# *Biochemical and molecular studies of some aspects of disease resistance in potato (solanum tuberosum L.)*

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# BIOCHEMICAL AND MOLECULAR STUDIES OF SOME ASPECTS OF DISEASE RESISTANCE IN POTATO (*Solanum tuberosum* L.)

A thesis submitted by Mohd Pu'ad Abdullah in accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham



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## ABSTRACT

Disease problems in crop plants are still a major threat to the agro-industry worldwide. Various strategies have been developed and evaluated in recent years. One strategy is to use naturally-occurring antipathogen factors such as lysozymes and chitinases in transgenic plants. In the present study, transgenic plants containing chick-egg white lysozyme (Lys 75) have been evaluated for lysozyme production *in planta*, sub-cellular localisation, and resistance to some potato pathogens, including *Phytophthora infestans* and *Erwinia carotovora* subsp. *atroseptica*, the two major potato pathogens worldwide. In addition, the evaluation of resistance was also undertaken for transgenic plants carrying other naturally-occurring antipathogen factors including a bean chitinase gene (BCH 35) and a snowdrop lectin gene (GNA 74). In order to accurately quantify the lysozyme production in Lys 75 plants, the turbidimetric lysozyme enzyme assay was optimised. Also, a modified substrate for the enzyme has been developed by covalently linked the *Micrococcus lysodeikticus* cell wall with a dye, remazol brilliant violet 5R to enable a colorimetric assay of the enzyme. In order to quantitatively assess resistance levels of the transgenic plant, a new method (leaf-bridge bioassay) for conducting and evaluating resistance *in planta* has been developed. All transgenic plants in tissue culture were tested for resistance using this technique. Evaluation of the progress of infection in detached leaves of Lys 75 showed that lysozyme gave some degree of protection against the bacterial pathogen, *Erwinia carotovora* subsp. *atroseptica* and the fungal pathogen, *Fusarium sulphureum*. Analysis of intercellular fluid from the Lys 75 leaves showed that more than 80% of the total lysozyme expressed in the leaf was located in the intercellular space which is a strategic place to combat pathogen attack. In contrast, the levels of protection in BCH 35 plants were relatively low compared with Lys 75. The progress of infection was delayed in BCH 35 leaves challenged with *F. sulphureum* only. No resistance at all was observed in GNA 74 to all the pathogens used. All the transgenic potato lines were susceptible to *P. infestans*.

Recently, a new strategy to combat disease problems has been suggested based on a 'durable resistance'. Potato variety `Stirling' which shows durable resistance in the field has been used to study the early biochemical and molecular events during elicitation of 'Stirling' cell suspension cultures with an elicitor mix derived from

infective units of a compatible strain of *P. infestans*. For comparison, an elicitor mix from an incompatible strain of *P. infestans* was also prepared and used. The mixed elicitor comprising zoospore extract, culture filtrate and mycelium homogenate induced defence responses in 'Stirling' cell suspension cultures as judged by the increase in PAL enzyme activity. PAL activity in 'Stirling' cells elicited with an elicitor mix derived from an incompatible strain of *P. infestans* was twice the activity in the compatible interaction. The peak levels in both types of interaction were at 6 h post-elicitation. An oxidative burst was demonstrated also in both types of interactions indicated by rapid release of $H_2O_2$ into the culture medium. The $H_2O_2$ level peaked at 2 h post-elicitation in both interactions before being reduced to its normal level at 4 h. The $H_2O_2$ released during incompatible interaction was twice the levels monitored in the compatible. A subtracted cDNA library of differentially expressed mRNAs during elicitation of 'Stirling' cell suspension cultures with the elicitor mix from a compatible strain of *P. infestans* was constructed using suppression subtractive hybridisation. Two cDNA clones, STS 42 and STS 52, relevant to the present study were identified and characterised. STS 42 showed high degree of similarity to potato leucine aminopeptidase gene which is induced in response to wounding. Gene expression studies using RT-PCR showed that the mRNA levels of STS 42 increased gradually throughout the 18 h elicitation. STS 51 was identified as a member of the ribonuclease T2 histidine proteins. It showed some degree of similarity to plant ribonucleases involved in self-incompatibility reactions during pollination. It has a site for tyrosine kinase phosphorylation at the hydrophilic region of the sequence and could possibly be involved in phosphorylation during signal transduction. mRNA levels of STS 51 were increased during the first 12 h of elicitation.

## DECLARATION

I confirm that no part of the material presented has previously been submitted for a degree in this or in any other university. If material has been generated through joint work, my independent contribution has been clearly indicated. In all other cases material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

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| | |
|---|---|
| **2,4-D** | *2,4-dichlorophenoxyacetic acid* |
| **AOS** | *Active oxygen species* |
| **ATP** | *Adenosine 5′-triphosphate* |
| **ATPase** | *Adenosine 5′-triphosphatase* |
| ***Avr* gene** | *Avirulence gene* |
| **bp** | *Base-pair* |
| **BSA** | *Bovine serum albumin* |
| **CaMV** | *Cauliflower mosaic virus* |
| ***Cf*** | *Cladosporium fulvum resistance gene* |
| **cfu** | *Colony-forming units* |
| **CAM** | *Chloramphenicol* |
| **CoA** | *Coenzyme A* |
| **D, kD** | *Dalton, kilodalton* |
| **DEPC** | *Diethyl pyrocarbonate* |
| **DNA, cDNA** | *Deoxyribonucleic acid, complementary DNA* |
| **Dnase** | *Deoxyribonuclease* |
| **DPI** | *Diphenylene iodonium* |
| **dsDNA** | *Double stranded DNA* |
| **$EC_{50}$** | *Concentration giving half-maximal response* |
| ***E. coli*** | *Escherichia coli* |
| **EDTA** | *Ethylenediaminetetra-acetate* |
| **ER** | *Endoplasmic reticulum* |
| **EtBr** | *Ethidium bromide* |
| **GNA** | *Galanthus nivalis agglutinin, snowdrop lectin* |
| **HCl** | *Hydrochloric acid* |
| **$H_2O_2$** | *Hydrogen peroxide* |
| **HR** | *Hypersensitive response* |
| **IDM** | *Integrated disease management* |
| **JA** | *Jasmonic acid* |
| **Kb** | *Kilobases* |
| **$K_m$** | *Michaelis constant* |
| **LAP** | *Leucine aminopeptidase* |
| **LB medium** | *Luria-bertaini medium* |
| **LRR** | *Leucine-rich repeat* |
| **MOPS** | *3-(N-morpholino)-propanesulfonic acid* |
| **MS medium** | *Murashige and Skoog medium* |
| **$\cdot O_2^-$** | *Superoxide radical* |
| **$\cdot OH$** | *Hydroxyl radical* |
| **ORF** | *Open reading frame* |
| **NAA** | *Naphthylacetic acid* |
| **$NAD^+$, NADH** | *Oxidised and reduced nicotinamide–adenine dinucleotide* |
| **NO** | *Nitric oxide* |
| **nt** | *Nucleotide(s)* |
| **PAGE** | *Polyacrylamide-gel electrophoresis* |
| **PAL** | *L-Phenylalanine-ammonia-lyase* |
| **PEG** | *Polyethylene glycol* |

| | |
|---|---|
| **PBS** | *Phosphate-buffered saline* |
| **PCD** | *Programmed cell death (apoptosis)* |
| **PCR** | *Polymerase chain reaction* |
| **Poly(A)$^+$ RNA** | *Polyadenylic acid* |
| **PR** | *Pathogenesis-related* |
| ***R* gene** | *Resistance gene* |
| **RIP** | *Ribosome inactivating proteins* |
| **RNA** | *Ribonucleic acid* |
| **mRNA,** | *Messenger RNA* |
| **rRNA** | *Ribosomal RNA* |
| **RNase** | *Ribonuclease* |
| **ROS** | *Reactive oxygen species* |
| **RT-PCR** | *Reverse transcriptase PCR* |
| **SA** | *Salicylic acid* |
| **SAR** | *Systemic acquired resistance* |
| **SCRI** | *Scottish Crop Research Institute* |
| **SCV** | *Settled Cell Volume* |
| **SD** | *Standard deviation* |
| **SDS** | *Sodium dodecyl sulphate* |
| **SOD** | *Superoxide dismutase* |
| **ssDNA** | *Single stranded DNA* |
| **SSC** | *(1x) 0.15M sodium chloride, 0.015M sodium citrate, pH 7.0* |
| **TAE** | *0.04M Tris-acetate, 0.001M EDTA, pH 8.0* |
| **Tc** | *Tetracycline* |
| **TE** | *10mM Tris-HCl (pH8.0), 1mM EDTA* |
| **$V_{max}$** | *Maximum velocity* |
| **var** | *Variety* |
| **UV** | *Ultraviolet* |
| **X-Gal** | *5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside* |

# GLOSSARY OF RELEVANT TERMS

**ANOVA.** This term is an acronym for a procedure entitled Analysis Of Variance. This procedure employs the statistic (F) to test the statistical significance of the differences among the obtained MEANS of two or more random samples from a given population.

**appressorium**. An enlargement on a hyphae or germ tube that attaches itself to the host before penetration takes place.

**autotrophic**. Capable of growth independent of outside sources of nutrients or growth factors.

**auxotroph**. A strain of microorganism lacking the ability to synthesise one or more essential growth factors.

**avirulent:** descriptive of a variant of a pathogenic organism that is incapable of causing severe disease. Synonymous with non-virulent, which is the preferred term because it avoids confusion in speech with ` a virulent `.

**biotrophic:** entirely dependent upon another living organism as a source of nutrients. The term is applicable to such organisms as mycorrhizal fungi, obligate parasites etc.

**blight**. A disease characterised by rapid and extensive death of plant foliage. A general term applied to any of a wide range of unrelated plant diseases.

**chlorosis**. The loss of chlorophyll from the tissues of a plant, resulting from microbial infection, viral infection, the action of certain phytotoxins, the lack of light, to magnesium or iron deficiency, etc. Chlorotic tissues commonly appear yellowish.

**compatible:** of a relation between host and pathogen in which disease can develop. When host and pathogen are incompatible disease does not develop, commonly because of hypersensitivity of the host.

**conidium**. A thin-walled, asexual spore that is borne exogenously on a conidiophore and is deciduous at maturity (pl. conidia)

**crop rotation**. The practice of growing a sequence of different crops on the same land in successive years or seasons; done to replenish the soil, curb pests, etc.

**cultivar:** (I) An assemblage of cultivated individuals which is distinguished by any characters (morphological, physiological, cytological, chemical or others) significant for the purposes of agriculture, forestry or horticulture and which, when reproduced (sexually or asexually), retains its distinguishing features. (2) variety. A cultivar may be a clone (*Solanum tuberosum* ` King Edward VII `).

**cyst**.: An encysted zoospore

**disease**. An abnormal condition of a plant in which its physiology, morphology, and/or development is altered under the continuous influence of a pathogen.

**elicitor**. A molecule produced by the host (or pathogen) that induces a response by the pathogen (or host).

**encysted**. Surrounded by a hard shell (cyst).

**factorial design**. A factorial design is used to evaluate two or more factors simultaneously. The treatments are combinations of levels of the factors. The advantages of factorial designs over one-factor-at-a-time experiments is that they are more efficient and they allow interactions to be detected.

**gene-for-gene concept:** the concept that corresponding genes for resistance and virulence exist in host and pathogen respectively.

**germ tube**. A short, hypa-like structure that develops from certain types of spores upon germination.

**germination**. In bacteria or fungi, the process by which a spore gives rise to a vegetative cell or +hypha.

**haustorium:** a simple or complex structure formed by an interaction between a branch of a fungal hypha and a host cell into which it has penetrated without causing lethal injury, generally assumed to provide a means by which a fungus absorbs nutrients from the host cells. (Pl. **haustoria**.)

**hypersensitive**. The state of being abnormally sensitive. It often refers to an extreme reaction to a pathogen (e.g., the formation of local lesions by a virus or the necrotic response of a leaf to bacterial infection).

**hypersensitivity**. The expression of extreme reactivity by a plant in response to a potential parasite or pathogen, the plant's response commonly serving to limit or prevent parasitisation/disease.

**infection:** the entry of an organism or virus into a host and the establishment of a permanent or temporary parasitic relationship.

**infective:** (i) of an organism or virus able to attack a host and cause infection. The term is frequently qualified, e.g. weakly or strongly infective. (ii) of a vector carrying or containing a pathogen and able to transfer it to a host plant causing infection

**inoculum:** material containing microorganisms or virus particles to be introduced into or transferred to a host or medium. The term can also refer to potentially infective material available in soil, air or water and which by chance results in the natural inoculation of a host.

**lectin**. A plant protein that binds to certain sugar residues.

**lesion**. A localised area of diseased or damaged tissue.

**median.** The median is one of several indices of central tendency that statisticians use to indicate the point on the scale of measures where the population is centred. The median of a population is the point that divides the distribution of scores in half. Numerically, half of the scores in a population will have values that are equal to or larger than the median and half will have values that are equal to or smaller than the median.

**mycelium**. A mass of hyphae, often used to denote all hypae comprising a thallus. (Pl. **mycelia**.)

**necrosis**. Localised death of cells or tissues.

**necrotic**. Dead.

**necrotroph**. (i). An organism that kills part or all of another organism before deriving nutrients from it (usually applied to plant pathogens). (ii). An organism that derives nutrients from dead plant or animal tissues, whether or not it is responsible for the death of those tissues.

**obligate**. Restricted to a particular set of environmental conditions, without which an organism cannot survive. (e.g., an obligate parasite can survive only by parasitising another organism.)

**obligate parasite**. An organism that is incapable of living as a saprophyte and must live as a parasite.

**oomycetes**. A class of aquatic and terrestrial fungi (subdivision Mastigomycotina) that typically produce oogonia and zoosporangia in which form zoospores having one anteriorly-directed tinsel flagellum and one posteriorly-directed whiplash flagellum.

**pathogenesis**. That portion of the life cycle of a pathogen during which it becomes, and continues to be, associated with its suscept.

**pathogenic**. Having the characteristics of a pathogen.

**pathogenicity**. The capability of a pathogen to cause disease.

**penetration peg**. In some plant parasitic fungi: The peg-like hypha emerging from an appressorium that penetrates the epidermal cell wall.

**phenotype**. The observable characteristics of an organism, either in total or with respect to one or more particular named characteristics.

**phytoalexin**. A low molecular weight, antimicrobial compound synthesised by and accumulating in higher plants exposed to certain microorganisms (pathogenic and nonpathogenic).

**polygenic**. A character controlled by many genes.

**race** A genetically and often geographically distinct mating group within a species; also a group of pathogens that infect a given set of plant varieties.

**resistance**. The ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor.

**—, acquired:** non-inherited resistance conferred by some predisposing treatment.

**—, field:** resistance which is observed under natural infection in field conditions but is not detected under the experimental conditions of resistance tests; it is expressed at low inoculum potentials and is likely to be markedly sensitive to environmental factors (cf. resistance, major gene). Horizontal resistance is commonly of this type, but the terms are not synonymous because horizontal resistance refers specifically to the absence of differential interactions between varieties of the host and races of the pathogen. The term field resistance does not carry any implication either as to the mechanism of resistance or to the manner in which it is genetically controlled: it is often polygenic but field resistance is not synonymous with polygenic resistance.

**—, general:** resistance to more than one disease. Sometimes used synonymously with race non-specific resistance but this use is not recommended.

**—, horizontal:** resistance which is evenly spread against all races of the pathogen It is quantitative; traits are results of mixtures of multiple gene products, therefore one observes a range of inheritance and genetic expression in the crop from a minimum to a maximum. This kind of resistance is more location specific, selected for over time and in the presence of local races of all pathogens, and is delimited by the crop's own range of cultivation. Horizontal resistance can be developed and is durable in potatoes, coffee, corn, and beans. Importantly,

not only is this kind of resistance long-lasting, but it greatly reduces or eliminates the need for pesticides. For example the potato cultivar "Alpha," a Dutch cultivar grown in the Toluca Valley of Mexico, originally bred in Europe with vertical resistance to late blight (*Phytophthora infestans*).

**—, race non-specific:** resistance to all races of a pathogen. The term is essentially synonymous with horizontal resistance and is preferred because it is more obviously descriptive of the condition to which it refers. This form of resistance is commonly, but not necessarily, polygenic and the two terms are not interchangeable. See also resistance, race specific.

**—, race specific:** resistance to some races of the pathogen, but not to others. Usually related to cases of oligogenic resistance where there is a gene-for-gene relationship between host and pathogen. The term is preferable to the synonymous vertical resistance because it is descriptive of the condition to which it refers. It should not be used as a synonym for oligogenic resistance, for the latter is not necessarily race specific; the terms relate to different attributes of resistance. See also resistance, race non-specific.

**—, vertical:** resistance to some races of a pathogen but not to others

**saprobe**. An organism that obtains its nutrients from non-living organic matter (commonly dead and decaying plant or animal matter) by absorbing soluble organic compounds. (Also **saprotroph; saprophyte**)

**somaclonal variation**. Variability in clones generated from a single mother plant, leaf, etc. by tissue culture.

**sporangium**. A **sac** that bears endogenous, asexual spores (sporangiospores). (Pl. **sporangia**.)

**spore**. A discrete sexual or asexual reproductive unit, usually enclosed by a rigid wall, capable of being disseminated.

**standard deviation (SD).** The standard deviation is one of several indices of variability that statisticians use to characterise the dispersion among the measures in a given population.

**statistical Significance.** A finding (for example the observed difference between the means of two random samples) is described as statistically significant, when it can be demonstrated that the probability of obtaining such a difference by chance only, is relatively low. Significant at the .05 level is the lowest threshold of significance to be used.

**suscept**. Any plant that can be attacked by a given pathogen; a host.

**susceptible**. Lacking the inherent ability to resist disease or attack by a given pathogen; not immune.

**susceptibility**. The inability of a plant to resist the effect of a pathogen or other damaging factor.

**symptom**. A visible abnormality in a plant that results from disease.

**virulence**. The degree of pathogenicity of a given pathogen.

**virulent**. Capable of causing a severe disease; strongly pathogenic.

**zoospore**. An asexual, motile spore that bears one or two flagella.

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# CHAPTER 1

# INTRODUCTION

## 1.1. A First Step Towards Studying and Solving Disease Problems

A major challenge to the agro-industry in the next decade will be to reduce the cost of crop production, increase productivity as well as adopting environmentally friendly management and practices. It is obvious that an effective way of managing agriculture in the fertile regions of the world is needed in dealing with a rapidly growing world population. In addition, global industrial activities may contribute to the shortage of labour and agricultural land. Disease and its management is one of the important factors contributing to a significant increase in cost of crop production and decrease in yields. Disease causes both direct and indirect losses. Direct losses refer to plant losses as casualties in the field and during storage of harvested materials while indirect losses refer to reduce productivity of mature plants due to various degrees of debilitation of plants and lowering the yield quality.

Integrated pest management (IPM) is so far the best way to control plant pest and diseases and has been widely adopted by farmers (Jacobsen, 1997). IPM is a multiple management or control strategy defined as "a sustainable approach to managing pests by combining biological, cultural, physical, and chemical tools in a way that minimises economic, health and environmental risks" (Jacobsen, 1997). For instance, farmers may adopt this approach by selecting a resistant variety of crop plants (biological control), together with good agricultural and cultural practices (i.e. sanitation, pruning, good irrigation) and limited usage of chemicals and pesticides. Although this method is being accepted and adopted to control plant diseases, there are still problems arising through its implementation. (Jacobsen, 1997)· Among these problems is public perception that the use of chemicals creates significant health and environmental risks and, also as a result of chemical usage an increasing number of plant pathogens become resistant to the pesticides applied (Jacobsen, 1997). Therefore, an alternative strategy that can help eliminate these problems is required. With the

· See Glossary of Relevant Terms

development of new technologies, especially in the area of plant molecular biology and genetic modification, and an increasing understanding of plant-pathogen interactions, a new definitive solution may soon become feasible. Using gene transfer technology, crop plants can be engineered for any desired traits such as disease resistance. This involves the introduction of foreign genes encoding a disease resistance factor into the crop plant genome which when transferred may enhance the disease resistance of the recipient plants. Conventional plant breeding is a very time-consuming process; from the very first crosses, back crossing and evaluation may take years to complete. In addition, not all good characteristics of the parent are inherited, leading to the development of new hybrids which lack one or two of the major desired characters. For instance, a given hybrid may perform better in terms of yield and quality but be very susceptible to certain diseases or pests. This is where genetic modification can play a role by introducing the lacking characteristics to the existing breeding line. Where the genes determining a characteristic are known and can be isolated, engineering multiple genes is still a difficult task compared with the transfer of only one or two genes.

There are several genes that have been isolated and display selected antipathogen activity such as chitinase (Benhamou *et al.*, 1993; Neuhaus *et al.*, 1991), glucanase (Melchers *et al.*, 1993) and lysozyme (Trudel *et al.*, 1992; During *et al.*, 1993). Constructs encoding some of these have already been transferred into plants and show encouraging results (Broglie *et al.*, 1991; During *et al.*, 1993; Trudel *et al.*, 1992; Zhu *et al.*, 1994). However not all such genes are suitable to be used to tackle all disease problems. Some pathogens may have a different mode of pathogenicity or lack certain targeting factors such as cell wall composition/structure. Therefore, an alternative factor may be required. Disease problems occur to all plants but they become important if the plant is a major crop in a certain region of the world. One of the most important crops in the world, the potato is greatly influenced in its annual production world-wide by a disease called lateblight. Losses due to lateblight are estimated to be about US $ 2 billion annually (Kamoun *et al.*, 1999).

## 1.2. Late Blight Disease of Potato and the Biology of its Causal Agent, *Phytophthora infestans*

Late blight disease is caused by a pathogen called *Phytophthora infestans* (Mont.) de Bary. The pathogen is a member of the oomycetes, a group of organisms which exhibit a fungal-like growth morphology in the Kingdom Protista (Paquin *et al.*, 1997). It is suggested that this pathogen might have different biochemical and genetic mechanisms during host-pathogen interaction compared with fungi (Kamoun *et al.*, 1999).

*P. infestans* is less well characterised compared with the true or higher fungi despite its economical importance. The reason for this may be due to a lack of fundamental information about the organism (growth requirements, structure, lifecycle) and confusion over its taxonomic status. Research towards an understanding of the biology of *P. infestans* has dramatically increased over the last 10 years not only because of the problem of late blight disease of potato but also because more advanced research tools have been discovered.

Basically, oomycetes like many other fungi exhibit two life cycles, namely an asexual cycle and a sexual cycle. Both cycles can be present either in the host plant tissue or in cultures of the organism (Cohen *et al.*, 1997). In plants, the infection process starts when fruit bodies of the pathogen (sporangia) dispersed by the wind or by "rain-splash", land on host tissues such as leaves and tubers. Under wet conditions and relatively high temperature, the sporangia germinate directly and penetrate the cuticle of the host tissues using a germ tube to infect the plants. At a relatively low temperature (3-8°C), motile biflagellate zoospores develop within the sporangia and are subsequently released into the surrounding area. Zoospores can swim and spread in the water films on leaves. Once the encysted spores adhere to a tissue surface, they germinate autonomously. Germination produces the germ tube which differentiates to form a structure called the appressorium which later develops a "penetration peg" and this enables host tissue penetration through an epidermal cell. Host-*P. infestans* interaction begins at this point and further disease development is totally reliant on the outcome of the defence response of the host. At the early stage of infection, there is no difference in the mode of infection between the compatible and incompatible hosts (Kamoun *et al.*, 1999).

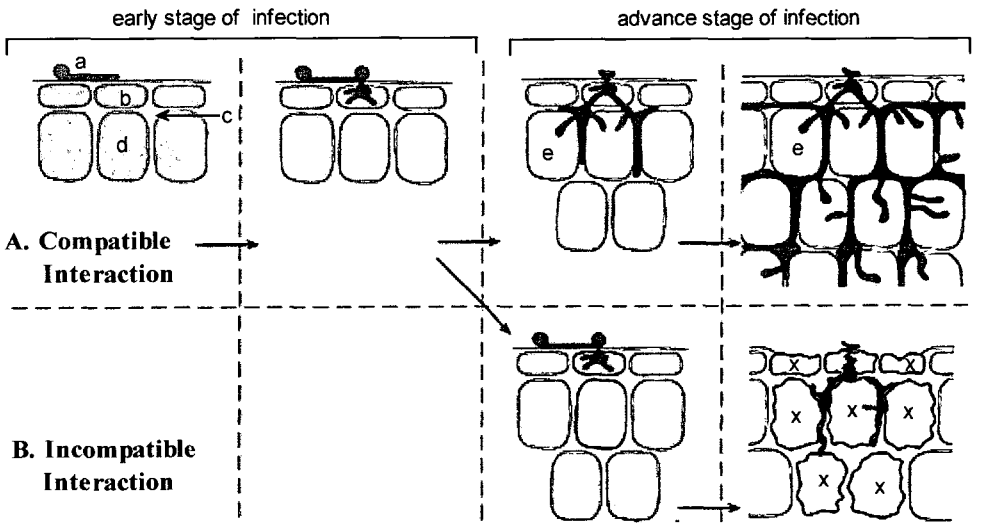


**Figure 1. Compatible and Incompatible Interactions between *Phytophthora infestans* and Potato.** Early stages of infection are similar in all types of interactions. The appressorium (a) develops into a penetration peg to penetrate the epidermal cell (b) of potato tissue. This mainly occurs on young leaves. In susceptible plants (**Panel A**), hyphae grow into the intercellular space (c) and form a feeding structure called haustoria (e) inside mesophyll cells (d). The hyphae branch and rapidly colonise the mesophyll tissue, which eventually diseased. In resistant plants, cells are activated after the penetration of the pathogen (**Panel B**), followed with the induction of the hypersensitive response (HR) in the neighbouring cells (x) which eventually die. (Modified from Kamoun *et al.*, 1999)

If the interaction is a "compatible" one, then the disease is fully developed and the organism is said to enter its biotrophic phase or disease cycle in the host tissue. Later an "infection vesicle" is formed in the infected epidermal cell and hyphae grow further into the neighbouring cells through intercellular spaces of mesophyll cell layers (Kamoun *et al.*, 1999) (Figure 1). A feeding structure called "haustoria" if formed may accelerate the damage to the host tissues leading to the widespread infection and death of the plant. Once the host plant has died, *P. infestans* grows saprophytically and sporangiospores are formed from the aerial hyphae where new sporangia are released.

In the incompatible interaction (resistant plant), the hyphae development in the potato tissues was limited due to the hypersensitivity reaction (HR) and the extent of the hyphae depends on the resistance levels of the potato plants (Kamoun *et al.,* 1999). Potatoes with high levels of resistance to *P. infestans* produce a group of cells displaying HR and in turn prevent the spread of the hyphae *in planta.*

### 1.3. Molecular Basis of Plant-Pathogen Interactions Following Elicitation and Infection

Like many other organisms, plants exhibit responses to pressures coming from the surrounding environments. Physiological stress, pest and pathogen attacks, and nutritional imbalance are just a few examples of pressures that plants must detect and respond to in order to survive. Since plants, unlike their animal counterparts cannot move to alleviate most stresses, they have other efficient ways to reduce the effects of stress. Extensive research over the past decades has shown that plants possess comprehensive and complicated defence networks that can be used to protect from or overcome stresses (reviewed in Hutcheson, 1998).

Interaction between plants and pathogens is a continuing process. The outcome varies depending on the compatibility status of the plant and the pathogen. In 'compatible-type' interactions, disease is developed, whereas in the 'incompatible-type', resistance occurs. In an incompatible interaction, the 'gene-for-gene' hypothesis, first formulated by Flor in the late 1940's applies. According to this hypothesis, for each dominant gene determining resistance in the host (plants) there is a corresponding dominant gene determining avirulence in the pathogen. Interaction between the two, the plant resistance (*R*) and the pathogen avirulence (*Avr*) genes, induces the activation of cellular defence responses in the host plants leading to resistance (reviewed in Ji *et al.*., 1998). In other words, for *R* gene-dependent resistance to occur in the plant, the pathogen must express the complementary *Avr* gene encoding a product that is recognised by the plant. Boller and Keen (1997) proposed an 'elicitor-receptor' model to explain this concept. In this model, a specific elicitor molecule encoded by the *Avr* gene is produced by the pathogen. Plants possessing the corresponding *R* gene respond to the presence of an elicitor by activating various plant defence response genes. Activation of these defence responses follows by pathogen recognition including elicitor perception and subsequent signal transduction processes. Most elicitors are

produced by the invading pathogen but plant chemicals released during the interaction can also function as an elicitor (Boller, 1995; De Wit, 1995). Elicitors include a wide range of compounds produced by the pathogen such as oligoglucans, ethylene, fatty acids, chitosan oligomers and polypeptides (reviewed in Hahn, 1996). Elicitor molecules can be race specific and produced by the pathogen *in planta* or they can be non-specific and enzymatically released from the surface of the pathogen during interaction (De Wit, 1995).

The best example supporting the 'elicitor-receptor' model is the tomato-*Cladosporium fulvum* pathosystem which involves the interaction between the fungal *Avr9* gene product, AVR9 elicitor peptide, and its corresponding tomato *cf-9* resistance gene product, Cf9 protein (van den Ackerveken *et al.*,1992). The CF9 protein confers resistance in the tomato against the fungal pathogen *Cladosporium fulvum.* Resistance, expressed as a HR followed by other defence responses, is based on recognition of an AVR9 elicitor by the receptor molecule, CF9 peptide, in the host tomato. Transgenic tobacco and potato plants carrying the *Cf*-9 gene exhibit a rapid HR to AVR9 peptide injection (Hammond-Kosack *et al.*, 1998). However, the signal transduction pathway leading to HR in this system is unclear (de Wit *et al.*, 1999). Studies on the AVR9-CF9 binding showed that the CF9 peptide is not the primary receptor for the AVR9 elicitor (Kooman-Gersmann *et al.*, 1996). This is because both the resistance and susceptible tomato genotypes contain a high affinity binding site (HABS) for the AVR 9 peptide in plasma membranes of mesophyll cells. Therefore, the AVR9 elicitor is suggested to form a complex with HABS (AVR9-HABS complex) on the membrane cells (de Wit *et al.*, 1999) and in turn interacts with the Cf9 protein. The HABS molecule is speculated to be a transmembrane LRR-receptor-like-kinase (de Wit *et al.*, 1999). Blatt *et al.* (1999) found that the AVR9 elicitor targets the $K^+$ channels in Cf-9 transgenic tobacco guard cells suggesting the involvement of $K^+$ channels in AVR9/Cf-9 signal transduction.

There are several combinations of *R/Avr* genes and their products have been identified as indicated in Table 1. The common feature for all known *R* gene proteins, is the presence of LRR motifs except for the tomato *Pto* gene which encodes a cytoplasmic serine/threonine protein kinase. However, the Pto still requires an LRR-containing protein, Prf, to function (Salmeron *et al.*, 1996). The LRR domain comprising 20 to 30

amino acids with leucine repeats and other hydrophobic residues. Ellis *et al.* (1999) demonstrated that variation in the LRR domain in several *R* genes isolated from the flax-flax rust system, the classical example of gene-for-gene of Flor (1971), determine the specificity of the interaction. However, Sakamoto *et al.* (1999) shows that when rice was induced for SAR with the chemical, probenazole, a gene containing a nucleotide binding site (NBS) and LRR was induced.

**Table 1. List of Isolated *R* genes and the Corresponding *Avr* Genes in Plants**

| R gene | Plant host | Pathogen | avr gene | Disease | Features Motifs |
|---|---|---|---|---|---|
| *Cf-2* | Tomato | *C. fulvum* | *avr2* | Leaf mould | LRR, TM, SP |
| *Cf-9* | Tomato | *C. fulvum* | *avr9* | Leaf mould | LRR, TM, SP |
| *L6* | Flax | *M. lini* | Unknown | Rust | LRR, NBS, Toll |
| *N* | Tobacco | TMV | Replicase? | Leaf mosaic | LRR, NBS, Toll |
| *Pto* | Tomato | *P. syringae pv. Tomato* | *AvrPto* | Bacterial speck | S/TPK, Myr |
| *RPS2* | Arabidopsis | *P. syringae pv.* Tomato | *AvrRpt2* | Bacterial speck | LRR, NBS, LZ |
| *RPM1* | Arabidopsis | *P. syringae pv.* Maculicola | *AvrRpt1, avrB* | Bacterial speck | LRR, NBS, LZ |
| *RPR1* | Rice | *Magnaporthe grisea* | unknown | Blast disease | LRR, NBS |

**Abbreviations:** LRR, leucine-rich repeats; TM, transmembrane domain; SP, signal peptide; NBS, nucleotide-binding site; S/T PK, protein kinase with serine/theorine specificity; Myr, myristoylation site; LZ, leucine zipper; Toll, homology to the Toll receptor. Adapted from Halterman and Martin (1997) with some additions.

From Table 1, at least two groups of *R* genes encode proteins that are released into the cytoplasm i.e cytoplasmic protein kinases and cytoplasmic kinases with a region of leucine-rich repeats and a nucleotide binding site. Therefore, plants may use various mechanisms in transducing the signal molecules to the cytoplasm. Halterman and Martin (1997) suggested that plant pathogens might use the same secretion system (type III) that has been identified in the human bacterial pathogen, *Yersinia pseudotuberculosis*. In this novel secretion system, the bacteria can deliver the virulence protein directly to the host (Galan and Collmer, 1999). It is known from the different types of elicitors and receptors isolated that most of them are proteins with no

enzymatic activity leading to the suggestion that protein-protein interactions are likely to take part actively during the perception process (Hutcheson, 1998). Ji *et al.* (1998) proposed a gene perception model to describe the early events during plant cells-pathogen interaction as shown in Figure 2.

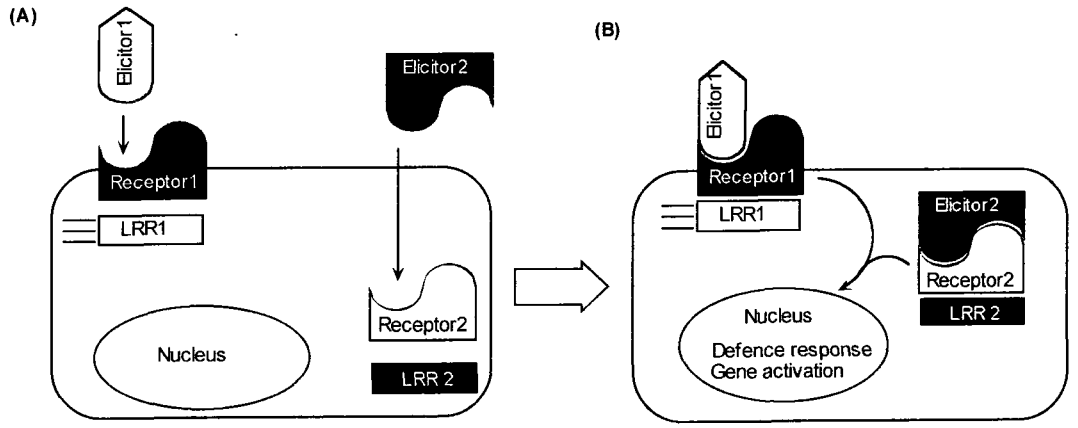


**Figure 2. Gene Perception Model.** The model proposed by Ji *et al.* (1998) involves the creation of receptor complexes in plant cells. "A cell expressing two different LRR disease-resistance gene proteins; LRR 1 is associated with the plasma membrane and LRR 2 is cytoplasmic. The cell also contains two specific receptor proteins for two different pathogen-produced specific elicitors. Again receptor 1 is localised to the plasma membrane and receptor 2 occurs in the cytoplasm. (**Panel A**) Following binding of either complementary specific elicitor, receptor 1 or receptor 2 recruits its corresponding LRR resistance gene proteins into a complex. The LRR protein complex is then and only then competent to transmit intracellular signals which result in nuclear defence response gene activation and disease resistance" (**Panel B**) (Adapted from Ji *et al.*, 1998).

## 1.4. Biochemical Basis of Plant-Pathogen Interactions Following Elicitation and Infection

During the plant-pathogen interactions in incompatible-type pathosystems, a number of defence responses are activated, depending on the type of incoming eliciting signals namely, primary response, secondary response and systemically acquired resistance (reviewed in Hutcheson, 1998). The primary response is localised at the infection site involving only a group of cells which are in direct contact with the pathogen. The response is often called the hypersensitive reaction (HR) and it produces visible

necrotic symptoms only at the site of infection (Dangl *et al.*, 1996). As a result of HR, numerous transfusable low molecular weight molecules called elicitors, derived either from the pathogen or as a result of its interaction with the plant cells are produced. These elicitors then activate secondary responses in the plant cells surrounding the infection. Some of these elicitors may also trigger a non-specific, a systemically acquired resistance (SAR) which enhances the whole plant against secondary infection from the same pathogen and/or different ones (Hutcheson, 1998).

There is considerable evidence that during HR, a burst of oxidative metabolism takes place, generating reactive oxygen species (ROS) derived from oxygen such as the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) (Lamb and Dixon, 1997; Lu and Higgins, 1998). This process, also called the oxidative burst; may require or induce other cell functions and metabolism such as an activation of $Ca^{2+}$ and anion channels, and normally occurs within minutes after the infection takes place (Levine, et al., 1994; Levine, *et al.*, 1996). ROS may directly trigger the HR and/or cell death and subsequently induces the defence-related genes (Levine *et al.*, 1994; *Desikan et al.*, 1998). Blocking the enzyme thought to be responsible for generating ROS, NADH oxidase using an inhibitor such as diphenylene iodonium (DPI), no ROS are generated and no defence responses take place (Tenhaken *et al.*, 1995; Dwyer e*t al.*, 1995). Recent studies have shown that $H_2O_2$ may act directly as a signal molecule to induce the expression of plant defence genes encoding various enzymes associated with aspects of resistance in plants such as PAL, glutathione S-transferase and anthranilate synthase, an enzyme involved in the biosynthesis of phytoalexins in *Arabidopsis* (Desikan *et al.*, 1998).

It has been reported that a plasma membrane-bound NADPH oxidase is activated during plants undergoing pathogen attacks (Jabs *et al.*, 1996; Draper, 1997). Keller *et al.*, (1998) isolated a plant homologue of the neutrophil NADPH Oxidase $gp91^{phox}$ gene subunit encoding a subunit of the plasma membrane protein with $Ca^{2+}$ binding motifs called *rbohA* in *Arabidopsis*. This finding strongly supports the earlier observations that NADPH oxidase rapidly generates $H_2O_2$ in the apoplast during the incompatible pathogen interaction (Lamb and Dixon, 1997; Keller *et al.*, 1998). The accumulation of $H_2O_2$ on the external face of the plasma membrane and apoplast could kill the pathogen directly and it also functions to repair the cell wall by cross-linking

the cell wall proteins through a peroxidase-dependent reaction (Lamb and Dixon, 1997). In addition $H_2O_2$ may also act as an intercellular signal to activate secondary defence reactions (Lamb and Dixon, 1997)

Several potential sources of ROS in cells have been reported (reviewed in Bolwell, 1996; Bolwell *et al.,* 1995; Bolwell and Wojtaszek, 1997; Wojtaszek, 1997). These pathways include the action of NADPH oxidase, the pH-dependent generation of $H_2O_2$ by cell wall peroxidase and by apoplastic oxalate oxidases. NADPH oxidase converts $O_2$ to $O_2^-$ which is followed by dismutation $O_2^-$ to $H_2O_2$. An increase in NADPH oxidase activity following either pathogen infection or elicitor treatment has been widely reported. For example, in potato tubers infected with an incompatible race of *P. infestans* (Doke, 1983), in soybean cell suspension cultures and leaf tissue challenged with an avirulent strain of *Pseudomonas syringae* pv. Glycinea; leaves of *Arabidopsis* inoculated with avirulent strain of *P. syringae* pv. Tomato; tobacco cell suspension cultures treated with fungal peptides, cryptogein (Simon-Plas, *et al.,* 1997). In addition to NADPH oxidase, there is the possibility that $H_2O_2$ might also be generated by a different route. Oxalate oxidase activity has been shown to increase when barley leaves are challenged with incompatible and compatible strains of powdery mildew fungus, *Blumeria graminis* f.sp. *hordei* (Thordal-Christensen *et al.* 1997). The transcripts of the enzyme increased after 6 h of inoculation in both incompatible and compatible interactions but sharply decreased only in the incompatible system. *In situ* localisation studies reveal that oxalate oxidase expression is localised at the site of contact between the epidermal cells undergoing HR and the adjacent mesophyll cells (Thordal-Christensen *et al.* 1997) the most suitable place for the $H_2O_2$ generation. They also found that the optimum pH for this enzyme is 3.2, leading to the hypothesis that signalling for HR involves an acidification of the apoplast compartment (Zhou, *et al.*, 1998).

Wojtaszek (1997) argued against the function of oxalate oxidase as a major source of $H_2O_2$ in plants as there was no report at that time of oxalate oxidase being isolated from dicotyledoneous plants, therefore, it was thought to be limited to cereals only. However, recent publications show that oxalate oxidase is in fact also present in dicotyledoneous plants. Membre *et al.* (1997) isolated cDNA clones for three germin-like genes (ATGER1, ATGER2 and ATGER3) from *A. thaliana* and also isolated a

*Brassica napus* cDNA clone, strongly homologous to ATGER1. They showed that ATGER1 is expressed during germination, ATGER2 strictly in embryos and ATGER3 in leaves and flowers. In barley, Zhou *et al.* (1998) found two oxalate oxidase genes in its genome but only one of them is induced during the interaction with the powdery mildew fungus, indicating that this enzyme is present in various isoforms for a different physiological function. In addition, the work at Durham University has confirmed that oxalate oxidase is at least in part an apoplastic enzyme (Croy RRD, pers. comm.).

A further report including the activation of $H^+$-ATPase in plants undergoing elicitation may be relevant to the suggestion that oxalate oxidase is a source for $H_2O_2$ in plants undergoing oxidative burst. The $H^+$-ATPase activity has been reported to increase in an incompatible interaction system between tomato cells with the fungal pathogen *Cladosporium fulvum* (Vera-Estrella *et al.*, 1992, 1994a, 1994b). An increase in plasma membrane $H^+$-ATPase activity leads to the acidification of the apoplast creating a more enzymatically-suitable environment for oxalate oxidase; the normal pH within the apoplast has been measured between 5.5 to 6.0 (Zhou, *et al.*, 1998). In addition, acidification of the apoplast could generate more free $Ca^{2+}$ and also solublise the oxalate, the substrate for oxalate oxidase (Zhou, *et al.*, 1998). Under normal conditions, most of the extracellular $Ca^{2+}$ binds to other molecules including pectates and oxalates making it inaccessible to the enzyme. Until more biochemical properties of this enzyme have been studied especially in dicotyledoneous plants, little can be said about its role, if any, in an oxidative burst.

It has been suggested that the plasma membrane $H^+$-ATPase possesses an inhibitory domain which regulates the ATP hydrolytic activity and $H^+$ pumping activity (Palmgren *et al.*, 1991). The enzyme can be activated by phosphorylation of a site within this domain. Recently, an *in vivo* phosphorylation site (phosphothreonine-948), involving the second residue from the C-terminal end of the $H^+$-ATPase protein has been identified and sequenced (Olsson *et al.*, 1998). This phosphorylation site, the first of its kind in plasma membrane $H^+$-ATPase binds to a receptor protein named 14-3-3, a highly conserved family of eukaryotic proteins with multiple regulatory functions (Olsson *et al.*, 1998). The 14-3-3 proteins were first demonstrated to be a receptor for fusicoccin, a toxin isolated from an almond tree fungal pathogen *Fusicoccum*

*amygdali* (Marra *et al.*, 1994; Oecking *et al.*, 1994) but later it was shown that there are also natural ligands for this receptor, regulating the $H^+$ pump by replacing the inhibitory domain of the enzyme (Jahn *et al.*, 1997; Baunsgaard *et al.*, 1998). If a suitable elicitor is available to bind to this ligand, a complex of $H^+$-ATPase/14-3-3 may be established and dephosphorylation would take place to activate the enzyme leading to $H^+$ proton export. At least two groups of researchers (Vera_Estrella *et al.*, 1994a; Xing *et al.*, 1996) have demonstrated that this enzyme is activated in tomato cells using an elicitor derived from an incompatible fungal pathogen, indicating that the enzyme is likely to be involved in signal transduction during the infection process.

One of the main characteristic features for plants undergoing HR is the accumulation of cytosolic $Ca^{2+}$ and some workers have suggested that $Ca^{2+}$ may play a central role in the activation of defence responses in plants (Mehdy, 1994; Jabs *et al.*, 1997). The most recent data showed that an elevation of free cytosolic $Ca^{2+}$ is the first sign of HR in the incompatible cowpea-*Uromyces vignae* pathosystem. As soon as the pathogen makes contact with the cell wall, an elevation of $Ca^{2+}$ is observed using a fluorescent calcium reporter dye (CG-1) and a confocal laser scanning microscope (Xu and Health, 1998). Soon after the pathogen enters the cell and grows in the lumen, the concentration returns to normal again. This observation is not detected in a compatible-type interaction (Xu and Health, 1998). When a $Ca^{2+}$ channel blocker is used in this system, no detectable $Ca^{2+}$ change is observed, and the HR is delayed indicating that a $Ca^{2+}$ influx is most likely to be facilitated by $Ca^{2+}$ channels. A novel elicitor-inducible $Ca^{2+}$ channel has recently been discovered by Zimmerman *et al.*, (1997) may be involved in $Ca^{2+}$ flux in these interactions. This channel is reversibly activated when parsley protoplasts are elicited by an oligopeptide elicitor derived from a cell wall protein of *Phytophthora sojae*. In addition, it has structural features for elicitor-receptor binding to take place indicating that its activity is receptor-orientated and, therefore, is likely to be involved in the signalling cascade and triggering defence responses (Zimmerman *et al.*, 1997).

This finding together with NADPH oxidase isolated by Keller, *et al.* (1998) may help us to understand some of the steps in the mechanism of oxidative burst in plants undergoing pathogen attacks in incompatible-type pathosystems. The significance of the NADPH oxidase lies in its large hydrophilic N-terminal domain containing two

$Ca^{2+}$ -EF hand motifs indicating a potential direct regulation by the $Ca^{2+}$ (Keller, *et al.* 1998). In addition, the structure of the enzyme also supports its predicted function as its N terminal domain contains no hydrophobic stretches, strongly indicating that the extended domain projects into the cytosol (Keller, *et al.*, 1998). Therefore, the resistance gene-mediated recognition of pathogen proteins could activate the elicitor-activatible $Ca^{2+}$ channel causing an influx of $Ca^{2+}$ ions which in turn activates the NADPH oxidase to generate ROS (Hutcheson, 1998). A model for elicitation of active defence responses in a plants system comprising of six known *R* genes and their *avr* genes including downstream components proposed by Hutcheson (1998) is shown in Figure 3.

Another possible $H_2O_2$-generating enzyme that has been reported in addition to the two discussed above, is a cell wall-bound peroxidase which is an important component of plant stress responses (reviewed in Bolwell, 1999; Wojtaszek, 1997). Bestwick *et al.* (1998) showed an early increase in peroxidase activity during the nonhost HR of lettuce leaf inoculated with a wild strain of *Pseudomonas syringe (p.s.) phaseolicola* and the peroxidase activity was restricted to the cell wall at the site of infection. Localisation studies showed that the increase of peroxidase at the infection site was accompanied by the accumulation of $H_2O_2$ confirming the presence of peroxidase-dependent oxidative burst (Brown *et al.*, 1998). The peroxidase has an optimum pH ranging from 4.5 to 6.5 depending on the substrate used for the assay (Bestwick *et al.*, 1998). This is only slightly lower than the normal pH range in the apoplast indicating that this enzyme might have a multifunctioning activity depending on the physiological status of cells. A superoxide-binding intermediate that responsible to generate ROS in the peroxidase-dependent system requires neutral to alkaline pH (reviewed in Bolwell, 1999). Previous studies showed that this enzyme takes part in some other cell activities associated with defence responses in plants such as in the cross-linking of cell wall proteins and other wall components (Iiyama *et al.*, 1994), and also in regulating $H_2O_2$ levels (Mittler *at al.*, 1998). The activity of cytosolic ascorbate peroxidase, a key enzyme in scavenging $H_2O_2$ has been demonstrated to increase in plants under environmental stresses (Mittler and Zillinkas, 1992; Jimenez *et al.*, 1997). Studies of different groups showed that $H_2O_2$ induces apoptosis and defence responses in bean and tobacco (Levine *et al.*, 1994; 1996; Draper, 1997; Shirasu *et al.*, 1997). Inhibition of ascorbate peroxidase and catalase by salicylic acid (SA) during plant-

pathogen interactions leads to an apoptosis and results in an increase of $H_2O_2$ production and an activation of defence responses (Draper *et al.*, 1997; Rao *et al.*, 1997; Shirazu *et al.*, 1997).

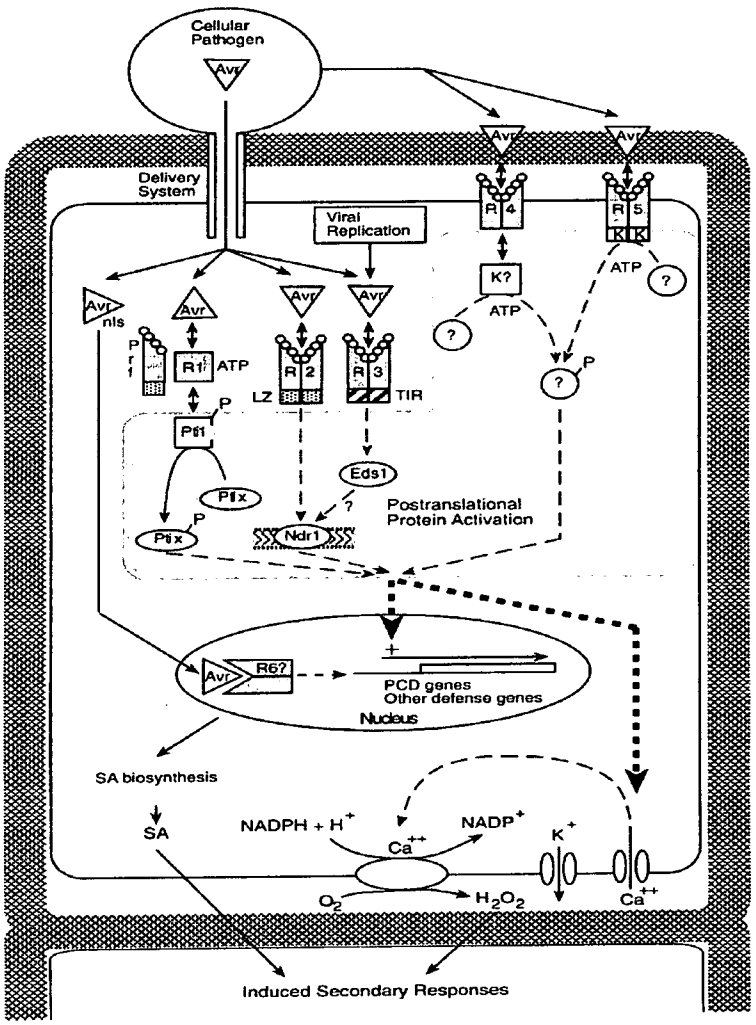


**Figure 3. Model for Elicitation of Active Defence Responses in Plants.** The model is proposed by Hutcheson (1998). Products of avirulence genes (Avr; triangles) are delivered into the apoplast or into the cytoplasm of plant cell by a pathogen-encoded delivery system, such as the *hrp*-encoded protein translocation complex. Viral replication (box) may also product proteins with avirulence activity. (Key: 1 Avr, triangles = products of avirulence gene; <--> , 2 headed arrow = reversible binding of avirulence products to resistance gene products; small linked circles = LRR domain; Shaded box = Six classes of resistance genes products; Signal transduction pathways = shaded region, involving posttranslational protein activation events; ------- = postulated pathway; ? = hypothetical components; K = protein kinase; LZ=leucine zipper; nls= nuclear localisation signal; SA= salicylic acid; TIR, Tol/IL01R Signal transduction domain) (Adapted from Hutcheson, 1998).

Inhibition of ascorbate peroxidase and catalase by salicylic acid (SA) during plant-pathogen interactions leads to an apoptosis and results in an increase of $H_2O_2$ production and an activation of defence responses (Draper *et al.*, 1997; Rao *et al.*, 1997; Shirazu *et al.*, 1997). This suggests that $H_2O_2$ may act as a signal in triggering apoptosis. Takahashi *et al.* (1997) provided further proof using transgenic tobacco plants with antisense catalase. The transgenic plants which exhibit catalase levels reduced by more than 90% and showed enhanced resistance to tobacco mosaic virus, expressed very high levels of PR-1 proteins, SA and developed necrosis at the site of infection. This suggests that SA may be responsible for the induction of resistance. Mittler *et al.* (1998) suggested that the transgenic plants used in Takahashi *et al.* (1997) could perform better if antisense for ascorbate peroxidase genes was used. This was suggested for two reasons, first ascorbate peroxidase possesses a higher affinity towards $H_2O_2$ compared with catalase and secondly the $H_2O_2$ is first transfused to the cytosol where ascorbate peroxidase is abundantly available while it needs to enter the peroxisome to reach the catalase.

From these results, it was predicted that the cytosolic ascorbate peroxidase would not be increased during the infection process as SA formed during the interactions (Hammond-Kosack and Jones, 1996) could inhibit its activity. However, SA is shown to possess only a weak inhibitory effect on ascorbate peroxidase (Kvaratskhelia *et al.*, 1997; Tenhaken and Rubel, 1997) leading to the suggestion that other mechanisms may be involved. Mittler *at al.* (1998) suggested a so-called 'post-transcription suppression mechanism' to explain the involvement of ascorbate peroxidase in regulating $H_2O_2$ in incompatible interaction-type pathosystem. They studied the expression patterns of cytosolic ascorbate peroxidase in tobacco undergoing apoptosis and found that the mRNA levels of the cytosolic ascorbate peroxidase were induced but the translation of mRNA was inhibited avoiding the accumulation of ascorbate peroxidase that could inhibit $H_2O_2$ accumulation and disable the HR.

The major function of $H_2O_2$ and SA in plant-pathogen interactions is yet to be established. Pontier *et al.* (1998) in a series of comprehensive experiments argued against the function of $H_2O_2$ or/and SA as key signal molecules triggering PCD. Using a transgenic tobacco carrying *hsr203*, a plant gene expressed during incompatible plant-pathogen interactions, they showed that none of the potential effectors for HR or

resistance tested (i.e. $H_2O_2$, SA, methyl jasmonate, and 2,6-dichloro-isonicotinic acid) were able to activate the gene promoter. In contrast, all bacterial and viral pathogens known to induce HR in tobacco, as well as inducers for HR-like responses (i.e. harpin and elicitin) were able to activate the promoter. In contrast, heavy metals that can cause leaf necrosis did activate the gene promoter, leading to the suggestion that this gene might be a useful marker for detecting PCD in plants caused by other death-triggering extracellular agents (Pontier *et al.*, 1998). Three reasons were suggested to explain this phenomenon, first the tissue culture system used by most of the previous experimenters did not represent the actual whole plant system fully leading to an incomplete expression of certain key pathways; secondly, that $H_2O_2$ is not a key determinant in cell death; and finally, a pathway not involving $H_2O_2$ may be responsible for activating their gene. This is the first experiment confirming that $H_2O_2$ does not trigger HR or apoptosis during an incompatible plant-pathogen interaction. Interestingly, active oxygen intermediates resulting from localised HR in Arabidopsis leaves, induced secondary oxidative bursts in discrete cells at sites distant from HR leading to a low frequency systemic resistance (Alvarez *et al.*, 1998). Probably $H_2O_2$ is part of the general-type resistance network such as SAR as the oxidative burst is not limited to the incompatible-type interaction. Alvarez *et al.* (1998) also showed that SAR appears before SA accumulation is detected, questioning whether SA is a main key triggering signal for SAR (Ji *et al.*, 1998).

$H_2O_2$ alone can be seen as an instant antimicrobial agent that can kill the pathogens directly. This is important because not all pathogens are biotrophs or hemibiotrophs, some are necrotrophs. This latter group of pathogen can propagate whether the host is dead or alive. *P. infestans* is one of the plant pathogens belongs to this group. Normally necrotrophs kill the cells they enter by various toxin and necrotic enzymes and then metabolise the cell content for their survival. Therefore, the plants cell needs to kill the pathogen as soon as it attaches to the cell wall before any toxic substances are released into the cell by the pathogen. Cell death either as a result of HR or PCD is simply not effective or too late in responding to this type of pathogen. The secondary responses as a result of HR or PCD such as the production of PR- proteins such as glucanase or chitinase are even less effective being synthesised much later. Cell death could in fact benefit the necrotrophs rather than to prevent their spread.

Evidence discussed earlier (Xu and Health, 1998) suggested that the level of $Ca^{2+}$ increased once the pathogen attaches to the cell and this led to the production of $H_2O_2$. This indicates that the cells are 'primed' ready to respond to an incoming pathogen attack. In contrast, HR or PCD can block the flow of nutrients from the plant to the pathogen leading to a killing of the invading biotrophs or hemibiotrophs. These pathogens can only grow in living hosts because they require nutrients produced in living cells.

As mentioned earlier, plants possess a complex multilayer defence network in order to survive from pathogen attacks. The second layer of protection or the secondary reaction following infection depends on the outcome of the primary reaction (signal exchanges between host cells and the pathogen leading to HR). The secondary responses can be likened to a factory to produce suitable products that have been signalled during the primary responses. Among the chemicals or biochemical pathways that are activated are those involved in the production of PR-proteins (van Loon *et al.,* 1985), activation of phytoalexin biosynthesis pathway (Dixon *et al.,* 1995), and the production of hydrolytic enzymes such as chitinase and glucanase (Kombrink *et al.,* 1991).

Phytoalexins are defined as low molecular-weight antimicrobial compounds that are both synthesised and accumulated in plants after exposure to micro-organisms (reviewed in Strange, 1998). Therefore, any chemicals that are not actively synthesised by the plants metabolic pathways, are excluded from this group but they are classified as a passive biochemical defence. The existence of phytoalexins was first postulated by Muller and Borger around 1940. In an experiment using an avirulent strain of *P. infestans* and potato tubers they found interesting observations; the tuber cuts that were inoculated with the incompatible race of *P. infestans* before failed to display the usual symptoms of late blight when inoculated with the virulent strain of the pathogen, or even with an other pathogen like *Fusarium caeruleum* (reviewed in Strange, 1998).

