TOWARDS THE DEVELOPMENT OF A GENE-MEDIATED TRANSFORMATION SYSTEM FOR THE POTATO LATE BLIGHT PATHOGEN PHYTOPHTHORA INFESTANS

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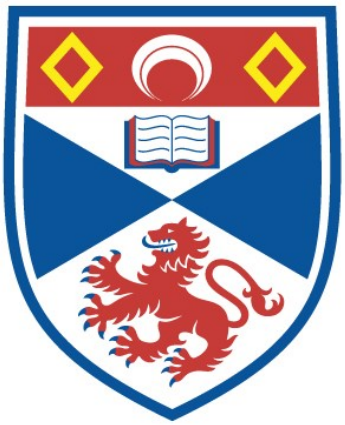

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TOWARDS THE DEVELOPMENT OF A GENE-MEDIATED TRANSFORMATION SYSTEM FOR THE POTATO LATE BLIGHT PATHOGEN *PHYTOPHTHORA INFESTANS.*

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Submitted in accordance with the requirements
for the degree of Doctor of Philosophy

The University of St. Andrews
Department of Biochemistry and Microbiology

September 1990

Declaration for the Degree of Ph. D.

I Richard Philip Moon hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date

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ABSTRACT

The aims of this project were to develop the necessary techniques and components for achieving gene-mediated transformation in *Phytophthora infestans*. Protoplasting and regeneration procedures were developed for encysted zoospores and sporangia. The presence of $CaCl_2$ in the protoplasting osmoticum was found to increase regeneration frequencies to over 90% and 70% for cyst and sporangial protoplasts respectively. Published methods for mycelial protoplasts were improved to give regeneration frequencies of 25-30%. Attempts were made to develop repair of uridine auxotrophy as a transformation marker but isolation of uridine auxotrophs by positive selection with 5-fluoro-orotic acid and filtration enrichment was unsuccessful. Various antibiotics were tested on three isolates of *P. infestans* for their suitability as potential transformation selection agents. Hygromycin B, G-418 and phleomycin were found to give clean backgrounds at low concentrations. Genes encoding resistance to these antibiotics and placed under the control of a variety of fungal, bacterial and viral transcription control sequences were used in transformation experiments employing $CaCl_2$/PEG treatment, lithium acetate/PEG treatment and electroporation to introduce the plasmid DNAs into cyst, sporangial or mycelial protoplasts. No unequivocal evidence for transformation was obtained. To circumvent the possibility that failure to generate transformants using these plasmids was due to non-recognition of the heterologous promoter/terminator sequences *P. infestans* genes were isolated from a gene library. Two different actin genes (*actA* and *actB*) and one glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) were characterised. All three genes are actively transcribed in mycelia but only at a low level in the case of *actB*. *actA* and *actB* have CCAAT motifs in their 5' noncoding regions but lack introns in their coding regions. The actins encoded by *actA* and *actB* are highly diverged from actins of other eukaryotic microbes suggesting the evolutionary lineage of the Oomycetes is distinct from that of the higher fungi. All three genes, but especially *actA* are strongly biased in their codon usage, a feature of highly expressed genes in other eukaryotes. The promoters of *actA* and *gpd* seem suitable for directing expression of hybrid selectable marker genes.

ACKNOWLEDGEMENTS

I would first like to thank my supervisors, Dr. Jim Kinghorn of the University of St. Andrews and Dr. Jim Duncan of the Scottish Crop Research Institute at Invergowrie for giving me the opportunity to carry out this work. I would also like to thank the SERC for providing my studentship and Dr. Sarah Jane Gurr for her encouragement during my first year. I am particularly indebted to Dr. Shiela Unkles and Dr. Andrew MacCabe for their patience and much advice. The construction of the *P. infestans* gene library, cloning of the actin and *gpd* genes, associated Northern and Southern blots and sequencing of *actA* and *actB* were performed by Dr. Shiela Unkles, as was part of the sequencing of *gpd*. Development of the protoplasting system for sporangia and transformation experiments using sporangial protoplasts were performed by Alison Campbell at SCRI. Former colleague Debra Gould performed some of the antibiotic MIC tests and much of the mutant isolation work. Genomic DNA of *P. infestans* isolate E13a was a gift from Dee Carter, Department of Biology, Imperial College of Science and Technology, London.

I am grateful to many friends and colleagues including Janet Macro, Dr. Michael Abberton, Euan Duncanson, Michael Whitehead, Maureen Riach, Mandy Gilkes, Dennis Kirk, Carolyn Grieve, Eddie Campbell, Dr. Paul Montague, Alison Campbell and Mark Errington who between them, and in various ways, helped make my stay in St. Andrews truly memorable. That I retain most of my sanity is due largely to Kim Hawker who through our Munro-bagging sorties provided timely reminders that Life isn't only found in axenic culture.

Finally I pay tribute to my family, particularly my mother and stepfather, for were it not for their help and encouragement over the years, particularly at the low points, I would have had a great time sailing round the world.

ABBREVIATIONS

A	adenine
ATP	adenosine triphosphate
bp	base pair(s)
C	cytosine
cm	centimetres
^{o}C	degrees centigrade
DNA	deoxyribose nucleic acid
EDTA	ethylenediamine tetra-acetic acid
et al.	*et alia* (and others)
Fig.	Figure
5-FOA	5-fluoro-orotic acid
g	gram(s)
G	guanine
x g	times force of gravity (centrifugation)
h	hour(s)
kb	kilobase(s) (pairs)
kV	kilovolts
L	litres
M	molar
µCi	microcuries
µg	micrograms
µl	microlitre(s)
mM	millimolar
mm	millimetres
mg	milligrams
ml	millilitre(s)
min.	minutes
M_r	relative molecular weight
mRNA	messenger RNA
N	any nucleotide
nm	nanometres
No.	number
nt	nucleotides
OD600	optical density at 600 nanometres (wavelength)

PEG 6000	polyethylene glycol of molecular weight 6000
pers. comm.	personnal communication
pg	picograms
R	any purine
RNA	ribose nucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SV40	Simian Virus 40
T	thymine
Tris	Tris (hydroxymethyl) aminomethane
UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume
X-gal	5'-bromo-4'-chloro-3'-indolyl-β-D-galactopyranoside
Y	any pyrimidine

Chemical symbols have their usual meaning.

Restriction enzymes are abbreviated in diagrams as follows:

B	*Bam*HI
Bg	*Bgl*II
E	*Eco*RI
H	*Hin*dIII
S	*Sal*I
X	*Xho*I

Amino acids are abbreviated in text and figures as follows:

Amino acid	One letter code	Three letter code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

CONTENTS

Chapter 1

INTRODUCTION

1.1 THE GENUS *PHYTOPHTHORA*

1.1.1 *GENERAL*

Phytophthora infestans belongs to a genus of Oomycete fungi which includes some of the worlds most damaging plant pathogens. They were first identified over a century ago when *P. infestans* was described as the type species (de Bary, 1876). *Phytophthora* species cause disease on a wide range of host plants, including major food crops, forest trees, subtropical and tropical fruit trees, berries, nut trees and ornamental plants. Nearly 150 years after the Irish potato famines *P. infestans*, the causative agent of late blight, remains an important pathogen. The genus as a whole currently accounts for about one third of the world fungicide market.

Phytophthora is renowned for its inter- and intra-species variation in many aspects of its genetics, physiology and biochemistry (see Erwin, 1983). The genus contains both homothallic and heterothallic species, some of the latter having extremely limited geographic distribution of one mating type with respect to the other and others having a more even distribution of mating types. *P. infestans* has only two known hosts while *P. cinnamomi* and *P. palmivora* each have host ranges of several hundred species, yet have only a few of these in common. *P. infestans* and *P. palmivora* are air- and water-borne pathogens, principally of the aerial parts of their hosts, whereas *P. cinnamomi* is principally soil-borne, attacking roots. Although the genus has been studied for over a century, little is known of their biochemistry and virtually nothing of the molecular basis of their pathogenicity or virulence. To study the genetic basis of pathogenicity and virulence in this genus is to study genetically heterogeneous pathogen populations interacting with a genetically and physically heterogeneous environment. This fact distinguishes these investigations from those

of near isogenic strains of 'model' eukaryotic organisms and will continue to be source of new and unforseen challenges in the future as it has in the past.

1.1.2 *LIFE CYCLE*

The life cycle of *Phytophthora* (Fig. 1.1) has sexual and asexual stages which are primarily diploid, polyploid or aneuploid (Sansome, 1977; Sansome & Brasier, 1973; Tooley & Therrien, 1987).

In the asexual cycle the mycelium produces sporangia - large, polynucleate, single cells which, in *P. infestans*, can be dispersed by air currents or water droplets (Fig. 1.2 (a)). These cells may either germinate directly by formation of a germ tube, or indirectly, by cytoplasmic differentiation and release of zoospores through an apical pore, by an osmotically-governed, pressure-driven mechanism. The form of germination is largely determined by environmental factors, the most important being water status. 100 % of *P. infestans* sporangia can be induced to release zoospores when incubated at an optimum temperature (12-16^0 C) in double distilled water for more than 80 min.. At lower water potentials direct germination of the sporangium replaces zoospore release. Sporangia remain viable on the sporangiophore for only a limited period (Fig.1.2(b)) and as they age they lose the ability to produce zoospores (Gisi, 1983).

Zoospores are motile, predominantly uninucleate, kidney-shaped cells possessing one tinsel flagellum, responsible for propulsion, and one whiplash flagellum, responsible for producing changes in direction (Carlisle, 1983). The cell envelope of *Phytophthora* zoospores is very thin (50-100 nm.) and in some species is known to have a 'fuzzy coat' of unknown function and composition covering the plasma membrane (Bartnicki-Garcia & Wang, 1983). Lacking a cell wall, zoospores need to osmo-regulate by means of a water-extruding vesicle to maintain their integrity. Before germination, motile zoospores undergo an encystment process that involves remarkable architectural changes. The flagella are either shed or retracted and the cell rounds up. The contents of numerous vesicles and other structures are

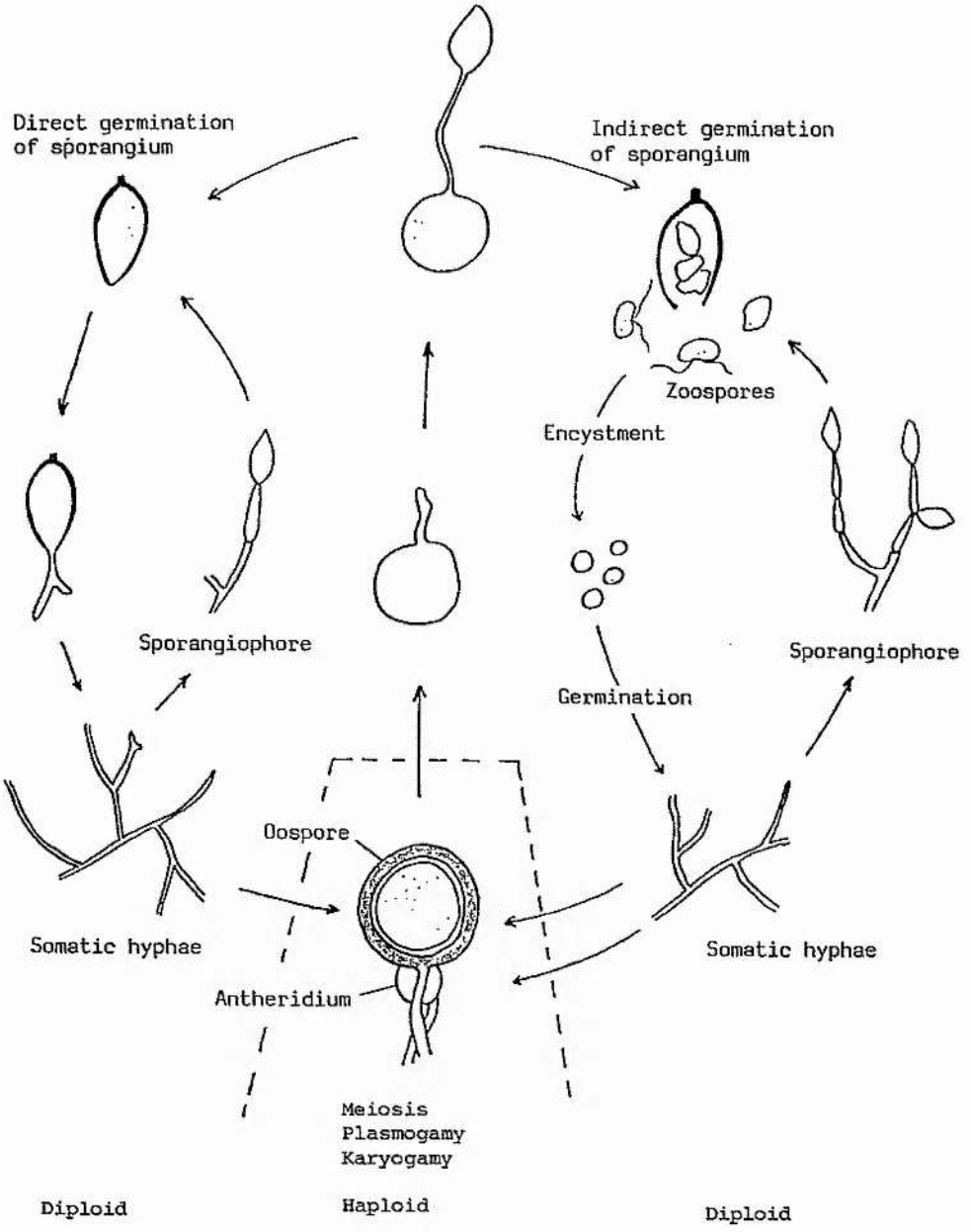

Fig. 1.1 Life cycle of *Phytophthora*. Asexual reproduction proceeds through the germination of diploid, multinucleate sporangia either directly, or indirectly by release of motile zoospores. Direct germination leads to the establishment of somatic mycelium which may produce more sporangia which can themselves germinate either directly or indirectly. Zoospores released by indirect germination undergo encystment before germinating to give rise to mycelium which in turn may sporulate, completing the cycle. In heterothallic species such as *P. infestans*, sexual reproduction normally requires the participation of two opposite mating types, the A1 and A2 which hormonally stimulate formation of haploid gametes in each other. The diploid oospore formed from fusion of the male antheridium and the female oogonium may be a self or a cross of the parental isolates.

Figure 1.2 Reproductive structures of *P. infestans*.

(a) and (b): Scanning electron micrographs of sporangia produced from a sporulating lesion on a detached potato leaf 3 days after inoculation with encysted zoospores of a compatible race of *Phytophthora infestans*.

(a) A young sporangium from near the edge of the lesion. Bar = 10 μm.

(b) An older sporangium from near the centre of the lesion.

Bar = 100 μm

(c) An oospore of *Phytophthora infestans*. The thick wall is clearly visible. Scale:

10 μm = ——


MODE 02 50 001


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extruded onto the outer surface of the cell covering it with an amorphous coat, within and under which a micofibrillar wall is synthesised. All this is achieved without a requirement for protein synthesis. For a short period during the early stages of encystment the zoospore becomes adhesive and sticks to any surface it comes into contact with. However, this stage soon passes and mature cysts lose this property (Bartnicki-Garcia & Wang, 1983). Encystment can be triggered by a range of stimuli including agitation, rise in temperature, fall in pH or rise in osmotic potential and may become visibly apparent within seconds of the initial stimulus. Shortly after encystment the cell germinates and can infect any available host tissue.

P. infestans is a heterothallic species, requiring two bisexual mating types (designated A1 and A2) for sexual reproduction. Some homothallic or self-fertile isolates can produce gametes in the absence of stimulation from another individual but their sporangia and zoospores seem to be inviable (Shaw, 1987). In crosses either gamete may come from either parent or alternatively parents may self (Shattock *et al.*, 1986b). The male antheridium and female oogonium contain the only haploid nuclei of the life cycle and these fuse to form the diploid oospore, a thick-walled survival structure (Fig. 1.2(c)) which may lie dormant for many years before germinating to give rise to a sporangium or infect a host directly, thus renewing the infection cycle (J. Duncan per. comm.).

Until very recently (Shattock *et al.*, 1987) the use of the sexual cycle in genetical experiments was considerably hindered by prolonged oospore dormancy, low germination rates and a lack of suitable genetic markers. Such difficulties, characteristic of the genus, delayed settlement of the long debate over the ploidy of the Oomycetes until the mid-1970's, when it was shown that they have patterns of Mendelian inheritance typical of a diploid (reviewed by Shaw, 1987).

Both A1 and A2 mating types were discovered in Central Mexico in 1956 where they were found to occur with equal frequency in the field. However, only the A1 mating type was found among isolates from North America, Western Europe, South Africa, and the West Indies and sexual reproduction was assumed to be unimportant

in these regions. The reason(s) for the highly restricted distribution of the A2 mating type remains a mystery as it seems improbable that only the A1 mating type was introduced outside Mexico. Then, in the early 1980's the A2 mating type was discovered in field isolates in Switzerland, Scotland, England and Wales (Hohl & Iselin, 1984; Malcolmson, 1985; Tantius *et al.*, 1986). It is unclear whether the arrival of the A2 mating type in Europe is a recent development or whether its presence has simply been unrecognised for a long time. In either case its presence may have important implications for the versatility of the pathogen population in responding to the introduction of new potato cultivars.

Cytogenetic studies suggest that mating type in heterothallic species of *Phytophthora* is determined by a two-allele locus such that the diploid somatic cells are heterozygous for one mating type (probably A2) and homozygous for the other (Sansome, 1980; Shaw, 1983). This hypothesis is supported by genetical evidence: only A1 progeny are obtained from selfed A1 parents but selfing A2 isolates yields A1 and A2 progeny (Shattock *et al.*, 1986a; 1986b).

1.1.3 *GROWTH REQUIREMENTS AND CULTURE OF* P.infestans

The basic nutritional requirements of *P. infestans* and its growth responses to various nitrogen and carbon sources, vitamins, reducing agents and 'growth factors' have been much studied (reviewed by Hohl, 1983). Such investigations revealed *P. infestans* to be a fastidious organism with marked interisolate variation in growth responses to various nutrients. For many years this complexity frustrated attempts to develop a defined 'minimal' medium on which all or at least most isolates could thrive and so 'natural' media such as pea broth and potato dextrose agar, on which *P. infestans* grows quite well, continue to be the principal type of culture media. However, growth on defined media was sufficient to determine the basic nutritional requirements of *P. infestans*. In general glucose and sucrose are significantly better carbon sources than other mono-, di-, tri- or polysaccharides. The only known vitamin requirement is for thiamine and although sterols are not required for growth

they are essential for asexual and sexual reproduction (reviewed by Ribeiro, 1983 and Elliott, 1983 respectively). A number of compounds have been found to stimulate growth on defined media but not usually for all isolates. Like some other members of the genus, *P. infestans* has lost the ability to utilise nitrate as a sole nitrogen source. Various workers have recommended supplying nitrogen in an organic form, as a cocktail of up to several amino acids although opinions often differed over which ones were 'good' nitrogen sources. Hohl (1975) formulated a medium containing minerals, several amino acids, sucrose, thiamine, guanine, lecithin and cholesterol, which supported reasonable growth of the isolates tested. Huang *et al.* (1980) noted that pH and the concentrations of calcium and organic acid in a defined medium containing glucose and minerals, interacted to significantly affect the amount of growth obtained from ammonium or various amino acids as single nitrogen sources. When these three parameters were optimised and a single amino acid used as the nitrogen source this simple medium supported growth of the test isolates that was comparable to that obtained on natural media. With ammonium as the nitrogen source growth was also significantly improved compared to media either lacking organic acid or with suboptimal pH or calcium concentrations.

1.1.4 *PHYLOGENY OF THE OOMYCETES*

The "zoosporic fungi" have traditionally been grouped into the subdivision Mastigomycotina of the kingdom Fungi (Sparrow, 1973), in which the organisms are placed according to their type of flagellation (Table 1.1).

Oomycete zoospores possess a posteriorly directed whiplash flagellum and an anteriorly directed tinsel flagellum.The Hyphochytriomycetes are a small Class whose zoospores have only an anteriorly directed tinsel flagellum suggesting they may be related to the Oomycetes and at some point in evolutionary history they lost the whiplash flagellum (Barr,1981). Zoospores of the Class Plasmodiophoromycetes have two whiplash flagella which emerge on different sides. Views are divided on the origin of this group, it has been suggested on the basis of

Table 1.1 Classification of the zoosporic fungi in the kingdom fungi.

Kingdom Fungi			
Division Eumycota (True Fungi)			
Subdivisions:	Ascomycotina		
	Basidomycotina		
	Zygomycotina		
	Mastigomycotina	Classes:	Oomycetes
			Hyphochytriomycetes
			Plasmodiophoromycetes
			Chytridiomycetes
Division Myxomycota (Slime molds)			

cytological data that they may be related to the Oomycetes or the Myxomycetes, whereas others consider them to be more closely related to ciliated protozoa (Barr,1981). Chytridiomycete zoospores have a single whiplash flagellum and may be the ancestral group to the Zygomycetes and higher fungi (Ascomycotina, Basidiomycotina and Deuteriomycotina) (Barr, 1983), but there is no agreement on the origin of this group. It is clear that this classification scheme bears little resemblance to actual phylogenetic relationships between these Classes and there are a number of structural and biochemical traits that suggest that the Oomycetes had separate evolutionary origins to the zygomycetes and higher fungi.

With the lack of a complete fossil record, the identification and reliability of characteristics used to assess evolutionary relationships is a problem for taxonomists attempting to determine phylogenies based on phenotypic criteria. Deciding which characters are taxonomically useful and the relative weight that should be given to each of them makes the construction of phylogenetic trees a process in which complete objectivity is difficult to achieve and so a consensus phylogeny for eukaryotes based on phenotypic markers has never emerged. Conserved amino acid and nucleic acid sequences have become widely used as evolutionary probes in attempts to construct phylogenetic trees which reflect true genotypic similarities between organisms.

Ribosomal RNAs (rRNAs) in particular are useful for this purpose as they are universally distributed and functionally equivalent in all known organisms. Although comparisons of 5S and 5.8S rRNAs have been used to construct phylogenies (Walker, 1985; Huysmans & de Wachter, 1986; Hori & Osawa, 1987), they have the disadvantage of relatively small size which compromises their statistical reliability when measuring very close or very distant relationships (Sogin *et al.*, 1987). The 16S-like or small-subunit (SSU) rRNAs are large enough to make statistically accurate comparisons and the existence of highly conserved and partially conserved sequence elements make them particularly useful for measuring both close and distant phylogenetic relationships (Woese, 1987). The large-subunit 23S-like rRNA is also useful for similar reasons but the relatively low number of published sequences limits their usefulness at present (Cedergren *et al.*, 1988).

The use of protein or nucleic acid sequences has not been a panacea for the construction of phylogenetic trees. The construction of phylogenies using sequence comparison data is sensitive to the assumptions inherent in the method of analysis and the type of sequence analysed (Gillespie, 1986; Cedergren *et al.*, 1988; Felenstein, 1988; Jin & Nei, 1990), so that a number of differing trees are possible from similar data sets of analogous sequences or from different molecules analysed by similar methods (Woese, 1987; Walker, 1985; Gunderson *et al.*, 1987; Hori & Osawa, 1987; Sogin *et al.*, 1989; Cedergren *et al.*, 1988; Perasso *et al.*, 1989). However, the consensus of such analyses that include representatives of the Oomycetes is that they have a separate and distinct lineage from the ascomycetes, basidiomycetes and zygomycetes (Walker, 1985; Gunderson *et al.*, 1987; Hori & Osawa, 1987; Cedergren *et al.*, 1988). The Oomycetes may instead be closely related to the brown (Phaeophyta) and golden (Chrysophyta) algae (Elwood *et al.*, 1985; Sogin *et al.*, 1986; Gunderson *et al.*, 1987; Bhattacharya & Druehl, 1988; Cedergren *et al.*, 1988) or to the Cryptophyta and cellular slime molds (Hori & Osawa, 1987).

The interpretation of rRNA sequence data that the Oomycetes and higher fungi have separate evolutionary lineages is also supported by biochemical and

Table 1.2 Biochemical differences between the Oomycetes and the higher fungi and zygomycetes.

	Oomycetes	Ascomycetes	Basidiomycetes	Zygomycetes	Reference
Cellulose present in cell walls	+	-	-	-	Griffin, 1981
Thymidine kinase	+	-	-	-	Griffin, 1981
Ability to synthesize sterols	-	+	+	+	Griffin, 1981
Storage polysaccharide	mycolaminaran	glycogen	glycogen	glycogen	Griffin, 1981
Lysine biosynthetic pathway[1]	DAP	AAA	AAA	AAA	Vogel,1964
NADP-linked GDH[2]	-	+	+	-	LeJohn, 1974
Type of NAD-linked GDH	III	I	?	?	LeJohn, 1974
L (+) or D (-) LDH[3]	D (-)	L (+)	L (+)	D (-)	LeJohn, 1974
Size of large rRNA (x 10^6 daltons)	1.40-1.43	1.28-1.32	1.30-1.33	1.32-1.34	Lovett & Haselby, 1971

Notes

1. DAP - Diaminopimelic acid
   AAA - α-aminoadipic acid
2. GDH - Glutamate dehydrogenase
3. LDH - Lactate dehydrogenase

morphological data (Table 1.2 and Fig.1.3). The lysine biosynthetic pathway proceeds along the diaminopimelic acid (DAP) pathway in bacteria, cyanobacteria, Chrysophyte, Phaeophyte, and Chlorophyte algae, vascular plants and Oomycetes but by the α-aminoadipic acid (AAA) pathway in the higher fungi and euglenoids (Vogel, 1965). Glycogen is a widely distributed reserve polysaccharide among fungi. It has been found in ascomycetes, basidiomycetes, zygomycetes and chytridiomycetes. Oomycetes lack glycogen but instead use β-1,3-linked glucans and mycolaminarin for this role. The organisation of the *trp1* gene of *Phytophthora parasitica* represents a new class in fungi, previously only known from prokaryotes (Karlovsky & Prell 1989). According to Cavalier-Smith (1987) the 'original' common ancestral eukaryote synthesised lysine by the AAA pathway and possessed no mitochondria or chloroplasts. The higher fungi retained the AAA pathway but this was replaced in the ancestors to the Oomycetes, Phaeophyte and Chrysophyte algae and others by the DAP pathway, acquired from cyanobacterial symbionts that evolved into chloroplasts (Fig. 1.3). Evolution of the Oomycetes from a heterokont algal ancestor which lost chloroplasts is also supported on the basis of their mastigonemes ('hairs') on the anterior flagellum, flagellum rootlet morphology (Barr, 1981) their tubular mitochondrial cristae and the similarity of their kinetids to those of the Phaeophyte and Chrysophyte algae.

The apparent existence of a wide evolutionary gap between the Oomycetes and the green plants, animals and higher fungi raises interesting questions about the genomic organisation and regulation of gene expression in these and related organisms. Little work of this kind has yet been done on the Chrysophytes, Phaeophytes or Oomycetes and it would be interesting to determine if their genomic organisation is different again from that in other groups, which are already known to contrast strongly (Kinghorn, 1987).

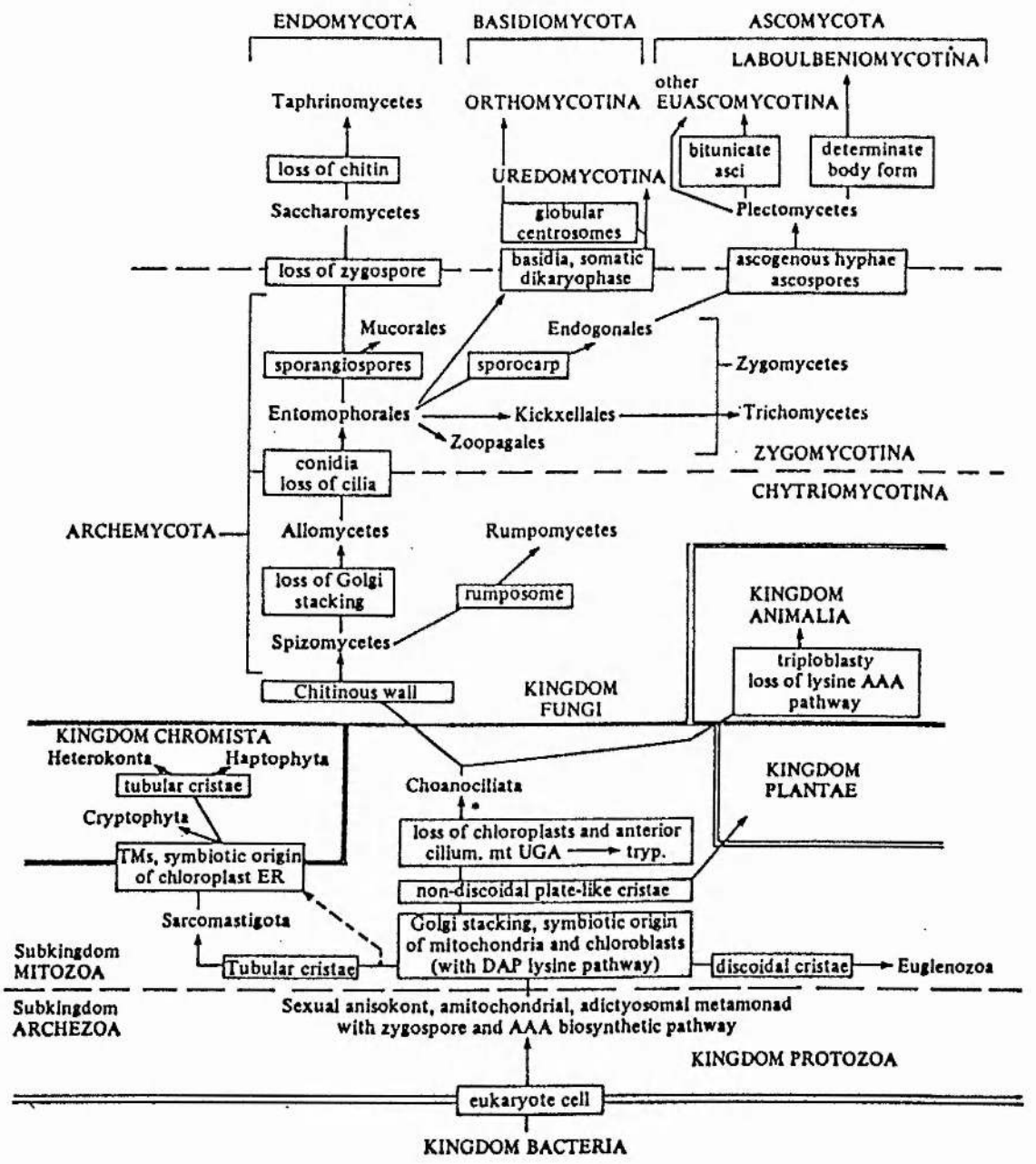


Fig. 1.3 Phylogenetic relationships of the fungi. The subkingdom Archezoa contains the four protozoan phyla totally without mitochondria or chloroplasts. The α-aminoadipic acid (AAA) lysine biosynthetic pathway was retained by Fungi and euglenoids, but replaced in Chromista and Plantae by the diaminopimelic (DAP) pathway from the cyanobacterial symbiont that evolved into chloroplasts. The DAP pathway in the Pseudofungi, their mastigonemes (TMs) on the anterior cilium, and their tubular cristae establish their origin from a photosynthetic heterokont. Fungi, Choanociliata and Animalia together form a clade, the Opisthokonta, whose common ancestor (asterisk) had a single, posterior cilium, but which (as shown by the presence of a second non-ciliated centriole) itself evolved from a biciliated anisokont by losing the anterior cilium. Reproduced, with permission, from Cavalier-Smith (1987).

1.2 WHY DEVELOP A TRANSFORMATION SYSTEM FOR *P. infestans* ?

1.2.1 *THE POTATO/*P. infestans *RESISTANCE-VIRULENCE INTERACTION*

Higher plants have two mechanisms of resistance to pathogens.

(a) General resistance, ('horizontal' resistance) which is influenced by many genes and may result in a reduction in the establishment of infection, rate of tissue invasion and/or level of pathogen sporulation on infected tissue and may involve structural features of the host, preformed chemical inhibitors of pathogen functions, induced structural barriers and hypersensitive reactions (HR) (characterised by loss of electrolytes, rapid cell death, tissue browning) and the accumulation of phytoalexins (low molecular weight antimicrobial compounds). The polygenic nature of general resistance is thought to make it particularly effective in controlling disease as the pathogen population has to change at many loci to adapt to the host. Plant breeders now focus their efforts at improving crop disease resistance on this kind of protection.

(b) Specific resistance, which is usually monogenic and known to operate only through the HR and accumulation of phytoalexins. Unlike general resistance it is effective only against certain races of a pathogen species. Pathogen races that do not elicit an HR in a particular cultivar are termed compatible, those that do provoke an HR are said to be incompatible. Eleven specific resistance determinants that help protect the Mexican wild potato species *Solanum demissum* against infection by *P. infestans* have been identified and soon after their discovery some of these resistance or R genes were introduced into the commercial potato species *S. tuberosum* by conventional breeding techniques in attempts to develop cultivars resistant to late blight. Individual cultivars may carry no or many R genes but varieties that did carry them still succumbed to late blight in the field. It soon became apparent that there were races of *P. infestans* capable of overcoming the resistance imparted by any R gene. A system of nomenclature for isolates of *P. infestans* where races are classified

according to the R gene resistance(s) they can overcome was developed (Black, 1952; Black *et al.*, 1953). A set of eleven race specific virulence determinants corresponding to the eleven R genes of potato was proposed for *P. infestans* establishing the elements for a so-called gene-for-gene resistance-virulence system. Because classical genetic studies on *P. infestans* were difficult to perform until recently, genetic data on the characteristics of virulence determinants is still incomplete. There is some evidence that virulence in *P. infestans* against different potato R genes may not be uniformly controlled by single recessive genes as in a classic gene-for-gene interaction (Keen, 1982) but may involve recessive or dominant, single or multiple genetic determinants, in different cases (Spielman *et al.*,1989; 1990 and references therein). However like many other host-parasite systems the potato-*P. infestans* interaction exhibits reciprocal race-cultivar characteristics and is generally considered to be a gene-for-gene system.

But what of the nature of the products of virulence and avirulence genes? It has been suggested that avirulence genes in gene-for-gene systems encode glycosyl transferase enzymes involved in the addition of unique sugar residues by specific linkages to nascent glycosyl chains that are destined to become the carbohydrate side chains of the surface and extracellular glycoproteins of the fungal pathogen. This model proposes that these unique sugar residues in specific linkages are recognised by resistance gene receptors which invoke the HR-phytoalexin response. Recessive pathogen virulence alleles would produce defective transferase proteins incapable of efficiently catalysing addition of the appropriate sugar to the surface carbohydrate resulting in non-recognition by the corresponding resistance gene receptor (Keen, 1982).

An alternative model proposes that all races of *P. infestans* are capable of initiating an HR-phytoalexin response but compatible races of the pathogen are able to suppress hypersensitivity, cell death, necrosis and phytoalexin accumulation (Kuc *et al.*, 1979) possibly through the mediation of specific water soluble glucan inhibitors originating from the pathogen (Doke *et al.*, 1979).

Reservations have been expressed about both these and other models of the resistance-virulence interaction (Keen, 1982; Callow 1987) but as yet there is insufficient hard information about the nature and mode of action of race-specific HR elicitors/suppressors in plant-fungal interactions generally to draw any firm conclusions about what role the products of virulence and avirulence genes in *P. infestans* actually play.

Although genes for specific resistance at present have limited value as the principal genetic components for disease resistance in crop plants, a greater understanding of the molecular biological basis of gene-for-gene interactions in general may lead to improved disease control methods and/or better designed and more rational approaches to the development of new disease resistant cultivars for many important agricultural crops. Apart from the fact that the potato is one of the world's major food crops the *P. infestans*-potato interaction has a number of advantages as a model for studying gene-for-gene interactions. *P. infestans* is culturable, sexual and asexual stages of the life cycle can be manipulated experimentally and an increasing number of genetic markers, including antibiotic resistance (Shattock & Shaw, 1975) and isozymes (Tooley *et al.*, 1985), are becoming available for use in classical genetic experiments. The results of tests for race-R gene specificities are reasonably clear and reproducible and virulence can be tested convieniently using a detached leaf assay which accurately reflects responses of whole plants (Spielman *et al.*, 1989)

A combination of the recent development of recombinant DNA technologies together with more traditional biochemical methods promises to enable the characterisation of the role of virulence/avirulence loci of *P. infestans* in the pathogenesis of potato late blight at a level not previously possible. A gene mediated transformation system is an essential tool for the realisation of this goal.

1.2.2 *STRATEGIES FOR ISOLATING VIRULENCE AND AVIRULENCE GENES*

In cases where virulence is recessive to avirulence, loci encoding avirulence could be isolated by transforming a multivirulent race of *P. infestans* with a gene library of a Race 0 isolate. Transformants isolated by means of a selectable marker could be screened for incompatibility against appropriate potato R gene differentials in detached leaf tests (Spielman *et al.*, 1989). Conversely any dominant virulence genes could be isolated by transforming a Race 0 isolate with a gene library from a multivirulent isolate and screening transformants for aquired virulence. Because of the large size of the *P. infestans* haploid genome (about 2.4×10^8 bp - see Section 6.1) screening the equivalent of one haploid genome in a plasmid-based genomic DNA library for up to 11 loci would require testing each of approximately 60,000 transformants in 11 separate leaf tests (assuming an average insert size of 4 kb). Isolation of even a few loci would be a very laborious task. This could be reduced if cosmid based gene libraries were used, alternatively a cDNA library could be screened. Methods are available which allow the synthesis of cDNAs which are complete copies of mRNA except for a few nucleotides at the 5' end (Sambrook *et al.*, 1989). Insertion of such cDNAs, which would lack the 5' cap of the mRNA molecule, into a cloning site just 3' of a partial *P. infestans* promoter (i.e. the 5' non-coding region up to and including the point of transcription initiation) to produce a functional gene would reduce the number of transformants that would be required to be screened in leaf tests to perhaps only a few percent of the number if a genomic library were used. This approach would be applicable where virulence or avirulence was determined by single genes. A genomic library would have to be screened where virulence or avirulence was encoded by linked genes at one locus. It is clear that further classical genetical information on the nature of virulence/avirulence determinants in *P. infestans* would be of immense value for the formulation of appropriate cloning strategies.

