost their stability after 2 and 3 transfers respectively whilst the rest of the transformants were stable.

Table 6.3 Stability test results of the six transformants

Generation	Tr-1	Tr-2	Tr-3	Tr-4	Tr-5	Tr-6
1	Stable	Stable	Stable	Stable	Stable	Stable
2	Stable	Stable	No	Stable	Stable	Stable
3	Stable	Stable		Stable	Stable	No
4	Stable	Stable		Stable	Stable	
5	Stable	Stable		Stable	Stable	

6.4 Discussion

In these experiments a vector containing a modified hygromycin resistance gene was used for transformation of *R. solani*. The initial experiment was to confirm that the binary vector pJW2.2hyg was functional by transforming it into *U. cynodontis*. In this transformation system it proved to be slightly more efficient than the pBINHL1 vector.

The hygromycin resistance gene in pJW2.2 hyg has introns inserted both upstream and downstream of the *hph* coding region (Scholtmeijer *et. al.*, 2001). Previous experiments with *S. commune* have shown that these introns are required for efficient expression of the *hph* gene in *S. commune* (Scholtmeijer *et. al.*, 2001). Versions of the gene containing only a single intron in either the 5' or the 3' position gave lower levels of expression than the version with introns in both locations resulting in a partially resistant phenotype. It was shown previously that introns were necessary for efficient accumulation of mRNA of *SC3*, *SC6*, *ABH1* and *GFP* in *S. commune* (Lugones *et. al.*, 1999). However, whilst introns are necessary for expression of the *hph* gene in *S. commune*, they are not required for expression in *U. cynodonti* (this study), *U. maydis*

(Wang *et al.*, 1988), or *U. violaceae* (Bej and Perlin, 1989). Moreover, prokaryotic genes eg., kanamycin resistance have previously been expressed in basidiomycetes without requiring modifications such as the insertion of introns (Randall *et al.*, 1991).

It has previously been shown that the occurrence of AT-rich stretches at the 5' end of the coding region of various prokaryotic genes (β -glucuronidase, β -galactosidase, *hph*), causes truncation of mRNA in these stretches when the genes are introduced into eukaryotic organisms (Schuren and Wessels 1994; 1998). In phleomycin resistant transformants of *S. commune*, expression of a linked hygromycin resistance gene was only observed after the number of AT base pairs in the 5' region of the *hph* coding sequence were reduced (Scholtmeijer *et al.*, 2001). Similar observations were made when the α -galactosidase (*agIA*) gene from *Cyamnopsis tetragonoloba* was expressed in *Aspergillus niger* (Gouka *et al.*, 1996).

The SCR122 transformants were capable of growth when transferred to fresh selection plates, and in four of the transformants the transformed phenotype was stable during growth in the absence of selection. In the remaining two transformants the resistance phenotype was lost after two transfers in non-selective medium. Loss of the transformed phenotype has been described in a number of other systems and is more usually associated with meiosis (Keller *et al.*, 1991). Transgene DNA is usually stable during mitotic growth, however Renno *et al.*, (1992) reported the deletion of transgene sequences during mitotic growth of *Penicillium chrysogenum*. They concluded that it was due to recombination between tandem copies. Keller *et al.*, (1991) reported similar phenomena in *Cochliobolus heterostrophus*.

The presence of the transgene in the transformants was confirmed by PCR with two pairs of primers, the Hyg primers for the *hph* gene fragment, and the M13 primers which amplify a larger region containing the entire chimeric *hyg* gene from pHYM2.2. Whilst five of the six transformants showed a positive reaction with the Hyg primers, only three were positive with the M13 primers. The negative results obtained with the M13 primers may be due to transfer of incomplete T-DNA strands. The transfer process initiates with a cut in one strand at the right border and continues until the transfer apparatus encounters

the left border sequence where it makes a second single strand cut to release the T-DNA strand (Gelvin, 2000). However, if it encounters any sequences that resemble the left border sequence, then transfer may terminate prematurely resulting in the transfer of an incomplete T-DNA strand. Such events are commonly observed during plant transformation experiments (Gelvin, 2000). To establish whether this is the case with the *R. solani* transformants we would need to analyse the transformants for the presence of T-DNA sequences adjacent to the left border. The Southern blotting experiment also showed the presence of the *hph* gene in the transformants. However, it would not detect the presence of incomplete T-DNA strands.