There is still an unclear relationship between the oxidative burst and phytoalexin accumulation in plants or cell suspension cultures undergoing a pathogen attack or elicitation. Guo *et al.* (1998) found that $H_2O_2$ produced in soybean cell suspension cultures following *Pseudomonas syringae* pv *glycinea* harbouring an avirulence gene (*avrA*) was not sufficient to induce the accumulation of isoflavonoid phytoalexin. An

accumulation of phytoalexins is always accompanied with the induction of the key enzyme in the phenylpropanoid biosynthesis pathway, PAL and chalcone synthase (Lo and Nicholson, 1998). ). PAL converts L-phenylalanine to *trans*-cinnamic acid, an intermediate in the formation of a large variety of plant-specific phenylpropanoid derivatives, including lignin, flavanoids, stilbenes, coumarins (Joos and Hahlbrock, 1992).

Jabs *et al.* (1997) showed that by treating parsley (*Petroselinum crispum*) cell suspension cultures with a fungal elicitor, an active oxygen species is detected, followed by activation of defence genes and then followed by phytoalexin biosynthesis. These were confirmed through inhibition studies using NADPH oxidase inhibitor and a $Ca^{2+}$ ion channel blocker. Both treatments gave no production of an active oxygen species, no defence response and no phytoalexin biosynthesis (Jabs *et al.*, 1997) indicating that $Ca^{2+}$ is needed for the production of active oxygen species and subse, but not $H_2O_2$ triggering the activation of defence leading to phytoalexin biosynthesis (Jabs *et al.*, 1997). The finding that $H_2O_2$ is not involved in triggering the defence response has been confirmed by Pontier, *et al.* (1998) as discussed earlier

In potato tubers, phytoalexin production (i.e. sesquiterpenoids) occurs through the terpenoid biosynthesis pathway (Kuc, 1982) The same observation was also reported by Rohwer *et al.* (1987). They showed that the accumulation of sesquiterpenoid phytoalexin is more rapid (i.e. within the first 24-48 h of infection) in incompatible interactions than the compatible ones. However, they failed to detect the sesquiterpenoid phytoalexin in infected leaves, in either the incompatible or compatible interactions indicating that this compound may not be actively involved in defending the potato from *P. infestans* infection. Inoculating a potato tuber with sporangia of *P. infestans* resulted in an activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Choi *et al.*, 1992), one of the key enzymes in the sesquiterpenoid phytoalexin biosynthesis pathway. HMGR catalyses the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid, the rate-limiting step in terpenoid biosynthesis. In plants, terpenoid compounds play important roles in mediating plant growth and development, electron transport, photosynthesis, and disease resistance (Denbow *et al.*, 1996). HMGR and PAL are mildly induced at the late stage of normal HR response or after SA treatment when compared with the PR-gene expression

(Kang *et al.,* 1998). When potato tuber discs were treated with eicosapentaenoic acid (EPA), an elicitor found in *P. infestans*, HMGR gene transcripts level increased. This increase paralleled that of the cytosolic glyceraldehyde-3-phosphate dehydrogenase (Laxalt *et al.,* 1996). HMGR and PAL activities are also reported to be induced in potato tubers in an incompatible reaction with *P. infestans* (Yoshioka *et al.,* 1996).

Another common product of secondary responses of host pathogen interaction (i.e. in incompatible systems) is pathogenesis-related (PR) proteins (van Loon, 1985). PRs have been defined as plant proteins that accumulate after a pathogen attack or related situations. PR proteins were originally grouped into five families (van Loon, 1985) but van Loon *et al.* (1994) have proposed a unified nomenclature for the classification of PR proteins comprising of 11 families of proteins (PR-1 to PR-11). This classification is based on their known or predicted amino acid sequence, serological relationship and/or enzymatic or biological activity. Some members of the families (PR-1, PR-2, PR-3, PR-4, PR-5) have an antimicrobial activity and are thought to play a role in plant defence by inhibiting the pathogen growth (Boller, 1993). Only two groups, PR-2 (β-1.3-glucanases) and PR-3 (chitinases) display specific enzymatic activities (van Loon *et al.,* 1994). Some proteins, belonging to groups PR-1, PR-4 and PR-5, have been shown to have antifungal activities but the mechanism of action and the catalytic activity, are unknown (Niederman *et al.,* 1995). Examples of PR-5 proteins with antifungal activity are permatin (Roberts and Selitrennikoff, 1990), osmotin (Singh *et al.,* 1987) and thaumatin (Koiwa *et al.,* 1997; Zhu *et al.,* 1995). PR proteins are normally detected abundantly at the site of infection although they are also induced systemically and found in uninoculated tissues though to a lower level (Stinzi *et al.,* 1993).

In the potato, there are several reports dealing with PR-protein induction. For instance, Schroder *et al.* (1992) reported an accumulation of chitinases and β-1,3-glucanases in potato leaves infected with *P. infestans*. They found a similar, strong and co-ordinated induction of 1,3-β-glucanases and chitinases in compatible and incompatible interactions of two selected potato cultivars infected with appropriate races of the fungus. Accumulation of the protein is preceded by progressive activation of the corresponding gene, commencing near the infection sites and spreading rapidly throughout the whole infected leaf as well as to adjacent, non-infected leaves

(Schroder *et al.,* 1992). In contrast to β-1,3-glucanase, PAL is accumulated more rapidly and remains restricted to the infection site (Schroder e*t al.*, 1992).

### 1.5. Strategies in Developing Transgenic Plants with Disease Resistance

Transgenic plants are now becoming a common way of expressing the gene of interest into the plant. Many potential genes associated with disease resistance have been isolated in recent years. This development may help to improve crop production in crop varieties which lack certain disease resistance while at the same time maintaining good agronomical traits. In conventional breeding, there is no guarantee that all the good characters of the parent are inherited in the new hybrid. Often breeders manage to develop good hybrid plants in terms of yield and quality which lack a desired disease resistance. This is where the use of transgenic technology might be useful.

As the understanding of various aspects of plant-pathogen interactions is increasing, the prospect of being able to engineer transgenic plants using different strategies becomes feasible. These strategies are developed mainly based on an understanding of the plant defence mechanisms in responding to pathogen attacks such as the production of phytoalexin, PR proteins, antimicrobial substances and an interaction between *R/Avr* genes as discussed previously.

#### 1.5.1. Exploitation of Genes Encoding Enzyme-Degrading Pathogen Cell Walls

Among the major PR proteins induced during plant-pathogen interaction events are proteins that posses fungal cell wall degrading enzyme activities, glucanases and chitinases. Chitinase or poly [1,4-(N-acetyl-β-D glucosaminade] glucanohydrolase (EC 3.2.1.1.4.) catalyses the hydrolysis of β-1, 4 linkages in chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine (Boller *et al.*, 1983). Since chitin is one of the major components of the cell walls of many fungi, constituting 3-60% of the dry weight of cell wall (Wessel and Sietsma, 1981), the presence of chitinase when plants are under attack is likely to destroy the pathogen. The effectiveness of chitinase in destroying fungal cell walls are shown in several *in vitro* studies involving fungal species such as *Trichoderma viride, Alternaria radicina* (Melchers *et al.*, 1994); (Schlumbaum *et al.*, 1986) and *Fusarium solani* (Sela-Buurlage *et al.*, 1993). The chitinase isolated from *Trichoderma harzianum* seems to have a very potent antifungal

activity compared with the plant counterpart and is also effective against a wider range of fungal pathogens (Lorito *et al.*, 1993).

Engineering the plant to produce high, constitutive levels of chitinase as a component has been reported. For instance, Broglie *et al.* (1991) successfully transferred and expressed a bean vacuolar chitinase gene under the control of a strong constitutive promoter (Cauliflower mosaic virus (CaMV) 35S) in tobacco and canola (*Brassica napus* cv. Westar) plants. The transgenic tobacco seedlings showed an increased ability to survive in soil highly infested with the fungal pathogen *R. solani*. Crop losses due to the same disease in the transgenic canola plants were also significantly decreased. Using these transgenic canola plants, Benhamou *et al.* (1993) showed that the hyphae of *R. solani* in infected transgenic canola plants were severely altered showing symptoms ranging from increased vacuolisation to cell lysis. Fungal colonisation was restricted to the cortex tissue but on some occasions the damaged and wall fragments of the fungal cells are found in the xylem vessels (Benhamou *et al.*, 1993).

Using non-plant sources of chitinase, the endochitinase genes isolated from the fungus, *Trichoderma harzianumbacteria* (Lorito *et al.*, 1998) have been used to construct transgenic tobacco and potato plants using CaMV35S promoter which showed high resistance to a wide spectrum of pathogens including both foliar and soilborne origins. The level of resistance was complete or nearly complete to the soilborne pathogen, *Rhizoctonia solani* and foliar pathogens *Alternaria solani*, *Botrytis cinerea* and *Alternaria alternata*. This is the first report where transgenic plants encoding a single gene for chitinase showed a high level of resistance to a wide range of pathogens. The reason for this may arise from the fact that the mycoparasitic-origin of the chitinase is far more potent compared with the plant-origin as shown in the earlier experiment where the level of effectiveness can be up to 100 times compared with the corresponding plant chitinase (Lorito *et al.*, 1993).

Another cell-wall degrading PR-protein induced in plants during pathogen attacks is β-1,3-glucanase or glucan endo β-1,3-glucosidase (EC 3.2.1.39) (Collinge and Slusarenko, 1987). It catalyses the hydrolysis of β-1,3-glucan, another major component of cell walls of many fungi (Wessel and Sietsma, 1981). This enzyme is

also induced in response to elicitor treatment (Kombrink *et al.,* 1988), and in response to exposure to ethylene (Mauch and Stachelin, 1989). *In vitro* studies of purified β-1,3-glucanases for antifungal activity on *Fusarium solani* showed that the class I β-1,3-glucanases have potent antifungal activity (Sela-Buurlage *et al.,* 1993; Melchers *et al.,* 1993) and in combination with class I chitinase, synergistic effects were observed (Sela-Buurlage *et al.,* 1993). The observations that β-1,3-glucanase and chitinase react synergistically *in vitro*, together with induction of the activities in hypersensitive reaction indicated that these enzymes play a role in non-specific disease resistance.

Combining these two enzymatic activities together by crossing transgenic parental lines of rice exhibiting strong constitutive expression of an alfalfa acidic β-1,3-glucanase and a rice basic chitinase, respectively, showed an enhanced disease resistance toward the fungal pathogen *Cercospora nicotianae* (Zhu *et al.*, 1994). Enhanced protection of the $T_2$ hybrids compared with either of the transgenes alone against *C. nicotianae* was concluded by the delay in the appearance of the first symptoms and the size and number of leaves lesions developed. The $T_2$ hybrids heterozygous for both transgenes were more resistant to infection compared with the homozygous hybrids (Zhu *et al.*, 1994) indicating that the synergistic effects observed *in vivo* were maintained *in planta.* A similar phenomenon has also been demonstrated when a transgenic tobacco line expressing a combination of barley class-II chitinase and barley class-II β-1,3-glucanase under the control of CaMV 35S-promoter was challenged with the *Rhizoctonia solani* (Jach *et al.*, 1995). The possible explanation for this phenomenon is that the combined enzymatic actions of the chitinase and glucanase on the fungal cell walls may enhance the substrate accessibility and accelerate cell wall damage leading to complete cellular lysis (Zhu *et al.*, 1994). In addition, the resulting fungal cell wall breakdown products that come from the glucan and chitin may act as active elicitors to trigger the plants cellular defence network. Both chitin and glucan fragments are active elicitors of plant defence system (Dixon and Lamb, 1990).

### 1.5.2. Exploitation of Non-Enzymatic Antimicrobial Protein

Other combinations of antimicrobial proteins have been used in protection strategies. For instance, combining thionin isolated from wheat or barley with 2S albumin isolated from radish and oilseed rape, and barley trypsin inhibitors lowered the

concentration required for 50% inhibition of fungal growth by up to 73-fold (Terras *et al.*, 1993). Thionins are well known small polypeptides (about 5kDa) found in different tissues of many plant species and shown to have a potent antimicrobial activity, especially the thionins isolated from wheat (purothionins) and barley (hordothionins) (Bohlman, 1994; Florack and Stickema, 1994). The proteins are cysteine-rich and the toxicity results from the destruction of the pathogen cell membranes.

Its function as a plant protein has been enlightened by the recent finding that the transgenic plants expressing thionin isolated from Arabidopsis (THI2.1) produce no transcripts for pathogenesis-related PR-1, PR-5, or the pathogen-inducible plant defensin (Pdf1.2) when challenged with *Fusarium oxysporum* f sp *matthiolae*, indicating that all of the observed effects in the overexpressing lines are most likely the result of the toxicity of the THI2.1 thionin system (Epple *et al.*, 1997). Earlier work on the overexpression of α-hordothionin in tobacco under the control of CaMV35S promoter showed enhanced resistance to at least one bacterial pathogen, *Pseudomonas syringae* (Carmona *et al.*, 1993) and a broad range of disease resistance may come into prospect if an appropriate partner of antimicrobial genes is selected as quoted earlier in the *in vitro* studies. The latter have shown that the antimicrobial activity of thionin against *F. solani* was increased when it was combined with a non-specific lipid transfer protein (LTP).

Another candidate for engineering disease resistance in transgenic plants is ribosome-inactivating proteins (RIPs) (reviewed in Stirpe, *et al.*, 1992; Barbieri *et al.*, 1993). This type of protein has been isolated from a wide variety of plant species and it functions by enzymatically cleaving a specific RNA-N glycosidic bond in eukaryotic ribosomes making them unable to bind the elongation factor 2 during the protein translation. Two types of RIPs (type I and type II) have been identified so far. Type 1 RIPs are single enzymatically active polypeptide chains of Mr about 26 000-32 000. They are glycosylated and have relatively low cytotoxic activity. Type II RIPs are heterodimers comprising of an active A chain which is functionally equivalent to a type 1 RIP and is linked to a sugar-binding B chain. They can also be glycosylated. Type II RIPs are very toxic to whole cells due to lectin moiety. RIPs have been reported not to inactivate ribosome from some plants, but show varying degrees of

activity towards ribosome's of distantly related species including fungi (Robert *et al.*, 1986; Stirpe and Hughes, 1989). For instance the cytosolic Type 1 RIPs from the starchy endosperm of cereals are not significantly active on plant ribosomes *in vitro* (Taylor *et al.*, 1994), whereas the purified RIPs from barley seed inhibits the growth of fungi as reported in Leah *et al.* (1991). This finding has led to the use of RIPs in plant protection by expressing the barley RIP cDNA under control of the wound-inducible promoter of the potato *wun1* gene in barley (Logemann *et al.*, 1992). The transgenic barley produced showed limited resistance to the soil-borne pathogenic fungus *R. solani* without influencing normal plant growth. In addition, the combinatorial expression of RIP and chitinase from barley using the CaMV 35S-promoter has been reported to enhance resistance against the same fungus (Jach *et al.*, 1995).

### 1.5.3. Exploitation of *R/Avr* Genes

The genetic breakthrough by Flor in the 1940s, triggered scientists to study the plant-pathogen interactions especially in incompatible pathosystems, leading to the isolation of several *Avr* and *R* genes (See Table 1 for the list of some of the isolated *R* and *Avr* genes so far). These genes are responsible for the activation of HR in plants undergoing pathogen attack as discussed previously. In nature, a plant with an *R* gene would display a HR if attacked by a pathogen possessing the corresponding *Avr* gene leading to resistance in the plant.

A transgenic plant could be developed by transferring the *R* or *Avr* genes into a given plant. Hammond-Kosack *et al.* (1998) developed transgenic tobacco and potato lines using a *Cf-9* gene (*R* gene) encoding an extracytoplasmic leucine-rich repeat (LRR) protein that confers resistance in tomato to a race of the fungus *Cladosporium fulvum* that expresses the corresponding avirulence gene A*vr9*. Both the transgenic potato and tobacco, showed a rapid HR when injected with Avr 9 peptide.

On the other hand, Tang *et al.* (1999) constructed transgenic potatoes by transferring the avirulent gene, *avrPto*. As discussed earlier, *avrPto* is an avirulence gene isolated from the bacterial pathogen, *Pseudomonas syringae*. The matching *R* gene in potato, *Pto* encodes a cytoplasmic serine/threonine protein kinase which is activated when it interacts with the corresponding AvrPto protein from the pathogen (Loh and Martin, 1995). The transgenic potatoes show high levels of SA (up to 6 x) in the leaves

compared with the control non-transgenic plants. In addition, all the PR proteins (PR-1 – PR-5) tested including the basic and acidic form are increased constitutively in response to infection (Tang *et al.,* 1999). The transgenic plants were not only resistant to *P. syringe* as expected but also other pathogens such as *Xanthomonas campestris* and *Cladosporium fulvum* indicating a race non-specific nature of the transgenic plants. This finding confirms the work of Hammond-Kosack *et al.* (1998); the transgenic plants encoding avirulence gene show resistance to a wide range of pathogen (race non-specific) due to the expression of general defence response in plants.

### 1.5.4. Exploitation of Genes for Phytoalexin Production

The production of phytoalexins in plants involves a series of enzymatic steps encoded in a collection of genes attempting to engineer transgenic plants producing a phytoalexin using a single gene appears difficult. However, a recent attempt to do this using the gene encoding the enzyme stilbene synthase in plants has shown some promising results (Hain *et al.,* 1993). Stilbene synthase is an enzyme involved in biosynthesis of the stilbene phytoalexin, resveratrol. This compound has been shown to have antifungal activity in peanut, grapevine and pine (Hain *et al.,* 1993). The gravewine gene encoding this enzyme was transferred to tobacco, and demonstrated some enhanced degree of resistance towards the fungal pathogen *Botrytis cinerea* (Hain *et al.,* 1993). However, when the same gene was transferred to a tomato plant, resistance was not observed against *B. cinerea* as expected rather some degree of resistance to *P. infestans* was discovered. (Thomzik *et al.,* 1997).

### 1.5.5. Exploitation of Genes for Production of Active Oxygen Species

As discussed earlier, active oxygen species especially $H_2O_2$ have a direct antimicrobial effect (Peng and Kuc, 1992). The levels increase in plants undergoing pathogen attacks. Active oxygen species are also involved in the covalent crosslinking of cell wall proteins (May *et al.*, 1996). Based on its potential function in defending the plant from pathogen attacks, Wu et al. (1995) successfully developed transgenic potatoes using a glucose oxidase gene isolated from *Aspergillus niger*. Glucose oxidase, in the presence of glucose and molecular oxygen produces $H_2O_2$. $H_2O_2$ levels were increased in both leaves and tubers of the transgenic potato plants (Wu *et al.*, 1995). Plants

challenged with the potato pathogens, *E. catovora* subsp. *Carotovora, Verticillium dahliae* and *P. infestans* showed enhanced resistance. They found that the transgenic plants were effective *against E. carotovora* subsp. *carotovora* in both aerobic and anaerobic conditions. Interestingly, the increase of $H_2O_2$ resulted in increased SA levels several fold in the leaf tissue (Wu *et al.*, 1997). The mRNA level for two other PR proteins, anionic peroxidase and acidic chitinase are also induced. These results suggest that the elevated sublethal levels of $H_2O_2$ are sufficient to activate an array of host defence mechanism leading to the $H_2O_2$-mediated disease resistance in transgenic plants (Wu *et al.*, 1997).

### 1.6. The overall conclusions from the literature review

1. Crop losses due to various plant diseases significantly reduce crop production worldwide. Various strategies have been developed and evaluated and these include the use of conventional approaches (i.e. integrated disease management) and the more advanced genetic modification techniques using transgenic technology. Integrated disease management is still the best way of controlling disease problems while transgenic technology still remains to be evaluated thoroughly.

2. Several transgenic plants have been developed to contain genes that target a specific site in the pathogen cell and are potentially effective against a wider range of pathogen. However, none of these transgenic plants are effective against a wider range of pathogens. None of them has been extensively tested in the field.

3. The development of transgenic plants containing an avirulence gene (*Avr*) rather than an *R* gene seems promising because the resulting transgenic plants show race non-specific resistance and this should be effective against a wide range of pathogens. This is in contrast to the use of *R* gene where the resulting transgenic plants tend to be race-specific and this has proved to be unreliable for a sustainable resistance and is also limited to a specific pathogen only. The use of *Avr* genes in transgenic plants is a very recent development in this area and therefore it is premature to conclude their effectiveness before extensive field trials have been carried out.

4. Less than 10 pairs of *R/Avr* gene have been identified and these are mostly in Solanaceous plants. Two of them have been extensively studied and these give some clues to the mechanism behind the 'incompatible interaction' between plants and pathogens. This is only part of a possibly more complex defence network in plants.

5. There have been some advances in the understanding of the molecular mechanism behind the incompatible plant-pathogen interactions (based on the existence of a specific elicitor produced by pathogen and a receptor in the plant cells; gene-for gene), The system is more complex than anticipated. Several speculative models are also suggested for the gene perception and transduction.

6. The biochemical mechanism of the plant-pathogen interaction is rather complicated and no conclusive result can be drawn. In incompatible interactions between plant and pathogen, HR is certain to be the outcome. The mechanism behind the development of HR is still unclear and whether it is part of the PCD. The involvement of oxidative burst associated with HR, and the importance of this phenomenon in plant defence response, remains to be clarified. The results of the various findings are not clear enough to conclude a direct involvement of oxidative burst in defence response in plants. This is partly due to the various systems used by different groups such as tissue culture system vs whole plant, elicitors vs viable infective unit, specific elicitors vs general elicitors. At this stage, it is speculated that oxidative burst is a general mechanism in plants towards stress.

7. Late blight disease is one of the most important diseases in potato and tomato worldwide. The development of a strategy to combat this disease is challenging because i) there is less understanding of the biology of the pathogen ii) the pathogen is complex i.e. exists in various races and mating types iii) genetically it is highly variable due to migration and this leads to sexual and asexual reproduction from various races and mating types which are geographically different. iv) the causal agent is not a true fungus or bacteria rather it is a group related to brown or red algae which rarely infest plants, and therefore the mechanism of disease development and resistance may be unique from the existing understanding of plant resistance to pathogens mainly bacteria and fungi.

8. Due to the complexity of the pathogen, it has been recommended that the development of resistance to this pathogen should be based on the 'durable-resistance' mechanism. One of the potato varieties developed at SCRI, namely 'Stirling' exhibits this phenomenon and came top in the recent global initiative on late blight (GILB) survey to study the 'genotype x environment' interactions. However, the biochemical and molecular mechanism of durable resistance is poorly understood because it is only recently (within the last 5 years) that this strategy was recommended to solve late blight disease by either a conventional breeding system or genetic modification.

### 1.7. Objectives of the Present Research

The specific objectives for this study were as follows:-

1. To investigate the effectiveness of lysozyme as a potential antipathogen factor in transgenic plants using Lys 75, a transgenic potato line carrying a chick-egg white lysozyme gene. In addition, transgenic potatoes carrying other naturally-occurring antipathogen factor, chitinase (BCH 35) and snowdrop lectin (GNA 74) genes were investigated.

2. To optimise and develop a sensitive assay for lysozyme in transgenic plants.

3. To develop a convenient technique *in vitro* to test transgenic plants for resistance to a range of pathogens including *Fusarium sulphureum*, *Rhizoctonia solani*, *Erwinia carotovora* subsp. *atroseptica* and *Phytophthora infestans*.

4. To study some biochemical changes in response to elicitation in cell suspension cultures using *P. infestans* elicitor preparations.

5. To construct a subtracted cDNA library representing mRNAs that are differentially expressed during elicitation in 'durable-type' interaction between 'Stirling' cell suspension cultures and elicitor mix of *P. infestans*.

6. To identify and characterise some of the selected cDNA closes.

7. To investigate the expression of genes corresponding to the selected, characterised cDNA clones.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1. Chemicals, Media and Biological Reagents

All general chemicals, unless otherwise stated, were obtained from Sigma Chemicals Co., St Louis, USA and BDH Chemicals Ltd, Poole, UK and were of analytical grade or the best grade available. The following materials were purchased from various sources.

Bacteriological Peptone (MC 24) was from Amersham, Bury, UK.

Chlorofrom:Isoamylalcohol (Ready Red™ 130321) and AquaPhenol™ (130181) were from Appligene, Gaithersburg, USA.

Commercial bleach, Clorox was from Safeway, Durham, UK.

Murashige and Skoog medium (26-100-24) from ICN Biomedicals, Inc., Ohio, USA.

Nutrient Agar (CM3), Nutrient Broth (CM4), Potato Dextrose Agar (CM139), Bacteriological Agar No. 1 (L11), Trypton (L42) and Yeast Extract (L21) were from Oxoid Ltd., Hampshire, UK.

Phytagel™ (P8169) and Agarose (A-6013) were from Sigma Chemical Co., St Louis, USA.

Silica fines were kindly provided by Dr B. Stanchev of the University of Durham, Department of Biological Sciences

Trizol™ Reagent (15596-026) was from Life Technologies, New York, USA.

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· See Glossary of Relevant Terms

### 2.1.2. Kits, Enzymes and Markers

Access RT-PCR System (Promega A1250) was purchased from Promega UK Ltd., Southampton, UK.

Advantage™ cDNA PCR Kit (K1905-y) and PCR-Select™ cDNA Subtraction Kit was purchased from Clontech Laboratories UK Ltd., Basingstoke, Hants, UK.

DNA and RNA ladder were from Life Technologies, New York, USA.

QuantumRNA™ 18S Internal Standard (Ambion 1718) was from Ams Biotechnology (Europe) Ltd., Oxfordshire, UK.

mRNA isolation kits (1741 985), and restriction endonucleases were supplied by Boehringer Mannheim UK Ltd., East Sussex, UK.

Random Primer Labelling (Rediprime™ 1633) was from Amersham International Plc., Buckinghamshire, UK.

RNase A (000354) was from Qiagen Ltd., West Sussex, UK.

Topo™ TA Cloning Kit (pCR®II-Topo Vector – 45-0640) was from Invitrogen BV, CH Groningen, The Netherlands.

### 2.1.3. Equipment and Laboratory Supplies

10-μm Polypropylene Mesh (6838000) and Laboratory Sealing Film were from Whatman International Ltd., Maidstone, UK.

25-mm Cellulose Acetate 0.22-μm Membrane Filter was from Oxoid Ltd., Hampshire, UK.

Benchtop Centrifuge, Avanti™ 30 was from Beckman Interments Inc., Palo Alto, USA.

Carbon Steel Surgical Blade 10a (0102) was from Swann-Morton® Ltd., Sheffield, UK.

Film Cassette, KodakX-Omatic™ with KodakX-Omatic™ Regular Intensifying Screens was from Eastman Kodak Co., Rochester, NY, USA.

Fixer and Developer were from Photosol Ltd., Essex, UK.

Hybridizer, Techne HB-ID was from Techne (Cambridge) Ltd., Cambridge, UK.

Medical X-Ray Film (100NIF 18 x 24 cm) was from Fuji Photo Film Co., Ltd., Tokyo, Japan.

Mixer, Vortex-Z Genie™ was supplied by S.H.Scientific, Northumberland, UK.

Nylon membrane, Hybond™-N was from Amersham International Plc., Buckinghamshire, UK.

PCR Amplification Machine, Hybaid™(Omn-E) was supplied by Life Sciences International UK Ltd., Hampshire, UK.

Polaroid Black and White Instant Film (8.5 x 10.8 cm) was from Fabriquĕ au Royaume-Uni par Polaroid (UK) Ltd., Hertfordshire, UK.

Rap PVC film, Clingorap™,was from Terinex Ltd., Bedford, UK.

UV Transilluminator (300 nm) was from San Gabriel, California, USA.

### 2.1.4. Bacterial and Fungal Potato Pathogens

Potato pathogens used were provided by Dr Helen Stewart of Scottish Crop Research Institute in Dundee, Scotland. The pathogens were *Phytophthora infestans* compatible strain (race 1, 3, 4, 7, 10, 11; designated as SC95 in this thesis) and incompatible strain (race 1, 2, 3, 4, 7; designated as IC2 in this thesis), *Fusarium sulphureum*, *Erwinia carotovora* subsp. *atroseptica* and *Rhizoctonia solani*.

### 2.1.5. Potato Materials

Non-transgenic and transgenic potato lines of the variety Desiree containing a chick lysozyme gene were kindly provided by Dr R.R.D.Croy of the Department of Biological Sciences, University of Durham. Tubers of Stirling and Bintje potato varieties were kindly provided by Dr Helen Stewart of Scottish Crop Research

Institute in Dundee, Scotland. Transgenic potato plantlets containing bean chitinase (BCH 35) and encoded GNA (GNA 74) genes were kindly provided by Dr A.Gatehouse of the Department of Biological Sciences, University of Durham. A complete list of the different potato varieties and lines are shown in Table 2 below.

**Table 2. List of Potato Materials Used in the Current Project.**

| Potato Tubers (Varieties – Lines) | Status | Source |
|---|---|---|
| Desiree - Lys 3, 7, 16, 25, 27, 41, 44, 47, 68, 70, 74, [a]75, 78, 84, 86, 93, 100, 130. | Transgenic – contain chick egg white lysozyme gene | Dr R. Croy, University of Durham. |
| [a]Desiree Control | Non-transgenic | Dr R. Croy, University of Durham. |
| [b]Desiree - BCH 35 | Transgenic – contains bean chitinase gene | Dr A. Gatehouse, University of Durham. |
| [a]Desiree - GNA 74 | Transgenic – contains Snowdrop lectin gene | Dr A. Gatehouse, University of Durham. |
| [a]Binje | Susceptible to *P. infestans* | Dr H. Stewart, SCRI, Scotland. |
| [a c]Stirling | Show durable resistance | Dr H. Stewart, SCRI, Scotland. |

[a]Initiated into plantlets by direct bud cultures.
[b]Supplied as a tissue cultured plantlet
[c]Initiated into cell suspension cultures

## 2.2. Methods

### 2.2.1 Plant Tissue Culture-Related Techniques

#### 2.2.1.1. Preparation of Tissue Culture Media

Throughout the whole project, Murashige and Skoog (MS) Medium from ICN was used for plant tissue culture. The medium was prepared according to the manufacturers recommendation with the addition of appropriate plant growth regulators as indicated. Suspension medium (SMS) for cell suspension cultures was supplemented with 2.0 mg $L^{-1}$ 2,4-D and 0.1 mg $L^{-1}$ kinetin and adjusted to pH 5.7 with 1 M NaOH. Callusing medium (CMS) for growing potato callus from leaf or tuber explants was prepared the same way except 0.8 % agar or 2 g $L^{-1}$ Phytagel was added as a solidifying agent. For the maintenance of axenic potato plant cultures MS medium without growth regulator

(OMS) and solidified with 2 g $L^{-1}$ Phytagel was used. Transgenic potato plant cultures were maintained on the same medium containing 150 μg $L^{-1}$ kanamycin (KOMS).

### 2.2.1.2. Leaf Explant Sterilisation

Leaf explants were cut into small segments of approximately 15 mm x 15 mm and then the surface was sterilised by gently shaking it in 70% (v/v) ethanol for 10 s, followed by 20% (v/v) of Clorox with addition of 1 to 2 drops of Tween 20, a wetting agent for 10 min. The explants were then extensively washed with sterile distilled water for at least 6 times to make sure no residual disinfectant remained. The sterile explants were then placed in a sterile petri dish containing a layer of sterile filter paper to absorb any remaining water before they were transferred onto the OMS medium.

### 2.2.1.3. Micropropagation of Potato Plantlets from Tuber Tissues

Micropropagation of potato from tuber was carried out using an organ culture (bud culture). A potato tuber containing several good buds ('eyes') was selected and washed thoroughly but carefully to avoid damaging the bud tissues. To further reduce the bacteria and fungal spores contaminants, the tuber was soaked in a beaker where tap water was running overnight. Then, the tuber was dried in open air before it was sliced at the bud area. The slice was about 2 to 3 cm in diameter and 1 cm thick at the middle. Each slice contained only one tuber bud and they were then surface-sterilised as mentioned for leaf explants in section 2.2.1.2. Following sterilisation, the tuber slices were trimmed into a square shape with approximately 1 cm width x 1 cm length x 0.75 cm height with the bud on the top. The bud was transferred into a jar containing OMS medium and sealed with parafilm. The jar was placed in a tissue culture growth room under a regime of 16h light/8h dark. Newly developed contaminant-free shoots were excised and transferred onto a fresh OMS medium to develop a potato plantlet. Plantlets were ready to be propagated when they produced 4 or 5 leaves.

### 2.2.1.4. Maintenance of Potato Plantlets

Plantlets were propagated using nodal cuttings. Prior to this, all leaves were removed and cut so that each cutting contained only one node. They were then transferred onto the fresh OMS medium. For transgenic potatoes, a KOMS medium was used. Depending on the size of the jar, 3 to 6 nodes were occupied in each jar. The jar was then sealed with parafilm and at the sealing area, a few holes were made to prevent

heavy condensation. All plantlets were kept in the tissue culture growth room under the same environment as described in section 2.2.1.3. This maintenance procedure was carried out every 4 weeks.

### 2.2.1.5. Callus Initiation and Maintenance

Calli were initiated from sterile young healthy leaves (2 first leaf of tissue culture-grown plantlets) of potato varieties Stirling. The leaves were chopped into small pieces (about 4 mm x 4 mm) and transferred onto CMS medium in petri dish. The plate was then incubated in the dark at room temperature (20 °C) to induce calli formation. Calli formed were isolated from the explant and transferred onto fresh CMS medium. They were maintained by subculturing fortnightly in CMS medium in petri dishes and incubated as before.

### 2.2.1.6. Induction and Maintenance of Potato Cell Suspension Cultures

Cell suspension cultures were initiated from a healthy, actively-growing, friable callus of Stirling. The calli were suspended in a 250-mL Erlenmeyer flask containing 10 volume of SMS medium relative to the initial weight of the callus. The flask was incubated at room temperature with continuous agitation at 110 rpm and under a regime of 16h light/8h dark. An observation was made everyday to spot possible contamination in the flask and to detect the development of cells. The newly formed suspension cultures which appeared within the first two weeks of incubation were separated from the remaining calli by filtering through a 450-μm sieve. The suspension was left to settle for about 5 to 10 min before three quarters of the medium was slowly discarded. A small portion of the cells was pipetted onto a microscope slide for microscopy observation. Then, the discarded medium was replaced with fresh SMS medium, and put back on a shaker run at a speed and condition described earlier in this section. The same procedure was repeated every week. The cells went through several passages before they were used for any experimental purposes. Two methods of determining cell growth in suspension cultures namely, direct microscopy examination to observe the presence of embryogenic cell clumps and cell growth plot for estimation of growth rate and doubling time were carried out. Healthy and fully developed cell suspension cultures after being filtered at 450 μm should have appeared under the microscope as clumps of 4 to 10 cells. In most cases, a clear small dark spot of nucleus can be clearly seen within the transparent cytoplasm.

The pattern of cell growth was made by plotting the data of settle cell volume (SCV) that was measured every two days after subculture for a period of 14 days. The SCV was done using a sterile 50-mL falcon tube. The suspension was poured into the tube and left to settle for about 10 min before the volume of the cell was recorded. Healthy cell suspension cultures were representing with sigmoid-type growth profile within the two weeks of incubation period.

The suspension was maintained by subculturing at least 1/10 volume of the cell in relation to the total volume of the medium weekly. Cell cultures in their early exponential growth phase (approximately 3d after subculturing) were used for all experiments.

### 2.2.2. Microbiological Methods

#### 2.2.2.1. Purification of *Phytophthora infestans* Cultures

Purification of contaminated *P. infestans* cultures is a difficult task because the pathogen is relatively weaker *in vitro* and grows slowly on Rye A (see section 2.2.2.2) agar, and therefore, can be easily overgrown by contaminants. All (most) cultures of *P. infestans* supplied showed evidence of contamination. Contaminated *P. infestans* cultures were purified either by plating the contaminated cultures onto *P. infestans* selective media or by direct inoculation of the pathogen onto a potato leaf, or usually by a combination of the two methods. The selective medium was Rye A comprising rifamycin, ampicillin and nystatin, and was recommended by Dr. Jenny P. Day of Dept. of Biological Sciences, University of Bangor, Wales. The medium was prepared by adding 1 mL of the antibiotic mixture (250 mg rifamycin, 200 mg ampicillin and 500 mg nystatin dissolved in 10 mL dimethyl sulphoxide and filter-sterilised) into 500 mL autoclaved Rye A medium (50 °C). Generally, the pathogen grew slowly on this selective media. The second method was a direct inoculation of the contaminated cultures onto a sterile potato plantlet growing in tissue culture or potato leaf. The potato cultivar Bintje was most suitable for this because Bintje does not posses any *R* gene and the *P. infestans* strains should be able to infect this variety regardless of their compatibility status. Diseased tissue was then excised from the leaf and transferred onto a selective medium, and observed daily to monitor its growth. New mycelial growth of the pathogen was then isolated as soon as they appeared onto a fresh Rye A plate and maintained as described in section 2.2.2.1.

### 2.2.2.2. Maintenance of Fungal and Bacterial Pathogens

*F. sulphureum* was grown on a Potato Dextrose Agar (PDA) and subcultured every week. *P. infestans* was cultured on a Rye A agar and subcultured every two weeks. The bacterial pathogen, *E. carotovora* subsp. *atroseptica* was streaked on a Nutrient Agar (NA) and subcultured every two weeks. All cultures were incubated at 15 $^{0}$C in the dark. The commercial media were prepared according to the recommendation of the manufacturers. Rye A agar was prepared based on the procedure of Caten and Jinks (1968). Briefly, sixty grams of rye grain was washed thoroughly and soaked in 500 mL distilled water for 36 h after which the supernatant was poured off and retained. To the remaining swollen grains, 400 mL distilled water was added. The grains were then macerated by heating at 50 °C in a water bath for about 3 h. The extract was then filtered and the sediment discarded. The original supernatant was added to the filtrate, together with 15 g agar and 20 g sucrose, and the whole was made up to one litre with distilled water. The medium was autoclaved at 115 kPa and 121 °C for 15 min. Table 3 shows the summary of the handling procedures of the pathogens used in this project.

**Table 3. Summary of Maintenance Procedures for Potato Pathogens.**

| Pathogen | Growth Medium | Maintenance | Storage Procedure |
|---|---|---|---|
| *E. carotovora* subsp. *atroseptica* | NA and/or NB | Subculture weekly on NA and incubate at 15°C in the dark. | Culture in NB containing 40% (v/v) final concentration of glycerol and kept at –80°C. |
| *P. infestans* | Rye A agar | Subculture fortnightly on Rye A agar and incubate at 15°C in the dark. | Culture on Rye A slope, overlaid with sterile mineral oil and kept at room temperature. |
| *F. sulphureum* | PDA | Subculture fortnightly on PDA and incubate at 15°C in the dark. | Culture on PDA slope, overlaid with sterile mineral oil and kept at room temperature. |
| *R. solani* | PDA | Subculture fortnightly on PDA and incubate at 15°C in the dark. | Culture on PDA slope, overlaid with sterile mineral oil and kept at room temperature. |

### 2.2.2.3. Preserving Viable Pathogens

Generally, preserving microorganisms involves a method which can slow down the metabolic activity and physical growth of the organism as well as to prevent the culture from dehydration due to long storage. The method used in this study was mineral oil preservation which is one of the reliable preserving techniques for fungi.

Pathogens were preserved in an appropriate medium as indicated in Table 3. The medium was prepared as a slant (30° to the horizontal) in 30-mL universal bottles. Small amount of fungal inoculum was inoculated onto the middle of the slant and incubated with the cap loose at 15 °C in the dark until the mycelial about to cover the whole surface of the slant. Sterile mineral oil (liquid paraffin with specific gravity 0.830-0.890) was then added to the cultures to make a layer of mineral oil of about 10 mm thick from the edge of the agar. The mineral oil was first autoclaved twice at 121 °C for 15 min before use. Applying too much mineral oil (i.e. > 10 mm) could cause the fungi to die due to insufficient oxygen while in contrast too little oil (< 10 mm) could cause evaporation if mycelial or agar is accidentally exposed. The bottles were then stored with their caps loose in appropriate racks at room temperature in the dark.

Subculturing preserved fungal cultures was done by removal of a small amount of mycelial on a mounted needle and draining away as much oil as possible. Then the fungus was inoculated onto fresh medium (see Table 3) and incubated at 15°C in the dark. During incubation, the plates were orientated at 30°C to the horizontal to allow any excess oil to drain down the slope so that the fungus can grow in the opposite direction. Newly grown cultures were then re-isolated from the edge of the colony to fresh medium.

*Erwinia carotovora* subsp. *atroseptica* was preserved in a nutrient broth containing 40% (v/v) final concentration of glycerol and kept at –80°C. After long storage, the bacteria were first inoculated onto a sterile leaf using the leaf-bridge bioassay to check and restore the pathogenicity of the pathogen. This step may not have been necessary but was used routinely as a precautionary step to ensure the pathogen was almost at its highest possible virulence.

### 2.2.2.4. Induction of Sporulation of Fungal Pathogens

The fungal pathogens used for spore production were *P. infestans* and *F. sulphureum*. In theory, any fungi can be induced to produce spores in vitro if they are left to grow for extended periods on a nutrient-containing plate without subculturing. Reproductive growth of *P. infestans* and *F. sulphureum* were induced by subculturing mycelial plugs onto plates of Rye A and PDA, respectively. The plates were incubated at 15 $^{0}$C in the dark until sporulated. Sporulation was determined by cutting a small portion (approximately 1 cm x 1 cm in size) of mycelial block of the cultures. Using a sterile 200-µL pipette tip, 150 µL distilled water was applied on the surface of the mycelial block by sucking up and down using the pipettor for several times. Then, a drop of the washing solution was observed under a microscope to detect the presence of either sporangia for *P. infestans* or conidia for *F. sulphureum*.

### 2.2.2.5. Spore Production and Germination Test

Sporangia, spore-fruiting bodies of *P. infestans* were induced in Rye A agar. Sporangia formed were harvested by washing the mycelia of four-week-old cultures with sterile distilled water. Following harvest, sporangial suspensions were placed in a refrigerator at 4°C for 60 minutes to liberate zoospores. The zoospores were counted using a haemocytometer and then an appropriate dilution was made to obtain a final concentration of 1000 zoospores mL$^{-1}$. Viability was confirmed by spreading 200 µL of a diluted spore suspension onto a Rye A agar. The plate was incubated at 15°C for 24 h to germinate the spores and the number of colonies counted and compared with the total spore plated.

The *F. sulphureum* spores were prepared by prolonged incubation of cultures on PDA plates at room temperature for four weeks. Sterile distilled water was added aseptically to the plate which was then placed on an orbital shaker running at 50 rpm for 15 min to liberate spores from the mycelia. The spores were counted as before and diluted to 1000 spores mL$^{-1}$. Viability was tested as described for *P. infestans*.

### 2.2.2.6. Preparation of Mycelial Plugs

Mycelial plugs were prepared from actively growing cultures which were estimated when the size of the colony reached about half of the plate. A sterile cork borer of 50-

mm diameter was used to make the plug at the advancing edges of the fungal colony which represented mycelium of the same age.

#### 2.2.2.7. Determination of Colony Forming Units in *Erwinia carotovora* subsp. *atroseptica*

The preparation of suspensions of the bacterial pathogen, *Erwinia carotovora* subsp. *atroseptica*, was carried out by growing the pathogen in nutrient broth for 12 h at 15°C. Colony forming units were determined by plating out a serial dilution of a stock culture. Stock culture was prepared by growing a single colony of *Erwinia carotovora* subsp. *atroseptica* in 100 µL of nutrient broth for 24h at 15°C. Aliquots of serial dilution (100 µL) were spread evenly over NA plate in triplicate. The plates were incubated at 15°C for 24-48 h for colony formation. Colonies formed from each plate were counted and averaged, and the number of cells in the original stock culture calculated.

### 2.2.3. Phytopathology Methods

#### 2.2.3.1. Maintaining Phytopathogenicity of Potato Pathogens

The pathogenicity of the potato pathogen was assessed from time to time by infecting a potato leaf (var Desiree) using the leaf-bridge bioassay technique (see section 2.2.3.2). This is to maintain the virulence of the pathogens throughout the experimental period. Disease symptoms and progress were monitored and compared with the previous data to check any changes in disease development especially after long storage. Tissue developing disease symptoms were excised and transferred onto a suitable medium (see Table 3) and incubated at 15°C in the dark. If there were no significant changes in disease development detected, the newly isolated cultures were used for experimental purposes and preserved for future use as described in section 2.2.2.6., otherwise the pathogen was re-infected on plant tissues as described earlier.

#### 2.2.3.2. The Development of the Leaf-Bridge Bioassay

The leaf-bridge bioassay method was developed in order to evaluate resistance in transgenic plants. Details of the technique were discussed in Chapter 4 (section 4.2.1). Briefly, detached potato leaf was inoculated with pathogen inocula at the tip and disease progress towards the other end of the leaf was monitored. A three compartment petri dish was used in which to place the leaves with two compartments

filled with OMS medium. The leaf was orientated so that the petiole immersed into the medium and the leaf tip lay down in the empty compartment (details in Chapter 4).

#### 2.2.3.3. Optimisation of Inoculum Density in the Leaf-Bridge Bioassay

In order to challenge the leaflets with an appropriate pathogen density, each pathogen preparation (spores, bacterial cultures) was diluted to give a series of different concentrations of inocula. The inocula were first counted either directly by counting using a haemocytometer for fungal spores or by colony forming units determination for *Erwinia carotovora* subsp. *atroseptica*. Each concentration of pathogen was then inoculated onto the leaflet in three replicates and disease development and severity were monitored and measured over the course of 7 to 14 days depending upon the pathogen used. Inoculated leaves were incubated at 15°C for disease development. Disease progress was monitored as described in Chapter 4 (section 4.2.8 and 4.2.9).

#### 2.2.3.4. Assessing Transgenic Potatoes Encoding Lysozyme, Chitinase and GNA

The transgenic plants assessed in this study were Lys 75 (containing lysozyme gene), BCH 35 (containing bean chitinase gene) and GNA 74 (containing snowdrop lectin gene). The control used was a non-transformed potatoes var. 'Desiree'. The plantlets of each transgenic and control were propagated by nodal cutting on OMS medium as described earlier (section 2.2.1.4). For each transgenic line, and the control, 25 plantlets were prepared in tissue culture jars (maximum of 2 plantlets in each jar). The plantlets were allowed to grow for 2 weeks under conditions described earlier (section 2.2.1.4), before they were ready for assessment. Three first leaves of identical size from the apical were harvested for each plantlet. The leaves were immediately transferred in a three compartments petri dish as described in section 4.2.1 (chapter 4). For each transgenic line, and the control 50 leaves were randomly placed in 25 petri dishes for use in resistance assessment with each petri dish containing two leaves. They were incubated in an incubator (16h light at 15°C) for 3 days. After 3 days, any unhealthy leaves were removed.

For each transgenic line and the control, 36 leaves from 18 petri dishes were inoculated with the pathogen inocula. Six petri dishes from each line (transgenic and control), were not inoculated and used as a negative control. The leaves were then incubated using the same conditions described earlier. Three time points were used to harvest the inoculated leaves. At each time, 12 leaves from 6 petri dishes were

harvested and disease symptoms were recorded before they were processed (section 2.2.2.4 though 2.2.2.7) to determine the magnitude of infection. In this experiment, all three transgenic plants were assessed together and therefore only one set of controls was necessary for each time point.

### 2.2.3.5. Determining the Magnitude of Infection

Theoretically, the pathogen movement *in planta* mimics the growth pattern of the pathogen *in vitro* (fungal mycelia spread evenly in all directions). The movement of the mycelia *in vitro* is linear provided no inhibitor is present in the area of the advancing mycelia. Because the inoculation site was located at the leaf tip, the pathogen moved toward the petiole. The progress of the mycelial growth in the leaf was rated using a 1 to 5 scale with 5 represented as the largest magnitude of infection. This was carried out by sampling leaf disc - 5 discs of 50 mm diameter leaf disc 3 mm apart across the midrib of the leaf. The 5 leaf discs produced were then incubated using a suitable medium (see Table 3) for pathogen growth. Each hole carried a point (from 1 to 5) with hole 1 (the nearest point from the inoculation site) scaled as 1, 2 for the next point until 5 for the last point as described in detail in Chapter 4 (section 4.2.1, 4.2.2 and 4.2.3). The data was recorded and subjected to statistical analysis.

### 2.2.3.6. Statistical Data Analysis

All data collected in the pathogen testing experiments (the means for the magnitude of infection) were analysed using a two-way analysis of variance (ANOVA) to show any significant differences in the experimental means. This was done by using a statistical computer program, Minitab version 12.2 (Minitab Inc). Details on the description of the statistical test used and the assumptions made are available in section 5.1.3 (Chapter 5).

### 2.2.3.7. Preparation of *P. infestans* Elicitors

Three types of elicitors were prepared from the *P. infestans* cultures. These preparations were i) culture filtrate ii) mycelium homogenate and iii) zoospore extract. Culture filtrate consisted of materials secreted into liquid Rye B medium in which *P. infestans* was cultured for four weeks. Four 100-mL Erlenmeyer flasks containing 25 mL liquid Rye B agar were inoculated with two mycelial plugs (0.5 cm in diameter) of *P. infestans.* They were incubated at 15 °C in a temperature-controlled orbital shaker run at 110 rpm for 4 weeks. The medium was separated from the mycelia by filtering

through a filter paper (Whatman No. 3). The filtrate (about 100 mL) was dialysed for 48 h against two changes of distilled water (2 litres) before it was concentrated by freeze drying. The concentrated material (about 4 mL) was then filter-sterilised using a 0.22-μm nitrocellulose membrane filter and stored at –20°C. The preparation was checked for elicitor activity as described in section 2.2.3.8 The remaining mycelia were rinsed thoroughly with distilled water and then dried on a filter paper (Whatman No. 3) in air. They were then frozen in liquid nitrogen and ground to a powder using mortar and pestle. The resulting powder was homogenised with 5 mL of phosphate buffer (100 mM, pH 7.0) before being centrifuged at 12, 800 x g for 15 min. The mycelium extract was filter-sterilised and stored at –20 °C for future use.

Zoospore extract was prepared from three-week-old cultures of *P. infestans* grown on Rye A plates as described in section 2.2.2.4. The *P. infestans* plates were thoroughly flooded with 5 mL sterile distilled water and gently shaken for 2 to 3 minutes. The resulting suspension of sporangia was then transferred to a 15-mL falcon tube and incubated at 4 °C for 2 h in order to liberate the zoospores. The zoospores were separated from the immature sporangia and mycelial debris by centrifuging in a 2-mL Eppendorf tube with an integral 10-μm filter. This allowed the zoospores to pass through but retained the debris. Aliquots of the zoospore preparations were observed under the microscope (x 100) and zoospores counted using a haemocytometer. Approximately, 2 x $10^{12}$ viable zoospores were used for the elicitor preparation. The zoospores were pelleted and then resuspended in 1.0 mL sterile phosphate buffer (100 mM, pH 7) using a sterile mortar and pestle. To ease the extraction a small amount of sterile acid-washed sand was added. The extract was then centrifuged at 12 800 x g for 10 min and the supernatant was stored at –20°C until required. All these crude elicitor preparations were subject to an elicitor activity test as described below (section 2.2.3.7).

### 2.2.3.8. Optimisation of Elicitation in Potato Cell Suspension Cultures

Phenylalanine-ammonia-lyase (PAL) enzyme activity was used as a general indicator for the presence of elicitor activity in the elicitor preparations described before (section 2.2.3.6). Elicitation was carried out using potato cell suspension cultures (var. Stirling). Serial dilutions (1:100, 1:1000, 1:10000) of each elicitor preparation were tested against the Stirling cell suspension cultures for induction of PAL activity. Four

time points (0.6.12 and 18 h) were used to monitor the increase in PAL activity following each treatment. Each of these samples were processed as described in section 2.2.4.1 and assay for the PAL activity (section 2.2.4.5). The highest dilution in each elicitor preparation that activated the PAL activity was used as a standard for treatment. An elicitor mix comprising of equal activities of the three elicitor preparations was prepared and the concentration optimised for elicitation of cell suspension culture as before.

### 2.2.4. Enzyme- and Protein-Related Techniques

#### 2.2.4.1. Extraction of Protein and Enzymes from Potato Leaves and Cell Suspension Cultures

Cells from 1 to 5 mL aliquots of suspension cultures were collected by filtration on a filter paper (Whatman No. 3) under gentle vacuum. They were then rinsed several times with cold (4 °C) distilled water and frozen immediately in liquid nitrogen until required. Leaf discs cut from potato leaves under study were washed and dried between two layers of filter paper (Whatman No. 3). Then they were frozen in a liquid nitrogen, and stored at $-80^0$C.

PAL enzyme preparation was made by homogenising the frozen material in 2 volumes of cold extraction buffer (4 °C) containing 0.1 mM EDTA, 5 mM mercaptoethanol and 50 mM Tris-HCl, pH 8.5. The sample was first powdered with liquid nitrogen using mortar and pestle before addition of the extraction buffer. The crude extract was then centrifuged at 12, 800 x g for 15 min at 4 °C. The supernatant was saved and assayed for enzyme activity immediately. The remaining enzyme preparation was frozen in liquid nitrogen and kept at –20 °C. Other enzymes preparation were extracted in the same way but using an extraction buffer containing 0.1 mM EDTA, 5 mM mercaptoethanol and 50 mM phosphate buffer, pH 7.5.

#### 2.2.4.2. Soluble Protein Determination

Bradford assay system (Bradford, 1976) was used to determine the amount of soluble protein present in the crude extract. Standard curves were constructed using bovine serum albumin (BSA) with protein amounts ranging from 0.0 to 100 μg $mL^{-1}$. Appropriate dilutions were made for each sample so that the protein amount in the sample when assayed was within the linear range of the standard curve.

### 2.2.4.3. Turbidimetric Assay of Lysozyme

The lysozyme activity in the crude extract was determined using an optimised turbidimetric assay. The assay mixture contains 900 mL suspension of *M. lysodeikticus* cell-walls (0.5 mg $mL^{-1}$) in 0.05 M sodium-acetate buffer pH 6.0 and 100 µL of crude extract of the sample. The reduction in absorbance was immediately monitored at a wavelength of 500 nm for 1 min following the addition of the extract. The substrate was pre-incubated at 35 $^0$C before the addition of enzyme to make sure that the temperature for the enzyme reaction was optimised from the start.

### 2.2.4.4. Colorimetric Assay of Lysozyme

The assay was based on a substrate, remazol brilliant violet 5R developed during this study and is discussed in more detail in section 3.2.4. Except the substrate, the assay mixture and condition was the same as for the turbidimetric assay. The concentration of the substrate was 5 mg $mL^{-1}$ in 0.05 M Sodium-acetate buffer pH 6.0. The assay was run for an hour and the colour developed was measured at 550 nm. The activity was expressed as µg lysozyme per hour per mg protein. Standard amounts of chick lysozyme was used to calibrate the assay.

### 2.2.4.5. Assay for Phenylalanine-Ammonia-lyase (PAL)

Treated and control cell suspension cultures were processed and extracted for crude PAL enzyme preparation as described in section 2.2.3.1. The crude enzyme extract (100 µL) was then incubated at 40°C with 900 µL 12.1 mM L-phenylalanine (prepared in 50 mM Tris-HCl, pH 8.5). A control assay was performed using 100 µL of crude extract incubated with 900µL 12.1 mM D-phenylalanine. The formation of cinnamic acid was monitored by sampling 100 µL of the enzyme mixture together with its corresponding control every 30 min, and the absorbance was measured at 290 nm. PAL activity was expressed as changes in absorbance at 290 nm $h^{-1}$ $mg^{-1}$ protein as follows:

$$\frac{(\Delta A_{290}\ L-\text{Phe}/60\,\text{min} \quad - \quad \Delta A_{290}\ D\text{-Phe}/60\,\text{min})}{ug\ \text{protein}}$$

### 2.2.4.6. Malate Dehydrogenase Enzyme Assay

Malate dehydrogenase activity was measured by incubating 25 µL intercellular fluid (section 2.2.4.8) with 50 µL of 200 µM oxaloacetate, 300 µL of 100 µM NADH and 600 µL of 50 mM Tris HCl, pH 7.7. The mixture was incubated at room temperature and the reduction in optical density was continuously measured at 340 nm for 1 min. The specific activity was calculated as the initial rate of the enzyme per mg of soluble protein.

### 2.2.4.7. Assay for $H_2O_2$

The accumulation of $H_2O_2$ was measured using the peroxide-mediated oxidation of $Fe^{2+}$, followed by the reaction of $Fe^{3+}$ with xylenol orange as described by Jiang et al. (1990). This method has been shown to be reproducible within 0.1 to 1 µM $H_2O_2$ concentration range by Marre *at al.* (1998). The reaction mixture (total volume of 1 mL) contained 100 µM xylenol orange, 250 µM ferrous sulphate, 100 µM sucrose, 25 mM $H_2SO_4$ and 500 µL sample in 1000 µL cuvette. The mixture was incubated at room temperature for 45 min and at the end of the reaction time, the formation $Fe3^{+}$-xylenol orange complex was measured at 560 nm.

### 2.2.4.8. Vacuum Infiltration: Preparation of Intercellular Fluid from Leaves of Mature Transgenic Potato Plants

Leaves from glasshouse-grown plants were cut into 1.5 x 1.5 cm strips and the leaf pieces infiltrated with PBS buffer at 4°C 3x for 30 seconds. The leaf pieces were taken out from the buffer, gently blotted dry, rolled and transferred to 0.5-mL Eppendorf tubes with a small hole in the base. The tubes were then placed into intact 1.5-mL Eppendorf tubes. This assembly was centrifuged at 805 x g, 4°C for 10 minutes. The supernatant recovered intercellular fluid was immediately frozen in liquid nitrogen and stored at -20°C until required.