1.3 GENETIC TRANSFORMATION SYSTEMS

DNA transfer in eukaryotes is an indispensable tool in the study of their molecular genetics and biochemistry. The power of this technique is reflected in the rapidly expanding range of organisms for which gene-mediated transformation systems have been developed. Some of the most extensively studied organisms in this respect are the higher fungi. Probably more is known about gene structure and expression in these organisms than in all other eukaryotic microbes combined (Kinghorn, 1987 and references therein), and while it is helpful to draw on this knowledge in developing a transformation system for *P. infestans*, it should be remembered that extrapolations may not always be appropriate.

1.3.1 *ELEMENTS REQUIRED FOR A TRANSFORMATION SYSTEM*

A transformation system which is to be developed for the purpose of isolating avirulence/virulence genes from *P. infestans* should have the following characteristics:

1. It should be easily applicable to any isolate of interest.
2. It should be efficient enough to permit the the screening of gene libraries.

All gene-mediated transformation systems require three major components: a method for introducing exogenous DNA into suitable cells, a marker gene that confers a selectable phenotype and functional transcription signals to direct expression of the marker gene.

(a) Introduction of DNA to cells.

P. infestans has three walled and one wall-less cell type - the mycelium, sporangium, cyst and motile zoospore respectively - that can each be produced in sufficient quantities in pure culture to be useful as potential recipients for recombinant DNA. Several methods have been used to introduce recombinant DNA into cells of lower eukaryotes, including, $CaCl_2$/PEG treatment, lithium acetate treatment, electroporation, liposome fusion, micro-injection and bio-ballistics.

Certain techniques, such as $CaCl_2$/PEG treatment and liposome fusion, require an exposed cell envelope and so walled cells may need to be protoplasted before DNA can be introduced. Experience has shown that for a variety of practical and biological reasons no method is universally applicable to all organisms and the precise conditions under which DNA uptake is achieved must be determined empirically in each case.

(b) Selectable markers

Selectable markers used in transformation of eukaryotic microbes are usually of two principal types, repair of auxotrophy and antibiotic resistance. Transformation from auxotrophy to prototrophy normally requires the isolation of a suitable mutant strain to act as a recipient for the wild-type gene. Such mutants are usually recessive and are difficult to isolate from *P. infestans* due to diploidy and its fastidious nutritional requirements. However there are cases where mutation to auxotrophy leads to concomitant resistance to an otherwise toxic metabolite analogue, thus allowing large numbers of mutagenised cells to be screened by positive selection. Examples of this include the isolation of fungal mutants deficient in nitrate reductase through chlorate resistance (Cove, 1976) and in acetate utilisation through fluoroacetate resistance (Apirion, 1965), and the isolation of uridine and lysine auxotrophs through 5-FOA resistance (Boeke *et al.*, 1984) and α-amino-adipate utilisation (Chattoo *et al.* , 1979) respectively.

Use of an antibiotic resistance gene as a selectable marker has the advantages of not usually requiring mutant isolation and conference of a positively selectable dominant or semi-dominant phenotype. The coding region of most antibiotic resistance genes used in transformation systems are of bacterial origin and some, such as the *hph* (hygromycin B resistance), *kan* (kanamycin resistance) and *neo* (neomycin resistance) genes, have been shown to function in a range of phylogenetically distant hosts. Oligomycin C interacts with the ATP synthase subunit 9 protein of the F_0 region of the mitochondrial ATP synthetase complex thereby inhibiting synthesis of ATP by oxidative phosphorylation. Resistance to oligomycin C

provided an homologous selectable marker system for *Aspergillus nidulans* (Ward *et al.*, 1986), *A. niger* (Ward *et al.*, 1988) and *Penicillium chrysogenum* (Bull *et al.*, 1988) by transformation of a wild-type oligomycin C-sensitive strain to oligomycin C resistance ($OliC^R$) using a mutant ATP synthase subunit 9 allele. This gene is semidominant so mutants carrying a resistant allele can be screened for directly. As the ATP synthase subunit 9 protein forms part of a complex assembly in the mitochondrial membrane it is unlikely to be functional in a heterologous host. Indeed the *Aspergillus nidulans* $OliC^R$ gene, *OliC31,* does not appear to function in *A. niger,* the homologous gene being required for transformation of this species to $OliC^R$ (Ward *et al.*, 1988). Although functional constraints may preclude the use of heterologous $OliC^R$ genes as transformation markers, ATP synthase subunit 9 genes in other species are probably highly conserved at the amino acid level (Sebald & Hoppe, 1981) and may be of use as heterologous probes to screen a gene library.

(c) Functional transcription signals

To improve expression the transcription signals of bacterial antibiotic resistance genes are usually replaced with eukaryotic or viral ones. Among the ascomycetes interspecific gene expression is generally quite permissive (e.g. Turgeon *et al.*,1985; Hartingsvedlt *et al.*, 1987; Kolar *et al.*, 1988; Campbell *et al.*, 1989; Daboussi *et al.*, 1989; Unkles *et al.*, 1989; Churchill *et al.*, 1990) and this often allows a vector constructed for one host to be used in another. A particularly successful example of this is the pAN7-1 vector (Punt *et al.*, 1987) which confers hygromycin B resistance to several filamentous ascomycetes (Oliver *et al.*, 1987; Cooley *et al.*, 1988; Farman & Oliver, 1988; Comino *et al.*, 1989; Osiewacz & Weber, 1989). In addition various lower eukaryotes are able to express genes driven by animal viral or bacterial transcription signals (Barclay *et al.*, 1983; Sreekrishna *et al.*, 1983; Bull & Wooton, 1984; Hasnain *et al.*, 1985; Revuelta & Jayaram, 1986; Manavathu *et al.*, 1988; Randall *et al.*, 1989).

While the use of heterologous constructs is attractive as an initial strategy, the literature supports the intuitive hypothesis that transformation is more likely to be

achieved when the transcription signals come from a closely related species or the host itself, rather than a distant relative. Recognition of heterologous promoters and genes is an unpredictable phenomenon in fungi (e.g. Ballance *et al.*, 1983; Casselton & de la Fuente Herce, 1989; Randall *et al.*, 1989; Wostemyer *et al.*, 1987; Revuelta & Jayaram, 1986; Whitehead, 1990) and it is generally felt that homologous ones also tend to give higher transformation efficiencies, although difficulties in standardising experiments mean. there are few published reports of rigorous comparisons. Nevertheless there may be a double advantage in employing *P. infestans* transcriptional control sequences to direct expression of a heterologous antibiotic resistance gene and this would have the added advantage of avoiding uncertainty about the efficacy of at least this aspect of transformation experiments before transformants were actually isolated. Such sequences may be obtained by (a) functional complementation of auxotrophic mutations in *Escherichia coli* or yeast (Munoz-Rivas *et al.*, 1986; Revuelta & Jayaram, 1987) (b) screening a promoter library (Turgeon *et al.*, 1987; Arnau *et al.*, 1988), or (c) screening a genomic library, either conventionally with a heterologous gene fragment or with a degenerate oligonucleotide probe derived from amino acid sequence comparison data (Reichardt *et al.*, 1988). Of these three approaches, the one least dependent on uncertainties such as heterologous expression is the latter, and so, in view of the uniqueness of the Oomycetes and the lack of knowledge of their genetics, this is arguably the more favoured strategy for isolating *P. infestans* transcription signals.

Chapter 2

MATERIALS AND METHODS

2.1 CHEMICALS AND EQUIPMENT

All chemicals were obtained from BDH plc., U.K. or Sigma Chemical Co., U.K. and were of analytical grade, unless specified otherwise.

Centrifugation was routinely performed using a Sorvall RC-5C centrifuge fitted with either a SS34 or GS3 fixed angle rotor (Du Pont plc., U.K.). Ultracentrifugation of plasmid and genomic DNA was performed using a Sorvall OTB65B ultracentrifuge fitted with a T-865.1 fixed angle rotor (Du Pont plc., U.K.). Microfuge tubes were centrifuged in a Centrifuge 5415 microfuge (Eppendorf, F.R.G.). Other centrifuges were used where specified.

2.2 MYCOLOGICAL AND BIOCHEMICAL TECHNIQUES

2.2.1 *STRAINS*

The following strains were used.

(a) *P. infestans.*	Isolate	Mating type	Race
	ATCC48720	A1	0
	I230	A1	1,3,4,7,8,10,11
	I259	A2	1,2,3,4,7,8,10,11
	ATCC36609	A2	2,4

ATCC48720 and ATCC36609 were obtained from the American Type Culture Collection. Isolates I230 and I259 are single-zoospore isolates obtained from plant material at the Scottish Crop Research Institute. Isolates were subcultured by mass-transfer and stocks maintained on Rough Rye Agar (RRA) slopes for up to six months

or on RRA slopes under glycerol for longer term storage. All cultures and stocks were incubated at 18-20° C.

(b) *Aspergillus nidulans*.

Strain	Genotype (nomenclature according to Clutterbuck,1984)
G191	*pyrG89, fwA1, pabaA1*
G34	*yA, methH2, argB2*

Cultures were incubated at 30° C. Liquid cultures were shaken in a rotary incubator at 250 rpm.

2.2.2 *MEDIA*

(a) Pea broth (PB)

This was the standard growth medium used where high levels of sporulation were not required and was prepared as follows: 250 g of frozen peas (Birds Eye Walls plc., UK) were brought to boiling point in 600 ml of distilled water, simmered for approximately 3 min. and allowed to cool. The peas were removed by passage through 4 layers of muslin and discarded. The volume of the liquid was made up to 1 L with distilled water. Where required, the medium was solidified with 1.2 % (w/v) No. 2 agar (Lab M plc., U.K.).

For selective media, drugs and antimetabolites were added to molten pea broth agar at 55° C. Hygromycin B (Calbiochem Co., U.S.A.), G-418 (Gibco plc.,U.K.), and phleomycin (Cayla, France) were added as filter sterilised aqueous solutions. Oligomycin C (Sigma plc., U.K.) was dissolved in absolute ethanol, chloramphenicol was dissolved in 40% ethanol and 5-fluoro-orotic acid was added as a solid. In growth inhibition tests for oligomycin C and chloramphenicol ethanol controls were included to account for any inhibition by the ethanol. For these absolute ethanol was added to molten PBA at 55° C to a concentration of 2 % (v/v) (the maximum amount that was added in kill curves) before pouring.

(b) Rough rye agar

Rough rye agar (RRA) was used as the sporulation medium for *P. infestans* and was prepared as follows: 60 g of rye grains were soaked in 700 ml of distilled water for

36 h and then boiled for 1 h . The softened grains were blended in the liquid, 20 g of sucrose added and the volume made up to 1 L. The medium was solidified with 1.2 % (w/v) No. 2 agar (Lab M plc.,U.K.).

(c) Complete medium (CM)

This was used for the growth of *A. nidulans* and contained (per L): D-glucose, 10 g; mycological peptone, 2 g; yeast extract,1 g; casein hydrolysate, 1 g; salt solution, 20 ml; trace element solution, 1 ml; vitamin solution, 1 ml. The pH was adjusted to 6.5 and agar added to a concentration of 1.5 % (w/v) where required.

(d) Stock solutions

(i) Salt solution for CM contained (per L): KCl, 26 g; $MgSO_4.7H_2O$, 26 g; KH_2PO_4, 76g.

(ii) Trace element solution for CM contained (per L): $Na_2MO_7O_{24}.2H_2O$, 1.1g; H_3BO_4, 11.2 g; $CoCl.6H_2O$, 1.6 g; $CuSO_4.5H_2O$, 1.6 g; EDTA, 50 g; $MnCl_3.4H_2O$, 5 g; $ZnCl_2.7H_2O$, 22 g. The solution was heated to boiling, cooled to 60° C and adjusted to pH 6.5 with 1 M KOH.

(iii) Vitamin solution for CM contained (per L): Aneurine, 1.5 g; biotin, 2.5 g; nicotinic acid, 2.5 g: choline.HCl, 20 g; para-aminobenzoic acid, 0.8 g; pyridoxine.HCl, 1.0 g; riboflavin, 2.5 g; pantothenate (Ca salt), 2.0 g.

2.2.3 *CELL COUNTS*

Counts of zoospore, sporangium and protoplast suspensions were performed using a modified Fuchs Rosenthal haemocytometer (ARH plc., U.K.) of grid dimensions 0.2 mm x 0.0625 mm. Counts of the whole grid were multiplied by 555.6 to give the number of cells/ml.

2.2.4 *PREPARATION OF SUSPENSIONS OF SPORANGIA*

A RRA petri dish (82 mm diameter, 3-4 mm depth of agar) was inoculated with two blocks (approximately 5 x 5 x 5 mm) of RRA, 4-5 cm apart, containing mycelium from a stored slope culture and was incubated until growth was confluent. Further RRA plates were inoculated as above with squares (5 x 5 mm) of agar containing

mycelium from this 'starter' culture and grown until maximum sporulation was reached (10 day or 14 day, A1 and A2 mating type isolates respectively). Sporangia were harvested by pouring approximately 10 ml of distilled water onto the culture and gently rubbing the mycelium with a glass rod. The liquid was poured off the culture, through nylon cloth (30 µm pore size; Henry Simon plc., U.K.) to remove hyphal debris, if necessary. The sporangial suspension could be concentrated by centrifugation at 500 x g for 5 min. or by allowing to settle out for 20 min. and pouring off excess water.

2.2.5 *PREPARATION OF SUSPENSIONS OF ZOOSPORES*

Motile zoospores were obtained either by chilling a suspension of harvested sporangia at 4° C for 2-3 h or by flooding RRA cultures with SDW, both prechilled to 4° C, and incubated at 4° C for 2-3 h. Sporangia could be largely separated from motile zoospores by passing the suspension through nylon cloth (20 µm pore size; Henry Simon plc., U.K.). Motile zoospores were induced to encyst by vortexing the suspension for 90 sec.. Cyst suspensions were concentrated by centrifugation at 8,000 x g, 4° C for 10 min.. Aggregation of cysts into large clumps during centrifugation could largely be avoided by allowing the cyst suspension to stand for 10-15 min. after vortexing.

2.2.6 *PRODUCTION OF PROTOPLASTS FROM MYCELIUM*

Mycelium, grown from a relatively heavy inoculum of sporangia in 40 ml of PB in a 250 ml conical flask, was harvested after 36-48 h incubation on a single layer of muslin and washed with SDW followed by sterile protoplasting buffer. The mycelium was transferred with sterile forceps to a sterile 250 ml conical flask containing 25 ml of protoplasting buffer; 10 mg/ml NovoZym 234 (Novobiolabs, Novoindustries, Denmark). Care was taken to avoid compressing the mycelium as this reduced digestion. The flask was gently mixed on a platform shaker for 80-90 min at room temperature. Digestion was usually incomplete by this time and the

debris was removed by filtration through a sterile glass sintered funnel (BDH Chemicals plc., U.K.. P160 size), into two 25 ml plastic universal bottles. The protoplasts were washed with sterile protoplasting buffer, by centrifugation (40 x g, 5 min., 20° C) three times in a bench-top centrifuge fitted with a swing bucket rotor (M.S.E. Scientific Instruments, U.K.. Model: Minor), to remove the NovoZym 234.

2.2.7 *PRODUCTION OF PROTOPLASTS FROM ENCYSTED ZOOSPORES*

The encysted zoospore suspension was washed twice with protoplasting buffer by centrifugation (10,000 x g, 10 min.) and resuspended in 5 ml of protoplasting buffer containing 50 mg/ml NovoZym 234. The suspension was swirled gently at 25° C for 2 h, then washed twice, by centrifugation (40 x g, 5 min., 20° C) in a bench-top centrifuge fitted with a swing bucket rotor (MSE Scientific Instruments plc., U.K.. Model: Minor), in protoplasting buffer and resuspended in an appropriate osmoticum.

2.2.8 *PRODUCTION OF PROTOPLASTS FROM SPORANGIA*

Sporangia were harvested from 10 day old petri dish cultures in 10 ml of protoplasting buffer and passed through nylon cloth (53 µm pore size. Henry Simon plc., U.K.) to remove hyphae. The suspension washed with protoplasting buffer by centrifugation (670 x g, 10 min.). The pellet was resuspended in 10 ml of protoplasting buffer containing 20 mg/ml NovoZym 234 and incubated with gentle swirling at 25° C for 24 h. Suspensions were then extensively washed, by centrifugation (49 x g, 10 min., 20 ° C) in a bench-top centrifuge fitted with a swing bucket rotor (MSE Scientific Instruments plc., U.K.. Model: Centaur 2), in protoplasting buffer before being resuspended in an appropriate osmoticum.

2.2.9 *REGENERATION OF* P. infestans *PROTOPLASTS.*

Protoplasts were washed free of salt, by centrifugation three times (40 x g, 5 min., 20° C) in a bench-top centrifuge fitted with a swing bucket rotor, resuspending the pellet in a sugar solution isotonic to the protoplasting osmoticum (0.9 M mannitol

for mycelial protoplasts; 1.0 M sorbitol for sporangial and cyst protoplasts). Suitable aliquots of protoplasts were added to liquid or solid regeneration media.

2.2.10 *FLUORESCENCE MICROSCOPY*

Cell walls were stained with Calcofluor White (American Cyanamid Co., U.S.A.) added to suspensions of sporangia, zoospores, cysts and protoplasts at a final concentration of 0.1 % (w/v). Suspensions were examined by UV fluorescence microscopy using a microscope (Olympus, Japan. Model: BH-2) fitted with a UV Excitation Dichromic Mirror Assembly with a Y495 supplementary barrier filter and illuminated with a high pressure mercury lamp.

2.2.11 *UV MUTAGENESIS*

10 ml of concentrated suspensions of encysted zoospores were exposed to UV light (Hanovia Lamps plc., U.K.. Model: 112F-0003), while being mixed in an open glass petri dish, for periods inducing kill levels of 75 % or 99 % as determined from a UV exposure kill curve. Actual kill levels in each experiment were confirmed by haemocytometer counts of aliquots of exposed cysts after incubation in PB for 18-24 h.

2.2.12 *NITROSOGUANIDINE MUTAGENESIS*

Encysted zoospores were mixed in a solution of 60 µg/ml N-methyl-N'-nitro-N-nitroso-guanidine (NTG) (Sigma Chemical Co. plc., U.K.. Batch no. 65F-3663-1) to produce a kill rate of 99 %. NTG was diluted to less than 5 µg/ml and removed by washing the cysts by centrifugation (3,000 x g, 10 min., 20° C) in bench-top centrifuge (Wifuge, U.K.. Model: 500E). Actual kill levels in each experiment were confirmed by haemocytometer counts of mutagenised cysts after incubation in PB; 20 mM uridine for 18-24 h.

2.2.13 *MUTANT SCREENING*

P. infestans isolate ATCC48720 was used in attempts to obtain oligomycin C resistant mutants and isolates ATCC48720 and ATCC36609 in attempts to isolate 5-FOA (Fluorochem Co., U.S.A.) resistant mutants. Mutagenised cysts were screened for oligomycin C resistance by plating out on PBA containing 20 µg/ml oligomycin C (Sigma Chemical Co. plc., U.K.). Uridine auxotrophs were screened for by (a) plating out on PBA containing 500 µg/ml 5-FOA (both isolates) or (b) incubating mutagenised cysts in PB for 48 h, then filtering the cell suspension through 20 µm pore size nylon cloth to remove any actively growing cells. Cells that passed through the cloth were plated onto PBA containing 5-FOA or 20 mM uridine (both isolates).

2.3 RECOMBINANT DNA TECHNIQUES

2.3.1 *BACTERIAL STRAINS*

The following strains were used.

Escherichia coli	Strain	Genotype (nomenclature according to Bachmann, 1983)
	DH5α	*supE*44, Δ*lac*U169, (ϕ80 *lacZ* ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1. (Sambrook *et al.*, 1989)
	LE392	*hsdR*514, *supE*44, *supF*58, *lacY*1, *galK*2, *galT*22, *metB*1, *trpR*55. (Sambrook *et al.*, 1989).

Strain DH5α was used as a host for harbouring recombinant DNA plasmids and strain LE392 was used as a host for growth of bacteriophage λ. Strains were stored in media plus glycerol to 14 % (v/v) at -70° C. Single colonies of bacterial strains were reisolated for new cultures by scraping the surface of frozen cell stocks and spreading on a fresh LBA plate. Cultures were incubated at 37° C.

Table 2.1. Plasmids used in transformation experiments.

Selection System	Plasmid	Gene	Promoter	Terminator	Used to transform	Other sequences	References
Hygromycin B	pAN7-1	*hph*	*A. nidulans* (*gpdA*)	*A. nidulans* (*trpC*)	Several ascomycetes	-	Punt *et al.* (1987)
	pROH1	*hph*	*C. acremonium* (IPNS)	*E. coli*	*C. acremonium*	-	Harford (Glaxochem plc.) (per. comm)
	pLG90	*hph*	*S. cerevisiae* (*CYC1*)	*S. cerevisiae* (*CYC1*)	*S. cerevisiae*	2 μm *ORI* *URA3*	Gritz & Davies (1983)
	pRD1	*hph*	*P. megasperma* (actin)	*A. nidulans* (*trp C*)	No reports	-	Dudler, Switzerland (per. comm.)
G-418 [1]	pEB10	*kan*	*E. coli*	*E. coli*	*N. crassa*	*N. crassa am*	Bull & Wooton (1984)
	pBC10	*kan*	*E. coli*	*E. coli*	*N. crassa*	*N. crassa am*	Bull & Wooton (1984)
	pJL2	*kan*	*E. coli*	*E. coli*	*Phycomyces blakesleeanus*	*URA3* *Phycomyces* ARS	Revuelta & Jayaram (1986)
	pSV2*neo*	*neo*	SV40 early	*E. coli*	*Achlya ambisexualis* Mammalian cells	SV40 *ori* SV40 polyA site	Manavathu *et al.* (1988)
Phleomycin	pAN8-1	*ble*	*A. nidulans* (*gpdA*)	*A. nidulans* (*trp C*)	*A. niger*	-	van den Hondel, Netherlands (per. comm.)
	pUT332	*ble*	*S. cerevisiae* (*TEF1*)	*S. cerevisiae* (*CYC1*)	*S. cerevisiae*, *Fulvia fulva*	-	Cayla Co., France Oliver, U.K. (per. comm.)
	pUT701	*ble*	*Streptomyces*	*S. cerevisiae* (*CYC1*)		-	Cayla Co., France

Notes

1. *neo* encodes aminoglycoside-3'-phosphotransferase I from Tn*5*.

kan encodes aminoglycoside-3'-phosphotransferase II from Tn*903*(formerly Tn*601*).

2.3.2 *PLASMIDS*

Plasmid pUC13 was used in subcloning DNA fragments from recombinant bacteriophage λ DNA. The plasmids listed in Table 2.1 were used in attempts to transform *P. infestans* with antibiotic resistance genes controlled by heterologous signals. They were gifts from the corresponding authors listed in the table.

2.3.3 *BACTERIOPHAGE*

A Lambda DASH *Bam*H1 replacement vector kit (Stratagene plc., U.K.) was used in the construction of a *P. infestans* genomic library.

2.3.4 *MEDIA*

(a) Luria broth

Luria broth (LB) contained (per L): tryptone 10 g, yeast extract 5 g, NaCl 5 g. This was solidified with 1.2 % No. 2 agar (Lab M, U.K.) where required. Luria broth and agar (LBA) were the standard media used for bacterial cultures. For the selection of *E. coli* transformants ampicillin (Sigma plc., U.K.) was added to LB or molten LBA at 55° C, from a 25 mg/ml filter sterilised aqueous stock solution, to a final concentration of 50 μg/ml and 100 μg/ml respectively.

(b) SOC medium

SOC medium was used in the expression step of *E. coli* transformations and contained (per L): tryptone, 20 g; yeast extract, 5 g; NaCl, 0.58 g; KCl, 1.86 g; $MgCl_2.6H_2O$, 2.03g; $MgSO_4.7H_2O$, 2.46 g; glucose, 3.60 g.

(c) NYZDT broth

NYZDT broth powder (Gibco BRL plc., U.K.) was made up according to the manufacturer's instructions. This medium was solidified with 1.2 % agar (Lab M, U.K.. No.2) or 0.7 % agarose (Bethesda Research Laboratories Life Technologies Inc., U.S.A.. Ultrapure Grade) when used for Bottom or Top agar respectively in the propagation of bacteriophage λ-infected LE392 cells.

2.3.5 *PHENOL EXTRACTION AND ETHANOL PRECIPITATION OF DNA*

Equal volumes of phenol reagent (phenol, 100 g; chloroform, 100 ml; isoamyl alcohol, 4 ml; 8-hydroxyquinoline, 0.1 g) saturated with 10 mM Tris-HCl, pH 7.5 were added for the extraction of proteins from nucleic acid solutions. The phases were separated by centrifugation at 14,000 x g for 15 min. and the upper aqueous phase retained. Following phenol extraction, the DNA solution was usually extracted in the same way with chloroform reagent (chloroform:isoamyl alcohol (24:1)) to remove traces of phenol. The phases were separated by centrifugation in a bench top centrifuge (Wifuge plc., U.K.. Model: 500E) at 4,000 rpm for 5 min. and the upper aqueous phase retained.

Three methods were used to precipitate DNA from solution. (a) The DNA solution was made to 0.3 M with 3 M sodium acetate (pH 5.6), 2 vol. of 96 % (v/v) ethanol (-20° C) added and mixed. This was chilled at -20° C overnight or at -70° C for 30 min.. (b) After dilution with 3 vol. of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA), DNA dissolved in CsCl solutions from density gradients was precipitated by the addition of 0.05 vol. of 8 M LiCl, 2 vol. of absolute ethanol (-20° C) and incubation at -20° C for at least 2 h. (c) The DNA solution was chilled on ice and overlaid with an equal volume of 30 % polyethylene glycol (PEG) 8000 (Sigma Chemical Co. plc., U.K.); 1.5 M NaCl. The layers were slowly mixed together such that the DNA precipitated at the interface. The mixture was then incubated on ice for 1 h.

The precipitates were recovered by centrifugation at 14,000 x g, 4 ° C for 15-20 min. and the pellet rinsed in 70 % (v/v) ethanol, dried under vacuum and resuspended in an appropriate vol. of SDW or TE buffer.

2.3.6 *DNA ELECTROPHORESIS*

Horizontal agarose gels (0.8 % (w/v)) were prepared and run in TEA electrophoresis buffer (40 mM Tris; 20 mM sodium acetate; 1.8 mM EDTA; pH 7.8). Ethidium bromide was included in the gel at a concentration of 0.5 μg/ml. DNA molecular weight standards were provided by *Hin*dIII digestion of bacteriophage λ

DNA. DNA samples were mixed with 0.1 vol. of electrophoresis loading buffer (0.25 % bromophenol blue; 60 % sucrose; 100 mM EDTA), and electrophoresis continued until the bromophenol blue dye had travelled to within the last third of the gel. DNA binding ethidium bromide in gels was visualised under short wavelength UV light and photographed using a MP-4 Landcamera and Polaroid 667 film (Polaroid plc, U.K.).

2.3.7 *RESTRICTION ENZYME DIGESTION OF DNA*

Restriction enzyme (Northumbria Biologicals plc., U.K.) digests were performed according to the manufacturer's recommendations. A 2-5 fold excess of enzyme in a typical reaction volume of 20 ml was used to restrict plasmid and bacteriophage λ DNA and a 5-10 fold excess of enzyme in a 400 ml volume was used for genomic DNA. When further manipulations were to be performed following restriction enzyme digestion, phenol extraction and one sodium acetate/ethanol precipitation were carried out.

2.3.8 *ISOLATION OF RESTRICTION FRAGMENTS*

In most cases digested DNA was electrophoresed in a TEA buffered agarose gel until the desired fragment was resolved and could be excised and placed in a preweighed microfuge tube. The DNA was recovered using PrepaGene (Northumbria Biologicals plc., U.K.) according to manufacturer's instructions.

DNA fragments of < 500 bp, or in bands of low concentration, were recovered by electroeluting the DNA from the excised piece of agarose into a small vol of sterile TE buffer in a sealed dialysis bag. The DNA was concentrated by sodium acetate/ethanol precipitation.

2.3.9 *CONSTRUCTION OF HYBRID PLASMIDS*

Fragments of *P. infestans* DNA were amplified using plasmid vectors in *E. coli* strains.

(a) Ligation of DNA into plasmid vectors

Linearised plasmid vectors were prepared for ligation by dephosphorylation of 5' ends by incubation with calf intestinal phosphatase (CIP) (Boehringer Mannheim (Diagnostics and Biochemicals) plc., U.K.) according to Sambrook *et al.*, (1989).

DNA ligations were done using a 10 x ligase buffer giving a final concentration of 50 mM Tris-HCl, pH 7.8 @ 11° C; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM ATP; 100 µg/ml B.S.A.; 100 ng of the prepared vector, samples of purified *P. infestans* DNA fragment or total digested recombinant bacteriophage λ clone DNA (such that the vector was in 2-8 fold molar excess) and 0.1 units (cohesive end ligation) or 1 unit (blunt end ligation) (manufacturer's definitions) of T4 DNA ligase (Northumbria Biologicals plc., U.K.) in a final volume of 10 ml. Control ligations of CIP-treated linearised vector and non-CIP-treated linearised vector were also set up. Ligation reactions and controls were incubated at room temperature for 3 h followed by 4° C overnight. Suitable aliquots of the ligation mixtures and controls were used to transform *E. coli* strain DH5α

2.3.10 *ESTIMATION OF DNA CONCENTRATION*

The concentrations of plasmid DNA solutions were estimated by measuring the absorbance (Pye Unicam plc., U.K.. Model: SP6-550) of a 1/50 or 1/200 dilution of the plasmid at 260 nm against a TE buffer blank, in quartz glass cuvettes (Hellma plc., U.K.). DNA concentration was calculated from the equation OD_{260} x 50 x dilution factor = DNA concentration (µg/ml).

2.3.11 *PREPARATION OF COMPETENT CELLS*

10 ml of LB was inoculated with a single colony of *E. coli* strain DH5α and grown at 37° C at 250 rpm overnight in an orbital shaker. 0.8 ml of this culture was inoculated into 100 ml of LB and grown as before to an OD_{600} of 0.15 to 0.20. The cells were incubated on ice for 10 min. and centrifuged at 12,000 x g, 4° C, 1 min..The supernatant was discarded and the pellet resuspended in 25 ml of ice cold 100 mM

$MgCl_2$. The cells were again pelleted at 12,000 x g, 4° C, 1 min. and the supernatant discarded. The cells were resuspended in 25 ml of ice cold 100 mM $CaCl_2$, incubated on ice for at least 1 h, and pelleted as before. The supernatant was discarded and the cells resuspended in 5 ml of ice cold 100 mM $CaCl_2$; 14 % (v/v) glycerol. The cells were stored in 200 µl aliquots at -70° C and remained competent for at least a month.

2.3.12 *GENETIC TRANSFORMATION OF* E. coli

200 µl of competent cells from storage at -70° C were thawed on ice. Plasmid DNA was added and mixed by gentle pipetting. The cells were incubated on ice for 20 min., then heat shocked for 2 min. at 42° C and 0.8 ml of SOC medium added. After an expression period of 1 h at 37° C, an appropriate dilution series was made into SOC medium, the cells plated out onto selective LBA medium and incubated overnight at 37° C.

2.3.13 *PREPARATION OF PLATING BACTERIA AND INFECTION WITH BACTERIOPHAGE λ*

From a single colony, *E. coli* strain LE392 was grown overnight with shaking at 30° C in 25 ml of LB supplemented with 0.2 % maltose and 10 mM $MgSO_4$. The cells were pelleted at 1000 x g for 10 min. and gently resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5 (~8 x 10^8 cells/ml). These cells could be stored at 4° C for up to 5 day or, if maximum efficiencies were required, used fresh. 150 µl of plating cells were inoculated with phage in a 7.5 cm x 1.2 cm capped tube and preincubated for 20 min. at 37° C with gentle shaking to allow attachment of the phage to the cells. 3 ml of Top agar at 48° C was added, mixed by inversion and overlaid onto prewarmed plates of Bottom agar.

2.3.14 *PREPARATION OF RECOMBINANT BACTERIOPHAGE λ DNA*

This was based on the method of Zabarovsky and Turina (1988). The recombinant bacteriophage λ clone was plated out at a low enough plaque density to allow the isolation of a single plaque plug which was used to inoculate 10 ml of NYZDT medium containing 4 x 10^7 plating bacteria. The culture was shaken vigorously for 6-12 h at 37° C until the cells had lysed. 100 ml of chloroform were added, the lysate shaken for a further 2 min. and then centrifuged at 3,000 x g at room temperature for 10 min. to remove cell debris. 100 ml of 1 M $MgSO_4$ was added and the lysate titred for plaque-forming units (pfu). The lysate could be stored at 4° C with 0.5 ml of chloroform for 10 day without a significant reduction in titre.

The following day, a single colony of LE392 was grown in 20 ml of LB at 37° C, 250 rpm to an OD_{600} of 1.0, centrifuged at 3,000 x g for 10 min. and resuspended in 10 ml of 10 mM $MgSO_4$. From the 10 ml lysate 5 x 10^8 pfu of bacteriophage were added, to give a cell:bacteriophage ratio of approximately 30:1, and allowed to adsorb to the cells by incubation at 37° C for 10 min.. The whole suspension was inoculated into 500 ml LB;10 mM $MgSO_4$ and shaken vigorously for 10-12 h at 37° C until lysis occurred. 10 ml of chloroform was added and the culture shaken for a further 5 min.. Cell debris was removed by centrifugation at 3,000 x g, 4° C for 10 min. and solid PEG 6000 and solid NaCl added to the supernatant to final concentrations of 10 % (w/v) and 1 M respectively. The mixture was chilled on ice for 30 min., then centrifuged at 10,800 x g, 4° C for 10 min. and the pellet resuspended in 4.5 ml TE; 10 mM $MgCl_2$. After one chloroform extraction this suspension was centrifuged at 35,000 rpm in a L7-65B ultracentrifuge fitted with a SW65 swing bucket rotor (Beckman Instruments Co. U.S.A.) for 20 min. at room temperature and the pellet resuspended in 600 µl of TE; 10 mM $MgCl_2$. This was centrifuged for 2 min. in a microfuge, 6 µl of 10 % SDS and 8 µl of 0.5 M EDTA pH 8.0 added to the supernatant and incubated at 70° C for 15 min.. Following two phenol extractions and one chloroform extraction the DNA was sodium acetate/ethanol precipitated and resuspended in 800 µl of TE. This typically yielded several hundred µg of DNA.

2.3.15 *LARGE SCALE PREPARATION OF PLASMID DNA*

Two methods were used for large scale purification of plasmid DNA.

(a)Alkaline lysis method.

This was based on the method of Sambrook *et al.* (1989). A 2 L conical flask containing 500 ml of LB plus 50 µg/ml ampicillin was inoculated with 1 ml of an overnight culture of an *E. coli* strain carrying the required plasmid. This was incubated at 37° C, 250 rpm, overnight. The cells were pelleted by centrifugation at 10,800 x g for 5 min. at 4 ° C, resuspended in Solution I (50 mM glucose; 25 mM Tris-HCl, pH 7.5; 10 mM EDTA) and stood at room temperature for 5 min.. 100 ml of freshly prepared Solution II (0.2 M NaOH; 1 % SDS) were added and the mixture inverted gently several times and then stood on ice for 10 min.. To this, 150 ml of ice cold Solution III (3 M potassium acetate; 11.5 % (v/v) glacial acetic acid) were added, the suspension inverted sharply several times and stood on ice for 10 min. before being centrifuged at 12,200 x g, 4° C for 10 min.. The supernatant was carefully decanted, 0.6 vol. of ice cold isopropanol added to it, mixed well, centrifuged at 12,200 x g, 4° C for 10 min. and the pellet dissolved in 8 ml of TE buffer. This was phenol extracted once and then incubated with 50 mg/ml RNase A (Sigma Chemical Co. plc., U.K.) at 37° C for 1 h. At the end of the incubation the mixture was extracted twice with phenol:chloroform and then once with chloroform. The DNA was sodium acetate/ethanol precipitated, the pellet washed with 70 % ethanol, dried and resuspended in 1 ml of TE. The DNA was further purified by CsCl density gradient centrifugation.

(b) Qiagen method.

This was based on the protocol recommended for use with Qiagen ion exchange columns (Diagon, F.R.G.). 250 ml of LB plus 50 µg/ml ampicillin was inoculated with 0.5 ml of an overnight culture of an *E. coli* strain carrying the required plasmid. This was incubated at 37° C, 250 rpm, overnight. The cells were pelleted by centrifugation at 10,800 x g for 5 min. at 4 ° C and resuspended in 18 ml of an ice cold solution of 50 mM Tris-HCl, pH 7.4. 2 ml of freshly prepared 20 mg/ml lysozyme

solution were added and the suspension incubated on ice for 10 min.. 5 ml of 0.5 M EDTA (pH 8.0, adjusted with NaOH) solution were added and incubation on ice continued for 10 min.. 1 ml of a 2 % Triton X-100 solution was added and incubation on ice continued for 1 h. The mixture was centrifuged at 14,000 x g, 4° C for 45 min. to remove cell debris and chromosomal DNA. The supernatant (cleared lysate) was collected and treated with RNase A (final concentration 20 µg/ml) for 30 min. at 37° C. Proteinase K (freshly prepared) was added to a final concentration of 10 µg/ml and incubation at 37° C continued for 30 min.. The mixture was centrifuged at 14,000 x g, 4° C for 10 min. and 25 ml of the cleared lysate transferred to a new tube. To this, 5.5 ml of 5 M NaCl and 2.5 ml of 1 M MOPS, pH 7.0 were added. Plasmid DNA was recovered from this solution using Qiagen ion-exchange columns according to manufacturer's instructions.