An alternative explanation for the negative results with the M13 primers is that the amplification of such a large region (3kb) may simply have been too inefficient. DNA preparations vary considerably in the extent to which they inhibit PCR (O'Brien, pers com). Amplification of longer regions would be expected to be more sensitive to the effects of inhibitors than amplification of short regions. Cell wall polysaccharides such as those found in plant and fungal cell walls are potent inhibitors of PCR amplification (Demeke and Adams, 1992).

In the previous experiments on transformation of *R. solani*, protoplasts (Robinson and Deacon, 2001) failed to obtain stable transformants, and showed that the transgene did not integrate into the endogenome. We obtained similar results with *A. tumefaciens* mediated transformation with an unmodified hygromycin gene (Ch 4). However, in the experiments described in this chapter we did obtain stable transformants with a gene modified for enhanced expression, and the results show that the transgene is integrated into the chromosome. Why should modification of the gene lead to enhanced integration. An obvious possibility is that it contains sequences that may be homologous to sequences on the chromosome and this promotes integration of the plasmid into the endogenome by homology dependent recombination. The results of a number of studies have shown that integration of transforming DNA in fungi occurs mainly through homologous recombination (Farman and Oliver, 1992; Fincham, 1989; Keller *et. al.*, 1991; Mellon *et. al.*, 1987; Gouka *et. al.*, 1999). Higher transformation frequencies can be achieved by

having homologous sequences on the vector and the host chromosome. This has also been reported with the *A. tumefaciens* fungal transformation protocol.

A comparison of transient vs stable expression of transgenes in plants has revealed that most of the DNA transferred to plant cells is not integrated into the genome and is lost through successive cell divisions (Gelvin, 2000). For many plant species T-DNA integration is the limiting step for transformation. It has been estimated that the stable transformation of apple decreased approx 10,000 fold from the initial transformation event (Maximova *et al.*, 1998). That T-DNA does not integrate efficiently in yeast cells has been shown by a 500 fold higher transformation frequency obtained with a replicative vector compared to a non replicative version of the same vector (Piers, *et al.*, 1996). Given these results, the results with the pBINHL vector in this study, and the results of Robinson and Deacon (2001) who found only non integrative transformation events in *R. solani* we would expect that stable transformation resulting from integration of pJW2.2hyg would be a rare event and that the predominant type of transformant would be transient transformants in which the vector is not integrated. This was observed with the isolates 1342, ST-11-6, and RH165. However with SCR122 we did obtain stable transformants and showed that the vector was integrated.

Host proteins also play a role in integration. Ecotypes of *Arabidopsis thaliana* show variation in their susceptibility to tumour formation by *A tumefaciens*. Mysore *et al.*, (2000) have provided evidence that a histone protein may be at least partially responsible for this variation.

Chapter 7 General Discussion

The aim of this research was to develop a transformation system for the phytopathogenic fungus *Rhizoctonia solani*. The reasons for developing a transformation system for this pathogen are to overcome the problem of the lack of markers to study the genetics and pathology. Very little is known about gene transfer systems in this pathogen, and phenomena such as transfer of mitochondria and mitochondrial plasmids, and cytoplasmic dsRNA's have not been studied despite the fact that these can make an appreciable contribution to the phenotype of the pathogen (Adams 1996). The availability of isolates differentially marked with antibiotic genes would enable the study of such systems.

The genetics are further complicated by the fact that the vegetative mycelium contains 6-12 nuclei per cell (Flentje and Stretton, 1970) which may differ considerably in their vegetative (non sexual) genes. Thus there is a very high capacity for generating phenotypic variation by heterokaryon formation, and the available evidence suggests that all field isolates are heterokaryotic (Bolkan and Butler, 1974). It has been suggested that genetic changes might be induced by shifts in the balance of nuclei in cells of the vegetative hypha perhaps in response to selective pressure (Adams 1996). Similar shifts in the balance of different mitochondria resulting in a mycelium that is a genetic mosaic. Confirmation of these 'speculations' requires the ability to be able to differentially label nuclei.

The transformation system will also allow us to probe mechanisms of pathogenicity and virulence. Using antisense technology (Bramlage *et al.*, 1998) or gene disruption techniques we can switch off expression of classes of genes or individual genes (Sweigard *et al.*, 1992, Tenhave *et al.*, 1998).

Previous reports had indicated that traditional methods of transformation were likely to be unsuccessful due to degradation of the DNA (Robinson and Deacon, 2001).