### 2.2.4.9. Elicitation of 'Stirling' Cell Suspension Cultures

Stirling cell suspension cultures, grown to semi-log phase (approximately three days after subculturing to a fresh medium) were filtered aseptically through a 450-µm sieve to remove the large cell aggregates. The filtrate, comprising of small cell aggregates were left to settle for about 10 min and then the upper medium was removed slowly without disturbing the settled cells. Fresh sterile medium was then added to the cells and gently shaken. The medium was removed from the suspension as described earlier

and the cells resuspended again in fresh medium so that the final SCV in each treatment was 5 mL in a total of 50 mL of medium. The cells were then left to grow for three days before elicitation. Elicitation was initiated by adding a mix elicitor (diluted to 1000 times in final concentration) and incubated on an orbital shaker as described in section 2.2.1.6. The cell suspensions were sampled after 6, 12, 24 and 48 h after elicitation for protein analysis.

### 2.2.5. General Molecular Biology Methods

#### 2.2.5.1. Preparation of RNase-free Glassware and Plasticware

Glassware and metal items were dry-sterilised at 180°C for at least 4 h. Plasticware was either supplied pre-sterile or was soaked in water containing 0.1% (v/v) diethylpyrocarbonate (DEPC) at 37°C for 2 h to inactivate nucleases before autoclaving. Certain apparatus that could not be dry-sterilised was treated in the same way as the plasticware.

#### 2.2.5.2. Preparation of RNase-free Distilled Water and Reagents

RNase-free water were prepared by adding DEPC to a final concentration of 0.1% (v/v) to deionised water, left at 37°C for 2 h or overnight at room temperature and then autoclaved for 30 min to decompose residual DEPC. Whenever possible, solutions for RNA work were treated with 0.05% (v/v) DEPC in distilled water at 37°C for 2 h and autoclaved prior to use. For solutions which could not be treated with DEPC (i.e. those containing chemicals with amine groups) or autoclaved (i.e. volatile materials such as ammonium acetate), these were prepared in DEPC-treated distilled water and prepared from components as nuclease free as possible.

#### 2.2.5.3. Bacterial Culture Preparation and Storage

Bacterial cultures were prepared by inoculating 5 mL of Luria-Bertani (LB) broth with a single colony picked from a fresh agar plate or 10 µL of previously frozen stored cells of the appropriate strain. For plasmid-containing strains, the broth was supplemented with an appropriate antibiotic (eg. 100 µg $mL^{-1}$ ampicillin) depending on the antibiotic-resistant gene carried by the specific plasmid. Cultures were incubated at 37°C on an orbital shaker running at 160 rpm overnight. For growth on solid media, 15 g $L^{-1}$ agar was added to the LB medium with or without added antibiotics.

Bacterial colonies for regular use were stored on plates, inverted and sealed with parafilm at 4 °C for up to a month or two. For long-term storage, bacterial lawns grown from a single colony were transferred into a culture tube containing 2 mL aliquots of a solution of 40% (v/v) glycerol in LB broth, vortexed thoroughly and stored at –80°C.

**2.2.5.4. Isolation of Plasmid DNA**

Single colonies of bacteria containing the appropriate plasmid were cultured in 15-mL falcon tubes containing 5 mL LB broth and incubated at 37°C on an orbital shaker running at 160 rpm overnight. Plasmid DNA isolation was carried out using alkaline lysis method. Bacteria were harvested by centrifugation at 3000 x g for 5 min. The supernatant was poured off and the bacterial pellet was drained at room temperature by inverting the tube over paper towels for a few minutes.

The pellet was resuspended in 180 µL of ice-cold TGE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), mixed thoroughly and transferred into a 1.5-mL Eppendorf tube containing 20 µL lysozyme (10 mg $mL^{-1}$, freshly prepared in TGE solution). The suspension was mixed and incubated at room temperature for 5 min. Then, 400 µL freshly prepared solution containing 0.2 M NaOH and 1% (w/v) SDS was added. The suspension was mixed by inversion several times and kept on ice for 5 min. This was followed by the addition of 300 µL cold 3 M potassium acetate (mixture of 600 µL 5M potassium acetate, 115 µL glacial acetic acid and 285 µL $H_2O$) and mixed thoroughly by inversion and gentle vortexing. The tube was incubated on ice for 10 min and then centrifuged at room temperature for 10 min at 12, 000 x g. The supernatant was transferred into a new 1.5-mL Eppendorf tube and 0.5 mL (or equivalent to 0.6 volumes) of isopropanol was added and mixed thoroughly by inverting and vortexing. The DNA was then recovered by centrifuging at 12, 000 x g for 5 min at room temperature. The supernatant was poured off and the pellet was washed by slowly adding 0.5 mL 70% (v/v) ethanol. The ethanol was slowly discarded and the pellet was dried at room temperature by inverting over paper towels for 10 to 15 min. The DNA was resuspended in 100 µL 1x TE buffer. At this stage, the DNA was digestible by most restriction enzymes for a quick DNA analysis.

For analyses that require pure DNA or for DNA sequencing, the plasmid DNA was further purified from RNA and proteins. RNA was removed by adding 2 µL RNase A (10 mg mL-1) and incubating for 15 min at room temperature. Residual protein was removed by phenol extraction i.e. by vortexing the sample with 100 µL buffered phenol for 10 s. The DNA was recovered by centrifugation at 12, 000 x g at room temperature for 3 min where top aqueous phase containing the DNA was saved. The DNA was further purified from any remaining proteins, carbohydrates and phenol residues by chloroform extraction i.e. by mixing and vortexing with 100 µL chloroform:isoamyl alcohol (24:1) for 5 s. The DNA was recovered by centrifugation at 12, 000 x g for 1 min and the top aqueous phase was transferred into a new tube.The DNA was finally precipitated by adding and mixing with 15 µL 3M sodium acetate (pH 5.5), followed by 250 µL (equivalent to 2.5 volumes) 95 % (v/v) ethanol at –20 °C. The DNA was allowed to precipitate at –80°C for 30 min or overnight at –20°C. The DNA was pelleted by centrifugation at 12, 000 x g at room temperature for 15 min, then dried at room temperature by inverting the Eppendorf tube over paper towels for 15 min. The DNA was resuspended in 30 µL 1x TE buffer and store at –20°C or – 80 °C until required.

### 2.2.5.5. DNA Agarose Gel Electrophoresis

Electrophoresis of DNA was carried out in horizontal agarose gels submerged in 1x Tris-acetate with EDTA (TAE) running buffer (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA). Gels of the appropriate concentration were prepared according to Sambrook et al. (1989). Ethidium bromide to a final concentration of 1.0 µg $mL^{-1}$ was added to both the gel and the tank buffer. DNA samples were prepared by mixing with 0.3 volumes of agarose beads (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 30% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, 0.2% (w/v) agarose – autoclaved, then extruded through a syringe and fine needle when set). The samples with an appropriate DNA marker were loaded onto the gel and electrophoresed at 5 V $cm^{-1}$ until the bromophenol blue dye migrated two-thirds the length of the gel. DNA bands were detected by the orange fluorescence of the EtBr DNA complex under UV light illumination at 254 nm wavelength. Gels were photographed through a red-orange filter (Kodak 23A Wratten) and Polaroid Type 667 (3000 ASA) film or images captured using Gel Doc 1000 system (Bio-Rad )

### 2.2.5.6. Isolation of DNA from Agarose Gels

DNA fragments were isolated from agarose gels using silica fines to bind DNA. The DNA band of interest was excised from the gel using a scalpel which was flamed prior to use to avoid any nucleases contamination, and put in a 1.5-mL Eppendorf tube containing 1 mL of gel solubilising buffer (90.8% w/v sodium iodide solution, saturated with sodium sulphite). The mixture was incubated at 70°C for 10 min or until the gels pieces had completely dissolved. After cooling, 10 µL of a 50% (v/v) suspension of silica fines was added and the tube was gently shaken for 10 min. The bound DNA was recovered by briefly centrifuging the tube and the supernatant was discarded. The pellet was washed twice with 0.5 mL of 70% (v/v) ethanol and the DNA was recovered by a brief centrifugation. The pellet was air dried for 15 min and then resuspended in 15 µL sterile distilled water. The suspension was incubated at 37°C for 45 min to release the bound DNA from the silica and then spun to recover the DNA solution.

### 2.2.5.7. Southern Transfer of DNA from Gel to a Nylon Media

Gels containing DNA were blotted onto hybond™-N (Amersham Life Science RPN 203N) nylon filter by capillary blotting as described by Sambrook *et al.* (1989). Prior to blotting, the DNA in the agarose gel was denatured by gently shaking in denaturing solution (1.5 M NaCl, 0.5 M NaOH, 1 mM EDTA) twice for 15 min. The gel was neutralised twice for 15 min in neutralising solution (3 M NaCl, 0.5 M Tris-HCl pH 7, 1 mM EDTA) followed by equilibration in 10x SSC (3 M NaCl, 0.3 M Sodium citrate adjusted to pH 7 with HCl) for 15 min. The gels were placed on the apparatus as described by Sambrook *et al.* (1989) except nappy liners were used instead of paper towels. Blotting was allowed to proceed at room temperature for 15-18 h with 10x SSC as a transfer buffer. After blotting, the position of the wells on the membrane was marked with a pencil. The membrane filter was then air dried for 15 min and then immobilised by UV cross-linking for 30 s. The blotted gel was checked under the UV light to ensure DNA transfer was complete. The membrane filter was wrapped in aluminium foil and stored at 4°C until the hybridisation was performed.

### 2.2.5.8. Analysis of DNA by Southern Hybridisation

The membrane filters containing blotted DNA were placed in a hybridisation container (Techne) containing preheated (65°C) prehybridisation solution (5x SSC, 5x

Denhardt's solution, 100-200 μg $mL^{-1}$ herring sperm DNA). The prehybridisation was allowed to proceed for at least 2 h in the hybridisation oven (Techne) at 65°C. This solution was then replaced by hybridisation solution (5x SSC, 2x Denhardt's solution, 100 μg $mL^{-1}$ herring sperm DNA plus labelled probe) and hybridisation was carried out overnight or longer at 65°C. The hybridisation solution was then removed and the membrane filters were washed once for 10 min with 2x SSC, 0.1% (w/v) SDS at 65°C and twice for 10 min each with 0.2x SSC, 0.1% (w/v) SDS. The membrane filters were removed, wrapped in a wrap plastic and then audioradiographed.

#### 2.2.5.9. Audioradiography

Audioradiography was carried out to trace $^{32}P$-labelled probe on the membrane filters. Filters probed with $^{32}P$-labelled DNA were placed in an exposure cassette. In a dark room with a red-safe light on, a pre-flashed X-ray film (Fuji RX) was placed between the filter and an intensifying screen within the cassette and audioradiographed at –80°C. The film was developed in Kodak X-Omat developer at room temperature for 2 min, washed with water thoroughly, and then fixed with Kodak fixer for 2 min before again thoroughly washed with water. The film was air-dried at room temperature.

### 2.2.6. Preparation of Subtracted cDNA library of Elicited 'Stirling' Cell Suspension Cultures

#### 2.2.6.1. Strategy for Construction of a Subtracted cDNA Library

The cDNA library of elicited Stirling cell suspension cultures was constructed using a technique called suppression subtractive hybridisation (SSH) described by Diatchenko *et al.* (1996). The method was based on the two vital steps i.e. i) suppression PCR to selectively amplify the target, differentially-expressed cDNA fragments and ii) subtraction hybridisation to enrich the low abundance cDNA fragments by normalising the more abundant cDNAs (see Fig. 2 for the flow chart of various steps involved in the technique). During the hybridisation process, the low abundance differentially-expressed cDNA fragments are enriched more than 1000-fold (Diatchenko *et al.*, 1996).

In the current study, a Clontech PCR-Select cDNA Subtraction kit (K 1804-1) based on this technique was used. All procedures and instructions supplied in the Clontech manual were followed unless otherwise stated. The PCR-select technique is complex

and the reader is directed to the Clontech wwwsite (http://www.clonetech.com) and the original paper about the method by Diatchenko *et al.* (1996) for full details. Only a brief description of the technique as adopted to the present study is provided here. The strategy for constructing the subtracted cDNA library is shown in Figure 4 below.

The whole procedure started from the preparation of poly (A) RNA from two different tissues under comparison i.e. elicited cell suspension cultures and the corresponding non-elicited control cells. Following the procedure described by Diatchenko *et al.* (1996), the elicited cell material was designated as the `tester' and the control tissue material as the `driver' (Figure 4). Preparation of intact RNA is vital in this technique as degraded RNA fragments present in either of the two preparations may wrongly be amplified during the enrichment process and therefore be present in abundance in the subtracted cDNA library leading to a false positive clone.

### 2.2.6.2. Preparation of Elicited 'Stirling' Cell Suspension Cultures for cDNA library

Two 250-mL Erlenmeyer flasks containing 50 mL filtered (450 µm) and newly subcultured cell suspension cultures were prepared as described in section 2.2.1.6 and incubated to mid-log growth phase. One set was elicited with the prepared mixed elicitor of *P. infestans* (section 2.2.3.8) at a final dilution of 1000 times (designated as tester cells) and the other one was added with 100 µL of distilled water (driver cells). The cell suspension cultures were then incubated for 6 h on an orbital shaker under the same conditions described earlier (section 2.2.1.6). The cells were collected by filtering through sterile filter paper (Whatman No. 3) under vacuum for about 30 second. During filtration the cell aggregates were rinsed with DCPC-treated distilled water several times. Semi-dry cell aggregates of both tester and driver were immediately frozen in liquid nitrogen and stored at –80°C until required.

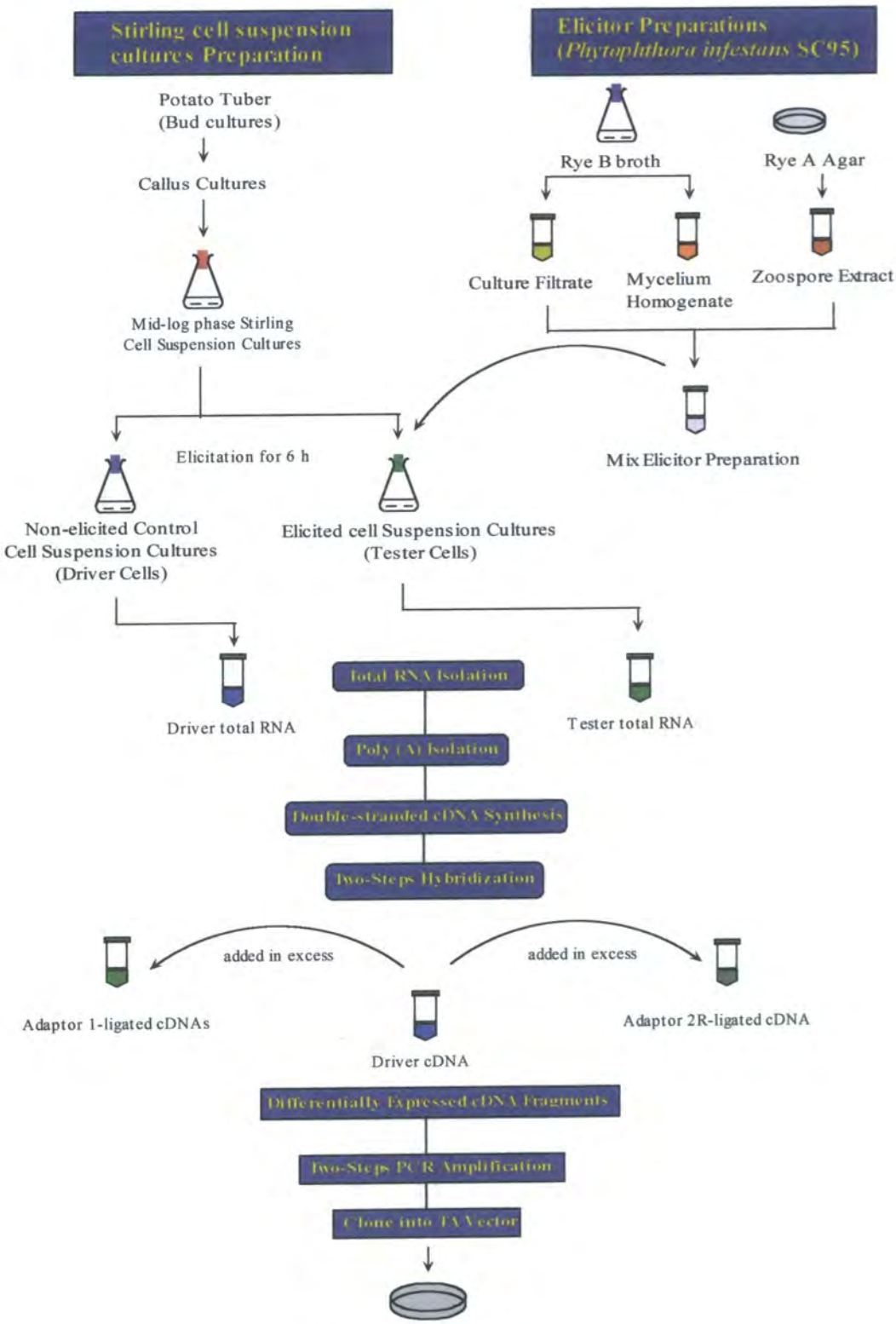


**Figure 4. Flow Chart of Steps and Strategy Designed to Construct the Subtracted cDNA Library from Elicited 'Stirling' Cell Suspension Cultures.**

### 2.2.6.3. Isolation of Total RNA from Potato Cells Grown in Suspension Culture

Total RNA was isolated using Trizol reagent (GIBCO BRL), a mono-phasic solution of phenol and guanidine isothiocyanate. The method used was based on the single-step RNA isolation developed by Chomezynski and Sacchi (1987). Frozen samples (1 g) of tester and driver were homogenised separately in 10 mL Trizol reagent according to the manufacturers instructions. During sample homogenisation, Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Prior to the addition of chloroform, the 10 mL cell homogenate was aliquotted (1 mL) into 10 Eppendorf tubes. Addition of chloroform followed by centrifugation, separates the solution into an aqueous and organic phase. RNA remains exclusively in the aqueous phase. After recovery of the aqueous phase, the RNA was recovered by precipitation with isopropanol. The isolated RNA samples were then checked for purity and integrity (section 2.2.6.3). Pure and intact total RNA samples of tester and driver were stored in DEPC-treated distilled water at – 80°C until used.

### 2.2.6.4 Verification of RNA Quality

The integrity of total RNA was checked by running on a 1.2 % (w/v) agarose gel alongside the corresponding RNA ladder. The size of distinct 18S and 28S RNAs are approximately 2000 and 4500 bases, respectively. The purity was checked by spectrophotometer at A260 and A280. The desired ratio of absorbances (A260/A280) should be 2.0 $\pm$ 0.05. In addition, the sample was also scanned between 240 and 320 nm to produce a characteristic clean "sigmoid like" curve which indicates the highest purity.

### 2.2.6.5. Quantitation of RNA Preparation

RNA samples were appropriately diluted with sterile DEPC-treated distilled water and the absorbance measured at 260nm using an acid-washed quartz cuvette. RNA concentration was calculated using the following formula:

$$\text{RNA } \mu g\ mL^{-1} = A_{260} \times 40 \times DF$$

Where A = absorbance at 260nm; 40 = extinction coefficient of RNA; DF = dilution factor.

### 2.2.6.6. Purification of Poly ($A^+$) RNA

Poly ($A^+$) RNA of tester and driver was prepared from 500 μg total RNA of corresponding cell aggregates using mRNA Isolation Kits (Boehringer) according to the manufacturer's instructions. The system relies on base-pairing of the poly (A) residues at the 3'ends of the mRNAs and a biotin-labelled oligo$(dT)_{20}$ probe which can be captured (immobilised) on streptavidin magnetic particles using a magnetic separator (Pharmacia). Non-polyadenylated RNA species (rRNAs and tRNAs) were not bound and easily washed off. The purity of the isolated poly (A) samples were electrophoresed on 1.2 % (w/v) gel to check any traces of the two major ribosomal RNAs, 18S and 25S. The concentration was determined in the same way as for the total RNA as described in section 2.2.6.4.

### 2.2.6.7. Concentrating Poly ($A^+$) RNA

In order to suit the initial concentration of poly (A) required to synthesis cDNA strands in the Clontech PCR Select kit, the two total RNA preparations (tester and driver) were concentrated to 2 μg $\mu L^{-1}$ using ethanol precipitation. To a sample of poly ($A^+$) RNA, 0.1 volume of 2.5 M sodium acetate (pH 5.2) was added and thoroughly mixed. This was followed with the addition of 2.5 volume of 95% (v/v) ethanol. The mixture was vortexed, pulse-spun and incubated at – 20°C overnight. The resulting precipitate of RNA was spun at 12,0000 x g for 30 min at 4°C, followed with 2x washing with 80% (v/v) ethanol. After each washing, the precipitate was collected by centrifuging at 12,000 x g for 15 min at 4°C. The pellet was air dried for 5 min and then dissolved in a small volume of sterile DEPC-treated distilled water so that the final concentration was 2 μg $\mu L^{-1}$. The concentrated poly (A) samples were immediately frozen in liquid nitrogen and stored at –80°C until required.

### 2.2.6.8 First- and Second-Strand cDNA Synthesis

First- and second strand cDNA synthesis was performed with each experimental tester and driver poly $A^+$ RNA according to the Clontech PCR kit instruction manual. Initial concentration of tester and driver poly $A^+$ RNA used was 2.0 μg $\mu L^{-1}$ and these were reverse transcribed using AMV reverse transcriptase enzyme supplied with the kit. The resulting double stranded cDNA (ds cDNA) were electrophoresed to estimate size range of ds cDNA products synthesised.

### 2.2.6.9 *Rsa* I Digestion and Adapter Ligation

The first step in the SSH technique is to generate restriction fragments in both tester and driver cDNA using a four-based cutting restriction enzyme, *Rsa* I (GTAC). This is possible because the cDNA synthesis primer was designed to have a single site of *R*sa I and therefore, the ds cDNA produced for both tester and driver contained multiple *Rsa* I sites. The blunt end *Rsa* I-digested tester cDNA fragments are to ligate to the ends of specifically designed adapters (adapter 1 and 2B) which are contained in the *Rsa* I site at one end. The driver cDNA fragment on the other hand was used in the hybridisation process that was described in more detailed in section 2.2.6.11 below.

### 2.2.6.10. Preparation of Adapter-Ligated Experimental Tester cDNA

Clontech PCR Select subtraction kit provides two unique ds adapters (adapter 1 and adapter 2R – see Figure 5) to be used in the ligation process with only *Rsa* I-digested tester cDNA. These adapters were designed to have three regions corresponding to a PCR primer 1 at the 5'-sticky end, nested PCR primer 2 and the *Rsa* I- specific end at the 3'-blunt end. The adapter 2R is identical except that the 3'-blunt end region sequence corresponding to the nested PCR primer 2R. Both ends of the adapter sequences (adapter 1 and 2R) do not have a phosphate group at the 5' end, so only the longer strand of each adapter is covalently attached to the 5'-ends of the cDNA fragments.

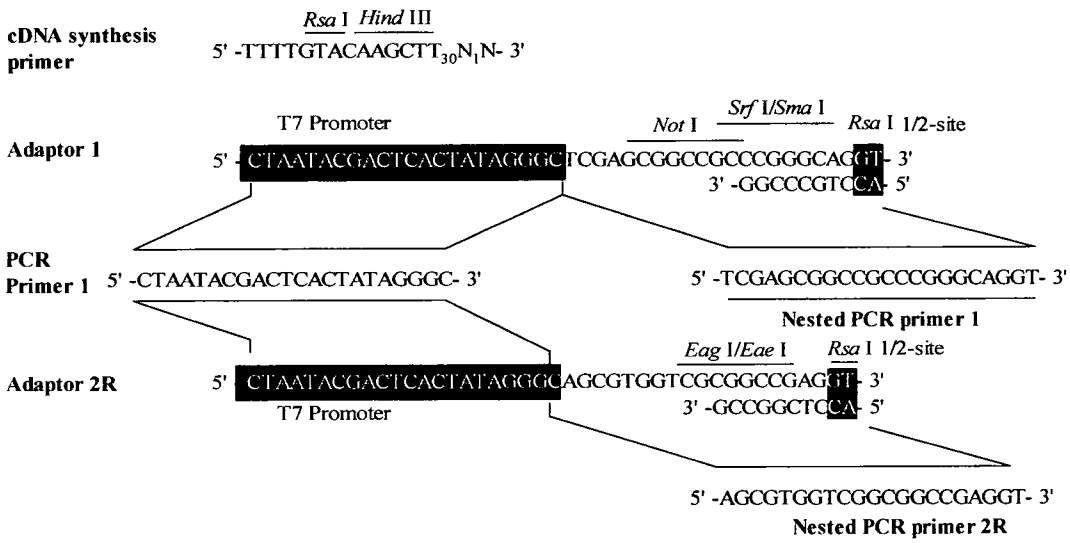


**Figure 5. Primers and Adapters Used in the Clontech PCR-Select Protocol** (Adapted from Clontech Manual).

Two populations of adapter-ligated tester cDNA fragments corresponding to adapter 1 and adapter 2R were prepared at this stage as instructed in the Clontech PCR Select manual. The idea of creating two populations of cDNA fragments with different adapters is to serve as templates for the two steps hybridisation which are responsible in enrichment of low abundance and normalising the high abundance cDNA fragments, and selective PCR amplification at the later stage of the protocol (Figure 6).

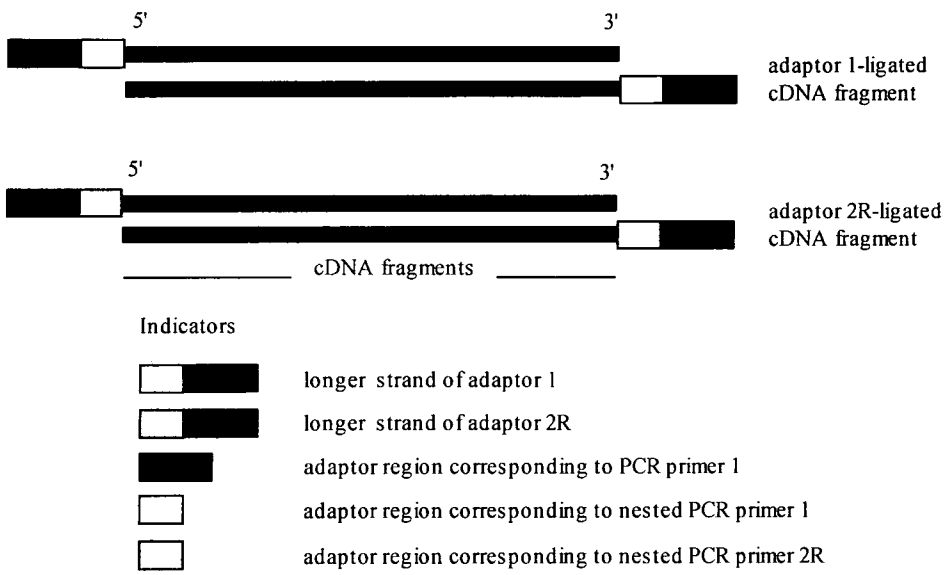


**Figure 6. Adapter-Ligated Tester cDNA Fragments Formed after the Ligation Process.**

### 2.2.6.11 Subtractive Hybridisation

The first hybridisation was carried out separately for the two adapter-ligated tester cDNA fragments prepared earlier (section 2.2.6.9). An excess of the driver cDNA fragments was added to each of the adapter-ligated tester cDNA fragments and incubated for 8 h under conditions described in the Clontech manual. This step should not exceed 12 h to avoid all of the ss adapter-ligated tester cDNA molecules hybridised with one another and leaving none for the second hybridisation. On the other hand, if the incubation time is too short, the ss tester fraction may not be completely equalised, and rare, differentially expressed genes will be lost or undetectable. During the incubation, four possible types of molecules were produced as shown in Figure 7 below.

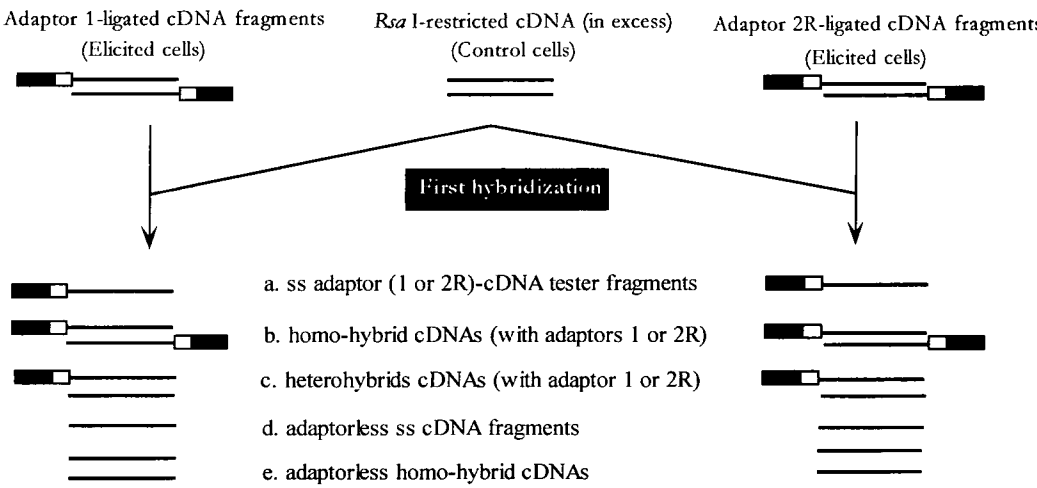


**Figure 7. The Possible Molecules Produced During the First Hybridisation in Clontech PCR Select Protocol.** The driver cDNA fragments were supplied in excess to enrich the low abundant ss cDNA fragments (type a). During hybridisation the low abundant ss tester cDNA fragments was 'normalised' i.e. the concentration of low and high abundance cDNAs becomes approximately similar. This occurs because the rate of reannealing process to generate homo-hybrid cDNAs (type b) is higher for the more abundant molecules (Diatchenko *et al.*, 1996). The non-target common cDNAs formed heterohybrid (type c) with the driver cDNA (Adapted from Clontech Manual).

In the second hybridisation, the two samples from the first hybridisation (see Figure 8) were quickly mixed together and fresh denatured driver cDNA was added to further enrich the fraction from differentially expressed genes. The low-abundance, differentially expressed ss cDNAs (type a molecules) anneal to form the only ds cDNA molecules with the two adapters at their 5'-ends derived from adapter 1-ligated cDNA and adapter 2R-ligated cDNA fragments. This feature is useful in the next step, pre-amplification using PCR as the primers used (nested primer 1 and nested primer 2R) correspond to the outer part of the two adapters 1 and 2R, respectively (Figure 8). The second hybridisation was carried out for 16 h to allow the low abundance, differentially expressed ss cDNA molecules to anneal.

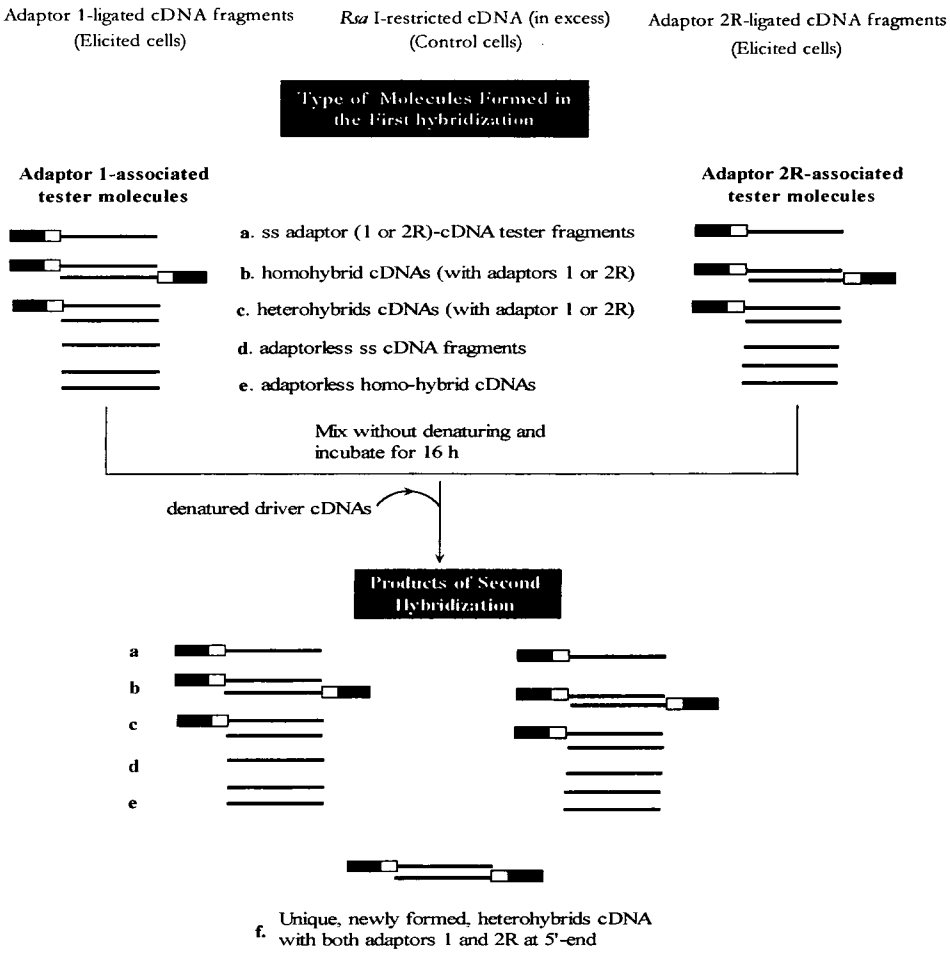


**Figure 8. The Products of the Second Hybridisation Process.** Newly formed heterohybrid comprising region for both adapters 1 and 2R. (corresponding to PCR primer 1, nested primer 1 and 2R). This becomes a template for PCR amplification which will enrich the molecule further. (adapted from Clontech manual)

### 2.2.6.12. PCR Amplification

Before proceeding with the PCR, the sticky ends of the molecules were filled in using DNA polymerase (Figure 9). In the first round of PCR, only ds cDNAs with two different annealing sites were exponentially amplified. The specific differentially-expressed molecules were further enriched and the background was largely reduced in the second PCR where nested primer was used. PCR products were electrophoresed on 1.4% (w/v) agarose gel.

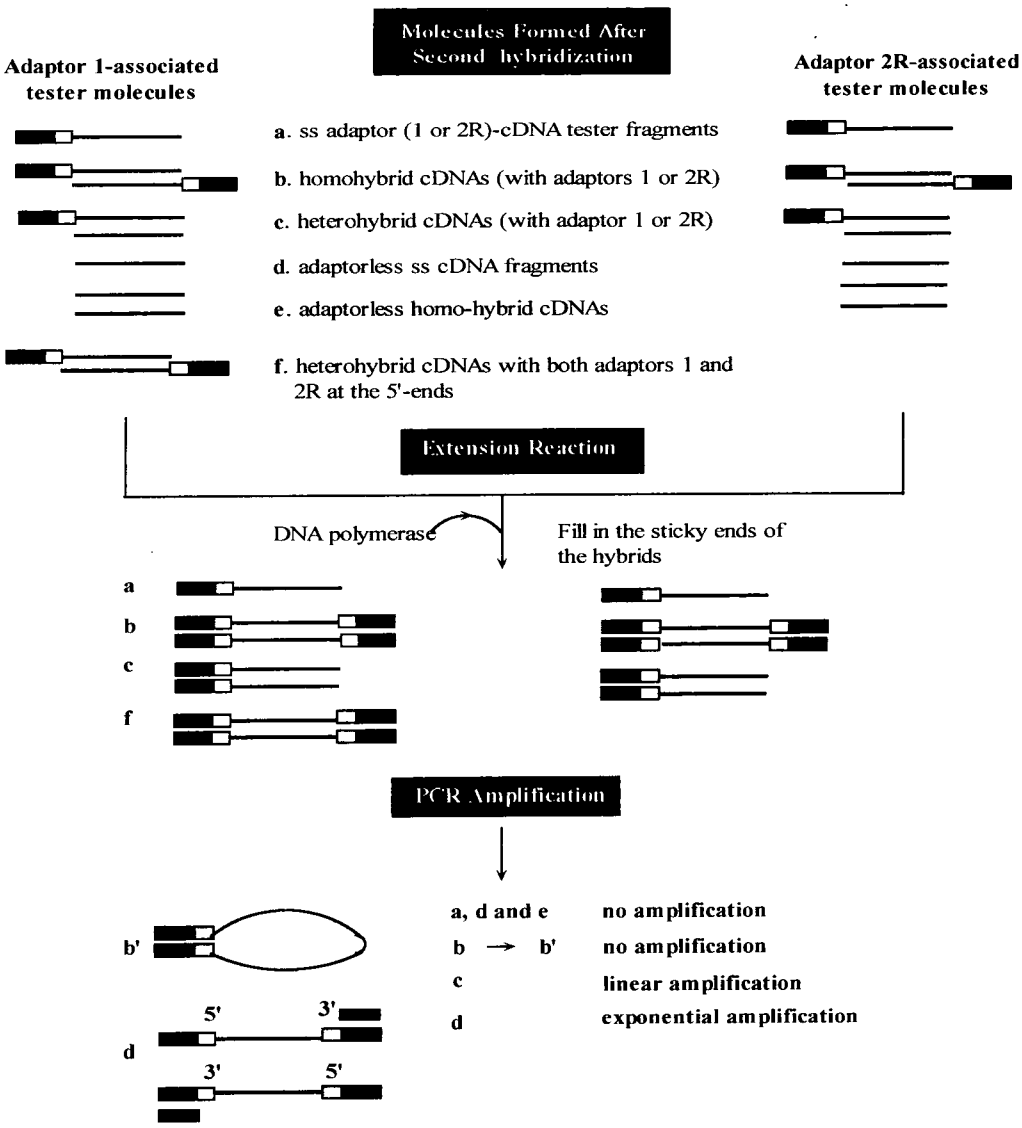


**Figure 9. Extension Reaction and PCR Amplification of the Products of the Second Hybridisation.** Prior to PCR, the sticky ends of the hybrids were filled by the activity of DNA polymerase to generate template for PCR amplification. All type a, d and e molecules cannot be amplified lack of primer annealing sites. Most type b molecules form a "pan-like structure" and thus cannot be amplified. Type c molecules have only one primer annealing site and can only be amplified linearly. The only molecules that can be amplified exponentially is type f because both of the primers annealing sites are present.

### 2.2.6.13 Cloning of PCR products

Products from the secondary PCR were inserted directly into pCR 2.1 using a TOPO TA Cloning® kit (Invitrogen K4500-01) following the manufacturers instructions. Using this kit, the Taq polymerase-amplified PCR products were directly inserted into

a linearised plasmid vector (pCR 2.1-TOPO) with a single overhanging 3' thymidine (T) residues. This allows PCR inserts containing a single 3-deoxyadenosine (A) due to terminal transferase activity within the Taq polymerase, to ligate efficiently with the vector.The TOPO-cloning reaction was transformed into One Shot™ Competent Cells supplied in the kit according to the instructions. Transformed cells were plated out on LB medium covered with a layer of X-gal (40 µL of 40 mg $mL^{-1}$ 50, spread evenly on the surface of the medium) and containing 50 µg mL ampicillin. The plate was then incubated overnight at 37 °C. Selection for transformants was carried out by blue/white colour selection screening. Ampicillin resistant transformants were transferred, using sterile toothpicks onto a new LB plate containing ampicillin and X-gal as described earlier.

### 2.2.6.14. Screening of Differentially-Expressed Clones

Selected clones (40) from the subtracted cDNA library were hybridised with DNA probe prepared using the product of second PCR (nested PCR) in the PCR Select™ protocols. The PCR products contain most of the subtracted cDNA fragments and also some common fragments for both the tester and driver cDNAs. The probe was prepared using a random priming method as described in section 2.2.6.15 below. Hybridisation and related methods were undertaken using the earlier mentioned procedures (section 2.2.5.6 to 2.2.5.9).

### 2.2.6.15. Random Primed Labelling of cDNA Fragments

Subtracted cDNA fragments (5 µL) were restricted with *Rsa* I for 30 min at 37°C then followed with denaturation of enzyme at 65°C for 10 min. The *Rsa* I-restricted cDNA fragments (20 µL) were then cut with *Ega* III to remove the vector sequence by incubating for 1 h at 37°C. The restricted fragments were then precipitated with 96 % (v/v) ethanol and then diluted to a final volume of 45 µL. The cDNA was denatured at 98°C for 5 min, centrifuged briefly before being added to the labelling mix prepared from the Rediprime™ labelling kit (Amersham International Plc.). The next steps followed the exact recommended procedure as in the Rediprime™ labelling kit.

### 2.2.6.16. Screening for Ribosomal RNA Clones

The subtracted cDNA fragments were screened for rRNA cDNAs using a pea genomic fragment containing a ribosomal repeat unit. The clone, pHA 1 (Figure 10) was kindly provided by Dr R. Croy, Department of Biological Sciences, University of Durham.

The clone was grown in LB medium containing 30 μg $mL^{-1}$ chloramphenicol overnight and the plasmid (pACYC 184) DNA was isolated using the method described in section 2.2.5.4. The DNA was then digested with *Hind* III and then electrophoresed on agarose gel (section 2.2.5.5). The band corresponding to the ribosomal repeat unit was cut and the DNA isolated (section 2.2.5.6), labelled (section 2.2.6.15) and used as a probe.

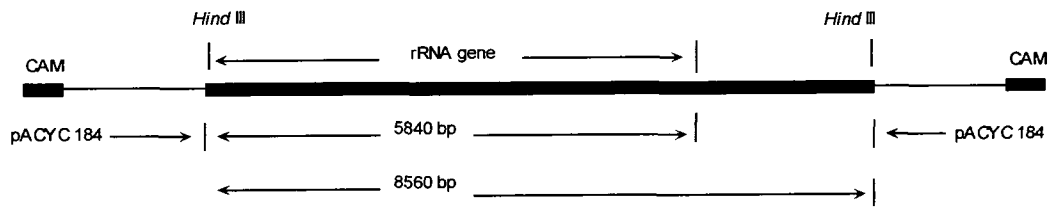


**Figure 10. Map of the pHA 1 Construct.** It is a pea (*P. sativum* cv Alaska) genomic clone containing 8.56 kb genomic fragment which include 5.84 kb of a ribosomal repeat unit (28S and 18S). The plasmid vector is pACYC184 which carries a chloramphenicol-resistance gene (CAM). Clone originally produced by Dr. R. Cuellar, Ph.D. thesis, Stanford University, 1982.

The DNA blots from the previous southern hybridisation experiment (section 2.2.6.14) were used with some additional blots for other cDNA fragments. The hybridisation was carried out in the same way as described earlier (section 2.2.5.8).

### 2.2.6.17. DNA Sequencing and Sequence Handling

Sequence determination was carried out using an automated Applied Biosystems Model 373 sequencer (Stretch Version) by the DNA Sequencing Service, Biological Sciences, University of Durham. DNA sequences were edited for removal of any primer or vector sequences associated with the sequence using EditSeq program from DNAStar suite (Lasergene). DNA sequence comparisons with those in the primary databases, Genbank at National Centre for Biotechnology Information (NCBI), Maryland, USA and EMBL at European Bioinformatics Institute, Cambridge, England were carried out using their corresponding search engines BLAST 2 (http://www.ncbi.nlm.nih.gov/BLAST) and FASTA 3 (http://www2.ebi.ac.uk/fasta3), respectively. Predicted protein sequences were compared with the Swissprot database

using the FASTA 3 programs available through the European Bioinformatics Institute, Cambridge, UK.

Protein patterns were scanned with the PROSITE database at the University of Geneva and conserved regions were searched using BLOCKS database at the Fred Hutchinson Cancer Research Centre, Seattle, USA. Transmembrane protein was predicted using the SOSUI predicted method available online at SOSUI WWW Server (http://www.tuat.ac.jp/~mitaku/adv_sosui/). Hydropathy protein profiles of the predicted protein sequence were plot using PROTEAN program from DNAStar suite. Multiple alignment of DNA and protein sequences was carried out using the Clustal W method (Thompson *et al.,* 1994) from the demo version of PROMEGA program (Oxford Molecular, Cambridge, UK). The potential coding sequence was determined based on the Starts Stops Codon method and Borodovsky Coding Prediction technique using the GENEQUEST program from DNAStar suite.

### 2.2.6.18 Confirmation of Some Differentially-Expressed Clones using RT-PCR

#### 2.2.6.18.1. Preparation and Treatment of Total RNA

Stirling cell suspension cultures were elicited using mix elicitor of *p. infestans* (section 2.2.6.2) and sampled at 6, 12 and 18h after elicitation. Total RNA from elicited potato cell samples was isolated using the method described in section 2.2.6.3. The preparation was then treated with 10 U of DNase I in a final concentration and incubated for 15 min at 37°C. This was then followed by addition of EDTA to 2.5 mM and incubation at 70°C for 15 min to inactivate the enzyme. Total RNA was then precipitated using the sodium acetate/ethanol precipitation as mentioned in section 2.2.6.7. RNA was quantified (section 2.2.6.5) and checked for integrity (section 2.2.6.4). RNA samples were frozen in liquid nitrogen and stored at –80°C until required.

#### 2.2.6.18.2. cDNA Synthesis

All cDNA synthesis reactions were carried out with M-MLV reverse transcriptase (Promega M1701). RT reaction for all RNA samples were assembled on ice in a thin wall PCR tube comprising 2.5 µg total RNA, 1 µL dNTP mix (10mM each dNTP), 200 ng random primer (decamers) and nuclease-free $dH_2O$ up to final volume of 15 uL. The RT mixtures were then heated at 70°C for 5 min to denature the RNA and chilled in ice immediately. To these RT mixtures, the remaining RT components (4 µL

5X RT-PCR buffer, 200 U M-MLV reverse transcriptase) were added, mixed gently and spun briefly. The reverse transcription was carried out at 42°C for 1h.

### 2.2.6.18.3. Determination of the Optimum Cycle for RT-PCR

In PCR, reaction products accumulate over a cycle at a rate dependent on the amplification efficiency. The relationship between reaction products and cycle numbers is linear only for certain numbers of cycles when the efficiency is at its maximum level. In other words, the line "cycle vs. product" remains straight only in a number of cycles before it becomes flat. It is very important to terminate the reaction before it reaches a point when the rate of product accumulation slows or "plateau".

A PCR cocktail for 10 samples was assembled to contain 25 µL PCR buffer (10X), 5 µL of the corresponding RT reaction (from 2.2.6.17.2 – in this study only the control sample was used), 20 µL dNTP mix (10 mM each dNTP), 1 µL *Tfi* DNA polymerase (5 u $\mu L^{-1}$), gene specific primer pair to a final concentration of 0.4 µM, 15 µL $Mg^{2+}$ and nuclease-free $dH_2O$ to 250 µL. The cocktail was aliquotted (25 µL each) into 10 identical PCR tubes and then overlaid with ≈ 40 µL mineral oil and run on the thermocycler. Amplification was undertaken for 31 cycles by denaturation at 94°C for 30s, annealing at 60°C for 50s and extension at 72°C. The sample was removed from the thermocycler and placed on ice after each odd numbered cycle starting with cycle 15 and ending with cycle 33. One fifth of the PCR products were run on 1.5 % (w/v) agarose gel alongside with 1 Kb DNA marker. The band intensity was quantified using BioImage™ Advanced Quantifier (B.I. System Inc.). The graph "signal intensity on ethidium bromide agarose gel" vs. cycle numbers was plotted. This graph was used as a guideline to choose the optimal cycle numbers when actual RT-PCR was set-up.

### 2.2.6.18.4. Preparation of RT-PCR Internal Standard

RT PCR internal standard used in this study was a 18S rRNA (QuantumRNA™ 18S Internal Standard kit, Ambion 1718). The kit comes with a primer pair for 18S rRNA and its corresponding 18S Competimer™. The 18S Competimer are modified at their 3' ends to block extension by DNA polymerase. By mixing 18S primers with increasing amounts of 18S Competimer, the overall PCR amplification efficiency of 18S cDNA is reduced without the primers becoming limiting and without loss of

relative quantitation (information from Ambion's QuantumRNA™ 18S Internal Standard Instruction manual).

Ambion recommended that the ratio of 18S primer and 18S Competimer is established for each gene-specific primer although in their hands the 3:7 (18S primer: 18S Competimer) is appropriate for most gene (information from Ambion's QuantumRNA™ 18S Internal Standard Instruction manual). Pilot experiments were carried out to test the suitable 18S primer and 18S Competimer for a gene-specific primer pair. The procedure described in the Ambion's QuantumRNA™ 18S Internal Standard Instruction manual was followed for the pilot experiment.

# CHAPTER 3

# ASSESSMENT OF LYSOZYME EXPRESSION IN TRANSGENIC POTATO PLANTS

## 3.1 Introduction

Part of the development of the current research programme was to assess existing transgenic plants encoding various types of antimicrobial agents. Suitable genes may be used in combination to enhance potato plants against major potato pathogen infection especially the causal agents of late blight disease, *P. infestans* and blackleg disease, *E. carotovora.* One of the first antipathogen factors that was engineered in potato plants in the Department of Biological Sciences, University of Durham is chick egg-white lysozyme. Transgenic potato plants were provided which had been previously characterised and known to be expressing lysozyme protein. Further lysozyme expression studies in the transgenic plants were conducted in order to relate the synthesis of the enzyme to resistance to pathogen attack. This involves the use of accurate assay to estimate enzyme activity in transgenic plants. Although antibodies were available against chicken egg-white lysozyme, enzyme assays were thought to be more appropriate since it is the enzyme action which provides the anti pathogen activity. Transgenic plants were subjected to several pathogen challenges including from *P. infestans, E. carotovora, F. sulphureum and R. solani.*

In general, an enzyme assay is the measurement of a chemical reaction, which might involve measuring the formation of the product or the removal of a substrate from the reaction mixture. In the case of lysozyme, the standard assay is slightly unusual in that the substrate comprises of *Micrococcus lysodeikticus* cell walls, and the enzyme activity is measured by the hydrolysis of this substrate[·]. Lysozyme cleaves the β(1,4) linkage between adjacent N-acetylmuramic and N-acetylglucosamine residues of the cell wall component, peptidoglycan, a polysaccharide structure of the *M. lysodeikticus* cell walls (Figure 11). Cell wall fragments in the reaction mixture scatter light and when they are broken open less and less light is being scattered. The decrease in light

· See Glossary of Relevant Terms

scattering (i.e. the clearing of the reaction mixture) is used to assay the enzyme as suggested by Shugar (1952).

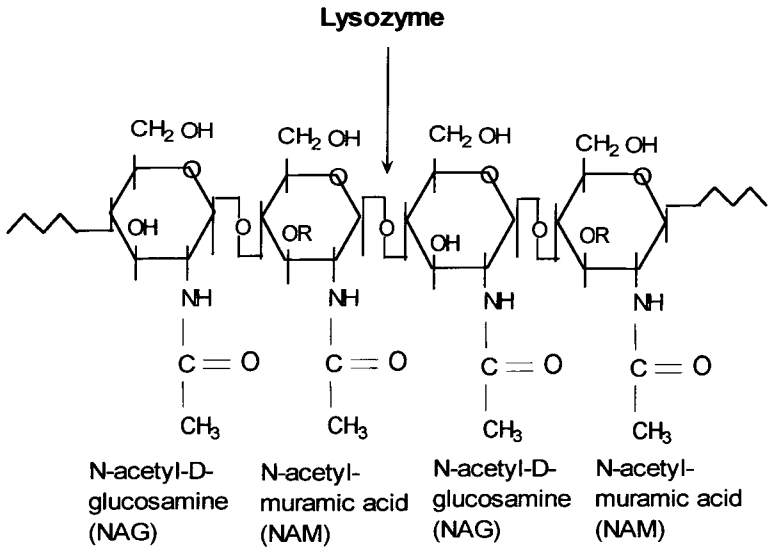


**Figure 11. The structure of part of the cell wall component of the *M. lysodeikticus*.** Lysozyme cleaves at β(1,4) glycosidic linkage, connecting the C1 carbon of NAM to the C4 carbon of NAG as indicated by the arrow.

Because of its simplicity, this assay has been widely used to estimate the activity of lysozyme derived from various tissues. Although the assay may probably work for any sources of lysozyme, the optimisation of the system to meet certain demands such as low concentration of the enzyme in tissue under study was essential in order to enhance the sensitivity of the assay. It should be noted that in actual fact none of the plants subsequently studied had low levels of lysozyme expression. In this part of the study, the lysozyme enzyme assay based on the hydrolysis of bacterial cell wall, *M. lysodeikticus* was optimised for assaying low levels of lysozyme derived from potato transgenic plants. In addition, a new method based on the release of soluble dye, Remazol Brilliant 5R violet from a synthetic substrate of *M. lysodeikticus* cell wall-Remazol violet when hydrolysed by lysozyme was developed and compared with the standard method.

## 3.2 Results and Discussion

### 3.2.1 Optimisation of Lysozyme Turbidimetric Assay for Detecting Low Levels of Enzyme in Plant Tissues

It was essential for accurate, reproducible estimates of the lysozyme activity in the transgenic plants to be certain that the lysozyme enzyme assays were optimised for assay time, lysozyme concentration [E], and temperature. This is important because enzyme assays are sensitive over a limited range. The optimisation was based on the assumption that equilibrium conditions in which the formation and dissociation of lysozyme-*M. lysodeikticus* complex were established very rapidly in the reaction mixture compared with the formation of the product, disaccharide (NAM-NAG). This is necessary in order to measure the initial velocity (v) which occurred at a constant concentration of substrate (*M. lysodeikticus*). The initial velocity at any time depends on the concentration of lysozyme-*M. lysodeikticus* complex. Because of equilibrium conditions (lysozyme + *M. lysodeikticus* <–> lysozyme - *M. lysodeikticus* complex), the complex lysozyme-*M. lysodeikticus* can also be expressed as lysozyme and *M. lysodeikticus* concentration in the reaction mixture. Therefore, velocity of the lysozyme reaction is always proportional to the lysozyme concentrations in the reaction mixture as represented by the Henri-Michaelis-Menten equation below. Using the Henri-Michaelis-Menten with the assumption described earlier, the measurement of lysozyme concentration at any time is possible by measuring the true initial velocity of the reaction.

$$v = \frac{[S]V_{max}}{K_m + [S]} = \frac{[S]K_p[E]_t}{K_m + S}$$

The relationship between velocity and lysozyme concentration in the reaction mixture is linear only if the true initial velocity is measured. This was measured before a significant amount of *M lysodeikticus* cell wall was hydrolysed as velocity varies with substrate concentration as shown by the Henri-Michaelis-Menten above. In other words, the assay period must be short enough to ensure only a small fraction of the *Micrococcus* cell-walls were hydrolysed so that the only limiting factor in the system was lysozyme concentration. Therefore, the velocity of the lysozyme reaction at different concentrations (0.25, 0.5, 2, 4, 8 μg $mL^{-1}$) were monitored after 1 min of reaction, and followed with 4 min intervals for a period of 4 min. The hydrolysis of the

*M. lysodeikticus* cell walls depend on the initial concentration of lysozyme in the assay mixture as shown in Figure 12. The lines were linear at the beginning of the reaction process (shortly after reaction started) for all of the lysozyme concentrations tested, suggesting that the amount of substrate (*M. lysodeikticus* cell walls) was still available in excess and that the lysozyme concentration was the only limiting factor in the assay mixture. When incubation time increased, the amount of substrate hydrolysed was significant and both the lysozyme and the substrate became limiting factors in the assay.

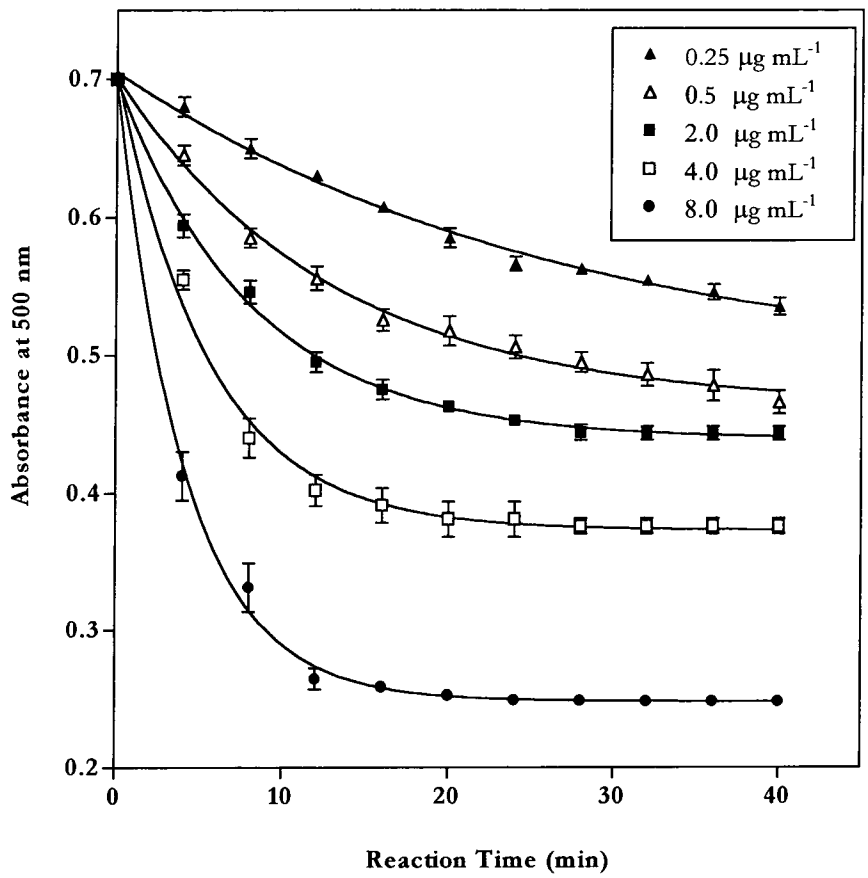


**Figure 12. Hydrolysis of *Micrococcus lysodeikticus* Cell Walls at Different Concentrations of Lysozyme.** The concentration of the substrate was fixed at 0.5 mg $mL^{-1}$. The absorbance was monitored after 1 min reaction and then 4 min intervals. Data are the mean of three replicate samples and error bars represent standard deviation. Lines plotted are the best fit calculated by Prism 2 (GraphPad Inc.).

By plotting another graph to represent the relationship between velocity (ΔAbsorbance at 550 $min^{-1}$) and lysozyme concentrations (Figure 13), the initial velocity of the

lysozyme reaction could be determined. Figure 13 shows clearly that during the first minute of the reaction starting, the reaction velocity (i.e. initial velocity) was proportional to all the lysozyme concentrations tested except at 8 μg $mL^{-1}$, judging by the linearity of the regression lines.