2.3.16 *CsCl DENSITY GRADIENT CENTRIFUGATION OF PLASMID DNA*

The DNA solution was made up to 17.7 ml with SDW and 18.5 g of CsCl (Bethesda Research Laboratories Life Technologies Inc., U.S.A.. Ultrapure Grade) were dissolved in this, followed by 400 µl of 0.1 % (w/v) ethidium bromide. The solution was divided between two Sorvall 11.5 ml polyallomer ultracentrifuge tubes (Du Pont plc., U.K.) which were topped up with liquid paraffin and sealed. The tubes were centrifuged at 50,000 rpm at 20° C for 24 h. Resolved bands of DNA in the density gradient were visualised under UV light and the lower band corresponding to closed circular plasmid DNA was removed from the tube by side puncture using a hypodermic needle and syringe. Ethidium bromide was removed from the plasmid DNA solution by repeated extraction with butan-1-ol. CsCl was removed by dialysis against 2.5 L of TE at 4° C for 24 h with four changes of buffer. The DNA was concentrated by sodium acetate/ethanol precipitation and resuspended in an appropriate volume of TE. The yield was usually at least several hundred µg of DNA.

2.3.17 *PREPARATION OF* P. infestans *AND* A. nidulans *GENOMIC DNA*

(a) Large scale preparation of genomic DNA.

A. nidulans genomic DNA was prepared by the RNA method (see below) from 18 h CM cultures grown from an inoculum of conidia.

For large scale DNA extraction of *P. infestans* genomic DNA, mycelial mats were produced by growing an inoculum of sporangia in 40 ml of PB in 250 ml conical flasks for 8-12 day. The mats were harvested on a single layer of muslin and washed thoroughly with SDW. Excess water was gently squeezed from the mycelium which was then wrapped in foil and immediately frozen in liquid N_2. The mycelium was normally stored at -70° C. Three methods for the large scale preparation of *P. infestans* genomic DNA were tried in an attempt find one suitable for obtaining DNA that was reasonably pure and could be efficiently cut with restriction enzymes.

(i) Cetyltrimethylammonium bromide (CTAB) method.

4-6 g of lyophilised mycelium was ground in liquid N_2 in a mortar and pestle. The powder was resuspended in 10 ml of prewarmed extraction buffer (0.7 M NaCl; 1 % CTAB (Sigma Chemical Co. plc., U.K.); 50 mM Tris-HCl, pH 8.0; 250 mM EDTA, pH 8.0; 1 % 2-mercaptoethanol) at 65° C in a sterile 250 ml conical flask. After incubating for 1 h the suspension was cooled to room temperature and extracted with 1 vol. of chloroform:octanol (24:1) by gentle mixing until the two phases combined, and then centrifuged at 16,000 x g for 20 min. (at above 15° C to prevent CTAB precipitating). The top aqueous layer was removed and extracted twice more. DNA was precipitated from the final supernatant by the addition of 1.2 vol. of ice cold isopropanol and mixed by several sharp inversions. The mixture was centrifuged at 12,000 x g for 15 min. at 4° C yielding an upper white cloudy layer, a small oily bottom layer and a large pasty pellet. The pellet was gently dissolved in SDW and the DNA further purified by bisbenzimide (Sigma Chemical Co. plc., U.K.) CsCl density gradient centrifugation. This typically yielded up to several hundred µg of DNA.

(ii) Sarkosyl/SDS method.

Approximately 5 g wet weight of mycelium was ground in liquid N_2 with a sterile mortar and pestle. The powder was transferred to a glass universal bottle and 18 ml of SET buffer (0.5 M sucrose; 25 mM Tris-HCl, pH 7.5; 20 mM EDTA) added. Sarkosyl (BDH Chemicals plc., U.K.. Biochemical Grade) and SDS were added to final concentrations of 4 % (v/v) and 0.5 % (w/v) respectively and the mixture incubated at 60° C for 1-1.5 h. The mixture was centrifuged at 14,000 x g, 4° C for 10 min., the supernatant recovered, brought to 35° C and Proteinase K (Boehringer Mannheim, (Diagnostics and Biochemicals) plc., U.K.) added to a final concentration of 200 µg/ml. This was then incubated at 35° C for 5-16 h. The extract was cooled on ice and the DNA PEG/NaCl precipitated. After centrifugation at 14,000 x g, 4° C for 20 min. the pellet, plus any material floating at the surface, was resuspended in 8 ml of 50 mM Tris-HCl, pH 8.0; 10 mM EDTA and extracted once with phenol. RNase A was added to the DNA solution to a final concentration of 50 µg/ml and incubated at 37° C for 1 h before being phenol extracted 4-5 times or until the interface was clear. An equal volume of chloroform was added and the mixture centrifuged at 14,000 x g at room temperature for 1 h. The aqueous phase was then removed and the DNA sodium acetate/ethanol precipitated. The DNA was resuspended in an appropriate volume of TE buffer and further purified by bisbenzimide/CsCl density gradient centrifugation. The yield was typically around 150 µg of high molecular weight DNA.

(iii) RNA method.

This was based on the method of Kolar *et al.* (1988). Approximately 10 g wet weight of mycelium was ground in liquid N_2 using a sterile mortar and pestle. The powder was suspended in 15 ml of freshly prepared TEN buffer (0.2 M Tris-HCl, pH 8.5; 0.05 M EDTA; 0.25 M NaCl; 48 mg/ml *p*-aminosalicylic acid (Sigma Chemical Co. plc., U.K.); 8 mg/ml tri-isonaphthalene sulphonic acid (sodium salt) (Kodak Laboratory and Research Products, U.K.) (the TEN buffer was stood on ice for 15 min. before use, to allow the precipitate to settle out). The extraction mixture was phenol

extracted once and then the phenol phase re-extracted twice at 68° C with an equal volume of TEN buffer. The aqueous supernatants were pooled and extracted twice with phenol and once with chloroform. The DNA was sodium acetate/ethanol precipitated three times to further purify it and finally resuspended in an appropriate volume of TE buffer. Co-purified RNA was removed by treatment with RNase A followed by one phenol and one chloroform extraction.

(b) Small scale isolation of genomic DNA

This was based on the method of Leach *et al.* (1986).

Mycelium was harvested on Whatman No. 1 filter paper (W. & R. Balston plc., U. K.). 200 mg wet weight of mycelium was lyophilised or diced with a scalpel and placed in a 12 x 75 mm glass tube. 0.7 ml of LETS buffer (0.1 M LiCl; 10 mM EDTA; 0.5 % SDS; 10 mM Tris, pH 8.0) was added followed by glass beads (0.45 mm) to the top of the liquid. The tube was covered with parafilm and vortexed for 1-2 min. at top speed. 1 ml of Tris-saturated phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added and the tube vortexed at medium speed for 20 sec.. The tube was centrifuged at 3000 rpm for 5 min. in a bench top centrifuge (Wifuge, U. K.. Model: 500E). 500 µl of supernatant was transferred to a microfuge tube and the DNA precipitated with 1 ml of 96 % ethanol at -70° C for 15 min.. The DNA was pelleted by centrifugation at full speed 4° C for 15 min., the pellet dried and resuspended in 40 µl of TE buffer. The resultant DNA was quite dirty and so restriction enzyme digests were done in a volume of 400 µl using 10 µl of DNA solution to 25 Units of restriction enzyme plus 20 µg of RNase A.

2.3.18 *CsCl DENSITY GRADIENT CENTRIFUGATION OF GENOMIC DNA*

This procedure was based on that of Garber & Yoder (1983). *P. infestans* genomic DNA was dissolved in 13.832 ml of TE buffer followed by 17.08 g of CsCl (Bethesda Research Laboratories Life Technologies Inc., U.S.A.. Ultrapure Grade). Once these were dissolved 0.168 ml of 10 mg/ml bisbenzimide (Sigma Chemical Co. plc., U.K.) solution was added and the mixture divided between two Sorvall 11.5 ml

polyallomer ultracentrifuge tubes (Du Pont plc., U.K.). The tubes were topped up with liquid paraffin, sealed and centrifuged at 50,000 rpm, 20° C for 24 h. Two bands were resolved, a thin upper band of mitochondrial DNA and a broader lower band of nuclear DNA. The bands were isolated separately and individually recentrifuged with the same concentration of CsCl but without additional bisbenzimide. The DNA bands were isolated once more and the bisbenzimide removed by repeated extraction with CsCl-saturated isopropanol. The CsCl was removed by precipitating the DNA with LiCl/ethanol and thoroughly rinsing the pellet with 70 % (v/v) ethanol. The DNA was finally resuspended in an appropriate volume of TE buffer.

2.3.19 *RADIO-LABELLING OF DNA WITH ^{32}P*

(a) Labelling of double stranded DNAs.

Double stranded DNA was labelled using a Multiprime kit (Amersham International plc., U.K.) according to the manufacturer's instructions. Typically 50 ng of DNA were labelled with 30 μCi of [α-^{32}P]dCTP. Unincorporated nucleotides were removed by passing the products of the labelling reaction down a NICK column (Pharmacia LKB Biotechnology, Sweden), eluting with TE. The probe was boiled for 3 min. and quenched on ice to denature it before hybridisation.

(b) Labelling of oligonucleotides.

Oligonucleotide probe mixtures were labelled as follows:

Oligonucleotides	1 μl (300 ng)
1 M Tris-HCl, pH 7.6	5 μl
50 mM $MgCl_2$	10 μl
200 mM 2-mercaptoethanol	5 μl
T4 polynucleotide kinase	5-10 units
[γ-^{32}P]ATP	50 μCi
SDW	to 50 μl

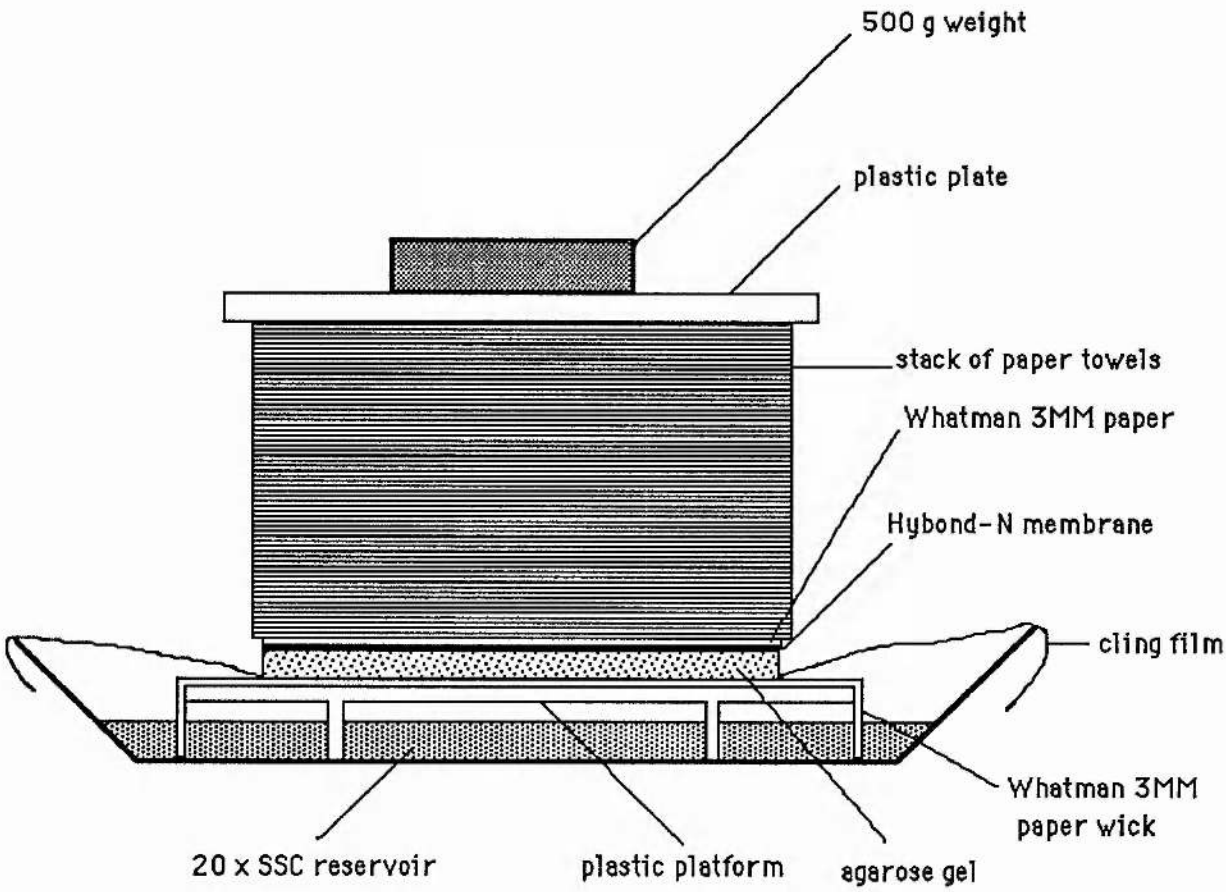


Figure 2.1. Arrangement for Southern transfer.

The reaction mixture was incubated at 37° C for 1 h. Removal of unincorporated radiolabel was not necessary.

2.3.20 *SOUTHERN TRANSFER AND HYBRIDISATION*

(a) Stock solutions.

(i) 20 x SSPE	NaCl	3 M
	NaH_2PO_4	0.2 M
	EDTA	25 mM
		pH 7.4 (with NaOH)

(ii) Heat denatured herring sperm DNA.

Herring sperm DNA (Sigma Chemical Co., U.K.. Type XIV) was dissolved in SDW to a concentration of 10 mg/ml and sheared by ultrasonication and passage through a 17-gauge hypodermic needle several times. The solution was then stored at -70° C until required. Before addition to prehybridisation solution the required volume of herring sperm DNA stock solution was denatured in a boiling water bath for 10 min. and then quenched on ice for 5 min.. The herring sperm DNA was then added to the rest of the prehybridisation solution ingredients.

(b) DNA transfer

DNA fragments were separated by agarose gel electrophoresis after which any unused parts of the gel were trimmed away and the gel rinsed in distilled water. The gel was then sequentially soaked in several gel volumes of depurination solution (1 M HCl), denaturing solution (0.5 M NaOH; 1.5 M NaCl) and neutralising solution (0.5 M Tris-HCl, pH 7.0; 3.0 M NaCl) for 20, 40 and 40 min. respectively, rinsing with distilled water between each. A rectangle of Whatman 3 MM paper (W & R Balston plc., U.K.) was wetted in 20 x SSC (3 M NaCl; 0.3 M trisodium citrate), laid over a plastic platform so that it overhung at each end to act as a wick, and placed in a plastic tray containing 20 x SSC (Fig 2.1). Any air trapped between the paper and the platform was removed by rolling a clean glass rod over the surface. The gel, with its original underside now uppermost, was placed on the paper and any trapped air removed as

before. Exposed surfaces of 3 MM paper and the 20 x SSC reservoir were covered with cling-film to minimise evaporation and short circuiting of the flow of 20 x SSC from the 3 MM paper to upper layers. A piece of Hybond-N (Amersham International plc., U.K.) nylon hybridisation membrane, cut to size, was laid over the gel and rolled over with a glass rod to ensure the removal of air bubbles and good contact with the gel. A dry piece of 3 MM paper of the same size was placed over the nylon membrane and this in turn was covered with a 10 cm high stack of paper towels also of the same dimensions. A plastic plate and a 500 g weight were placed on top of the stack. Transfer of the DNA was allowed to proceed overnight. The paper towels and 3 MM paper above the gel were removed and the position of the gel slots marked on the nylon membrane with a pencil. The gel was discarded and the nylon membrane allowed to air dry on a sheet of 3 MM paper at room temperature. The membrane was wrapped between two layers of cling-film, placed DNA side down on a UV transilluminator (Ultra-violet Products, Inc., USA. Model: TM 40) and exposed to short wave-length UV light for 3 min. to covalently link the DNA to the membrane. The filter was either probed immediately or stored at -20° C in cling-film until required.

(c) Hybridisation

The Hybond-N membrane was incubated in approximately 0.2 ml of prehybridisation solution (5 x SSPE; 6 % PEG 6000; 0.5 % skimmed milk; 1 % SDS; 0.1 % sodium pyrophosphate; 0.25 mg/ml heat- denatured herring sperm DNA) per square centimetre of membrane for a minimum of 3 h at a suitable temperature (Table 2.2) in a shaking waterbath. The ^{32}P-labelled probe (denatured if necessary) was added (for oligonucleotide probes the volume of prehybridisation solution was usually reduced to 10 ml before hand) and incubation continued overnight. Unhybridised probe was removed by rinsing the filter in large volumes of washing solution (Table 2.2) at the same temperature used for hybridisation for 20 min. in each solution until the desired stringency was reached.

Table 2.2 Hybridisation and washing conditions for Southern transfer membranes

Probe type	Temperature of prehybridisation, hybridisation and washing[a]	Washingsolutions[b]
(i) Gene fragment		
Heterologous	56° C	5 x SSC; 0.1 % SDS; 0.1 % SSPE
		3 x SSC; 0.1 % SDS; 0.1 % SSPE
		1 x SSC; 0.1 % SDS; 0.1 % SSPE
Homologous	65° C	5 x SSC; 0.1 % SDS; 0.1 % SSPE
		3 x SSC; 0.1 % SDS; 0.1 % SSPE
		1 x SSC; 0.1 % SDS; 0.1 % SSPE
		0.2 x SSC; 0.1 % SDS; 0.1 % SSPE
		0.1 x SSC; 0.1 % SDS; 0.1 % SSPE
(ii) Oligonucleotide mixture	$(Td_{min.} - 15)^{\circ}$ C	5xSSC;0.1 % SDS; 0.1 % SSPE

Notes: (a) $Td_{min.}$ is the temperature in ° C at which half of the oligonucleotide-genomic DNA duplexes are disassociated as calculated for the oligonucleotide with the lowest GC content and is given by the formula $Td_{min.} = 2(A+T)+4(G+C)$ (Zeif & Geliebter, 1987). If background radiation on the membrane was unacceptably high the washing temperature was raised in steps of 3° C until the level was sufficiently reduced.

(b) Where applicable, washing solutions were used in order of decreasing SSC concentration until background radiation on the membrane was insignificant.

When it was required to rehybridise a membrane with another probe the previous one was stripped off by boiling the membrane in a large volume of 0.1 % SDS for 10 min.. Drying out of hybridised membranes was avoided as this could make subsequent removal of the probe difficult.

(d) Autoradiography

Following hybridisation and washing, membranes were sealed in plastic bagging, placed in an autoradiography cassette fitted with intensifying screens and covered with either Fuji RX film or the more sensitive Kodak XAR5 film according to

the relative strength of signal expected. Loaded cassettes were stored at -70° C until the film was removed and processed in a Fuji RG II X-ray film processor.

2.3.21 *SCREENING OF THE* P. infestans *GENE LIBRARY*

(a) Plaque DNA transfer

Bioassay plates containing the gene library were chilled to 4° C for 1 h to firm up the agar and a 22 cm x 22 cm Hybond-N nylon membrane (Amersham International plc., U.K.) laid on top. Pin pricks were made in each corner of the filter to aid subsequent orientation of the membrane with the plate. After 1 min. the filter was removed from the agar and placed plaque side up onto Whatman 3 MM (W & R Balston plc.,U.K.) paper saturated with denaturing solution for 20 sec., then onto Whatman 3 MM paper saturated with neutralising solution for 2.5 min.. The membrane was transferred to a large volume of 3 x SSC and washed for 1 h at room temperature with several changes of solution and then air dried on a sheet of Whatman 3 MM paper. The membrane was sandwiched between two sheets of cling film and the plaque side exposed to shortwave-length UV light for 3 min. to bind the DNA to the nylon. This procedure was repeated for three further replicate membranes except they were left in contact with the plaques on the bioassay plate for 3, 5 and 7 min. respectively. Replicate membranes for each plate were numbered sequentially 1-4.

(b) Hybridisation of the membranes

(i) Oligonucleotide probes

Replicate membranes Numbers 1 and 3 were incubated at a suitable temperature (see Table 2.3) overnight in a shaking waterbath in 505 ml of prehybridisation solution which was replaced with 250 ml of fresh solution the next day. The products of three oligonucleotide labelling reactions were passed down a NICK column (Pharmacia LKB Biotechnology, Sweden) and the oligonucleotide fraction added to the prehybridisation solution. Incubation of the membranes was continued overnight. The membranes were washed in 6 x SSC; 0.05 % sodium pyrophosphate at a suitable temperature (Table 2.3) until the background level of radiation on the membranes

was acceptable, as measured by a hand-held Geiger counter (Mini-instruments plc., U.K.. Type 5.10). The membranes were autoradiographed at -70° C as indicated in Table 2.3.

Table 2.3 Conditions for hybridisation, washing and autoradiography of oligonucleotide-probed library membranes.

Oligonucleotide probe	Prehybridisation and hybridisation temperature	Washing temperature	Autoradiography (time and film type)
726C (β-tubulin) [1]	30° C	42° C	6 days. Fuji RX II

Notes.

1. Synthesised by OSWEL DNA Service, University of Edinburgh.

(ii) Gene fragment probes

Replicate membranes Numbers 2 and 3 were incubated in 505 ml of prehybridisation solution for 3 h at 54° C in a shaking waterbath. The denatured ^{32}P-labelled probe was added to this and incubation continued overnight. The membranes were washed in washing solution to an appropriate stringency (Table 2.3) at 54° C and then autoradiographed at -70° C with Kodak XAR5 film for 3 day.

2.3.22 *SCREENING OF POSITIVE PLAQUES*

Positive plaques identified from the autoradiogram were picked and resuspended in 1 ml SM buffer (100 mM NaCl; 10 mM MgCl2; 0.05 % gelatin; 10 mM Tris-HCl, pH 7.6), treated with chloroform to kill bacterial cells, and replated on petri dishes of Bottom agar at a suitable plaque density. Bacteriophage λ DNA was transferred to 82 mm diameter Hybond-N disc membranes (Amersham

International plc., U.K.) as above (Section 2.3.21) but with transfer times of 1 min. and 5 min. for duplicates. Membranes were treated with denaturing solution and neutralising solution as above and transferred to 3 x SSC; 0.1 % SDS. Bacterial debris was removed by gently rubbing the membrane between two gloved hands and rinsing in 3 x SSC;0.1 % SDS for 1 h with several changes of solution. Membranes were finally rinsed in 3 x SSC, air dried on Whatman 3 MM paper (W & R Balston plc., U.K.) and exposed to UV light as before.

(a) Oligonucleotide probes.

The membranes were prehybridised for 3 h at 40° C (β-tubulin screen), the products of one oligonucleotide labelling reaction added without separation and incubation continued overnight. The membranes were washed in 6 x SSC; 0.05 % sodium pyrophosphate at 37° C for 1-2 h and then at the hybridisation temperature for 20-60 min.. The membranes were autoradiographed with Kodak XAR5 film for 6-7 day. The procedure was repeated until a single pure positive plaque could easily be isolated.

(b) Gene fragment probes.

Disc membranes were prehybridised, hybridised and washed in the same way as the primary library screen membranes.

2.3.23 *DNA SEQUENCING*

DNA was subcloned into pUC13 and the insert sequenced by means of the dideoxy chain termination method using a Sequenase II kit (United States Biochemical Co., U.S.A.), according to the manufacturer's recommendations. Oligonucleotide primers, other than the 'universal' and 'reverse' primers supplied with the kit, were purchased from Dr. A. Hawkins of the Department of Genetics, University of Newcastle.

(a) Solutions.

(i) 40 % acrylamide solution	Acrylamide	38 g
	NN'-Methylenebisacrylamide	2 g
	Distilled water	to 100 ml

The solution was deionised by mixing with 25 g of 'Amberlite' resin (BDH Chemicals plc., U.K.. Analytical Grade) for 30 min. The 'Amberlite' was removed by filtration and the solution stored at 4° C in the dark.

(ii) Gel solution	40 % acrylamide solution	100 ml
(for electrophoresis of	10 x TBE	20 ml
oligonucleotide primers).	Urea	100 g
	Distilled water	to 200 ml

The solution was stored in the dark at 4° C.

(iii) Sequencing gel solution	Urea	430 g
	10 x TBE	100 ml
	40 % acrylamide solution	150 ml
	Distilled water	to 1 L

The solution was stored in the dark at 4° C.

(iv) Sample loading buffer	Deionised formamide	8 ml
(for oligonucleotide	0.5 M EDTA (pH 7.0)	100 µl
primers)	Bromophenol blue	5 mg
	Xylene Cyanol FF	5 mg
	Distilled water	to 10 ml

(b) Purification of oligonucleotides

Oligonucleotides, supplied as an orange-brown residue, were taken up in 100 µl of SDW, precipitated by addition of potassium acetate (to 0.25 M), 3-4 vol. of ethanol and incubated at -70° C for at least 2 h. The precipitate was pelleted by centrifugation dried briefly under vacuum, without washing in 70 % ethanol. The pellet was resuspended in 10 µl SDW.

One of a pair of gel-casting glass plates were siliconised with Repelcote (BDH Chemicals plc., U.K.) and assembled with 0.4 mm spacers. 70 ml of gel solution, 520 µl of 10 % ammonium persulphate and 52 µl of TEMED were added, mixed quickly and the solution poured between the glass plates. A 'sharks tooth' comb (4 mm wells) was positioned upside down at the top of the gel and the glass plates clamped to hold it in position. After allowing the gel to set for 1 h the comb was inverted and the top of the gel washed with 1 x TBE. The gel was prerun with 1 x TBE at 2 kV for 40 min.. The wells were washed once more prior to sample loading to remove urea.

2 µl of the oligonucleotide solution was mixed with 2 µl of loading buffer and heated at 95° C for 2 min. The samples were loaded immediately, leaving an empty lane between samples. The samples were run for approximately 3 h until the bromophenol blue had migrated about 30 cm from the sample wells. The glass plates were carefully separated, leaving the gel attached to the non-siliconised one. Cling film was stretched over the gel and the orientation of the of the wells marked on it. The gel and cling film were peeled off the glass plate and a second sheet of cling film laid over the gel to form a wrinkle-free sandwich.

The bands were observed under a 254 nm wave-length UV lamp (UV Products Inc., U.S.A.. Model: UVGL-58) and the position of the band at the top of the ladder marked. An arbitrary estimate of the amount of DNA contained in the band was made on a scale of 1 to 5. A gel slice containing the band was cut out and placed in an eppendorf tube containing 100 µl of SDW and incubated at room temperature for at least 2 h. The eluted oligonucleotide was desalted using a spun column (Sambrook *et al.*, 1989) and its concentration measured by absorbance at 260 nm on a SP6-550 spectrophotometer (Pye Unicam plc., U.K.).

(c) Preparation of sequencing gels

Sequencing was performed using a Sequi-Gen sequencing rig (Biorad Laboratories plc., U.K.) which was prepared and assembled according to manufacturer's instructions. The bottom of the gel space was sealed using 50 ml of sequencing gel solution + 120 µl of 25 % ammonium persulphate (freshly prepared) +

120 µl of TEMED (Sigma Chemical Co. plc., U.K.) which was allowed to set for approximately 30 min.. The gel was poured using 120 ml of sequencing gel solution +120 µl of freshly prepared ammonium persulphate +120 µl of TEMED. Two 0.4 mm thick 'sharks tooth' combs were inserted at the top of the gel space to a depth of 5 mm and clamped in place. The gel was allowed to set for at least 1 h before the sharks tooth combs were removed and the top of the gel irrigated with 1 x TBE. The sharks tooth combs were replaced so the points of the teeth just touched the gel. The gel was prerun at 2 kV with 400 ml of 1 x TBE in the bottom buffer reservoir and approximately 1.4 L in the top reservoir until the gel temperature had reached 55° C.

(d) Electrophoresis and autoradiography

Samples were heated to 90° C for 2 min. immediately prior to loading. The top of the gel was irrigated with 1 x TBE to flush away urea that had diffused out of the gel before 2 µl of each sample was loaded. Electrophoresis was continued for approximately 3 h, adjusting the voltage as necessary to maintain the gel temperature to within 50-55° C. The electrophoresis buffer was discarded down a sink designated for disposal of radioactive waste. The gel rig was dismantled leaving the gel adhered to one glass plate which was then gently agitated in 1 L of 10 % acetic acid for 20 min. to leach out the urea. After draining the glass plate and gel a sheet of 3 MM paper (W & R Balston plc., U.K.) cut to size was laid over the gel and smoothed over with a 1 ml glass pipette. The gel was carefully lifted off the glass plate, attached to the paper, and placed in a gel drier set at 80° C for 45 min.. The dried gel was placed in an autoradiography cassette lacking intensifying screens and exposed to Fuji RX film at room temperature for 20-36 h. The film was processed using a Fuji RG II X-ray film processor.

2.3.24 *COMPUTING*

DNA and amino acid sequence comparisons and sequence motif searches were performed using the DIAGON and ANALYSEQ programmes in the STADEN protein and nucleic acid sequence analysis package written by Roger Staden, Department of Molecular Biology, University of Cambridge.

2.3.25 *CONTAINMENT AND SAFETY*

All experiments described in this thesis were conducted with reference to the Genetic Manipulation Advisory Group's guidelines on safety and containment conditions for such work. All procedures fall within the category of Good Microbiological Practice. Chemicals were used and disposed of according to the Control Of Substances Hazardous to Health (COSHH) regulations.

Chapter 3

MUTANT ISOLATION STUDIES

3.1 INTRODUCTION

Most selectable marker systems based on transformation to prototrophy require the isolation of suitable auxotrophic mutant strains to act as recipients for a gene that complements the defect. Examples of such systems that are applicable to many fungi are those in which acetamide (Tilburn *et al.*, 1983), acetate (Hargreaves & Turner, 1989) or nitrate utilisation (Unkles *et al.*, 1989) or uridine monophosphate (UMP) (Begueret *et al.*, 1984; Ballance & Turner, 1985; Banks & Taylor, 1988), leucine (Beggs, 1978; van Heeswijck & Roncero, 1984), methionine (Iimura *et al.*,1987), tryptophan (Case *et al.*,1979; Yelton *et al.*, 1984; Binninger *et al.*, 1987) or arginine (Buxton *et al.*, 1985) prototrophy is the selectable phenotype. The acetamide utilisation system is unlike the others in that it does not necessarily require the isolation of a mutant strain. Because the conversion of acetamide to ammonium takes place in a single step this system can be used in species or strains which are naturally unable to efficiently utilise acetamide as a sole nitrogen source (Kelly & Hynes, 1985; Turgeon *et al.*, 1985; Beri *et al.*, 1987).

The use of marker systems based on some aspect of nitrogen metabolism is difficult in *P. infestans* due to its fastidious nitrogen requirements. *P. infestans* is unable to utilise nitrate as a sole nitrogen source and many strains grow poorly on ammonium (Hohl, 1983), the product of acetamide catabolism. Inter-isolate variability in growth responses to other nitrogen sources such as amino acids also makes selectable marker systems requiring the use of nitrogen-defined media and/or mutants of nitrogen metabolism unattractive for development in *P. infestans*.

A system based on transformation to UMP prototrophy, although requiring isolation of an auxotrophic mutant strain (*pyr* $^-$), does not demand the stringent

restrictions on media composition required by systems based on amino acid biosynthesis. Ultimately, the loss of the UMP biosynthetic pathway results in an inability to produce cytidine triphosphate (CTP) and deoxythymidine monophosphate (dTMP) which are necessary for synthesis of nucleic acids. Therefore the growth medium need only be deficient in CTP and dTMP precursors from downstream of the pathway lesion to permit positive selection of prototrophic transformants. *pyr*$^-$ mutants can be grown on such a medium supplemented with uridine. The formation of a *P. infestans* homozygous *pyr*$^-$ mutant directly by mutagenesis requires either all copies of a given gene to be 'hit' or recombination between mutant and wild type alleles resulting in the generation of an auxotroph. In either case it is probable that large numbers of cells would need to be screened. Fortunately fungal mutants in orotidine-5'-phosphate decarboxylase and orotate phosphoribosyl transferase can be positively screened for by resistance to 5-fluoro-orotic acid (5-FOA) (Boeke *et al.*, 1984; Razanamparany & Begueret, 1986).

Oligomycin C resistance (OliC^R) could be developed as a homologous transformation system for *P. infestans* (see Section 1.3.1). The advantage of Oli C^R over repair of uridine auxotrophy as a selectable marker is that it is not necessary to obtain a mutant recipient strain in every *P. infestans* isolate of interest. The ATP synthase subunit 9 genes of *Aspergillus nidulans*, *A. niger* and *Penicillium chrysogenum* are nuclear encoded and translated on cytoplasmic ribosomes whereas in *Saccharomyces cerevisiae* the gene is located and translated in the mitochondrion. The mitochondrial genomes of *Neurospora crassa* and *A. nidulans* contain DNA sequences similar to the nuclear ATP synthase subunit 9 gene but it is not known if they are expressed (Ward & Turner, 1986; Bull *et al.*, 1988). The cellular location of the analogous gene in *P. infestans* is not known but there may be important implications for phenotypic expression if it is located in the mitochondrion. It is unlikely that the product of a mitochondrial gene would be targeted to the mitochondrion if the transforming DNA is maintained outwith the organelle, autonomously or as a result of nuclear chromosomal integration - the fate of

transforming DNA in most transformation systems for ascomycetes (e.g. Ballance, 1986), basidiomycetes (e.g. Binninger *et al.*, 1987) and Oomycetes (Manavathu *et al.*, 1988; H. Prell, per. comm.). It may be possible to clone and fuse a signal sequence to the gene so the gene product is targeted to the mitochondrion but this has been not been a particularly successful approach in other lower eukaryotes (Boynton *et al.*, 1988). Recently, bioballistics - transformation by means of DNA coated microprojectiles - has been shown to be one of the few techniques whereby mitochondria can be transformed thus opening the way for direct complementation of mutations in the mitochondrial genome (Johnston *et al.*, 1988).

The lack of a manipulable haploid stage in the life cycle of *Phytophthora* has inhibited genetic characterisation of the genus. In spite of this difficulty some induced auxotrophic and/or antibiotic resistant mutant strains have been obtained in *P. megasperma* f. sp. *glycinea* (Layton &Kuhn, 1988), *P. megasperma* var. *sojae* (Long & Keen, 1977), *P. capsici* (Castro *et al.*, 1971), *P. drechsleri* (Castro *et al.*, 1971; Shaw & Khaki, 1971), and *P. infestans* (Shattock & Shaw, 1975). The antibiotic resistant mutants were isolated by direct selection and the auxotrophic mutants through conventional forms of filtration enrichment or rescue techniques. Elliott and MacIntyre (1973) obtained methionine auxotrophs of *P. cactorum* through a different method, by selfing colonies derived from mutagenised zoospores and screening the progeny for segregation of auxotrophic mutations. Unfortunately such an approach is much more difficult for a heterothallic species such as *P. infestans*. There are no reports in the literature of the successful isolation of auxotrophic mutants of *P. infestans* although they have been isolated in other heterothallic species of *Phytophthora* (Castro *et al.*, 1971; Shaw & Khaki, 1971).

As with the auxotrophic and antibiotic resistant mutants isolated in other species of *Phytophthora*, the phenotypes of the antibiotic resistance mutants isolated by Shattock & Shaw (1975) could be due to mutation in any of a number of genes and so it is not possible to estimate from any of these studies an approximate frequency at which *pyr* $^-$ or Oli C^R mutants might arise in *P. infestans*. However, the data in Table 3.1

Table 3.1. Isolation frequencies of antibiotic resistant mutants.

Organism	Antibiotic	Approximate number of cells screened per mutant isolated
Aspergillus niger [1]	Oligomycin C	5×10^7
P. drechsleri [2]	Actidione	3.45×10^8
	Chloramphenicol	1.67×10^9
	Tetracyclin	1.67×10^9
P. infestans [3]	Streptomycin	4.17×10^{10}
	Chloramphenicol	4.17×10^{10}

Notes:

1. Ward *et al.* (1988)
2. Shaw & Khaki (1971)
3. Shattock & Shaw (1975)

do suggest that quite substantial numbers of cells would probably need to be screened to obtain these mutants even by conventional standards of microbial mutant isolation.

3.2 RESULTS

3.2.1 *IDENTIFICATION OF A SUITABLE MEDIUM FOR THE ISOLATION OF* P. infestans *URIDINE AUXOTROPHS*

The growth responses of isolate I230 to various nitrogen sources were tested using a defined medium based on that of Huang *et al.* (1980) containing sodium nitrate, ammonium sulphate, and various amino acids as nitrogen sources to a total nitrogen concentration of 10 mM. Plates of these media were inoculated with mycelial plugs, taken from PBA cultures, and encysted zoospores washed by centrifugation with SDW. The plates were incubated for 14 days at 18° C.

Mycelial inoculum was unable to utilise nitrate as a sole nitrogen source but grew slowly and densely with ammonium as the nitrogen source. The colony diameter and the density of mycelial growth from plug inocula on media containing sodium aspartate, arginine, sodium glutamate, glutamine or arginine as sole nitrogen sources were highly variable (Fig. 3.1). None of these media, nor one supplemented with all five amino acids together, supported macroscopic growth from encysted zoospore inocula (data not shown). Reports on the development of this type of medium either describe only the growth of mycelial inocula (Huang *et al.*, 1980) or do not state the type of inoculum used (Hwang *et al.*, 1965). The growth achieved by isolate I230 was considered insufficient for this type of medium to be used in screening for mutants of *P. infestans*.