A. tumefaciens transformation seemed a better option as it transferred the DNA in a linear fashion which is more conducive to integration, and in addition the transferred DNA is accompanied by integration proteins which assist integration into the host genome. We developed vectors containing the hygromycin selectable gene under regulation of the *gpd* promoter and *SC3* terminator of *Schizophyllum commune* which belongs to a basidiomycetes. Because the perfect stage of *R. solani*, *Thanatephorus cucumeris* is a basidiomycete these promoters are more likely to function in *R. solani*. We also made vectors in which ascomycete promoters were used to drive the selectable marker gene. Transformation experiments with *F. oxysporum* confirmed that the system was working and that the *A. tumefaciens* strains were capable of transferring DNA to a fungus, and that the DNA would integrate into the genome. However the basidiomycete promoters did not function in *F. oxysporum* (an ascomycete). We therefore tested a second control species, the basidiomycete *U. cynodontis*. With this recipient we found that whilst the basidiomycete promoters resulted in transformants, the ascomycete promoters did not.

Although we obtained transformant colonies with the pBINHL vector the transformants were unstable in that they grew to a certain size then showed no further growth. Although it was not confirmed, the interpretation is that the vector would persist for several generations in a transient extrachromosomal state and that the protein product of the *hph* gene was translocated along the hyphae to the growing tip. *R. solani* has a very efficient mechanism of translocation (Deacon 1996). The amount of protein product translocated would however decrease with distance from the source and hence when a certain size was reached the concentration would no longer be sufficient to confer resistance. Transient transformation is well recognized in plant transformation and is characterized by the persistence of the gene for a short time, and lack of the ability to replicate and transmit the gene to subsequent generations (Gelvin, 2000). Transient transformation has also been observed in the fungi *Puccinia graminis* (Schillberg *et al.*, 2000) and *Glomerulla cingulata* (Rikkerink *et al.*, 1994).

We did obtain stable transformants of one isolate SCR122 with the modified *hph* gene under control of a basidiomycete promoter. The modifications included changing a number of AT bp in the 5' coding region of the *hph* gene to GC bp, and the insertion of artificial introns into the 5' and 3' UTR (Scholtmeijer *et al.*, 2000). Why did this vector give stable transformants when the pBINHL vector did not? The *hph* gene in pBINHL was expressed as indicated by the appearance of resistant colonies on the selective plates, therefore the difference is unlikely to be due to expression of the *hph* gene. Moreover it was only in one isolate that stable transformants were obtained, in all other isolates the pJW2.2hyg vector behaved as the pBINHL. A possible explanation for the generation of stable transformants in SCR122 is that pJW2.2hyg contains a sequence with homology to the genome of SCR122, and that this is not present in pBINHL1 or in the other isolates of *R. solani*. The presence of homology between vector and insert has been observed to increase the transformation efficiency (Gouka *et al.*, 1999; Farman and Oliver, 1992). It would be interesting to test this by inserting chromosomal fragments from various isolates into the vector.

An alternative possibility for developing a transformation system would be to construct extrachromosomal replicative vectors. In yeast, extrachromosomal vectors give 100 to 1000 fold higher transformation efficiencies compared to integrating vectors (Fincham 1989). Initially the extrachromosomal vectors were based on the 2 μ plasmid of yeast, subsequent improvements involved the insertion of a yeast chromosomal replication sequence into the vector resulting in 1000 fold increase in transformation efficiency (Stinchcomb *et al.*, 1979). The development of extrachromosomal vectors for filamentous fungi has lagged behind that of yeast however vectors have been developed for a number of species. The addition of telomere sequences to vector sequences has resulted in extrachromosomal replication in *Podospora anserina* (Javerzat *et al.*, 1993), *Pestalotiopsis microspora* (Long *et al.*, 1998), and *Colletotrichum gloeosporoides* (Poplawski *et al.*, 1997). The use of such vectors increased the transformation efficiency 10 to several hundred fold. Randall *et al.*, (1991) described the construction of an extrachromosomal vector for *Phanaerochaete chrysosporium* by incorporating a segment

of a *P. chrysosporium* plasmid into the vector. The potential for developing such vectors for *R. solani* is quite high as plasmids are widespread in this species (Miyasaka *et al.*, 1990; O'Brien, unpublished). Although useful, extrachromosomal vectors are limited in their application to studying the genetics of *R. solani* since they may be able to move independently of chromosomal transfer (Poplawski *et al.*, 1997).

In conclusion, the results of this project have demonstrated that stable transformation of *R. solani* is feasible, and that it depends critically on the isolate used as recipient, and the promoter used to drive expression of the selectable marker. The frequency of transformation is relatively low due to poor integration of the transforming DNA. Future efforts should be directed at developing vectors with homology to the endogenome, or extrachromosomal replicating vectors.

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