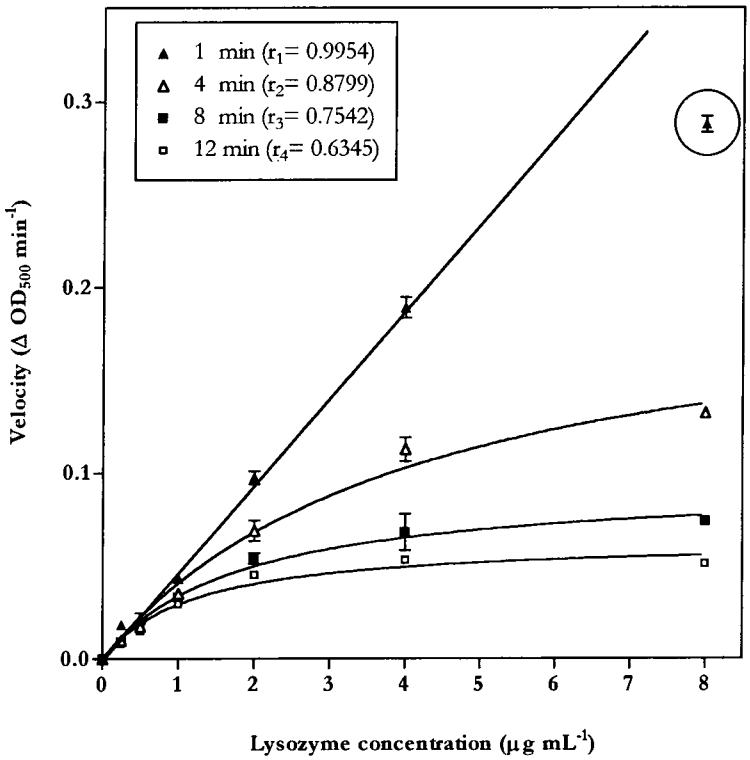


**Figure 13. Reaction Velocity as a Function of Enzyme Concentration.** Data are means of three replicate samples and error bars represent standard deviation. R square values for each of the regression lines are given in brackets (r1 – r4). For r1, the best fit line was obtained by excluding the last point (lysozyme concentration at 8 μg $mL^{-1}$ as circled in the graph).

Lysozyme concentration in the assay mixture was no longer proportional to the reaction velocity if the assay was run beyond 1 min and this would result in an underestimate of the true lysozyme activity in the assay. With this time limit, the maximum lysozyme concentration can be measured only 6 μg $mL^{-1}$ after which the relationship between reaction velocity and lysozyme concentration in the assay was no longer linear. Assaying lysozyme beyond the valid range and limit resulting in inaccurate determination of the true lysozyme activity, and in turn give a false result for the level of lysozyme expressed in the transgenic plants under study.

The assay system was further optimised by estimating the optimum concentration for the substrate used in the assay. This is important to make sure that the amount of substrate in the assay mixture was enough before it became a limiting factor in the assay. In addition, the substrate *M. lysodeikticus* cell walls was insoluble, and only a small fraction of the cell walls fragments was actual substrate of the enzyme. A range of different *M. lysodeikticus* cell wall concentrations were used in the assay at a fixed lysozyme concentration. The result (Figure 14) shows that the relationship between reaction rate and substrate concentration was represented by a typical right rectangular hyperbola curve.

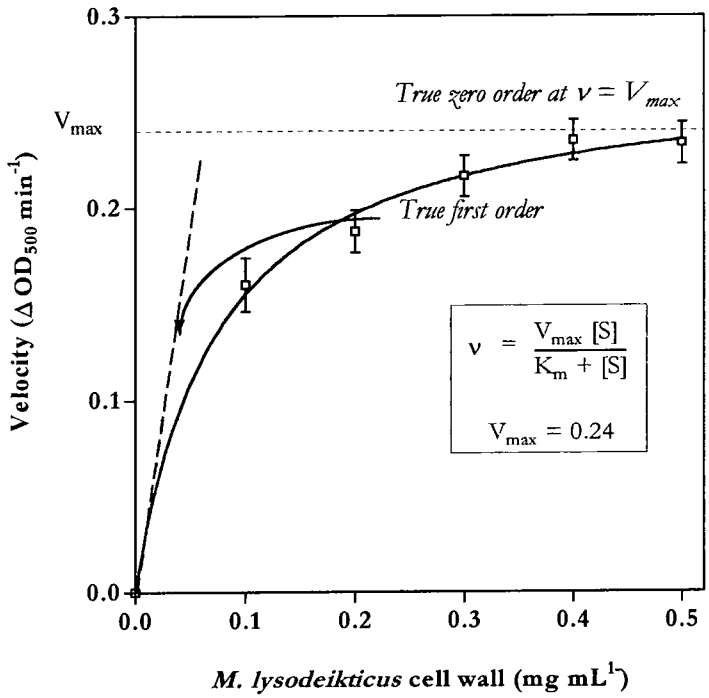


**Figure 14. Effect of *M. lysodeikticus* Cell Wall Concentration on the Activity of Lysozyme.** Data are means of three replicate samples and error bars represent standard deviation.

The curve clearly shows two different reaction orders at the substrate concentrations between 0.1 to 0.5 mg $mL^{-1}$. At very low concentrations of substrate (<< 0.1 mg $mL^{-1}$), first order reaction kinetics occurred and when the substrate concentration approached saturation (> 0.5 mg $mL^{-1}$), the zero reaction order appeared. In practice, velocity can be considered as a true initial rate of the enzyme reaction only if the substrate

concentration remains constant over the assay time, i.e. only if a small fraction of *M. lysodeikticus* cell wall is hydrolysed. This can only be achieved if the enzyme is working under the zero order reaction kinetics or when the reaction is substrate-independent. In this assay system, the reaction mixture should contain a concentration of *M. lysodeikticus* cell walls of at least 0.5 mg $mL^{-1}$. If this requirement is followed, initial velocity (v) is close to $V_{max}$ at any lysozyme concentration during the first minute of reaction. Assaying the enzyme at different temperatures ranging from 30 to 40°C showed typical enzyme temperature optimum at 35 °C as shown in Figure 15. This is because an increase of temperature caused an increase in kinetic energy of the molecules in the reaction mixture resulting in more collisions between the lysozyme molecules and cell wall fragments of *M. lysodeikticus*. At concentration beyond 35 °C, a decrease in activity was observed because of the thermal denaturation of the lysozyme protein. Optimised lysozyme assay system should contain at least 5 mg $mL^{-1}$ *M. lysodeikticus* cell-wall in 50 mM Sodium-acetate buffer pH 6.0.

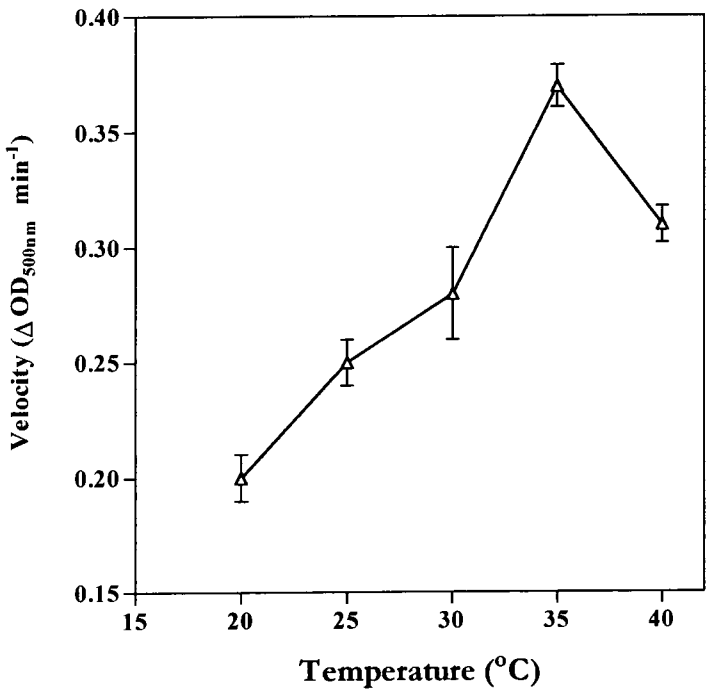


**Figure 15. Optimum Concentration of Lysozyme in Turbidimetric Assay Method.** Each point represent a mean of three replicates samples and error bars are standard deviation. This was assay using the optimum assay system which contained 0.5 μg $mL^{-1}$ *M. lysodeikticus* cell-wall in 50 mM Sodium-acetate buffer, pH 6.0 and 2 μg $mL^{-1}$ chick egg-white lysozyme, assayed for 1 min.

The lysozyme must be adjusted so that the final concentration in the assay mixture does not exceed 6 μg $mL^{-1}$. The mixture should be assayed for 1 min at 37 °C. Using *M. lysodeikticus* cell as a substrate of lysozyme did not significantly change the kinetics of the enzyme although it was water-insoluble substrate. In this respect, the cell walls behaved in much the same way as a 'normal' soluble substrate.

### 3.2.2. Development of a Convenient Colorimetric Lysozyme Assay

#### 3.2.2.1. Introduction

One of the drawbacks of the turbidimetric assay method is the spectrophotometric readings tend to fluctuate a little especially when the lysozyme concentration is very low. This is due to the insoluble nature *M. lysodeikticus* cell wall substrate. To provide a simple solution which was at least of the equivalent sensitivity as the existing method an alternative assay method was investigated to solve the problem. With only a limited choice of assay method developed for lysozyme so far, most researchers are quite satisfied with the turbidimetric assay because it is rapid, easy to set-up and is reasonably sensitive. The alternative methods such as fluorometric assays in which bacterial cell walls are labelled with fluorescamine or fluorescein isothiocyanate (Maeda, 1980) need an expensive spectrofluorimeter to detect the fluorescent product and involve complicated procedures to prepare the substrate free from contaminating label. The dye-labelled substrate (chitin-Remazol brilliant violet 5R) prepared using the suggested protocol by Wirth and Wolf (1990) was not working in the current study. This could be due to low chitinase activity in chick egg-white lysozyme. Therefore chitinase substrates are of limited value. In addition, the preparation of the soluble chitin-remazol violet 5R-labeled substrate was complicated and took as long as 3 days for completion.

#### 3.2.2.2. Preparation of Remazol Brilliant Violet 5R-*M. lysodeikticus* Cell Wall Substrate

Using the same labelling principle described by Wirth and Wolf (1990), the *M. lysodeikticus* cell wall was labelled with remazol brilliant violet 5R and the subsequent assay system worked well. Preparing the substrate involved linking up *M. lysodeikticus* cell walls with remazol brilliant violet 5R dye using a simple process presented in a flow diagram in Figure 16. The modification of the substrate is based on the capability of remazol brilliant violet 5R dye to modify the hydroxyl group of

sugars (Wirth and Wolf, 1990). Therefore, sugar residues present in the peptidoglycan of the *M. lysodeikticus* cell wall are thought to be labelled with this dye. The substrate was purple in colour and during the enzyme reaction, dye was released by lysozyme action producing short, soluble fragments from the cell wall labelled with remazol violet 5R. After centrifugation, the dye released was measured spectrophotometrically at its optimum absorption, 550 nm.

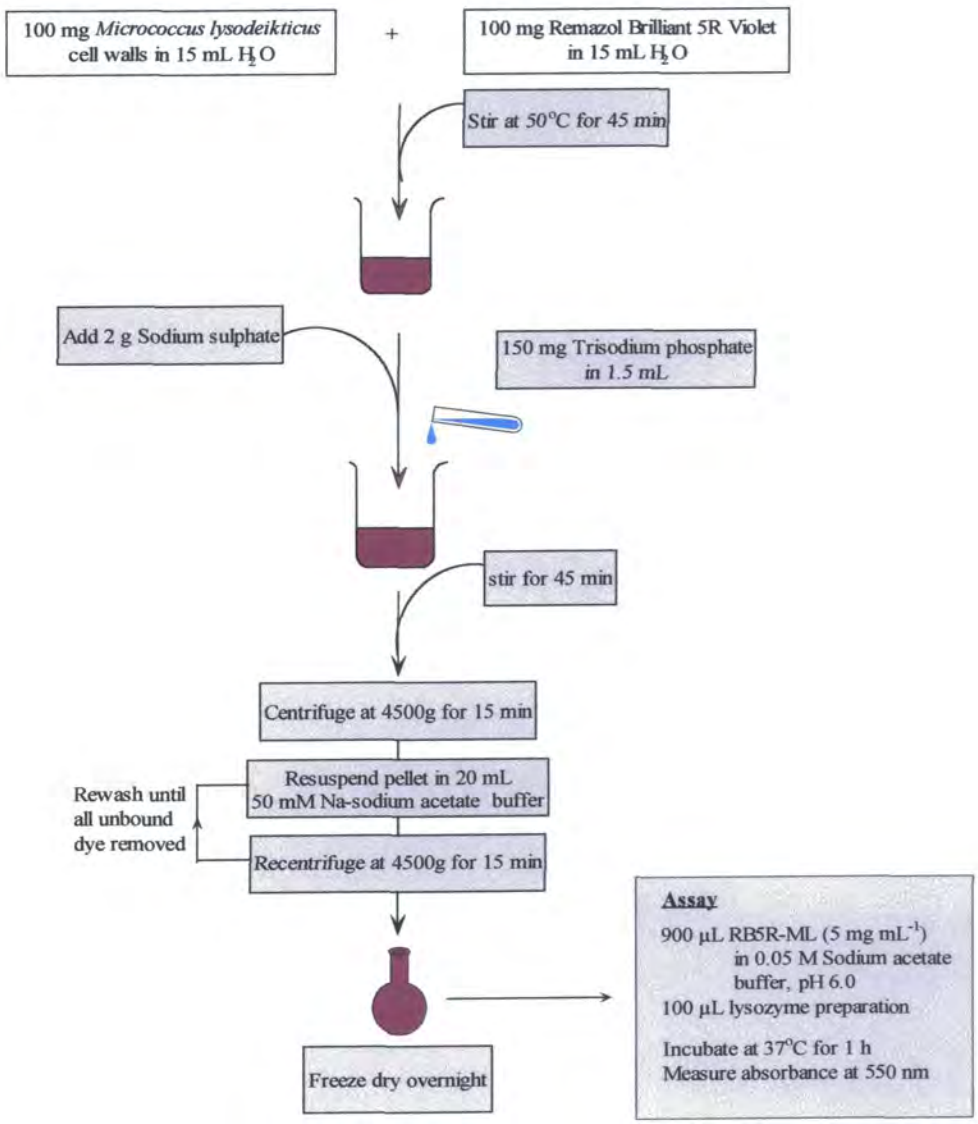


**Figure 16. Schematic Showing the Preparation of Modified *M. lysodeikticus*-cell Wall-Remazol Brilliant 5R Substrate.**

The optimum absorption of remazol brilliant violet 5R maximum was determined using different solutions including distilled water, 50 mM acetate buffer at pH 5.8 and

50 mM phosphate buffer at pH 7.5 and 50 mM Tris-HCl buffer at pH 8.0. The substrate was stable for at least 4 weeks storage in buffer solutions of pH values ranging from 5.8 to 8.0 at 4°C. The colour released from the substrate on storage was less than 4%.

### 3.2.2.3. Optimisation of Lysozyme Assay for Remazol Brilliant Violet 5R-*Micrococcus lysodeikticus* Cell Wall Substrate

With the new substrate produced, the lysozyme assay system again needed to be optimised. Optimisation followed the same line of experiments described for the turbidimetric method (section 3.2.1). In this assay, the product of the reaction, the soluble purple-coloured remazol brilliant violet 5R as a result of the complex substrate hydrolysis was measured at 550 nm.

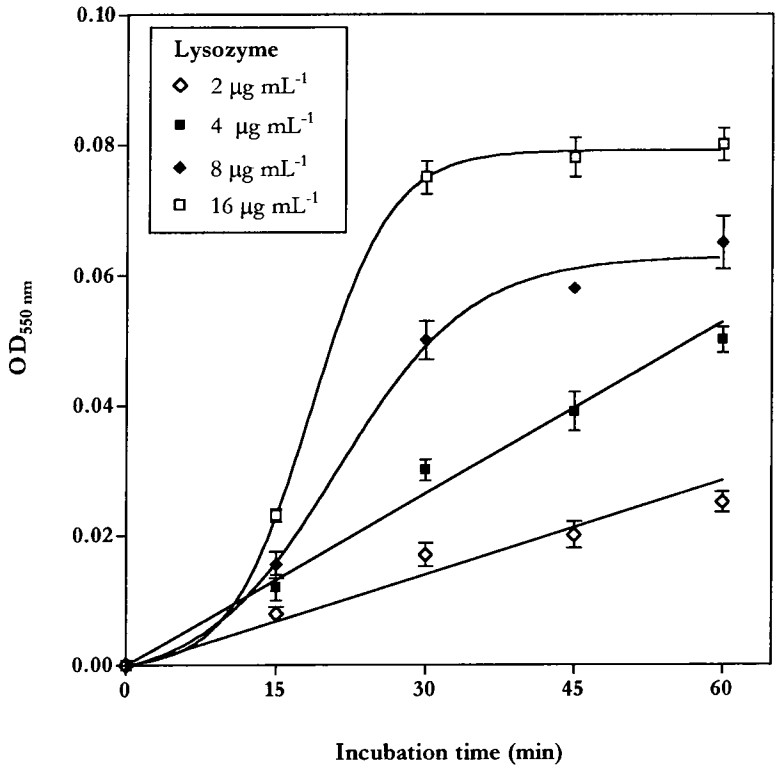


**Figure 17. The Relationship Between Colour Development and Lysozyme Concentration in a Colorimetric Assay for Lysozyme.** The lysozyme was assayed in the present of fixed concentration of remazol brilliant violet 5R-*M. lysodeikticus* at 3 mg $mL^{-1}$ in 50 mM sodium-acetate buffer, pH 6.0, incubated at 37°C for 15, 30, 45 and 60 min. Data are mean of three replicate samples with standard deviation.

The relationship between colour development and lysozyme concentration measured at different incubation periods is shown in Figure 17. The colour formation during an hour incubation period was linear for lysozyme concentrations of 2 and 4 μg $mL^{-1}$. This indicates that during the incubation period of 1 h, the amount of substrate (3 μg $mL^{-1}$) in the assay mixture using lysozyme concentrations of 2 and 4 μg $mL^{-1}$ were still within the limit in which the hydrolysis of the labelled-*M. lysodeikticus* cell wall was linear over the incubation time. Therefore, the lysozyme concentration used in this system should not exceed the limit of 4 μg $mL^{-1}$ if the incubation time was 1 h. This was further explained in Figure 18 below.

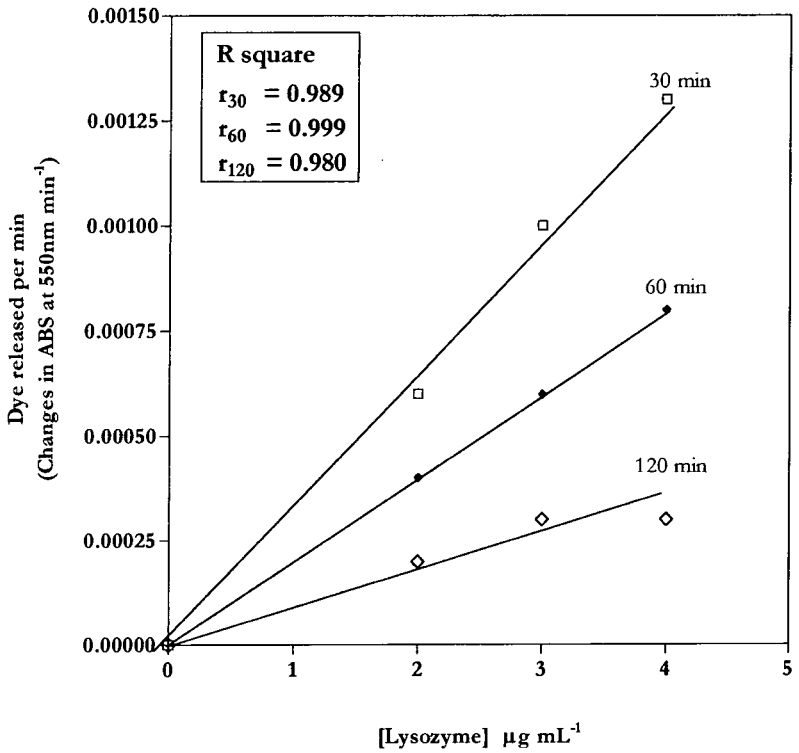


**Figure 18. Correlation Between the Velocity and Lysozyme Concentration in the Lysozyme Colorimetric Assay.** The lysozyme at 2 and 4 μg $mL^{-1}$ was assayed in the present of fixed concentration of remazol brilliant violet 5R-*M. lysodeikticus* at 3 mg $mL^{-1}$ in 50 mM sodium-acetate buffer, pH 6.0, incubated at 37°C for 30, 60 and 120 min. Data are mean of three replicate assays with their corresponding regression lines and R square values.

The relationship between velocity and lysozyme concentration was linear only if the lysozyme was assayed at a concentration of 4 μg $mL^{-1}$ or below (Figure 18). The velocity of the lysozyme reaction was proportional to the amount of lysozyme in the

assay mixture up to 2 hours of incubation period. During this period, the formation of a small soluble fragment of remazol brilliant violet 5R-labelled *M. lysodeikticus* was constant judging by the regression lines and their corresponding $r^2$, indicating that the velocity measured was an actual initial velocity. Figure 18 also shows that lysozyme was best assayed for 1 h if 3 μg $mL^{-1}$ remazol brilliant violet 5R-labelled *M. lysodeikticus* cell walls substrate was used in the assay mixture containing 4 μg $mL^{-1}$ or less lysozyme. The optimum substrate required for the assay system was determined using 4 μg $mL^{-1}$ lysozyme and incubation period of one hour at 37°C. The substrate concentrations tested were between 0 and 5 mg $mL^{-1}$. Figure 19 shows the typical right rectangular hyperbolic curve representing a first order reaction at low substrate concentration and changing to zero order at a saturating substrate concentration.

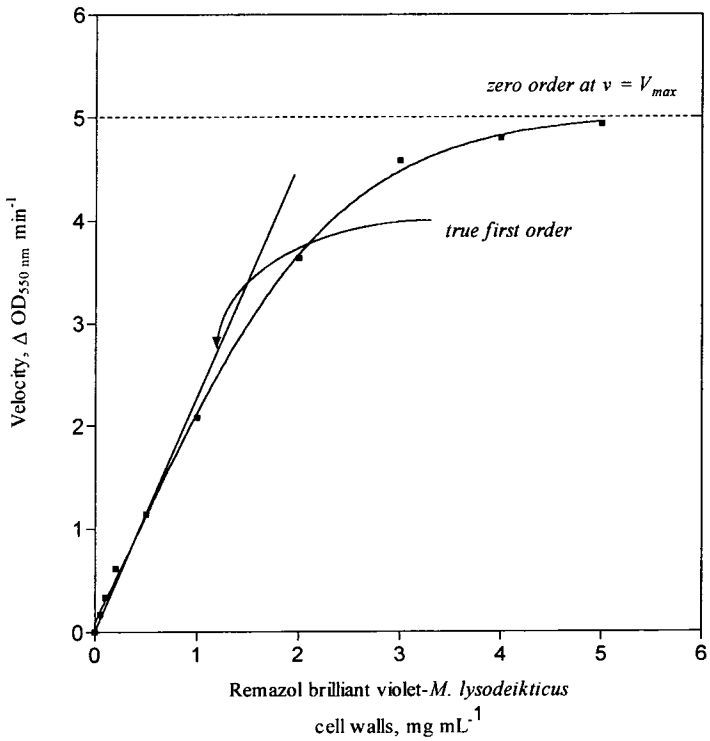


**Figure 19. Effect of Substrate Concentration on the Activity of Lysozyme in a Colorimetric Assay Method.** The assay mixture contained 4 μg $mL^{-1}$ lysozyme in different concentrations of substrate in 50 mM Sodium acetate buffer, pH 6.0. Data are means of three replicate samples and error bars represent standard deviation.

This profile was the same as the velocity plot as a function of substrate concentration in the turbidimetric assay system described earlier (section 3.2.1). The optimum

substrate concentration under these conditions was 5 mg $mL^{-1}$ where velocity was constant and independent of substrate concentration (remazol brilliant violet 5R-*M. lysodeikticus* cell wall) in the assay mixture over the incubation period of 1 h. Using optimum conditions, a standard curve for the amount of dye released in the reaction mixture with varying lysozyme concentration was established (Figure 19).

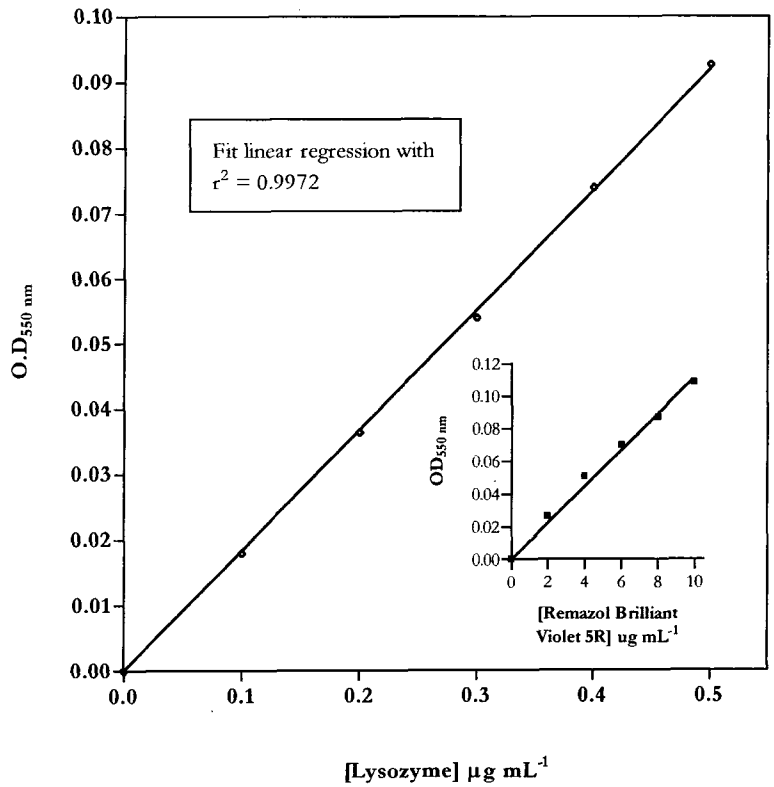


**Figure 20. Remazol Colour Development as a Function of Lysozyme Concentration in the Colorimetric Assay Method.** Concentration of chicken lysozyme down to 0.1 µg $mL^{-1}$ was used in the construction of the standard curve. Assay detail as for Figure 16. Each point is the mean of three replicate samples and is presented as a regression line.

The curve was a linear correlation between the release of remazol brilliant violet 5R dye into the assay mixture and the concentration of lysozyme used. The relationship was near perfect judging by the correlation coefficient ($r^2$ = 0.9972) of nearly 1. The assay was reproducible and highly sensitive. It is capable of detecting lysozyme levels at concentrations down to 0.1 µg $mL^{-1}$.

### 3.2.2.4. Comparison between Turbidimetric and Colorimetric Assays for Lysozyme

The main objective of these experiments was to determine the best substrate for lysozyme based on the *M. lysodeikticus* cell walls and remazol brilliant violet 5R-*M. lysodeikticus* cell wall substrates. A range of substrate concentrations for both *M. lysodeikticus* cell wall and RBV-5R-ML substrates were used to assay the enzyme. The resulting data were analysed using Lineweaver-burk reciprocal plots to find the corresponding $K_m$ and $V_{max}$ for the substrates. Table 4 shows the summary for some important parameters of the lysozyme assays using both substrates.

**TABLE 4. Comparison Between Two Substrates for Lysozyme**

| | **Substrate** | |
|---|---|---|
| | ***M. lysodeikticus* cell wall** | **Remazol Brilliant Violet 5R labelled *M. lysodeikticus* cell wall** |
| $K_m$ | 0.07 | 2.47 |
| $V_{max}$ | 0.24 | 3.00 |
| $V_{max}/K_m$ | 3.28 | 1.21 |
| Sensitivity[a] | 0.25 μg $mL^{-1}$ | 0.1 μg $mL^{-1}$ |

[a]Based on the lowers lysozyme concentration tested in this experiment.

In general a Michaelis constant, $K_m$ indicates the affinity of substrate for a particular enzyme. The $K_m$ values for the enzyme were 0.07 and 2.47 when assayed using *M. lysodeikticus* cell wall and *M. lysodeikticus* labelled with Remazol Brilliant Violet 5R, respectively. This demonstrated that the pure *M. lysodeikticus* cell wall has the highest apparent affinity for the enzyme. In equilibrium state (lysozyme + remazol labelled *M.* lysodeikticus cell wall <--> lysozyme-remazol labelled *M. lysodeikticus* cell wall complex), the position of the equilibrium is not only dependant on the free remazol labelled *M. lysodeikticus* cell wall available in the assay mixture but also depends on the strength of the bonds between the two molecules (lysozyme and its substrate). It could be that the bonds between the two molecules were weak, and therefore the equilibrium tends to move to the left and this needs a relatively high concentration of substrate to achieve 50% saturated. One possible explanation to this could be that the labelling process may have structurally changed the substrate due to the addition of

remazol brilliant violet 5R and various chemical treatments during the process. However, this does not mean the assay is not sensitive because as long as the enzyme is measured within the range limit, its reaction velocity is always proportional to the lysozyme concentration in the assay mixture, and this represents true lysozyme activity.

### 3.2.3. Assessing Lysozyme Expression in Different Lines of Transgenic Potatoes Grown in Glass House Conditions

At the start of this project, 18 transgenic potatoes and one non-transgenic control lines were kindly provided by Mrs E.J.Croy as stored tubers, and planted in the glass house. These lines were already selected and characterised as to lysozyme levels of expression. The transgenic potato contained a chick egg-white lysozyme gene construct. The gene construct contained a double enhancer CaMV 35S promoter and CaMV termination signals as well as the template cDNA coding sequence for chick lysozyme with its own leader sequence (see Figure 40 in Chapter 5 for the gene construct). The level of lysozyme expression in leaves were determined using the optimised turbidimetric assay method because when the plants were ready for sampling, the colorimetric lysozyme substrate was still under development. Lysozyme activity was measured six weeks after planting. Leaves were sampled and selected to include only those that appeared healthy, undamaged and of identical size and shape i.e. of the same physiological age.

Figure 21 shows the overall results of these assays for level of lysozyme expression in these lines. The level of lysozyme expressed in each plant was compared with the non-transgenic line using Dunnett's pairwise comparison and Turkey's multiple comparison following one-way analysis of variance (ANOVA). Dunnett's test which is specifically designed to compare treatment samples against one single control was used to compare the levels of lysozyme expressed in the transgenic lines against the control non-transgenic plant. On the other hand, Turkey's test which was designed to compare all treatment pairs was used to compare the level of lysozyme expression in each transgenic line against each other. By using Turkey's test it is possible to group the transgenic lines based on the level of lysozyme expression.

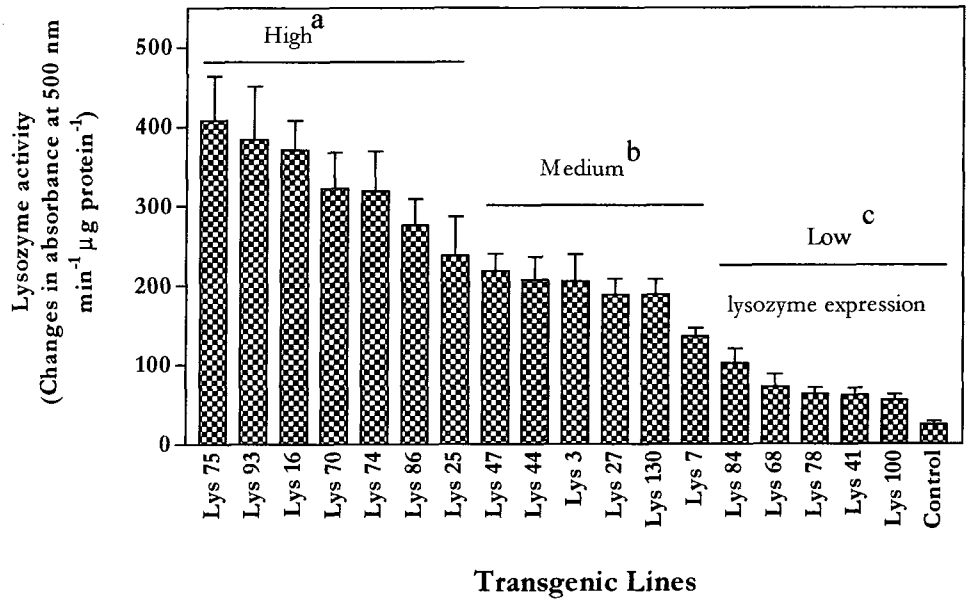


**Figure 21. Graphical Presentation of the Lysozyme Expression in Transgenic Potato.** Based on Turkey's and Dunnett's post-test following a one way analysis of variance, three groups were identified to have no significant difference among them ([a]Higher; [b]Medium; [c]Low lysozyme expressing lines; see Table 5). Lysozyme was assayed by turbidimetric method in extracts from leaves of near identical physiological age. Results shown are the mean lysozyme activities from triplicate assays of four independent samples.

Table 5 shows that five transgenic lines showed no significant difference in mean levels of lysozyme expression compared with the non-transgenic plant. These are designated Lys 100, 84, 68, 78 and 41. The rest of the lines showed mean levels of lysozyme expression significantly higher compared with the control (Lys 75, 93, 16, 70, 74, 86, 25, 47, 44, 3, 27, 130 and 7).

**TABLE 5. Rankings of the Lysozyme Expression of the Transgenic Potato Lines Encoding Lysozyme.**

| Transgenic Lines[p] | Lysozyme Expression[q] | Multiple comparison after one way ANOVA at p<0.05 | |
|---|---|---|---|
| | | Turkey's Test[r] | Dunnett's Test[s] |
| Lys 75 | 407.55 | A | ** |
| Lys 93 | 384.14 | Ab | ** |
| Lys 16 | 370.48 | Abc | ** |
| Lys 70 | 321.91 | Abcd | ** |
| Lys 74 | 319.24 | Abcde | ** |
| Lys 86 | 276.35 | Abcdef | ** |
| Lys 25 | 237.23 | Abcdefg | ** |
| Lys 47 | 218.49 | Bcdefgh | ** |
| Lys 44 | 206.88 | Cdefghi | ** |
| Lys 3 | 205.52 | Cdefghij | ** |
| Lys 27 | 188.78 | Defghijk | * |
| Lys 130 | 188.52 | Defghijkl | * |
| Lys 7 | 135.78 | Ghijklm | * |
| Lys 84 | 102.11 | Ghijklm | N |
| Lys 68 | 72.21 | Hijklm | N |
| Lys 78 | 63.40 | Hijklm | N |
| Lys 41 | 61.74 | Hijklm | N |
| Lys 100 | 54.88 | Hijklm | N |
| Control | 24.52 | Klm | N |

[p]The transgenic lines were grown in a glass house and the leaves were sampled six weeks after planted.

[q]Lysozyme activity was determined using a turbidimetric assay method. The activity was expressed as a changes in absorbance at 500nm per min per mg soluble protein.

[rs]Common letters in the same column do not differ significantly according to Turkey's (r) and Dunnett's (s) multiple comparison following the one way analysis of variance at p=0.05. Dunnett's test was applied specifically to compare the transgenic lines with the control.

** highly significance at $p < 0.01$

* significant at $p < 0.05$

From Turkey's multiple comparison test analysis, the transgenic lines can be categorised into three i.e. low, medium and high lysozyme expressors as shown in Figure 21. Within the group (either low, medium or high), no significant difference was observed in the mean levels of lysozyme expressed at $p < 0.05$ level.

As mentioned earlier in this section, the newly developed substrate (remazol brilliant violet 5R-*M. lysodeikticus* cell wall) was not ready when the leaves of the planted transgenic lines were about to be harvested for lysozyme assay. Therefore, when the

substrate was ready, some of these lines were also assayed using the new assay method for comparison of its sensitivity against the turbidimetric assay method. Selected transgenic lines representing candidates of low, medium and high lysozyme expressors were assayed using this method. The pattern of lysozyme expression among the selected transgenic lines was almost the same compared with the earlier data as shown in Table 6.

**Table 6. The Expression of Lysozyme in Different Lines of Lysozyme-Encoding Transgenic Potato Assayed Using the Colorimetric Technique.**

| Transgenic Lines[p] | Lysozyme Expression[q] | Multiple comparison after one way ANOVA | |
|---|---|---|---|
| | | Turkey's Test[r] | Dunnett's Test[s] |
| Lys 16 | 12.07 | A | ** |
| Lys 93 | 11.44 | Ab | ** |
| Lys 75 | 9.94 | Abc | ** |
| Lys 44 | 7.87 | C | ** |
| Lys 100 | 3.49 | D | Ns |
| Control | 1.95 | D | |

[p]The transgenic lines were grown in the glass house and the leaves were sampled eight weeks after planted.

[q]Lysozyme was assayed using Remazol brilliant violet 5R-*M. lysodeikticus*. The activity was expressed as μg lysozyme $h^{-1}$ $mg^{-1}$ soluble leaf protein.

[rs]Common letters in the same column do not differ significantly according to Turkey's (r) and Dunnett's (s) multiple comparison following the one way analysis of variance at p=0.05. Dunnett's test was applied specifically to compare the transgenic lines with the control.

** highly significance at $p < 0.01$

ns not significant at $p <= 0.05$

The lysozyme levels were slightly different as Lys 16 shows the highest followed with Lys 93 and Lys 75 although statistically the difference between their means was not significant. Lys 100 remained at the bottom of the table with no significant difference in mean compared with the non-transgenic line. The slight difference in lysozyme patterns and levels may be due to differences in age of samples, and also perhaps difference in sensitivity between the two assay methods.

### 3.2.4. Expression of Lysozyme in Leaf Tissues of Transgenic Potato During Plant Development

Since the CaMV promoter is known to be affected by developmental stage selection of leaves of equivalent physiological ages it was critical for comparative purposes. To assess the possible effect of development stage on the accumulation of the lysozyme product in the cell, the lysozyme activity at different leaf development was carried out.

Figure 22 (Panel A) shows the level of lysozyme expression at different stages of leaf development using leaves sampled from six-week-old plants.

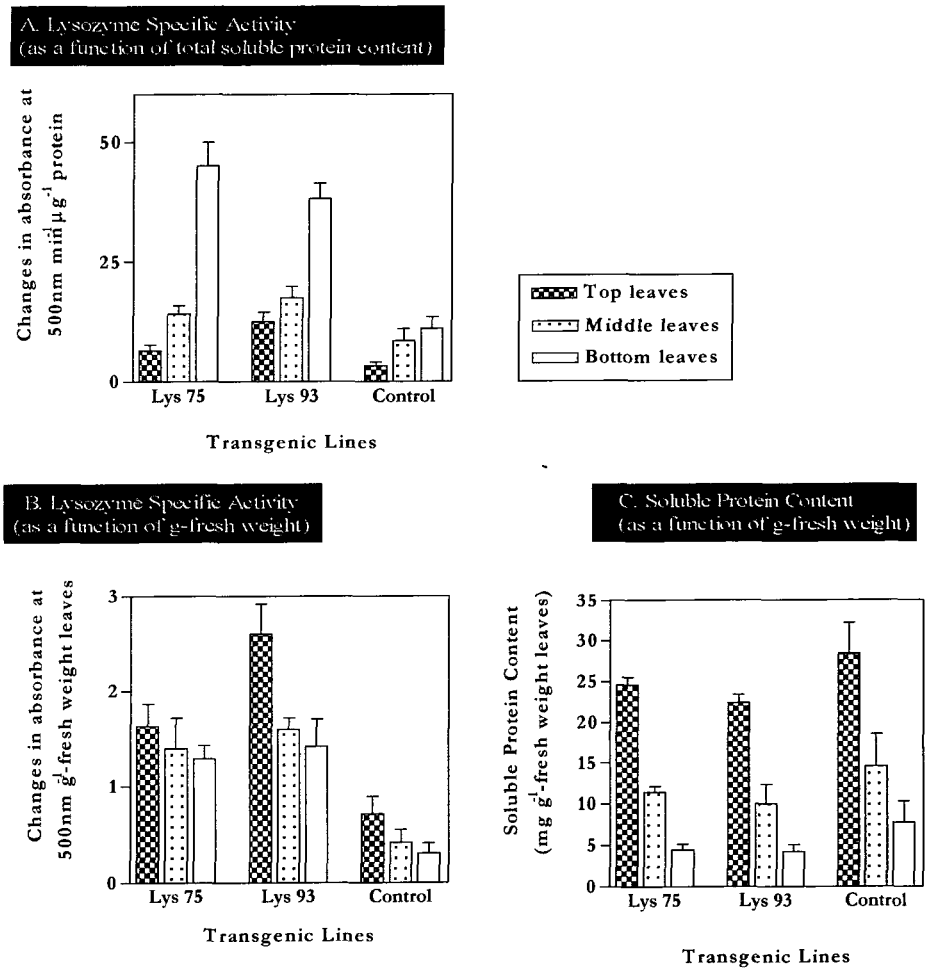


**Figure 22. Lysozyme Expression in Selected Lysozyme-Transgenic Potato Lines at Different Stages of Leaf Development. Panel A,** The lysozyme activity expressed as function of total soluble protein content. **Panel B,** Lysozyme activity expressed as a function of g-fresh weight of leaf tissues. **Panel C,** The soluble protein content in the leaf tissues as a function of g-fresh weight of the leaf tissues. Each value is the mean of three replicate assays of two independent samples. Error bars show the corresponding standard deviation.

The highest levels of lysozyme specific activity (activity in a µg leaf soluble protein) were recorded in leaves at the lower part of the plant followed by leaves in the middle and upper positions. This result indicates that the relative amount of lysozyme in mature leaves was higher than that in new young leaves. However, this does not imply that the plants are actively producing new lysozyme protein in the old leaves as results

in Figure 22 (Panel B) may indicate. The lysozyme activity is relatively low in leaves at the lower part compared with the upper part if the lysozyme activity is expressed on a fresh weight basis. The soluble protein contents were also relatively low in the old leaves indicating that protein synthesis was no longer highly active (Figure 24, Panel C). The higher level of lysozyme activity recorded in the old leaves suggests the pre-accumulation of lysozyme protein in the intracellular space and remains stable for a long time. This is useful for prolonged maintenance of protection against pathogens.

### 3.2.5 Accumulation of Lysozyme in Intercellular Space of Leaf Tissues

In the previous analysis of the lysozyme expression pattern during development under glass house conditions, lysozyme activity was higher as the leaves aged. This indicates that lysozyme protein was accumulating during leaf growth. In chicken, the lysozyme is extracellular as is most lysozymes in other animal tissues. This is not known in plants although earlier work (Mrs E. Croy, personal communication) showed some extracellular. Investigation of the distribution of lysozyme in leaves of a transgenic potato was carried out by vacuum infiltrating leaves of Lys 75 and a non-transgenic control line with 50 mM phosphate buffer, pH 7.2 containing 100 mM $CaCl_2$ to collect as much protein as possible out of the intercellular space. Lys 75 line was chosen simply because it was the highest lysozyme expressor amongst all the lines. To make sure that the collected protein was originally from intercellular space, a cytoplasmic contamination test was carried out. The test was carried out by assaying one of the abundant cytoplasmic enzymes, malate dehydrogenase (MDH) in the intercellular fluid following vacuum infiltration. Earlier work (Tetlow and Farrar, 1993) showed that only 0.01% of MDH is present in intercellular fluid of barley after vacuum infiltration. Figure 23 shows the percentage of lysozyme in leaf tissue present in the intercellular wash fluid.

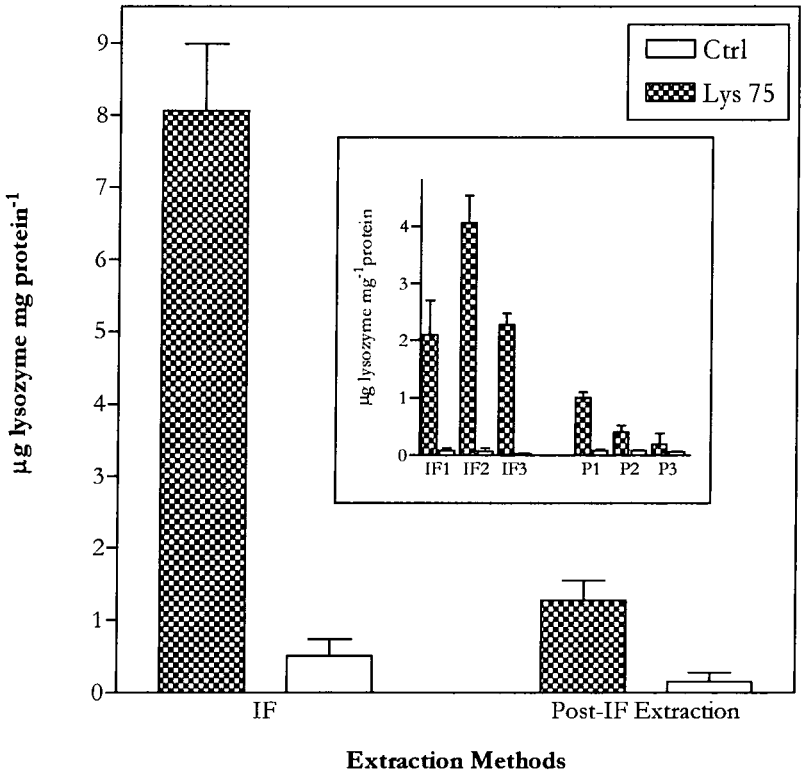


**Figure 23. The Level of Lysozyme in the Intercellular Fluid of Transgenic Potato Plants.** Each bar represents the mean of two replicate samples and each was sequentially vacuum extracted for the intercellular fluid (IF1, IF2 and IF3) and followed with three sequential total protein extractions (P1, P2 and P3). IF in the big graph is the mean of IF1, IF2 and IF3 in the small graph. Post-IF-extraction is the mean of P1, P2 and P3. Error bars show standard deviation.

Less than 0.1 % of cytoplasmic marker, MDH was in the intercellular wash fluid indicating that the method used to extract proteins from intercellular space did not remove any appreciable amounts of contaminating cytoplasmic protein (Figure 24). More than 86 % of lysozyme activity in transgenic potato Lys 75 was found in the intercellular fluid. This result supports the earlier idea that lysozyme was accumulating in the extracellular compartment (apoplast) of the leaf tissue.

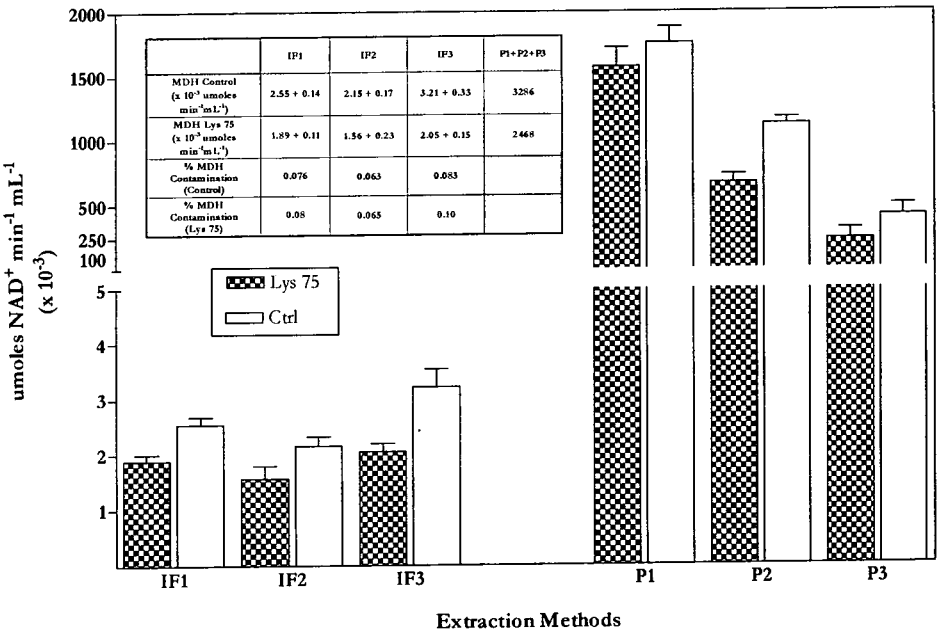


**Figure 24. The Level of Cytoplasmic Malate Dehydrogenase in the Intercellular Fluid (IF1, IF2, IF3) from Leaf Following Vacuum Infiltration Compared with the Levels in the Total Protein Extracts (P1, P2, P3).** The small table (inset) presents the details of the calculation of the enzyme and its distribution.

The expression of lysozyme in the intercellular space of transgenic potatoes was also reported by During *et al.* (1993) although the level of expression was very low (0.001% of total soluble protein). Their transgenic potato was developed using the bacteriophage T4 lysozyme under the control of CaMV 35S promoter with an α-amylase to direct into the secretory pathway.

### 3.3. Conclusions

Lysozyme can be assayed both using the turbidimetric and colorimetric methods. The optimised turbidimetric method can detect lysozyme as low as 0.25 μg $mL^{-1}$. On the other hand, colorimetric assay using a new substrate Remazol brilliant violet 5R-*M. lysodeikticus* was also very sensitive and the lowest lysozyme level tested in this study was 0.1 μg $mL^{-1}$.

Using both assays, the lysozyme encoding transgenic potato lines can be categorised into three groups i.e. higher expressor, moderate and low performing lines. The higher expressors were Lys 75, 16, 93, 16, 70, 74, 86 and 25 which made up of 38.89% of the

lines tested. The rest were moderate (33.33%) and low-performing (27.78%) lines. Lysozyme expressed in these transgenic plants was accumulating and more than 86% of the total lysozyme expressed was accumulated in the intercellular space. This feature is an advantage for preventing the plant from pathogen attack. The lysozyme expression patterns in both tissue culture and green house environment were the same. The levels of expression remained high after several growing sessions indicating the stability of the gene construct.

# CHAPTER 4

# DEVELOPMENT OF AN *IN VITRO* BIOASSAY METHOD FOR TESTING DISEASE RESISTANCE IN TRANSGENIC PLANTS

## 4. 1 Introduction

One of the major steps in engineering transgenic plants for disease resistance is to test the effectiveness of the new protein against the target pathogen. This involves the exposure of the plants to the pathogen in a suitable infective unit such as spores, fungal hyphae and bacterial cells in a small-scale controlled environment growth room or in open field plots. The two types of trials have advantages and disadvantages. The major advantage of the field trial is that the transgenic plants have the opportunities to interact with the many complex environmental elements including climate variation that may affect the overall performance of the plants. Some transgenic plants that have been tested satisfactorily in the growth room may not perform the same in the field.

However, not all people have the opportunity to run such full-scale field or growth room trials due to lack of facilities, high cost of operation, lack of assistance and time. As a field trial is always conducted in an open area there is also the risk of spreading a new pathogen (or race of pathogen) into an area if it is not conducted properly. In addition, large numbers of plants are needed in field trials in order to ensure a sufficient sample size for adequate statistical studies to ensure the results are statistically significant. This is even more critical when the difference between the control and the experimental mean is predicted to be low. Potentially huge numbers of the planted materials are needed. There is no simple rule to accurately determine the right sample size for a field trial but in general the smaller the difference between the compared means then the larger the sample size is (Jerrold, 1996). This is obviously difficult to meet especially when the starting material is in limited supply.

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· See Glossary of Relevant Terms

A further problem concerning plant resistance tests in the field is the task of trying to ensure even distribution of a pathogen inoculum (challenge) between the population of experimental plants. In field trials this is normally achieved by distributing infected plants evenly within the trial plots such as by using infector plants to generate inoculum (Dorrance, *et al.,* 1998; Stewart *et al.,* 1983). In growth room trials spraying of pathogen inoculum is carried out (Stewart *et al.,* 1983). In both cases delivery of a pathogen challenge is highly variable. Some plants may have received an excess amount of the pathogen while the other may not receive enough inoculum to initiate disease. These problems coupled with variation in the levels of protein expression within the transgenic plants may contribute to large experimental errors and difficulties in quantifying disease development. There is also a growing concern about environmental impacts caused by openly testing transgenic plants. After considering all these problems, a semi-quantitative method was specifically designed for a small-scale transgenic plant susceptibility test in our laboratory. The method is applicable to any disease resistance test for different type of transgenic plants and is conveniently carried out in a normal growth room or illuminated incubator. The Department of Biological Sciences has no dedicated glasshouse or environmental room for pathogen work. Neither the Department nor the Botanic Gardens have adequate experimental plots for pathogen field trials nor for genetically-modified plants. The method is particularly useful where only limited plant material is available or where facilities are not available. This method is also useful for testing transgenic plants for pathogen resistance.

## 4.2 Results and Discussion

### 4.2.1 Preparation and Setting-up the Technique

The technique, later described as the leaf-bridge bioassay, was developed initially using the lysozyme transgenic plants from tissue culture. It was shown previously in chapter 3 (section 3.2.4) that the lysozyme protein in the leaf of transgenic plants containing the lysozyme gene was accumulating over the growing period. This may reflect the behaviour of the constitutive promoter used in the gene construct where the protein was continuously produced during growth of the leaves as well as low protein turnover at the site of deposition. Detached leaves from actively growing plants support the growth and spread of pathogens provided the leaves are maintained in

culture media. Furthermore, the previous work on the distribution and stability of lysozyme enzyme in transgenic leaves indicated that the detached leaves could be used for pathogen bioassays to test the efficacy of antimicrobial agents expressed in transgenic plants.

In the leaf-bridge bioassay method, detached leaves from transgenic plants grown in tissue culture were used. The leaves were placed in a three-compartment petri dish supplied with solidified, quarter-strength Murashige and Skoog (MS) medium (Figure 25).

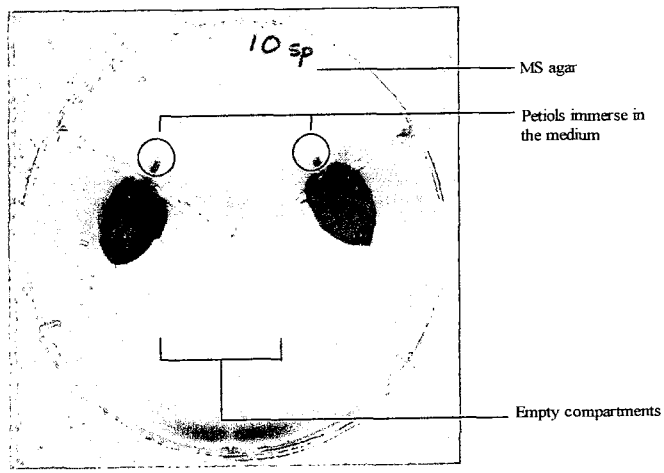


**Figure 25. The Leaf-Bridge Bioassay Set-up.** The orientation of the leaves in a three-compartment petri dish. The upper compartment contained 2% (w/v) Phytagel in hormone-free quarter-strength MS medium supplemented with 2% (w/v) sucrose.

The MS agar was used to support the leaves and also to supply water and basic nutrients needed to prolong life of the leaf *in vitro*. Although any type plant nutrients may be supplied, a medium similar to the tissue culture medium for maintaining the plantlets was preferred and gave the best results. In this study, a hormone-free quarter strength MS medium supplemented with 20 g $L^{-1}$ sucrose and 2 g $L^{-1}$ Phytagel was used. The sterile medium was poured into one compartment of the three-compartment petri dish used for the bioassay.

Plants used for these bioassays were produced by micropropagation to allow bulking of materials for multiple assays and to synchronise growth. Leaflets of identical size

and position on the plants were taken from 14-day-old axenic potato plantlets growing in tissue culture. The leaves were cleanly cut with a sterile scalpel blade at the base of the petiole to avoid damaging the tissue. The excised leaflets were examined to ensure they were free from any physical damage caused by cutting. Any such damaged material was discarded. For each set of assays, two leaflets were orientated in the bioassay plate as shown in Figure 25. The leaflets were arranged so that the end of the petiole was completely embedded in the agar medium and the rest of the leaf was raised over the partition to make contact with the base of the plate at the leaf tip (Figure 26).

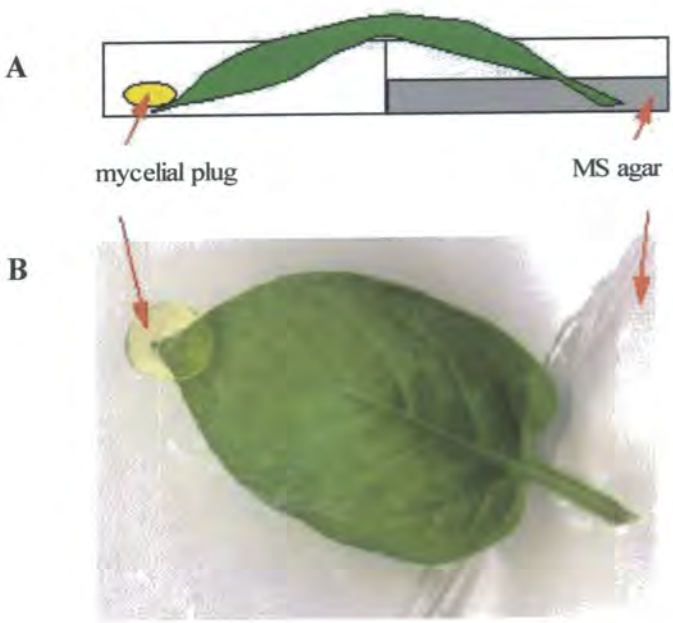


**Figure 26. The Assembly of the Leaf-Bridge Bioassay.** The leaflet was arranged so that the end of the petiole was embedded in the nutrient agar and the rest of the leaf was raised over the partition to make contact with the base of the plate at the leaf tip (A and B).

Pathogen inoculum either in the form of an agar plug of mycelium from an actively growing fungal plate (section 2.2.2.6) or a suspension of fungal spores (section 2.2.2.5) or bacteria (section 2.2.2.7) was positioned at the end of the abaxial leaf surface as shown in Figure 27. The plates were sealed using parafilm and vented with 3 small holes to allow air exchange. The inoculated leaflets were incubated in a refrigerated incubator at 15°C and the appearance of disease symptoms were observed

daily and the progress of infection was estimated every two days until the control plants were fully colonised by the pathogen. This normally took about 7 to 10 days depending on the leaf size and the pathogen used.