PBA was tested for its ability to support growth of fungal uridine auxotrophs. Conidia of a uridine auxotrophic strain of *A. nidulans*, defective in orotidine-5'-phosphate decarboxylase (strain G191), and a uridine prototrophic strain (strain G191), were plated onto two media: (i) PBA containing 0.2 mM arginine, 0.33 mM methionine and 0.8 mg/ml para-aminobenzoic acid and (ii) the same medium but

additionally supplemented with uridine to 20 mM. Arginine, methionine and para-aminobenzoic acid were required to accommodate other auxotrophic mutations in these strains. Both strains grew well on uridine-supplemented PBA but only the uracil prototrophic strain (G34) grew at all on unsupplemented PBA (Fig. 3.2). 20 mM uridine did not impair the growth of *P. infestans* mycelial or cyst inocula (data not shown) and so it was concluded that uridine-supplemented PBA would be suitable for the selection of *P. infestans* orotidine-5'-phosphate decarboxylase mutants and non-supplemented PBA would be suitable for the selection of uridine prototrophic transformants.

3.2.2 *KILL CURVES FOR 5-FOA AND OLIGOMYCIN C*

The Minimal Inhibitory Concentration (MIC) of 5-FOA for encysted zoospores was determined for *P. infestans* isolates ATCC48720, I259, I230 and ATCC36609 (Table 3.2). ATCC48720 was used initially in mutagenesis experiments, being the best sporulating isolate then available. Later, ATCC36609, a higher sporulating isolate was obtained and used in subsequent mutagenesis experiments. The respective concentrations of 5-FOA in Table 3.2 were used to select for mutants.

Table 3.2 Minimum inhibitory concentrations of 5-FOA and oligomycin C (μg/ml).

Isolate	ATCC48720		I259		I230		ATCC36609	
Cell Type[1]	M	Z	M	Z	M	Z	M	Z
5-FOA	ND	500	ND	700	ND	500	ND	700
Oligomycin C	20	20	2	<1	20	20	ND	ND

Notes.

1. M: mycelial plug inocula
   Z: encysted zoospore inocula

ND: Not Determined

The MIC of oligomycin C was determined for encysted zoospore and mycelial plug inocula of isolates ATCC48720, I259 and I230 (Fig. 3.3 and Table 3.2). An oligomycin C concentration of 20 µg/ml was chosen to select for resistant mutants of isolate ATCC48720.

3.2.3 *MUTAGENESIS AND SCREENING*

Table 3.3 summarises attempts to isolate uracil auxotrophic and oligomycin C resistant mutants.

Table 3.3 Summary of mutagenesis experiments.

Method	Total number of zoospores screened	Total number of survivors screened
Oligomycin C resistance		
Non-mutagenized cells selected directly with antibiotic	5.37×10^7	Not applicable
UV mutagenesis (90% kill)	6.43×10^8	6.43×10^7
TOTALS	6.97×10^8	1.18×10^8
Uridine auxotrophy		
UV mutagenesis (99% kill) + selection with 5-FOA	1.8×10^9	1.8×10^7
NTG mutagenesis (99% kill) + selection with 5-FOA	1.5×10^9	1.5×10^7
NTG mutagenesis (99% kill) + filtration enrichment	9×10^8	9×10^6
TOTALS	4.2×10^9	4.2×10^7

No putative mutants of either type were isolated from these experiments.

3.3 DISCUSSION

Failure to isolate either 5-FOA resistant or Oli C^R mutants may have been principally due to screening insufficient numbers of encysted zoospores. The isolates used in this work did not sporulate as well as those used by Shattock & Shaw (1975) and the difficulty in obtaining even comparable numbers of zoospores was a limiting parameter in the screening experiments.

The main reason for developing a transformation system for *P. infestans* is to employ it as a tool in the investigation of virulence loci. Therefore an important requirement of any system developed for this purpose is that it should be usable in virtually any isolate. The practical difficulties in obtaining a suitable UMP biosynthetic mutant in every isolate or Race of interest make development of a system based on transformation to uridine prototrophy nonviable for *P. infestans*. The development of the oligomycin C resistance system remains feasible as once an Oli C^R ATP synthase subunit 9 gene is isolated it can be used as a marker in any isolate that is sensitive to the antibiotic. However, isolation of such a gene would probably require the screening of much larger numbers of zoospores than were screened here and this would be greatly facilitated by the availability of isolates with higher sporulation levels than those used in this work.

Fig. 3.1 Growth of isolate I230 on a minimal medium (MM) (Huang *et al.*, 1980). The MM contained no nitrogen source except as indicated.

(a)

A. PBA B. MM C.MM + NO3

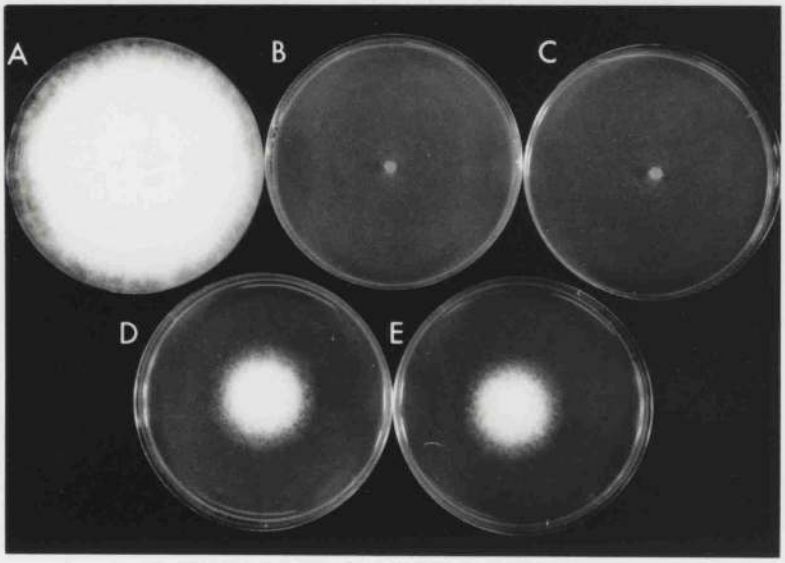
D. MM + NH4 E. MM + NH4 +NO3

(b)

A. PBA

B. MM C. MM + Asp D. MM + Glu

E. MM + Asn F. MM + Arg G. MM + Gln


A
B
C
D
E

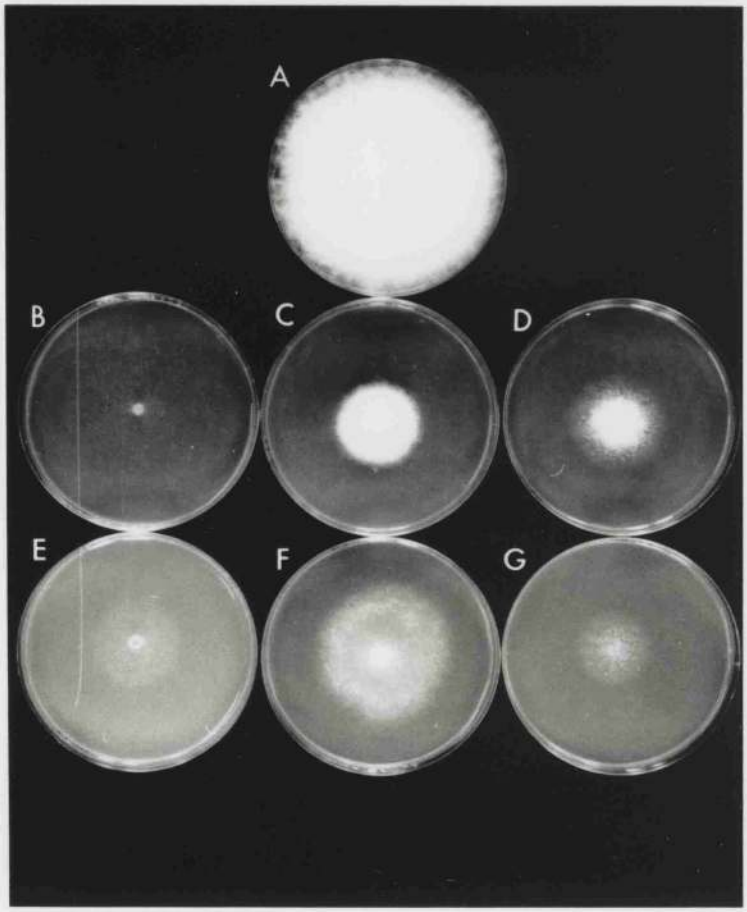

A
B
C
D
E
F
G

Figure 3.2. Growth of conidiospores of *Aspergillus nidulans* uridine prototrophic (G34) and uridine auxotrophic (G191) strains on PBA alone (left) or PBA supplemented with 20 mM uridine (right). Background growth of the auxotrophic strain on PBA alone is completely absent.

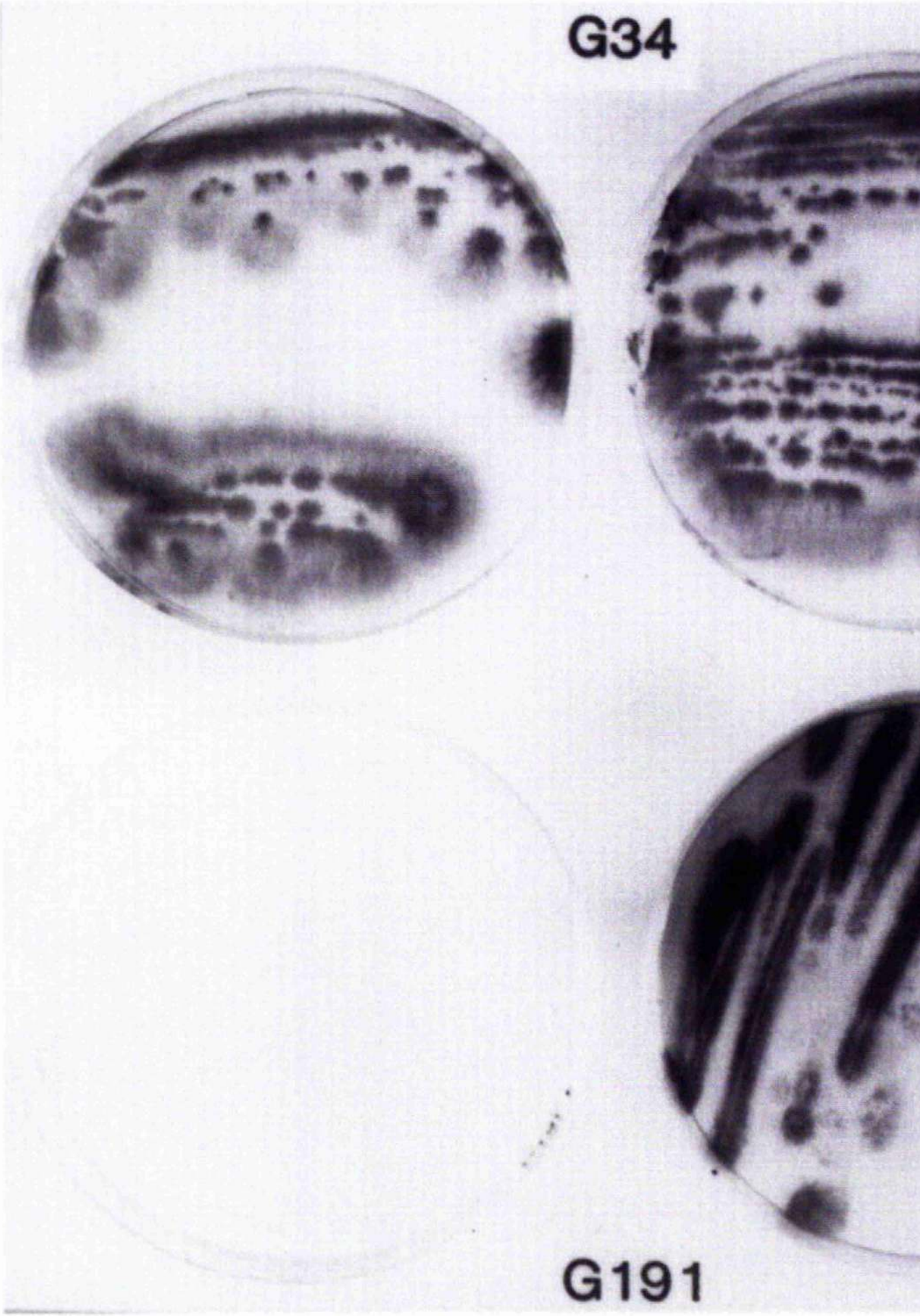

G34
G191

Figure 3.3 Growth inhibition of 3 isolates of *P. infestans* by oligomycin C. Oligomycin C concentrations (µg/ml) are as indicated below.

Isolate ATCC48720

0 Ethanol control

0.2 1 5

10 15 20

Isolate I259

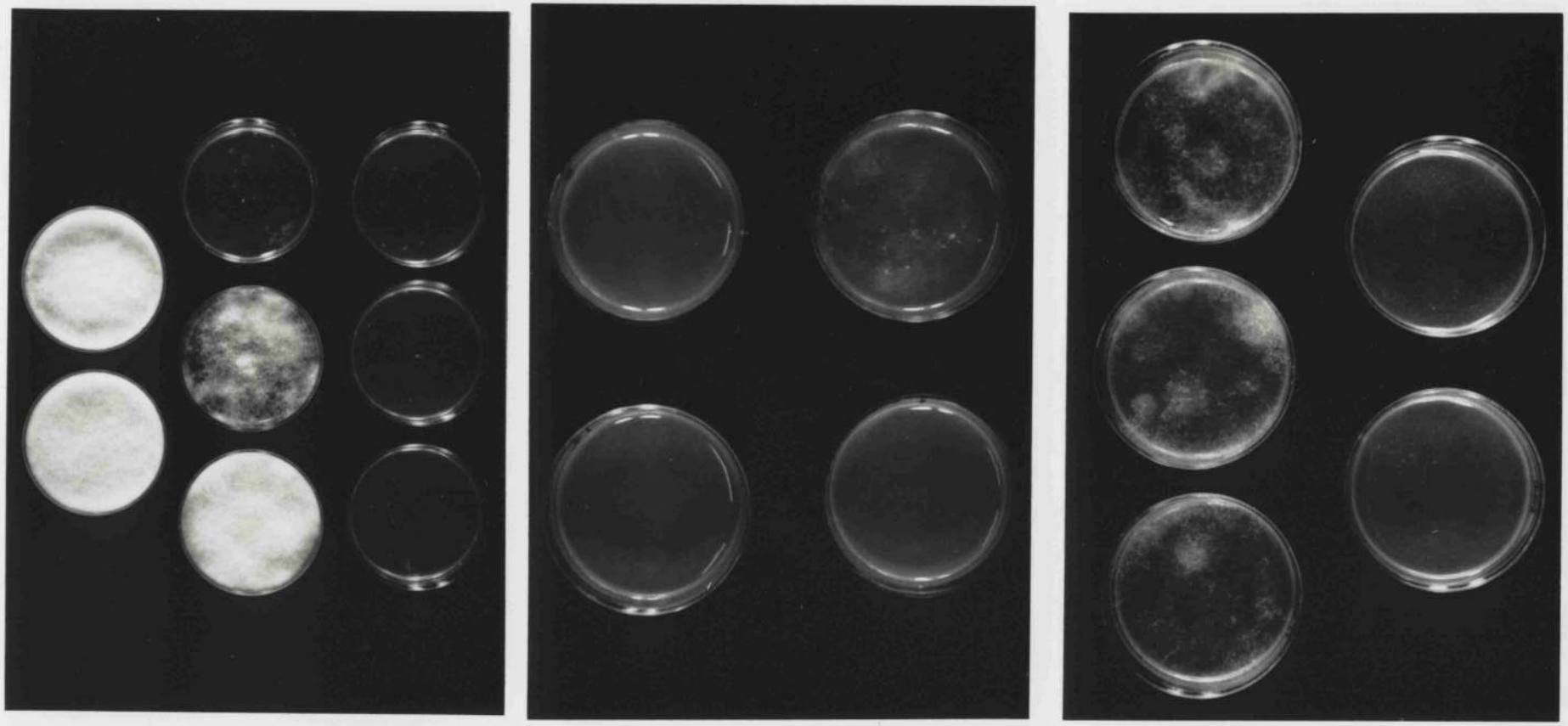
12 5

1 0

Isolate I230

0 Ethanol control 5

10 20



Chapter 4

PROTOPLASTING AND REGENERATION STUDIES

4.1 INTRODUCTION

Although protoplast formation from *P. cinnamomi* and *P. parasitica* were first reported over two decades ago (Bartnicki-Garcia & Lippman, 1966; 1967) it is only comparatively recently that protoplast formation with subsequent regeneration have been reported for *Phytophthora* species - for *P. infestans* (Pesti & Ferenczy, 1979), *P. parasitica* (Jahnke *et al.*, 1987), *P. megasperma* f. sp. *glycinea* and *P. megasperma* f. sp. *medicaginis* (Layton & Kuhn, 1988). Protoplast formation and regeneration have also been reported for the Oomycetes *Achlya ambisexualis* (Manavathu *et al* ., 1988) and *Pythium* PRL2142 (provisionally identified as *Pythium acanthicum* Drechs. (Haskins, 1963)) (Sietsma & de Boer, 1973).

80-90 % of the dry weight of cell walls of *Phytophthora* spp. consists of β-linked glucose polymers (glucans) with small amounts of other carbohydrates. These glucans are of two types, a β-1,3 linked non-cellulosic fraction which is highly branched (β-1,6 links) and a smaller, β-1,4 linked cellulosic fraction. The ratio of non-cellulosic to cellulosic glucans varies between 2:1 and 10:1 by dry weight according to species and cell type (Bartnicki-Garcia & Wang, 1983).

Obviously, efficient digestion of such cell wall material requires selection of an appropriate enzyme preparation. Table 4.1 lists some of the types of enzyme preparations that have been used for the formation of protoplasts from various Oomycetes. Commercial enzyme preparations are convenient but the efficacy of some of these in digesting fungal cell walls is known to vary from batch to batch (Hamlyn *et al.*, 1981; Jahnke *et al.*, 1987).

Table 4.1. Enzyme preparations used for formation of protoplasts from Oomycetes.

Enzyme preparation	Activities present	Source	Supplier	Species protoplasted	Cell type	Reference
NovoZym 234	β-1,3 glucanase α-1,3 glucanase chitinase	*Trichoderma*	Novo Biolabs, Denmark	*P. parasitica*	mycelium	Jahnke *et al.*, 1987
Driselase	laminarinase cellulase xylanase	*Irpex lacteus*	Sigma Chemical Co., U.S.A.	*P. megasperma* f. sp. *glycinea* *P. megasperma* f. sp. *mediginis* *Achlya ambisexualis*	mycelium mycelium mycelium	Layton & Kuhn, 1988 Manavathu *et al.*, 1988
Snail digestive juice extract	N. D.	*Helix pomatia*	Non-commercial	*P. infestans*	mycelium	Pesti & Ferenczy, 1979
Helicase	laminarinase cellulase	*Helix pomatia*	l'Industrie Biologique Francaise, France	*Pythium* PRL2142	mycelium	Sietsma & de Boer, 1973
Cellulase	laminarinase cellulase	*Aspergillus niger*	Nutritional Biochemicals Co., U.S.A.			
Bacterial enzymes	β-1,3 glucanase β-1,6 glucanase endocellulase	*Streptomyces*	Non-commercial	*P. parasitica* *P. cinnamomi*	mycelium mycelium	Bartnicki-Garcia & Lippman, 1966; 1967

A wide range of osmotic stabilisers have been employed in the formation of fungal protoplasts including mineral salts, sugars and sugar alcohols (reviewed by Davies, 1985). The optimal type and concentration of the stabiliser is influenced by the species the protoplasts are derived from and the nature of the enzyme preparation used. Sietsma & de Boer (1973), using a combination of snail gut enzymes and cellulases obtained from *Aspergillus niger*, found mineral salt osmotic stabilisers, particularly NaCl, gave much higher protoplast yields than sugar or sugar alcohol osmotic stabilisers, which were strongly inhibitory to protoplast formation. Jahnke *et al.* (1987) observed that the formation of protoplasts from *P. parasitica* using NovoZym 234 was optimal in $CaCl_2$ solutions and was inhibited in mannitol solutions. Pesti & Ferenczy (1979), using snail gut enzymes, also found lower yields of *P. infestans* protoplasts were produced in sugar or sugar alcohol solutions than in $CaCl_2$ ones. However relatively high yields of protoplasts have been obtained from *Achlya ambisexualis* and from *P. megasperma* f. sp. *glycinea* and *P. megasperma* f. sp. *medicaginis* with mannitol as the osmotic stabiliser using Driselase (Manavathu *et al.*, 1988; Layton & Kuhn, 1988). Insufficient comparative data is available to determine whether differences in the performance of these enzyme preparations are due simply to the influence of the type of osmotic stabiliser or whether there are species-dependent effects.

Culture age is known to influence protoplast yields from many fungi (Davies, 1985). The yield of *P. parasitica* (Jahnke *et al.*, 1987) and *Pythium* PRL2142 protoplasts from cultures older than 24-36 h falls rapidly with time. Pretreatment of older *Pythium* PRL2142 mycelium with Triton X-100, SDS or sodium deoxycholate strongly stimulated subsequent protoplast formation but such treatment was unnecessary with very young mycelium (Sietsma & de Boer, 1973).

Mannitol or mannitol + $CaCl_2$ are the most frequently used osmotic stabilisers for the regeneration media of Oomycete protoplasts. Regeneration of *Pythium* PRL2142 mycelial protoplasts is completely inhibited in media osmotically buffered with the salts NaCl, KCl, $MgSO_4$ or NH_4Cl. However the inclusion of 0.1M $CaCl_2$ in

the mannitol-buffered regeneration medium of *P. infestans* and *P. parasitica* mycelial protoplasts more than doubled regeneration efficiencies compared to media buffered with mannitol alone (Pesti & Ferenczy, 1979; Jahnke *et al.*, 1987).

From the above reports it appears that protoplast formation in *P. infestans* may be strongly influenced by the type of protoplasting osmoticum and culture age. Efficient regeneration may depend on the type of regeneration osmoticum and whether it includes $CaCl_2$.

4.2 RESULTS

A high efficiency protoplasting and regeneration system for *P. infestans* did not exist prior to this work. The best regeneration frequency obtained previously was only 8 % - for mycelial protoplasts (Pesti & Ferenczy, 1979). Sufficient numbers of sporangia and zoospores of *P. infestans* can be obtained for even a relatively low efficiency transformation system and as they are specially equipped biochemically for germination and differentiation it was felt that protoplasts of these cells may be more successfully regenerated into macroscopic colonies than protoplasts derived from mycelium. In addition, attempts were made to improve the efficiency of mycelial protoplast formation and regeneration. For the purposes of this work the term 'protoplast' describes cells which (a) rapidly lyse in water, (b) fuse in polyethylene glycol (PEG) solutions and (c) either lack UV-fluorescence (sporangial protoplasts) or have diminished UV-fluorescence localised to small patches of the cell surface (cyst and mycelial protoplasts), in solutions of Calcofluor White, a fluorochrome with specificity for β-linked glucans (Maeda & Ishida, 1967).

4.2.1 *FORMATION AND REGENERATION OF PROTOPLASTS FROM ENCYSTED ZOOSPORES*

Encysted zoospores were suspended in 5 ml of various mannitol or KCl solutions (0.2 M - 1.2 M) containing 50 mg/ml NovoZym 234 and mixed on a platform shaker at room temperature. Digestion of the cells was followed by Calcofluor White staining. After digestion for 3 h a substantial amount of fluorescence remained over the entire surface of cells digested in mannitol solutions. No further experiments were conducted on these cells. Digestion was more rapid and complete in KCl solutions. Cells lysed on transfer to water after digestion for 1 h even though Calcofluor White fluorescence was present over the entire cell surface at this stage. After digestion for 2 h most Calcofluor White-stained cells lacked UV-fluorescence, others had a slightly speckled appearance under UV illumination. A small proportion of cells had small patches of UV-fluorescence where they had adhered to one another during centrifugation, affording protection to the cell wall at the point of contact. The overall amount of fluorescence in the cell population varied according to the concentration of KCl in which the cells were protoplasted, with a minimum at 1.0 M KCl as judged visually. Protoplasts were washed free of NovoZym 234 by centrifugation using an isotonic KCl solution. Protoplast concentrations were measured by haemocytometer counts (Fig. 4.1) and 0.1 ml aliquots from the three highest yielding osmotica (0.8 M, 1.0 M and 1.2 M KCl) were added to 2.5 ml of pea broth variously emended with mannitol or sorbitol (0.2 M - 1.2 M). Figures 4.2 and 4.3 show the percentage of digested cells that regenerated and produced branched hyphae or germ tubes more than three times the diameter of the cyst as measured by haemocytometer counts. From these results 1 M KCl and PB + 0.8 M sorbitol were selected as the protoplasting and regeneration osmotica respectively as this combination gave the highest overall yield of regenerated cells from the original suspension of cysts although PB + 0.8 M mannitol is probably equally suitable as a regeneration medium.

Following the observations that the presence of $CaCl_2$ in the regeneration medium increases regeneration frequencies of *P. infestans* and *P. parasitica*

mycelial protoplasts (Pesti & Ferenczy, 1979; Jahnke *et al.*, 1987) the responses of cyst protoplast formation and regeneration to $CaCl_2$ were investigated. Using osmolality curves, protoplasting osmotica containing KCl + $CaCl_2$ isotonic to 1 M KCl were produced so as to eliminate osmolality as a variable parameter of the experiments. Cysts were digested in these osmotica as before. The pinheads of fluorescence seen on cells protoplasted in KCl solutions were absent from cells protoplasted in osmotica containing $CaCl_2$ indicating cell wall digestion was improved. $CaCl_2$ has previously been noted to improve the protoplasting efficiency of some enzyme preparations (Davies, 1985). The cyst protoplasts were washed free of mineral salts and NovoZym 234 with 0.9 M sorbitol (isotonic to 1 M KCl) and 0.1 ml aliquots added to 2.5 ml of PB variously emended with sorbitol. Figure 4.4 shows the effect of $CaCl_2$ in the protoplasting osmoticum on protoplast yield and regeneration. Protoplast yield was not significantly affected by the presence of $CaCl_2$ in the protoplasting osmoticum but subsequent regeneration frequencies were substantially increased compared to protoplasts formed in 1 M KCl. From these results 0.71 M KCl; 0.2 M $CaCl_2$ was selected as the protoplasting osmoticum for encysted zoospores in future experiments. Figure 4.5 illustrates the formation and regeneration of protoplasts from zoospores.

4.2.2 *FORMATION AND REGENERATION OF PROTOPLASTS FROM SPORANGIA*

Protoplasting experiments on sporangia were conducted by A. Campbell at the Scottish Crop Research Institute. As with encysted zoospores, the concentration of KCl not only affected protoplast stability but also appeared to influence the rate of cell wall digestion (Fig. 4.6). Although comparable yields of protoplasts were obtained in 1 M KCl as at other concentrations it was the only concentration at which virtually complete digestion of sporangia cases occurred. For this reason 1 M KCl was selected as the protoplasting osmoticum in further studies. The thick sporangium wall requires digestion for 24 h at room temperature before it is completely broken down (Fig. 4.7). As with encysted zoospores, not all the sporangia are recovered as

protoplasts, typically 30 % are destroyed. Figure 4.8 shows the regeneration of sporangial protoplasts produced in 1 M KCl or KCl + $CaCl_2$ solutions isotonic to 1 M KCl and regenerated in PB variously emended with sorbitol. The highest regeneration frequency for protoplasts formed in 1 M KCl was obtained in PB + 1 M sorbitol, approximately 30 % in this experiment. Sporangial protoplasts formed in KCl + $CaCl_2$ solutions had higher regeneration frequencies in PB + 1 M sorbitol than protoplasts formed in 1 M KCl. Regeneration frequencies of over 80 % were obtained for protoplasts produced in 0.71 M KCl; 0.2 M $CaCl_2$ and regenerated in PB + 1 M sorbitol. Some experiments showed a variation in percentage protoplast yield with varying $CaCl_2$ concentration, and in these cases 0.71 M KCl; 0.2 M $CaCl_2$ was the optimal osmoticum (data not shown). In contrast to the results of Pesti & Ferenczy (1979) and Jahnke *et al.* (1987) the effect of $CaCl_2$ in the regeneration medium was extremely inhibitory to regeneration of sporangial protoplasts. In this experiment $CaCl_2$ was added to PB + 1 M sorbitol so the osmolality of the regeneration medium increased with increasing $CaCl_2$ concentration. High osmolality in regeneration media appears to reduce regeneration frequencies of sporangial protoplasts as judged from the low regeneration frequencies of sporangial protoplasts observed in 1.5 M sorbitol (Fig. 4.8). However, the low concentration of $CaCl_2$ required to dramatically reduce regeneration suggests it may be an effect of the mineral salt itself rather than simply osmolality. 1 M NaCl (isotonic to 1 M KCl) was also used as a protoplasting osmoticum but although protoplast yields were comparable to those obtained with 1 M KCl or KCl + $CaCl_2$ solutions, subsequent regeneration frequencies in PB + 1 M sorbitol were substantially lower (Fig. 4.9).

From these results 0.71 M KCl; 0.2 M $CaCl_2$ and 1 M sorbitol were selected as the protoplasting and regeneration osmotica respectively for sporangia as this combination gave the highest overall yield of regenerated cells from the original suspension of sporangia.

The formation and regeneration of sporangial protoplasts is illustrated in Figure 4.10.

4.2.3 *FORMATION AND REGENERATION OF PROTOPLASTS FROM MYCELIA*

Previous workers have found 0.35 M $CaCl_2$ to be the optimal osmoticum for protoplasting mycelia of *P. infestans* and *P. parasitica* (Pesti & Ferenczy, 1979; Jahnke *et al.*, 1987). Protoplast formation from mycelia of *P. infestans* isolate ATCC48720 in 0.35 M $CaCl_2$ and 0.71 M KCl; 0.2 M $CaCl_2$ was compared. Approximately equivalent wet weights of 4 day mycelia were digested in 25 ml of 0.35 M $CaCl_2$ or 0.71 M KCl; 0.2 M $CaCl_2$ plus 10 mg/ml NovoZym 234. After digestion for 3 h in 0.35 M $CaCl_2$ virtually no mycelial debris remained and 4.1×10^7 protoplasts had been produced. Large mycelial fragments remained in the KCl + $CaCl_2$ osmoticum after the same time and the protoplast yield was only 5.9×10^6. Therefore 0.35 M $CaCl_2$ was selected as the protoplasting osmoticum in further experiments. The regeneration of mycelial protoplasts in various liquid and solid regeneration media was also compared (Fig. 4.11). Protoplasts were washed three times in 0.9 M mannitol (isotonic to PB + 0.8 M mannitol) to remove $CaCl_2$ and NovoZym 234 prior to inoculation into regeneration media. Of the liquid media PB + sorbitol or mannitol in the concentration range 0.8 M-1.0 M gave the highest regeneration efficiencies. Due to clumping of some cells it was not possible to count the actual number of regenerated protoplasts on solid media. No significant differences in the proportion of clumped cells or the sizes of the clumps was apparent between the media so the results in Figure 4.11, although underestimates of actual regeneration frequencies, were presumed to reflect actual differences in the ability of the different media to support regeneration. The highest regeneration frequencies in liquid media were observed in PB emended with sorbitol or mannitol in the concentration range 0.8 - 1.0 M. In contrast to the findings of Pesti & Ferenczy (1979) and Jahnke *et al.* (1987), both liquid and solidified PB + 0.4 M mannitol + 0.1 M $CaCl_2$ supported only low levels of regeneration.

4.3 DISCUSSION

Encysted zoospores, sporangia and mycelia of *P. infestans* isolate ATCC48720 can be protoplasted and regenerated at high efficiencies. The optimal conditions for each system are summarised in Table 4.2.

Table 4.2 Summary of optimal protoplasting and regeneration conditions.

Cell type	Encysted zoospores	Sporangia	Mycelia
Protoplasting osmoticum	0.71 M KCl; 0.2 M $CaCl_2$	0.71 M KCl; 0.2 M $CaCl_2$	0.35 M $CaCl_2$
NovoZym 234 concentration (mg/ml)	50	20	10
Digestion time (h)	2	24	1.5
Yield	50 %	70 %	~3.6×10^6 per culture
Regeneration osmoticum	0.8 M sorbitol	1.0 M sorbitol	0.8 M mannitol
Regeneration efficiency	~90 %	~80 %	~27 %

Many isolates of *P. infestans* sporulate poorly but as very high protoplast viability is possible the operation of a transformation system in such isolates should not be compromised by low regeneration frequencies, unless the transformation protocol itself is seriously detrimental to cell viability. Although precise figures for protoplast yield and regeneration vary between experiments the overall conclusions reached in these investigations have been confirmed in transformation experiments employing these protoplasting and regeneration systems.

The batch number of the NovoZyme 234 was found to be very important for the formation of protoplasts from sporangia. Batch no. 1961 (no longer available) was used in all the experiments on sporangia described in this chapter. A more recent batch (no. 2416) did not protoplast sporangia of isolate ATCC48720 even after 48 h incubation but was equally efficient as batch no. 1961 in protoplasting encysted zoospores and mycelia.

The suggestion that inclusion of $CaCl_2$ in regeneration media is detrimental to colony establishment was not unambiguously confirmed in the experiments with sporangial protoplasts as osmolality was not a controlled parameter. However, growth inhibition appears to be a general effect of the presence of mineral salts in regeneration media on protoplasts of isolate ATCC48720, as maximum regeneration frequencies are not achieved if there is significant carry over of (NovoZym 234 - free) protoplasting osmoticum into the regeneration medium. The best way to ensure this is to wash the protoplasts by centrifugation with a suitable sorbitol or mannitol solution prior to inoculation into regeneration medium. Washing the protoplasts with PB + sorbitol or mannitol results in the formation of a precipitate, caused by calcium, in which the protoplasts become embedded making accurate haemocytometer counts impossible.

Jahnke *et al.* (1987) reported decreases in the ability of regenerated mycelial protoplasts of *P. parasitica* to establish macroscopic colonies on solid media the longer they were incubated in liquid medium before hand. Such a test was not applied to *P. infestans* protoplasts in this work but protoplasts continued to grow vigorously in liquid regeneration media for at least several days. Growth appeared just as abundant as in media inoculated with similar numbers of intact sporangia. Although this assessment was subjective substantial reduction in cell viability on prolonged incubation in liquid media was not obvious.

Regeneration of cyst and sporangial protoplasts sufficient for the cells to survive inoculation onto plant material is achieved within 18 h, allowing rapid manipulation and screening of isolates. A protoplasting and regeneration system for sporangia and

encysted zoospores has the advantage of permitting transformation and other protoplast manipulations to be performed on cells harvested from sporulating lesions on plant stems and leaves. This approach may be useful for Oomycetes, such as the downy mildews, which are obligate pathogens.

The improvements in regeneration efficiencies for *P. infestans* mycelium makes the use of these cells in transformation experiments viable. The cultures used to produce mycelium for protoplasts in these and other experiments were each inoculated with sporangia harvested from one Rough Rye Agar culture of isolate ATCC48720. It was apparent that inoculum density was a major limiting factor on protoplast yield. This is the principal reason for the modest mycelial protoplast yields obtained in this work. Other forms of inoculum such as mycelial blocks or mycelium fragmented in a blender may be used. Mycelial block inocula are unsuitable for this work because relatively little new mycelium is produced in the short incubation periods appropriate for protoplasting. This is probably due to a slow initial growth rate from the mycelial blocks and the relatively low numbers of growth points. Exponentially growing mycelial fragments obtained by successive homogenisation of liquid cultures (e.g. Jahnke *et al.*, 1987) are probably the most reliable and powerful form of inoculum since they can be easily produced in large quantities from even the poorest sporulating isolates. One drawback of this technique is the ease with which cultures can be contaminated, particularly in laboratories where other highly sporulating and more vigorous fungi are being cultured.

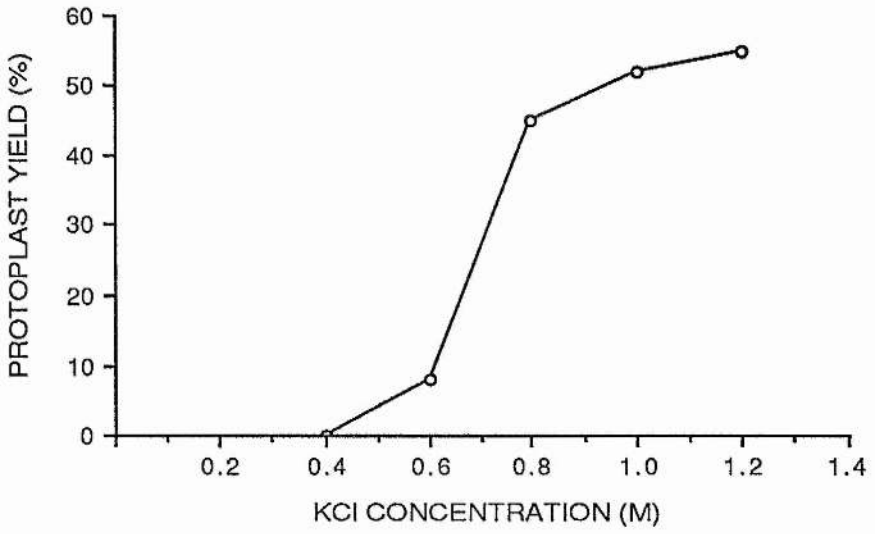


Figure 4.1. Effect of KCl concentration on cyst protoplast yield. Data are means of two counts.