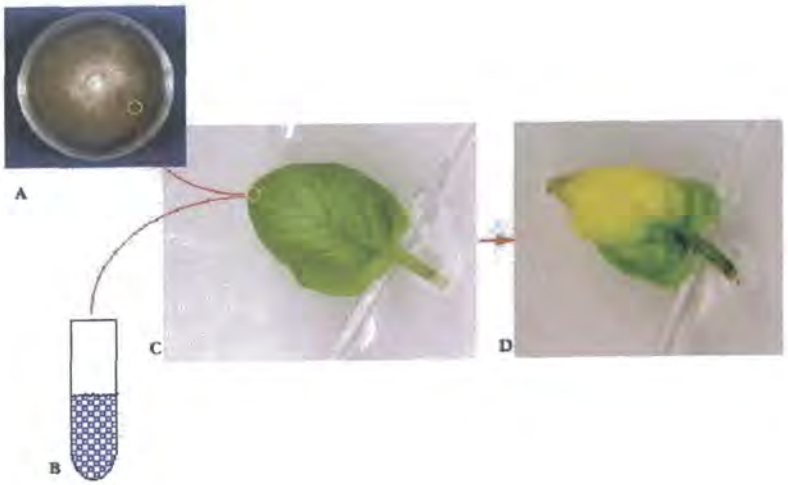


**Figure 27. Inoculation of Potato Leaf with Pathogen Inocula in the Leaf-Bridge Bioassays.** Source of inoculum is either a mycelial plug (A), a suspension of bacterial culture and spores (B). The inoculum was applied at the tip of the leaf (C) and incubated at room temperature for symptom development (D).

The petiole was in contact with the agar medium containing nutrients and water to maintain leaflet growth. Under these conditions leaflets remained healthy for at least 3-4 weeks. The remaining surfaces of the leaf were free from contact with medium or plastic surfaces. This was important to avoid any growth and spread of pathogen between leaf and plastic or agar surfaces. Instead the arrangement in the leaf-bridge ensured that the pathogen entered the leaf tissues and progressed internally through the leaf.

### 4.2.2 Quantitative Measurement of the Magnitude of Infection for Fungi

Disease progress in a leaf infected with fungal pathogens were more precisely monitored by following the movement of mycelial towards the opposite end. This was done by cutting infected leaves into five sections along the midrib using a 5-mm cork borer. The leaf disc represents the amount of mycelial at each section covering both

the vascular bundle and leaf laminae area. Each leaf section or disc was numbered, placed in PDA agar and incubated at 15° C to facilitate the fungal growth from each section. Disease progress was scored using a 0-5 scale according to the presence or absence of fungal growth on each disc. This scale represents linear progress of the fungal mycelium towards the uninoculated end of the leaf as shown in Figure 28.

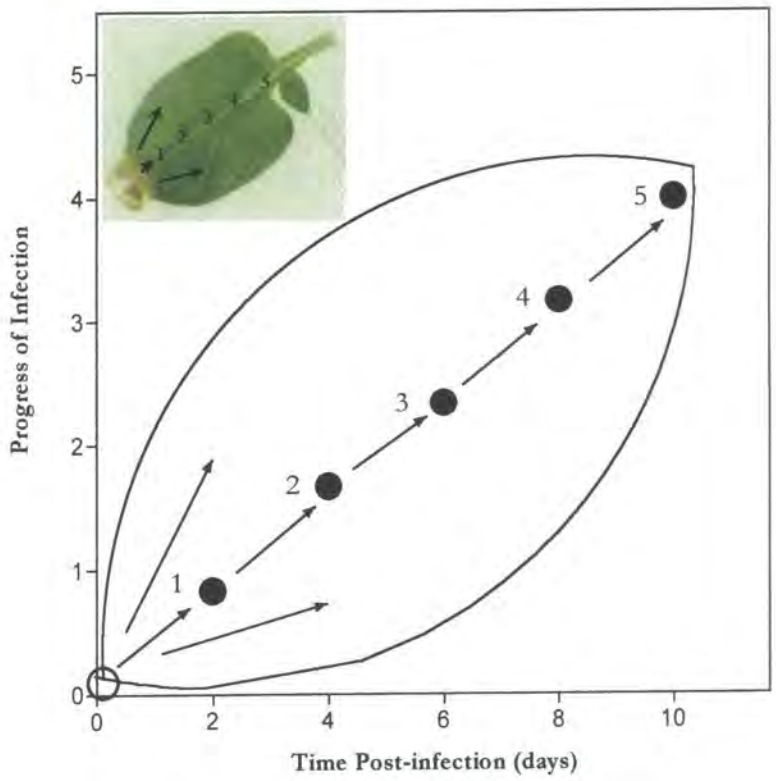


**Figure 28. Graphical Presentation of the Growth Progress of Mycelia in a Single Potato Leaf in the Leaf-Bridge Bioassay.** The black circles indicate the corresponding scale of the progress of infection. The open circle indicates the initial point where inocula were applied. The arrows showed possible directions of mycelial movement inside the leaf tissue. The assumptions in this model are that i) the pathogens grow indigenously, ii) the pathogens move in random directions inside the leaf tissues and iii) the mycelial movement is proportional to the incubation time under optimised inoculum density. The progress of infection across the leaves was determined by making 5 leaf discs as indicated by the black circles, and incubated for pathogen growth. The score was given based on the presence of the pathogen in the disc that was the farmost from the inoculation site. The magnitude of infection at a particular time point was the average of the progress of infection across the leaves at the corresponding time point.

### 4.2.3. Quantitative Measurement of the Magnitude of Infection for Bacteria – *Erwinia carotovora* subsp. *atroseptica*

The growth progress of the bacterial pathogen in the inoculated leaf was determined based on the average number of bacteria present in each of the 5 leaf discs sampled at each time point as shown in Figure 29. Counts were performed at four time points during 10 days incubation period. At each time point, six or more leaves based on the sample size of the experiment were harvested. Five discs from each leaf were cut using a 5-mm cork borer as for the fungal pathogen described earlier (section 4.2.2).

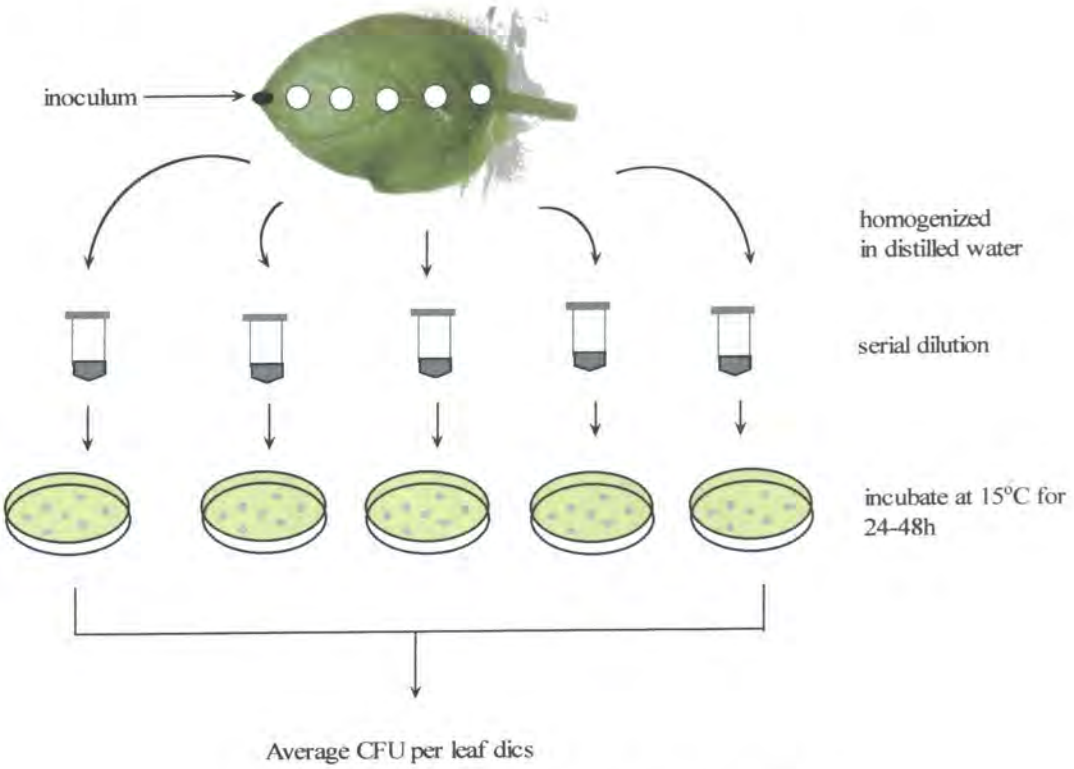


**Figure 29. Flow Diagram of the Procedure Used for Assessing Progress of Bacteria in the Leaf-Bridge Bioassay.**

The leaf discs were homogenised and diluted in distilled water before being incubated at 15°C for CFU determination (section 2.2.2.7). In this method, the mean CFU per disc obtained represents the severity of infection at a particular time. To simplify the analysis further, each mean of CFU per disc was rated according to a scale 1 to 6 as shown in Table 7. The scale represents log CFU mean at a particular time point and this number gives an indication about the multiplication speed of the pathogen throughout the leaf.

**TABLE 7. The Scale Used for Determining Magnitude of Infection in Leaf-Bridge Bioassay Using Bacteria.**

| Range of CFU count per disc | Scale (log CFU[a]) |
|---|---|
| 0-10 | 1 |
| 11-100 | 2 |
| 101-1000 | 3 |
| 1001-10000 | 4 |
| 10001-100000 | 5 |
| 1000000 | 6 |

a. log CFU for scale at each range was rounded to a maximum number so that the log CFU in proportional to the incubation time using an optimum numbers of bacterial inoculum.

### 4.2.4 Optimisation of the Leaf-Bridge Bioassay Technique

Challenging transgenic plants encoding microbial agents involves the choice of an effective plant inoculation procedure which ideally should be as near to the initial disease development in nature as possible. Any procedures which may potentially damage any plant leaf during the inoculation process should be avoided as this may create a new disease result from the introduction of pathogens into tissues which they would not normally colonise. Also, mechanical damage may induce a defence response. In this method, where leaflets from young potato tissue-cultured plants were used, optimisation was necessary to achieve suitable interaction between the potato plants and the corresponding pathogen. The optimisation involved the determination of the inoculum density required for the pathogen challenge process. The results presented here were obtained using the potato leaflets infected with *Erwinia carotovora* subsp. *atroseptica, Fusarium sulphureum, Rhizoctonia solani, and P. infestans.* With fungal species (except *R. solani*), suspension of spores were used since these were relatively easy to quantify (viable spores $mL^{-1}$) and pathologically, this is the infective stage of the pathogen.

### 4.2.4.1 Induction of Sporulation and Germination of *Fusarium sulphureum*

*Phytophthora* species produce sporangia, spore-fruiting from which zoospores were released. These are spores that can infect potato tissues and initiate late blight disease. Induction of sporulation of *F. sulphureum* was carried out as a time course of formation of conidia, an elongated curve-shape spore on a quarter strength PDA medium and it is called spore only throughout this thesis. Spores produced were counted at weekly intervals for a period of one month. Figure 30 (Panel A) shows that the production of spores increased over this period. This pattern is expected and is a well-known characteristic of most fungal species grown *in vitro*. Generally, fungal growth moved from vegetative growth towards the spore-productive stage as the nutrients in the medium decreased. It was also important in this study to check on the viability of the spores produced. The ability of the conidia to germinate was also shown to increase as the cultures aged (Figure 30, Panel B).

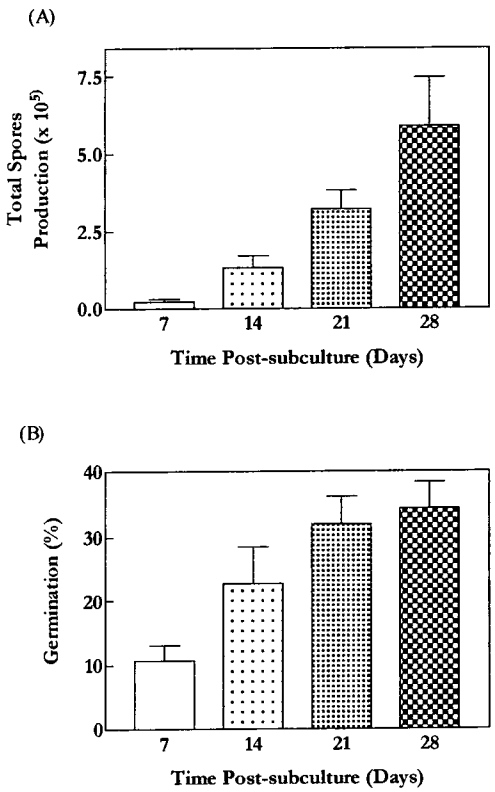


**Figure 30. Time-course of *Fusarium sulphureum* Spore Production *in vitro* (Panel A) and the Actual Number of Germinated Spores (Panel B).** Spore production was induced in a quarter-strength PDA at room temperature and germination was carried out on a PDA. Data shown are the means of four replicates in this particular experiment and error bars represent standard deviation.

The numbers of spores germinated were just above 10% for the 7-day-old spore, increased to 25 % at day 14 and just above 35% at the end of week 4. This result was essential to estimate the actual viable spore number for use as an inoculum in this bioassay technique.

#### 4.2.4.2. Induction of Sporulation and Germination of *Phytophthora infestans*

The sporangia were produced in a plate containing Rye A agar medium. The production was very low during the first week of growth and only slightly increased at the end of week 2 as shown in Figure 31 (Panel A). The highest level of production was recorded after 4 week of subculture. The sporangia produced at each time point are subject to zoospore liberation at 4 °C for 3 h before they were spread on a Rye A agar medium to germinate. It was hard to find zoospores from the 7-day-old sporangia, and after 3h of incubation at 4 °C most of the sporangia are still intact. Spreading this out directly onto the medium showed no sign of colony formation after 48 h of incubation. The release of zoospores can be easily detected on the suspension of sporangia aged 14 days and above. The percentage of germination is low for the sporangia harvested at day 14 but dramatically increased after 3 weeks (Figure 31, Panel B). This reflects the degree of maturity of the sporangia produced at each time point and the ability to release the zoospores.

Although sporangium itself is an infection unit of the pathogen, the zoospores are more likely to form and involve in the disease process in the presence of water. In dealing with the motile zoospores, it is extremely important that the whole leaflet is dry before inoculation is carried out. This is to ensure that the disease is only started at the inoculation site to allow the mycelial moving towards the other end of the leaf linearly. The zoospores can easily infect the leaf at various points if a layer of water is made available through the evaporation process during the incubation. This can be avoided by making sure that there are enough ventilation holes made before the plate is incubated. If the multipoints disease initiation were detected during the incubation, the leaf should not be included for the analysis.

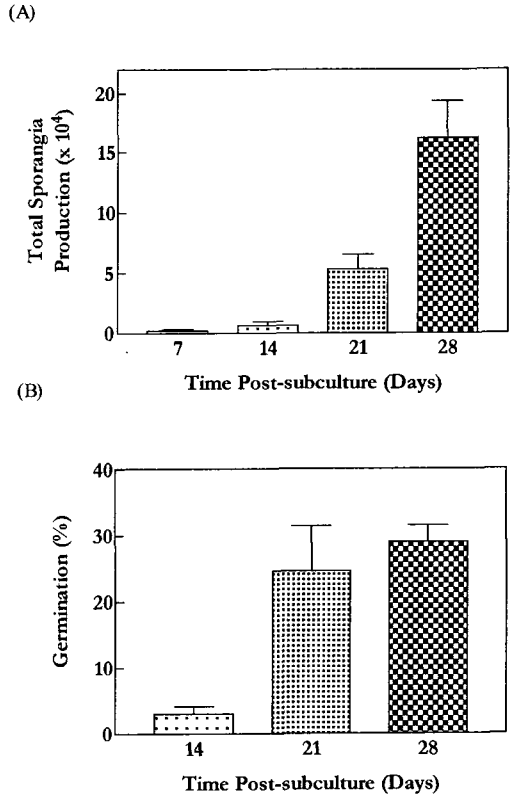


**Figure 31. Time-Course Measurement of Sporangia Production *in vitro* (Panel A) and the Actual Germination Rate of the Spores (Panel B).** Sporangia was induced on a Rye A agar at 15 °C and germination was carried out using a known number of zoospores on the Rye A agar. Data are the means of four replicates and error bars represent standard deviation in this particular experiment.

### 4.2.4.3. Preparation of Inoculum from *Erwinia carotovora* subsp. *atroseptica* cultures

The estimation of bacteria cell count was made by direct counts of the bacterial cells at time points during incubation or by plating out samples of a serial dilution of bacterial suspension to form a single colony. In this study the bacteria density was determined using a colony forming unit and the relationship between the CFU and the increasing medium density as a result of cell multiplication was established as shown in Figure 32. Using this standard curve, the inoculum density of actively growing *E. carotovora* subsp. *atroseptica* cultures was estimated.

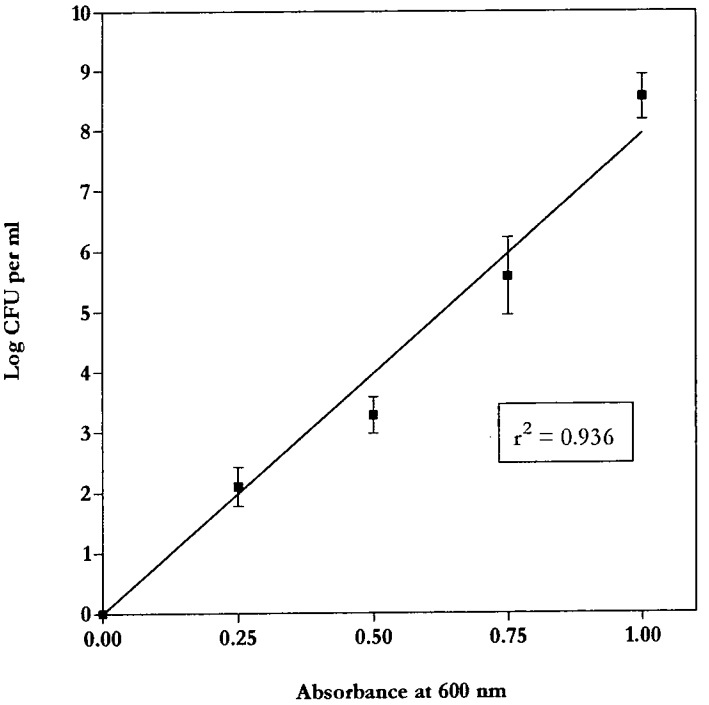


**Figure 32. The Relationship between Absorbance and Colony-Forming units of the *Erwinia carotovora* subsp. *atroseptica*.** The pathogen was cultured in a broth containing 1% (w/v) Trypticase Soy and 1% (w/v) glucose. Data are means of four replicates samples and error bars represent standard deviation.

### 4.2.4.4. Effect of Inoculum Density of *Fusarium sulphureum* on the Magnitude of Infection

For the testing, leaflets were carefully selected from a batch of non-transgenic plant and positioned into the three-compartment petri dish as described earlier (Figure 25 and 26). Individual leaflets were inoculated at the tip with different densities of spore suspensions ranging from 10 to 10 000 spores per point. The plates were incubated at 15 °C in a refrigerated incubator for disease development. Disease progress was monitored both by the visual observations and quantitative measurement of the progress of mycelial growth inside the tissue. Infected regions of leaves were indicated by the discoloration of the diseased tissues started from the inoculation point towards the other end of the leaf over the incubation period of 7 days as shown in Figure 33.

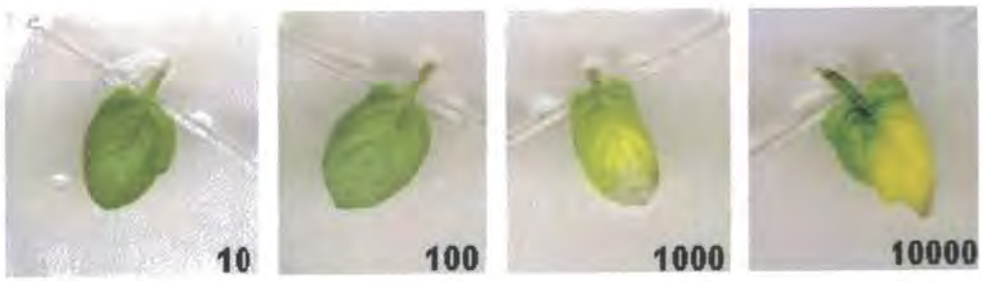


**Figure 33. Progress of Disease Symptoms Caused by *F. sulphureum* on Leaves of non-Transgenic Control Potato Leaflets.** The leaves were challenged with different numbers of variable spores. Disease development was scored after 7 days of incubation.

Leaflets challenged with 1000 or more viable spores per inoculation showed disease symptoms as early as 4 days after being inoculated. However, within this period, there was no visible difference between leaves challenged with 100 spores inoculation or less. After 10 days of incubation, leaves challenged with 1000 and 10000 spores per inoculation were fully colonised. At the same period, leaves challenged with 100 spores or less showed limited symptom development with only a small, yellowish brown lesion and limited growth of mycelium at the inoculated point were observed. There was no detectable visual symptom on leaves infected with 10 spores per inoculation during the whole experimental period. Results from disease incidence as shown in Figure 34, demonstrated that the disease occurrence among leaflets challenged with 100, 1000 and 10000 spores per inoculation were 31.3%, 81.3% and 100%, respectively. These figures were obtained from 16 leaflets randomly selected from equivalent position on 8 different non-transgenic control plants challenged with the corresponding spores densities. Disease incidence was recorded as positive when one or more leaf discs obtained from 7-day-challenged leaves showed growth of *F. sulphureum* (section 4.2.2) and calculated as shown in the equation below.

$$\text{Disease Incidence} = \frac{\text{Number of leaflets developing disease symptom}}{\text{Total number of leaflets challenged}}$$

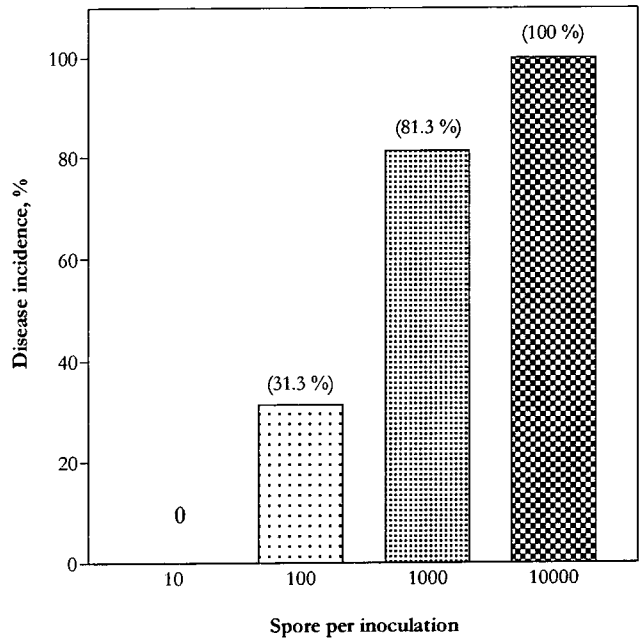


**Figure 34. Disease Incidence in Non-transgenic Potato Leaflets Inoculated with Different Numbers of *F. sulphureum* Spores Scored after 14 Days of Inoculation.** Data represent the percentage of leaflet showing disease symptoms at the end of day 14 of incubation period. The total numbers of leaves inoculated was 16 per challenge.

Quantitative assessment of disease development as measured by the progress of the mycelial towards the other end of the leaf showed that the relationship between the severity of infection and incubation period was nearly linear when the leaflets were challenged with 1000 spores for the time period of 10 days (Figure 35). The region of infected tissue was no longer proportional to the incubation time if more or less 1000 spores were used to challenge the leaves. This relationship provides a possibility of quantifying and scoring the disease incidence across the leaflet over the period of 10 days because a simple linear regression model could be applied in the calculation of disease progress. In order to apply the simple linear regression model, the number of *F. sulphureum* spores required to develop disease symptom in potato leaflet was 1000 per point and harvested within the incubation period of 10 days.

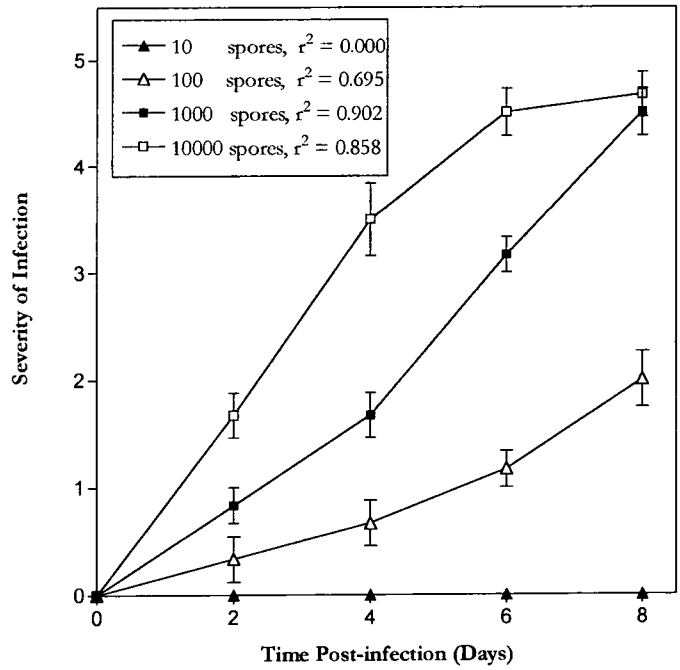


**Figure 35. Effects of *F. sulphureum* Spores Density on Mycelial Progress in Leaf Tissue Using the Leaf-Bridge Bioassay Method.** Each leaf was challenged with the amount of spores ranging from 10 to 10 000 as indicated in the graph. Data are the means of 10 replicate leaves and error bar is the standard deviations.

### 4.2.4.5. Effect of Inoculum Density of *Phytophthora infestans* on the Magnitude of Infection

*P. infestans* is a well-known pathogen for the potato causing late blight to the leaf. Infection units for this pathogen can be any of their vegetative and reproductive tissues including spores and hyphae. Zoospore was chosen to infect the leaf in this method simply because the disease is more likely to spread through the zoospores in nature and also the spores can be produced and quantified in the laboratory. Challenging the leaflets with different densities of zoospores resulted in different rates of disease development. At a zoospores density of 10 per site, the disease symptom was almost invisible throughout the incubation period of 10 days (Figure 36). Disease symptom was clearly seen in leaves challenged with 100 or more zoospores per site after 7 days of the incubation period.

The symptoms were developed from a small dark spot lesion at the site of inoculation and then spread across the leaves where more lesions developed especially on the leaves challenged with 1000 or more zoospores. Severed infected leaf tissue showed discoloration, chlorosis and water-soak lesions scattered on the leaves.

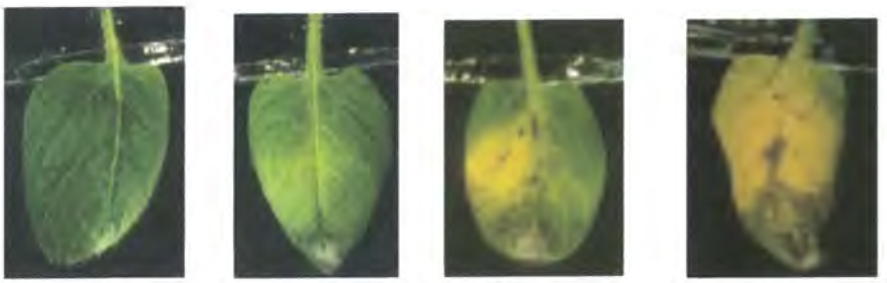


**Figure 36. Effect of Different Zoospore Densities on Disease Progress Using the Leaf-Bridge Bioassay Method.** The zoospores were applied at the leaf tip and incubated at 15°C for 10 days. These symptoms were taken after 7 days of incubation.

The percentage of disease incidence was increased up to 40% when the zoospores challenge was increased to100. At zoospore densities beyond 1000 per inoculation, the disease incidence after 10 days of inoculation reached 100 %. Regression analysis of the two lines corresponding to the data at 1000 and 10 000 zoospores density showed good linear relationships between disease progression and incubation time with $r^2$ values of 0.926 and 0.950, respectively as shown in Figure 37.

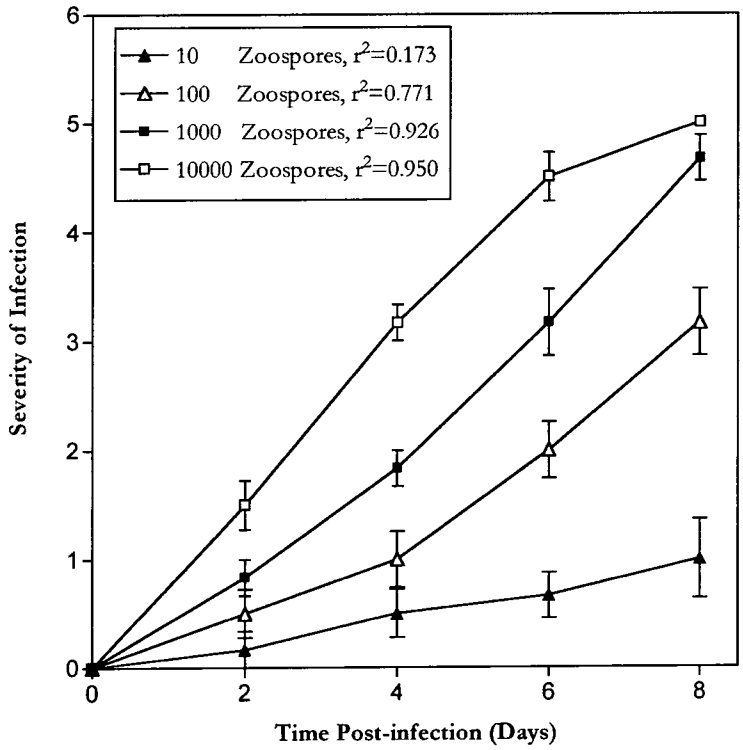


**Figure 37. The Magnitude of Infection Caused by Different Densities of *Phytophthora infestans* Zoospores in the Leaf-Bridge Bioassay Method.** Each leaf was challenged with the amount of zoospores ranging from 10 to 10 000 as indicated in the graph. Data are the means of 10 replicate leaves and error bar is the standard deviations.

### 4.2.4.6. Effect of Inoculum Density of *E. carotovora* subsp. *atroseptica* the Magnitude of Infection

*E. carotovora* subsp. *atroseptica* infections proceeded very rapidly in leaflets inoculated with 10 000 CFUs per site and the whole leaf turned dark brown and soft as shown in Figure 38, just 4 days after inoculation. The disease started with a small water-soaked lesion around the inoculation site and then followed with the discoloration of the tissue to turn dark brown and at the later stage the whole leaf became soft. This development can be seen in leaflets inoculated with low density bacterial cells at 100 to 1000 CFUs per site. Disease incidence in leaflets inoculated with more than 1000 CFUs per site showed a 100 % level after 5 days of inoculation whereas the leaflets inoculated with less than 100 CFUs recorded less than 50 %.

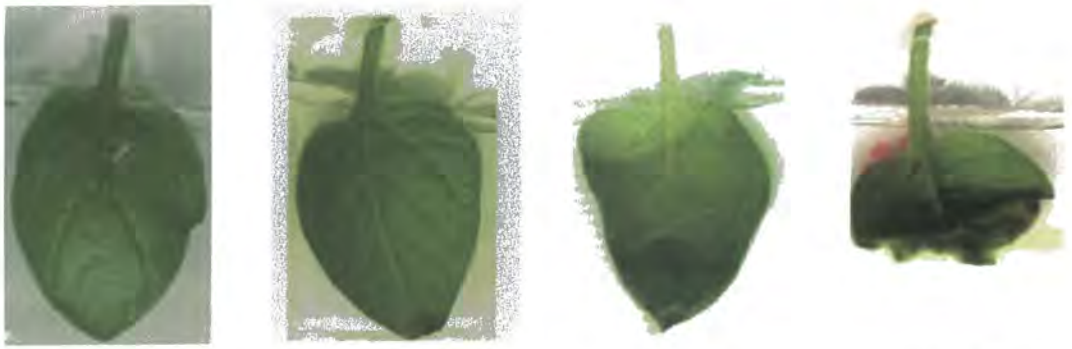


**Figure 38. Effect of Different *Erwinia carotovora* subsp. *atroseptica* Inoculum Density on Disease Development.** Black water-soak lesions, the typical symptoms for this disease clearly appeared early in the assay at the site of inoculation. This symptoms were recorded at days 4 days after pathogen challenge.

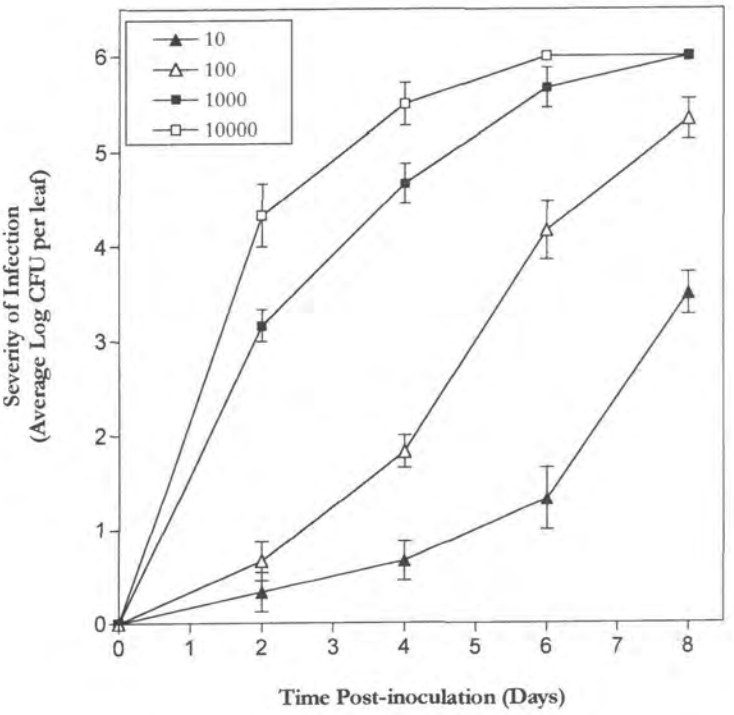


**Figure 39. Effect of Different CFUs of *Erwinia carotovora* subsp. *atroseptica* on Disease Development in the Leaf-Bridge Bioassay.** For each leaf, 5 discs (5 mm in diameter) were analysed and average for CFU. This value represents the average numbers of bacteria in each leaf. Data shown here are the means of six replicates leaves and error bars are standard deviation.

It was hard to establish a linear relationship between inocula density and disease severity in leaves infected with *E. carotovora* (Figure 39). The relationship seems to be non-linear and this makes a system used to scale disease severity using a linear-based classification inappropriate.

Optimisation of the technique for fungal pathogen *Rhizoctonia solani* did not follow the same line of the previous procedure described. In this pathogen, standard 5-mm diameter mycelial plug was used as a source of inocula. This is because the pathogen is a complex fungus producing no spores but different resting bodies *in vitro* when the cultures aged. The inocula were prepared using a 5-mm diameter cork borer from cultures of less than a week old to ensure the homogeneous form of the pathogen. They were then applied to the tip of the leaf and disease development monitored until the whole leaf became diseased or fully colonised with the fungus. Results obtained indicate that to fully colonise the leaf, the fungus needs 10 days or more of an incubation period. Disease incidence at this time point reached to a level of 100% and when the incubation period extended to 14 days all leaves were fully colonised.

### 4.3. Conclusion

The leaf-bridge bioassay described here is a simple and easy to set-up method for testing transgenic plants against pathogen of interest in the laboratory. It is particularly useful if the planting materials are limited and the field trial is not possible. With some simple optimisation to determine the progress of mycelium movement inside the host tissues for a particular plant-pathogen interaction, the method can be used to test transgenic plants against almost any type of microbial pathogens. The magnitude of infection measured, as a progress of mycelium movement inside the leaf tissues toward the petiole is proportional to the incubation time. Using a suitable scale as presented here, the magnitude of infection between leaf samples can be estimated and compared.

# CHAPTER 5

# EVALUATION OF TRANSGENIC POTATO PLANTS FOR DISEASE RESISTANCE

## 5.1. Introduction

Plants defend themselves from invading pathogens by activating a number of defence responses. These may include the reinforcement of cell wall components (Bradley, *et al.,* 1992), the synthesis of phytoalexins (Abenthum *et al.*, 1995) and the induction of various defence-related genes (Baillieu, *et al.* 1995). One commonly induced resistance mechanism in response to pathogen infection is the induction of pathogenesis-related proteins such as chitinases and glucanase (reviewed in Bowles, 1990). It has been shown that two of the induced proteins (i.e. chitinase and glucanase) have antimicrobial activities (Schlumbaum *et al.,* 1986; Mauth *et al.,* 1988) which target specific sites in fungal cell walls to demolish the integrity of the cell. Based on this principle, the plant defence system can be manipulated to produce high, constitutive levels of antimicrobial proteins which mimics and enhances the natural plant-pathogen interactions. Any genes encoding cell wall degrading enzymes (such as lysozyme, chitinase and glucanase) may be used to enhance resistance in plants. Such a strategy has been discussed earlier in section 1.5.1 and 1.5.2 and has shown some promising results, although none of them has been fully evaluated in the field or for effectiveness against wider ranges of plant pathogens.

In the Department of Biological Sciences, University of Durham this approach has been developed over the last 10 years and several transgenic crop plants, expressing different types of anti-microbial and anti-insect proteins, have been produced and are now undergoing various trials to test their performance. A part of the present research programme involved an assessment of three types of transgenic potato plants encoding potential antimicrobial proteins*. The transgenic plants include lines expressing lysozyme (Lys 75), chitinase (BCH 35) and GNA lectin (GNA 74). Apart from Lys 75 which was described in details earlier (section 3.1), the rationale for the selection of

* see Glossary of Relevant Terms

BCH 35 and GNA 74 were: i) BCH 35 contains bean chitinase gene which is a well-known example of pathogenesis-related (PR) protein while GNA has demonstrated anti-metabolic effects on insects (Gatehouse *et al.,* 1997), and is suspected of having antimicrobial activity also (J. Gatehouse, pers. comm.) ii) the BCH 35 and GNA 74 plants have been undergoing continuous propagation in tissue culture in Durham for several years and their levels of gene expression (protein accumulation) have been carefully monitored.

Chitinase is a member of PR protein group 3 which exhibits differential chitinase and weak lysozyme activities (Stintzi *et al.*, 1993). Some of them, either alone or in combination with 1,3-β-glucanases, have been shown to have enhanced antifungal activity *in vitro* and in transgenic plants (see section 1.5.1 for details of the chitinase-encoded transgenic plants). Generally, the antifungal activity of chitinase is due to the ability of this enzyme to hydrolyse homopolymer chitin (N-acetylglucosamine residues with β-1,4-linkages), a major structural component of fungal cell walls (Jach *et al.*, 1995).

GNA 74 contains the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA). Lectins, in general are proteins which specifically bind (or crosslink) carbohydrates. Recently, Peumans and van Damme (1995) defined plant lectins as proteins that bind specific mono- and oligosaccharides and possess at least one catalytic domain. GNA lectin, which was first isolated by van Damme *et al.* (1987) from the bulbs of snowdrop, specifically binds to mannose residues. The physiological functions of plant lectins remain unclear. It has been shown to be effective in controlling sap-sucking insects (Gatehouse *et al.*, 1997; Tang *et al.*, 1999). The insecticidal activity of GNA is due to the ability of the lectin to bind to the brush border membrane of insect larval guts which in turn disrupts the digestive capacity of the larval midgut (Fitches and Gatehouse, 1998).

Various studies have been done on the antifungal effects of lectins and the results were mixed. For instances, Mirelman *et al.* (1975) showed that wheat-germ agglutinin (WGA), a chitin-binding lectin from wheat embryos inhibited the growth of *Trichoderma viride*, a non-pathogenic fungus used in the test, at concentrations ranging from 600 to 1200 μg $mL^{-1}$. However, Schlumbaum *et al.* (1992) using the

same WGA and fungus as used by Mirelman *et al.* (1975), showed that at concentrations up to 25 mg $mL^{-1}$, purified WGA did not inhibit the growth of *T. viride*. In addition, all the commercial preparations of purified chitin-binding lectins (from wheat germ, tomato, potato, pokeweed, gorse, and concanavalin A, bean phytohemmaagglutin A, peanut agglutinin and osage orange lectin) failed to inhibit the growth of this fungus. The antifungal activity showed in Mirelman *et al.* (1975) was due to a chitinase contamination during purification steps using affinity chromatography (on separose-2-acetamido-N-(5-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine) as shown by Schlumbaum *et al,* (1986). On the other hand, Does *et al.* (1999) recently showed that isolectin I purified from stinging nettle inhibits the growth of various fungi i.e. *Botrytis cinerea, Trichoderma viride* and *Colletotrichum lindemuthianum* but the effect was only temporary indicating the presence of an adaptation mechanism in these fungi to the lectin. So far, there is no published report on the antifungal activity of GNA lectin and it was therefore of interest to investigate this using the existing transgenic potatoes GNA 74 to the selected potato pathogens used in the study.

### 5.1.1. Notes on the Transgenic Plants

The selection of Lys 75, BCH 35 and GNA 74 transgenic lines was based on high expression levels among the available lines within the corresponding transgenic group. Expression levels were either measured as a part of this study (Lys 75) or expression data was provided as measured by others (BCH 35 and GNA 74). This allowed for the assessment of the highest available expressing lines for the degree of resistance over control (non-transgenic) plants to the major potato pathogens such as *E. carotovora* and *P. infestans.* It was beyond the scope of this programme to establish the relationship between the level of gene expression in each transgenic plant and the level of resistance to each of the pathogen studied, and to test the efficacy of the strategy.

The transgenic potatoes carrying lysozyme gene were kindly provided by Mrs E.J.Croy as stored tubers, and planted in the glasshouse. These lines were already selected and characterised as to lysozyme levels of expression assessed by both enzyme activity and immunoblots using antilysozyme antibodies. The Lys 75 line was specially chosen based on its high lysozyme expression level (E.J.Croy; pers. comm.)

which was confirmed in the current study. The Lys 75 contained a chick egg-white lysozyme gene construct. The gene construct contained a double enhancer CaMV 35S promoter and CaMV termination signals as well as the template cDNA coding sequence for chick lysozyme with its own leader sequence (Figure 40).

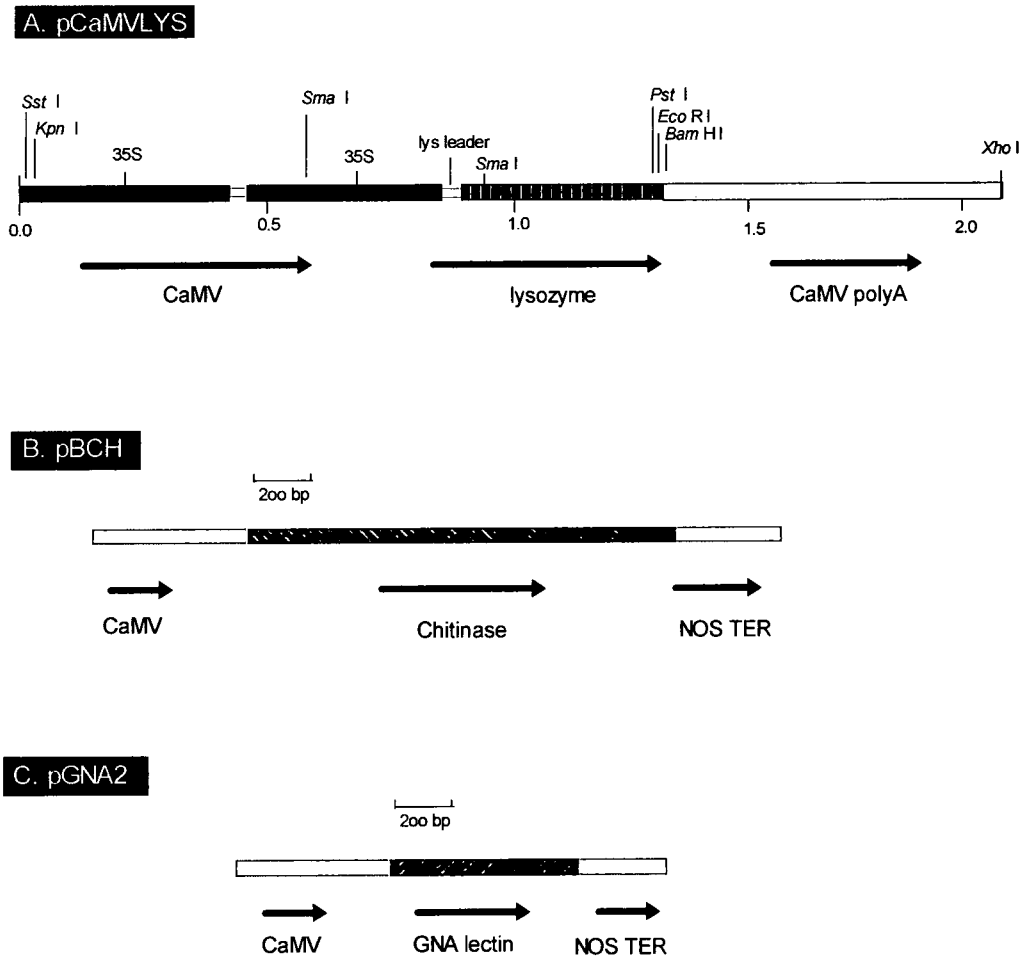


**Figure 40. Structure of Chimaeric Gene Constructs for the Transgenic Plants.** A. pCaMVLYS gene construct contained a double enhancer CaMV 35S promoter and CaMV termination signals as well as the template cDNA coding sequence for chick lysozyme with its own leader sequence. B. pBCH gene construct contained a CaMV 35S promoter, nopaline synthase terminator (NOS TER) and the template cDNA coding sequence for bean chitinase. C. pGNA2 contained a CaMV 35S promoter, NOS TER and the template cDNA coding sequence for snowdrop lectin.

The lines BCH 35 and GNA 74 have been characterised and kindly provided by Dr Angharad Gatehouse of Department Biological Sciences, University of Durham. The BCH 35 contained a bean chitinase gene construct. The gene construct contained a CaMV 35S promoter, a nopaline synthase terminator (NOS TER) and a template cDNA coding sequence for bean chitinase. The GNA 74 contained a snowdrop lectin gene construct. The construct contained the coding sequence for snowdrop lectin, NOS TER and the expression was driven by the CaMV 35S promoter (Gatehouse *et al.*, 1997). The lines tested, BCH 35 and GNA 74 were the highest expressors amongst the lines as assayed by immuno-dot blot using antibodies raised against each of the purified proteins (Dr Angharad Gatehouse, pers. comm.).

### 5.1.2. Notes on the Leaf-bridge Bioassay, Assumptions and Data Collection

All plantlets used for the resistance evaluation (Lys 75, BCH 35, GNA 74 and the non-transgenic control) were maintained in tissue culture by micropropagation as described previously (section 2.2.1.4). They were grown in the same batch so that the plantlets produced for the evaluation were in equivalent ages (section 4.2.1). The evaluation of these transgenic plants against potato pathogens (*F. sulphureum*, *P. infestans*, *R. solani* and *E. carotovora* subsp. *atroseptica*) was carried out using a leaf-bridge bioassay as previously described (section 2.2.3.2. Leaves of identical size from each transgenic plant were cut and positioned into a three-compartment petri dish as in section 2.2.3.2. Plates containing the leaves were pre-incubated at room temperature for 48 h to minimise any effects resulting from the leaf cutting and mechanical wounding response. Also, damaged leaves due to mishandling during set-up could be identified and eliminated during the pre-incubation period. Therefore, monitoring the leaf development during the first 48 h after set-up was crucial.

The variable measured was the magnitude of infection at each time point. The word infection applied here was the entry of inocula into plant tissues and the establishment of a permanent or temporary parasitic relationship as adopted from a guide to the use of terms in plant pathology at the British Society for Plant Pathology website (http://www.bspp.org.uk/fbpp.htm). In the present study, the presence of fungal mycelium or bacterial cells inside the leaf tissues after inoculation indicated an infection. The magnitude of infection at a particular time point was quantified as described in section 4.2.4.4. In quantifying the magnitude of infection, three

assumptions were applied: i.e. i) the pathogens grow endogenously ii) the pathogen movement inside the plant tissues is in a random direction and iii) the progress of infection inside is essentially proportional to the incubation time. The magnitude of infection was scored between 0 and 5 according to the semi-quantitative method described in section 4.2.4.4. The smaller the score, the better the performance of the plant would be. For the purpose of describing the graphs and statistical analysis, the word "scoring value" is used throughout and this is the magnitude of infection.

### 5.1.3. Notes on the Experimental Design, Statistical Analysis and Graphical Presentation of the Data

There are two factors under consideration in this experiment i.e. i) genetic material of the potato plantlets and ii) post-inoculation time. There are 4 levels in the first factor (i.e. Lys 75, BCH 35, GNA 74 and the non-transgenic control plantlets) and 3 levels in the second factor (3, 5 and 7 days after inoculation). Therefore the suitable experimental design used in this experiment was 4 x 3 factorial design with unequal replication. The unequal replication could not be avoided as some of the prepared "pre-incubation" leaflets were not suitable for the test and excluded from the whole batch of experiments. This was due to various factors such as stress resulting in leaf wilting, and mishandling of the leaves during the leaf-bridge bioassay preparation. In this experiment 4 potato pathogens were used. There were *F. sulphureum*, *P. infestans*, *R. solani* and *E. carotovora* subsp. *atroseptica.* Only one pathogen was used in each batch of experiment and therefore, 4 independent experiments were undertaken throughout the evaluation process.

Data obtained was statistically analysed based on the experimental design applied in the experiment. Because the two factors involved in the experiment may or may not be responsible to the outcome of the variable under consideration (i.e. the magnitude of infection), then the data was analysed using a two-way or two-factor analysis of variance (ANOVA) to confirm this. For the ANOVA test, the following assumptions were applied i.e. i) all leaves were randomly selected from "pre-defined" positions of leaves of plantlets (tissue culture material) grown in the same batch ii) the determination of magnitude of infection in each leaf was independent of one another i.e. the magnitude of infection was only monitored once in each leaf sample.

The result of the test indicates if there are any significant differences in the magnitude of infection among the levels for the factors under consideration. However, this test does not indicate which level of the factor is actually responsible to make the significant differences in the magnitude of infection, and therefore, a multiple comparison was employed. The multiple comparison used was a Dunnett's multiple comparison test. The Dunnett's test was chosen because it was specifically designed to compare all treatment means i.e. the magnitude of infection in leaves of Lys 75, BCH 35 and GNA 74 against a single control mean i.e. the magnitude of infection in leaf of non-transgenic control. All these tests were carried out using a Minitab version 12.2 (Minitab Inc). Typically, in many sciences, results that yield $P<=0.05$ are considered borderline statistically significant. Results that are significant at $P<=0.01$ are commonly considered statistically significant, $P<=0.005$ or $P<=0.001$ are called highly significant. These categories were used for the interpretation of the data in the present study.

In addition to statistical analysis, a graphical presentation for the distribution of the scoring values in each time point was plotted using a Box- and -Whiskers plot (GraphPad PRISM, GraphPad Software Inc.). The Box- and -Whisker plot was used to show the characteristics of the scoring values such as the "box", and the "Whisker" line. The box represents a range in which the scoring values are heavily scattered The upper and the bottom lines of the box are defined by the 25'th and 75'th percentile, respectively. In other words, the 25'th and 75'th percentiles lines show 25% and 75% of the scoring values lies below these lines, respectively. The median line which indicates 50% of the scoring values lie below the corresponding line and the mean of the scoring values are both positioned within the box. Therefore, the distribution of scoring values within the box is important for comparison. The whiskers on the other hand, are the lines that extend from the top and bottom of the box to the adjacent values. The adjacent values are the lowest and highest scoring values within the permitted lower and upper limits.

## 5.2. Results and Discussion

### 5.2.1. Expression of Lysozyme in Tissue-Cultured Transgenic Potatoes

As stated earlier Lys 75 was provided as a stored tuber and this was put back in the tissue culture for resistance tests. It was of interest to know if the lysozyme expression

in this transgenic line was still maintained at a high level after prolonged tuber storage and therefore lysozyme activity was monitored in tissue culture before being tested. This is in contrast with the other two transgenic lines, GNA 74 and BCH 35 which have been undergoing continuous gene expression studies in tissue culture by Dr. Angharad Gatehouse's Group, Department of Biological Sciences, University of Durham. These two lines (GNA 74 and BCH 35) were the higher expressors amongst the lines and kindly recommended and provided to be tested by the above-mentioned group.

The lysozyme activity in Lys 75 was measured using the colorimetric assay as described earlier (section 2.2.4.4). As the lysozyme levels were higher in leaves at the bottom of Lys 75 plants grown in glass house conditions, it was important to check if the same variation in levels occurred in plants grown in tissue culture. This was important information for selecting equivalent leaves for the pathogen testing. Three different leaves i.e. the second, third and fourth from the top of a 14-day-old plantlet were sampled and assayed separately for lysozyme. The three were selected based on their near identical size which is one of the criteria for selecting leaves for the pathogen testing. If there is significant variation amongst the leaves from the same plant some caution is needed in selecting leaves samples so that no systematic bias is introduced during sampling and preparing the leaf-bridge bioassay.

Figure 41 shows that the differences between the means of lysozyme activity in these leaves were not statistically significant at $p<0.05$, level unlike the situation in the glasshouse grown plants. This could be that the accumulation of lysozyme in the apoplast was still low within the first two weeks compared with glasshouse grown plants which were more mature. Most of the leaves at these positions were of near identical sizes. This is important because it is assumed that the same leaf size represents the same physiological stage of the plant and it also provides an identical surface area for scoring the infection in the leaf-bridge bioassay.

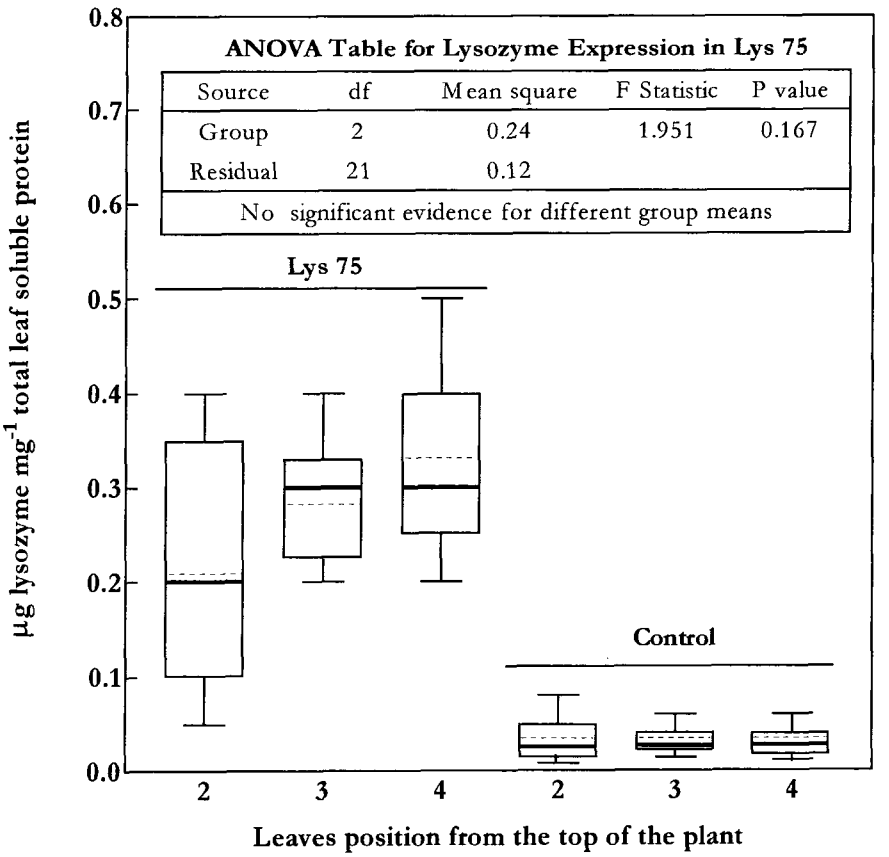


**Figure 41. Lysozyme Expression Levels in Leaf Tissues of Lys 75 in Tissue Culture.** The expression levels of lysozyme in different leaf positions in Lys 75 plantlets are presented as a Box- and -Whiskers plot. The plot shows the distributional characteristics of the data (i. the lowest value within lower limit, ii) highest value within upper limit, iii) first quartile iv) third quartile, median and mean. The thick line drawn across the box is the median and the dotted lines as a mean of the data. The bottom of the box is at the first quartile, and the top is at the third quartile value. The whiskers are the lines that extend from the top and bottom of the box to the adjacent values. The adjacent values are the lowest and highest observations within the lower and the upper limits. The ANOVA test shows that the differences between the mean of lysozyme levels in leaves from positions 2, 3 and 4 are not significant.

In general, most of the transgenic potato lines used in the current study, Lys 75, BCH 35 and GNA 74 showed little morphological differences amongst them in tissue culture. This was especially noticed in BCH 35 and GNA 74 lines where slightly small leaves were produced compared with the Lys 75. The morphological differences noticed here were unlikely to be due to abnormalities but rather due to the growing conditions. The GNA 74 and BCH 35 were previously propagated in jars containing 4 to 6 plantlets therefore, this could probably account for the production of undersized leaves as plantlets compete for space and nutrients. When these plantlets were maintained in a pair in the tissue culture jar, they performed to as near an identical morphological appearance as the Lys 75. All potato lines were propagated this way (one pair of plantlets per tissue culture jar) to maintain the optimal leaf sizes for resistance evaluation.

### 5.2.2. The Performance of the Transgenic Potatoes Lys 75, BCH 35 and GNA 74 against *Fusarium sulphureum*

#### 5.2.2.1. Brief Visual Description of Disease Symptoms

The first disease symptom in control leaves appeared after 3 days of inoculation. This was a discoloration of leaves starting from the inoculation site and then spread into wider areas toward the other end of the leaf after 5 days of incubation period. At the end of the incubation period (7 days), most leaves turned into yellowish brown. Also, aerial growth of the pathogen was observed on most leaves especially at the heavily infected area.