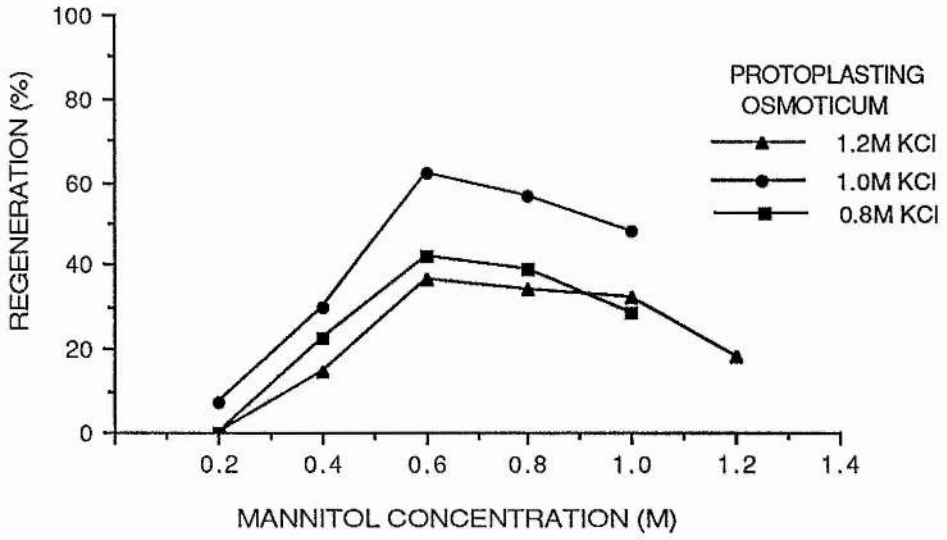


Figure 4.2. Regeneration of cyst protoplasts in PB emended with various concentrations of mannitol. Data are means of two counts.

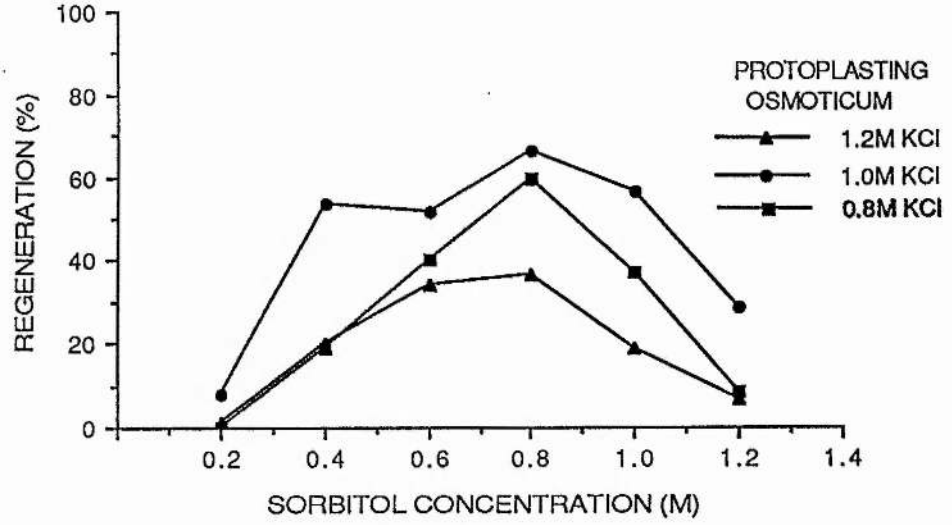


Figure 4.3. Regeneration of cyst protoplasts in PB emended with various concentrations of sorbitol. Data are means of two counts.

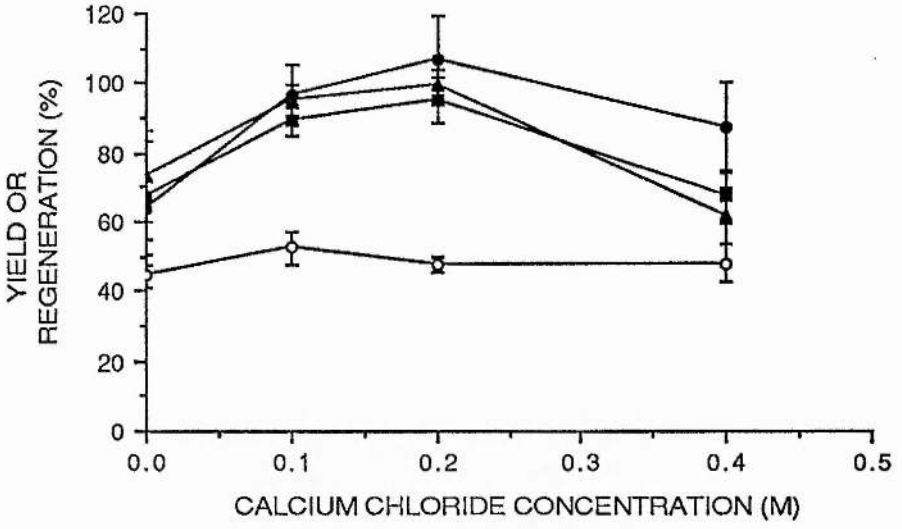


Figure 4.4. Effect of $CaCl_2$ concentration on cyst protoplast yield and regeneration. Protoplasts were formed in KCl + $CaCl_2$ solutions isotonic to 1 KCl (○) and regenerated in the following media: PB + 1M sorbitol (▲), PB + 0.8 M sorbitol (●), PB + 0.6 M sorbitol (■). Data are means of 5-7 counts. Bars represent standard errors with confidence limits of 95%.

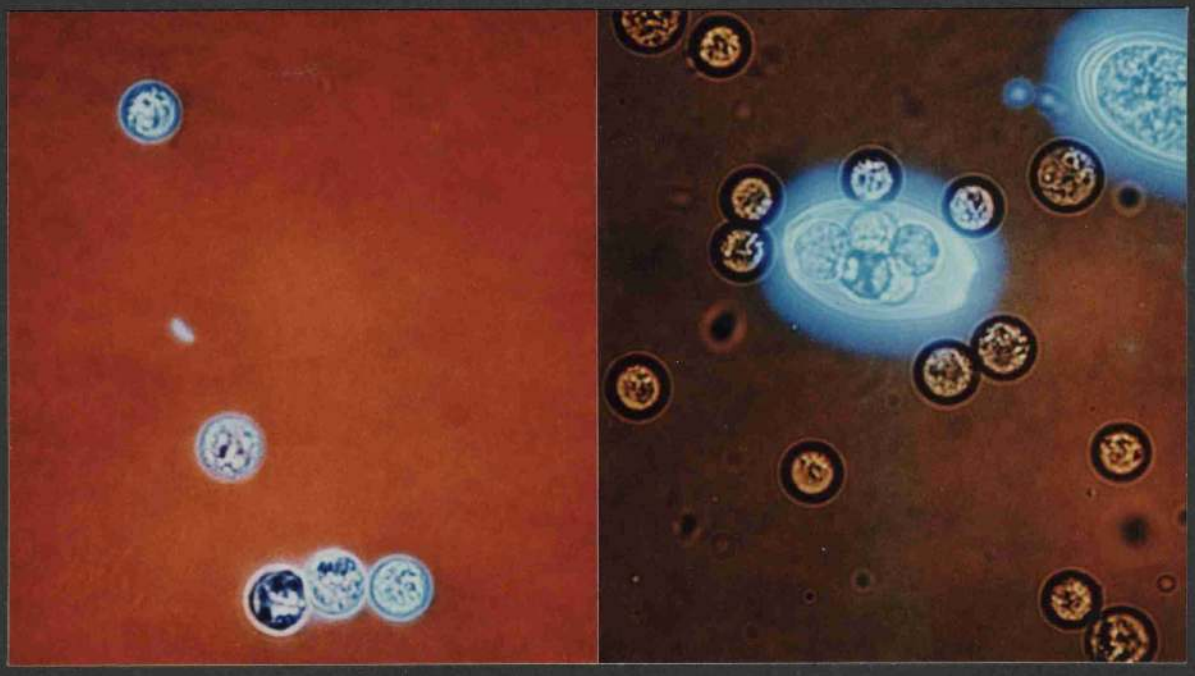
Figure 4.5. Stages in the formation and regeneration of protoplasts from encysted zoospores. Cells were stained with Calcofluor White and viewed under combined UV and visible light.

(Top left) A zoospore about to undergo encystment.

(Top right) The same cell 3 min. later. The cell begins to fluoresce as the cell wall is synthesised.

(Bottom left) Encysted zoospores after 1.5 h digestion with NovoZym 234 in 1 M KCl. Pinheads of fluorescence can be seen when viewed under UV light only (not shown). These are absent from cells digested in 0.71 M KCl; 0.2 M $CaCl_2$.

(Bottom right) Protoplasts of encysted zoospores 1 h after removal of NovoZyme 234. The cell wall is quickly regenerated but this does not in itself indicate the cell's ability to develop into a macroscopic colony.



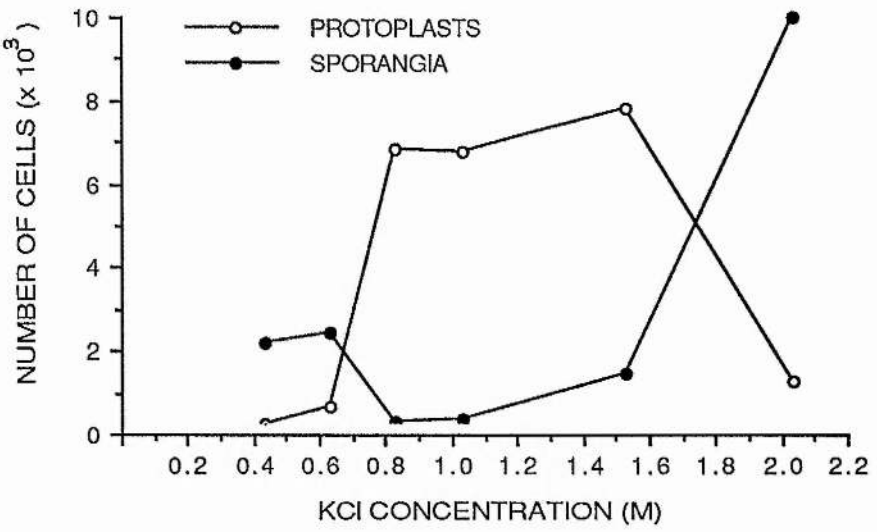


Figure 4.6 Effect of KCl concentration on protoplast yield. All treatments contained the same number of sporangia. Data are the means of two counts.

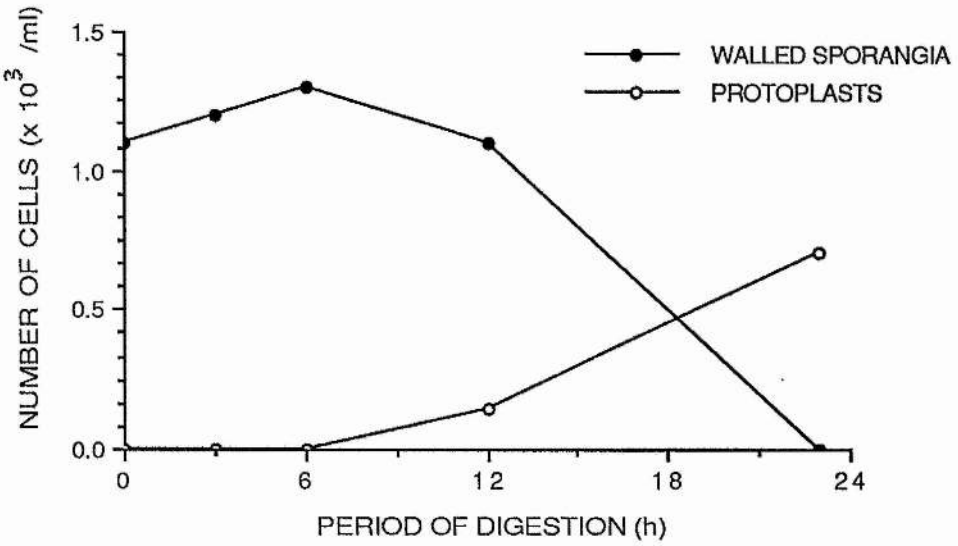


Figure 4.7. Time course for conversion of sporangia to protoplasts. Data are means of two counts.

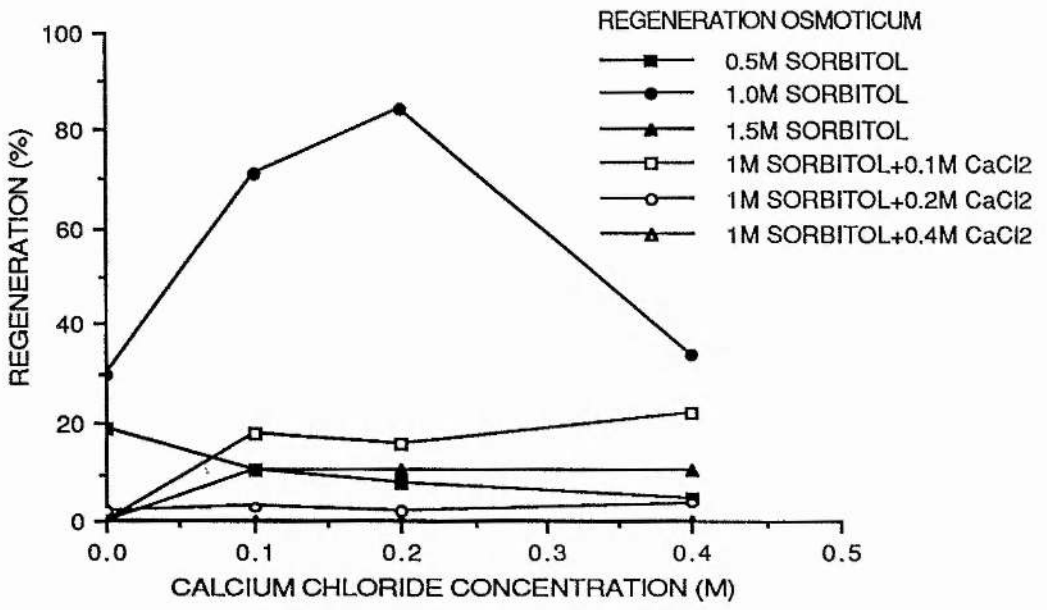


Figure 4.8. Effect of $CaCl_2$ on sporangial protoplast regeneration. Sporangia were protoplasted in 1 M KCl or KCl + $CaCl_2$ solutions isotonic to 1 M KCl. Protoplasts were regenerated in PB emended with various concentrations of $CaCl_2$ and/or sorbitol. Data are the means of two counts.

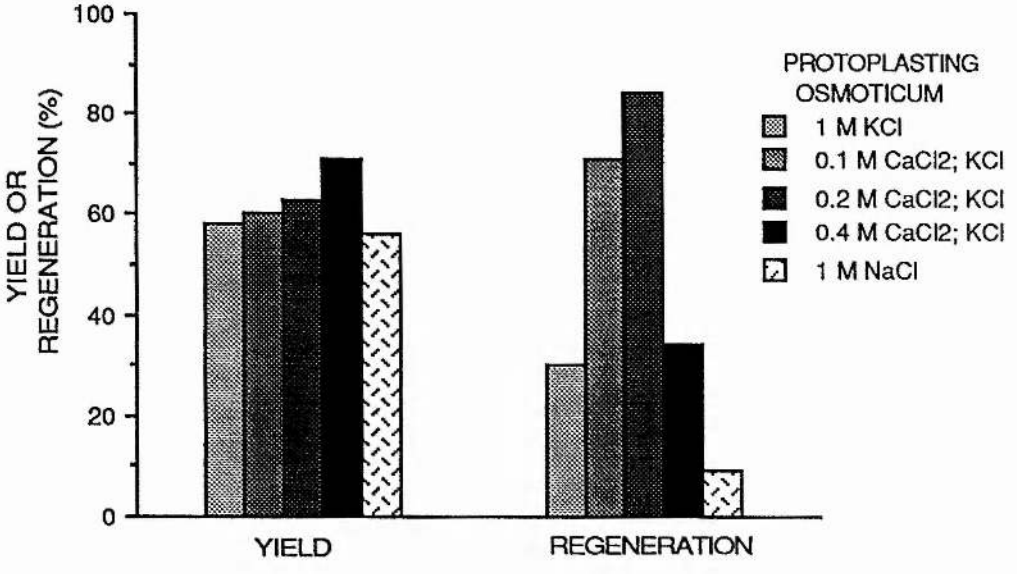


Figure 4.9. Comparison of sporangial protoplast yields in various protoplasting osmotica and subsequent regeneration in PB + 1 M sorbitol. Data are means of two counts.

Figure 4.10. Formation and regeneration of protoplasts from sporangia. Cells in the top photographs are viewed under visible light only. Cells in the bottom photographs were stained with Calcofluor White and viewed under combined UV and visible light (left) or UV light alone (right).

(top left) Sporangia before digestion with NovoZym 234.

(top right) Sporangia after 24 h digestion in 1 M KCl. Note that the cell walls are completely digested away.

(bottom left) Sporangial protoplasts after removal of NovoZym 234. Regeneration and germination are relatively rapid.

(bottom right) Substantial growth is established after 24 h.

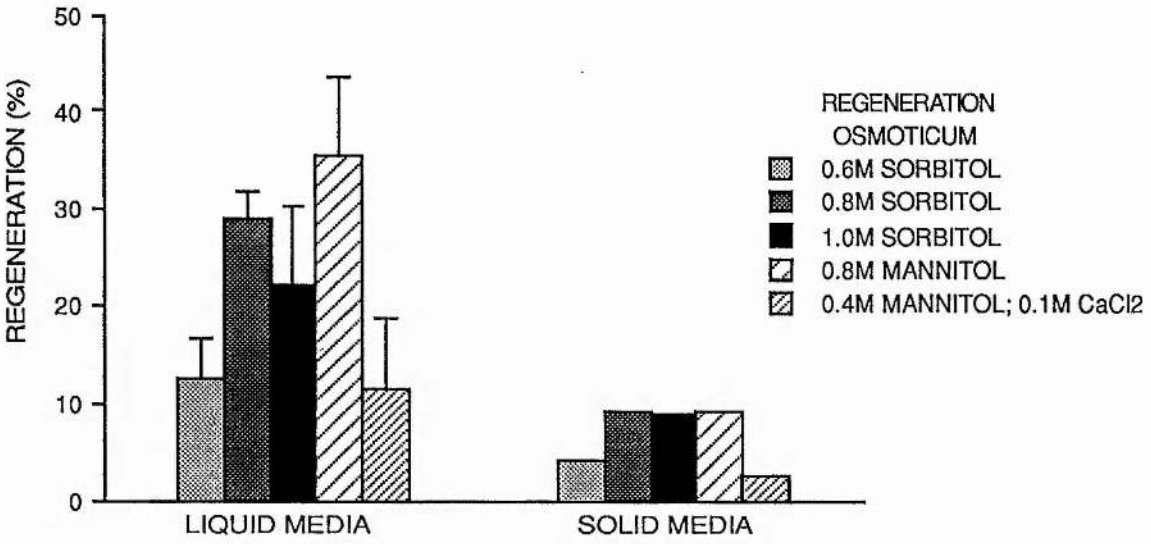
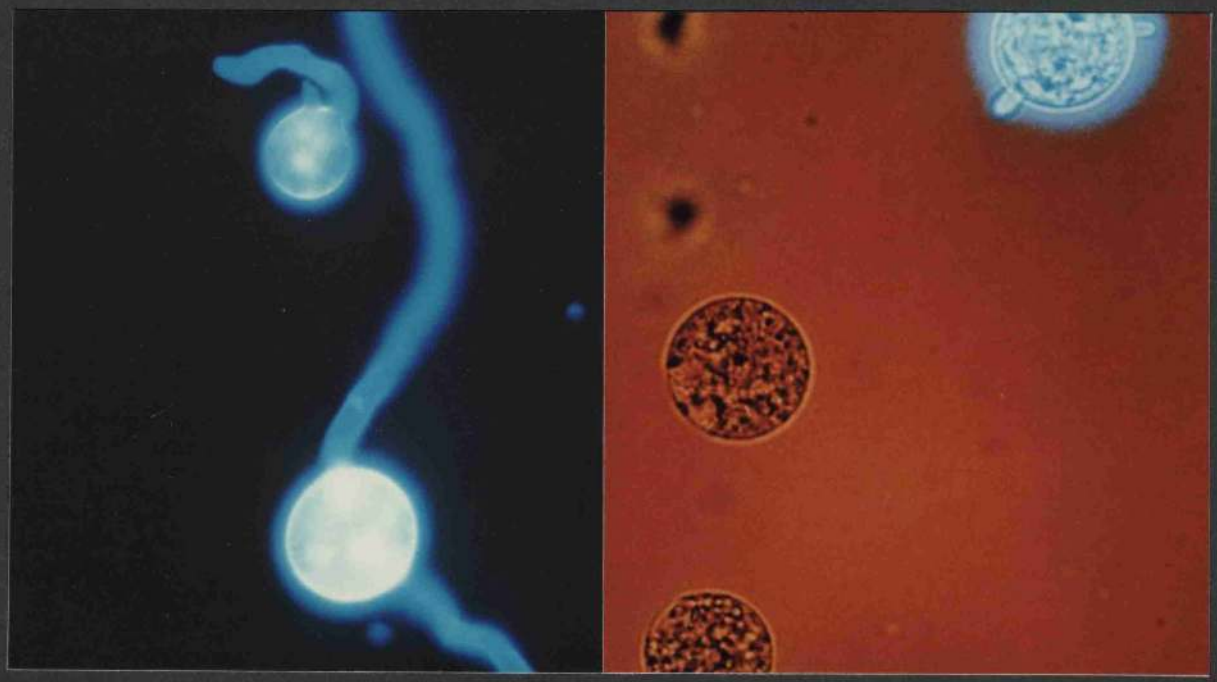
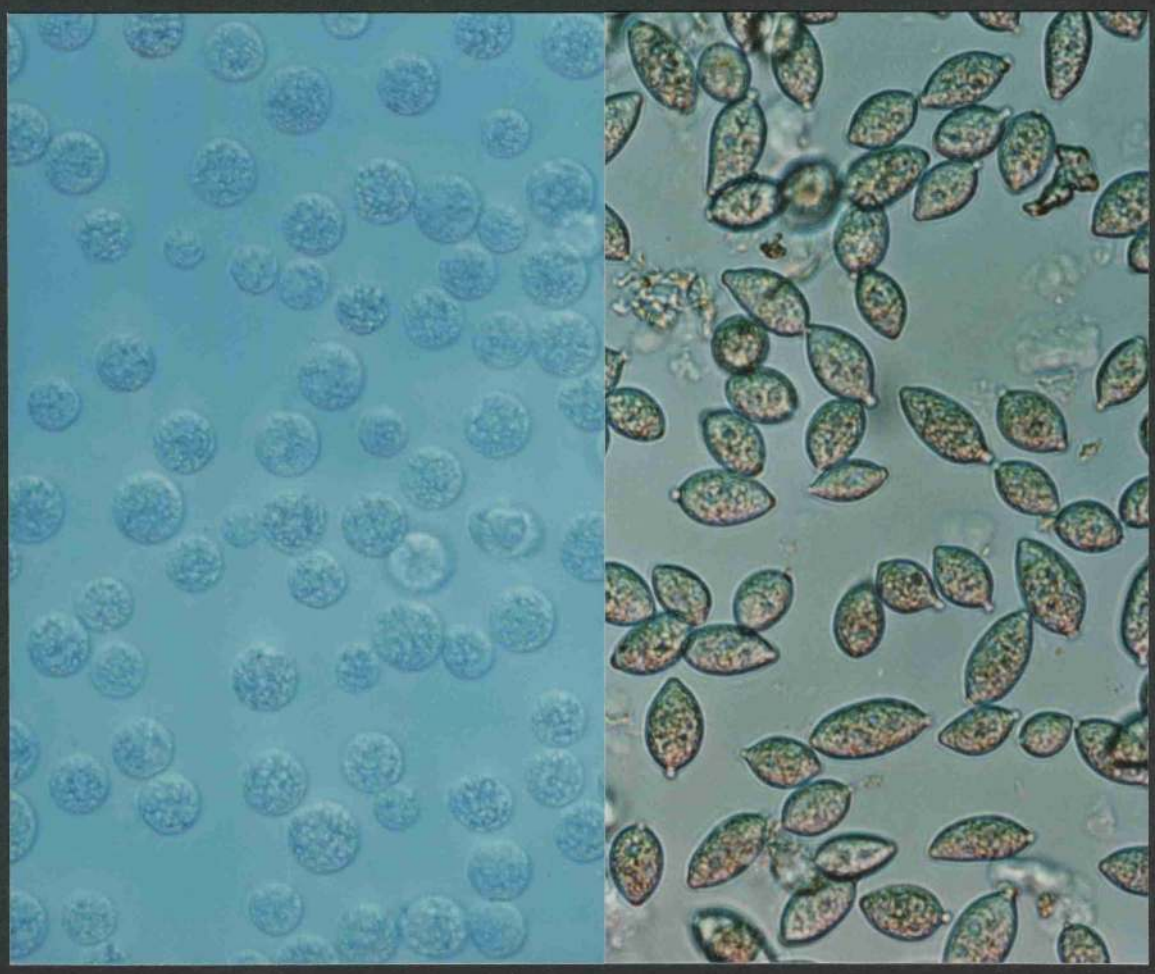


Figure 4.11 Regeneration of mycelial protoplasts in PB or PBA emended with various osmotica. Protoplasts were formed in 0.35 M $CaCl_2$. Data are means of five counts and two counts for liquid and solid media respectively. Bars represent standard errors with confidence limits of 95%.





Chapter 5

TRANSFORMATION STUDIES

5.1 INTRODUCTION

Probably the biggest obstacle to developing a transformation system for a virgin organism such as *Phytophthora infestans*, is that most results obtained are negative and so there is little information to interpret to aid rational modification of procedures. In addition constraints on resources, including time, do not permit the testing of all transformation methods and their variations and so investigations have to be limited to those strategies that are perceived to have the best chance of success. As the available information on which such an assessment will be based is less than comprehensive, strategy formulation necessarily involves an element of educated guesswork based on what is known for other organisms.

A variety of techniques have been used to introduce DNA into the cells of eukaryotic microbes including $CaCl_2$/PEG treatment, electroporation, lithium acetate/PEG treatment, micro-injection and bioballistics but none of these has been universally transferable to other organisms. $CaCl_2$/PEG treatment is the most commonly used method, it has the advantages of being relatively inexpensive and is, for most organisms, a fairly rapid and convenient way of producing large numbers of transformants, although transformation efficiencies can be variable for reasons which are often difficult to discover. Lithium acetate/PEG treatment was developed as a method for transforming cells of *Saccharomyces cerevisiae* without the need for protoplast formation (Ito *et al.*, 1983). The technique has since been used in the ascomycetes *S. carlsbergensis* (Sakai & Yamamoto, 1986), *Neurospora crassa* (Dhwale *et al.*, 1984), *Colletotrichum trifoli* (Dickman, 1988), and the basidiomycetes *Coprinus cinereus* (Binninger *et al.*, 1987) and *Ustilago violacea* (Bej & Perlin, 1989). To what extent induction of competency by this method is dependent upon cell wall

composition is not known and so its efficacy with Oomycete species is uncertain. Electroporation, in which exogenous DNA is introduced to cells through transient 'pores' induced in the plasma membrane by electrical pulses, is a technique that is widely used for bacterial, mammalian and plant cells. It has also been applied to yeast and filamentous fungi (Richey *et al.*, 1989; Simon & McEntree, 1989; Ward *et al.*, 1989) and shows promise as an easily applied technique which may provide high efficiency transformation. Electroporation was used to generate transformants of *Phytophthora parasitica* (H. Prell, pers. comm.). Although it is sometimes a highly efficient method in terms of the number of cells manipulated, transformation by micro-injection is a labour intensive method that yields relatively low numbers of transformants and is not therefore a feasible technique for gene library screening, an important requirement of a transformation system for *P. infestans*. Transformation with DNA-coated microprojectiles (bioballistics) is another method recently applied to lower eukaryotes but up until the present has been little used in organisms other than plants and so its efficacy as a high transformation efficiency technique is untested. However it does appear to have a special advantage in achieving *in situ* transformation of mitochondria and chloroplasts, which are difficult to reproducibly transform by other methods (Johnston *et al.*, 1988; Boynton *et al.*, 1988)

As isolation of *pyr* or OliC^R mutants in *P. infestans* was unsuccessful it was decided to attempt transformation of *P. infestans* using bacterial structural genes encoding antibiotic resistance as dominant selectable markers. There are many levels at which transformation with a heterologous structural gene may fail to be expressed phenotypically including: rearrangement of the DNA producing a nonfunctional gene or gene product, degradation of the foreign mRNA transcript, secondary structure formation in the mRNA which is inhibitory to translation by the host, incorrect folding of the nascent polypeptide leading to a non-functional protein or sensitivity of the gene product to host proteases. Some bacterial antibiotic-resistance genes such as those for hygromycin phosphotransferase (*hph*), aminoglycoside phosphotransferase (3') I (*kan*), aminoglycoside phosphotransferase (3') II (*neo*) and

phleomycin/bleomycin binding protein (*ble*) have been expressed in a wide range of phylogenetically distant hosts when driven by appropriate transcriptional control sequences, indicating they are not prone to such problems and encourages the view that their functionality in *P. infestans* is likely. Some of these marker genes are available as hybrid constructs, fused to to a variety of terminator and/or promoter sequences taken from viral and eukaryotic genes, especially fungal ones. The availability of such constructs has helped illuminate the unpredictable nature of gene expression by heterologous control sequences in fungi. Current understanding of the nature of sequence components essential for transcription initiation and termination among eukaryotic microbes other than yeast is generally rather poor (Montague, 1987) so prediction of what a suitable source of a heterologous promoter and terminator for a transformation system for *P. infestans* might be, is not possible beyond the intuitive hypothesis that sequences from a close relative are more likely to be functional than those from a remote one. However the functionality of promoter and terminator sequences from a distant relative is not precluded as there are many examples of this but it is not clear whether these instances are due to the purely fortuitous presence of host-like promoter or terminator elements or are due to conservation of sequence elements between the organisms. Attempts at transformation using heterologous promoters and terminators, even from distantly related species, are obviously worthwhile as success may obviate the need to clone and engineer homologous transcription signals, a substantial undertaking in itself.

Of particular relevance to this project is the transformation of the Oomycete *Achlya ambisexualis* to G-418 resistance using the plasmids pSV2*neo* and pSV2*neo*2μm. The latter construct contained the *Saccharomyces cerevisiae* 2μm-circle origin of DNA replication which enabled mitotically stable maintenance of G-418 resistance. Transformation efficiencies of approximately 40-50 transformants/μg were obtained with each plasmid. The *neo* gene was fused to the SV40 virus early promoter and T antigen gene terminator and polyadenylation signal. Northern blot analysis revealed a larger than expected *neo* mRNA transcript

suggesting the possibility of vector regulated alternate initiation and/or termination of the mRNA transcript or the use of host regulatory elements to effect transcription of an integrated genome. The *neo* gene was not detected extrachromosomally by Southern analysis but plasmid rescue of functional *neo* gene into *E. coli* was successful albeit with considerable DNA rearrangement (Manavathu *et al.*, 1988). Expression of functional herpes simplex virus (HSV) thymidine kinase was also achieved in *A. ambisexualis*, using the metallothionine I promoter from mouse. Although there was no direct evidence that transcription was driven by the heterologous promoter in either of these cases, that transformation was achieved with a heterologous marker gene and control sequences encourages the view that heterologous transformation of *P. infestans* is also feasible.

Hygromycin B, G-418, and chloramphenicol principally act by interfering with protein synthesis. The products of transformation marker genes encoding resistance to these antibiotics inactivate them by catalysing structural modifications. Phleomycin and the related antibiotic bleomycin appear to act through single DNA strand scission following sequence-selective binding (Fox *et al.*, 1987). The product of the phleomycin/bleomycin resistance gene *ble* functions as a binding protein and has very high affinity for these antibiotics, thus preventing them from interacting with DNA. Methotrexate acts through high affinity but reversible binding to dihydrofolate reductase (DHFR) thus blocking the conversion of dihydrofolate to tetrahydrofolate, which is required as a co-enzyme in nucleic acid biosynthesis. In yeast sulphonamide is required to block synthesis of dihydrofolate, probably at the dihydropteroate synthetase level, so that growth is inhibited by the action of methotrexate (Zhu *et al.*, 1985; Carramolino *et al.*,1989). This selection system has been used in animals, plants and fungi with methotrexate resistant transformants being generated by supplying cells with either extra copies of a DHFR gene (Zhu *et al.*, 1985) or a mutant DHFR gene whose product is insensitive to methotrexate (Pua *et al.*, 1985; Isola *et al.*, 1989).

5.2 RESULTS

Hygromycin B, G-418, phleomycin, chloramphenicol and methotrexate were tested for toxicity to *P. infestans*. Background growth from encysted zoospores could not be satisfactorily suppressed in *P. infestans* isolate ATCC48720 using methotrexate in combination with sulphanilamide (data not shown) and so this system was not pursued further. As mentioned previously, the study of virulence in *P. infestans* will involve investigations of a genetically heterogeneous 'wild' population and so to be useful in this work a transformation selection system will need to be generally applicable. For this reason hygromycin B, G-418, phleomycin and chloramphenicol were tested on mycelium and encysted zoospores of three isolates. Figures 5.1, 5.2, 5.3 and 5.4 show the plate tests performed for encysted zoospores of isolate ATCC48720 and Figures 5.5, 5.6, 5.7 and 5.8 show the growth inhibition curves for mycelial plug inocula of three isolates. The results are summarised in Table 5.1.

Table 5.1 Minimum inhibitory concentrations (μg/ml) of various antibiotics for three isolates of *P. infestans*.

Isolate	ATCC48720		I259		I230	
Inoculum	M	Z	M	Z	M	Z
Hygromycin B	100	50	5	10	100	25
G-418	4	4	4	2	4	1
Phleomycin	300	150	400	100	200	40
Chloramphenicol	600	600	1000	1000	1000	600

The minimal inhibitory concentration of an antibiotic was defined as the minimum concentration which prevented measurable fungal radial growth from a hyphal plug (M) or which prevented macroscopic growth from encysted zoospores (Z), after 12-15 days incubation at 18-20° C.

Table 5.2 Plasmids used in transformation experiments.

Selection System	Plasmid	Gene	Promoter	Terminator	Used to transform	Other sequences	References
Hygromycin B	pAN7-1	*hph*	*A. nidulans* (*gpdA*)	*A. nidulans* (*trpC*)	Several ascomycetes	-	Punt *et al.* (1987)
	pROH1	*hph*	*C. acremonium* (IPNS)	*E. coli*	*C. acremonium*	-	Harford (Glaxochem plc.) (per. comm)
	pLG90	*hph*	*S. cerevisiae* (*CYC1*)	*S. cerevisiae* (*CYC1*)	*S. cerevisiae*	2 µm *ORI* *URA3*	Gritz & Davies (1983)
	pRD1	*hph*	*P. megasperma* (actin)	*A. nidulans* (*trp C*)	No reports	-	Dudler, Switzerland (per. comm.)
G-418 [1]	pEB10	*kan*	*E. coli*	*E. coli*	*N. crassa*	*N. crassa am*	Bull & Wooton (1984)
	pBC10	*kan*	*E. coli*	*E. coli*	*N. crassa*	*N. crassa am*	Bull & Wooton (1984)
	pJL2	*kan*	*E. coli*	*E. coli*	*Phycomyces blakesleeanus*	*URA3* *Phycomyces* ARS	Revuelta & Jayaram (1986)
	pSV2*neo*	*neo*	SV40 early	*E. coli*	*Achlya ambisexualis* Mammalian cells	SV40 *ori* SV40 polyA site	Manavathu *et al.* (1988)
Phleomycin	pAN8-1	*ble*	*A. nidulans* (*gpdA*)	*A. nidulans* (*trp C*)	*A. niger*	-	van den Hondel, Netherlands (per. comm.)
	pUT332	*ble*	*S. cerevisiae* (*TEF1*)	*S. cerevisiae* (*CYC1*)	*S. cerevisiae*, *Fulvia fulva*	-	Cayla Co., France Oliver, U.K. (per. comm.)
	pUT701	*ble*	*Streptomyces*	*S. cerevisiae* (*CYC1*)		-	Cayla Co., France

Notes

1. *neo* encodes aminoglycoside-3'-phosphotransferase I from Tn*5*.

kan encodes aminoglycoside-3'-phosphotransferase II from Tn*903*(formerly Tn*601*).

Hygromycin B, phleomycin and particularly G-418 were effective against each of the isolates at low concentrations and gave very clean backgrounds. These antibiotics were considered good selection agents for a transformation system. Chloramphenicol was toxic to these isolates but high concentrations were required to substantially reduce persistent background growth. This system was considered unsuitable for further development.

The plasmids listed in Table 5.2 were obtained for use as potential transformation vectors for *P. infestans*. These plasmids were purified using CsCl density gradient centrifugation or Qiagen ion-exchange columns as transformation efficiency can be affected if plasmid preparations of low purity are used (Buxton & Radford, 1984; Randall *et al.*,1989; Wnendt *et al.*, 1990).

All transformation experiments were performed on *P. infestans* isolate ATCC48720. Three basic techniques were used in attempts to transform *P. infestans*: $CaCl_2$/PEG treatment, lithium acetate/PEG treatment and electroporation. Figures 5.9, 5.10 and 5.11 outline the methodologies used for the $CaCl_2$ and lithium acetate/PEG methods. As transformation in at least some fungi is clearly influenced by both protocol and strain, it seemed prudent to try different $CaCl_2$/PEG protocols with *P. infestans*. Many filamentous fungal transformation procedures, including that for the Oomycete *Achlya ambisexualis* (Manavathu *et al.*, 1988), are variations of Method I-like protocols. Method III is based on that of Yelton *et al.* (1984), for *Aspergillus nidulans*, modifications of which have been used to transform other higher fungi (van Hartingsveldt *et al.*, 1987; de Ruiter-Jacobs *et al.*, 1989; Wang *et al.*, 1988). Method II-like protocols give higher transformation efficiencies than Method III-like protocols for all tested strains of *A. niger* and most strains of *A. oryzae* (Campbell *et al.*, 1989; Unkles *et al.*, 1989) although certain strains of *A. oryzae* do not seem transformable by this method (Campbell & Macro, unpublished observations). Protocol IV is based on the lithium acetate/PEG method of Ito *et al.* (1983). Table 5.3 details the transformation experiments performed. More than 5×10^5 viable protoplasts were selected for transformation in each of the experiments.

Table 5.3 Summary of $CaCl_2$/PEG and lithium acetate/PEG transformation experiments for *P. infestans* isolate ATCC48720.