The development of disease symptoms and their appearance in transgenic leaves (Lys 75, BCH 35 and GNA 74) was almost similar when compared with the control, except for less severe disease symptoms in Lys 75 and BCH 35 lines. After 3 days of inoculation, most of the Lys 75 and BCH 35 leaves developed black spots near to the inoculation site. In some leaves, discoloured tissue, a yellowish green area starting from the inoculation site was developed. At day 5, most leaves in both Lys 75 and BCH 35 developed discoloured tissues about half the size of the leaf starting from the inoculation site. At days 7, the discoloured tissues turned into a yellowish- brown area about two third of the size of the leaf. An aerial growth of *F. sulphureum* on the leaf surface especially at the severely diseased area was also observed in some leaves. The disease symptoms for GNA 74 were almost similar when compared with the control.

### 5.2.2.2. Description of the Box-Whisker Plot for the Distribution of Data

Figure 42 (Plot A-D) shows the distribution of scoring values in each of the transgenic leaves (Lys 75, BCH 35 and GNA 74) in comparison with the control. The boxes which represent the heavily scattered scoring values in each leaf, were overlapped at day 3 post-inoculation as shown in Figure 42 (Plot A). In addition, the median for all leaves was the same. However, the boxes for Lys 75 and BCH 35 which completely overlapped between 0 and 1, were better than the boxes for GNA 74 (0.5 to 1) and control (0.5 to1.5). This indicates that the Lys 75 and BCH 35 showed some degree of resistance against the pathogen, *F. sulphureum* at this stage.

At time point 2 (5 days after inoculation), the boxes for Lys 75, BCH 35 and GNA 74 overlapped each other and their 75'th percentile levelled with the 25'th percentile of the control (Figure 42, Plot B). The boxes for Lys 75 and BCH 35 were completely overlapped between 1 and 2 and this was better than the GNA 74 (1.5 to 2) and the control (2 to 3). Again, this indicates the presence of inhibitory effects on the growth of the pathogen within leaf tissues of the Lys 75 and BCH 35. The GNA 74 also showed some inhibitory effects compared with the control although less compared with the Lys 75 and BCH 35.

After 7 days of inoculation, the Lys 75 performed well in comparison with the other transgenic lines judged by the box size ranging between 2.5 and 3 (Figure 42, Plot C). This was better than BCH 35 (2.5 to 3.5) although they share the same median line. The scoring values for GNA 74 were heavily scattered and completely overlapped within the range of the box for control (3 to 4).

The overall distribution of the scoring values for all plants regardless of the time points were shown in Figure 42 (Plot D). This was the combined scoring value from time point 1, 2 and 3. All boxes representing the distribution of the scoring values for the plants were overlapped as well as the Whisker lines. However, the box for Lys 75 (1 to 2) was better than BCH 35 (1 to 3), GNA 74 (1-3) and control (0-3.5). It was clear that, when the scoring values were combined, the overall magnitude of infection was reduced compared with the value, at a specific time point, indicating the importance of the right time point used for measuring the magnitude of infection in leaf-bridge bioassay.

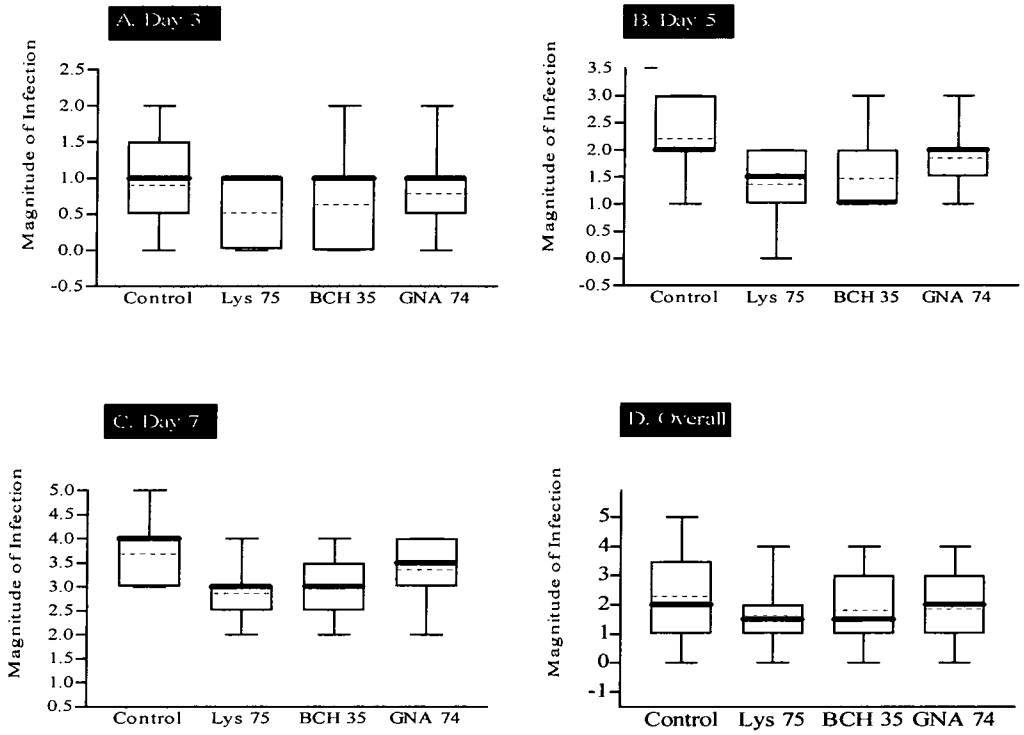


**Figure 42. Graph Showing the Progress of *Fusarium sulphureum* Mycelium in all the Transgenic and Control Leaves.** Data are presented as a Box Whiskers plot at each time point started from day 3 post-inoculation (Plot A), day 5 (Plot B) and day 7 (Plot C). Plot D represents the overall effect of pathogen to the leaves during the incubation period. This was the combined data at each time point and therefore the sample size was 3 times larger then the other plots (Plots A, B and C). The sample sizes for Lys 75, BCH 35, GNA 74 and the control was 12, 10, 8 and 9, respectively. Box-and-whisker plot shows the distributional characteristics of the data such as i). the lowest value within lower limit, ii) highest value within upper limit, iii) the 25'th percentile iv) the 75'th percentile v) the medians and vi) the means. A thick line drawn across the box is a median and the dotted line is a mean of the data. The bottom of the box is at the 25'th percentile, and the top is at the 75'th percentile. The whiskers are the lines that extend from the top and bottom of the box to the adjacent values. The adjacent values are the lowest and highest observations that are still inside the lower and the upper limits of the data. The extreme points i.e. values that lie beyond the limit if any are indicated as a star.

### 5.2.2.3. Statistical Analysis of the Magnitude of Infection

Table 8 summarises the statistical analysis of the results of the leaf-bridge bioassays with the three Lys 75, BCH 35 and GNA 74 lines against *F. sulphureum*. Data presented in this table were the means for scoring values (called "the mean score") in leaves Lys 75, BCH 35 and GNA 74 in comparison with the control at different time points after inoculation with spores of *F. sulphureum*.

**TABLE 8. The Performance of Different Transgenic Potato Lines Grown in Tissue Cultures against *Fusarium sulphureum* Infection as Assayed by the Leaf-Bridge Bioassay.**

| Transgenic Lines[a] | Number of Leaves/Time Points[b] | Magnitude of infection[c] | | |
|---|---|---|---|---|
| | | Time Post-inoculation (Days) | | |
| | | 3 | 5 | 7 |
| Control | 9 | 0.92 | 2.25 | 3.75 |
| Lys 75 | 12 | 0.50 | 1.42* | 2.83** |
| BCH 35 | 10 | 0.67 | 1.50 | 3.00* |
| GNA 74 | 8 | 0.75 | 1.80 | 3.40 |

[a]Each plant in each transgenic line was selected randomly a single batch of two-week-old plantlets grown in tissue culture.

[b]Similar sized leaflets from each individual plant were used for the tests. Leaves were fully developed and sampled only from position numbers 2,3 and 4 from the top of the plant.

[c]The magnitude of infection at a particular time point was quantified by monitoring the progress of infection towards the uninoculated end of the leaf and the magnitude of infection was scored using a scale of 1 to 5 as detailed in the text. Each presented value is the mean score of the magnitude of infection at a particular time point. Data were subjected to two-way ANOVA and the mean score of the transgenic lines at each time point was compared with the non-transgenic control line using Dunnett's post-test.

* mean score was significant at $p<=0.05$ compared with control within column.

**mean score was highly significant at $p<=0.01$ compared with control within column.

After 3 days of inoculation, the mean score for Lys 75 was lower (0.50) compared with the control leaves (0.92). The difference in the mean scores however was not statistically significant as indicated by the analysis. However, when infection progressed, the mean score for Lys 75 was lower as the differences between mean scores of the Lys 75 and the control were significant. The differences between the

mean scores were signed at both 5 and 7 days post-inoculation with significant levels at $P$=0.025 and $P$=0.01, respectively. This indicates that lysozyme enzyme significantly reduced the magnitude of infection of the pathogen *F. sulphureum* in Lys 75 leaves.

For BCH 35, no significant differences can be signed in mean scores of the BCH 35 and the control after 3 days of inoculation. The mean score for the BCH 35 line showed some degree of reduction after 5 days of incubation compared with the control but the differences were not significant. The differences in mean scores for the BCH 35 and the control were close to the level of acceptance ($P$=0.061) at this stage. At time point 3 (7 days after inoculation), the differences between mean scores of the BCH 35 and the control were nearly significant ($P$=0.053). This result suggests that chitinase has some inhibitory effect on the magnitude of infection in BCH 35 leaves.

For GNA 74, the differences between mean scores were not significant compared with the control leaves at any time points suggesting that GNA lectin in GNA leaves has no inhibitory effect on the growth of *F. sulphureum.*

### 5.2.3. The Performance of the Transgenic Potatoes Lys 75, BCH 35 and GNA 74 against *Rhizoctonia solani*

#### 5.2.3.1. Brief Visual Description of Disease Symptoms

The development of disease symptoms for *R. solani* infection in control leaves was initiated from the inoculation site after 3 days of inoculation. This was a yellowish brown lesion at the area where the mycelial plug was placed. On some leaves, the fungus tends to grow on the leaf surface and this caused some problems for quantifying the magnitude of infection using the leaf-bridge bioassay method. To avoid false positive results, leaf discs were only made from mycelial-free areas of the leaf. After 6 days of inoculation, the leaf appeared to be dry and some parts of the leaf turned into greenish-brown. These symptoms were further developed and at the end of 9 days post-inoculation, a more intense symptom appeared. The leaf became dry and turned completely brown. The development and the appearance of disease symptoms in the BCH 35 and GNA 74 leaves were almost similar to the control. In Lys 75 leaves, less severe disease symptoms were observed especially after 6 days of inoculation as compared with the control.

### 5.2.3.2. Description of the Box-Whisker Plot for the Distribution of Data

Figure 43 (Plots A-D) shows the distribution of scoring values in the transgenic leaves (Lys 75, BCH 35 and GNA 74) in comparison with the control. At time point 1 (3 days post-inoculation) (Figure 43, Plot A) the boxes for Lys 75, BCH 35 and GNA 74 were overlapped and their upper 75'th percentiles lines levelled with the bottom 25'th percentile line for the control. The box which represents Lys 75 (0 to 1) was better than BCH 35 and GNA (0.5 to 1). The Lys 75 showed some inhibitory effects on the magnitude of infection of *R. solani* compared with the control (box range, 1 to 1.5).

At time point 2 (6 days after inoculation), the distributions of scoring values for Lys 75, BCH 35, GNA 74 and the control were nearly identical to the scoring distributions at time point 1 as shown in Figure 43 (Plot B). The boxes representing the transgenic lines were overlapped and their 75'th percentile lines levelled to the 25'th percentile line of the control plant. The scoring value distributions for the Lys 75 and the BCH 35 were similar at this stage except the Lys 75 has a higher mean score. They obviously showed some degree of inhibitory effects on the growth of *R. solani.* GNA 74 on the other hand, showed less inhibitory effects to the *R. Solani* growth as judged by its mean score which was closer to the control line.

At time point 3 (9 days after inoculation), the boxes for Lys 75 and BCH 35 were overlapped and their 75'th percentiles lines levelled to the 25'th percentile line of the control and the GNA 74 (Figure 43, Plot C). At this stage, the Lys 75 was better in terms of resistance to *R. solani* infection compared with the other two transgenic lines. This was judged by the range of the box (2 to 3 for Lys 75, 2.5 to 3 for BCH 35 and 3 to 3.5 for GNA 74) and also the 75'th percentile line which levelled to the median line of BCH 35. The overall distribution of the scoring values as shown in Figure 43 (Plot D), differentiate Lys 75 from the other lines. The scoring values for Lys 75, were heavily scattered in the range of 1 to 2 compared with the control box (1 to 3). On the other hand, the boxes for the BCH 35 and GNA 74 were identical (1 to 3) and this was completely overlapped with the control box. This indicates Lys 75 has some inhibitory effects on the growth of *R. solani.* As a conclusion, the Lys 75 and BCH 35 showed some degree of resistance to *R. solani* infection especially after 5 and 7 days of incubation. However, the difference in mean scoring values for these lines

compared with the control line were subjected to statistical analysis to determine the significance.

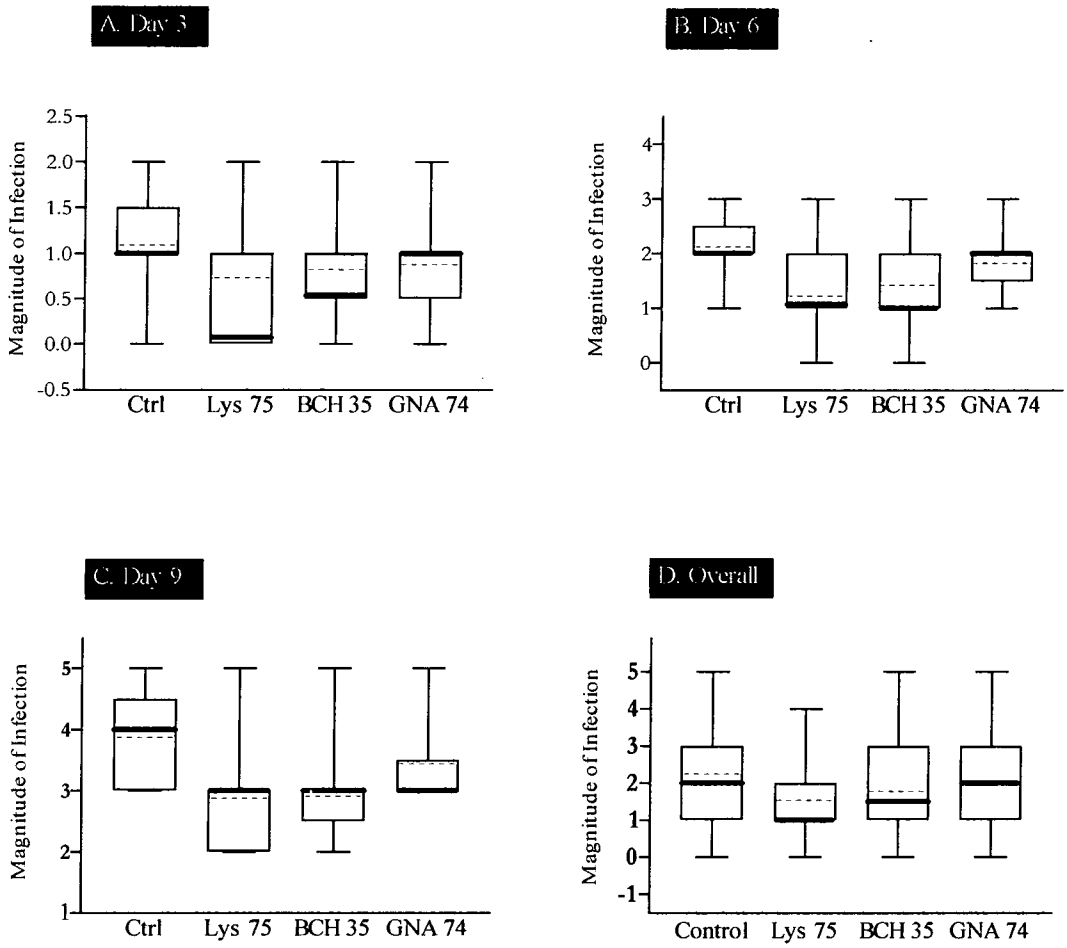


**Figure 43. The Performance of Various Transgenic Potato Lines against *Rhizoctonia solani* Infection.** Data are presented as a Box Whiskers plot at each time point started from day 3 post-inoculation (plot A), day 5 (plot B) and day 7 (plot C). Plot D represents the overall effect of pathogen to the leaves during the incubation period. This was the combined data at each time point and therefore the sample size was 3 times larger then the other plots (Plots A, B and C). The sample sizes for Lys 75, BCH 35, GNA 74 and control leaves at each time point were 12, 10, 8 and 10, respectively. The description of indicators within the graphs is the same as in Figure 42.

### 5.2.3.3. Statistical Analysis of the Magnitude of Infection

Table 9 shows the mean scoring values for the magnitude of infection of *R. solani* on Lys 75, BCH 35 and GNA 74 leaves in comparison with the control leaves. Dunnett's multiple comparison indicated that there were no statistical evidence that differences between mean scores of the transgenic lines and the control were significant for all the transgenic leaves after 3 days of incubation.

For Lys 75, the difference in mean score compared with the control line reached the borderline of significance ($P$=0.0564) after 6 days of incubation. This was further improved when infection progressed. The reduction in mean score in Lys 75 was significant compared with the control leaves after 9 days of inoculation ($P$=0.0119). For the BCH 35 and GNA 74, the reduction in the magnitude of infection was not significant at any of the points.

There was a potential problem when testing leaves with *R. solani* using the leaf-bridge bioassay method. The fungi tend to grow on the surface of the leaf instead of growing through the tissue. This leads to a false negative observation if precautions are not taken. One possible explanation for this is because the source of the inoculum used was a mycelium agar plug. The nutrient within the agar plug supported the continued growth of the fungus for some time before the infection process took place. The range of scores at each time point may reflect this situation where a higher range of data was recorded compared with the scores observed in other pathogens, as shown by the "whisker" line in Figure 43. While the use of spore was impossible in *Rhizoctonia* sp., applying a small size of mycelial plug probably reduced the problem. In addition the sample size would need to be increased in order to sign significant differences and improve the sensitivity of the statistical analysis.

**TABLE 9. The Performance of Different Transgenic Potato Lines Grown in Tissue Culture against *Rhizoctonia solani* Infection as Assayed by the Leaf-Bridge Bioassay.**

| Transgenic Lines[a] | Number of Leaves/Time Points[b] | Magnitude of Infection[c] | | |
|---|---|---|---|---|
| | | Time Post-inoculation (Days) | | |
| | | 3 | 6 | 9 |
| Control | 10 | 1.17 | 2.17 | 3.92 |
| Lys 75 | 12 | 0.75 | 1.33 | 2.92* |
| BCH 35 | 10 | 0.83 | 1.42 | 3.08 |
| GNA 74 | 8 | 0.90 | 1.82 | 3.75 |

[a]Each plant in each transgenic line was selected randomly a single batch of two-week-old plantlets grown in tissue culture.

[b]Similar sized leaflets from each individual plant were used for the tests. Leaves were fully developed and sampled only from position numbers 2,3 and 4 from the top of the plant.

[c]The magnitude of infection at a particular time point was quantified by monitoring the progress of infection towards the uninoculated end of the leaf and the magnitude of infection was scored using a scale of 1 to 5 as detailed in the text. Each presented value is the mean score of the magnitude of infection at a particular time point. Data were subjected to two-way ANOVA and the mean score of the transgenic lines at each time point was compared with the non-transgenic control line using Dunnett's post-test.

* mean score was significant at $p<=0.05$ compared with control within column.

**mean score was highly significant at $p<=0.01$ compared with control within column.

### 5.2.4. The Performance of the Transgenic Potatoes Lys 75, BCH 35 and GNA 74 against *Phytophthora infestans*

#### 5.2.4.1. Brief Visual Description of Disease Symptom

The first disease symptom was noticed after 3 days of inoculation. Most leaves showed discoloration of tissue starting from the inoculation point and then developed patches of water-soaked lesions surrounded by greyish-green tissue. These lesions expanded rapidly into a large, dark brown greasy-like appearance. The mycelial appeared to grow on the leaf surface of heavily infected tissues. In both the transgenic and non-transgenic leaves, the symptoms were severe with most of the leaves becoming completely diseased at the end of the 8 day of incubation period.

### 5.2.4.2. Description of the Box-Whisker Plot for the Distribution of Data

Figure 44 (Plots A-D) shows the scoring values distribution in each leaf challenged with *P. infestans*. At the first time point (4 days after inoculation), the boxes for all lines overlapped each other and the median lines for all plants levelled at 1 (Figure 44, Plot A).

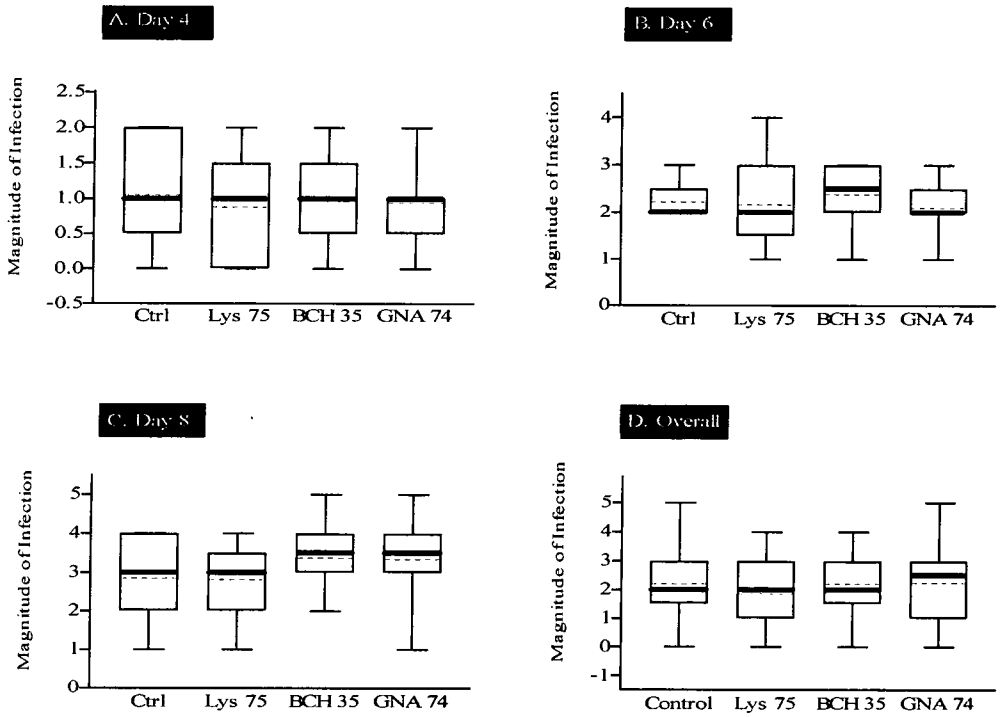


**Figure 44 (Plots A-D). The Performance of Various Transgenic Potato Lines against *Phytophthora infestans* Infection.** Data are presented as a Box Whiskers plot at each time point started from day 3 post-inoculation (plot A), day 5 (plot B) and day 7 (plot C). Plot D represents the overall effect of pathogen to the leaves during the incubation period. This was the combined data at each time point and therefore the sample size was 3 times larger then the other plots (Plots A, B and C). The sample sizes for Lys 75, BCH 35, GNA 74 and control leaves at each time point were 11, 9, 8 and 12, respectively. The description of indicators within the graphs is the same as in Figure 42.

The Whisker lines for all leaves were the same, 2 for the upper point and 0 for the lower. There were no obvious differences in the pattern of scoring values distribution suggesting no inhibitory effects occurred to the growth of the pathogen. At time point 2 (6 days after inoculation), the distribution of scoring values in each plant were almost similar to the previous time point, except the median for BCH 35 was higher

than the other plants (Figure 44, Plot B). After 8 days of inoculation, the magnitude of infection of *P. infestans* in all the transgenic leaves were either similar (Lys 75) or worse (BCH 35, GNA 74) compared with the control (Figure 44, Plot C). The combined scoring values corresponding to all the time points in each plant clearly indicated that there were no differences in distribution patterns in all plants challenged with *P. infestans* (Figure 44, Plot D). This suggested that *P. infestans* was not affected by the extra proteins (lysozyme, chitinase, lectin) produced in the transgenic lines.

### 5.2.4.3. Statistical Analysis of the Magnitude of Infection

Table 10 shows the mean scores of *P. infestans* in Lys 75, BCH 35, GNA 74 leaves in comparison with the control. The differences in mean scores for the transgenic lines (Lys 75, BCH 35 and GNA 74) were not significant at any time points compared with the control.

**TABLE 10. The Performance of Different Transgenic Potato Lines Grown in Tissue Culture against *Phytophthora infestans* Infection as Assayed by the Leaf-Bridge Bioassay.**

| Transgenic Lines[a] | Number of Leaves/Time Points[b] | Magnitude of Infection[c] | | |
|---|---|---|---|---|
| | | Time Post-inoculation (Days) | | |
| | | 4 | 6 | 8 |
| Control | 12 | 1.08 | 2.25 | 2.92 |
| Lys 75 | 11 | 0.83 | 2.17 | 2.83 |
| BCH 35 | 9 | 1.00 | 2.42 | 3.42 |
| GNA 74 | 8 | 0.92 | 2.10 | 3.30 |

[a]Each plant in each transgenic line was selected randomly a single batch of two-week-old plantlets grown in tissue culture.

[b]Similar sized leaflets from each individual plant were used for the tests. Leaves were fully developed and sampled only from position numbers 2,3 and 4 from the top of the plant.

[c]The magnitude of infection at a particular time point was quantified by monitoring the progress of infection towards the uninoculated end of the leaf and the magnitude of infection was scored using a scale of 1 to 5 as detailed in the text. Each presented value is the mean score of the magnitude of infection at a particular time point. Data were subjected to two-way ANOVA and the mean score of the transgenic lines at each time point was compared with the non-transgenic control line using Dunnett's post-test.

* mean score was significant at $p<=0.05$ compared with control within column.

**mean score was highly significant at $p<=0.01$ compared with control within column.

The poor performance of all the transgenic lines challenged with *P. infestans* may be due to the inability of the antimicrobial proteins expressed to cause any physical damage to the cell wall of the pathogen. *P. infestans* is a not a true fungus and its cell wall contains no chitin but they share a similar component, β-1,3-glucans with most other fungi. It is interesting therefore, to observe that the BCH 35 leaves did not show any resistance to this pathogen when one might have expected hydrolysis of these cell wall components. It is possible that the complex structure of the cell wall prevented access of the chitinase to susceptible linkages. Chitinase is induced in potato plants challenged with zoospore and culture filtrate of *P. infestans* inoculation in several studies involving potato (Kombrink *et al.*, 1988). The level of chitinase protein induced was reported to be less compared with other PR proteins such as β-1,3-glucanase suggesting that chitinase may be involved in another defence response which has nothing to do with the cell wall of *P. infestans*

### 5.2.5. The Performance of the Transgenic Potatoes Lys 75, BCH 35 and GNA 74 against *Erwinia carotovora* subsp. *atroseptica*

#### 5.2.5.1. Brief Visual Description of Disease Symptoms

After 3 days of inoculation, most of the control leaves showed a disease symptom of *E. carotovora* infection. These were water-soaked lesions originally from the inoculation site to about half the size of the leaf. The lesions spread rapidly and in some cases turned the whole leaf into soft discoloured tissue within 5 days of infection. After 7 days of inoculation, all control leaves turned into soft-discoloured tissue. In Lys 75, the disease symptoms were noticeably less severe compared with the control indicating the inhibitory effects of lysozyme to *E. carotovora*. A few spots of dark lesions surrounded by a yellowish green chlorotic tissue were amongst the first symptoms to appear in Lys 75 after 5 days of inoculation. These symptoms have developed into water-soaked lesions after 7 days of inoculation. At this stage, some leaves showed completely soft-discoloured tissue as observed in the control leaves. The effects of *E. carotovora* infection in both the transgenic lines (BCH 35 and GNA 74) were severe. After 3 days of inoculation, most of the leaves in both lines produced water-soaked lesions of comparable size to the control starting from the inoculation site. After 5 days of inoculation, some leaves in these lines turned into soft-discoloured tissue. Severe damage to the leaves in both lines were observed after 7 days of inoculation.

### 5.3.5.2. Description of the Box-Whisker Plot for the Distribution of Data

Figure 45 (Plots A-D) show the distribution of scoring values at each time point in Lys 75, BCH 35, GNA 74 leaves in comparison with the control. At time point 1 (3 days after inoculation), the boxes representing all leaves were overlapped except for Lys 75 (Figure 45, Plot A). The upper 75'th percentile line for Lys 75 levelled to the 25'th percentile line of the control and the GNA 74, and to the median line for BCH 35. This indicates that some inhibitory effects occurred in the growth of *E. carotovora* in Lys 75 leaves.

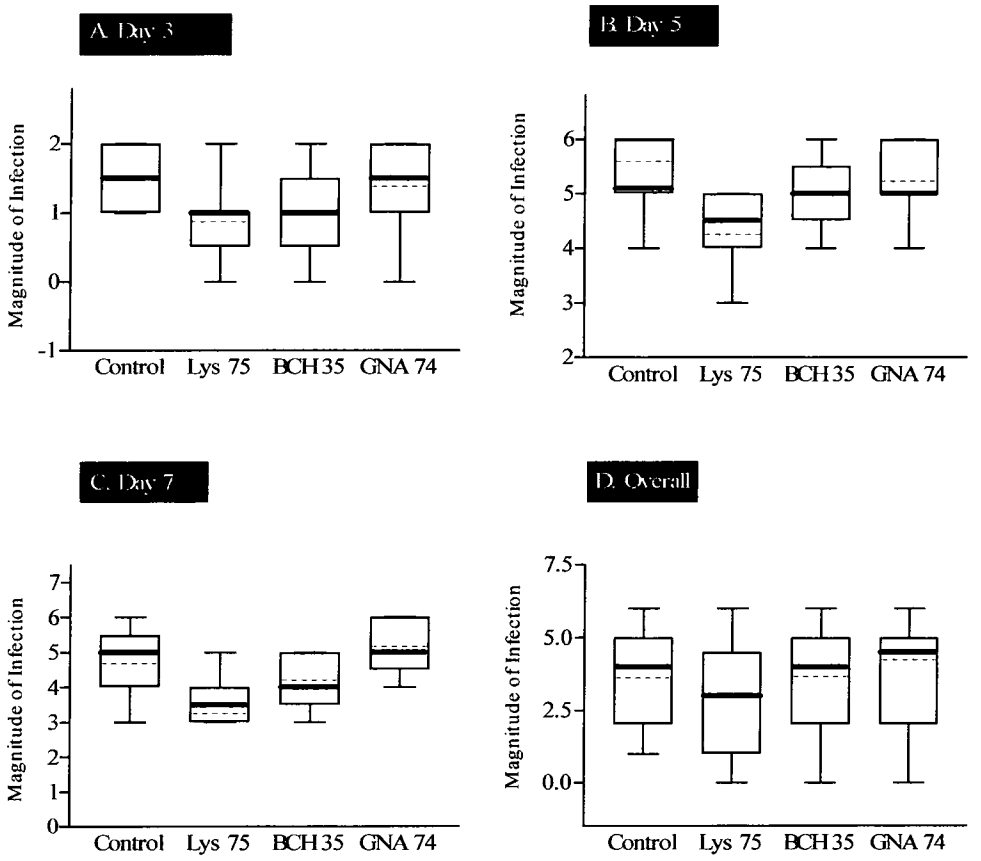


**Figure 45. The Performance of the Transgenic Potato Lines toward *Erwinia carotovora* subsp. *atroseptica* Infection.** Data are presented as a Box Whiskers plot at each time point started from day 3 post-inoculation (plot A), day 5 (plot B) and day 7 (plot C). Plot D represents the overall effect of pathogen to the leaves during the incubation period. This was the combined data at each time point and therefore the sample size was 3 times larger then the other plots (Plots A, B and C). The sample sizes for Lys 75, BCH 35, GNA 74 and control leaves at each time point were 10, 8, 7 and 10, respectively. The description of indicators within the graphs is the same as in Figure 42.

The inhibitory effects of Lys 75 were also clearly observed after 5 and 7 days of inoculation. The upper line of the boxes (75'th percentiles) were both levelled to the 25'th percentile line of the control leaves. The BCH 35 showed little difference at time points 5 and 7 as judged by the corresponding boxes. The median for BCH 35 at both time points levelled to the 25'th percentile of the control leaves. The GNA 74 leaves on the other hand, showed almost similar scoring distribution regardless of time points, suggesting that the lectin protein in GNA 74 leaves has no effect on the growth of *P. infestans.*

### 5.3.5.3. Statistical Analysis of the Magnitude of Erwinia carotovora Infection

Table 11 shows the result of challenging the transgenic lines with *E. carotovora.* Dunnett's multiple comparison showed no significant reduction in mean scores of the transgenic lines compared with the control leaves at time point 1 (after 3 days of inoculation).

**TABLE 11. The Performance of Different Transgenic Potato Lines Grown in Tissue Culture against *Erwinia carotovora* subsp. *atroseptica* Infection as Assayed by the Leaf-Bridge Bioassay.**

| Transgenic Lines[a] | Number Of Leaves/Time Points[b] | Magnitude of Infection [c] | | |
|---|---|---|---|---|
| | | Time Post-inoculation (Days) | | |
| | | 3 | 5 | 7 |
| Control | 10 | 1.51 | 5.50 | 4.75 |
| Lys 75 | 10 | 0.88 | 4.18* | 3.10* |
| BCH 35 | 8 | 1.00 | 5.00 | 4.16 |
| GNA 74 | 7 | 1.38 | 5.25 | 5.14 |

[a]Each plant in each transgenic line was selected randomly a single batch of two-week-old plantlets grown in tissue culture.

[b]Similar sized leaflets from each individual plant were used for the tests. Leaves were fully developed and sampled only from position numbers 2,3 and 4 from the top of the plant.

[c]The magnitude of infection at a particular time point was quantified by monitoring the progress of infection towards the uninoculated end of the leaf and the magnitude of infection was scored using a scale of 1 to 5 as detailed in the text. Each presented value is the mean score of the magnitude of infection at a particular time point. Data were subjected to two-way ANOVA and the mean score of the transgenic lines at each time point was compared with the non-transgenic control line using Dunnett's post-test.

* mean score was significant at $p<=0.05$ compared with control within column.

**mean score was highly significant at $p<=0.01$ compared with control within column.

Significant reduction in mean scores was only observed in Lys 75 after 5 days of inoculation ($P$=0.0136) showing that lysozyme confers significant resistance to *E. carotovora*. This effect was further confirmed in the later stage of incubation period where $P$ value remained significantly low ($P$=0.0342). On the other hand, no significant reduction in mean scores was observed in both the BCH 35 and GNA 74 leaves at all time points, suggesting that the chitinase and the snow drop lectin were both ineffective against this bacteria.

### 5.3. Conclusion

Leaf-bridge bioassay provides a quantitative way of evaluating transgenic plants against pathogen attack. Using this technique, the Lys 75 line showed significant resistance to *F. sulphureum* and *E. carotovora* subsp. *atroseptica*.. Good performance was also observed against *R. solani*. The BCH 35 showed some degree of resistance to *F. sulphureum* and no resistance to other pathogens. GNA 74 showed no resistance to any of the pathogens tested. None of the transgenic plants showed any resistance to *P. infestans* which is one of the major potato pathogens in the world.

# CHAPTER 6

# BIOCHEMICAL RESPONSES OF STIRLING CELL SUSPENSION CULTURES ELICITED WITH THE COMPATIBLE AND INCOMPATIBLE STRAIN OF *P. INFESTANS*

## 6.1. Introduction

The long-term objective of the present study was to develop potatoes that were resistant to the most important potato pathogens. Partial resistance to *E. carotovora,* one of the major potato pathogens and *F. sulphureum* has been shown in the lysozyme-transgenic plants, Lys 75 (Chapter 5). However, Lys 75 was not effective in preventing *P. infestans*, the causal agent of potato late blight disease. Late blight disease is the most important disease of potato responsible for the reduction of potato production world-wide (Fry and Goodwin, 1997). It is estimated that losses due to this disease alone are around 2 billion dollars annually (Kamoun *et al.*, 1999).

So far, none of the potato varieties developed through conventional breeding in various places around the world have the ability to prevent *P. infestans* infection. In addition, resistance mechanisms in potato to this pathogen are poorly understood. It is suggested that for long-term control of the disease, potatoes that possess a durable resistance need to be developed (Kamoun *et al.*, 1999). This is because durable resistance might be controlled by many genes which partially reduce the severity of the disease in the field. This strategy is better than the development of a potato variety in which resistance to *P. infestans* is controlled only by a single *R* gene due to rapid evolution of the pathogen. One of the potato varieties that show such resistance is "Stirling" (Dr Helen Stewart, pers. comm.).

There are several major molecular events that take place during host-pathogen interactions leading to defence responses in plants. These include the initiation and activation of signal transduction followed by many biochemical and physiological changes to suit the demands of the plant to overcome the invading pathogen (reviewed

· See Glossary of Relevant Terms

in Dixon and Lamb, 1990). One of the molecules responsible for the activation of the plant defence system is an elicitor. The term elicitor is used for molecules that stimulate any plant defence mechanism (Dixon and Lamb, 1990). There is a wide range of elicitors involved in plant-pathogen interactions ranging from glycopeptides, polypeptides, oligosaccharides and fatty acids (reviewed in Hahn, 1996). These elicitors are responsible for the induction of many defence mechanisms including the induction of a hypersensitivity response (Kamoun *et al.*, 1993; 1998), oxidative burst (Fauth, *et al.,* 1998; Nurnberger *et al.*, 1994) and the production of lignin and other structural materials (Oelofse and Dubery, 1996). Unfortunately, the *P. infestans* elicitors responsible for triggering defence responses in the potato are unknown. In order to study the response of potato cells to *P. infestans*, an elicitor derived from *P. infestans* cultures needed to be prepared. Because the identity of the elicitor is unknown, various types of *P. infestans* extracts were prepared from different stages of the growth cycle of the pathogen. The use of isolated elicitor preparations instead of the viable form of the pathogen avoided any complications when the plant cell suspension cultures were extracted for biochemical and molecular studies. Elicited cells were eventually used to prepare a subtracted cDNA library for the study of differentially-expressed genes during the compatible interaction of *P. infestans* and potato cell suspension cultures. In order to cover all possible sources of elicitor from different infective units and including specific and non-specific elicitors, a mixed elicitor preparation from culture filtrate, mycelial homogenate and zoospore extract of *P. infestans* was prepared. The mixed elicitor was prepared in a way that each source of elicitor in the mixture was present in an optimum and equivalent concentration for eliciting a response in the potato cell culture. This involved monitoring i) the induction of PAL activity as a biochemical marker of the activation of plant defence responses, ii) cell growth following elicitation. Induction of an oxidative burst, which is associated with the plant-pathogen interaction in an incompatible system, was also studied in this system. As a comparison, the oxidative burst was also monitored in an incompatible interaction of 'Stirling' cell suspension cultures with an elicitor mix isolated from an incompatible strain of *P. infestans*. In addition to elicitor, the viable zoospores of the pathogen were also used to study the oxidative burst.

### 6.1.1. Notes on the P. infestans Strain Used to Prepare Elicitors

The *P. infestans* strains used to prepare elicitors in the present study were SC 95 and IC2. SC 95 was compatible with 'Stirling' and IC2 was incompatible (details in section 2.2.2.1). Elicitors were isolated from different infective units of this pathogen i.e. zoospore, culture filtrate and mycelia homogenate. These elicitor preparations were designated as ZE (zoospore), CF (culture filtrate), MH (mycelia homogenate) and ME (elicitor mixed; combination of ZE, CF and MH). The incompatible-type elicitors were prepared in the same way as the compatible strain but using an incompatible strain of *P. infestans* isolate 13.2.3 of race 1,2,3,4 and 7. The *P. infestans* isolates were kindly provided by Dr Helen Stewart, Scottish Crop Research Institute, Dundee, Scotland. The elicitors were prepared once as in section 2.2.3.7, stored at –20°C and used throughout the whole project.

### 6.1.2. Note on the Optimisation of Elicitor Preparation for Use in Elicitation of Stirling Cell Suspension Cultures

In order to achieve the maximum response to elicitation in the potato cell suspension cultures, a serial dilution of each separate elicitor preparation was made and tested against the cells. PAL activity, as a general indicator of defence response activation was used as a biochemical marker. The objective was to select the lowest elicitor concentrations needed to induce maximum PAL activity with minimal (none) effect on the cell growth i.e. to minimising effect of any toxic components in the extract. PAL activity was measured after 6h of elicitation because a preliminary experiment undertaken in the present study showed that PAL was induced to its maximum level after 6 h of elicitation in both compatible and incompatible interactions of Stirling cell suspension cultures and the corresponding elicitors.

## 6.2. Results and Discussion

### 6.2.1. Optimisation of Elicitor Preparations from a Compatible Strain of *Phytophthora infestans* for Elicitation Studies

Figures 46 and 47 show the PAL activities and cell growth of Stirling cell suspension cultures after being treated with different elicitors of *P. infestans.* PAL activity varied when different dilutions of the different types of elicitor were added to the cells. The activities increased when the culture filtrates (CF) of *P. infestans* were added to the cell suspension cultures at 1:100 and 1:1000 dilutions but deceased at 1:10000 dilution

(Figure 46, Panel A). On the other hand, the cell growth was reduced when the higher concentration of CF was added to the cell cultures (Figure 47, Panel A). The reduction of cell growth indicated the possible presence of growth inhibiting factors (toxins) in the CF. This was not entirely unexpected as crude CFs were used in these experiments and a wide range of potentially active or toxic components are released into the culture filtrates of actively growing fungal pathogens as well as the elicitor molecules. Various types of secondary metabolites may function as necrotic factors which could kill the cells. It was essential to avoid the effects of these factors which may be associated with cellular lysis.

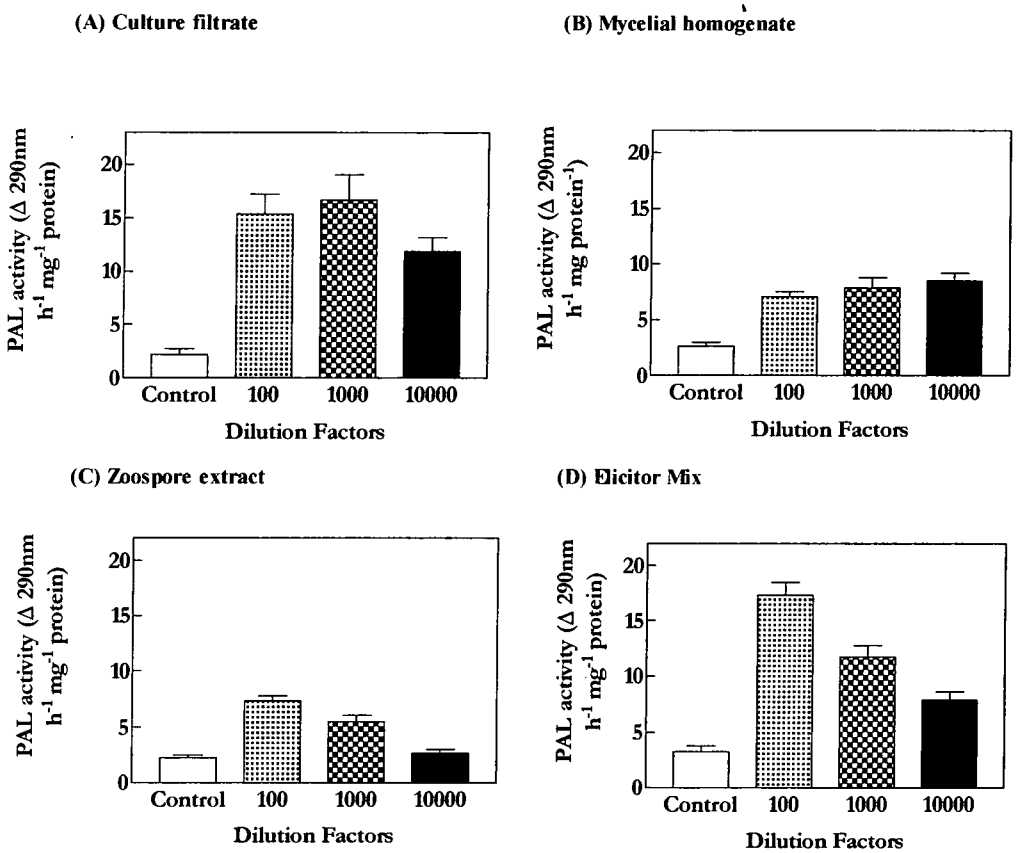


**Figure 46. Effect of Different Elicitor Preparations Isolated from Different Infective Units of a Compatible Strain of *P. infestans* on PAL Activity in Stirling Cell Suspension Cultures.** The enzyme activity was assayed after 6h of incubation. Data are the means of triplicate determinations and the error bars are the corresponding standard deviation.

Figure 46, (Panel B) shows that the activities of PAL varied slightly when mycelial homogenate (MH) at different dilutions were added to the cultures. The cell growth

was inhibited at high MH concentration (1:100 dilution) again indicating the presence of a necrotic factor inhibiting the growth of the cells (Figure 47, Panel B). When elicited with zoospore extract (ZE), maximum PAL activity was measured at 1:100 dilution (Figure 45, C). PAL activity was decreased when cells were treated with more diluted ZE (1:1000 and 1:10000 dilutions). There were almost no changes in cell growth when cells were treated with different concentrations of ZE as shown in.

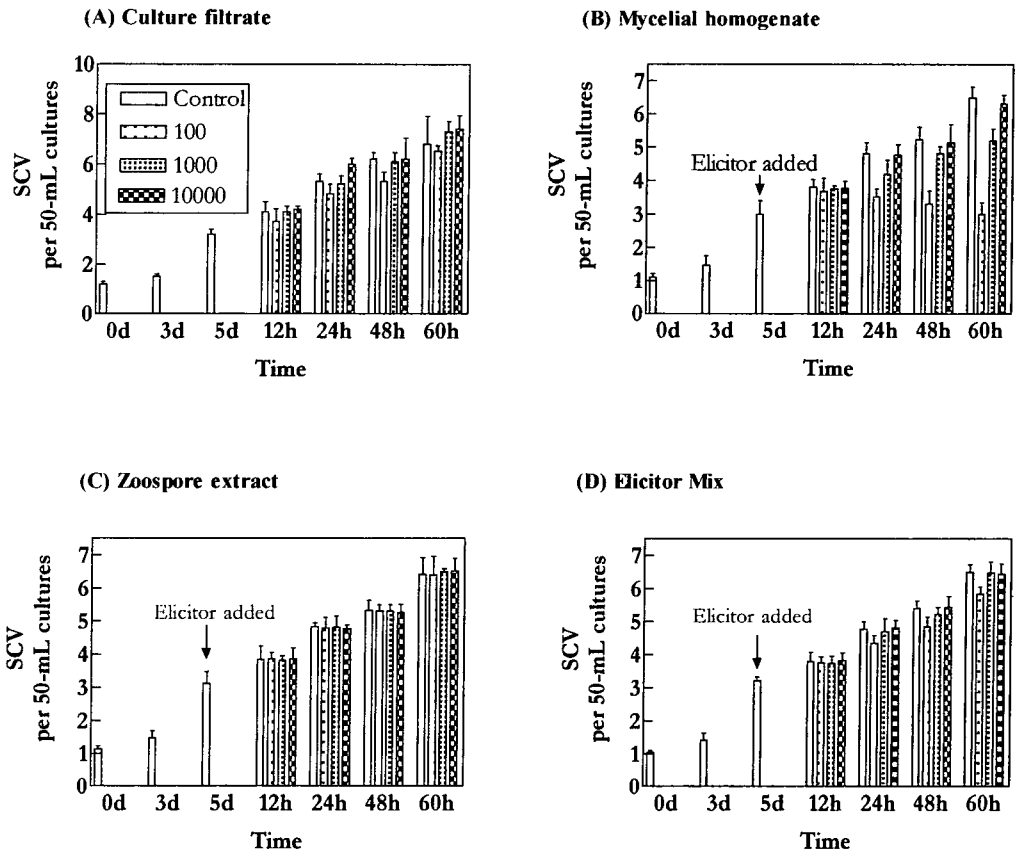


**Figure 47. Effect of Elicitor Preparations from Different Infective Units of Compatible Strain of *P. infestans* on the Growth of Stirling cell suspension cultures.** The elicitation was undertaken in actively growing cell suspension cultures and the settled cell volume was determined as described in section 2.2.3.8. Data are the means of triplicate determinations and the error bars are the corresponding standard deviations.

Figure 46 (C), suggesting either no inhibition factor may be present in the preparation or the concentration of crude preparation may contain low active elicitor or other inhibition factor. Regardless of the type of elicitor used, PAL activity responded

substantially indicating the non-specific nature of this enzyme towards a stress environment. Figures 46 (Panel D) and 47 (Panel D) show the PAL activities and cell growth of Stirling cell suspension cultures when treated with different dilutions of elicitor mix (ME) comprising CF (at 1:10000 dilution), MH (at 10000 dilution) and ZE (at 1:100 dilution) in a similar proportion. PAL activities were relatively lower when more diluted ME were added to the cultures (Figure 46, Panel D). The cell growth was slightly affected by ME at 1:100 dilution but no obvious differences could be observed when lower ME concentrations were used (Figure 47, Panel D).

The preparation of elicitors from an incompatible strain of *P. infestans* followed the same procedures used in the preparation of elicitors from a compatible strain. These elicitor preparations (CF, MH, ZE and ME of incompatible strain) were added to Stirling cell suspension cultures to monitor the PAL activities and cell growth. The results were similar to those obtained previously using the same types of elicitor preparations from a compatible strain of *P. infestans* (results not shown).

These experiments show that all elicitor preparations induced PAL activities in Stirling cell suspension cultures. These occur in both elicitor preparations either from the compatible or incompatible strains of *P. infestans*. It was obvious that an unknown growth inhibiting factor (s) may be present in CF and MH but none in ZF. The ME was prepared to comprise CF, MF and ZF in the same proportions; each at a dilution that exhibits minimum inhibiting effects to the cell growth. The 1:1000 dilution of the ME was used throughout the study.

### 6.2.2. Time Course of Changes in PAL Activity during Elicitation

It was important to check the kinetics of the induction of PAL activity to ensure that the required period for activation of other genes was reached. PAL activity was monitored in cells following elicitation for up to 48 h. PAL activities rose to a maximum level by the sixth hour post-elicitation in both compatible- and incompatible-elicited cells (Figure 48). With the elicitors from the incompatible strain, the activity increased rapidly and to a noticeably higher level compared with elicitors from the compatible strain. A further difference was observed in that PAL activities decreased sharply with the elicitor from the incompatible strain whereas in the compatible interaction the activity decreased slowly throughout the 48 h incubation period. The time-course of PAL induction reported here is similar to other

observations either in a tissue culture or in whole plant systems in the potato and some other plant species. For example, (Schmidt *et al.*, 1998) showed that PAL activity rises to maximum level in potato cell suspension cultures (*var.* Desiree) between 5 to 10 h after elicitation with a culture filtrate of *P. infestans* (Schmidt *et al.*, 1998). The compatibility status of the interaction in their study is not mentioned and therefore the specificity of the PAL response to elicitors from compatible or incompatible strains of *P. infestans* in the present study could not be compared. However, Awan *et al.* (1997) reported that PAL activity in suspension cultures of a resistant potato variety is higher compared with the susceptible cells when elicited with the same culture filtrate of *P. infestans.*

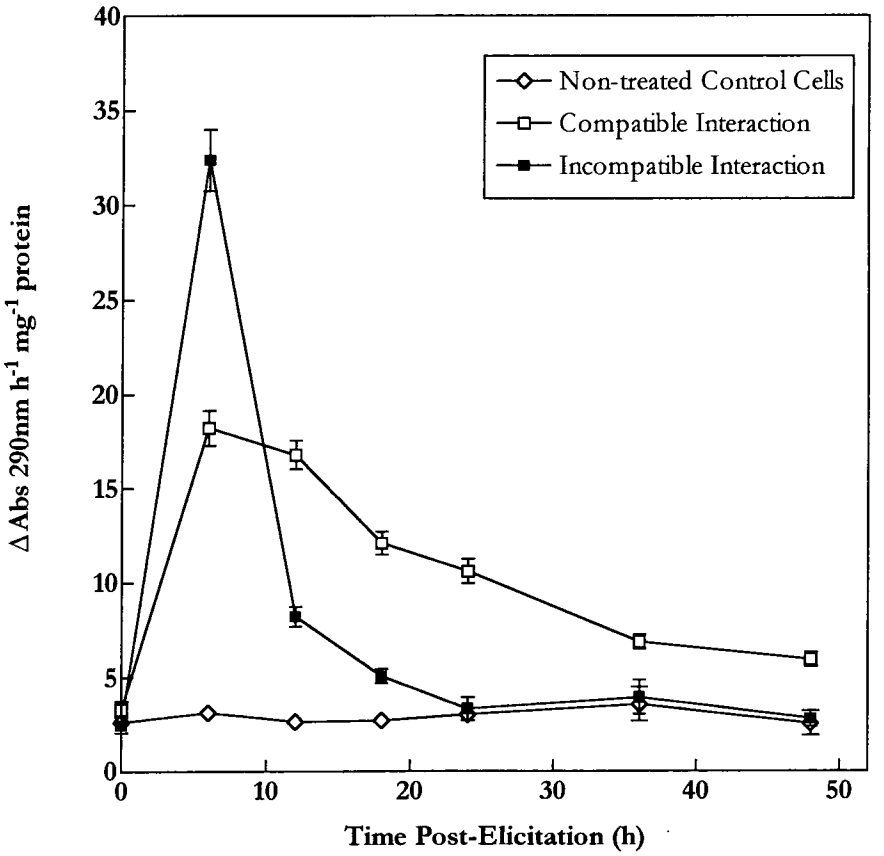


**Figure 48. Time course of induction of PAL enzyme in Stirling cell suspension cultures elicited with elicitors derived from compatible and incompatible strains of *P. infestans*.** Data are the means of triplicate measurements with error bars indicating standard deviation.

The maximum activity in resistant cells was more than twice that of the susceptible variety and reached a maximum level 15 h after elicitation (Awan *et al.* 1997). In the

present study the incompatible interaction induced PAL levels 60% higher than the compatible interaction. Apart from the difference in magnitude of PAL activity between resistant and susceptible cells there is no clear indication as to whether this enzyme is responsible for recognition of disease resistance in plants. PAL was also induced using yeast extract, a non-specific elicitor, in suspension cultures of *Glehnia littoralis* (Kitamura *et al.*, 1998) and also in plants undergoing stress or pathogen attack (Dixon *et al.,* 1995). These observations support the idea of the non-specific nature of PAL responses. The wide range of PAL inducers ranging from abiotic to biotic stresses may be explained by the many numbers of intermediates that are associated with the phenylpropanoid pathway. Some of these intermediates are involved in disease resistance while others are involved in wounding and development. For instance, the increase in PAL activity is concurrent with the accumulation of 4-hydroxybenzaldehyde, 4-hydroxybenzoate, and N-4-coumaroyl- and N-feruloyltyramine into the cell wall and secretion of N-4-coumaroyl- and N-feruloyltyramine into the culture medium of potato cell suspension cultures (Schmidt *et al.*, 1998). These phenolic compounds are thought to be involved in cell wall reinforcement and may also affect fungal growth in the apoplastic space (Schmidt *et al.*, 1998). The secretion of N-4-coumaroyl- and N-feruloyltyramine into the medium following elicitation (Schmidt *et al.*, 1998) may have explained the rapid browning of the culture medium when cell suspension of 'Stirling' was treated with elicitor preparations derived from *P. infestans.*

In the present study a mixed elicitor was used to elicit the cell culture to ensure that any stage-specific elicitors were present. None of the work previously reported has identified an elicitor that is responsible for the induction of defence reaction in potatoes. Elicitin (INF), an elicitor isolated from a culture filtrate of *P. infestans* (Kamoun *et* al., 1993) does not cause any hypersensitive response when applied to the potato but rather to other species such as tobacco, radish and turnip cultivars (Kamoun *et al.*, 1993). INF1 is thought to be one of the components responsible in non-host resistance of tobacco (tobacco is a non-host for *P. infestans*) to *P. infestans* (Kamoun *et al.*, 1997; 1998). This elicitor is expressed in the mycelium of *P. infestans* grown in various culture media but is not expressed in sporangia, zoospores, cysts, and germinating cysts, and during the host-pathogen interactions INF1 is induced only in the later stages of disease development i.e. when necrosis is occurring (Kamoun *et al.*,

1998). It could not be a specific elicitor not only because it fails to induce a hypersensitivity response but also in the way in which INF1 is produced. To be specific, an elicitor must be produced before the disease is developed. One of the most likely candidates is the zoospores as the infection process originally starts from this infective unit. Zoospores infect the leaf tissue by producing cysts, followed by the production of a germinating tube that is responsible for penetrating the tissue. During this process which takes only a few hours, some sort of endogenous elicitor could be produced which was responsible for determining the specificity of the interaction with the plant cells leading to either resistance or susceptibility.

### 6.2.3. Oxidative Burst in Suspension Cells of Potato Following Elicitation and Infection

A further indication of plant cells responding to elicitor or pathogen attack or stress is the 'oxidative burst'. This response is a burst of oxidative metabolism leading to the release of free radicals such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($^{\cdot}OH$) (reviewed in Bolwell, 1995; Wojtaszek, 1997). This substance is thought to be associated with defence reaction in plants specifically responsible in inducing hypersensitivity reaction as well as a participant in cell strengthening/repair and directly as a defence substance (antimicrobial factor). Elicitation was undertaken using mixed elicitors derived from compatible and incompatible strains of *P. infestans*, whereas infection was initiated using viable zoospores. The release of $H_2O_2$ in the culture medium was then monitored as described in section 2.2.4.7.

Figure 48 shows that the levels of $H_2O_2$ rapidly rose and reached a maximum level after 2 h using both elicitor preparations. This rise was followed by a sharp decrease and the levels diminished to basal values within a further two hours. $H_2O_2$ production was similar in both elicited cultures except that the peak level using a compatible elicitor was lower than that with the incompatible elicitor. The levels of $H_2O_2$ production in cell cultures treated with elicitors derived from the compatible and incompatible strains of *P. infestans* are similar to what was observed in PAL production in the corresponding cells; higher levels of PAL and $H_2O_2$ in cell cultures treated with the elicitor mixed derived from an incompatible strain of *P. infestans* compared with the compatible elicitor. Previous work has shown that the PAL gene can be activated as early as 10 to 20 min after elicitation in alfalfa cell suspension

cultures (Hi *et al.*, 1996). The rapid accumulation of $H_2O_2$ after elicitation as demonstrated in this study has also been reported in other systems. For instance, Jabs *et al.*, (1997) showed that by treating parsley cell suspensions (*Petroselinum crispum*) with fungal elicitor, an active oxygen species including $H_2O_2$ are detected before phytoalexin is synthesised.

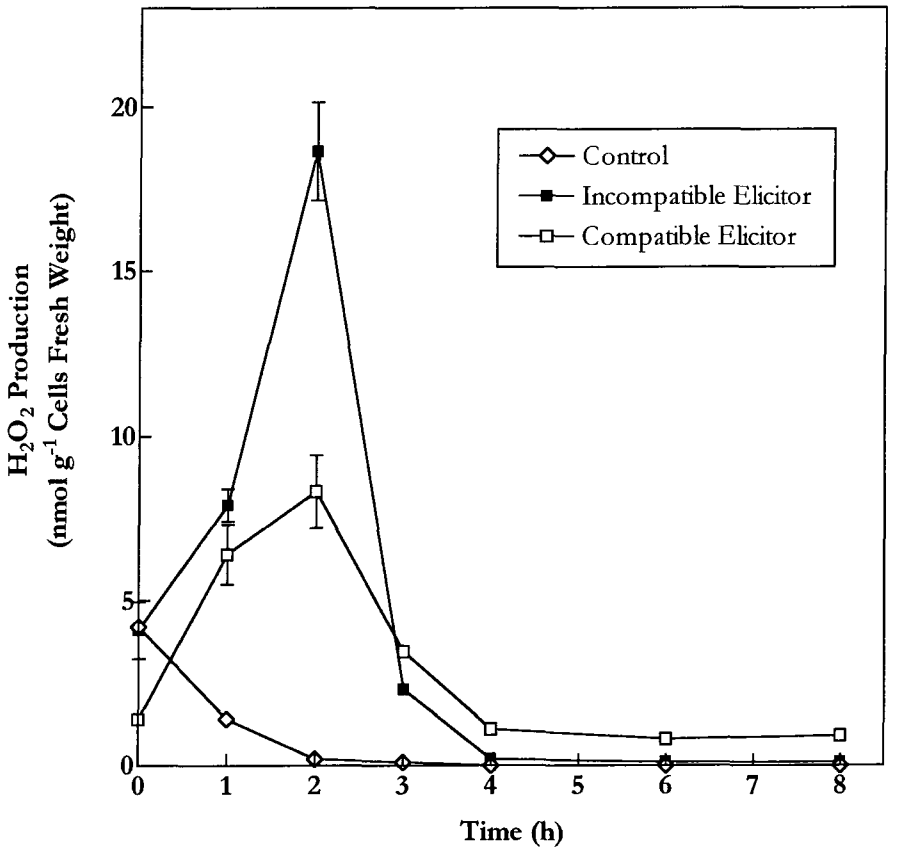


**Figure 49. $H_2O_2$-Levels Arising from 'Oxidative Burst' in Stirling Cell Suspension Cultures Induced by Incompatible and Compatible Elicitor Preparation Derived from *P. infestans*.** Aliquots of the media were sampled at each time point and assayed for $H_2O_2$. Data are the means of triplicate determinations and error bars represent the standard deviations.

High $H_2O_2$ production in incompatible cells during the first 2 h of elicitation may be related with the increase in pH in the medium as shown in Figure 50. External alkalisation as a result of changes in the ionic balance of the cells is a common phenomenon in suspension cells following incompatible elicitation (Bourque *et al.*, 1998; Mathieu *et al.*, 1996). Elicitation of suspension cells of tobacco by elicitin for

instance resulted in $Ca^{2+}$ influx and a corresponding extracellular medium alkalisation (Bourque *et al.*, 1998). In contrast, in the compatible-elicited cell suspension cultures the pH of extracellular medium decreased in this study. The pH steadily decreased until about 4 h post-elicitation before increasing back towards normal or slightly higher pH values after 4-12 h of elicitation. Higher pH observed at the later stage of this interaction seemed to have no effects on $H_2O_2$ accumulation indicating that the two events are probably independent at least in the later stages of the interaction.

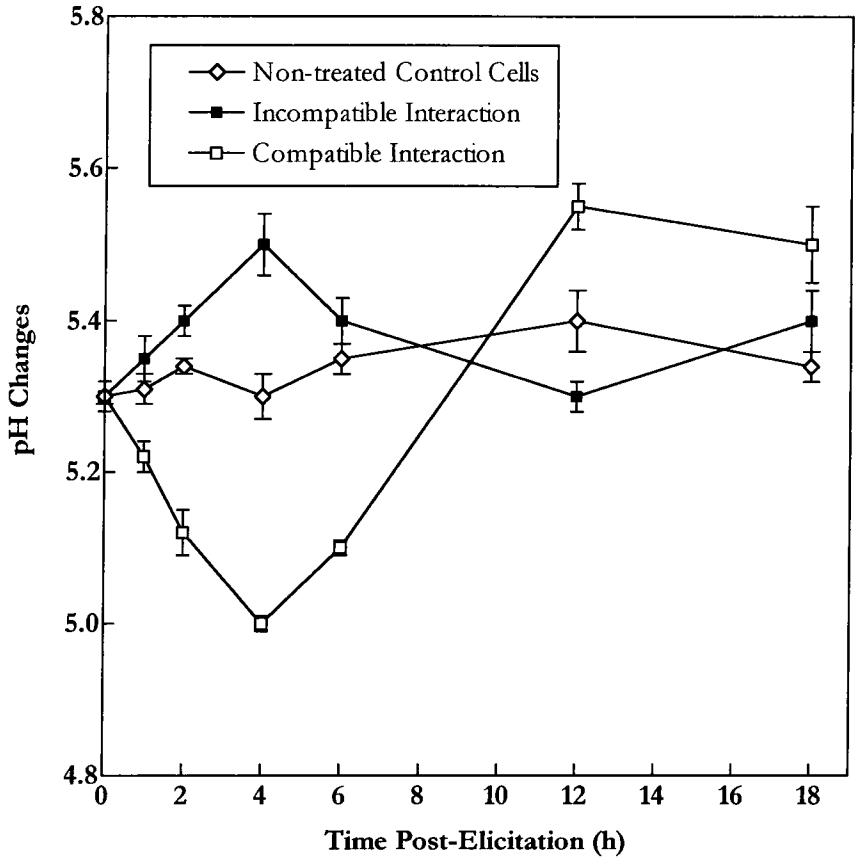


**Figure 50. Time-Course of Changes in pH of the Media in Stirling Cell suspension Cultures Following Elicitation by Elicitor Preparations from Compatible and Incompatible Stains of *P. infestans*.** Data are means of duplicate measurements with error bars showing standard deviation.

The accumulation of $H_2O_2$ in the media of cells occurred only at the first 2 h and this effect was diminished completely after 4 h regardless of the compatibility of the interaction. This indicates a corresponding effect i.e. secretion of a factor or an enzyme

which removes the $H_2O_2$. The significance of the $H_2O_2$ increase in the defence response of Stirling cell suspension cultures to elicitation in the present study is unclear. This is because i) the levels of $H_2O_2$ increased in Stirling cell suspension cultures treated with elicitors derived from compatible and incompatible strains of *P. infestans* and ii) $H_2O_2$ is produced not only as a result of pathogen attack (Able *et al.*, 1998) but also by abiotic stresses such as mechanical damage (Gus-Mayer *et al.*, 1998; Cazale *et al.*, 1998) and variation in the osmotic pressure (sucrose) of the culture medium (Cazale *et al.*, 1998). Study on transgenic tobacco carrying *hsr203*, a plant gene expressed during incompatible plant-pathogen interactions, demonstrated that $H_2O_2$ is unable to activate the gene promoter indicating that $H_2O_2$ is not the key messenger molecule responsible in activating defence responses in plants (Pontier *et al.*, 1998).

Investigations on the levels and patterns of $H_2O_2$ production in Stirling cell suspension cultures following inoculation with zoospores were also carried out in order i) to confirm the elicitation work and ii) to investigate if the process of plant-pathogen initial interaction produced a more potent elicitor. Infecting the Stirling cell suspension cultures with viable zoospores produced profiles of $H_2O_2$ production completely different from those observed during elicitation (Figure 51). At least three major peaks of $H_2O_2$ were clearly detected in both compatible and incompatible interactions. This pattern was not seen in suspension cells of potato treated with elicitor only. In the incompatible interaction the first 'burst' occurred after 1 h of inoculation and was followed by another two bursts at 3 h and 10 h post-inoculation, respectively. Previous study showed that in suspension cells of tobacco, two major bursts were reported to occur when the cells were infected with incompatible zoospores of *P. parasitica* var *nicotianae* based on the production of superoxide radicals (Able *et al.*, 1998). The first burst is reported to be small whereas the second one is much bigger. A similar profile was not observed in the compatible interaction (Abel *et al.*, 1998). Unlike Able *et al.*, (1998), in this study multiple peaks were recorded in both compatible and incompatible; three peaks of $H_2O_2$ accumulation were detected. The profile of $H_2O_2$ accumulation in both systems was nearly identical except that the magnitude of $H_2O_2$ production at 1 h post-inoculation was much higher in the incompatible interaction.

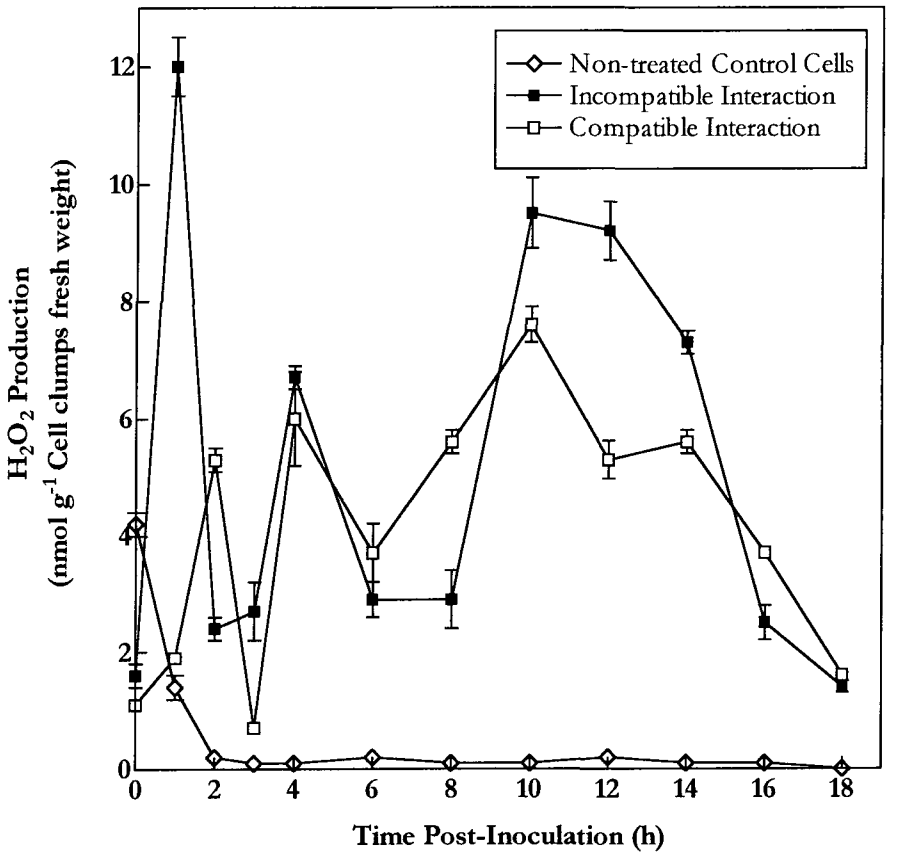


**Figure 51. $H_2O_2$-Levels Arising from Oxidative Burst in Stirling Cell Suspension Cultures Induced by Zoospores from Incompatible and Compatible Strains of *P. infestans*.** Aliquots of the medium were sampled at each time point and assayed for $H_2O_2$. Data are the mean of triplicate determinations and error bars represent standard deviation.

The $H_2O_2$ production in the two systems seemed also to be pH-dependent as the accumulation of $H_2O_2$ was concurrent with increased pH in the medium as shown in Figure 52. The multi-$H_2O_2$ peaks in both elicited cells could be explained by the

complexity of the pathogenesis process during the infection process but this is probably true only if the same pathogenesis occur in cell suspension cultures. In plants, this commences with the encystment of the wall-less zoospores to produce a spherical cyst that only then can attach to the host cell surface before the formation of a cylindrical germ tube takes place (Kamoun *et al.*, 1999).

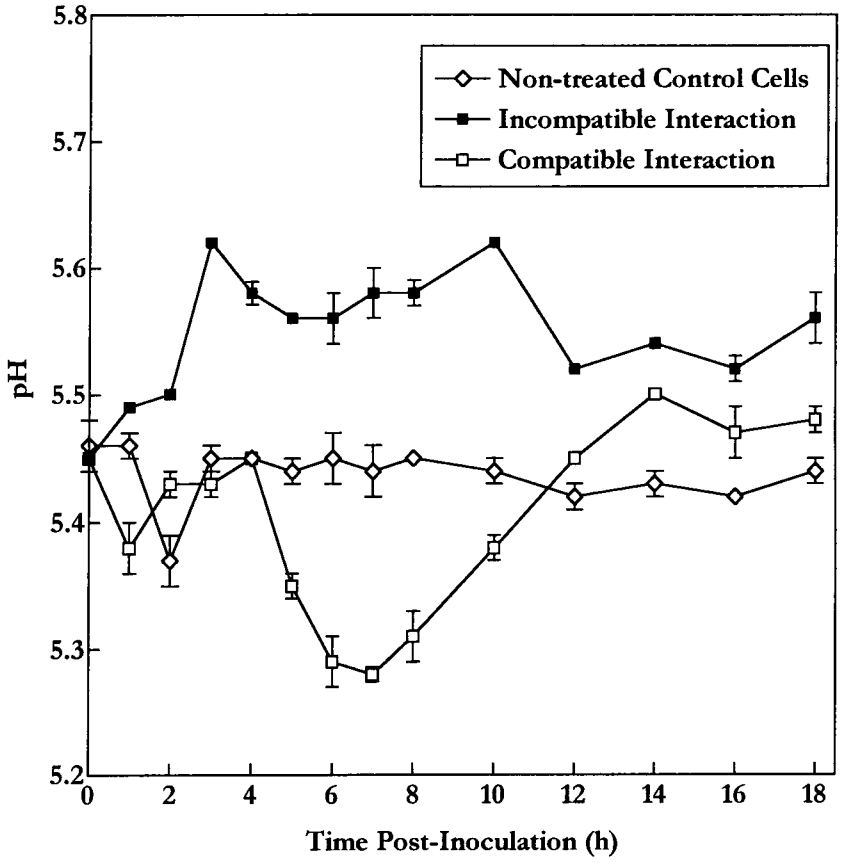


**Figure 52. Time-Course of Changes in pH of the Media in Stirling Cell Suspension Cultures Following Inoculation with Zoospores from Compatible and Incompatible Strains of *P. infestans*.** Data are means of duplicate measurements with error bars showing standard deviation.

The germ-tube apex starts to differentiate to produce appressorium only at a suitable site, a vesicular structure responsible for the formation of an infection tube that can penetrate the host cells. Inside the host cells, the pathogen forms a so-called infection vesicle and from this structure another one or two secondary hyphae germinate and grow into the intercellular space to form haustoria. If these processes occur in cell suspension cultures, then they may occur continuously throughout the incubation period as the cell culture system is not static nor differentiated as is the whole plant.

Some cysts may attach to the cell surface and start to penetrate the cells earlier than others. The accumulation of $H_2O_2$ at different time points may have been caused by the damage of the cells during the penetration of the pathogen or may be as a result of various mechanical changes that take place during the infection process.

### 6.3. Conclusion

All the crude elicitor preparations derived from *P. infestans* induced PAL activity in suspension cells of potato (var Stirling). This phenomenon indicates the presence of either specific or non-specific elicitors for this pathogen in the crude preparations. Increases in PAL activity alone is not sufficient to differentiate the specificity of the interaction that could help to relate PAL activity to disease resistance. PAL itself is controlling many intermediates, several of which may be involved in different developmental processes or responses to environmental stimuli.

The use of suspension cells in studying host-pathogen interaction using elicitor as an inducer probably mimics the actual plant-pathogen pathosystem provided an elicitor preparation is made from an appropriate strain capable of the induction of a defence response. This was difficult to verify in this study as none of the previous works in this area has actually succeeded in identifying a specific elicitor from *P. infestans*. The mixed elicitors used in this study comprised all possible sources of elicitor. By using a mixture of crude elicitor preparations all responses, either arising from specific or non-specific interactions, could be generated. Since it was not possible to investigate the effects much more using biochemical approaches, a molecular approach was devised. Such molecular approaches have the advantages of sensitivity, specificity and can be used globally for gene expression microarrays cDNA. The cDNA library resulting from the interaction may contain all possible genes that have been up- or down-regulated as a result of specific and non-specific interaction.

The impact of elicitation in cell suspension cultures is maximal compared with the whole plant system. This is understandable if we look at the number of cells in contact with elicitor molecules when they were first introduced into the medium whereas in plant tissue the number of cells elicited is extremely limited to the inoculation site. It is not surprising therefore if cells react to all sorts of changes that occur in the culture

medium and the magnitude of response is always detectable. Whether this is of help to the cells or otherwise is yet to be proved.

There was very little difference in the profile of $H_2O_2$ production in the two elicited cells. This pattern could be a result of the combination of mechanical stimulus during infection and also the elicitation itself. Gus-Mayer *et al.* (1998) showed that when suspension cells of parsley are stimulated with a tiny needle of the same diameter as the fungal hyphae, they observed results similar to those with elicitation. The cells responding to the needle by generating reactive oxygen species ($H_2O_2$, superoxide radical), translocation of cytoplasm and nucleus to the stimulated site as well as by the expressing of some elicitor-responsive genes. Therefore $H_2O_2$ accumulation could not be a specific indicator to relate to the levels of resistance in plants. In addition, the cell suspension cultures used in the present study was derived from Stirling, a variety of potato that possess the so-called 'durable resistance'. Infecting potato leaves of durable-type resistance variety with zoospores of *P. infestans* showed an induction of limited HR (Kamoun *et al.*, 1999).

# CHAPTER 7

## EARLY RESPONSES OF STIRLING CELL SUSPENSION CULTURES FOLLOWING ELICITATION WITH A MIXED ELICITOR FROM *PHYTOPHTHORA INFESTANS*

### 7.1 Introduction

One of the major problems in the potato industry is how to tackle the most destructive potato disease, late blight, caused by *P. infestans*, in an environmental friendly way. Potato breeding programmes directed towards a sustainable resistance to the late blight pathogen in many parts of the world are still unable to tackle the problem. One of the reasons is the lack of understanding of the mechanism of interaction between *P. infestans* and the potato. Several fundamental questions surrounding the interaction towards either disease development or resistance need to be investigated especially at the molecular level.

Disease resistance based on the incompatible interaction of pathogens and their corresponding hosts has proved to be less sustainable in the field. In most cases, the new resistant variety becomes susceptible after only a few generations due to adaptation that gradually occurs in the pathogens in order for them to survive. The pathogen itself probably possesses multilayered mechanisms to colonise plant tissues. As a consequence, plants have evolved multilayer defence systems to detect and combat the incoming pathogen. In other words, the interaction between plants and pathogens is a dynamic process and perhaps no single model is appropriate or can be used in describing the various pathosystems.

There are many potato clones that are already bred and used in producing the potato commercially and some of these showed a certain degree of resistance to *P. infestans*. After consultation with a plant pathologist at the SCRI (Dr Helen Stewart) the potato variety Stirling was chosen as an experimental variety.

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· See Glossary of Relevant Terms

This selection was based on the unique character of this variety i.e. 'durable resistance' to late blight disease. After years of field observations, the plant pathologists at SCRI have concluded that Stirling possesses the durable resistance (Stewart, pers. comm.). Durable resistance is a phenomenon in which the plant shows some degree of resistance to a compatible pathogen in the field and the resistance is sustainable (Stewart, pers. comm.). The mechanism behind this phenomenon is poorly understood and only recently scientists, after the failure of vertical resistance programmes, (based on gene-for gene hypothesis), have started to consider resistance strategies based on durable resistance. A call for the development of a potato variety with durable resistance was made in 1996, in the global initiative on late blight (GILB) survey (Birth, pers. comm.). Unfortunately the task is not straight forward, as a molecular basis for durable resistance is not available. Therefore, the first approach to study durable resistance must be to try to understand and establish the basis of the resistance.

As shown in the previous chapter, none of the genes that had already been incorporated into potato plants at Durham and tested in this study exhibited significant levels of resistance to *P. infestans* infection. Therefore, it was of interest to look at the molecular level to investigate perhaps biochemical and molecular events induced during the early interaction of *P. infestans* and its potato host. This could provide a basis for further studies in the future. The experimental system of choice for this study was potato cell suspension culture due to the convenience of such a system. This also avoided complications such as pathogen-contaminated plant tissue for the preparation of an RNA sample, since an elicitor mix was used instead of the viable *P. infestans* pathogen. In addition, during host-pathogen interactions, elicitor molecules produced by invading pathogens are responsible for the induction of intracellular signals by interaction with the corresponding cell-surface receptor molecule in the host plants. The cDNA library for differentially expressed genes during elicitation in Stirling cell suspension cultures was prepared using a Clontech PCR Select™ kit. The method based on the so called suppression subtractive hybridisation (SSH) described by Diatchenko *et al.* (1996) (section 2.2.6 for the details).

## 7.2. Results and Discussion

### 7.2.1. Construction of a cDNA library from Elicited Potato Cell Suspension Cultures

Double-stranded cDNA was synthesised from poly $(A)^+$ mRNA isolated from total RNA derived from Stirling cell suspension cultures treated with elicitor mixed as previously described (section 2.2.6.2). The isolated total RNA appeared to be intact as judged by the appearance of the 25S rRNA which is about one and the half of the size of 18S rRNA (Figure 53, Panel A). In addition, the 280:260 ratio of the total RNA samples measured using a UV spectrophotometer were about 2 indicating that the RNA preparations were pure. The poly $(A^+)$ mRNA preparations were also pure as judged by the appearance of mRNA smear without any visible trace of rRNAs (15S and 18S rRNAs) (Figure 53, Panel B).

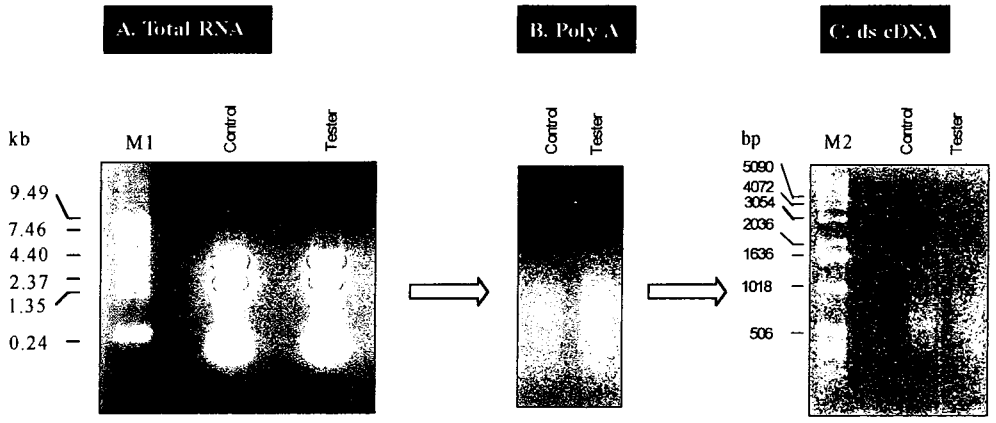



**Figure 53. Preparation of ds cDNA for Subtraction Hybridisation. Panel A.** Total RNA isolated from elicited (tester) and control (driver) "Stirling" cell suspension cultures using Trizol reagent. 100 mg cell aggregates yielded about 0.8 to 1 µg total RNA. Each lane contains 10 µg total RNA. **Panel B.** Poly $A^+$ of corresponding total RNAs isolated using streptavidin magnetic particles. 100 µg total RNA yielded about 2 to 4 µg poly $A^+$. Each lane contains about 1 µg poly $A^+$. **Panel C.** cDNA generated from 2 µg poly $A^+$ for the subtraction hybridisation. One tenth of the cDNA products were electrophoresed on each lane. **All Panels.** Samples were run on 1.2% (w/v) agarose/EtBr gels. For RNA sample, 1% (w/v) EtBr was added into the electrophoresis buffer to enhance the band. M: RNA ladder, M2 : 1 kb DNA ladder.

The ds-cDNAs produced for both control (driver) and elicited cells (tester) were in the size range of 0.2 – 1.6 Kb as estimated by agarose gel electrophoresis (Figure 53, Panel C) and these were used to construct the subtracted differentially expressed cDNA library. The subtracted cDNA library contained 85 clones. The low yield in the subtracted cDNA library is predicted because the normalisation process in the first hybridisation step of PCR-Select™ was prolonged to 12 h to reduce background.

### 7.2.2. Initial Screening of Differentially-Expressed Sequences in the cDNA Library

The resulting subtracted cDNA pool was greatly enriched for sequences expressed only in the elicited cells (tester). The library was screened for differentially expressed fragments using a probe prepared from the same subtracted cDNA used to construct the subtracted library. In theory this cDNA probe should contain all the differentially-expressed fragments and therefore should hybridise and select differentially expressed (subtracted) cDNA clones. The one drawback of using subtracted probes is that they typically result in a slightly higher false-positive rate than the unsubtracted probes (information from Clontech Webpage). However, using unsubtracted probes (made from cDNA before the hybridisation step) result in cDNAs corresponding to highly abundant mRNAs which will produce detectable hybridisation signal. Therefore, a further test (i.e. northern hybridisation or RT PCR) must be carried out to confirm the expression status of the clones. Figure 54 shows the signals produced from the hybridisation. Six clones amongst the most intense signals were selected and sequenced. These were STS 7, STS9, STS16, STS27, STS31 and STS41. Surprisingly, 5 of these clones were ribosomal RNA and only one non-rRNA sequence clone (STS 7) showed similarity to consensus of anti-apoptosis sequence.

There could be a number of explanations for this unexpected high rRNA representation in the selection of up-regulated clones. Ribosomal RNA is obviously present in large amounts and much of this is removed during the normalisation process of the first hybridisation step section. However, while the normalisation enriches for rare sequences (by preferentially removing abundant sequence), abundant sequences will often remain in excess and this is heavily influenced by the efficiency of the hybridisation process, the numbers of mRNAs differentially expressed in the elicited cells and relative success of the cDNA synthesis in both the tester and driver samples

(Clontech technical supports staff; pers. Comm.). It could be that the cDNA synthesis was not as efficient as expected as the cDNA products of both tester and driver were hardly visible on the agarose gel (Figure 53, Panel C). Rather than repeat the lengthy and expensive subtracted process and library construction it was decoded to screen out the rRNA cDNA clones.

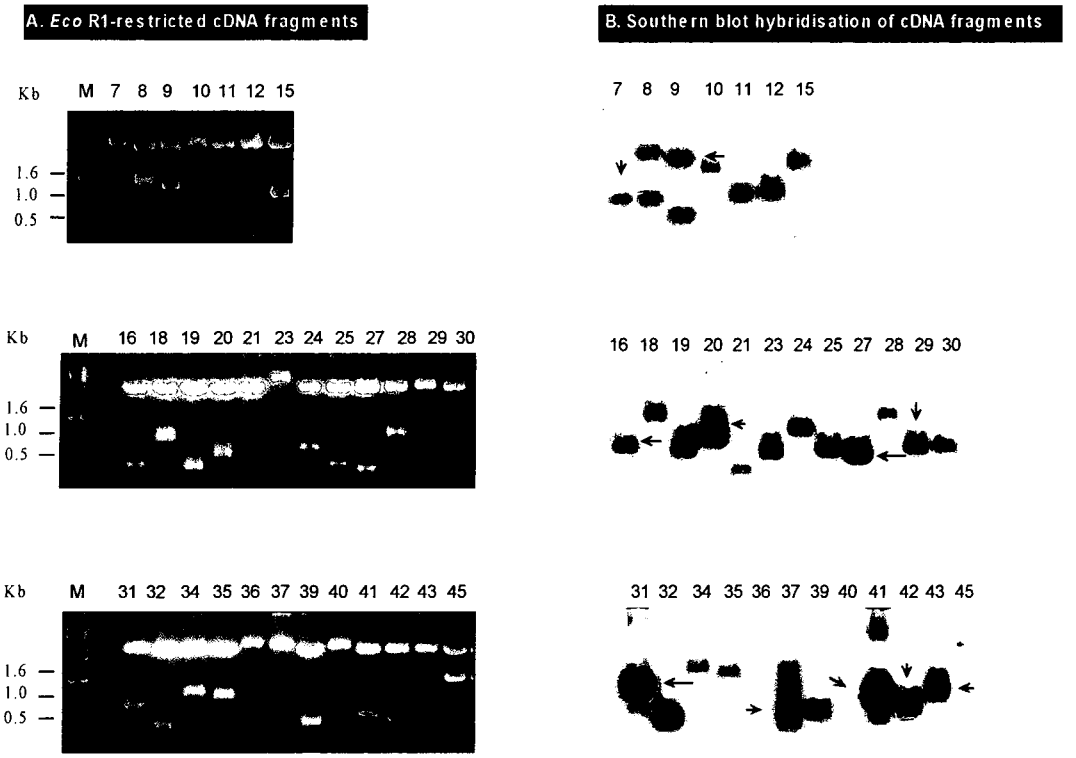



**Figure 54. Initial Screening of Subtracted cDNAs Showing Putative, Differentially Expressed cDNA Sequences.** Thirty one clones were processed for the screening. **Panel A.** *Eco*R1-restricted fragment were electrophoresed on 1.2% (w/v) agarose gel containing 0.1% (w/v) EtBr. M: 1kb DNA ladder. **Panel B.** cDNA fragments were transferred onto a Nylon membrane, Hybond™-N. The filter was hybridised with $^{32}$P-labelled subtracted cDNA, washed to a high stringency of 0.2x SSC and 0.1% (w/v) SDS at 65°C, exposed to Fuji x-ray film for 4h at –80°C. Clones representing mRNAs that are differentially expressed was hybridised with the probe. Some enhanced signals that indicate probable differentially expressed cDNA clones were indicated with arrows.

### 7.2.3. Screening for Ribosomal RNA Sequences in the cDNA Library

The ribosomal RNA sequence used to prepare the probe for screening was kindly provided by Dr. R. Croy of Department of Biological Sciences, University of Durham. The clone is a pea genomic clone containing 8.5 kb genomic fragment which includes

5.8 kb of a ribosomal repeat unit. The plasmid (pACVC 184) which carries genes for Cm and Tc resistance was cut with *Hind* III and the corresponding gene insert was isolated. The rRNA probe was prepared as earlier mentioned. Results obtained from this screening showed that only 8 out of the 69 selected clones were positives for rRNA as shown in Figure 55. These clones were eliminated from further studies.

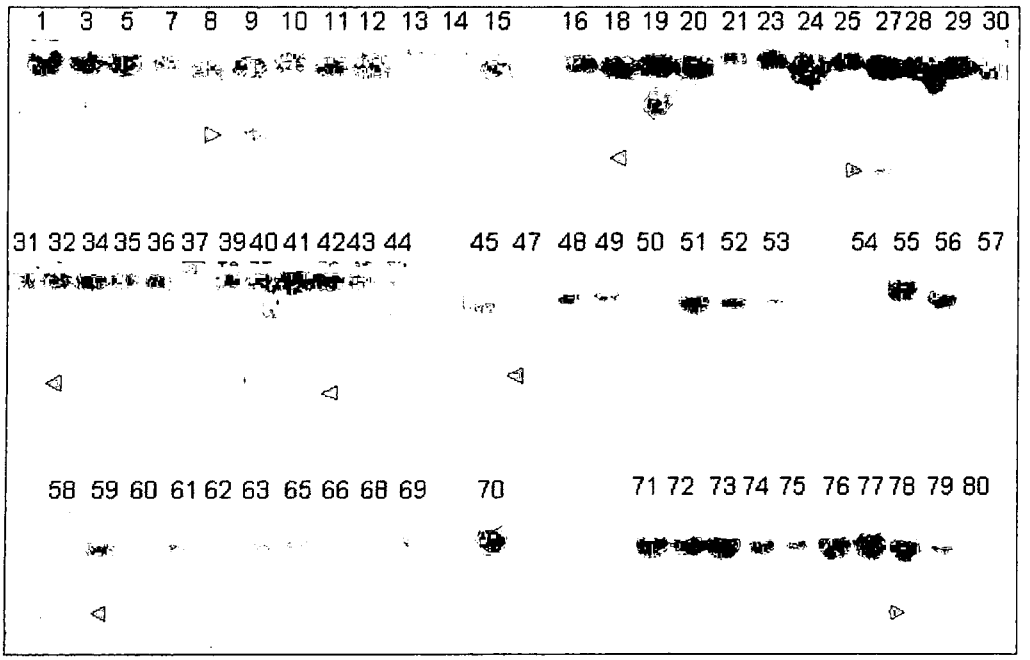



**Figure 55. Southern Blot Hybridisation of Subtracted Potato cDNAs with pHA 1.** The cDNA fragments were hybridised with a DNA probe made from pea genomic clone containing a ribosomal repeat unit using the same conditions as described in Figure 54 and exposed to x-ray film overnight. Eight positive rRNA clones found were marked with arrows.

### 7.2.4. Sequence Analysis of Isolated Non-rRNA Differentially-Expressed cDNA Sequences

#### 7.2.4.1. Notes on the Sequenced cDNA Clones

Table 12 shows the summary of properties of selected putative non-rRNA cDNA sequenced in the present study. Detailed descriptions of the clones STS 42 and STS 51 are presented in sections 7.2.4.2 and 7.2.4.3, respectively. These two clones are relevant to this study and were used for the gene expression study. The properties of the other 4 clones, STS66, 39, 7 and 25 are available under the Appendix section. These clones are new and showed no sequence similarity to known sequences

(proteins and nucleotide) in the Genbank and Swissprot databases except for STS 7 which showed some degree of similarity to a consensus sequence of anti-apoptosis (BLOCK search).

**Table 12. Summary of Properties of Selected Differentially Expressed cDNA Transcripts.** The information is retrieved from Genbank and Swissprot databases using BLASTN/X and FASTA 3 alignment search tools, respectively. Sequence comparison was carried out using default parameter settings for each alignment search tool.

| Clone | *bp* | Identified gene | Source | Accession No/Database | Similarity Score | Over-Expressed In |
|---|---|---|---|---|---|---|
| STS51 | 351 | S7-RNase (179 aa) | *Malus domestica* | 642043 (Genbank) | 51% identity in 35 aa overlap | Pathogen, wounding |
| STS42 | 538 | Leucine aminopeptidase (573 aa) | *Solanum tuberosum* | P31427 (Genbank) | 98% identity in 175 aa overlap | Pathogen, wounding |
| STS66 | 300 | Hypothetical protein (701 aa) | *Arabidopsis thaliana* | AAB63649 (Genbank) | 90% identity in 76 aa overlap | ? |
| STS39 | 453 | Pyruvate dehydrogenase subunit C (160 aa) | *Neisseria meningitidis* | 068418 (Swissprot) | 40 % identity in 48 aa overlap | ? |
| STS 7 | 266 | Undetectable sequence similarity | - | - | - | - |
| ST25 | 401 | Undetectable sequence similarity | - | - | - | - |

### 7.2.4.2. Sequence analysis of STS 42 clone

The STS 42 subtracted cDNA clone is 538 bp long. It is a truncated cDNA containing a 5' open reading frame of 527 bp which was also predicted to be a coding region of the gene using a combination of the start stop codon method and the Borodovsky prediction technique.

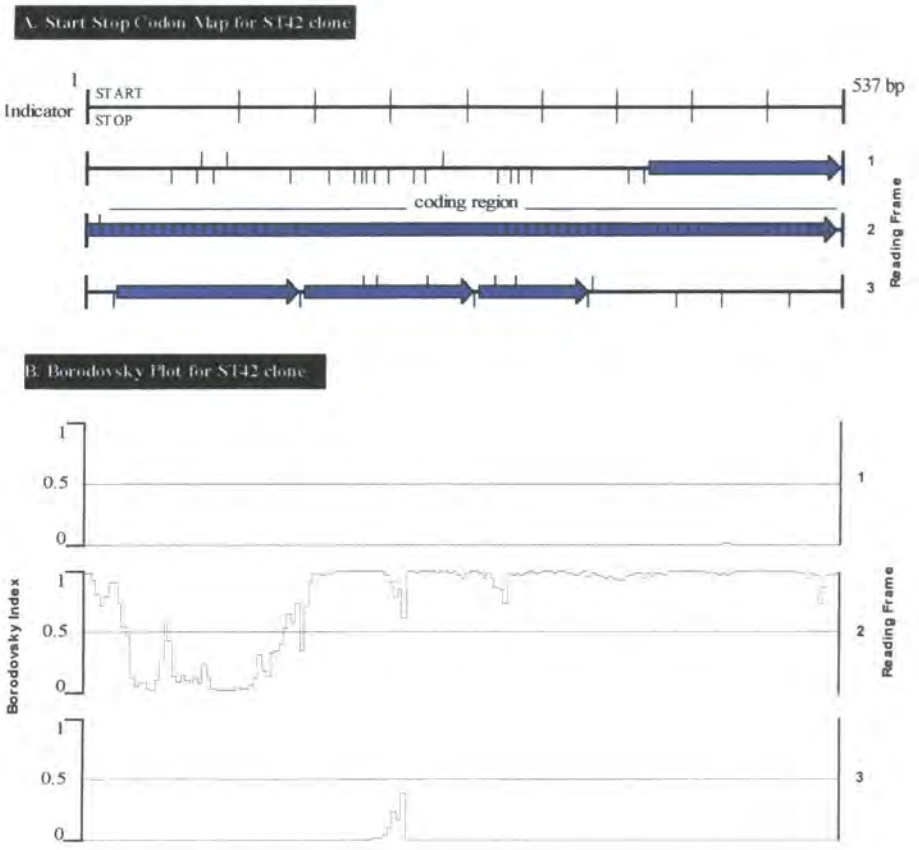



**Figure 56. Open Reading Frame Map and Coding Region of the STS 42 Clone.** The potential coding region from the six open reading frames was predicted using a combination of Start-Stop Codon and Borodovsky prediction techniques in the GeneQuest program of DNAStar (DNAStar Inc.). The overlap region of the corresponding reading frame between the two methods is likely to be the true coding region of the sequence. **Panel A.** All possible open reading frames (ORF) of the STS 42 clone based on the location of start and stop codons.. Bars above and below the line of the Start-Stop Codon frame indicate the position of start and stop codons, respectively. **Panel B.** The Borodovsky method evaluates coding potential, independent of identifying ORFs by analysing the sequence against data contained in statistical matrix files. The matrix used in this prediction was from tomato included with the GeneQuest program. The potato matrix is yet to be made available. (http://genemark.biology.gatech.edu/GeneMark/models_complete.html). A potential coding region is appeared as a sharply rising, flat topped peak in a line graph. Coding regions will have a value over the threshold of 0.5, and preferably, near 1. Only 3 reading frame were shown here as all the corresponding complementary reading frames showed insignificant signals based on the criteria stated above. Based on both prediction techniques the predicted coding region for the STS 42 clone was identified in the longest ORF of reading frame 2 starting at residue 10 as indicated as a thick bar.

Comparison of STS 42 amino acid sequence with proteins in Genbank revealed strong similarities to several plant leucine aminopeptidase (LAP) proteins including ones isolated from the potato. The STS 42 fragment shows the highest similarity with the LAP isolated from the potato (Genbank Accession No. X77015) (Herbers, *et al.*, 1994). The similarity started at the predicted start codon of the STS 42 sequence corresponding to the start codon of the potato LAP. In terms of size, the STS 42 clone is just under one-third of the complete potato LAP. Aligning the STS 42 sequence with the complete potato LAP protein shows 172 of the amino acids within the overlapping region (177 amino acids) was identical, 5 mismatches and 2 deleted. (Figure 57). It is unclear whether the mismatches are due to genuine differences between the two proteins or due to errors in the chemical processes during the library construction or sequencing. The STS 42 clone was sequenced once only. Table 13 presents some of the other LAP sequences that show high similarity to the STS 42 sequence.

```
Potato LAP   MATLRVSSLL ASSPSSLHCN PSVFTKCQSS 30
STS 42       MATLRVSSLL ASS--SLHCN PSVFTKCQSS 30

Potato LAP   PRWAFSFSVT PLCSRRSKRI VHCIAGDTLG 60
STS 42       PRWAFSFSVT PLCSRRSKRI VHCIAGDTLG 60

Potato LAP   LTRPNESDAP KISIGAKDTD VVQWQGDLLA 90
STS 42       LTRPNESDAP KISIGAKDTD VVQWQGDLLA 90

Potato LAP   IGATENDLAR DENSKFKNPL LQRLDSKLNG 120
STS 42       IGATENDLAR DENSKFKNPL LQQLDSKLNG 120

Potato LAP   LLSAASSEED FSGKSGQSIN LRLPGGRITL 180
STS 42       LLSAASSEED FSGKSGQSIN LRLPGGRITL 180

Potato LAP   VGLGSSASSP TSYHSLGEAA AAAAKSA..... 177
STS 42       VGLGSSASSP TSYHSLRKAA AAAANSA End 177
```

**Figure 57. Alignment of the Predicted STS 42 Amino Acid Sequence with Leucine Aminopeptidase (LAP) of Potato.** The potato LAP (Swissprot, accession no. P31427) is induced during wounding. (Herbers, *et al.*, 1994). The alignment was done based on the Clustal W method (Thompson, Higgins and Gibson, 1994) using Omiga 1.1.3, Oxford Molecular Ltd. Shaded boxes represent mismatch residues and dashes (-) indicate the gabs introduced to maximise alignment.

**Table 13. List of Other Leucine Aminopeptidase Sequences that Show High Similarity to STS 42 Clone.**

| Source | Swissprot Database (accession no.) | Similarity | Score | Inducer |
|---|---|---|---|---|
| Potato | P31427 | 96.1% identity in 177 aa overlap | $5.2e^{-63}$ | Wounding |
| Tomato | Q10712 | 90.4% identity in 177 aa overlap | $6.2e^{-59}$ | Wounding |
| Tomato | O24022 | 89.8% identity in 177 aa overlap | $1.1e^{-59}$ | Wounding |
| Tomato | G440604 | 90.7% identity in 75 aa overlap | $1.4e^{-20}$ $1.4e^{-20}$ | Pathogen |

LAP [EC 3.4.11.5] like many other aminopeptidases catalyses the cleavage of amino acids from the amino-terminal of proteins or peptides. This enzyme is found in many eukaryotic and prokaryotic organisms. In general little is known about the role of the enzyme in plants or other organisms although the enzyme was first discovered in 1929. Studies on bovine LAP shows that this enzyme is hexameric with two zinc-binding sites in each of its subunits (Carpenter and Vahl, 1973). Plant LAP was first cloned from *Arabidopsis* by Bartling and Weiler (1992). They showed that LAP forms a superfamily of highly conserved enzymes, spanning the evolutionary range from bacteria to animals and higher plants. Details of a comparative study of the enzyme isolated from *E. coli*, tomato and pig was reported recently (Gu *et al.*, 1999). The enzymes are very stable at high temperature and high pH. They found that the purified enzymes have the same enzyme and pH profiles regardless of their origin with an optimum activity at 60 °C and pH ranging from 7 to 11. This enzyme is inhibited in the presence of $Zn^{2+}$ and is strongly activated by $Mn^{2+}$ and $Mg^{2+}$. The preferred substrate has N-terminal Leu, Met and Arg residues (Gu *et el.*, 1999). It is interesting that two of these N terminal residues, Leu and Arg are not normally present in the cytoplasm and nucleus under normal conditions, and therefore also unlikely to be available in other organelles. Previous studies have shown that most of eukaryotic cytoplasmic and nuclear proteins have at their N-termini either Met, Ser, Thr, Val, Gly or Ala (Arfin and Bradshaw, 1988). Only abnormal proteins appear to have N-termini

other than these residues and are therefore, removed from the cellular compartments where they are located. Abnormal proteins are generated in plants from time to time as a result of various mechanisms including mutation, biosynthetic errors, free radical-associated damage and stress factors such as pathogen attack (Vierstra, 1996). In the current study, it was shown that hydrogen peroxide was released rapidly as a result of elicitation with a mixed elicitor preparation of *P. infestans* in potato cell suspension cultures. It was speculated that this phenomenon could cause damage to proteins which in turn change the integrity of the cells as a whole. Therefore, LAP could possibly be involved in removing damaged proteins although such a function has not been reported. This speculation is based on the substrate preference of LAP which is atypical for normal proteins except for proteins with methionine N-terminal. The possibility that LAP is part or a component of other proteolytic pathway in the cell can not be ruled out.

As mentioned earlier this enzyme is reported to be extremely stable at high temperature. Possessing an enzyme stable at high temperatures could be useful for plants especially if the enzyme might be involved in degrading damaged proteins due to various environmental stresses as described above. Plants can be exposed to all sorts of environmental situations ranging from cool to hot weather. Exposure to sunlight for a long period during day time is a potential danger that can damage plant proteins especially those proteins that are located near to the leaf surface. Protein denaturation is likely to occur. Damaged proteins may be accumulated so long as the plant is exposed to sunlight and this might endanger plant cell integrity if they are not removed. In this situation, plants may need an active enzyme to do the job and that enzyme is possibly LAP.

In the potato, LAP is induced in response to wounding and jasmonic acid (Herbers *et al.*, 1994). The same observation has also been reported in the tomato (Pautot *et al.*, 1993). Increase in LAP activities during wounding and jasmonic acid treatments can easily be understood because jasmonic acid is one of the components in wounding-associated pathway. Wounding or mechanical damage increases the level of abscisic acid (ABA) which in turn activates the jasmonic acid pathway (Damman *et al.*, 1997).

### 7.2.4.3. Sequence Analysis of STS 51 Clone

The STS 51 subtracted cDNA clone is 351 bp long and is a truncated cDNA containing only 3' coding and non-coding portions of the gene. A putative polyadenylation signal is located at the 3' end of the nucleotide sequence. It has a major interrupted open reading frame of 267 bp (Figure 58). This open reading frame which is predicted to be part of the coding region of the gene was shown to have a significant similarity to portions of the C-terminal region of plant ribonucleases (RNase) proteins when compared with protein sequences in the Genbank database using BLASTX search engine.

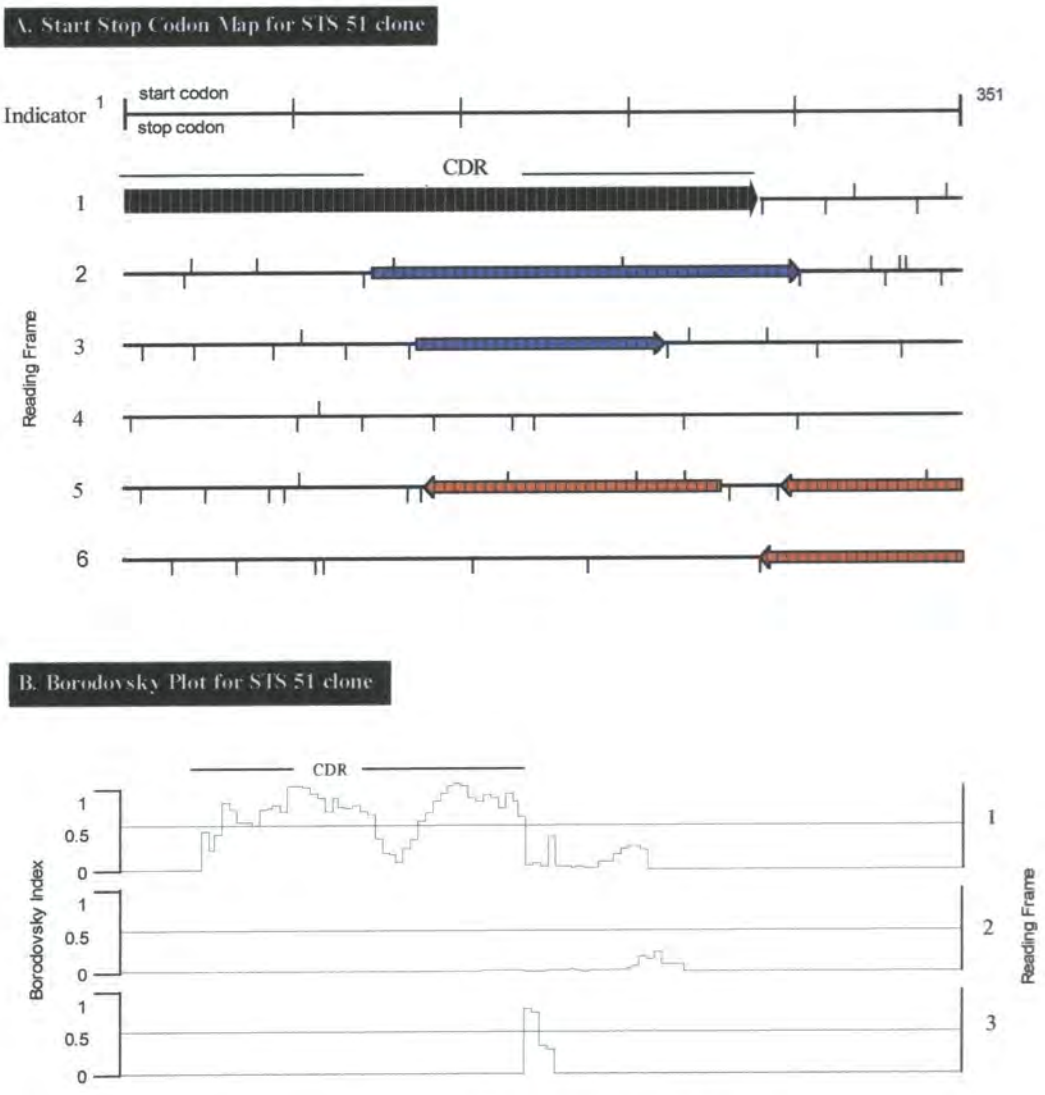



**Figure 58. Predicted Coding Region of the STS 51 Clone.** The predicted coding region was identified in reading frame 1 based on the Start Stop Codon method (**Panel A**) and the Borodovsky prediction technique (**Panel B**). Details of the methods was described in Figure 56.

BLOCK search revealed that the STS 51 fragment is a member of the ribonuclease T2 family histidine proteins 1 (consensus sequence LNEVtLCtyPNGTRFIsC). In several higher plants of the Solanaceae family RNase is involved in the self-incompatibility reaction during pollination (Haring *et al.*, 1990 McClure *et al.*, 1989). The single potential *N*-glycosylation site in the amino acid sequence of the STS 51 sequence is said to be conserved in almost all self-incompatibility RNases sequenced so far (Oxley *et al.*, 1996). However, the function of the *N*-glycans attached to the site is unknown and is not apparently related to the rejection of pollen (Parry *et al.*, 1998).

The STS 51 sequence also contains homologies to sequences of potential importance in other functions. A glycine-myristolation (consensus sequence G-{EDRKHPFYW}-X(2)-[STAGCN]-{P}) site is recognised at position 71 (Figure 59). The *N*-myristolation may be involved in the protein binding to the membranes. A potential tyrosine kinase phosphorylation site (consensus sequence [RK]-X(2,3)-X(2,3)-Y) is found at position 63. The presence of a tyrosine kinase phosphorylation site suggests that the STS 51 polypeptide could possibly form a part of a receptor molecule responsible for the signal transduction process which takes place in the early interaction between an elicitor molecule of the pathogen and the corresponding receptor molecule in the host plant cells. Phosphorylation is an important process in the initial transduction of the elicitation (Simon-Plas *et al.*, 1997). The self-incompatibility RNases are reported to be extracellular proteins (Galiana *et al.*, 1997), the presence of these two extra functional motifs and their function in the cell is difficult to predict.

The tyrosine kinase phosphorylation site in the STS 51 sequence was located at the hydrophilic region of the STS 51 sequence as shown in Figure 60. It is clear that the region in which the two motifs are located (position 63–76) is very hydrophilic. This is important because the phosphorylation by protein kinases during signal transduction process occur in the cytoplasm.

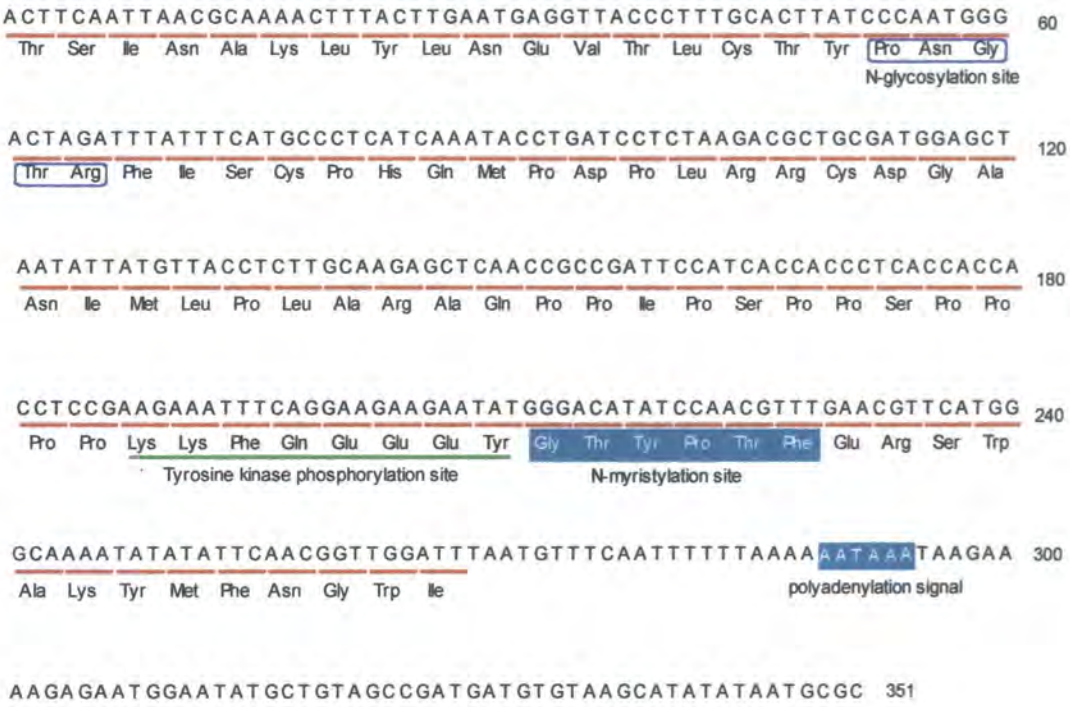



**Figure 59. Nucleotide Sequence and Deduced Amino Acid Sequence from the cDNA Clone STS 51.** The consensus polyadenylation signal is boxed in black and the available protein motifs are indicated with the corresponding labels.

At least 2 types of extracellular-self incompatibility like RNases are induced during the infection of *Phytophthora cryptogea* on tobacco leaves (Galiana *et al.*, 1997). The increase of RNases in the apoplast space is reported to be an important factor in reducing infection by zoospores of *P. cryptogea*. Infiltrating tobacco leaves with RNase reduced infection by this pathogen up to 90% (Galiana *et al.*, 1997). While the extracellular types of RNase are important in pollen rejection and defence response, little is known about the function of intracellular RNase. There is one report about the intracellular RNase in the bean, *Phaseolus vulgaris* (Walter *et al.*, 1996). This RNase is induced following various environmental changes and external stimuli related to pathogen-defence such as glutathione and salicylic acid.

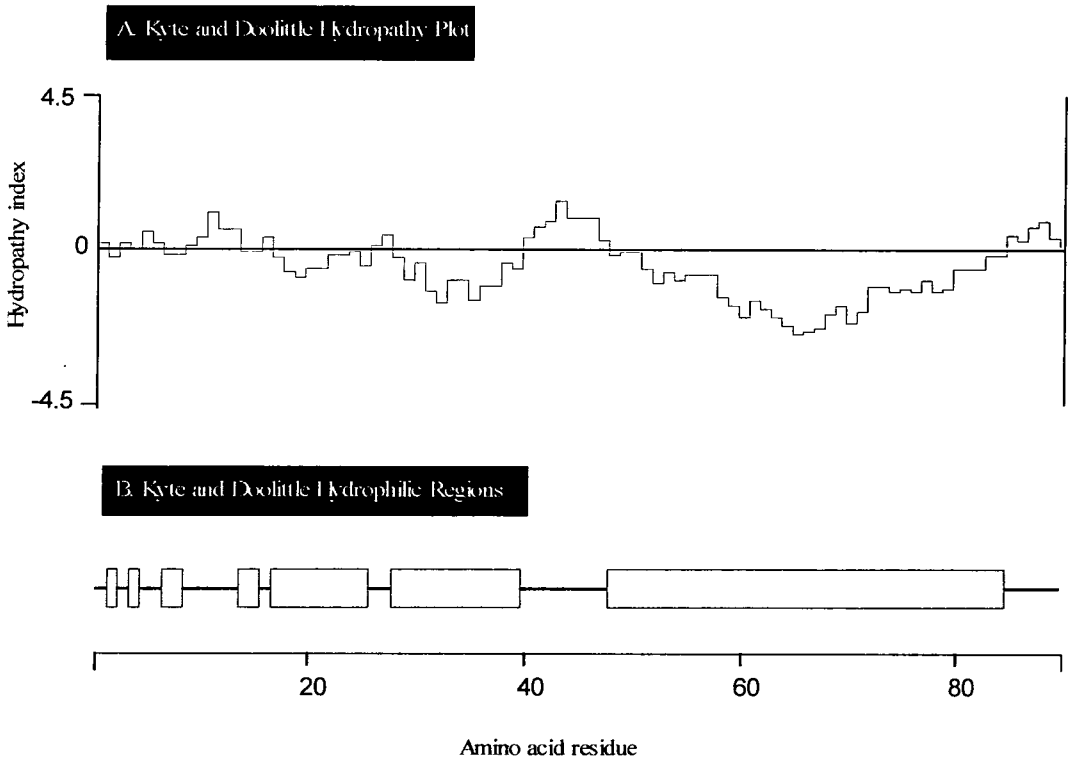



**Figure 60. A Kyte and Doolittle Hydropathy Plot of the STS 51 Clone.** The Hydropathy – Kyte-Doolittle method predicts regional hydropathy of proteins from their amino acid sequences, using the approach of Kyte and Doolittle (1982).