Cell Type	Protocol	Transformation Buffer[1]	Volume (µl)	Incubation Temperature[2]	Incubation Time (min.)[2]	PEG solution[3]	Incubation Temperature[4]	Incubation Time (min.)[4]	Expression/ Selection Method[5]	Expression Time[6]	Marker System and No. of Experiments Performed With Each Plasmid									
											Hygromycin B				G-418			Phleomycin		
											pAN7-1	pROH1	pLG90	pRD1	pEB10	pBC10	pJL2 pSV2*neo*	pAN8-1	pUT332	pUT701
Cyst protoplasts	I	KCl/Tris[a]; 10 mM $CaCl_2$	500	RT[c]	30	50 % PEG 6000; 10 mM $CaCl_2$	RT	30	A	18h	1	1								
"	"	KCl/Tris; 30 mM $CaCl_2$	"	"	"	As above	"	"	"	"	1	1								
"	"	As above	200	"	20	50 % PEG 6000; 30 mM$CaCl_2$	4°C	20	"	48								1	1	1
"	"	As above	200	"	"	25 % PEG 6000; 30 mM $CaCl_2$	RT	"	"	18	1	1			1	1				
"	"	As above	"	"	"	As above	4°C	"	"	"	1	1			2	2		1	1	1
"	"	As above	"	"	"	As above	"	45	"	"	1	1								
"	"	As above	"	"	"	50 % PEG 4000; 30 mM $CaCl_2$	RT	20	"	"					2	2				
"	"	As above	"	"	"	As above	4°C	"	"	"	3	3	3		2		2 2	4*	4*	4*
"	"	As above	"	"	"	As above	"	"	"	48								2	2	2
"	"	As above	"	On ice	45	As above	on ice	"	"	18					2		2 2	1	1	1
"	"	As above	"	RT	20	25 % PEG 4000; 30 mM $CaCl_2$	RT	"	"	"								2	2	2
"	"	As above	"	"	"	As above	4°C	"	"	"	1	1						3	3	3
"	"	KCl/Tris + 30 mM $CaCl_2$; 5 % PEG4000	"	On ice	"	As above	on ice	"	"	"	2	2								
"	II	KCl/$CaCl_2$/Tris[b] + 0.1 M Lithium acetate	"	RT	45	40 % PEG 4000; 0.1 M Lithium acetate	RT	"	"	"	2	2	2		2		2 2	2	2	2
Encysted zoospores	"	As above	"	"	60	"	"	"	"	3	1	1	1							
"	"	As above	"	"	30	"	"	"	"	"	1	1	1							
"	"	0.1 M Lithium acetate	"	"	"	"	"	60	B	18	2	2	2		2		2	2	2	2
Mycelial protoplasts	I	0.35 M $CaCl_2$;10 mM Tris-HCl, pH 7.5	500	"	"	25 % PEG 6000; 10mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5	"	"	"	24	2	2	2	1	2		2 2	2	2	2
"	III	0.8 M mannitol; 50 mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5	"	"	20	25 %/50 % PEG 6000; 50 mM$CaCl_2$; 10 mM Tris, pH 7.5	"	20	A	48	1	1	1	1	1		1 1	1	1	1
"	III	As above	"	"	"	"	"	"	"	24	1	1	1		1		1 1	1	1	1
Sporangial protoplasts	I	1 M KCl; 0.1 M $CaCl_2$; 10 mM Tris-HCl, pH 7.5	"	"	30	25 % PEG 6000; 10mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5	"	30	"	18	1	1	1		1		1 1			
"	"	As above	"	"	"	50 % PEG 6000; 10mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5	"	"	"	"	1	1	1					1	1	1
"	"	As above	"	On ice	"	25 % PEG 400; 10mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5 (2 vols)	"	1	"	"	1	1	1		1	1	1	1	1	1
"	"	As above	"	"	"	40 % PEG 400; 10mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5 (2 vols)	"	"	"	"	1	1	1		1	1	1			
"	"	As above	"	"	"	50 % PEG 400; 10mM $CaCl_2$; 10 mM Tris-HCl, pH 7.5 (2 vols)	"	"	"	"	1	1	1					1	1	1

Notes.

1-6 : See Figs. 5.9, 5.10 and 5.11 for full experimental details.

(a) KCl/Tris : 1M KCl; 10 mM Tris, pH 7.5

(b) KCl/$CaCl_2$/Tris : 0.64 M KCl; 0.2 M $CaCl_2$; 10 mM Tris, pH 7.5 (approximately isotonic to 1 M KCl)

RT : Room temperature (19-22° C)

* : Experimental conditions yielding putative transformants. See main text for details.

Plasmids encoding the same selectable marker were usually pooled and added to a population of cells together. All experiments included controls for viability and antibiotic sensitivity of cells passed through the transformation protocol in the absence of plasmid DNA. The experiments using sporangial protoplasts were performed by A. Campbell at the Scottish Crop Research Institute.

Three experiments yielded putative transformant colonies. These are indicated in Table 5.3 with an asterisk. Putative transformants derived from cyst protoplasts simultaneously exposed to the phleomycin resistance plasmids pAN8-1, pUT332 and pUT701 arose in two experiments (designated T36 and T38) out of four identical ones, no putative transformants were obtained from two further similar experiments in which the expression period was extended from 18 h to 48 h. One putative transformant colony was obtained in experiment T36 and three were obtained in experiment T38, after 6 days incubation. Three mycelial plugs were taken from different areas of the colony from experiment T36 of which one continued to grow well when subcultured onto PBA + 300 µg/ml phleomycin. One mycelial plug was taken from each 'transformant' colony from experiment T38 of which two continued to grow well when subcultured onto PBA + 300 µg/ml phleomycin. Mycelium from the three colonies which continued to grow was transferred to PB and to PB + 200 µg/ml phleomycin. Mycelium grew in all the PB liquid cultures but failed to do so in any containing 200 µg/ml phleomycin. Genomic DNA was prepared by the fungal miniprep. method from the PB cultures and from mycelium derived from a cell viability plate from experiment T36. No hybridisation to DNA of any of the putative transformants was detected (data not shown) and on this basis they were assumed to be false positives.

Putative transformant colonies arose in one experiment employing electroporation in which sporangial protoplasts were exposed to plasmid pRD1. DNA was prepared from these cultures and from a suitable negative control culture by the RNA method. Figure 5.12 shows the results of Southern analysis of these DNAs. A weak hybridisation signal was obtained against DNA from one of the putative

transformants but not against any others. The experiment was repeated and this also yielded putative transformant colonies but this time no hybridisation signals were detected in genomic DNA prepared from several of these colonies.

5.3 DISCUSSION

In attempts to transform *P. infestans* isolate ATCC48720 over 200 combinations of conditions were tried, involving different experimental designs and parameters, three different cell types of *P. infestans* and ten different antibiotic resistance constructs incorporating three different antibiotic resistance genes fused to a variety of bacterial, eukaryotic and viral transcriptional control sequences. As mentioned in the introduction to this chapter firm conclusions cannot be drawn from negative results in these kinds of experiments. However, in view of the variety of techniques and experimental conditions employed it seems unlikely that the lack of success in generating transformants was due simply to the inability of plasmid DNA to gain entry to the cells, particularly as some recent work by Peterson and colleagues has revealed the ability of motile zoospores to take up exogenous plasmid DNA. Southern analysis suggested that some of this was concatermerisation and also chromosomally integrated. Unfortunately neither stable nor transient expression of the heterologous marker genes carried on these vectors was observed (T. Peterson, pers. comm.). The expression of *hph, kan, neo* and *ble* in a diverse range of hosts argues against the coding region of any of these genes being inherently nonfunctional in *P. infestans*. Arguably the most likely explanation for failure to generate transformants in these experiments seems to be that the noncoding flanking regions of the various constructs are not functional in *P. infestans*, possibly in terms of initiation of transcription, or even ribosome binding.

Plasmids pSV2*neo* and pRD1 in particular, became available to us relatively late on in the course of these transformation experiments and so were not tested as

extensively as some of the other vectors. It seems unwise therefore to assume at this stage that these two constructs are nonfunctional in *P. infestans* isolate ATCC48720.

Due to slow growth genomic DNA yields from some cultures of putative transformants were very low. DNA was extracted from cells grown either in the complete absence of selection or at selection levels below those necessary to completely inhibit growth from the Wild-type. Loss of both autonomously replicating and chromosomally integrated transforming DNA has been observed from transformants of other fungi when selective pressure is removed (Banks, 1983; Sreekrishna *et al.*, 1984; Wostemeyer *et al.*,1987; Farman & Oliver, 1988; Churchill *et al.*, 1990). Other groups developing transformation systems for *Phytophthora* species have also encountered the phenomenon of colonies appearing on antibiotic selective media but which do not show hybridisation on Southern analysis (H. Prell, pers. comm.; O. C. Yoder, pers. comm.).

Transformation systems for basidiomycetes (Banks, 1983; Binninger *et al.*, 1987; Alic *et al.*, 1988; Banks & Taylor,1988; Wang *et al.*, 1988; Casselton & de la Fuente Herce, 1989; Randall *et al.*, 1989) are principally based on marker genes which have either an homologous promoter or a promoter from another basidiomycete. The only exception to this employed bacterial transcription sequences but was highly unusual in that evidence for gene-mediated transformation was restricted to rescue of intact transformation vector into *E. coli* at low frequency and absence of growth in negative controls - transforming DNA in the genomic DNA of transformants could not be detected by Southern analysis (Randall *et al.*, 1989).

Coprinus cinereus could not be transformed by either of two *A. nidulans* biosynthetic genes or by *hph* fused to the *Ustilago maydis hsp70* gene promoter. Therefore in *C. cinereus* at least, it appears that functionality of heterologous promoters shows a similar pattern to that generally observed in ascomycetes i.e. recognition of such sequences from closely related species but often not those from species outwith the hosts' Subdivision or from less closely species within the host's Subdivision.

Since the work in this chapter was performed transformation of *P. parasitica* to G-418 resistance has been achieved using the *neo* gene flanked by the 5' and 3' noncoding regions of the *P. parasitica TRP1* gene. The plasmid construct was introduced into mycelial protoplasts by electroporation and was integrated into the nuclear chromosomal DNA (Prell *et al.*, 1989). These results are very encouraging with regard to the prospects for achieving transformation in *P. infestans* and further highlight the desirability of obtaining homologous transcription control sequences from *P. infestans*.

Figure 5.1. Growth inhibition of encysted zoospores of isolate ATCC48720 by hygromycin B. Hygromycin B concentrations (μg/ml) are as follows:

0 5 10

15 20 30

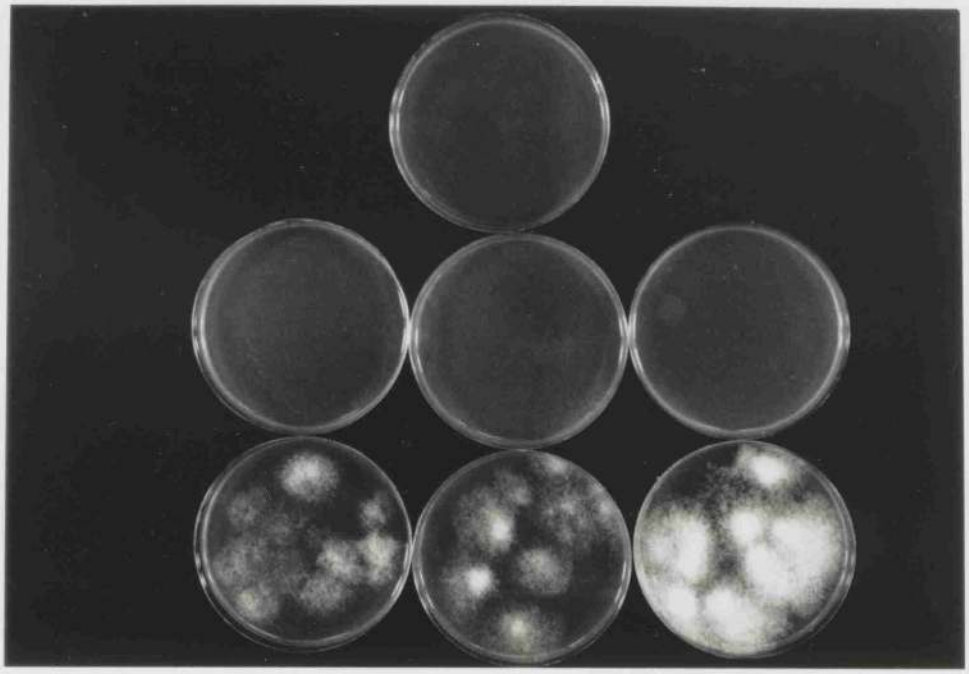
50 75 100

Figure 5.2. Growth inhibition of encysted zoospores of isolate ATCC48720 by G-418. G-418 concentrations (μg/ml) are as follows:

0 1 2

4 6 8

10



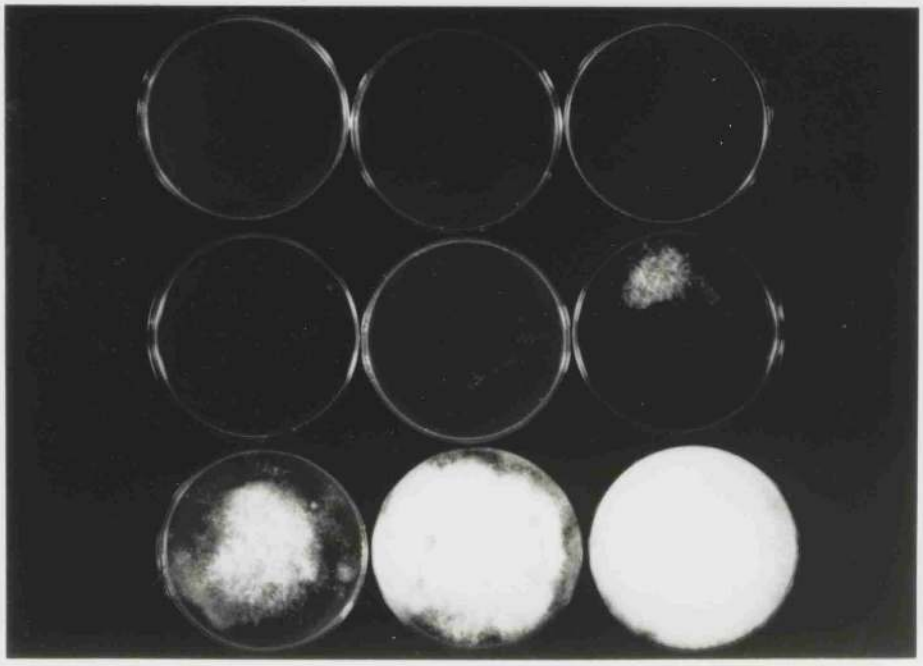


Figure 5.3. Growth inhibition of encysted zoospores of isolate ATCC48720 by phleomycin. Phleomycin concentrations (μg/ml) are as follows:

0

50 100 150

200 250 300

Figure 5.4. Growth inhibition of encysted zoospores of isolate ATCC48720 by chloramphenicol. Chloramphenicol concentrations (μg/ml) are as follows:

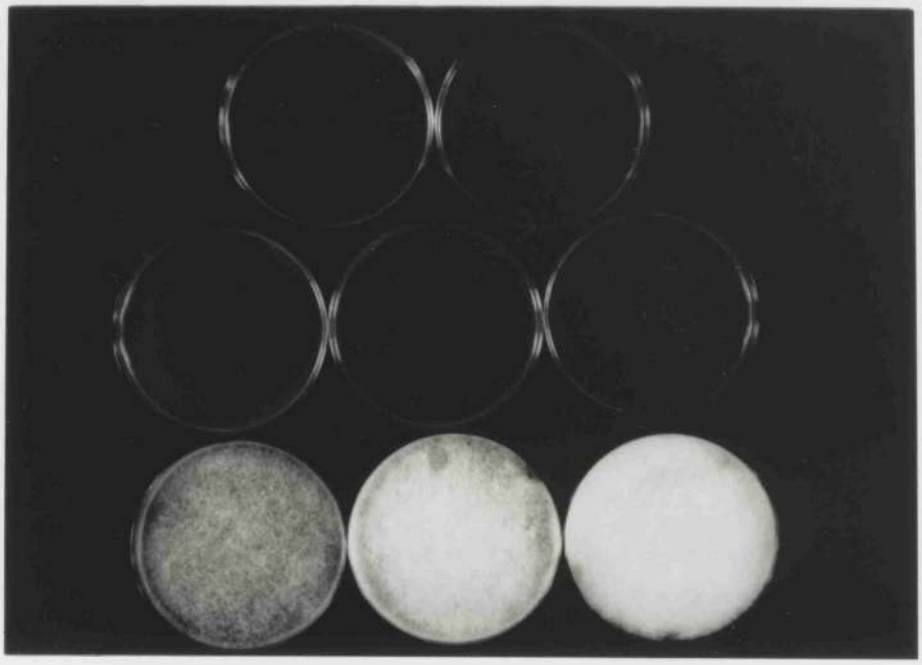
0 ethanol control[1] 100

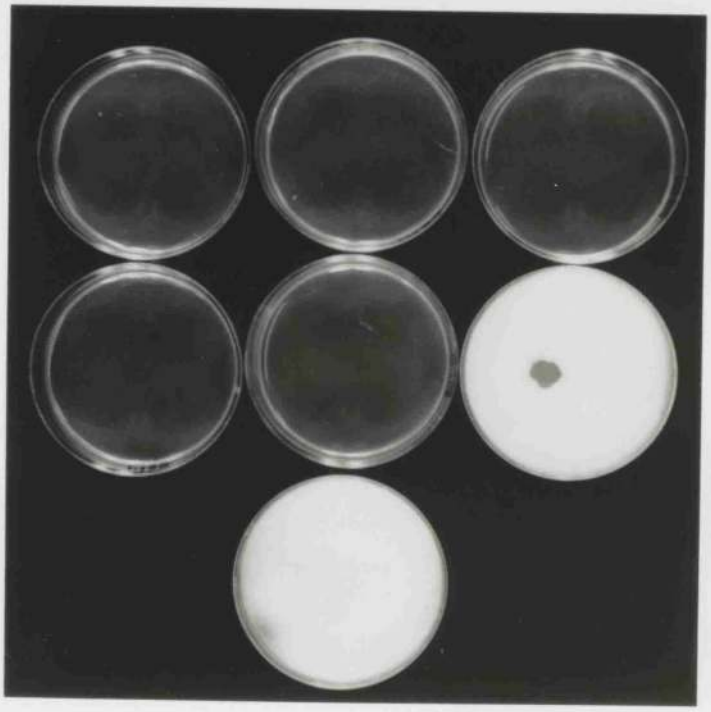
200 400 600

800 1000

Note:

1. See Section 2.2.2





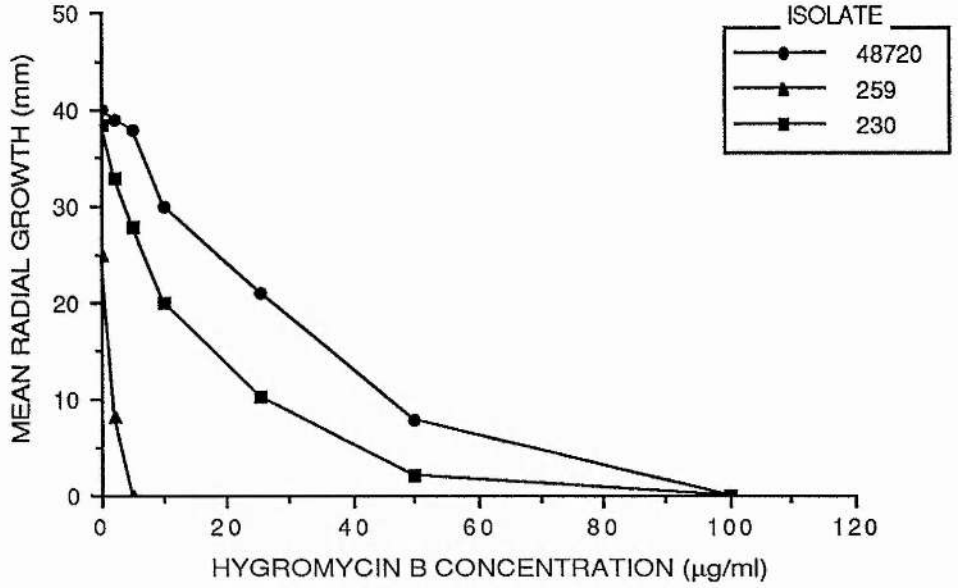


Figure 5.5. Inhibition of growth from mycelial plug inocula of three isolates of *P. infestans* by hygromycin B. Data are means of measurements from two or three plates. The experiments were repeated and similar results obtained.

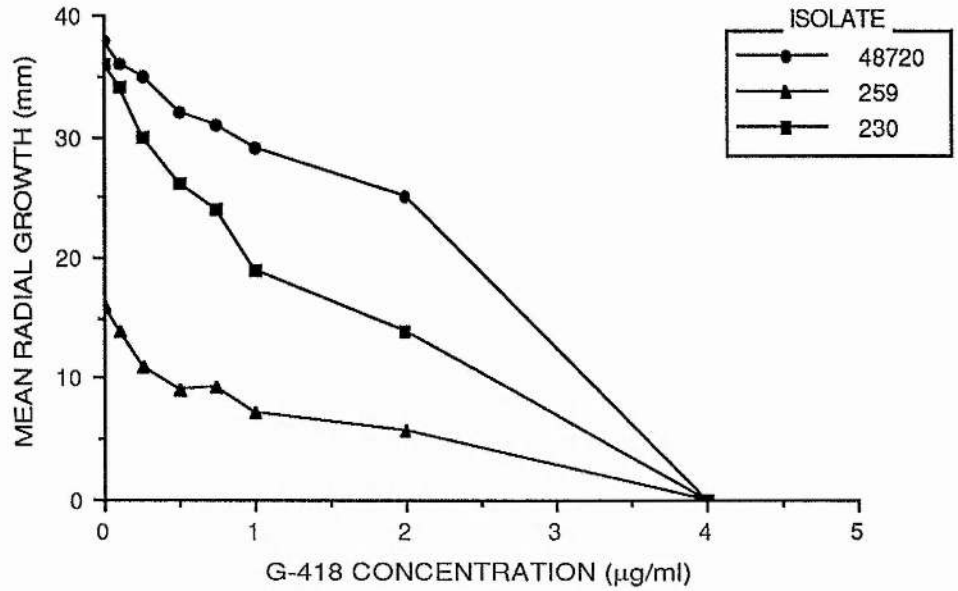


Figure 5.6. Inhibition of growth from mycelial plug inocula of three isolates of *P. infestans* by G-418. Data are means of measurements from two or three plates. The experiments were repeated and similar results obtained.

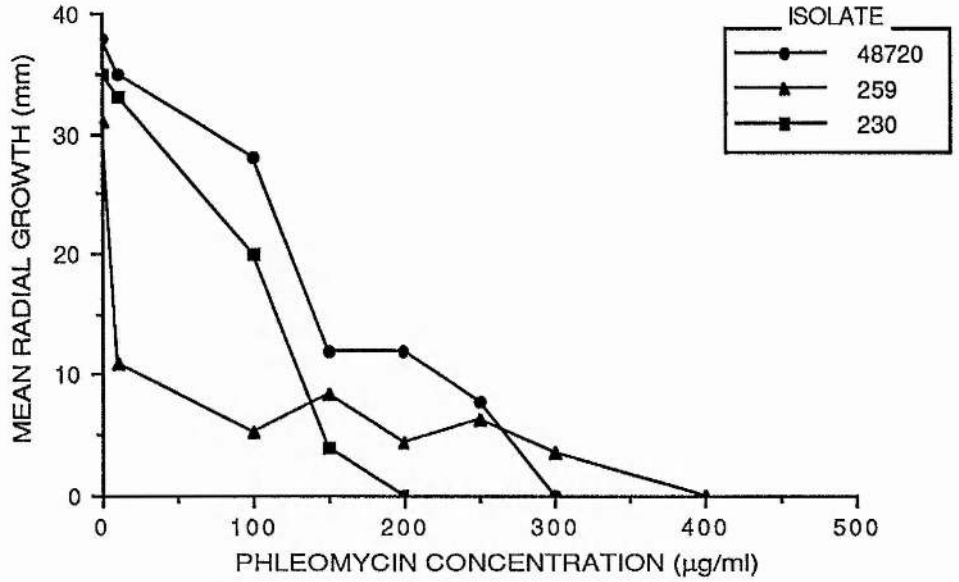


Figure 5.7. Inhibition of growth from mycelial plug inocula of three isolates of *P. infestans* by phleomycin. Data are means of measurements from two or three plates. The experiments were repeated and similar results obtained.

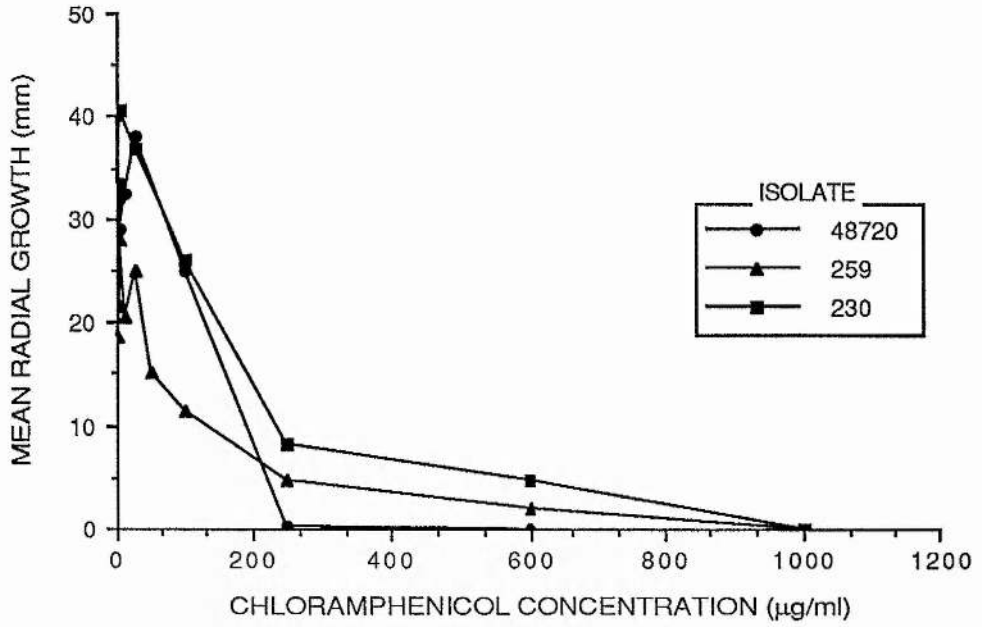
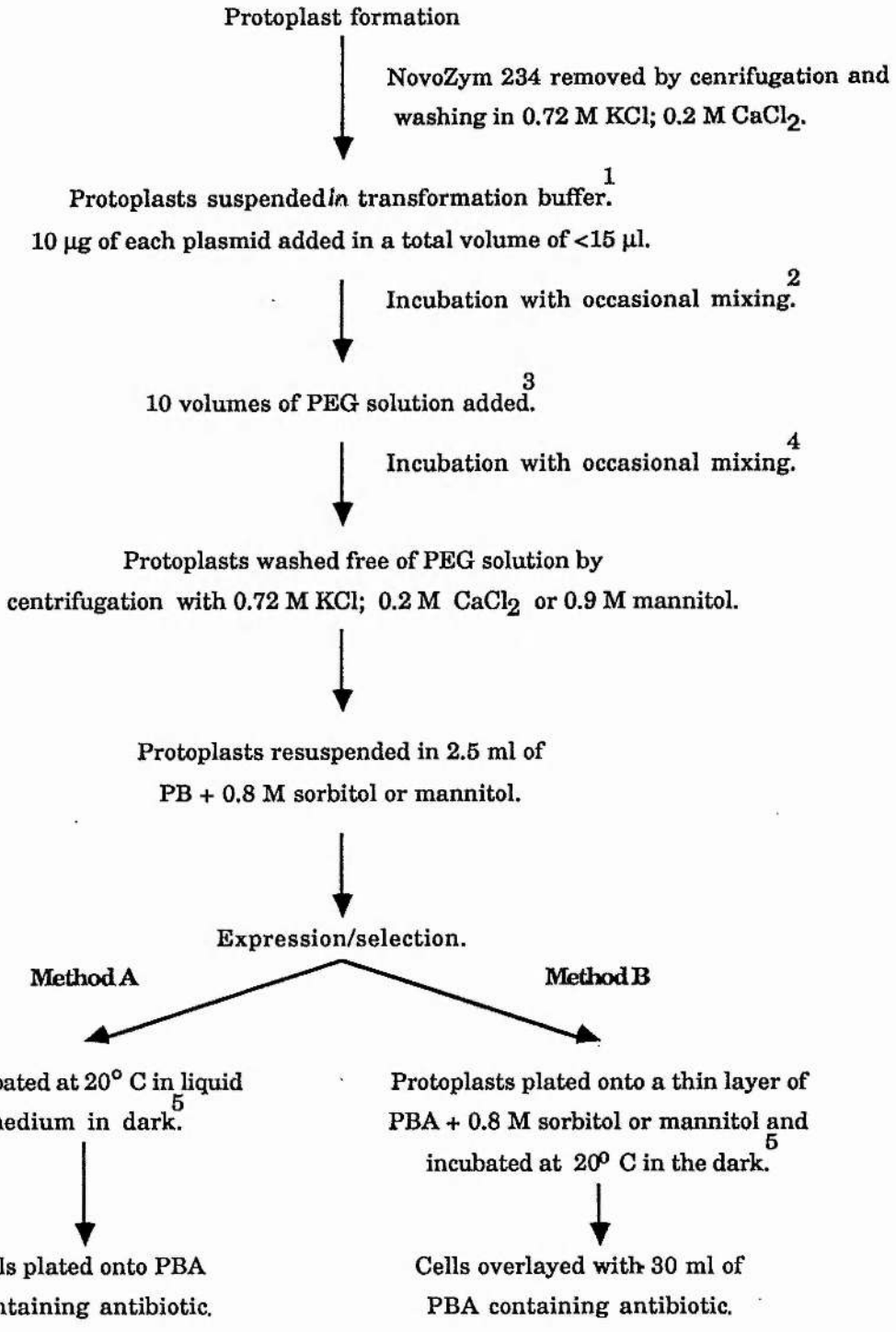


Figure 5.8. Inhibition of growth from mycelial plug inocula of three isolates of *P. infestans* by chloramphenicol. Data are means of measurements from two or three plates. The experiments were repeated and similar results obtained.

Figure 5.9 Outline of transformation Method I and Method II.

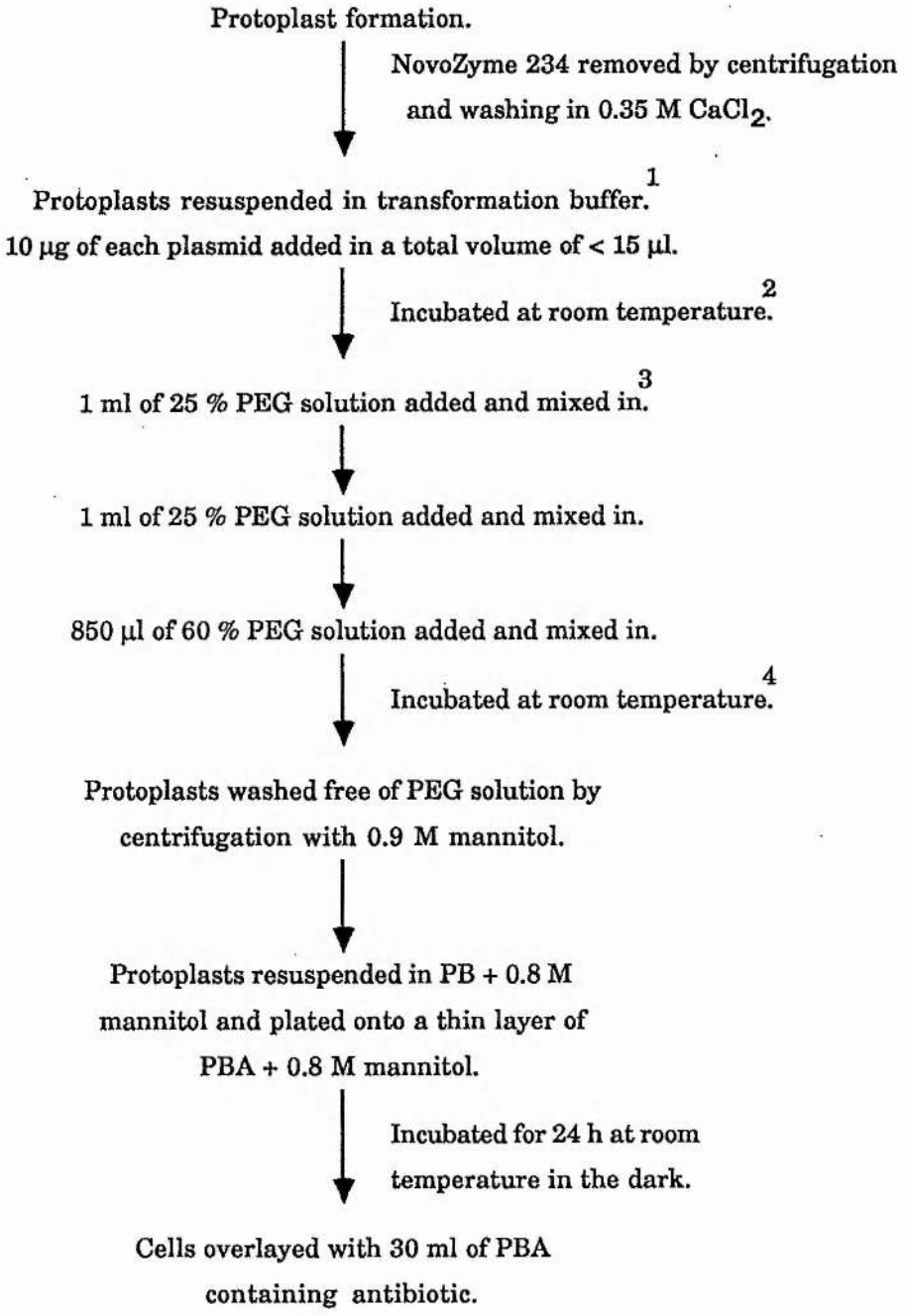


Notes.

1-5. See Table 5.3 for parameters of individual experiments.

* Method II is as Method I except 25 µl of PEG solution is added to protoplasts and plasmid prior to first incubation.

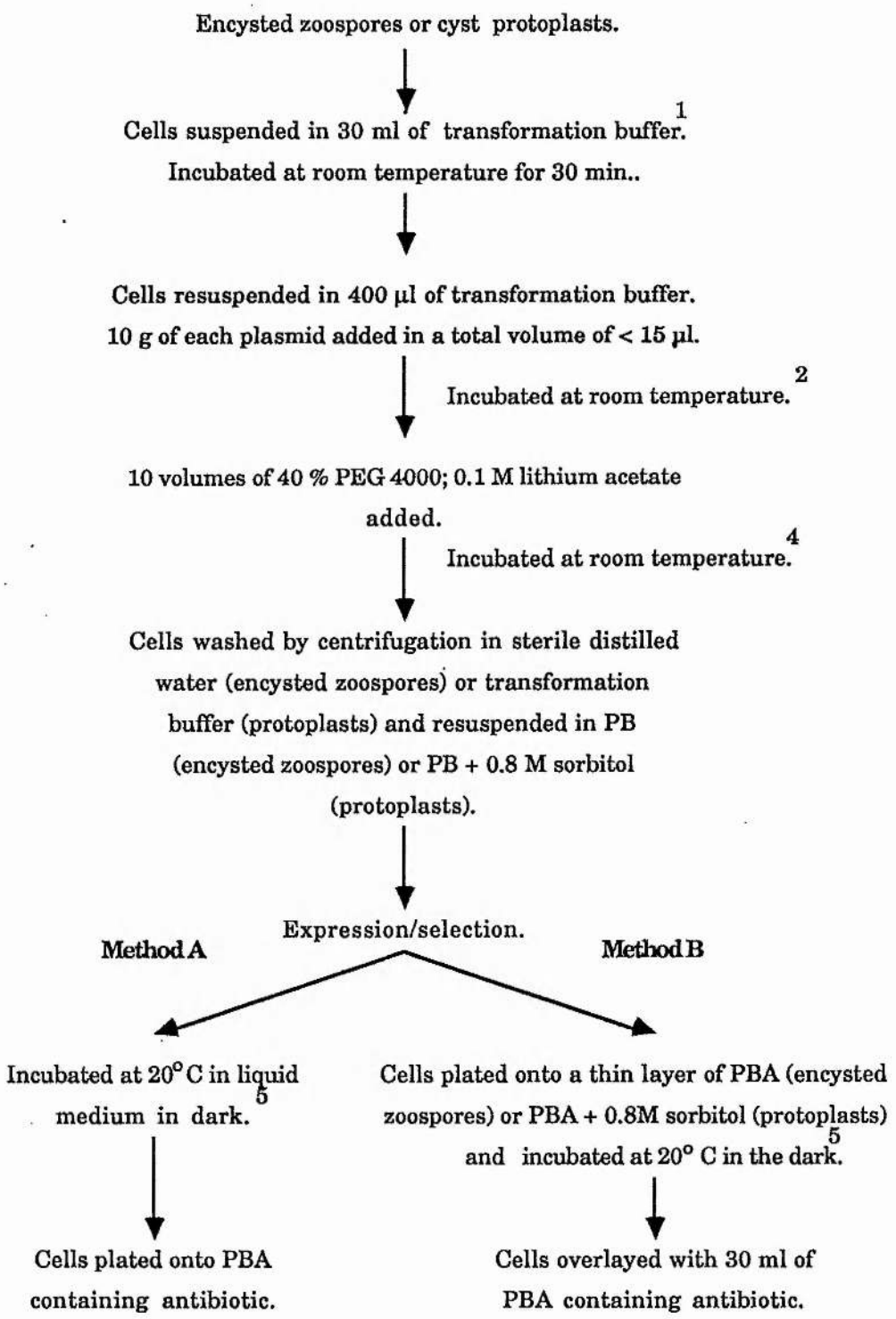
Figure 5.10 Outline of transformation Method III



Notes.