**Table 14. A selection of the Matches from a Blastx and Fasta Search with the cDNA Clone STS 51 Sequence.**

| Protein | Source | Accession No/Database | Similarity |
|---|---|---|---|
| S7-RNase (179 aa) | *Malus domestica* | A61820 (Genbank) | 51% identity in 35 aa overlap |
| S4-RNase precursor (233 aa) | *Antirrrhinum hispanicum* | Q38717 (Genbank) | 41% identity in 34 aa overlap |
| S4-RNase (223 aa) | *Pyrus pyrifolia* | BAA08474 (Genbank) | 47% identity in 34 aa overlap |
| S2-RNase (221 aa) | *Pyrus pyrifolia* | BAA08473 (Genbank) | 48% identity in 29 aa overlap |

Aligning of STS 51 amino acid sequence with other Solanaceae RNases showed that the overlapping region is from amino acid number 9 to 44 of the sequence and this

corresponds to the C-terminal regions of the Solanaceae amino acid sequences as shown in Figure 61. Therefore, STS 51 is predicted to be a RNase-like protein provided that there is some more of the STS 51 coding region upstream to the start codon.

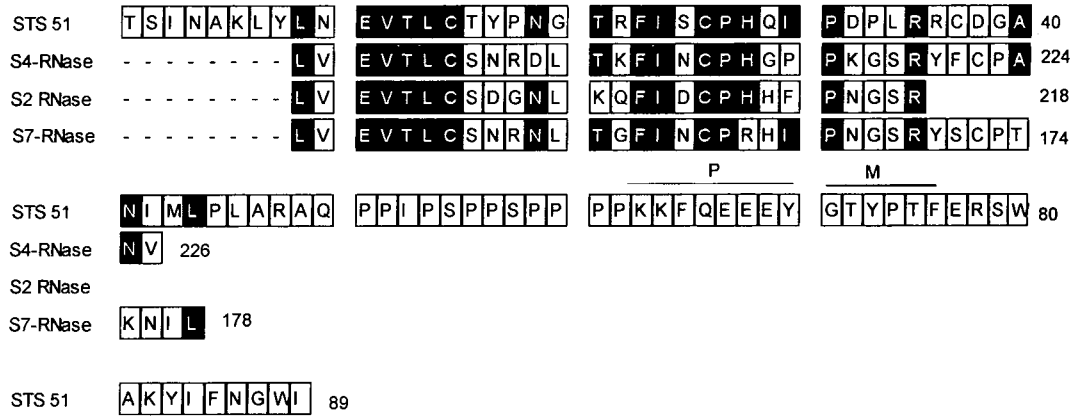



**Figure 61. Alignment of Deduced Amino Acid Sequence Encoded in the STS 51 with Partial Sequences of S2-RNase, S4-RNase and S7-RNase.** The clone is also aligned separately with chitin-lectin binding protein, a small protein of 27 amino acid sequence. Black shaded boxes represent identical residues. The alignment was done based on the Clustal W method (Thompson, Higgins and Gibson, 1994) using Omiga 1.1.3, Oxford Molecular Ltd.

### 7.2.5. Gene Expression Studies on the Selected cDNA Clones

The gene expression studies of the selected clones (STS 42 and STS 51) were first undertaken using a Northern blotting, but this failed to show significant results. It could be that the transcripts were too low to be detected by Northern hybridisation. Therefore, an RT PCR was chosen as it is a sensitive, quantitative detection system. RT-PCR was used initially in the construction of the library. Recent developments have improved the procedure such that it can be used to quantify the amount of a specific mRNA in small amounts of total RNA.

#### 7.2.5.1. Optimisation of the RT-PCR Technique

In PCR, the relationship between the product amplified is always in proportion to cycle numbers, a stage is reached when one or more of the reaction components such

as the primers or enzyme concentration become a limiting factor. It is very important to maintain the linearity of the relationship in a comparative study such as in gene expression studies using RT PCR. The results of gene expression studies using RT PCR partly depend on the amplified cDNA products which represent the corresponding mRNA levels. In the present study, the relationship between the PCR product development and the corresponding cycle numbers was established as shown in Figure 62.

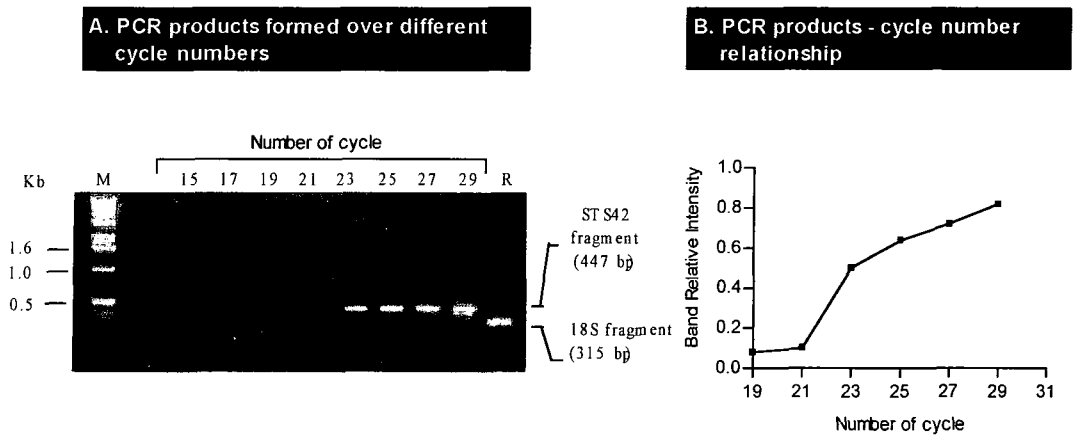



**Figure 62. Determination of Linear Range in RT PCR. Panel A.** Eight PCR tubes containing 25 µL aliquots of PCR mix (section 2.2.6.17.3), cDNA template and PAL primers were amplified by the indicated cycles. After the indicated cycle numbers, tubes were remove from the thermocycler and the products analysed by electrophoresis on 1.5 % (w/v) agarose gel. M: 1Kb DNA ladder. **Panel B.** The linear range of the reaction was determined by quantifying the band densities from a scanned gel using Bio Image™ Advanced Quantifier program (B.I. System Corp. ).

In this experiment, primer pairs for the PAL mRNA which was designed to flank an intron was used. Amplification of PAL transcripts from the genomic DNA would produce the PCR product a size larger than the amplified PAL product originally from the cDNA. The expected PAL product size from cDNA for this primer combination was 447 bp and the 'hot-start' amplification profile used was 30 s denaturation at 94°C, 50 s annealing at 60°C and 60 s extension at 72°C. As shown in Figure 62, the increase in the relative intensity of the band of the PCR product (PAL) over the cycle

numbers. There was no clear sign that the reaction reached to a 'plateau stage', a condition in which one or more of the reaction components in the mixture became a limiting factor, although some degree of reduction was observed after the 23rd cycle. For a precaution, all RT PCR experiments in the present study were terminated at the 23rd PCR cycle and if required (i.e. when the PCR products invisible or too faint), additional cycles were added.

The next step in the RT PCR optimisation was to determine the optimal ratio for 18S primer:Competimer™ combination used for an internal control (section 2.2.6.17.4). This is a primer combination for 18S rRNA endogenous standard for RT PCR from Ambion, Inc. The use of rRNA as an internal control for RT PCR is ideal because more than 95% of the total RNA population comprises of this species and, therefore, the quantitative variation between samples is likely to be low. Also, because of the abundance of this rRNA in total RNA preparations, the quantitation of initial input RNA for the RT reaction, is actually based on the amount of rRNA in the sample. In addition, there are reports that common internal RT PCR controls such as β-actin thought to be uniformly expressed, vary significantly from tissue sample to tissue sample and, therefore, should be used with caution (Murphy *et al.*, 1990; Gaudette *et al.*, 1991). The use of rRNA however, invites another complexity in that the abundance of the rRNA population means they can easily out compete the gene-specific product under examination. The components in the PCR reaction mixture can be easily exhausted due to the high demand from the rRNA population before the amount of product for the amplified gene-specific is achieved. Ambion Inc. introduced the Competimer™ technology which can be used to modulate the amplification efficiency of a PCR template without affecting the performance of other target sequences in the PCR reaction mixture (information is available from Ambion's QuantumRNA™ 18S Instruction manual and from the Ambion webpage http://www.ambion.com). Briefly, the 18S Competimer is a modified 18S primer with its 3' ends was chemically modified so that extension by DNA polymerase during PCR was impossible. By mixing 18S primers with increasing amounts of the 18S Competimer, the overall PCR amplification efficiency of 18S cDNA can be reduced in a controlled way.

Three different combinations of 18S primer:Competimer mixes were used as recommended in the Instruction manual, namely 3:7, 2:8 and 1:9. According to the Ambion, these ranges covered the detection of most levels of gene expression ranging from moderately expressed transcripts (3:7) to extremely rare transcripts (1:9). A primer pair for PAL enzyme transcripts was selected in this pilot experiment because this enzyme was known to be induced by the elicitor treatment as reported in Chapter 6. In addition, the primer was designed to flank an intron so that any PCR product amplified from the genomic DNA could be easily differentiated from the cDNA (transcript) template on the basis of size. The amplification was run up to 23 cycles based on conditions used in the earlier cycle number optimisation experiment.

Figure 63 shows that when the recommended 18S primer:Competimer for moderately expressed transcripts was included in the PCR mixture, the expected PCR product for PAL was not detectable when 20% of the PCR product was analysed on a 1.5% (w/v) agarose gel (Figure 63, A). Using the next recommended ratio (2:8; 18S primer:Competimer) for rare transcript, the PAL PCR product which was 447 bp in size was visible but faint when the same amount of product was electrophoresed on an agarose gel (Figure 63, C). Increasing the concentration of 18S Competimer (18S primer:Competimer; 1:9) in the PCR mixture improved the PAL product slightly (Figure 63, B). Therefore, the best ratio for this particular primer was 1 of 18S primer to 9 of 18S Competimer. This optimisation may be repeated when a new primer pair is used. Alternatively, one might start by using the 1:9 ratio for extremely rare messages and evaluate the outcome rather than repeating the whole combination of primer/Competimer for a new primer pair. This can reduce the cost of the expensive QuantumRNA 18S primer:Competimer™ and the other RT and PCR reaction components.

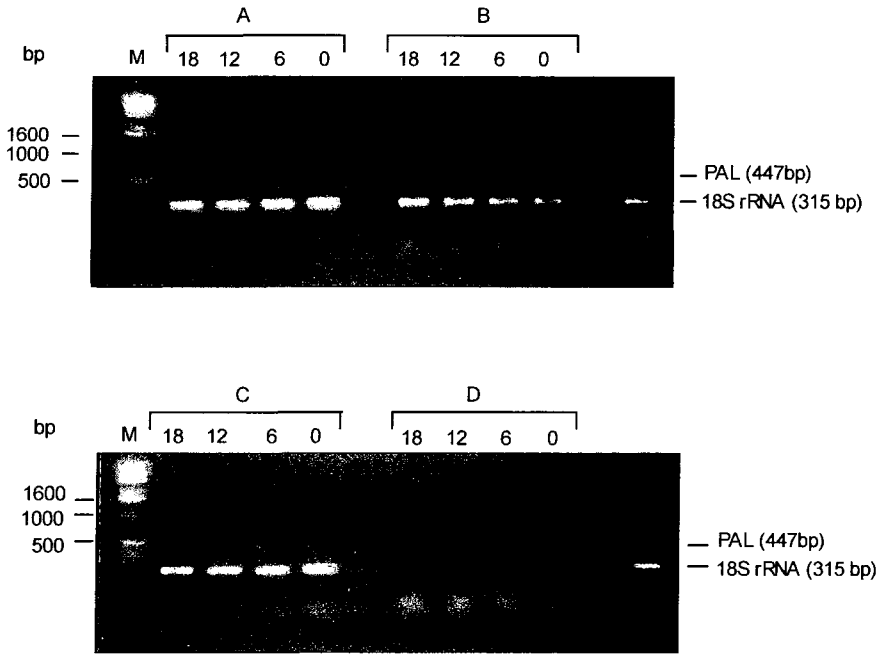



**Figure 63. Determination of Optimal Ratio of 18S Primer: Competimer™.** A PCR master mix comprising of reaction components and PAL primers was prepared and split into 16 aliquots. To these group (A, B, C) cDNA template corresponding to different experimental time points (0, 6, 12, 18h) were added. Group D was a 'no RT' control. To this group, the corresponding total RNA preparations (at 0, 6, 12, 18h) were added. A mixture of 18S primer:Competimer at different ratios of 3:7, 1:9 and 2:8 were added to groups A, B and C, respectively. The hot-start amplification was undertaken for 23 cycles.

### 7.2.5.2. Analysis of PAL Gene Expression

PAL enzyme was shown to be induced when 'Stirling' cell suspension cultures were treated with different *P. infestans* elicitors. This was detected using standard PAL enzyme assays. Primers based on the potato PAL gene sequence (Genbank Accession No. STLEAMPE) (Joos and Hahlbrock, 1992) were made to assay the transcripts using RT-PCR.

The elicitor and stress inducible enzyme, PAL used as a biochemical marker for elicitation in the present study showed the expected increase in PAL transcripts as shown in Figure 64. Transcripts for PAL increased 6 h after elicitation and remained detectable during the entire course of elicitation of 18 h. This increase is reflected in

the changes in PAL enzyme activity after elicitation as reported in the present study (Chapter 6). In this case PAL enzyme activity increased sharply and reached a maximum level after 6 h elicitation and then steadily decreased until the end of the elicitation period. The PAL transcripts detected followed a similar expression profile (Figure 64, Panel B).

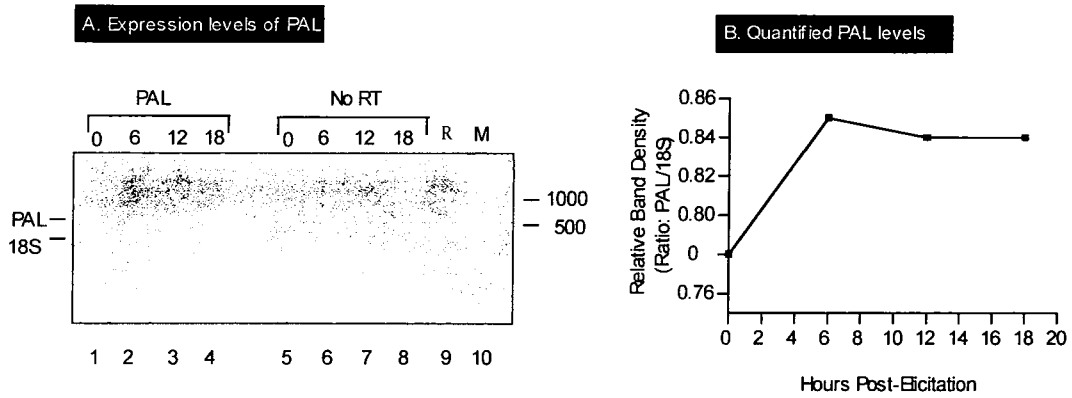



**Figure 64. RT PCR Amplification of mRNA for PAL Transcripts. Panel A.** RT PCR was undertaken using the optimised 23 cycles and QuantumRNA™ 18S internal standard (18S rRNA primer: Competimer™: 1:9). After 23 cycles of amplification, 20% of the PCR products were resolved on a 1.5% (w/v) agarose/EtBr gel. Lanes 1 to 8, labelled with 0, 6, 12 and 18 represent amplified cDNA from reverse transcribed RNA of elicited "Stirling" cell suspension cultures sampled at the indicated time (h). The "no RT" reactions were also run for all the corresponding samples to check if genomic DNA was amplified (lanes 5-8). Lane 9, labelled with R represents amplified 18S cDNA of control sample (RNA from mouse liver) supplied with the 18S rRNA internal control, lane 10 labelled with M represents DNA 1 kb ladder. **Panel B.** The intensity of band (as shown in Panel A) at each time points was quantified using a BioImage™ Advanced Quantifier (B.I. Systems Corp.), and the ratio (PAL: 18S rRNA was calculated.

### 7.2.5.3. Analysis of Gene Expression of the Selected Subtracted Clones

Three of the characterised cDNA clones namely STS 42 and STS 51 were selected for gene expression studies based on the sequence analysis of the corresponding sequence. As described earlier in this chapter (section 7.2.4.2), STS 42 is part of gene encoding LAP in plants which is induced by many environmental stresses such as wounding and

pathogen attack. This was highly relevant to the present study. STS 51 on the other hand, although similarities to known sequences of self-incompatibility plant ribonucleases was less strong than that of STS 42, was also important to the present study because it could be that STS 51 is only a small part of the gene in the same family of plant ribonucleases. There is growing evidence that ribonucleases may be involved in plant defence against pathogen attack as elaborated in section 7.2.4.3 and therefore of interest for the these studies. In addition, the third clone STS 7, which showed some similarity to consensus sequence for anti-apoptosis was also included.

#### 7.2.5.3.1. Gene Expression of the STS 42 Sequence

Figure 65 showed that the transcript for STS 42 did increase after 6 h of elicitation and continued to increase throughout the elicitation period. This result suggests that STS 42 which was part of the gene encoding LAP was up-regulated during elicitation of the "Stirling" cell suspension cultures. There was no trace of any amplification from the "no RT" control samples confirming that the amplified bands were from the genuine transcripts. Initial attempts to observe differences in amplified STS 42 transcripts from different samples using 28 cycle numbers, failed as the first results showed no noticeable differences. When the cycle numbers were reduced, the differences between the STS 42 transcripts from cDNA samples of different experimental time points were observed. Again, the right cycle numbers to amplify samples for comparative mRNA study was crucial for the success of the RT PCR.

LAP as mentioned earlier is a well-known stress-inducible enzyme and is very stable at a high temperature. However, the specific function of this enzyme in defence mechanisms against pathogens in plants is poorly understood. Pautot *et al.,* (1993) reported that tomato plants challenged with *Pseudomonas syringae* pv. *tomato*, a bacterial pathogen, showed an increased LAP mRNA level after 12 h of the challenge. However, challenging potato leaves with compatible and incompatible zoospores of *P. infestans* for 72 h did not show any elevation in LAP mRNA (Herbers *et al.,* 1994). This result is in contrast to the present study where STS 42 transcripts encoding LAP were shown to be induced in "Stirling" cell suspension cultures following elicitation. The reason for this increase was unclear and there is no report so far on the effect of elicitors on LAP expression either in whole plant system or in plant tissue culture.

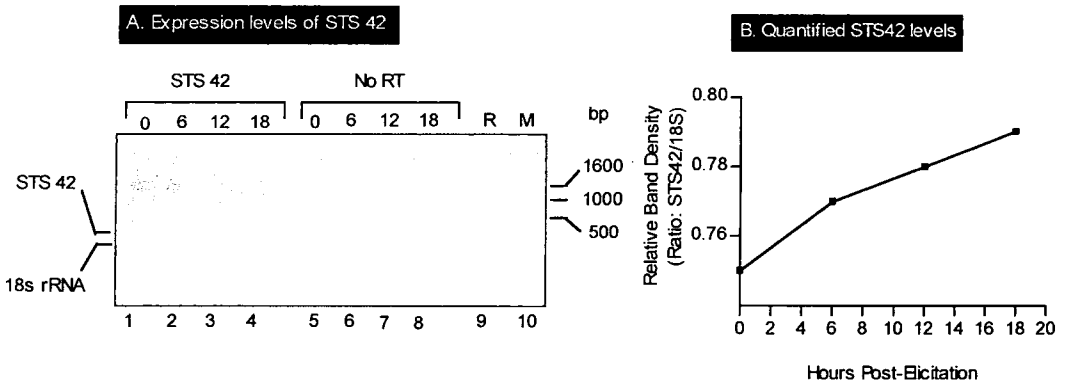



**Figure 65. RT PCR Amplification of mRNA for STS 42 Transcripts.** RT PCR was undertaken using the optimised 23 cycles and QuantumRNA™ 18S internal standard (18S rRNA primer: Competimer™: 1:9). After 23 cycles of amplification, 20% of the PCR products were electrophoresed on a 1.5% (w/v) agarose/EtBr gel. . Lanes 1 to 8, labelled with 0, 6, 12 and 18 represent amplified cDNA from reverse transcribed RNA of elicited "Stirling" cell suspension cultures sampled at the indicated time (h). The "no RT" reactions were also run for all the corresponding samples to check if genomic DNA was amplified (lanes 5-8). Lane 9, labelled with R represents amplified 18S cDNA of control sample (RNA from mouse liver) supplied with the 18S rRNA internal control, lane 10 labelled with M represents DNA 1 kb ladder. **Panel B.** The intensity of band (as shown in Panel A) at each time points was quantified using a BioImage™ Advanced Quantifier (B.I. Systems Corp.), and the ratio (STS 42: 18S rRNA was calculated.

At this stage, with little information on the behaviour of this enzyme in infected or elicited plant cells, it is difficult to explain the involvement of this enzyme in plant response to elicitation. However, there are several possible explanations based on the biochemical studies on elicited cells reported in chapter 6. Following elicitation, $H_2O_2$ was shown to be released very rapidly into the surrounding medium in cell suspension cultures. The accumulation of this toxic chemical at up to twice the normal concentration may have caused certain proteins to be damaged. LAP, although it has not been reported to function this way, could probably degrade those damaged proteins. The second explanation for the induction of LAP was that impure mixed elicitor used in this study may contain a trace of necrotic factors or toxin that affect the growth of the plant cells. This was demonstrated when the elicitor levels were optimised and showed that increases in cell settled volumes were depressed with the higher concentrations of elicitors. A major effect from such substances was unlikely as

the results from careful study of the effect of diluted mixed elicitor showed no effects on cell settled volumes. A third possibility, is that the STS 42 sequence may be slightly difference from the LAP sequence in Herbers *et al.* (1994) and therefore may behave differently. As mentioned before, the STS 42 clone is only a third of the full-length sequence of the potato LAP and therefore a complete sequence alignment could not be done. Obtaining the full-length sequence would be an objective for future work.

### 7.2.5.3.2. Gene Expression of the STS 51 Sequence

Figure 66 shows the RT PCR expression assay for STS 51 transcripts. No transcripts were observed from genomic DNA.. The STS 51 transcripts increased during the first 12 h elicitation as shown in Figure 66 (Panel B).

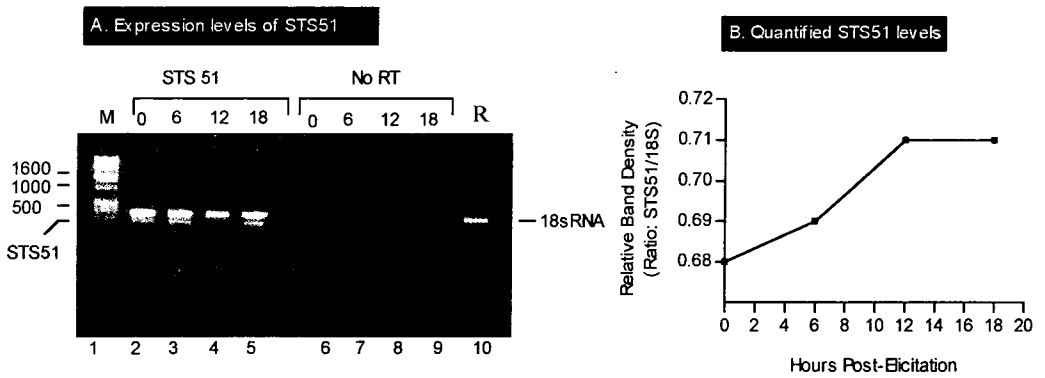



**Figure 66. RT PCR Amplification of mRNA for STS 51 Transcripts. Panel A.** RT PCR was undertaken using the optimised 23 cycles and QuantumRNA™ 18S internal standard (18S rRNA primer: Competimer™: 1:9). After 23 cycles of amplification, 1/10 of the PCR products were resolved on a 1.5% (w/v) agarose/EtBr gel. Lane 1, labelled with M represents 1 kb DNA ladder.Lanes 2 to 9, labelled with 0, 6, 12 and 18 represent amplified cDNA from reverse transcribed RNA of elicited "Stirling" cell suspension cultures sampled at the indicated time (h). The "no RT" reactions were also run for all the corresponding samples to check if genomic DNA was amplified (lanes 6-9). Lane 10, labelled with R represents amplified 18S cDNA of control sample (RNA from mouse liver) comes with the 18S rRNA internal control. **Panel B.** The intensity of band (as shown in Panel A) at each time points was quantified using a BioImage™ Advanced Quantifier (B.I. Systems Corp.), and the ratio (STS 51: 18S rRNA was calculated.

This observation is important because the STS 51 sequence contained a potential tyrosine kinase phosphorylation site. In plants, phosphorylation by protein kinases is one of the most common and important regulatory mechanisms in signal transmission (reviewed by Schenk, and Snaar-Jagalska, 1999). Some protein kinases are associated with pathogen resistance in plants (reviewed in Satterlee and Sussman, 1998). The presence of tyrosine kinase phosphorylation site especially at the hydrophilic region of the STS 51 sequence suggests that the STS 51 may be involved or is part of the signal transduction pathway in this system. Again, further work is needed to isolate the full-length sequence, study its behaviour in this and other situations. Also, studies on the patterns of protein phosphorylation is an obvious way forward.

#### 7.2.5.3.3. Gene Expression of the STS 7 Sequence

The transcript for STS 7 was not detected using the RT PCR assay. Information from the Ambion QuantumRNA Instruction manual suggested that this could have been due to incompatibility between the gene-specific primer (STS 7) and the 18S internal standard primer. However, amplification of the cDNA with the STS 7 primer without the internal 18S rRNA control gave the same result (results not presented).

## 7.3. Conclusion

Using the technique of PCR-select™ (Clontech PCR-Select™ cDNA Subtraction Kit K1804-1) at least two cDNA clones corresponding to known plant mRNAs were induced in response to stress such as wounding and pathogen attack. These clones STS 42 and STS 51 are both of possible significance to the current programme. STS 42 is part of a gene encoding LAP, a potentially important enzyme in protein degradation during cell-death. Increases in the level of STS 42 transcripts detected by RT-PCR suggest that this enzyme could possibly play a role during the interaction of potato cells with elicitors of *P. infestans* in a cell suspension system. This incidence and the behaviour of LAP has not so far been reported in a cell suspension culture. STS 51 on the other hand, may be involved in signal transduction established during the early stage of elicitation. The STS 51 clone has a potential site for tyrosine kinase phosphorylation which is involved in signal transduction. These isolated clones might be useful in further research into the durable resistance mechanism in potatoes. The increase in transcripts for both STS 42 and STS 51 clones proved that the cDNA

library constructed in this study contained differentially expressed fragments associated with events that takes place in early plant cell response to elicitation. The sample clones investigated confirmed up-regulation but a larger number need to be studied to estimate the degree of 'leakage'-sequences which are not up-regulated. The other sequences that have not been identified in the present studies can be used in the future. Because the cDNA library was constructed from Stirling cells after being treated with elicitors in suspension cultures, the remaining sequences may contain sequence information for other activated genes during the elicitation event. This information is crucial to understand the mechanism behind the durable resistance. One possible way of screening these sequences is by using the new technology called the cDNA microarray chips. Using this technique, one can assay the expression of thousands of genes in a single experiment or series of experiments. The chip which is a solid support (eg. a glass slide, nylon membrane) contained thousands of nucleic acid fragments such as cDNAs in an ordered layout. They can then be probed with a labelled sample, usually cDNA from the RNA sample of interest (eg. elicited RNA) and the control in the same way of the southern hybridisation. The relative spot intensities which correspond to the relative expression levels of the gene in the two RNA samples are then compared. In addition, future work should relate the sequences from this subtracted library from cell suspension cultures to the '*in planta*' situation.

Constructing a subtracted cDNA library using the PCR Select™ kit is relatively straight forward provided the initial RNA is good and not degraded. However, the screening process to identify the desired up-regulated clones can be problematic and time consuming. Even following the manufacturer's suggestions and guidelines for screening up-regulated clones for sequencing, most of the selected clones from the first round of screening are not at all relevant to this project because they are ribosomal RNA. Ribosomal RNA is obviously present in larger amounts despite the fact that much of this is removed during the poly A purification and then during the normalisation process of the first hybridisation step. However, while the normalisation enriches for rare sequences (by preferentially removing abundant sequence) an abundant sequence will often still remain in excess. The option to reduce the background by extending the first hybridisation incubation beyond the 8 hours as recommended by the manufacturer resulted in lower overall numbers of products

obtained as was found in the present study. This is a predictable effect – see the Clontech webpage (http://www.clontech.com).

Detecting the corresponding cDNA transcripts from the subtracted library using RT PCR may not be the best way to analyse large numbers of cDNA clones. This is simply because the procedure is complicated and needs various steps of optimisation before valid mRNA comparative studies can be done. In addition the method is relatively expensive compared with other conventional methods. However, this method is very useful if attempts to detect transcripts of interest by other methods such as northern hybridisation failed due to low message abundance. For people that do not have the appropriate radioactive facilities, this method is also very useful. In terms of sensitivity, RT PCR is better than northern hybridisation provided that all the optimisation steps and precautions are undertaken and followed before the actual experiment is carried out. Not only does the PCR cycle number need to be optimised, some other factors, such as priming efficiency, primer design and PCR product quantification need to be investigated and optimised.

# CHAPTER 8

## FINAL DISCUSSION AND FUTURE PROSPECTS

### 8.1. Background

When I came to Durham in 1996 from the University of Agriculture Malaysia, I brought with me several problems associated with the cocoa industry based on several years of work on aspects of the pathology of cocoa plants in the field. The problem is that we have developed several good quality cocoa clones after years of conventional breeding programmes which have good agronomic traits but these are scattered in different individual clones. In the end, as always, selected clones were chosen and crossed to produce new clones, perhaps with all the good characters inherited from their parent. Unfortunately, this is not always the case and, usually, the new clones only show some degree of similarity to their parent and sometimes totally new characteristics are developed. These new clones are subjected to screening, characterisation and field trials. These processes are time-consuming (3 – 10 years depending on the material), labour intensive and costly. Most of the time the demand for good quality cocoa clones is increasing especially when farmers or small holders cannot cope with the current pest and disease problems in cocoa. The major problems are due to the cocoa pod borer (an insect) and vascular streak dieback disease (a vascular fungal pathogen). In addition, large cocoa producers tend to switch to other crops such as oil palm to reduce the cost of operation due mainly to the high cost of the operation and management of these two problems. As a result, national cocoa production has decreased year after year without any signs of recovery. Therefore, any strategies learned from the present study that can be adopted to alleviate the pest and disease problems in the cocoa industry will be very useful especially in the molecular aspects of crop improvement. Engineering cocoa with multi-genes has always been my ambition, but, with the current controversies about genetically modified food and public scepticism in the UK, this strategy definitely needs to be re-evaluated and considered carefully[·].

[·] See Glossary of Relevant terms

How safe this extra genetic material is to the plant gene pools and the environment is still to be evaluated. The possibility of releasing unprecedented effects in the environment is already alarming with the recent report on the harmful effects of the BT gene on the monarch butterfly (Losey *et al.,* 1999). Most of the time, we assume that by introducing an extra gene we have equipped plants with some additional weapons to defend them against pathogen attacks but we sometimes do not realise that plants are living in an ecosystem with inter-relationships with each other and other living communities. For instance, many plants depend on other organisms to perform well and so too do other beneficial organisms, such as in the case of the symbiotic relationship between legume plants and a fungal *Rhizobium* to fix nitrogen, pollination by pollinating insects, butterflies and so on. What would happen to these beneficial organisms if the extra gene produces a new protein that could harm them. Until we have these doubts cleared-up and a set of standard bio-safety guidelines implemented world-wide especially for handling field trials of transgenic plants, then all procedures associated with the development of genetically modified plants should be restricted. Once the genetic pools are contaminated with transgenes, they will be in the environment forever, and the next generation will have to deal with the long-term consequences. However, this does not mean that we must stop our research in this direction because the technology is powerful and many improvements can be expected when a clearer picture of the whole plant genome is known. Producing the technology is one thing but implementing the technology and dealing with its associated effects is another that must be subjected to more debate.

With this background in view, the project presented in this thesis is a multidisciplinary study involving plant biochemistry, microbiology and pathology using various techniques such as plant tissue and cell culture, and molecular techniques. It was apparent in the final year of the project that its scope was too wide for a three-year PhD study to come out with complete and conclusive results. However, having experienced for the first time working with plant RNA, constructing my own cDNA library using recent technology i.e. subtraction hybridisation, studying gene expression using RT PCR, handling DNA sequences and the associated bioinformatics and working with transgenic plants are all valuable assets for me when I go back to Malaysia.

## 8.2. Potato as an Experimental Plant

The experimental plant used in the present study was the potato. The potato, although a relatively minor crop in Malaysia, is grown in the temperate highlands. The potato was chosen because it has been used as an experimental plant in the Department of Biological Sciences for years and several transgenic potato lines were available which were potentially suitable to be used in the programme. In addition, the potato is a relatively easy experimental plant to work with in tissue culture as well as producing short crop cycles in the field and glasshouse. To facilitate the study further, cell suspension cultures were adopted early in the programme. This greatly reduced the complexity of the plant system in studying the biochemistry and molecular biology aspects of the host-pathogen interactions. A homogeneous population of cells and being able to control and optimise the cell environment provided major advantages for looking at the impact of a single experimental parameter (i.e. elicitor molecule) on the biochemical and molecular responses of the cells. With these aspects taken into consideration the potato is a valuable model system for strategies involving other crops.

## 8.3. The Performance of the Lysozyme Transgenic Potato, Lys 75

Biochemical analysis of Lys 75, the transgenic potato line encoding lysozyme showed a promising performance in terms of stability, lysozyme expression and pathogen resistance. After at least 6 generations (Croy, R.R.D.; pers. comm.) the level of lysozyme expression in this transgenic line was still high. Studying its distribution showed that more than 80% of the expressed lysozyme was actually in the intercellular space. Therefore, the original animal signal peptide on the chick-egg white lysozyme gene was functioning correctly in plants in directing the newly synthesised lysozyme protein into the endoplamic reticulum (ER). Trudel *et al.* (1992) reported that their transgenic tobacco which encoded chick-egg white lysozyme with its original 18-amino acid signal peptide, showed some 10% of the total lysozyme expressed was in the intercellular space. However, During *et al.* (1993), using the T4-lysozyme gene in their transgenic potatoes, found the overall expression of lysozyme located in the intercellular space to be very low.

With the high levels of lysozyme found in the intercellular space of the transgenic potatoes (Lys 75), some degree of resistance to certain fungal and bacterial pathogens

in the present study was expected. Lys 75 does show levels of resistance to *F. sulphureum*, *E. carotovora* and *R. solani* when tested using the leaf-bridge bio-assay. Similar results against *E. carotovora* were also reported in transgenic potatoes containing T4 lysozyme (During, 1993). No report on the performance of transgenic tobacco containing chick-egg white lysozyme against tobacco pathogens was available (Trudel *et al.,* 1992). It is suggested that the resistance showed in Lys 75 plants is not only because of the hydrolytic properties of the lysozyme enzyme but more importantly its site of deposition. The intercellular space is the first location where most fungal and bacterial pathogens come into direct contact with plant cells and therefore the strategic place for such antimicrobial protein. In fact, some of the naturally-occurring antimicrobial proteins induced in plants in response to pathogen attacks such as chitinases and glucanases are extracellular proteins. It seems that the lysozyme enzyme is highly stable in Lys 75 because the activity of lysozyme in mature leaves was high compared with the younger apical leaves.

There were no obvious phenotypic abnormalities in Lys 75 line either in tissue culture plants or glasshouse materials compared with the control plants due to the expression of a chick-egg white lysozyme gene. These observations were made over three generations of Lys 75 plants grown in the glasshouses and more than two years tissue culture passages during the present study. Also, no phenotypic abnormalities were exhibited in other transgenic potato and tobacco developed by During *et al.,* (1993) and Trudel *et at.* (1992), respectively. Abnormalities in tissue cultured plants due to a somaclonal variation in Lys 75 is unlikely because this plant was put into tissue culture and maintained through internodal sections. Using such a technique without going through the disorganisation of the plant tissues (callusing or cell suspension), greatly reduced the possibility of a somaclonal variation in tissue culture as observed in Dobigny *et al.* (1994). There are several reports about abnormalities in transgenic potatoes either due to steps in tissue culture during the transformation process or other unknown factors (Belknap *et al.*, 1994; Conner *et al.*, 1994; Dobigny *et al.*, 1994). Belknap *et al.* (1994) reported a high percentage of undersized and malformed tubers in several transgenic potatoes which they evaluated for agronomic and quality varieties. In addition, some more obvious abnormalities in shoot appearance were also reported (Corner *et al.,* 1994). Bones *et al.* (1997) showed that transgenic potatoes containing the thioglucoside glucohydrolase gene produced significantly fewer tubers

compared with non-transformed control plants in three independent glasshouse experiments. There are several reports that clearly showed some abnormalities could be expected from the transgenic plants either through transformation, tissue culture steps such as regeneration and maintenance, and integration of a foreign gene.

### 8.4 The Leaf-Bridge Bioassay - a 'Safe' Way to Initially Test Disease Resistance in Transgenic Plants

The leaf-bridge bioassay for testing plant resistance to pathogens developed during the present project has been particularly useful for testing transgenic plants. Useful initially for testing especially when there is public concern about the safety of field trials for such plants. It cannot, however, substitute for full blown field trials to test interaction with the environment and field performance. Public scepticism is likely to continue for years until uncertainties about the safety of transgenic plants is resolved. Therefore, any alternative method for testing such plants is highly valuable. Although the leaf-bridge bioassay seems to be limited by leaf sizes due to the limited size of the petri dish, it could be easily extended or modified as long as the leaf is being supplied with suitable nutrients. The nutrient must not come in contact with the leaf laminae except through its petiole. This is to ensure that the pathogen (i.e. spore or mycelium) grows and spreads 'inside' the leaf tissues to the other end (petiole). Potential false positive results could be obtained if the mycelia colonised or spread over the leaf surface rather than infected the plant tissue. To avoid such a situation, it is advisable to use spores rather than a mycelial plug as an inoculum. Spores are also preferred because they are countable and tend to infect plants directly at the inoculation site. In addition, by using spores, a uniform standard pathogen challenge across several experimental leaves is possible. Quantifying the magnitude of infection, which is based on the rate of the pathogen movement towards the opposite end from the inoculation site, depends on three assumptions i.e. i) the pathogens are growing endogenously ii) their movement in plant tissues is in all directions and iii) the pathogen movement inside the tissue is proportional to the incubation time. With these assumptions, the semi-quantitative estimation of the magnitude of the infection can be made using the method described in Chapter 4. Since the technique uses detached leaves it is advised and possible to use reasonably large sample sizes to make sure a strong statistically significant result is obtained. Using large sample sizes enhances the power of the statistical test if small differences between the two experimental

populations are predicted. In addition, using a large sample size is not a problem in the leaf-bridge bioassay as the tissue culture materials can be easily propagated and also the technique does not require a large space for incubation.

### 8.5. Some Biochemical and Molecular Aspects of Durable Resistance

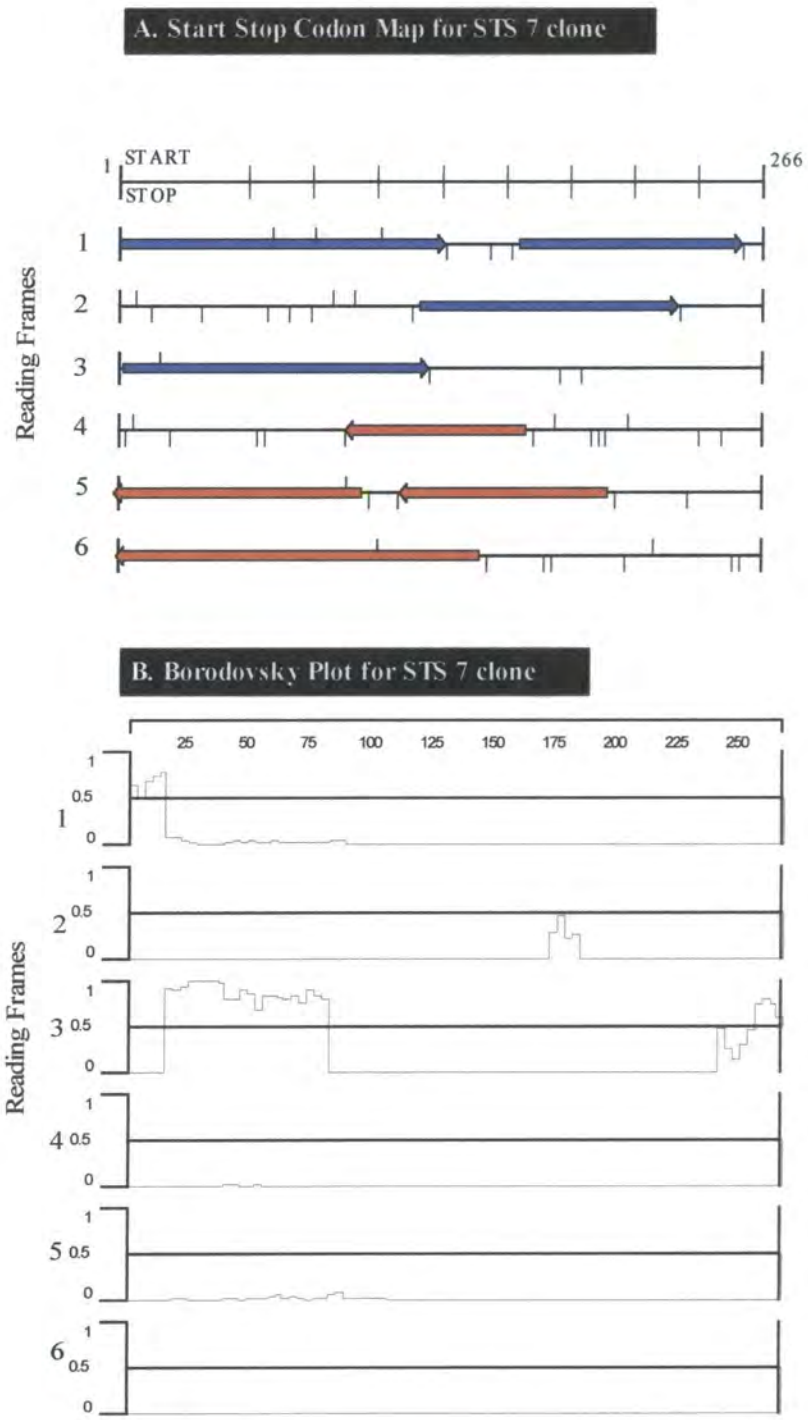
It was immediately obvious that none of the transgenic potatoes in the present study showed any signs of resistance to *P. infestans*. This is not entirely surprising because *P. infestans* is a unique potato pathogen, it is not a 'true' fungus and therefore has different properties to other fungi. The transgenic potatoes tested here express individual 'anti-pathogen' factors that target a specific, corresponding site in the pathogen cell. It is likely that the reason for their lack of effect in preventing *P. infestans* colonisation in the transgenic potatoes is due to the absence of the specific target in the *P. infestans* cell wall. Judging from the complexity of this organism i.e. genetic variability in the various forms and races, a lack of understanding about its biology and interaction with the host, elucidation of resistance factors and strategies in *P. infestans* could still be a long way off. Durable resistance manifested in the cultivar 'Stirling' is one of the best genetic materials to study *P. infestans* resistance. This is because Stirling shows some degree of resistance to compatible strains of *P. infestans* and it is sustainable. The resistance is suggested to be controlled by multiple genes (Stewart, H.; pers. comm.). The work started here would form an excellent basis for future research in this area.

The present studies provide some clues to the establishment of the early cell responses to elicitation. Clones identified in the present study such as STS 42 and STS 51 are both potentially valuable to understanding the process. The presence of STS 42 which encodes LAP, especially in the early interaction (i.e. 6 h) could be related to programme cell death and/or a hypersensitivity response as discussed before. This was supported by the demonstration of the rapid production of hydrogen peroxide during the first hour following elicitation. It would be interesting to investigate the molecules (eg. specific elicitor from *P. infestans*) that were responsible for triggering such a reaction. So far nothing is known about the exact elicitor molecule that triggers the defence responses in *P. infestans*-potato cell interactions. A small elicitor protein called elicitin (10 kb protein) isolated from *P. infestans* culture filtrate was shown to be a 'non-host type' elicitor that elicits defence responses in *N. benthamiana* rather

than in potato (Kamoun *et al.*, 1998). The product specified by the STS 51 clone on the other hand, could also be important because it was tentatively identified to be a member of the ribonuclease T2 family of histidine proteins 1 based on similarity to consensus sequence. Plant ribonucleases have been shown to be involved in defence reactions in plants (Galiana *et al.*, 1997). Plant ribonucleases are also more prominent in the apoplast of compatible host plants than incompatible-type host plants (reviewed in Green, 1994). Galiana *et al.* (1997) showed how extracellular ribonuclease in the apoplast reduced infection by *P. infestans* although the mechanism behind this is unclear. The possibility of producing transgenic potato plants expressing ribonucleases in the intercellular space has been suggested to combat *P. infestans* (Galiana *et al.*, 1997).

The subtracted cDNA library in the present study probably does not contain the whole story of *P. infestans*-potato cell interactions from the very first contact made between the two organisms to the manifestation of resistance i.e. producing PR-proteins which could last far beyond the 6 h used to construct the cDNA library. Therefore, transcripts in the cDNA library are most likely to represent some part of the initial process i.e. signal perception and transduction. In future, it would be interesting to construct further cDNA libraries covering the complete time frame from the very first moment of interaction until the appearance of durable resistance. This would give a clearer profile of the genes responsible for every process leading to durable resistance. Having a complete cDNA library is a valuable commodity for this process but without an efficient way of screening and identifying the clones, there is likely to be a severe delay in the discovery of urgently needed information to solve the late blight disease problems.

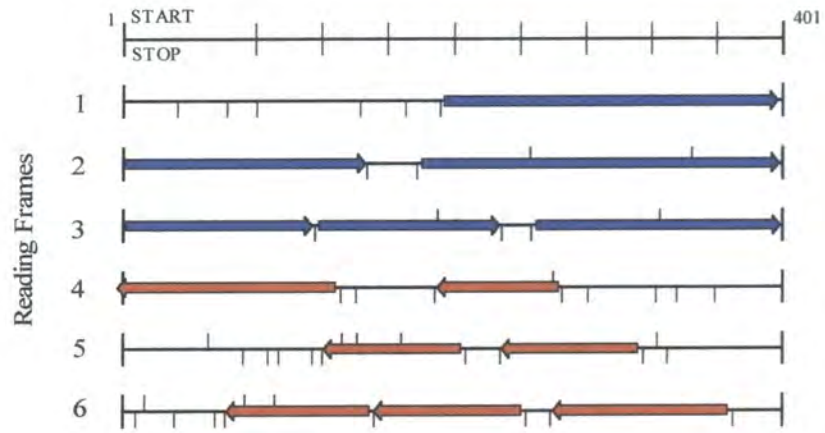
# APPENDIX 1.



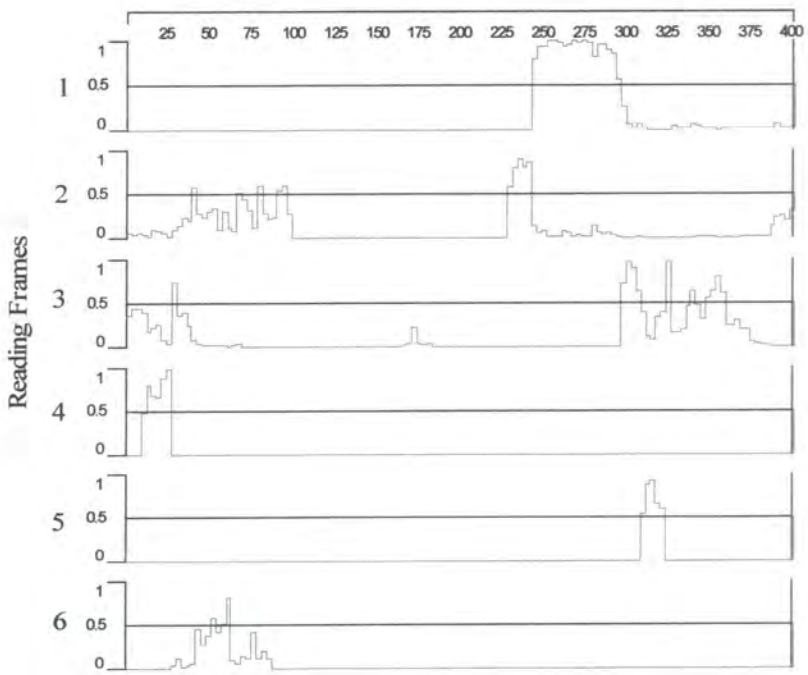

**Appendix 1. Predicted Coding Region of the STS 7 Clone.** The predicted coding region was identified in reading frame 1 based on the Start Stop Codon method (**Panel A**) and the Borodovsky prediction technique (**Panel B**). Details of the methods was described in Figure 56.

# APPENDIX 2

A. Start Stop Codon Map for STS 25 clone

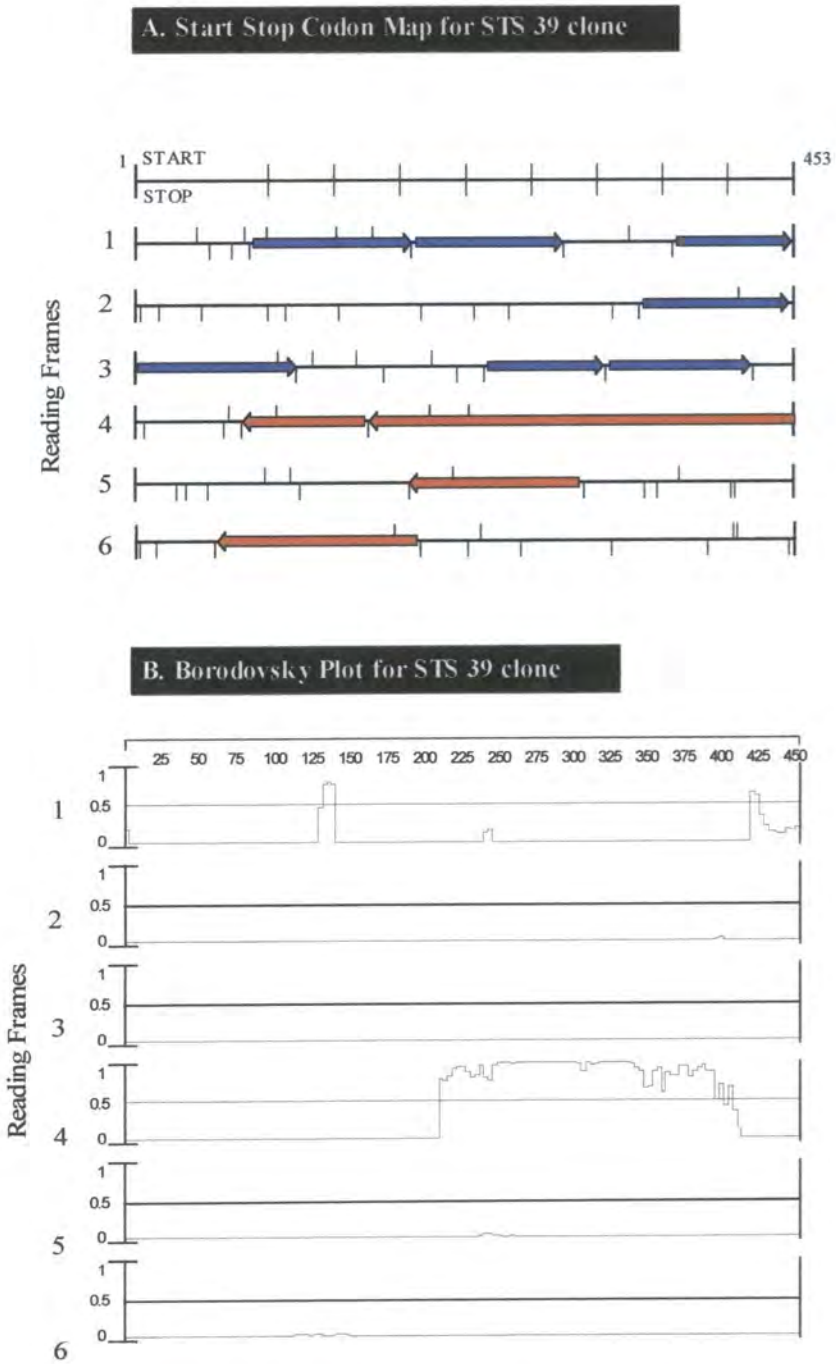



B. Borodovsky Plot for STS 25 clone



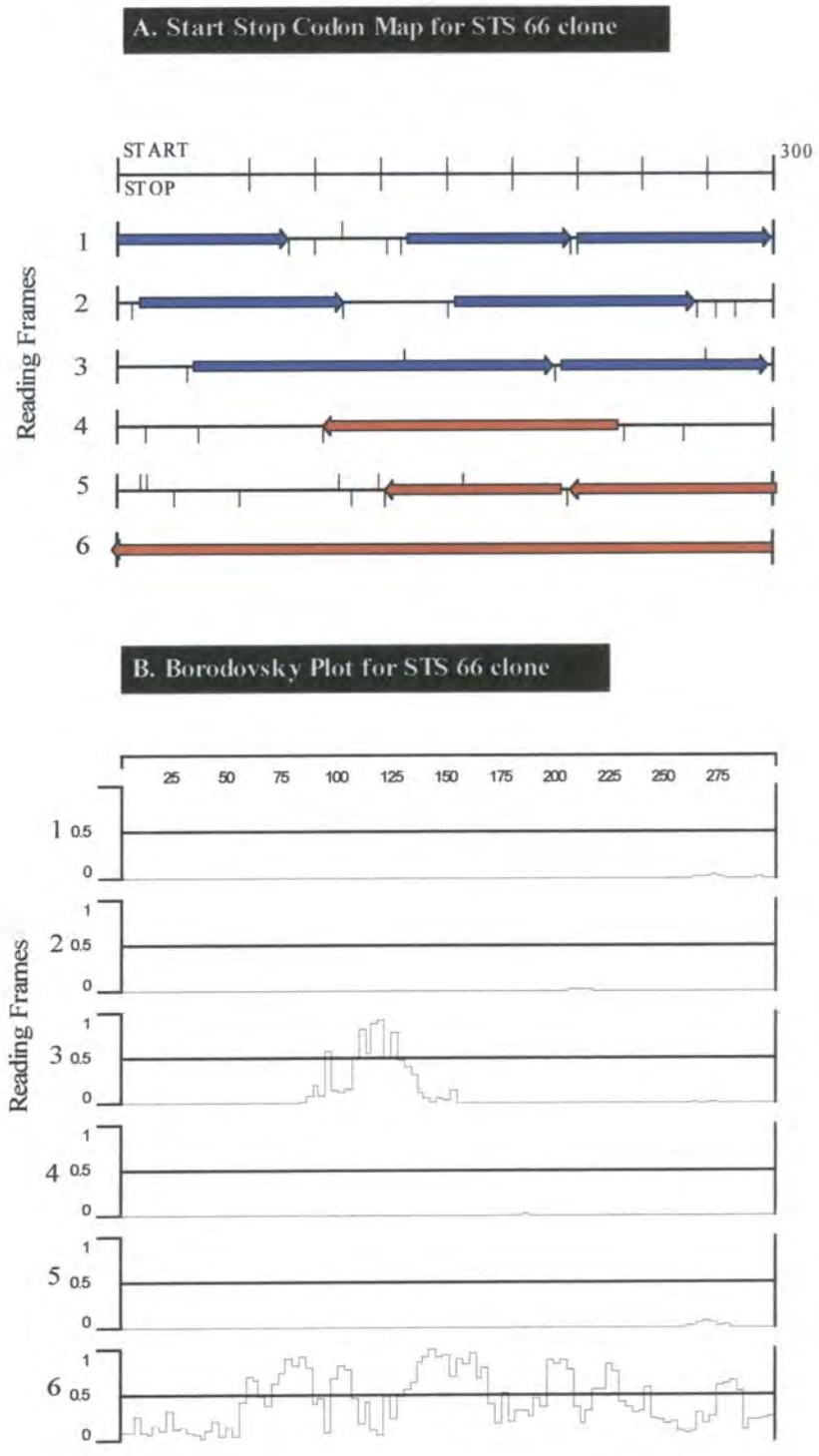

**Appendix 2. Predicted Coding Region of the STS 25 Clone**. The predicted coding region was identified in reading frame 1 based on the Start Stop Codon method (**Panel A**) and the Borodovsky prediction technique (**Panel B**). Details of the methods was described in Figure 56.

# APPENDIX 3




**Appendix 3. Predicted Coding Region of the STS 39 Clone.** The predicted coding region was identified in reading frame 1 based on the Start Stop Codon method (**Panel A**) and the Borodovsky prediction technique (**Panel B**). Details of the methods was described in Figure 56.

# APPENDIX 4




**Appendix 4. Predicted Coding Region of the STS 66 Clone**. The predicted coding region was identified in reading frame 1 based on the Start Stop Codon method (**Panel A**) and the Borodovsky prediction technique (**Panel B**). Details of the methods was described in Figure 56.

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