1-4. See Table 5.3 for parameters of individual experiments.

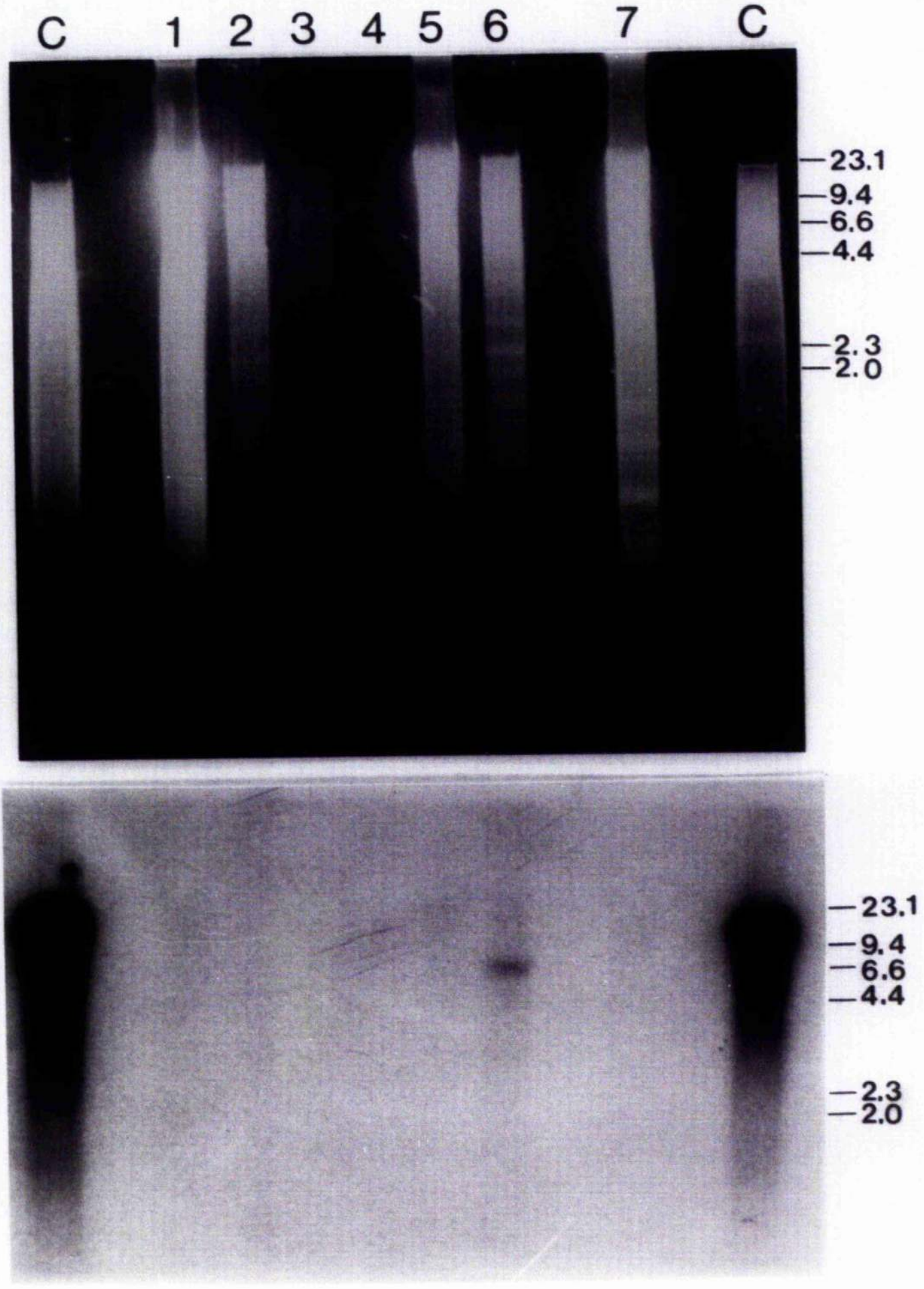
Figure 5.11 Outline of transformation Method IV



Notes.

1-5. See Table 5.3 for parameters of individual experiments.

Figure 5.12 Southern analysis of putative hygromycin B resistant transformants. Lanes 1-7 contain *P. infestans* genomic DNA. Lanes C contain genomic DNA of an *Aspergillus nidulans* pAN7-1 transformant. Genomic DNA was digested with *Hin*dIII, the fragments separated by electrophoresis in 0.7% agarose and transferred to a nylon filter. Prehybridisation, hybridisation and washing were as performed as described in Materials and Methods for homologous probes. The probe was pAN7-1 (on which pRD1 is based) linearised with *Hin*dIII. The filter was autoradiographed with Kodak XAR5 film for 17 days.


C
1
2
3
4
5
6
7
C
23.1
9.4
6.6
4.4
2.3
2.0
23.1
9.4
6.6
4.4
2.3
2.0

Chapter 6

ISOLATION AND CHARACTERISATION OF *P. infestans* GENES

6.1 INTRODUCTION

Attempts to generate *P. infestans* transformants with marker genes controlled by heterologous transcription signals were unsuccessful. To circumvent the possibility that *P. infestans* requires homologous transcription control sequences it was decided to isolate genes from a genomic DNA library of *P. infestans* as a source of such sequences. The nuclear haploid DNA content of *P. infestans* has been estimated to be approximately 0.26 pg which corresponds to about 2.4 x 10^8 bp. This is unusually large for a eukaryotic microbe and is even about 2.5 times larger than that of *P. megasperma* f. sp. *glycinea* (Tooley & Therrien, 1987; Tyler & Mao, 1989). The substantial size of the *P. infestans* genome effectively precludes the use of a plasmid as the cloning vector due to the relatively small average insert size such vectors can carry and the impracticable number of clones needed for sufficient coverage of the haploid genome. Therefore a bacteriophage λ cloning vector was selected for the construction of a gene library.

6.2 RESULTS

6.2.1 *CONSTRUCTION OF A* P. infestans *GENE LIBRARY*

Genomic DNA isolated from *P. infestans* isolate ATCC48720 by the RNA method was partially digested with *Sau*3A, size selected and cloned into a lambda DASH II vector (Stratagene plc., U.K.) according to the manufacturer's instructions. The

library contained approximately 3 x 10^5 clones of average insert size 17 kb, giving an estimated haploid genome coverage of 20 fold.

6.2.2 *SELECTION OF GENE PROBES*

Two types of probe were tested for cross-hybridisation to *P. infestans* genomic DNA, degenerate oligonucleotide probes derived from highly conserved amino acid motifs in β-tubulin and alkaline protease and gene fragment probes from highly conserved regions of genes for actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The probes were derived as follows.

```
                ValValGluProTyr
β–tubulin probe  GTNGTNGARCCNTAYAA
```

The sequence of this probe corresponds to a motif at position 179 in the amino acid sequence of the *Trypanosoma brucei rhodesiense* β–tubulin (Kimmel *et al.*, 1985).

```
                ThrSerMetAlaThrPro
Alkaline protease ACNTCNATGGCNACNCCNCA
```

The alkaline protease probe was derived from an amino acid motif at position 227 in the mature alkaline protease of *Aspergillus oryzae* (Tatsumi *et al.*, 1988). The probe for actin was the 0.83 kb *NcoI/KpnI* fragment of the *A. nidulans actA* gene (Fidel *et al.*, 1988). The probe for GAPDH was a 1 kb *EcoRI/BamHI* fragment of an *A. nidulans gpd* cDNA clone in pcDNA51a (Punt *et al.*, 1987).

Actin, β–tubulin and GAPDH are ubiquitous in eukaryotes and have domains which are highly conserved across wide evolutionary distances so their genes are suitable candidates for cloning using the above probes. Furthermore the genes for these proteins are constitutively or highly expressed in eukaryotes, an important consideration for the use of their promoters in a transformation system. Each of the above probes hybridised specifically to restriction enzyme-digested genomic DNA (Fig. 6.1 a-d). The actin probe also hybridised to a 1.8 kb mRNA transcript on a northern blot of total RNA from *P. infestans* isolate ATCC48720 (not shown). The probes for β-tubulin, actin, GAPDH genes were used to screen the gene library.

6.2.3 *GENE ISOLATION*

(i) β-tubulin probe.

Five clones (designated T1, T2, T3, T13 and T16) were isolated from the tertiary screen of the gene library. Three different restriction patterns were discernible among these clones. The oligonucleotide probe strongly hybridised, with equal intensity, to a 0.8 kb *Hin*dIII fragment in all of the clones and to one of three sizes of *Bam*HI fragment (not shown). The 0.8 kb *Hin*dIII fragment of clone T16 hybridised at high stringency (0.2 x SSC, 65° C) to the same size fragments that were identified by the oligonucleotide probe in the other four clones but did so less strongly to fragments in clones T1-T3 than to fragments in clones T13 and T16. This difference was not related to the amount of DNA in the bands indicating that the sequences of the 0.8 kb *Hin*dIII fragment in three clones are different but related to the sequences of the other two. The 0.8 kb *Hin*dIII fragment of clone T16 strongly back-hybridised to multiple bands in genomic DNA of isolate ATCC48720 at high stringency (0.2 x SSC, 65° C) showing that this and probably the other clones originated from *P. infestans*. The 0.8 kb *Hin*dIII fragments from clones T16 and T3 were inserted into pUC13 and partially sequenced. None of the predicted amino acid sequences in any reading frame on either strand shared homology with β-tubulin except for a Val Val Glu Pro Tyr Asn motif in both clones. There were numerous stop codons in the nucleotide sequences in all reading frames indicating that neither clone encoded protein.

(ii) Actin probe.

Six clones were isolated from the gene library using the *A. nidulans* actin probe. The probe specifically hybridised to a 0.8 kb *Hin*dIII fragment in five of the clones which itself back-hybridised to a band of this size in *Hin*dIII digested genomic DNA of isolate ATCC48720 and to an mRNA transcript of expected size (1.8 kb) on a northern blot of total RNA (Fig6.2 a and c respectively). These are probably the same bands identified by the *A. nidulans* actin probe. A 3.5 kb *Eco*RI fragment encompassing the *Hin*dIII fragment was subcloned into pUC13 and the resultant

plasmid designated pSTA31. The putative actin gene carried on this fragment was designated *actA*.

The sixth clone isolated with the *A. nidulans* actin probe encompassed a 20 kb *Hin*dIII and a 3 kb *Bam*HI fragment which hybridised strongly to this probe. The 3 kb *Bam*HI fragment back-hybridised to fragments of expected size in *Bam*HI digested genomic DNA of isolate ATCC48720 and to 9.2 and 6.0 kb bands in *Hin*dIII digested genomic DNA (Fig. 6.2b). The 3 kb *Bam* HI fragment also hybridised to an mRNA species of expected size but the signal was very weak, requiring an exposure 20 times longer than that for the *actA* 0.8 kb *Hin*dIII fragment probe before becoming visible on the autoradiograph (Fig. 6.2 c). The putative actin gene carried on this fragment was designated *actB*, inserted into pUC13 and the resultant plasmid designated pSTA30. Both *actA* and *actB* were sequenced (Figs. 6.3, 6.4 and 6.5).

(iii) GAPDH probe.

Eleven positive clones were isolated from the tertiary screen of the gene library. A 3.5 kb *Eco*RI fragment in each of five clones analysed hybridised specifically to the *A. nidulans gpd* probe (not shown). This fragment from one of the clones was inserted into pUC13 and the resultant plasmid designated pSTA33. Restriction enzyme digested genomic DNA of *P. infestans* isolate ATCC48720 was probed with linearised pSTA33 which hybridised at high stringency to multiple bands in each digest (Fig. 6.2d). The 3.5 kb insert in pSTA33 was partially sequenced.

6.2.4 *SOUTHERN ANALYSIS OF ACTIN GENES*

The 0.8 kb *Hin*dIII fragment of *actA* clearly identifies two bands in the *Bam*HI digested genomic DNA of *P. infestans* (Figure 6.2a). As *Bam*HI does not cut the *actA* clone only one band was expected. Similar amounts of DNA were run in each lane so the intensity of the 0.8 kb hybridising band in the *Hin*dIII digested genomic DNA suggests it is composed of comigrating bands.

*Hin*dIII does not cleave within the *actB* clone and so only one hybridising fragment was predicted in the *Hin*dIII digested genomic DNA in Figure 6.2b. Three

bands are predicted from the restriction map of *actB*, a 0.28 kb band and two bands larger than 0.5 kb. These are observed in Figure 6.2b. Two hybridising bands are observed in *Bam*HI digested genomic DNA where only one is expected from the restriction map (Fig.6.3).

The blots in Figures 6.2a and b were performed under similarly stringent conditions under which no cross hybridisation occured between *actA* and *actB*. The hybridising bands seen in addition to those predicted from the restriction maps of *actA* and *actB* may be due either to polymorphism or to gene families of very close nucleotide similarity.

6.2.5 *GENE STRUCTURE AND PRIMARY PROTEIN SEQUENCE OF* act A *AND* act B.

Figure 6.6 shows the inferred amino acid sequences of *actA* and *actB* aligned with those of actin genes from a variety of organisms. Extensive homology with these actins demonstrates both *P. infestans* genes also encode actin. The northern blot (Fig. 6.2c) and primer extension analysis (S. Unkles, per. comm.) show that *actA* is actively transcribed in mycelium. Primer extension analysis (S. Unkles, per. comm.) and the very weak signal on the northern blot for *actB* show that this gene is also transcribed in mycelium but at a low level. Further experiments are required to accurately locate the transcription initiation site(s) in *actB*.

(i) 5' noncoding region.

actA has two tracts of CT residues on either side of the transcriptional start site. A number of filamentous fungal transcription intiation sites are located near such pyrimidine rich motifs (Gurr *et al.*, 1987). There is a CCAAT motif at position -196, 125 nt upstream of the transcription initiation site in *actA* and at position -98 in the 5' noncoding region of *actB*. This motif is absent from the promoter region of the *P. megasperma* f. sp. *glycinea* actin gene (Dudler, 1990) but is present in multiple copies upstream from the 5' terminus of the mRNA of the inducible *Bremia lactucae hsp70* gene (Judelson & Michelmore, 1989). CCAAT motifs have been implicated in

developmental and tissue specific gene expression particularly in higher eukaryotes. There is a CCAATAAAG motif at position -125 in *actB*, similar to the CACATAATA motif at position -150 in the 5' noncoding region of the *P. megasperma* actin gene (Dudler, 1990). *actB* contains a putative 68 nt intron, immediately upstream of the translation initiation codon, with yeast-like 5' and 3' intron border sequences (Ballance, 1986). The putative intron sequence is slightly A+T rich (58.8%) as are plant introns (Shah *et al.*, 1983). There is no evidence that this sequence actually functions as an intron.

(ii) Translation initiation site environment.

The sequence of bases surrounding the translation initiation codon of *act A* (TCAAGATGGC) is very similar to the canonical ascomycete consensus sequence of translation initiation sites (TCACAATGGC) (Ballance,1986). In *actB* the sequence surrounding the the first codon (CAGTCATGGA) is closer to that found in many genes of higher eukaryotes (CCACCATGGC) (Ballance, 1986) although the match is rather poor.

(iii) Coding region.

Neither gene contains introns in the coding region as indicated by the continuity of homology between their inferred translation products with actins of other

Table 6.1 Comparison of G+C content of *Phytophthora* genes

Gene	G+C content of coding region (%)		G+C content of nuclear genome (%)	Reference
	Observed	Expected		
actA	58.9	48.36	54	Storck & Alexopoulos,
actB	52.48	48.14		1970
P. megasperma actin	57.27	48.11	48	Tyler & Mao, 1989
gpd	55.83	48.31		

organisms. Both *actB* and *actA* in particular display heavy codon bias (Tables 6.2 and 6.3) this being reflected in the G+C content of their coding regions, which although slightly higher than expected are reasonably close to that of the nuclear genome of *P. infestans* as a whole (Table 6.1).

Tables 6.4 and 6.5 show the positional distribution of each base in the codons of *actA* and *actB* respectively. In *actA* there is a marked avoidance of A in the third position and a low frequency of G in the second position. A similar pattern exists in *actB* but is less extreme. Codon bias in both genes results in a substantial proportion of their respective proteins being encoded by a small subset of codons. Eighteen of the possible sense codons are not used at all in *actA* and three are not used in *actB*. Codon bias is very pronounced for all multicodon amino acids in *actA* except Pro, for which 3 of the four possible codons are used with similar frequencies but the fourth, CCA, is not used at all. Only five codons in the whole of the *actA* structural gene have A in the third position. This avoidance of A in the 'wobble' base position can also be seen in *actB* (Table 6.3) although less stringently. In *actA* the most favoured codons are usually those ending with C or G. Noticeable exceptions to this trend are the CGT codon which is used for 61 % of Arg residues and the GGT codon which codes for 65.5 % of Gly residues. In *actB* CGT and CGC are the most favoured codons for Arg with those ending in G accounting for only 9.5 % of Arg residues. There is no significant codon bias for Gly.

(iv) 3' noncoding region.

TAA is used as the stop codon in both *actA* and *actB*. There is no AATAAA-like polyadenylation signal motif in the sequenced 3' noncoding region of either of the *P. infestans* actin genes. Although present in many genes of higher eukaryotes its absence from genes of lower eukaryotes is not unusual. There are however T rich regions which are often present in the 3' noncoding regions of fungal genes (Gurr *et al.*, 1987).

6.2.6 *GENE STRUCTURE AND PARTIAL PRIMARY PROTEIN SEQUENCE OF* gpd *FROM* P. infestans

Figure 6.7 shows the sequencing strategy used to reveal the presence of a GAPDH gene on the 3 kb *Bam*HI insert of pSTA33. The available sequence (Fig. 6.8) encompasses the last 239 amino acids of the protein, probably about 73 % of the total, and 607 nt of the 3' noncoding region. Northern blots show this gene is actively transcribed in mycelium (S. Unkles pers. comm.), that it encodes GAPDH is demonstrated by the extensive homology between the predicted amino acid sequence from this clone and that of the *A. nidulans* GAPDH gene (Fig. 6.9). There are at least four locations where the amino acid sequence of the *P. infestans* GAPDH diverges at residues which are conserved in GAPDHs of several species ranging from mammals to bacteria (Yarbrough *et al.*, 1987). These differences lie at positions corresponding to residues 115, 116,231 and 267 in the *A. nidulans* enzyme and with the exception of the latter are all conservative changes. The continuity of the translated *P. infestans* sequence with that of the *A. nidulans* GAPDH shows there are no introns in this region of the *P. infestans* gene. Like *actA* and *actB* translation is terminated with a TAA codon. A putative polyadenylation signal, TATAAA, is present in the 3' noncoding region at position 904.

6.3 DISCUSSION

6.3.1 *COMPARISON OF* actA *AND* actB *WITH ACTIN GENES OF OTHER ORGANISMS*

The homologies of *actA* and *actB* with actin genes of other organisms (Table 6.6 and Fig. 6.6) indicates that ACTB is more diverged from these actins than is ACTA with the exception of the *P. megasperma* actin with which it has a high degree of homology at the nucleotide and amino acid levels. Two possible explanations for the similarity between ACTB and the actin of *P. megasperma* are: (a) There was an exchange of genetic information between *P. infestans* and *P. megasperma* or their

ancestors after the time when the evolutionary lines represented by *actA* and the *P. megasperma* actin gene diverged. (b) Two actin genes arose by a gene duplication event in *P. infestans* and then rapidly diverged from one another, perhaps to fulfil different roles in the organism. The second possibility seems the most likely, the need to play different cellular roles such as in cytoskeletal structure, cell motility, chromosome movement and muscle contraction is thought to be one reason for the presence of multiple isoforms of actin in mammals, birds (Rubenstein, 1990) and perhaps higher plants (Hightower & Meagher, 1986). Multigene families for actin are found in a diverse range of eukaryotes and may reach sizes of up to twenty (Romans & Firtel, 1985). By comparison only a handful of organisms have been found to possess single actin genes e.g. yeast, *Aspergillus nidulans, Tetrahymena thermophila* and *Phytophthora megasperma* but this feature does not seem to be phylogenetically significant. The absence of introns in the coding regions of *actA* and *actB* and the actin gene of *P. megasperma* is a characteristic shared only with protozoan actin genes .

6.3.2 *NUCLEOTIDE CONTENT AND CODON USAGE IN* Phytophthora *GENES*

A consequence of codon bias in *Phytophthora* genes is that a very high percentage of amino acid residues are encoded by a remarkably small subset of codons (Table 6.7).

Table 6.8 lists the most favoured codon(s) for each amino acid in the *actA, actB* and *gpd* genes of *P. infestans* and the actin gene of *P. megasperma*. All these genes display marked codon bias, particularly *actA*. Of the 18 multicodon amino acids 14 are coded by the same one or two codons in all four genes. Among the remaining 4 multicodon amino acids there is little in common about the most prefered codon for Pro between the four genes, but a pyrimidine is usually the first and second most preferred base in the third position of the others. Gly in ACTB is the only amino acid for which there is no bias in codon usage (Table 6.3). As there are only two Gln residues in *gpd* it cannot be classified in these terms. All four genes show an

avoidance of A in third position of codons which is almost total in *actA*. There is only one instance where the most favoured codon for an amino acid ends with A - for Pro in *actB*. A bias against A in the third position of sense codons is also a feature of plant actin genes (Shah *et al.*,1983; Nairn *et al.*, 1988).

Figure 6.1 Cross-hybridisation of gene probes to enzyme restricted genomic DNA of *P. infestans*.

DNAs in (a) and (b): I - *P. infestans* isolate ATCC48720. II - *P. infestans* isolate E13a. DNAs in (c) and (d): An - *Aspergillus nidulans* Glasgow strain *biA*1. Pi - *P. infestans* isolate ATCC48720.

Probes: (a) β-tubulin oligonucleotide probe. (b) alkaline protease oligonucleotide probe. (c) *A. nidulans actA* gene fragment (see text). (d) *A. nidulans gpd* gene fragment (see text).

Hybridisation and washing conditions were as described in Materials and Methods except that an incubation temperature of 52° C was used throughout for the *gpd* probe.

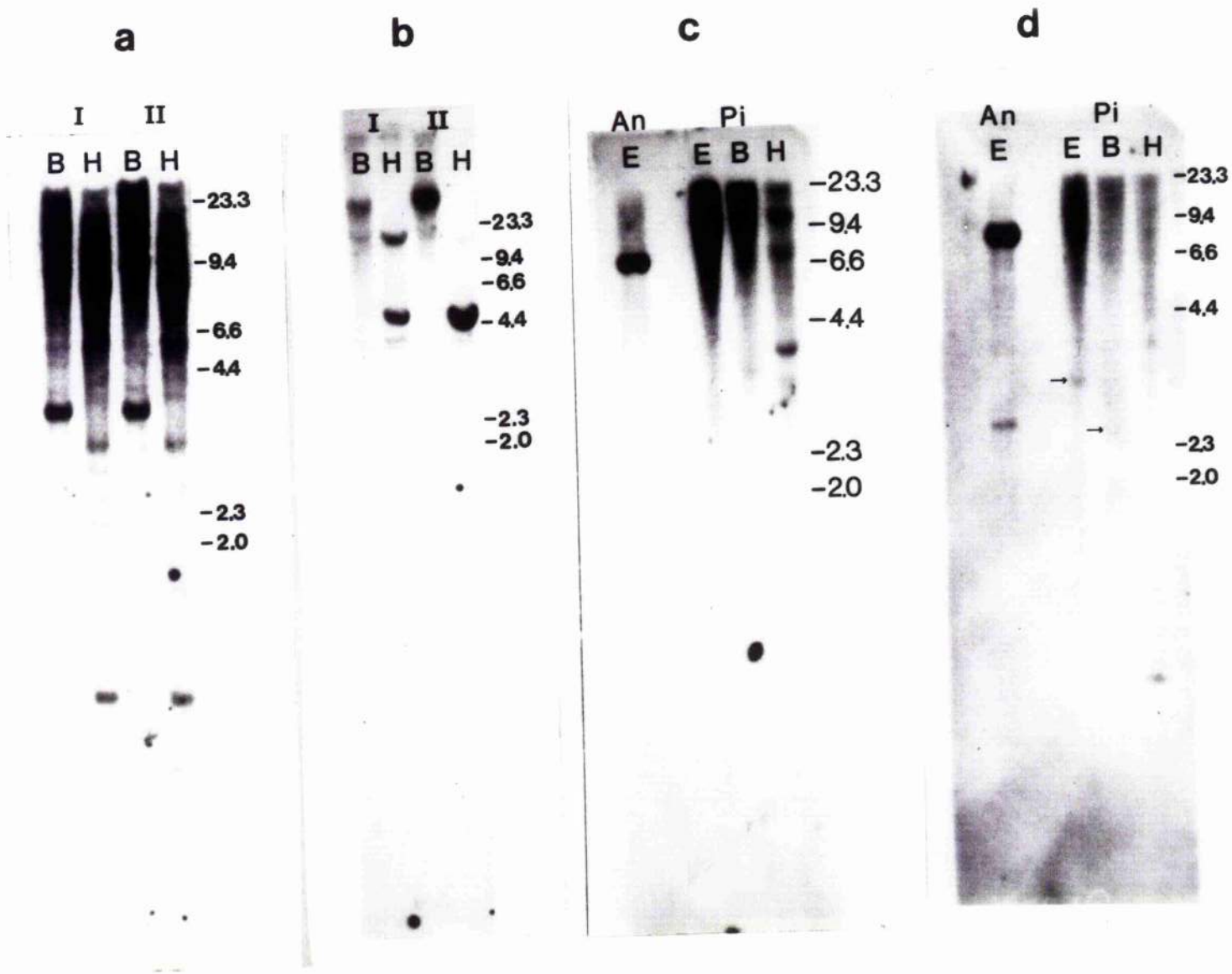

a
I
II
B H B H
-23.3
-9.4
-6.6
-4.4
-2.3
-2.0
b
I
II
B H B H
-23.3
-9.4
-6.6
-4.4
-2.3
-2.0
c
An
Pi
E
E B H
-23.3
-9.4
-6.6
-4.4
-2.3
-2.0
d
An
Pi
E
E B H
-23.3
-9.4
-6.6
-4.4
-2.3
-2.0

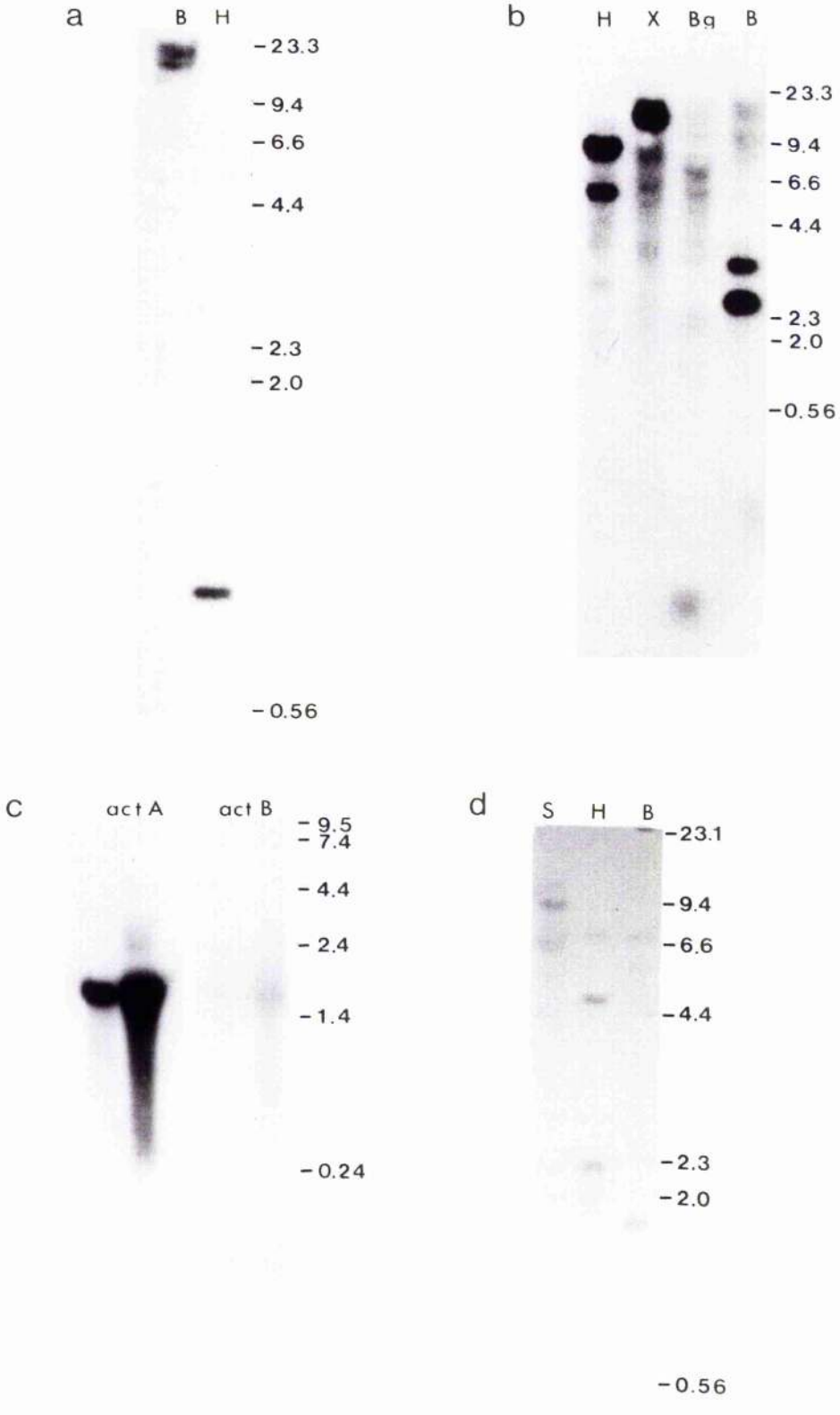
Figure 6.2 Hybridisation of *P. infestans* clones to nucleic acids from isolate ATCC48720. Hybridsation and washing conditions for Southern blots were as described for homologous probes in Materials and Methods.

(a) Back-hybridisation of 0.8kb *Hin*dIII fragment of *actA* to genomic DNA of *P. infestans*.

(b) Back-hybridisation of *Bgl*II fragment of *actB* to genomic DNA of *P. infestans*.

(c) Hybridisation of the 0.8 kb *Hin*dIII fragment of *actA* and the*Bgl*II fragment of *actB* to total RNA of *P. infestans* prepared by the method of Cathala *et al.* (1985). The left hand lane of each pair of tracks contained 10 μg of RNA, the right hand lane of each pair contained 40 μg of RNA which was electrophoresed in 1 % formaldehyde agarose (Davies *et al.*, 1986) and transferred to a nylon filter.

(d) Back-hybridisation of pSTA33 to genomic DNA of *P. infestans*.


a
B H
23.3
9.4
6.6
4.4
2.3
2.0
0.56
b
H X Bg B
23.3
9.4
6.6
4.4
2.3
2.0
0.56
c
act A
act B
9.5
7.4
4.4
2.4
1.4
0.24
d
S H B
23.1
9.4
6.6
4.4
2.3
2.0
0.56

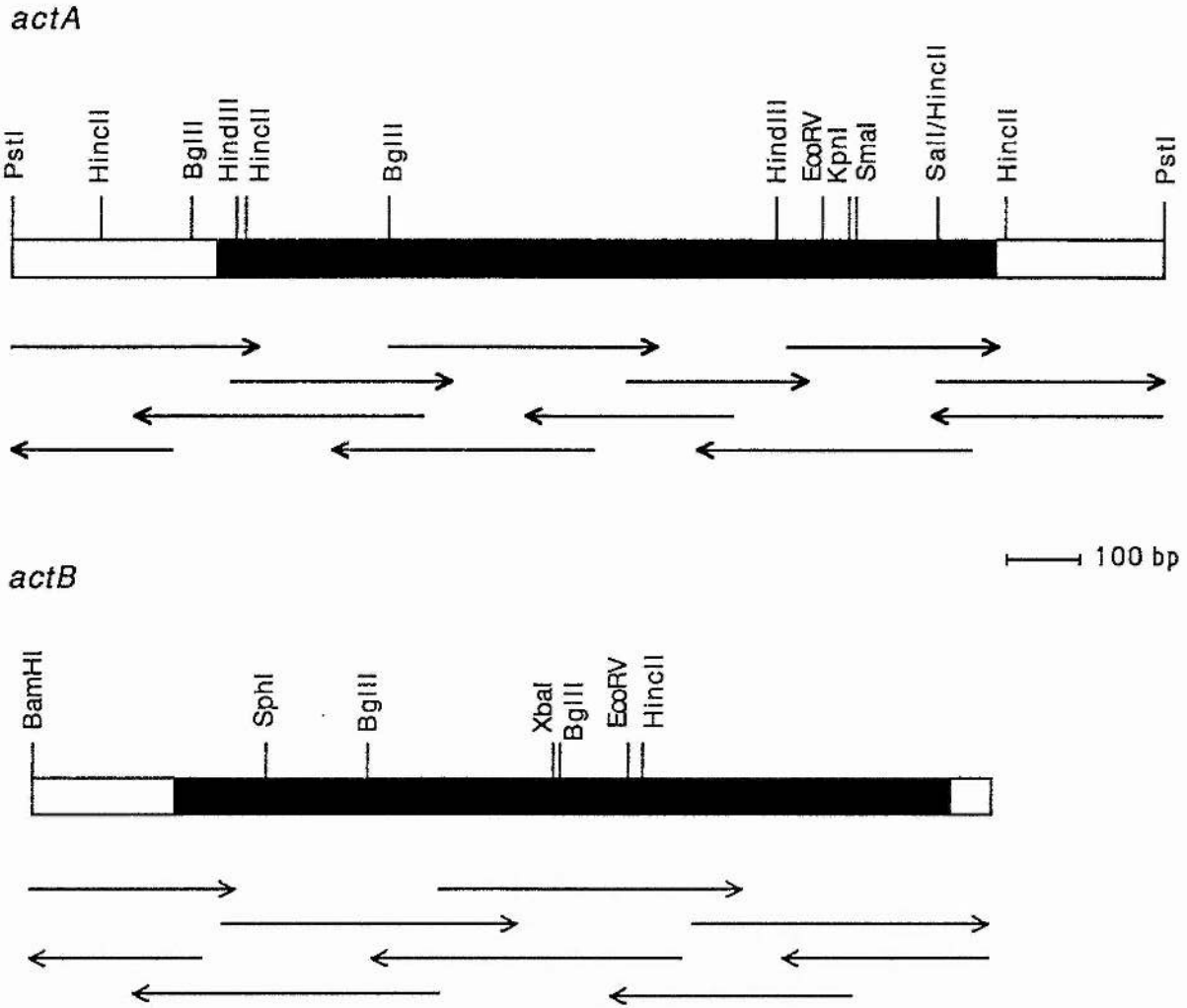


Figure 6.3 Restricton map and sequencing strategy for clones containing *actA and actB* genes. The solid black bar represents the approximate position of coding regions.

```
       CTGCAGCATGTGCTCGTCTATTTGAGGTGTTTGCCTCATCCAAACGGCCAGCGTCG -241
    GGTTGAAAACGAAACAGCCACCTCTTTCACTTGATTTCACTATGGCCAATGTTGCCGCTT -181
    CGCCTTCAGGCCTGTTGACATCCACCATTACACTTACTTCCCCTAGCCAAAAGGAGGGCT -121
                                                            ▼
    GACGTGTCCATCGTCGCTTCACCTCACAGTCTTGGCTCCCTCTTTGGCTCATTTCCCTTT  -61
    TCTTCCAGTTGGCAACAGATCTCCAAGCTCCCAAGCCTCCTAGCCCCTCCTTCAATCAAG   -1

  1  M  A  D  D  D  V  Q  A  L  V  V  D  N  G  S  G  M  C  K  A
    ATGGCTGACGATGATGTCCAAGCTTTGGTCGTTGACAACGGCTCCGGTATGTGCAAGGCC   60

 21  G  F  A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  R  P  K
    GGTTTCGCCGGTGACGACGCCCCGCGTGCCGTGTTCCCCTCGATTGTGGGTCGCCCCAAG  120

 41  H  L  G  I  M  V  G  M  D  Q  K  D  A  Y  V  G  D  E  A  Q
    CACTTGGGAATCATGGTGGGCATGGACCAGAAGGATGCCTACGTCGGTGACGAGGCCCAG  180

 61  S  K  R  G  V  L  T  L  K  Y  P  I  E  H  G  I  V  T  N  W
    TCCAAGCGTGGTGTGCTGACGCTTAAGTACCCTATTGAGCACGGTATTGTGACCAACTGG  240

 81  D  D  M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P  E
    GACGACATGGAGAAGATCTGGCACCACACCTTCTACAACGAGCTGCGTGTGGCCCCCGAG  300

101  E  H  P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  E  R  M
    GAGCACCCGGTGCTGCTTACCGAGGCCCCTCTTAACCCGAAGGCCAACCGTGAGCGCATG  360

121  T  Q  I  M  F  E  T  F  N  V  P  A  M  Y  V  N  I  Q  A  V
    ACGCAGATCATGTTCGAGACGTTCAACGTGCCCGCCATGTATGTGAACATCCAGGCCGTG  420

141  L  S  L  Y  A  S  G  R  T  T  G  C  V  L  D  S  G  D  G  V
    CTGTCCCTGTACGCCTCTGGCCGTACCACGGGCTGTGTGCTCGACTCTGGTGACGGTGTG  480

161  S  H  T  V  P  I  Y  E  G  Y  A  L  P  H  A  I  V  R  L  D
    TCCCACACTGTGCCCATCTACGAGGGTTACGCTCTTCCTCACGCTATCGTGCGTCTGGAC  540

181  L  A  G  R  D  L  T  D  Y  M  M  K  I  L  T  E  R  G  Y  S
    CTGGCTGGCCGCGACCTGACGGACTACATGATGAAGATCCTGACGGAGCGTGGTTACTCG  600

201  F  T  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  T  Y  I
    TTCACGACCACGGCCGAGCGCGAAATTGTGCGTGACATCAAGGAGAAGCTGACGTACATC  660

221  A  L  D  F  D  Q  E  M  K  T  A  A  E  S  S  G  L  E  K  S
    GCCCTGGACTTCGACCAGGAGATGAAGACTGCCGCCGAGTCGTCGGGTCTGGAGAAGAGC  720

241  Y  E  L  P  D  G  N  V  I  V  I  G  N  E  R  F  R  T  P  E
    TACGAGCTGCCCGATGGCAACGTTATTGTCATCGGTAACGAGCGTTTCCGTACCCCTGAG  780

261  V  L  F  Q  P  S  L  I  G  K  E  A  S  G  I  H  D  C  T  F
    GTGCTGTTCCAGCCCTCGCTCATCGGTAAGGAAGCTTCGGGTATCCACGAGTGCACGTTC  840

281  Q  T  I  M  K  C  D  V  D  I  R  K  D  L  Y  C  N  I  V  L
    CAGACCATCATGAAGTGTGATGTCGATATCCGTAAGGACTTGTACTGCAACATTGTGCTC  900

301  S  G  G  T  T  M  Y  P  G  I  G  E  R  M  T  K  E  L  T  A
    TCGGGTGGTACCACCATGTACCCGGGCATTGGCGAGCGGATGACCAAGGAGCTTACGGCT  960

321  L  A  P  S  T  M  K  I  K  V  V  A  P  P  E  R  K  Y  S  V
    CTGGCCCCGTCCACCATGAAGATCAAGGTGGTGGCCCCGCCTGAGCGCAAGTACTCGGTC 1020

341  W  I  G  G  S  I  L  S  S  L  S  T  F  Q  Q  M  W  I  S  K
    TCGGTGGTTCCATCCAGTCGTCGCTGTCGACGTTCCAGCAGATGTGGATCTCGAAGTGGA 1080

361  A  E  Y  D  E  S  G  P  S  I  V  H  R  K  C  F  *
    GCCGAGTACGACGAGTCTGGACCCTCGATCGTGCACCGCAAGTGCTTCTAAGCTGCCTGT 1140
    CCACGGGTCAACTGGACTTAGAATCTGGAGGAGAAATGCCGCTGCTCACGCGTGTGGCGT 1200
    CTTGAGGCTTAAGTGCTTCGATGCACGCGGGTAAGTGGTGGTGCCGTCCATTTACTTTTC 1260
    AAACCTTTCTTTGCTTTAATTTCTAAAATACTAGGATTGATGTTGTGATCTATCCGAGTG 1320
    TGCCTACTGTTCTCTTGTTTATGCCTGCATTGCCCTGAGAGTGCTTGTCACAAACGTGTC 1380
    GAAAGATAGCTTTGAACACAGACTAACAGTAAATTTAAAAGATCGATGTTAGAGTCACAC 1440
```

Figure 6.4 Nucleotide and predicted amino acid sequence of *actA*. Pyrimidine rich sequences and a putative CCAAT box in the 5' noncoding region are underlined. The transcription start site is indicated with an arrow.

```
                GGATCCTCGCGCTTGGATCTTGTGCAGCGTCAGCAGCGCTTGCTGCGTCGCTGA -181
GAGCTTCTTAGGCGCCTTTGGTTTACCCCCCAACATGAAGTCCGCTAGTGTGGCGCACAT -121
AAAGGCAACAAGTTGAAGAGGACCAATAGGCGATCCGACAGTTAGTTGAAGTATGTTGGT  -61
GTGTCAAAGTTCTCATTCTGCATTTTGTCTCGAAGGGCACAAGTTGCTAAGATTTCAGTC   -1

  1  M  D  D  D  I  Q  A  V  V  I  D  N  G  S  G  M  C  K  A  G
     ATGGACGACGATATTCAAGCCGTAGTAATTGACAATGGATCGGGAATGTGCAAGGCGGGG   60

 21  F  A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  M  P  K  H
     TTCGCTGGCGATGACGCCCCACGTGCCGTGTTCCCGTCCATTGTGGGCATGCCCAAACAC  120

 41  L  G  I  M  V  G  M  N  Q  K  D  A  Y  I  G  D  E  A  Q  A
     CTAGGTATCATGGTGGGAATGAACCAGAAAGACGCTTACATTGGGGATGAAGCGCAAGCG  180

 61  K  R  G  V  L  T  L  R  Y  P  I  E  H  G  I  V  T  N  W  D
     AAACGTGGCGTCTTGACGTTGAGATACCCAATTGAGCACGGGATCGTGACGAACTGGGAT  240

 81  D  M  E  K  I  W  S  H  T  F  Y  N  E  L  R  V  A  P  E  E
     GACATGGAGAAGATCTGGAGTCACACCTTCTACAACGAGCTGCGTGTAGCTCCAGAAGAG  300

101  H  P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  E  R  M  T
     CATCCAGTGCTCTTAACGGAGGCTCCACTCAACCCTAAAGCGAACCGCGAACGCATGACT  360

121  Q  I  M  F  E  T  F  N  V  P  A  M  Y  V  N  I  Q  A  V  L
     CAGATCATGTTCGAGACCTTCAATGTGCCAGCGATGTACGTAAACATCCAGGCGGTGCTG  420

141  S  L  Y  A  S  G  R  T  T  G  C  V  L  D  S  G  D  G  V  S
     TCGTTGTATGCCTCTGGTCGTACTACAGGCTGCGTACTCGACTCGGGTGATGGAGTCTCG  480

161  H  T  V  P  I  Y  E  G  Y  A  L  P  H  A  I  V  R  L  D  L
     CACACTGTACCTATTTACGAAGGTTATGCACTCCCCCACGCTATTGTGCGTCTAGATCTG  540

181  A  G  R  D  L  T  D  Y  M  M  K  I  L  T  E  R  G  Y  S  F
     GCTGGGCGAGACTTGACGGACTACATGATGAAGATTTTAACTGAGCGTGGGTACTCATTC  600

201  T  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  T  Y  V  A
     ACCACTACAGCCGAGCGAGAAATCGTGCGGGATATCAAGGAGAAGTTGACGTATGTAGCA  660

221  M  D  F  D  E  E  M  E  K  S  T  R  S  S  A  L  D  K  T  Y
     ATGGACTTTGACGAGGAGATGGAGAAATCTACTCGATCTTCAGCATTAGACAAGACGTAC  720

241  E  L  P  D  G  N  V  I  V  I  G  N  E  R  F  R  T  P  E  V
     GAACTGCCTGATGGGAACGTCATTGTGATCGGGAACGAGCGCTTCCGTACCCCTGAGGTG  780

261  L  F  N  P  S  M  I  G  R  E  C  S  G  V  H  E  C  A  F  Q
     CTATTTAATCCGTCCATGATTGGTCGCGAATGCTCTGGAGTTCACGAGTGCGCTTTCCAG  840

281  T  I  M  K  C  D  V  D  I  R  R  D  L  Y  S  N  V  V  L  S
     ACGATCATGAAGTGTGACGTCGATATTCGCCGGGACTTGTACAGCAACGTGGTGCTGTCC  900

301  G  G  S  T  M  F  P  G  I  G  E  R  M  T  K  E  V  I  K  L
     GGTGGCTCGACAATGTTCCCTGGGATTGGTGAGCGCATGACCAAGGAGGTGATTAAACTA  960

321  A  P  T  A  M  K  V  K  I  I  T  P  P  E  R  K  Y  S  V  W
     GCTCCAACGGCGATGAAAGTCAAGATCATCACGCCACCTGAACGCAAGTACTCGGTCTGG 1020

341  I  G  G  S  I  L  A  S  L  A  T  F  Q  H  M  W  I  S  K  T
     ATTGGAGGCTCGATTCTGGCCTCGCTGGCCACGTTCCAGCACATGTGGATCTCCAAGACC 1080

361  E  Y  D  E  S  G  P  S  I  V  H  R  K  C  F  *
     GAATACGATGAGTCGGGACCATCTATCGTCCACCGCAAGTGCTTCTAAGACTGCACGAG  1140
     TAGCCAATGTTCACACTTTGAGGCTAAGTGCT                             1173
```

Figure 6.5 Nucleotide and predicted amino acid sequence of *actB*. Putative transcriptional control elements are underlined. The borders of a putative intron are underlined with a dashed line.

```
                                                                                                    50
ACTA                MAD DDVQALV VDNGSGMCKA GFAGDDAPRA VFPSIVGRPK HLGIMVGMDQ
ACTB                -D-  -I--V- I--------- ---------- -------M-- --------N-
P. megasperma       -E-  -I--V- I--------- ---------- -------M-- --------N-
A. nidulans         -EE  E-A--- I--------- ---------- ---------R -H---I--G-
S. cerevisiae       -DS  E-A--- I--------- ---------- ---------R -Q------G-
O. fallax.          -S-   Q-TC- I-----VV-- ----E----- ---------- NVSALI-V-S
T. thermophila      --E SESP-I- I--------- -I-------- A--------- MP--------
A. castellanii I    -G-  E----- I--------- ---------- ---------R -T-V----G-
P. falciparum       -GE EV----- ------NV-- -V-------S ---------- NP------EE
H. attenuata        --- -E-A--- ---------- ---------- ---------R -Q-V----G-
D. discoideum A8    -GE  ------ I--------- ---------- ---------R -T-V----G-
A. thaliana         ---GE-I-P-- C---T--V-- ---------- ---------R -T-V----G-

                                                                                                   100
ACTA                KDAYVGDEAQ SKRGVLTLKY PIEHGIVTNW DDMEKIWHHT FYNELRVAPE
ACTB                ----I----- A-------R- ---------- -------S-- ----------
P. megasperma       ----I----- A-------R- ---------- -------S-- ----------
A. nidulans         --S------- ----I---R- -----V---- ---------- ----------
S. cerevisiae       --S------- ----I---R- ---------- ---------- ----------
O. fallax           ASE-L----- Q-----                        ---N-- --V----Q-D
T. thermophila      -EC---E--- A-----N--- --------DY ---------C -------T--
A. castellanii I    --S------- ----I----- ---------- ---------- ----------
P. falciparum       ---F------ T---I----- ---------- ---------- ------A---
H. attenuata        --S------- ----I----- ---------- ---------- ----------
D. discoideum A8    --S------- ----I----- ---------- ---------- ----------
A. thaliana         --------D- ----I----- -------N-- ---------- ----------

                                                                                                   150
ACTA                EHPVLLTEAP LNPKANRERM TQIMFETFNV PAMYVNIQAV LSLYASGRTT
ACTB                ---------- ---------- ---------- ---------- ----------
P. megasperma       ---------- ---------- ---------- ---------- ----------
A. nidulans         ---------- I---S---K- ---V------ --F--S---- ----------
S. cerevisiae       ---------- M---X--XKX ---------- --F--S---- ----S-----
O. fallax           ---I------ -S--T---K- ---------- --L--A---- ----SR----
T. thermophila      ---C------ Q---L---K- -KT------- -SF--A---- ----------
A. castellanii I    ---------- --------K- ---------T -----A---- ----------
P. falciparum       ---------- ----G----- ------S--- -----A---- ----S-----
H. attenuata        ---------- --------K- ---------S -----A---- ----------
D. discoideum A8    ---------- --------K- ---------T -----A---- ----------
A. thaliana         ---I------ --------K- ---------A -----A---- ----------

                                                                                                   200
ACTA                GCVLDSGDGV SHTVPIYEGY ALPHAIVRLD LAGRDLTDYM MKILTERGYS
ACTB                ---------- ---------- ---------- ---------- ----------
P. megasperma       ---------- ---------- ---------- ---------- ----------
A. nidulans         -I-------- T-V------F ------S-V- M--------L ----A----T
S. cerevisiae       -I-------- T-V----A-F S-----L--- ---------L ----S-----
O. fallax           -I-C-A---- T--------F SI---VS-IQ -------TFL A-L------N
T. thermophila      -I-V------ T--------- ------L-I- ---E--E-C --L-Y-I-LN
A. castellanii I    -I-------- T--------- ------L--- ---------L ----------
P. falciparum       -I-------- ---------- ------M--- -------E-L ----H----G
H. attenuata        -I-------- ---------- ------I--- ---------L ----------
D. discoideum A8    -I-M------ ---------- ------L--- ---------- ----------
A. thaliana         -I-------- ---------- ------L--- --------AL ----------
```

```
                                                                    250
ACTA                FTTTAEREIV RDIKEKLTYI ALDFDQEMKT AAESSGLEKS YELPDGNVIV
ACTB                ---------- ---------V -M---E--EK STR--A-D-T ----------
P. megasperma       ---------- ---------V -MN--E--EK --R--T-D-- ----------
A. nidulans         -S-------- -------C-V ----E--IQ- -SQ--S---- ------Q--T
S. cerevisiae       -S-------- -------C-V ----E---Q- --Q--SI--- ------Q--T
O. fallax           --SS--L--- -------CFV --NYESAL-Q SHD--QF--N ------K--T
T. thermophila      -SS------I -------C-V -I-YEA-L-A YK---XND-- -------T-T
A. castellanii I    ---------- -------C-V ----E---H- --S--A---- ------Q--T
P. falciparum       -S-S--K--- -------C-- --N--E---- SEQ--DI--- -------I-T
H. attenuata        ---------- -------S-V ----E---Q- --S--S---- ------Q--T
D. discoideum A8    ---------- -------A-V ----EA--Q- --S--A---- ------Q--T
A. thaliana         ---------- -------C-- ---YE--LE- -KT--SV--N ------Q--T

                                                                    300
ACTA                IGNERFRTPE VLFQPSLI GK EASGIHDCTF QTIMKCDVDI RKDLYCNIVL
ACTB                ---------- ---N--M- -R -C--V-E-A- ---------- -R---S-V--
P. megasperma       ---------- ---K--M- -R -CT-V-E-A- ---------- -R---N-V--
A. nidulans         -------A-K A-----VL -L -SG---VT-- NS-I-----V -----G---M
S. cerevisiae       -------A-- A--H--VL -L -SA--DQT-Y NS-------V --E--G---M
O. fallax           --S-----C-- Y--K-LEMN-R -LDS-Q-L-Y KS-QE----V -R---Q--I-
T. thermophila      VQDX---C-- L--K-AF- -- -FP---EL-- NS-------V ---P-N----
A. castellanii I    -------A-- A-----FL -M -SA---ET-Y NS-------- -----G-V--
P. falciparum       V------C-- A-----FL -- --A---TT-- NS-K------ -----G----
H. attenuata        -------C-- T-----F- -M -SA---ET-Y NS-------- -----A-T--
D. discoideum A8    -------C-- A-----FL -M -SA---ET-Y NS-------- -----G-VV-
A. thaliana         --S----C-- --Y---M- -M -NA---ET-Y NS-------- -----G----

                                                                    350
ACTA                SGGTTMYPGI GERMTKELTA LAPSTMKIKV VAPPERKYSV WIGGSILSSL
ACTB                ---S--F--- -------VIK ---TA--V-I IT-------- -------A--
P. megasperma       ---S--F--- -D-----M-K ---TA--V-I IT-------- -------A--
A. nidulans         ---------- SD--Q--I-- ----S--V-I I--------- -------A--
S. cerevisiae       ------F-S- A---Q--I-- ----S--V-I I--------- -------A--
O. fallax           -------E-- ---LL--IEN R--KSINV-V I-S-D-RFA- -R---T-T--
T. thermophila      ------F---- A--LS--VS- ----S----- ------R--- ----------
A. castellanii I    ------F---- AD--Q----- ---------I I--------- -------A--
P. falciparum       -------E-T ---L-RDI-T ---------- ---------- ----------
H. attenuata        ------F---- AD--Q--IS- ---P-----I I--------- -------A--
D. discoideum A8    ------F---- AD--N----- ---------I I--------- -------A--
A. thaliana         ------F---- AD--S--I-- ----S----- ---------- -------A--

                                                    376
ACTA                STFQQMWISK AEYDESGPSI VHRKCF
ACTB                A---H----- T--------- ------
P. megasperma       A---H----- TD-------- ------
A. nidulans         ---------- Q--------- ------
S. cerevisiae       T--------- Q-------X- --H---
O. fallax           ---AS---T- ED---N-A-- -----I
T. thermophila      ----T---T- ---------- ------
A. castellanii I    ---------- E--------- ------
P. falciparum       --------T- E--------- ------
H. attenuata        ---------- Q--------- ------
D. discoideum A8    ---------- E--------- ------
A. thaliana         --L-----A- ---------- ------
```

Figure 6.6 Comparison of the inferred amino acid sequences of *actA* and *actB* of *Phytophthora infestans* with actins from *Phytophthora megasperma* (Dudler, 1990), *Aspergillus nidulans* (Fidel *et al.*, 1988), *Saccharomyces cerevisiae* (Ng & Abelson, 1980), *Oxytricha fallax* (Kaine & Spear, 1982), *Tetrahymena thermophila* (Cupples & Pearlman, 1986), *Acanthamoeba castellanii* (Nellen & Gallwitz, 1982), *Plasmodium falciparum* (Wesseling *et al.*, 1988), *Hydra attenuata* (Fisher & Bode, 1989), *Dictyostelium discoideum* (Romans & Firtel, 1985) and *Arabidopsis thaliana* (Nairn *et al.*, 1988). Homologies are represented by a hyphen, X indicates sequence uncertainty.

Table 6.2 Codon usage in *actA*

F	TTT	0.	S	TCT	3.	Y	TAT	1.	C	TGT	2.
F	TTC	12.	S	TCC	6.	Y	TAC	14.	C	TGC	4.
L	TTA	0.	S	TCA	0.	*	TAA	1.	*	TGA	0.
L	TTG	3.	S	TCG	13.	*	TAG	0.	W	TGG	4.
L	CTT	5.	P	CCT	5.	H	CAT	0.	R	CGT	11.
L	CTC	3.	P	CCC	8.	H	CAC	9.	R	CGC	6.
L	CTA	0.	P	CCA	0.	Q	CAA	1.	R	CGA	0.
L	CTG	17.	P	CCG	6.	Q	CAG	9.	R	CGG	1.
I	ATT	7.	T	ACT	2.	N	AAT	0.	S	AGT	0.
I	ATC	19.	T	ACC	11.	N	AAC	10.	S	AGC	1.
I	ATA	0.	T	ACA	0.	K	AAA	0.	R	AGA	0.
M	ATG	16.	T	ACG	12.	K	AAG	21.	R	AGG	0.
V	GTT	2.	A	GCT	7.	D	GAT	6.	G	GGT	19.
V	GTC	6.	A	GCC	19.	D	GAC	19.	G	GGC	8.
V	GTA	0.	A	GCA	0.	E	GAA	2.	G	GGA	2.
V	GTG	20.	A	GCG	0.	E	GAG	24.	G	GGG	0.

TOTAL CODONS = 377
TOTAL AMINO ACIDS = 376.
MOLECULAR WEIGHT = 41853. HYDROPHOBICITY = -12.4

Table 6.3 Codon usage in *actB*

F	TTT	2.	S	TCT	5.	Y	TAT	3.	C	TGT	1.
F	TTC	11.	S	TCC	4.	Y	TAC	11.	C	TGC	5.
L	TTA	3.	S	TCA	2.	*	TAA	1.	*	TGA	0.
L	TTG	6.	S	TCG	9.	*	TAG	0.	W	TGG	4.
L	CTT	0.	P	CCT	6.	H	CAT	1.	R	CGT	7.
L	CTC	4.	P	CCC	2.	H	CAC	8.	R	CGC	8.
L	CTA	4.	P	CCA	9.	Q	CAA	2.	R	CGA	3.
L	CTG	7.	P	CCG	2.	Q	CAG	5.	R	CGG	2.
I	ATT	15.	T	ACT	6.	N	AAT	3.	S	AGT	1.
I	ATC	13.	T	ACC	6.	N	AAC	9.	S	AGC	1.
I	ATA	0.	T	ACA	3.	K	AAA	7.	R	AGA	1.
M	ATG	19.	T	ACG	10.	K	AAG	12.	R	AGG	0.
V	GTT	1.	A	GCT	8.	D	GAT	10.	G	GGT	7.
V	GTC	7.	A	GCC	7.	D	GAC	14.	G	GGC	6.
V	GTA	7.	A	GCA	3.	E	GAA	9.	G	GGA	7.
V	GTG	14.	A	GCG	7.	E	GAG	18.	G	GGG	8.

TOTAL CODONS = 376.
TOTAL AMINO ACIDS = 375.
MOLECULAR WEIGHT = 41958. HYDROPHOBICITY = -8.4

Table 6.4 Base distribution in *actA* codons

	T	C	A	G	
1	25.93	24.70	44.59	39.64	
2	45.27	28.05	52.70	17.16	
3	28.81	47.26	2.70	43.20	
Total	100%	100%	100%	100%	
1	16.71	21.49	26.26	35.54	= 100%
2	29.18	24.40	31.03	15.38	= 100%
3	18.57	41.11	1.59	38.73	= 100%
	21.49	29.00	19.63	29.89	Observed frequencies (%), overall totals.
	23.60	21.95	28.04	26.41	Expected frequencies (%) if no codon bias.

Table 6.5 Base distribution in *actB* codons

	T	C	A	G	
1	26.17	25.45	37.86	41.96	
2	44.14	32.36	40.36	19.24	
3	29.69	42.18	21.79	38.80	
Total	100%	100%	100%	100%	
1	17.82	18.62	28.19	35.37	= 100%
2	30.05	23.67	30.05	16.22	= 100%
3	20.21	30.85	16.22	32.71	= 100%
	22.70	24.38	24.82	28.10	Observed frequencies (%), overall totals.
	23.79	21.57	28.07	26.57	Expected frequencies (%), if no codon bias.

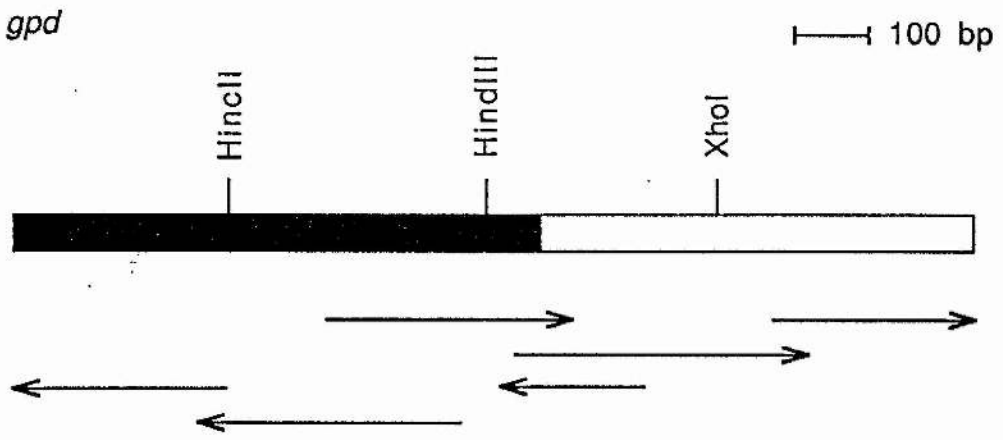


Figure 6.7 Restricton map and sequencing strategy for clone containing *gpd*gene. The solid black bar represents the approximate position of the coding region.

```
 89    E  Q  V  Q  Y  V  V  E  S  T  G  A  F  T  T  L  E  K  A
AGGAGCAGGTGCAATACGTGGTGGAGTCCACGGGTGCCTTCACGACCCTGGAGAAGGCAT  60

108 S  T  H  L  K  N  X  G  E  G  V  V  I  S  A  P  S  S  D  A
CGACCCACTTGAAGAACXGTGGTGAAGGAGTGGTGATCTCGGCTCCGTCCAGTGACGCCC 120

128 P  M  F  V  M  G  V  N  H  E  L  Y  E  K  N  M  H  V  V  S
CCATGTTCGTGATGGGCGTGAATCACGAGCTGTACGAGAAGAACATGCACGTGGTGTCGA 180

148 N  A  S  C  T  T  N  V  L  A  P  L  A  K  V  V  N  V  K  F
ACGCTTCGTGCACGACAAACGTCCTGGCCCCTCTGGCTAAGGTGGTGAACGTCAAGTTTG 240

168 G  I  K  E  G  L  M  T  T  V  H  A  V  T  A  T  X  X  T  V
GCATCAAGGAAGGCCTCATGACAACTGTCCACGCTGTGACTGCCACGCAXAAXACAGTTG 300

188 D  G  P  S  K  K  D  W  R  G  G  R  X  A  C  F  N  I  I  P
ACGGTCCTTCCAAGAAGGACTGGCGTGGTGGTCGTXXCGCTTGCTTCAACATCATCCCAA 360

208 S  S  T  G  A  A  K  A  V  G  K  V  I  P  S  L  N  G  K  L
GCTCTACTGGTGCTGCTAAGGCTGTGGGAAAGGTGATCCCGAGCCTGAACGGTAAGCTGA 420

228 T  G  M  S  F  R  V  P  T  A  D  V  S  V  V  D  X  T  A  R
CGGGCATGTCGTTCCGTGTGCCGACTGCTGATGTGTCTGTGGTGGACXTGACGGCTCGTC 480

249 L  V  N  P  A  S  Y  D  E  I  K  A  A  I  K  X  S  E  N  E
TGGTGAACCCTGCTTCGTACGACGAGATTAAGGCTGCCATTAAGXXGGCCAGTAACGAGA 540

269 M  K  G  I  L  G  Y  T  E  K  A  V  V  S  S  D  F  I  G  D
TGAAGGGCATTCTCGGCTACACAGAGAAGGCTGTGGTGTCGAGCGACTTTATCGGCGACT 600

289 S  H  S  S  I  F  D  A  E  A  G  I  A  L  T  D  D  F  V  K
CGCACTCGTCGATCTTTGATGCCGAGGCCGGTATTGCCCTGACAGATGACTTCGTGAAGC 660

309 L  V  S  W  Y  D  N  E  W  G  Y  S  S  R  V  L  D  L  I  E
TTGTGTCGTGGTATGACAATGAGTGGGGATACAGCTCCCGTGTGCTGGACCTGATTGAGC 720

329 H  M  V  K  N  E  *
ACATGGTTAAGAACGAGTAAGTTTGCACTCTAGGTGCTGCTTCTTTAGGCTTAAAGTACT 780
TGCGTTCGCGGCGGTGATCGTATTAGCCCCACGGTAGAAGTTTGCATTGTGGAGTATACG 840
ACTCACTCCCTTCTTATCAGAGCAACGTCAAAAAGTTTGCTGTTGTCTGTATCAATAGAA 900
GTATAAAGATCATGACAAACTGTACAAGTACGTATTGATGACCAGGTATGCCGCAATTTT 960
GGAAAACGTGCCACTGCGCGAGTGACTCGAGTATTGGTGACATCACACCATTCATGTTGC 1020
ATGTAGGTGACAAGCACGTAGCTCACCCATTTATGTCCAGCATGGAGGTGGCGGGCAATT 1080
ACCATTAGTAAATCAGTGTAAAACGTCGGCACCCACATCAGAAAGGATCAAGTAAAGTGG 1140
GTCTTTCTTCATATAGTCCACAGTCGCTTCCAATTTCCAAATCACCCAAGACCATTTTGG 1200
CGACAGTCCGGCCTAAACCTCGTACCTATCTGAGATAAACTGTGAATAACATTATCTCTC 1260
CGACGTATCTTATTTTTAGTATGTTGCGCACTAGCCAATGATAATCGTTTCTTATTCTTT 1320
ATAGCCAATTAAAGCGATCTCCGTT                                     1347
```

Figure 6.8 Partial nucleotide and predicted amino acid sequence of *gpd*. A putative polyadenylation signal is underlined.

```
                                                                      140
                      TG  F     A  H    GAK  V    P         V G N   Y
A. nidulans     DG AEYIVESTGV FTTQEKASAH LKGGAKKVVI SAPSADAPMF VMGVNNETYK
P. infestans    EQ VQ-V-----A ---L----T- --NXGEG--- ----S------ -----H-L-E

                                                                      190
                    SNAS CTTN LAP   KV    F I     MTTVH   T      D
A. nidulans       KDIQVLSNAS CTTNCLAPLA KVINDNFGII EGLMTTVHSY TATQKVVDGP
P. infestans      -NMH-V---- ----V----- --V-VK---K --------AV ---XXT----

                                                                      240
                  K  R  R  A   IIP  T GAAKA   V  P L G   GM A RVPT   S
A. nidulans       SAKDWRGGRT AATNIIPSST GAAKAVGKVI PSLNGKLTGM AMRVPTSNVS
P. infestans      -K-------X -CF------- ---------- ---------- SF----AD--

                                                                      290
                  D    L                 A  G    G L Y      V
A. nidulans       VVDLTVRTEK AVTYDQIKDA VKKASENELK GILGYTEDDI VSTDLNGDTR
P. infestans      ---X-A-LVN PAS--E--A- I-X-----M- -------KAV --S-FI--SH

                                                                      336
                 S  DA           VK    WYDNE GY    V DL
A. nidulans       SSIFDAKAGI ALNSNFIKLV SWYDNEWGYS RRVVDLISYI SKVDAQ
P. infestans      ------E--- --TDD-V--- ---------- S--L---EHM V-NE
```

Figure 6.9 Amino acid alignment of the *Aspergillus nidulans* GAPDH with the inferred amino acid sequence of part of the *P. infestans gpd* gene. Numbers refer to the amino acid position in the *A. nidulans* GAPDH. Homologies are represented by a hyphen, X indicates sequence uncertainty. Residues which are conserved in several higher and lower eukaryotes and in bacteria (Yarborough *et al.*, 1987) are shown above the *A. nidulans* sequence.

Table 6.6 Percentage homologies of various actin genes at the nucleotide and amino acid levels

	Percentage homologies			
	Nucleotides		Amino acids	
	actA	*actB*	ACTA	ACTB
Phytophthora megasperma	79.6	84.3	87.8	96.5
Aspergillus nidulans			82.4	78.4
Saccharomyces cerevisiae	71.4	69.9	82.4	80.0
Oxytricha fallax			67.0	65.6
Tetrahymena thermophila			76.9	72.8
Acanthamoeba castellanii r	80.8	73.1	87.8	82.7
Plasmodium falciparum			84.0	76.0
Hydra attenuata			87.8	82.2
Dictyostelium discoideum A8			87.5	82.7
Arabidopsis thaliana			85.1	79.5

Table 6.7 Comparison of proportion of protein encoded by most-used codons

Gene	No. of residues	Subset size for most-used codons[1]	No. of residues encoded by codon subset	Percentage of all residues
actA	376	23	302	80.3
actB	375	29	274	73.1
P. megasperma actin	375	24	276	73.6
gpd	239	23	128	53.6

Notes

1. This subset includes the codons listed in Table 6.8 plus those for single-codon amino acids, all four Gly codons for *actB* and both Gln codons for *gpd*.

Table 6.8 Comparison of codon usage in *Phytophthora* genes

Amino acid[1]	Gene											
	actA			*actB*			*P. megasperma* actin			*gpd*		
	No.[2]	Codon[3]	Freq.[4]	No.	Codon	Freq.	No.	Codon	Freq.	No.	Codon	Freq.
Phe	12	TTC	100.0	13	TTC	84.6	13	TTC	76.9	8	TTC	62.5
Leu	28	CTC	60.7	24	CTG	29.2	24	CTG	41.7	14	CTG	71.4
					TTG	25.0		TTG	37.5			
Iso	26	ATC	73.0	28	ATT	53.6	27	ATC	55.5	12	ATC	58.3
					ATC	46.4		ATT	44.5			
Val	28	GTG	71.3	29	GTG	48.3	28	GTG	57.1	29	GTG	82.8
Ser	23	TCG	56.3	22	TCG	40.9	20	TCG	45.0	23	TCG	82.8
Pro	19	CCC	42.0	19	CCA	47.4	19	CCG	36.5	8	CCT	37.5
											CCG	37.5
Thr	25	ACG	48.0	25	ACG	40.0	26	ACG	69.2	17	ACG	35.2
		ACC	44.0								ACA	29.4
Ala	26	GCC	73.0	25	GCT	32.0	26	GCC	42.3	23	GCT	56.5
					GCC	28.0						
					GCG	28.0						
Tyr	15	TAC	93.0	14	TAC	78.5	14	TAC	85.7	6	TAC	83.3
His	9	CAC	100.0	9	CAC	88.9	9	CAC	77.7	6	CAC	100.0
Gln	10	CAG	90.0	7	CAG	71.4	7	CAG	71.4	2	No bias	
Asn	10	AAC	100.0	12	AAC	75.0	13	AAC	84.6	12	AAC	83.3
Lys	21	AAG	100.0	19	AAG	63.2	16	AAG	80.0	17	AAG	100.0
Asp	25	GAC	76.0	24	GAC	58.3	21	GAC	87.5	13	GAC	76.9
					GAT	41.7						
Glu	26	GAG	92.3	27	GAG	66.7	19	GAG	73.3	14	GAG	85.7
Cys	6	TGC	66.7	6	TGC	83.3	6	TGC	100.0	2	TGC	100.0
Arg	18	CGT	61.1	21	CGC	38.1	21	CGC	52.4	5	CGT	100.0
					CGT	33.3						
Gly	29	GGT	65.5	28	No bias		28	GGC	60.7	18	GGT	44.4
											GGC	41.2

Notes.

1. Only amino acids with more than one possible codon are listed.
2. Number of residues in available protein sequence.
3. Preferred codon(s).
4. Percentage of residues of a given amino acid encoded by codon.

Chapter 7

DISCUSSION

At this point in time all the objectives of the project have been achieved save for actual generation of *P. infestans* transformants. High efficiency protoplasting procedures have been developed for encysted zoospores, sporangia and mycelia. The pattern of gene expression is certain to be different between these cell types and it will be interesting to discover whether the transformation efficiencies that can be achieved with them differ, for this or any other reason. The demonstration of uptake of exogenous DNA by motile zoospores of *P. infestans* (T. Peterson pers. comm.) and the successful transformation of mycelial protoplasts of *P. parasitica* (Prell *et al.*, 1989) show the cells of *Phytophthora* species can be made competant for DNA uptake and so there is no reason to suppose that transformation is not possible in *P. infestans*. Of the variety of methods available for the introduction of DNA into cells $CaCl_2$/PEG treatment and electroporation remain the techniques of choice for *P. infestans*. Both methods have been proven to work in Oomycetes (Manavathu *et al.*, 1988; Prell *et al.* 1989). The observation that motile zoospores of *P. infestans* can take up and chromosomally integrate exogenous plasmid DNA should not be neglected. Further investigations may prove to yield one of the quickest and simplest transformation procedures for any eukaryote.

Two types of transformation marker system were investigated in this work, repair of auxotrophy and antibiotic resistance. Attempts to isolate uridine auxotrophs by positive selection and filtration enrichment were unsuccessful and there are no reports of isolation of any auxotrophic mutants of *P. infestans*. The difficulties in isolating such mutants and the need to isolate, or generate by crossing, mutants in any isolate of interest, make the future development of a transformation system based on repair of auxotrophy unlikely. The development of transformation based on selection for oligomycin C resistance may be feasible and there may be some inherent

advantages in using an homologous selectable marker gene, e.g. high transformation efficiency or homologous site integration into the genome. However, the cellular location of the ATP synthase subunit 9 gene has to be determined beforehand. Should the native gene be located in the mitochondrion, surmounting the technical difficulties in directing the mutant gene product to its proper location in the mitochondrial membrane would probably not be considered worthwhile, particularly if an alternative transformation system were available.

Very high transformation efficiencies can sometimes be achieved in fungi using heterologous antibiotic resistance genes (Churchill *et al.*, 1990) and the development of this kind of system is relatively straightforward. Hygromycin B, G-418 and phleomycin have been identified as suitable selection agents for transformation in *P. infestans*. All gave clean backgrounds at selective concentrations for the three isolates tested suggesting they would be generally applicable, although MIC values must be determined in each case. Of the three antibiotics hygromycin B and G-418 may be considered the most suitable as they are effective at much lower concentrations than phleomycin and are also cheaper.

That transformation was not achieved using heterologous antibiotic resistance genes and transcriptional control signals is disappointing but perhaps not entirely unexpected. Similar situations have been found with other eukaryotic microbes, for example *Ustilago violacea* is the only basidiomycete to have been efficiently transformed using a marker gene driven by a non-basidiomycete promoter (Bei & Perlin, 1989) despite years of effort by several groups to obtain transformation in various species of the Subdivision. The frustrating experiences of many groups attempting to develop heterologous transformation in Oomycetes lends support to the conclusion of this work that promoter sequences from outside the Class are rarely if ever recognised (T. Peterson, pers. comm., H. Prell, pers. comm., O. C. Yoder pers. comm.). It is not unreasonable to hope that once marker genes driven by *P. infestans* transcriptional control sequences become available efficient generation of transformants will quickly follow, as was the case with *P. parasitica*.

The *actA*, *actB* and *gpd* genes of *P. infestans* show heavy codon bias which in other prokaryotes and eukaryotes correlates with high levels of expression (Anderson &Kurland, 1990). A strong promoter to drive a selectable marker gene is desirable for obvious reasons, but *actB* is not suitable for this purpose as it is expressed at very low levels in mycelium. Experiments are presently underway to determine if *actA* and *actB* are differentially expressed in sporangia and/or zoospores.

Methods of analysis are available that enable phylogenetic inferences to be drawn from comparisons of nucleotide sequences from highly conserved protein-encoding genes (Hightower and Meagher, 1986; Lake, 1987). One spin-off from the isolation of the actin and GAPDH genes from *P. infestans* may be a further clarification of the evolutionary position that the Oomycetes occupy in the eukaryotic family tree. However it may require many more Protistan sequences to become available before the answer to this puzzle emerges. At present it may be more meaningful to pose the question: to what groups are the Oomycetes <u>not</u> related. Nevertheless, for the time being this should be sufficient to appreciate the uniqueness of this intriguing group of organisms.

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